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Biology and characterisation of polyalanine as an emerging pathological marker

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Résumé

Dix-huit maladies humaines graves ont jusqu'ici été associées avec des expansions de trinucléotides répétés (TNR) codant soit pour des polyalanines (codées par des codons GCN répétés) soit pour des polyglutamines (codées par des codons CAG répétés) dans des protéines spécifiques. Parmi eux, la dystrophie musculaire oculopharyngée (DMOP), l'Ataxie spinocérébelleuse de type 3 (SCA3) et la maladie de Huntington (MH) sont des troubles à transmission autosomale dominante et à apparition tardive, caractérisés par la présence d'inclusions intranucléaires (IIN). Nous avons déjà identifié la mutation responsable de la DMOP comme étant une petite expansion (2 à 7 répétitions supplémentaires) du codon GCG répété du gène *PABPN1*. En outre, nous-mêmes ainsi que d'autres chercheurs avons identifié la présence d'événements de décalage du cadre de lecture ribosomique de -1 au niveau des codons répétés CAG des gènes *ATXN3* (SCA3) et *HTT* (MH), entraînant ainsi la traduction de codons répétés hybrides CAG/GCA et la production d'un peptide contenant des polyalanines. Or, les données observées dans la DMOP suggèrent que la toxicité induite par les polyalanines est très sensible à leur quantité et leur longueur.

Pour valider notre hypothèse de décalage du cadre de lecture dans le gène *ATXN3* dans des modèles animaux, nous avons essayé de reproduire nos constatations chez la *drosophile* et dans des neurones de mammifères. Nos résultats montrent que l'expression transgénique de codons répétés CAG élargis dans l'ADNc de *ATXN3* conduit aux événements de décalage du cadre de lecture -1, et que ces événements sont néfastes. À l'inverse, l'expression transgénique de codons répétés CAA (codant pour les polyglutamines) élargis dans l'ADNc de *ATXN3* ne conduit pas aux événements de décalage du cadre de lecture -1, et n'est pas toxique. Par ailleurs,

l'ARNm des codons répétés CAG élargis dans *ATXN3* ne contribue pas à la toxicité observée dans nos modèles. Ces observations indiquent que l'expansion de polyglutamines dans nos modèles *drosophile* et de neurones de mammifères pour SCA3 ne suffit pas au développement d'un phénotype.

Par conséquent, nous proposons que le décalage du cadre de lecture ribosomique -1 contribue à la toxicité associée aux répétitions CAG dans le gène *ATXN3*.

Pour étudier le décalage du cadre de lecture -1 dans les maladies à expansion de trinucléotides CAG en général, nous avons voulu créer un anticorps capable de détecter le produit présentant ce décalage. Nous rapportons ici la caractérisation d'un anticorps polyclonal qui reconnaît sélectivement les expansions pathologiques de polyalanines dans la protéine PABPN1 impliquée dans la DMOP. En outre, notre anticorps détecte également la présence de protéines contenant des alanines dans les inclusions intranucléaires (IIN) des échantillons de patients SCA3 et MD.

Mots-clés : Ataxie spinocérébelleuse de type-3 (SCA3), dystrophie musculaire oculopharyngée (DMOP), maladie de Huntington (MH), dégénération neuronale, inclusions intranucléaires (IIN), décalage du cadre de lecture ribosomique -1, ataxin-3, polyadenylate-binding protein nuclear 1 (PABPN1), huntingtin, polyglutamine, polyalanine, *ATXN3*, *PABPN1*, *HTT*.

Abstract

Eighteen severe human diseases have thus far been associated with trinucleotide repeat (TNR) expansions coding for either polyalanine (encoded by a GCN repeat tract) or polyglutamine (encoded by a CAG repeat tract) in specific proteins. Among them, oculopharyngeal muscular dystrophy (OPMD), spinocerebellar ataxia type-3 (SCA3), and Huntington's disease (HD) are late-onset autosomal-dominant disorders characterised by the presence of intranuclear inclusions (INIs). We have previously identified the OPMD causative mutation as a small expansion (2 to 7) of a GCG repeat tract in the *PABPN1* gene. In addition, we and others have reported the occurrence of -1 ribosomal frameshifting events in expanded CAG repeat tracts in the *ATXN3* (SCA3) and *HTT* (HD) genes, which result in the translation of a hybrid CAG/GCA repeat tract and the production of a polyalanine-containing peptide. Data from OPMD suggests that polyalanine-induced toxicity is very sensitive to the dosage and length of the alanine stretch.

To validate our *ATXN3* -1 frameshifting hypothesis in animal models, we set out to reproduce our findings in *Drosophila* and mammalian neurons. Our results show that the transgenic expression of expanded CAG repeat tract *ATXN3* cDNA led to -1 frameshifting events, and that these events are deleterious. Conversely, the expression of polyglutamine-encoding expanded CAA repeat tract *ATXN3* cDNA was neither frameshifted nor toxic. Furthermore, expanded CAG repeat tract *ATXN3* mRNA does not contribute to the toxicity observed in our models. These observations indicate that expanded polyglutamine repeats in *Drosophila* and mammalian neuron models of SCA3 are insufficient for the development of a phenotype.

Hence, we propose that -1 ribosomal frameshifting contributes to the toxicity associated with CAG repeat tract expansions in the *ATXN3* gene.

To further investigate ribosomal frameshifting in expanded CAG repeat tract diseases, we sought to create an antibody capable of detecting the frameshifted product. Here we report the characterization of a polyclonal antibody that selectively recognizes pathological expansions of polyalanine in the protein implicated in OPMD, PABPN1. Furthermore, our antibody also detects the presence of alanine proteins in the intranuclear inclusions (INIs) of SCA3 and HD patient samples.

Keywords : Spinocerebellar ataxia type-3 (SCA3), oculopharyngeal muscular dystrophy (OPMD), Huntington's disease (HD), neurodegeneration, intranuclear inclusions (INIs), -1 ribosomal frameshifting, ataxin-3, polyadenylate-binding protein nuclear 1 (PABPN1), huntingtin, polyglutamine, polyalanine, *ATXN3*, *PABPN1*, *HTT*.

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Nucleotides:

A	Adenine
G	Guanine
T	Thymine
C	Cytosine

Coding Sequences:

AAG	Lysine
ATG	Methionine
CAA	Glutamine
CAG	Glutamine
CGG	Arginine
CUG	Leucine
GCA	Alanine
GCG	Alanine
GGGGCC	Glycine-Proline

Abbreviations:

µg	Microgram
µm	Micrometer
ADH	Alcohol Dehydrogenase
ADNc	Acide Désoxyribonucléique Complémentaires

ALS	Amyotrophic Lateral Sclerosis
ARNm	Acide Ribonucléique Messager
ARX	Syndromic and Non-Syndromic X-Linked Mental Retardation
ATP	Adenosine Triphosphate
ATXN3	Ataxin-3 Gene
B3GAT	β-1,3 Glucuronyltransferase
BCL	B-Cell Lymphoma
BDNF	Brain Derived Neurotrophic Factor
bp	Base Pair
BSA	Bovine Serum Albumin
C57B16	C57 Black 6 Mouse
C9orf72	Chromosome 9 Open Reading Frame 72 Gene
CBP	CREB-Binding Protein
cDNA	Complementary Deoxyribonucleic Acid
CF	Ceavage Factor
CFTR	Cystic Fibrosis Transmembrane conductance Regulator
CHIP	C-Terminus of Heat Shock Cognate Protein 70-Interacting Protein
CK	Casein Kinase
CNS	Central Nervous System
CPSF	Cleavage/Polyadenylation Specificity Factor
CREB	cAMP-Response Element-Binding Protein
CstF	Cleavage Stimulation Factor
Ct	Threshold Cycle

C-terminal	Carboxyl-Terminal
DM	Myotonic Dystrophy
DMEM	Dulbecco's Modified Eagle Medium
DMOP	Dystrophie Musculaire Oculopharyngée
DNA	Deoxyribonucleic Acid
DRD-2	Dopamine Receptor D2
DRD-2	Dopamine Receptor D2 Gene
DRPLA	Dentatorubral-Pallidoluysian Atrophy
DsRed	*Discosoma sp*. Red Fluorescent Protein
E6-AP	E6-Associated Protein
ECL	Enhanced Chemiluminescence
EGFP	Enhanced Green Fluorescent Protein
EP	Equivalent Postnatal Day
ER	Endoplasmic Reticulum
ERAD	Endoplasmic Reticulum-Associated Degradation
expCAA	Polyglutamine-encoding expanded CAA *ATXN3* Trangene
expCAG	Polyglutamine-encoding expanded CAG *ATXN3* Trangene
FBS	Fetal Bovine Serum
FOXL2	Blepharophimosis, Ptosis and Epicanthus Inversus Syndrome Type II
FOXO	Forkhead Box O
FTD	Frontotemporal Dementia
FXTAS	Fragile X-Associated Tremor Ataxia Syndrome
GABA	Gamma-Aminobutyric Acid

gmr	Glass Multiple Reporter
Gp	Glycoprotein
HA	Influenza Hemagglutinin
HAP	Huntingtin-Associated Protein
HBSS	Hank's Balanced Salt Solution
HD	Huntington's Disease
HDAC	Histone Deacetylase
HIP	Huntingtin-Interacting Protein
hnRNP	Heterogeneous Ribonucleoprotein
HOXA13	Hand-Foot-Genital Syndrome
HOXD13	Synpolydactyly Type II
HRP	Horseradish Peroxidase
Hsc	Heat Shock Cognate Protein
Hsp	Heat Shock Protein
HTT	Huntingtin Gene
ICI	Intracytosolic Inclusion
IIN	Inclusions Intranucléaires
INI	Intranuclear Inclusion
InsP3R1	Type 1 Inositol (1,4,5)-Trisphosphate Receptor
IP_3	Inositol (1,4,5)-Trisphosphate
IT15	Interesting Transcript 15 Gene
JD	Josephin Domain
kDa	Kilodalton

LCL	Lymphoblastoid Cell Lines
lncRNA	Long Noncoding RNA
LSB	Laemmli Sample Buffer
MAP	Microtubule-Associated Protein
MBNL1	Muscleblind-Like 1
MH	Maladie de Huntington
MITOL	Mitochondrial Ubiquitin Ligase
MJD	Machado-Joseph Disease
ml	Millilitre
mM	Millimolar
MRI	Magnetic Resonance Imaging
mRNA	Messenger Ribonucleic Acid
mRNP	Messenger Ribonucleic Acid Ribonucleoprotein
NCoR	Nuclear Receptor Corepressor
NEDD8	Neural Precursor Cell Expressed Developmentally Down-Regulated 8
NES	Nuclear Export Signal
NGFR	Nerve Growth Factor Receptor
NGFR	Nerve Growth Factor Receptor Gene
NGS	Normal Goat Serum
NLS	Nuclear Localisation Signal
NMDA	*N*-Methyl-*D*-Aspartate
NR1	*N*-Methyl-*D*-Aspartate Receptor Subunit 1 Gene
N-Terminal	Amino-Terminal

OAT	Ornithine Aminotransferase
OD	Oligomerisation Domains
OPMD	Oculopharyngeal Muscular Dystrophy
PABPC1	Polyadenylate-Binding Protein Cytoplasmic 1
PABPN1	Polyadenylate-Binding Protein Nuclear 1
PABPN1	Polyadenylate-Binding Protein Nuclear 1 Gene
PACSIN	Protein Kinase C and Casein Kinase Substrate in Neurons
PAGE	Polyacrylamide Gel Electrophoresis
PAP	Polyadenylate Polymerase
PBS	Phosphate-Buffered Saline
PCAF	p300/CREBBP associated factor
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
PLIC1	Protein Linking IAP to the Cytoskeleton
PNS	Peripheral Nervous System
PRF	Programmed Ribosomal Frameshifting
PSD	Postsynaptic Density Scaffolding Protein
RAN	Repeat Associated Non-ATG
RE1/NRSE	Repressor Element 1/Neuron-Restrictive Silencer Element
REST/NRSF	RE1-Silencing Transcription Factor/Neuron-Restrictive Silencer Factor
RIPA	Radioimmunoprecipitation Assay
RNA	Ribonucleic Acid
RNAP	Ribonucleic Acid Polymerase

RP	Ribosomal Protein
rpm	Revolutions Per Minute
RRM	Ribonucleoprotein-Type RNA Binding Motif
RUNX2	Cleidocranial Dysplasia
SBMA	Spinal Bulbar Muscular Atrophy
SCA	Spinocerebellar Ataxia
scFv	Single-Chain Fv
SDS	Sodium Dodecyl Sulfate
SKIP	Ski-Interacting Protein
SMA	Spinal Muscular Atrophy
SNAP	Sensory Nerve Action Potential
SNP	Single Nucleotide Polymorphism
snRNP	Small Nuclear Ribonucleoproteins
SOD	Superoxide Dismutase
SOX3	X-Linked Hypopituitarism
SP	Specificity Protein
STEP	Striatal-Enriched Protein Tyrosine Phosphatase
STOP-CAA	Stop Modified Polyglutamine-encoding expanded CAG *ATXN3* Trangene
STOP-CAG	Stop Modified Polyglutamine-encoding expanded CAG *ATXN3* Trangene
TAFII	TATA-Binding Protein-Associated Factor
TBP	TATA-Binding Protein
ThT	Thioflavin T
TNR	Trinucleotide Repeat/ Trinucléotides Répétés

TrkB	Tropomyosin Receptor Kinase B
tRNA	Transfer Ribonucleic Acid
$tRNA^{Gln\text{-}CUG}$	Glutaminyl-Transfer Ribonucleic Acid
UIM	Ubiquitin Interaction Motif
UPP	Ubiquitin-Proteosome Pathway
US	United States
UTR	Untranslated Region
VCP	p97/Valosin-Containing Protein
VH	Variable Ig Heavy
VL	variable Ig Light
ZIC2	Holoprosencephaly

Contribution of authors

Chapter 2

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Chapter 3

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Rochefort: Construct technical assistance

Dion: Manuscript revision

McPherson: Antibody design and expertise

Rouleau: Supervision, manuscript revision

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Chapter 1 : Introduction

1.1 Trinucleotide repeat expansion diseases

The expansion of trinucleotide repeat (TNR) sequences within genes is a naturally occurring phenomenon in the human genome. On rare occasions; however, these expansion events have been shown to confer severe human phenotypes. TNR diseases are often categorised into two subclasses depending on the nature of the coding sequence concerned: polyglutamine [$(CAG)_n$] repeat expansion diseases; or polyalanine [$(GCN)_n$] repeat expansion diseases (Table 1.1).

Polyglutamine repeat expansion diseases comprise at least nine distinct adult-onset neurodegenerative conditions, including Huntington's disease (HD), spinal bulbar muscular atrophy (SBMA), spinocerebellar ataxia (SCA) types 1, 2, 3, 6, 7 and 17, and dentatorubral-pallidoluysian atrophy (DRPLA) (La Spada and Taylor, 2010; Orr and Zoghbi, 2007). The adult-onset disorder oculopharyngeal muscular dystrophy (OPMD), and eight other severe congenital conditions such as synpolydactyly type II (HOXD13), cleidocranial dysplasia (RUNX2), holoprosencephaly (ZIC2), hand-foot-genital syndrome (HOXA13), blepharophimosis, ptosis and epicanthus inversus syndrome type II (FOXL2), congenital central hypoventilation syndrome (PHOX2B), syndromic and non-syndromic X-linked mental retardation (ARX), and X-linked hypopituitarism (SOX3) currently account for the polyalanine repeat expansion diseases (Albrecht and Mundlos, 2005; Messaed and Rouleau, 2009).

Trinucleotide repeat instability depends on the nature of the repeat and its length. Polyglutamine repeat tracts are unstable in both somatic and germ cells, and the cause of their expansion likely involves one or more of the following processes: formation of unusual DNA structures and DNA slippage during lagging-strand synthesis; aberrant repair of unusual DNA mutagenic intermediates such as double-strand or single-strand breaks; or recombination within the repeats by interchromosomal strand annealing (Cleary and Pearson, 2005; Pearson et al., 2005). In contrast, polyalanine repeat tracts are mitotically and meiotically stable (Cleary and Pearson, 2005; Pearson et al., 2005), and the cause of their expansion is thought to arise from unequal crossing-over between two mispaired normal alleles (Nakamoto et al., 2002; Warren, 1997).

Table 1.1: Trinucleotide repeat expansion diseases

Disease	Locus	Gene	Protein	Protein Function	Repeat tract size		Reference
					Normal	Disease	
Polyglutamine expansion diseases							
Huntington's disease (HD)	4p16.3	*HTT*	Huntingtin	Signaling, transcription, transport	6-34	36-121	(1993)
Spinal and bulbar muscular atrophy (SBMA)	Xq12	*AR*	Androgen receptor	Steroid-hormone receptor	9-36	38-62	(La Spada et al., 1991)
Spinocerebellar ataxia type-1 (SCA1)	6p22.3	*ATXN1*	Ataxin-1	Transcription	6-39	40-82	(Banfi et al., 1994)
Spinocerebellar ataxia type-2 SCA2	12q24.13	*ATXN2*	Ataxin-2	RNA metabolism	15-24	32-200	(Pulst et al., 1996)
Spinocerebellar ataxia type-3/Machado-Joseph disease (SCA3/MJD)	14q32.12	*ATXN3*	Ataxin-3	Deubiquitinase activity, transcription regulation	10-51	55-87	(Kawaguchi et al., 1994)
Spinocerebellar ataxia type-6 (SCA6)	19p13.2	*CACNA1A*	α_{1A} calcium channel subunit	Voltage-sensitive channel activity	4-20	20-29	(Zhuchenko et al., 1997)
Spinocerebellar ataxia type-7 (SCA7)	3p14.1	*ATXN7*	Ataxin-7	Transcription	4-35	37-306	(Trottier et al., 1995)
Spinocerebellar ataxia type-17 (SCA17)	6q27	*TBP*	TATA box binding protein	Transcription	25-42	47-63	(Koide et al., 1999)
Dentatorubral-pallidoluysian atrophy (DRPLA)	12p13.31	*ATN1*	Atrophin 1	Transcription	7-34	49-88	(Koide et al., 1994)
Polyalanine expansion diseases							
Oculopharyngeal muscular dystrophy (OPMD)	14q11.2	*PABPN1*	Polyadenylate-binding protein nuclear 1	mRNA processing, transport	10	12-17	(Brais et al., 1998)
Synpolydactyly type II (HOXD13)	2q31.1	*HOXD13*	Homeobox D13	Transcription factor	15	22-29	(Goodman et al., 1997)
Cleidocranial dysplasia (RUNX2)	6p21.1	*RUNX2*	Runt-related transcription factor 2	Transcription factor	17	27	(Mundlos et al., 1997)
Holoprosencephaly (ZIC2)	13q32.3	*ZIC2*	Zinc finger protein of cerebellum 2	Transcription factor	15	25	(Brown et al., 2001)
Hand-foot-genital syndrome (HOXA13)	7p15.2	*HOXA13*	Homeobox A13	Transcription factor	18	24-26	(Goodman et al., 2000)
Blepharophimosis,/ptosis/ epicanthus inversus syndrome type II (FOXL2)	3q22.3	*FOXL2*	Forkhead transcription factor FOXL2	Transcription factor	14	22-24	(De Baere et al., 2001)
Congenital central hypoventilation syndrome (PHOX2B)	4p13	*PHOX2B*	Paired-like homeobox 2B	Transcription factor	20	25-29	(Matera et al., 2004)
Syndromic and non-syndromic X-linked mental retardation (ARX)	Xp21.3	*ARX*	Aristaless-related homeobox, X-linked	Transcription factor	12-16	20-23	(Stromme et al., 2002)
X-linked hypopituitarism (SOX3)	Xq27.1	*SOX3*	SRY-related HMG-box gene 3	Transcription factor	15	22-26	(Laumonnier et al., 2002)

Note: References indicate the first group to identify the causative mutation.

1.2 Spinocerebellar ataxia type-3

Spinocerebellar ataxia type-3 (SCA3), also known as Machado-Joseph disease (MJD), was originally described in families of Azorean descent (Nakano et al., 1972; Rosenberg et al., 1976; Woods and Schaumburg, 1972), and is currently deemed to be the most common form of SCA in the world (Ranum et al., 1995; Schols et al., 1995; Schols et al., 2004; Silveira et al., 1998). The disease is an autosomal-dominant spinocerebellar degeneration that presents a gait ataxia with pyramidal and extrapyramidal signs, peripheral amyotrophy, progressive external ophthalmoplegia, rigidity, and dystonia (Coutinho and Andrade, 1978). Cognitive deficits are not a feature of SCA3, even in advanced stages of the disease (Sudarsky et al., 1992). The age of onset has been documented to range from 4 to 70 years old, with a mean age of 40 (Carvalho et al., 2008; Coutinho, 1992), while survival time has varied from 7 to 29 years, with a mean of 21 years (Coutinho, 1992; Kieling et al., 2007). Most patients succumb to pulmonary complications and cachexia (Sequeiros and Coutinho, 1993; Sudarsky et al., 1992).

1.2.1 Clinical features

The differences in age of onset and survival time, along with the observed phenotypic variability (Nakano et al., 1972; Rosenberg et al., 1976; Woods and Schaumburg, 1972), help to illustrate the marked clinical heterogeneity associated with SCA3. To assist in the clinical classification of patients, Coutinho and Andrade (1978) characterised three distinct clinical subtypes based on the presence or absence of significant pyramidal and extrapyramidal signs. Type 1 ("Type Joseph") identifies with an early age of onset (often before 20 years old), and a swift progression of marked pyramidal (rigidity and spasticity) and extrapyramidal (bradykinesia and dystonia) signs, along with cerebellar ataxia and external ophthalmoplegia.

The most common subtype, type 2 ("Type Thomas"), is characterised by an intermediate onset (20 to 50 years old), cerebellar ataxia, external ophthalmoplegia, and pyramidal signs. Finally, type 3 ("Type Machado") presents with a later age of onset (40 to 75 years old), and is characterised by ataxia associated with peripheral alterations such as amyotrophy and motor neuronopathy. Patients that are classified as type 2 (in terms of symptoms) often progress to either type 1 or 3 in as few as four to five years, although, on occasion, some have remained in type 2 for over 20 years (Sequeiros and Coutinho, 1993). More recently, two additional subtypes have been added to the clinical classification: Type 4, the rarest subtype, which is associated with dopa-responsive parkinsonism, mild cerebellar deficits, and a distal sensorimotor neuropathy (Suite et al., 1986); and type 5, for cases resembling hereditary spastic paraplegia (Sakai and Kawakami, 1996).

Many SCA3 patients also suffer from sleep disorders thought to be resultant of the disease. Schöls and colleagues found that impaired sleep, reported as trouble falling asleep and nocturnal awakenings, was associated with older age, long-standing disease, and brainstem involvement (Schols et al., 1998). Such causes for these impairments include rapid eye movement sleep behaviour disorder (Friedman, 2002; Friedman et al., 2003), restless leg syndrome (D'Abreu et al., 2009b; Pedroso et al., 2011; Schols et al., 1998; van Alfen et al., 2001), and sleep apnea (D'Abreu et al., 2009b). Excessive daytime sleepiness is also common among patients (Friedman et al., 2003).

1.2.2 Imaging and neuropathological features

Neuroimaging and pathological studies performed on SCA3 patients have shown that the extent and localisation of neurodegeneration far exceeds its nomenclature. Magnetic resonance imaging (MRI) has been helpful in the diagnosis of patients, and it commonly reveals an enlargement of the fourth ventricle (Klockgether et al., 1998; Murata et al., 1998; Onodera et al., 1998). Quantitative MRI-based studies have identified atrophy of the medulla oblongata, pons, midbrain, thalamus, putamen, caudate nucleus, superior cerebellar peduncle, cerebellar vermis and hemispheres, and widespread cortical and limbic structures (D'Abreu et al., 2012; D'Abreu et al., 2011; de Oliveira et al., 2012; de Rezende et al., 2014; Klockgether et al., 1998; Murata et al., 1998; Yoshizawa et al., 2003). In addition, quantitative MRI-based studies have confirmed SCA3 patients also experience atrophy of the spinal cord, combined with anteroposterior flattening (Fahl et al., 2014; Lukas et al., 2008), and atrophy of deep white matter in the brainstem, lateral thalamus, cerebellar peduncles, and cerebellar hemispheres (Guimaraes et al., 2013; Kang et al., 2014; Lukas et al., 2006). Interestingly, the use of magnetic resonance spectroscopy has identified metabolic abnormalities in apparently normal deep white matter, suggestive of axonal dysfunction preceding atrophy (D'Abreu et al., 2009a).

The brain weight of SCA3 patients with advanced symptoms is considerably less than that of individuals with no previous history of neurological or psychiatric diseases (Iwabuchi et al., 1999). Macroscopic investigation reveals a depigmentation of the substantia nigra, as well as atrophic changes of the cerebellum, pons, medulla oblongata, medial cerebellar peduncle, and cranial nerves III to XII (Rub et al., 2003a; Rub et al., 2006; Rub et al., 2002; Rub et al., 2003b). Despite atrophy of the cerebellum, Purkinje cells and inferior olivary neurons are often spared

(Sequeiros and Coutinho, 1993). Neuropathological studies typically show neuronal loss of the cerebellothalamocortical and basal ganglia-thalamocortical motor loops, anterior horn cells and Clarke's column in the spinal cord, and the following systems: visual, auditory, somatosensory, oculomotor, ingestion-related (brainstem), vestibular (brainstem), precerebellar (brainstem), dopaminergic (midbrain), cholinergic (midbrain), noradrenergic (pontine), and GABAergic (thalamus) (Gilman, 2000; Hoche et al., 2008; Iwabuchi et al., 1999; Kumada et al., 2000; Robitaille et al., 1997; Rub et al., 2008). Myelin loss is also observed, affecting cerebellar, brainstem, and spinal cord white matter, cerebellar peduncles, the medial and lateral lemniscus, and the vestibulospinal, spinocerebellar, and spinothalamic tracts (Gilman, 2000; Hoche et al., 2008; Iwabuchi et al., 1999; Kumada et al., 2000; Robitaille et al., 1997; Rub et al., 2008).

1.2.3 Molecular genetics

The SCA3 locus has been mapped to chromosome 14q32.1 (Takiyama et al., 1993), and the gene identified as *ATXN3* (Kawaguchi et al., 1994). *ATXN3* comprises 11 exons within a 1,776 bp coding region containing one long open reading frame (Ichikawa et al., 2001; Kawaguchi et al., 1994). The causative mutation was shown to be an expansion of a polymorphic CAG repeat within exon 10, encoding for polyglutamine in the ataxin-3 protein (Ichikawa et al., 2001; Kawaguchi et al., 1994). This repeat is nearly a pure CAG tract [$(CAG)_2CAAAAG(CAG)_n$], interrupted by a single lysine codon (AAG) near the start of the repeat (Kawaguchi et al., 1994). The length of the CAG repeat within the normal allele varies greatly, ranging from 12 to 43 (Cancel et al., 1995; Limprasert et al., 1996; Maciel et al., 1995; Matilla et al., 1995; Matsumura et al., 1996a; Ranum et al., 1995; Sasaki et al., 1995; Takiyama et al., 1995), with lengths of 14 and 23 repeats being observed most frequently (Limprasert et

al., 1996). Conversely, expanded alleles have CAG repeat lengths that range from 61 to 87 (Cancel et al., 1995; Kawaguchi et al., 1994; Maciel et al., 1995; Matilla et al., 1995; Ranum et al., 1995; Schols et al., 1996; Silveira et al., 1996; Takiyama et al., 1995; Takiyama et al., 1997b). Although extremely rare, intermediate size alleles (45 to 56 CAG repeat lengths) have been observed in seven individuals, and associated with disease in six (Egan et al., 2000; Gu et al., 2004; Padiath et al., 2005; Takiyama et al., 1997a; van Alfen et al., 2001; van Schaik et al., 1997). The one unaffected individual was reported to have an allele with a CAG repeat length of 51, indicating the possibility of low penetrance among intermediate size alleles in SCA3 (Maciel et al., 2001).

An inverse correlation is found between the length of the CAG repeat tract within the expanded *ATXN3* allele and the age of onset for the disease, accounting for 50% to 75% of the observed variation (Maciel et al., 1995; Maruyama et al., 1995; Matsumura et al., 1996a). The length of the CAG repeat tract also determines the observed clinical subtype, with longer CAG repeat lengths conferring a more severe classification (type 1 *versus* type 2 or 3), and may associate with a faster disease progression (Maciel et al., 1995; Maruyama et al., 1995; Matsumura et al., 1996a). In addition, a gene dosage effect may be present in SCA3, as individuals homozygous for expanded *ATXN3* alleles present with an earlier age of onset and a more rapid progression than their heterozygous peers (Lerer et al., 1996; Sobue et al., 1996).

Repeat instability of the *ATXN3* gene is thought to be conferred by a single nucleotide polymorphism (SNP) immediately following the CAG repeat tract [$(CAG)_nC$ or $(CAG)_nG$)] (Limprasert et al., 1996; Matsumura et al., 1996b). Previous work has found that expanded

alleles exclusively contain the $(CAG)_nC$ SNP, while both the $(CAG)_nC$ and $(CAG)_nG$ polymorphisms were seen in normal alleles from SCA3 patients and control individuals (Limprasert et al., 1996; Matsumura et al., 1996b). Interestingly, the CAG tract in normal alleles with the $(CAG)_nC$ SNP were significantly longer than in the alleles with the $(CAG)_nG$ SNP (Limprasert et al., 1996; Matsumura et al., 1996b). Furthermore, the risk for intergenerational change in the expanded allele is greater in paternal than maternal transmission (Igarashi et al., 1996; Maciel et al., 1995; Manikandan et al., 2007). It is this intergenerational instability of the expanded allele that accounts for the phenomenon of anticipation occasionally seen in families with SCA3 (Coutinho and Sequeiros, 1981; Sequeiros and Coutinho, 1993).

1.2.4 Ataxin-3

Ataxin-3 is an evolutionarily conserved protein, with *ATXN3* orthologues identified in such eukaryotes as fungi, protozoans, plants, and animals (Albrecht et al., 2003; Costa et al., 2004; Linhartova et al., 1999; Rodrigues et al., 2007; Schmitt et al., 1997). In unaffected humans ataxin-3 has a molecular weight of 40 to 43 kDa, depending on the length of the polyglutamine repeat (Kawaguchi et al., 1994). It is a modular protein, located in both the cytoplasm and the nucleus, as well as mitochondria (Antony et al., 2009; Macedo-Ribeiro et al., 2009; Perez et al., 1999), and is ubiquitously expressed in cells and tissue throughout the body (Costa et al., 2004; Ichikawa et al., 2001; Paulson et al., 1997a; Schmidt et al., 1998; Trottier et al., 1998); however, levels of expression vary depending on the region (Trottier et al., 1998). The ataxin-3 protein encompasses a globular N-terminal Josephin domain (JD) with a papain-like fold, similar in structure and catalytic activity to cysteine proteases, combined with a flexible C-terminal tail containing ubiquitin interaction motifs (UIMs) and the polymorphic

polyglutamine tract (Albrecht et al., 2003; Goto et al., 1997; Masino et al., 2003; Scheel et al., 2003). Alternative splicing results in a C-terminal containing either two UIMs followed by the polyglutamine sequence and a hydrophobic amino acid stretch, or a C-terminal with a third UIM replacing the hydrophobic tail (Goto et al., 1997). Although both variants are detected in the brain, the three UIM variant is predominantly expressed and considered to be the more physiologically relevant isoform (Harris et al., 2010).

1.2.5 Normal cellular and physiological roles of ataxin-3

1.2.5.1 Involvement in the ubiquitin-proteasome pathway

The ubiquitin-proteasome pathway (UPP) is the principle mechanism used by cells for the catabolism of proteins. Many studies have provided evidence for ataxin-3 involvement with the UPP, in its ability to bind and cleave (deubiquitinate) polyubiquitin chains and polyubiquitinated proteins (Albrecht et al., 2003; Burnett et al., 2003; Chai et al., 2004; Doss-Pepe et al., 2003; Scheel et al., 2003). Ataxin-3 appears to function as an editor of the polyubiquitin chains added to target proteins during ubiquitination, shortening them to yield free ubiquitin instead of completely dismantling them (Burnett and Pittman, 2005; Kuhlbrodt et al., 2011; Nicastro et al., 2010; Scaglione et al., 2011; Winborn et al., 2008). Ubiquitination is the process in which one ubiquitin molecule (or a polyubiquitin chain) is covalently linked to one or more lysine residues of a target protein by an E3 ubiquitin ligase (Hershko and Ciechanover, 1998). Different linkage types confer specific functions: Lysine 48-linked polyubiquitin chains typically target proteins for proteasomal degradation (Chau et al., 1989; Finley et al., 1994); whereas lysine 63-linked chains play diverse roles in subcellular localisation (Weissman, 2001), membrane endocytosis (Mukhopadhyay and Riezman, 2007), DNA damage repair (Spence et

al., 1995), stress responses (Arnason and Ellison, 1994), and inflammation (Sun et al., 2004). Interestingly, ataxin-3 shows a strong preference for chains of four or more ubiquitin monomers, and lysine 48-linked polyubiquitin chains of four or more monomers are the ones involved in the targeting of proteins for proteasome degradation (Burnett et al., 2003; Chai et al., 2004; Winborn et al., 2008). Moreover, an *in vitro* study involving neuronal cells demonstrated that inhibiting the catalytic activity of ataxin-3 results in the accumulation of polyubiquitinated proteins (Berke et al., 2005). Collectively, these suggest that unlike the usual function of deubiqutinating enzymes to rescue target substrates from degradation, the deubiquitinase activity of ataxin-3 is associated with the delivery of the target substrates to the proteasome (Scaglione et al., 2011; Ventii and Wilkinson, 2008). In fact, ataxin-3 knockout mice show increased levels of ubiquitinated proteins when compared to their wild-type littermates (Schmitt et al., 2007), and the *Caenorhabditis elegans* ataxin-3 orthologue was shown to aid protein catabolism *in vivo* (Kuhlbrodt et al., 2011).

In certain instances, ataxin-3 is itself ubiquitinated. The protein is either mono- or oligo-ubiquitinated; however, the common form is monoubiquitinated (Berke et al., 2005; Todi et al., 2009). This posttranslational modification enhances the deubiquitinase activity of ataxin-3 toward ubiquitinated substrates and free polyubiquitin chains, independent of potential cofactors and interactors (Todi et al., 2010; Todi et al., 2009).

1.2.5.2 Involvement in transcription regulation

Another aspect of ataxin-3 function is believed to involve transcriptional regulation, likely as a transcriptional co-repressor *via* the modulation of histone acetylation and

deacetylation at selected promoters (Li et al., 2002a). Through interaction with the histone acetylase cAMP-response element-binding protein (CREB)-binding protein (CBP), p300 and p300/CBP associated factor (PCAF), ataxin-3 was shown to inhibit CREB-mediated transcription (Evert et al., 2006; Li et al., 2002a). Ataxin-3 also has the ability to inhibit p300-mediated histone acetylation by blocking access to histone actetylation sites, and to promote histone deacetylation by interacting with histone deacetylase 3 (HDAC3) and nuclear receptor co-repressor 1 (NCOR1) (Evert et al., 2006; Li et al., 2002a).

There is also evidence for ataxin-3 involvement in the cellular response to oxidative stress, as it has been shown to interact with and stabilise the forkhead box O (FOXO) transcription factor FOXO4 (Araujo et al., 2011). When cells experience oxidative stress, ataxin-3 and FOXO4 translocate to the nucleus and promote the transcription of the superoxide dismutase-2 (*SOD2*) gene, which in turn increases expression of the antioxidant enzyme SOD2 (Araujo et al., 2011).

1.2.5.3 Ataxin-3 interactors and protein homeostasis systems

Much work has been done to identify ataxin-3 interacting proteins, in the hope of identifying its biological functions. One such interacting protein is the ATPase p97/valosin-containing protein (VCP), which works coordinately with ubiquitinating complexes to shuttle polyubiquitinated substrates to the proteasome for degradation (Boeddrich et al., 2006; Doss-Pepe et al., 2003). The VCP/ataxin-3 complex may act to transfer ataxin-3 edited polyubiquitinated substrates directly to the proteasome or other proteasomal shuttling factors such as ubiquilin/PLIC1 and the human homologues of the yeast DNA repair protein Rad23,

HHR23A and HHR23B (Doss-Pepe et al., 2003; Heir et al., 2006; Kuhlbrodt et al., 2011; Wang et al., 2000). This complex may also function to regulate endoplasmic reticulum-associated degradation (ERAD), the process in which misfolded proteins in the ER secretory pathway are ubiquitintated and exported to the cytosol for proteasomal degradation (Doss-Pepe et al., 2003; Wang et al., 2006; Zhong and Pittman, 2006). It is still uncertain, however, if the VCP/ataxin-3 complex works to promote or inhibit ERAD (Wang et al., 2006; Zhong and Pittman, 2006). Interestingly, the VCP/ataxin-3 complex may also be associated with aging. Kuhlbrodt and colleagues have shown lifespan increases in *C. elegans* VCP and ataxin-3 double knockouts, and that the VCP/ataxin-3 complex regulates components of the insulin/insulin-like growth factor 1 signaling pathway – a pathway involved in lifespan regulation (Kuhlbrodt et al., 2011).

Another ataxin-3 interactor is C-terminus of heat shock cognate protein 70 (Hsc70)-interacting protein (CHIP) (Jana et al., 2005), an E3 ubiquitin ligase that has been linked to the pathology of several neurodegenerative diseases (Cantuti-Castelvetri et al., 2005; Howland et al., 2002; Krobitsch and Lindquist, 2000; Petrucelli et al., 2004; Qin and Gu, 2004; Shimura et al., 2004). Recent work has shown that monoubiquitinated CHIP forms an ubiquitination complex with ataxin-3, through which the deubiquitinase activity of ataxin-3 limits the length of polyubiquitin chains linked to CHIP substrates (Scaglione et al., 2011). Once the linkages have been formed ataxin-3 deubiquitinates CHIP, terminating the ubiquitination cycle (Scaglione et al., 2011). Conversely, CHIP has been shown to monoubiquitinate ataxin-3 at lysine 117 in the JD, enhancing its deubiquitinase activity (Todi et al., 2010; Todi et al., 2009).

Ataxin-3 also interacts with the ubiquitin-like protein neural precursor cell expressed developmentally down-regulated 8 (NEDD8), showing deneddylase activity *in vitro* (Ferro et al., 2007). Neddylation is a process similar to ubiquitination, in which the function of the target protein is regulated *via* conjugation with NEDD8. The ability of ataxin-3 to cleave isopeptide bonds between a substrate and NEDD8 provides evidence for its role in regulating neddylated complexes (Ferro et al., 2007).

Parkin, an E3 ubiquitin ligase involved in Parkinson's disease, also shows a functional interaction with ataxin-3. *In vitro*, parkin is able to self-ubiquitinate, forming lysine 27- and lysine 29-linked polyubiquitin chains which are known to target substrates for lysosomal and autophagic degradation (Shimura et al., 2000). Ataxin-3 is able to deubiquitinate self-ubiquitinated parkin, and while this does not affect its stability or turnover (Durcan et al., 2011), this action may control the number and linkage type of the polyubiquitin chains attached to parkin, and thus its targeted cellular pathway (Durcan et al., 2011).

1.2.5.4 Roles in cytoskeletal organisation and myogenesis

When the ubiquitin-proteasome pathway is compromised or overwhelmed, misfolded proteins are sequestered in perinuclear inclusions termed aggresomes (Johnston et al., 1998). Ataxin-3 is thought to help regulate aggresome formation through its interactions with the aggresome/cytoskeletal organisation components tubulin, dynein, microtubules, microtubule-associated protein 2 (MAP2), HDAC6, and protein linking IAP to the cytoskeleton (PLIC1) (Burnett and Pittman, 2005; Heir et al., 2006; Mazzucchelli et al., 2009; Rodrigues et al., 2010).

These interactions also seem necessary for proper skeletal organisation and assembly of focal adhesions (Rodrigues et al., 2010).

Given its association with skeletal organisation, there is also evidence for ataxin-3 involvement in myogenesis (do Carmo Costa et al., 2010). In order for myoblasts to differentiate into muscle fibers, both the remodelling of the cytoskeleton and the regulation of proteins involved in integrin-mediated signalling are essential (do Carmo Costa et al., 2010). Ataxin-3 interacts with the α5 integrin subunit, repressing this proteins degradation *via* its role in the UPP (do Carmo Costa et al., 2010).

1.2.6 Intracellular localisation and transport

Ataxin-3 shows great mobility throughout the cytoplasm and nucleus, and its transport across the nuclear membrane is aided by a functional nuclear localisation signal (NLS), 282RKRR285, and two nuclear export signals (NES), NES77 and NES141 (Antony et al., 2009; Macedo-Ribeiro et al., 2009; Tait et al., 1998). The main mechanism for the import of ataxin-3 into the nucleus, however, seems to be the phosphorylation of three serine residues by casein kinase 2 (CK2) – serine 236 in UIM1, and serine 340 and 342 in UIM3 (Macedo-Ribeiro et al., 2009; Mueller et al., 2009). Interestingly, the translocation of ataxin-3 to the nucleus also appears to be regulated by proteotoxic stimuli such as oxidative stress and heat-shock (Reina et al., 2010). There is debate on whether CK2-dependent phosphorylation participates under these conditions (Mueller et al., 2009; Reina et al., 2010); however, evidence shows that the nuclear

localisation of ataxin-3 upon heat-shock requires the phosphorylation of serine 111 in the JD (Reina et al., 2010).

1.2.7 Aggregation

In vitro studies have shown that ataxin-3 has a tendency to form aggregates in a process influenced by its N-terminal JD. Aggregation occurs through a single-step mechanism involving the self-assembly of JDs into dimers (Ellisdon et al., 2007; Ellisdon et al., 2006; Gales et al., 2005; Masino et al., 2004). These dimers then associate to form spheroidal oligomers, before elongating into classic beads-on-a-string fibrils. Ataxin-3 fibrils are SDS-soluble, Thioflavin T (ThT)-positive, and structurally resemble those of other self-associating amyloidogenic proteins. In cells, the ataxin-3 isoform bearing two UIMs (2UIM) exhibits a greater tendency to form aggregates than the three UIM (3UIM) isoform (Harris et al., 2010). Furthermore, the deubiquitinase activity of ataxin-3 is lost in fibrils, likely owing to the structural transition from α-helix to β-sheet (Masino et al., 2011b). Interestingly, the ubiquitination of ataxin-3 was shown to prevent JD self-assembly *in vitro*, thus preventing fibril formation and preserving its enzymatic function (Masino et al., 2011a).

1.2.8 Proteolysis

There is evidence from animal model and cell line studies that ataxin-3 is cleaved by caspases and possibly calpains (Berke et al., 2004; Colomer Gould et al., 2007; Jung et al., 2009; Wellington et al., 1998). Both caspase-1 and caspase-3 have been shown to successfully cleave ataxin-3; however, apoptotic cleavage occurs largely through the action of caspase-1, producing a polyglutamine-containing fragment in the process (Berke et al., 2004; Wellington et al., 1998).

Whether calpains actually participate in ataxin-3 proteolysis remains uncertain, as there is evidence for (Berke et al., 2004; Jung et al., 2009; Wellington et al., 1998) and against (Haacke et al., 2007) its involvement.

1.2.9 Degradation

The degradation of ataxin-3 has been shown to occur through both the UPP and autophagy, with the chosen method determined by the isoform involved (Berke et al., 2005; Harris et al., 2010). 2UIM ataxin-3, the less stable isoform, primarily undergoes polyubiquitination and shuttling to the proteasome for degradation. This happens through E3 ligase/shuttle protein complexes, including E4B/VCP, CHIP/heat shock protein 70 (Hsp70), and E6-associated protein (E6-AP)/Hsp70, and the endoplasmic reticulum-associated E3 ligase glycoprotein 78 (Gp78) (Jana et al., 2005; Matsumoto et al., 2004; Mishra et al., 2008; Ying et al., 2009). In contrast, the 3UIM ataxin-3 isoform is commonly degraded by macrophagy (Harris et al., 2010). Furthermore, the catalytic state of ataxin-3 may also regulate its degradation, as studies have revealed higher levels of catalytically inactive ataxin-3, which suggests slower proteasomal degradation (Todi et al., 2007).

1.2.10 Expanded ataxin-3 and disease pathogenesis

In SCA3, expansion of the polyglutamine tract in the C-terminal of ataxin-3 likely causes conformational changes in the protein, which would lead to alterations in its stability and degradation, aggregation, subcellular localisation, and molecular interactions with other proteins (Jana and Nukina, 2004). In turn, these affected properties would lead to a loss- and/or

gain-of-function, resulting in cellular dysfunction and the observed pathogenesis (Williams and Paulson, 2008).

1.2.10.1 Protein aggregates and intracellular inclusions

A common feature of all repeat expansion diseases is the presence of large macromolecular aggregates containing the disease protein. The initial observations made while examining SCA3 patient brain tissue were the presence of intranuclear inclusions (INIs) in disease vulnerable areas: ventral pons, substantia nigra, globus pallidus, dorsal medulla, and dentate gyrus (Paulson et al., 1997b; Schmidt et al., 1998). These INIs not only contained expanded ataxin-3, but also ubiquitin, heat-shock proteins, proteasome constituents, transcription factors, molecular chaperones, and other polyglutamine proteins (Chai et al., 1999a; Chai et al., 1999b; Chai et al., 2001; Paulson et al., 1997b; Schmidt et al., 1998). Newer techniques, however, have also identified INIs in unaffected brain areas (Rub et al., 2008; Rub et al., 2006; Yamada et al., 2002), suggesting that their presence alone does not determine the neuron's fate (Rub et al., 2006).

More recently, expanded ataxin-3-positive aggregates have been observed in the cytosol of neurons in SCA3 brain tissue (Hayashi et al., 2003; Yamada et al., 2004), along with axons in fiber tracts known to degenerate (Seidel et al., 2010). Intracytosolic inclusions test negative for ubiquitin (Yamada et al., 2002), whereas intra-axonal inclusions are ubiquitin-positive and contain nucleoporin p62 (Seidel et al., 2010). Furthermore, intra-axonal inclusions are thought

to interfere with axonal transport, impairing cellular functions and promoting degeneration (Seidel et al., 2010).

Although both normal and expanded ataxin-3 form aggregates, those formed by the expanded protein occur through a two-step mechanism (Ellisdon et al., 2006). Initially, expanded ataxin-3 associates into SDS-soluble fibrils through a process similar to, but quicker than normal ataxin-3 (Ellisdon et al., 2006). In the second step, hydrogen bonding between the glutamine main- and side-chains of the polyglutamine tract induces either a β-helical turn or hairpin conformation, resulting in the formation of SDS-insoluble aggregates (Natalello et al., 2011; Seidel et al., 2010; Sikorski and Atkins, 2005). Recently, the polyglutamine tract of disease-associated proteins has been predicted to self-associate through the formation of coiled-coils, suggesting that its interaction with natural coiled-coil partners could increase aggregation (Fiumara et al., 2010; Petrakis et al., 2013).

1.2.10.2 Proteolytic cleavage and the "toxic fragment" hypothesis

As has been suggested for other polyglutamine diseases, pathogenesis resulting from the proteolytic cleavage of expanded polyglutamine protein, termed the "toxic fragment" hypothesis, may also apply to SCA3 (Tarlac and Storey, 2003; Wellington et al., 1998). In the case of ataxin-3, proteolytic cleavage was shown to generate SDS-soluble 36 kDa C-terminal fragments containing the expanded polyglutamine tract (Goti et al., 2004; Ikeda et al., 1996; Paulson et al., 1997b). These C-terminal fragments have been detected in brain homogenates from SCA3 patients and transgenic mice (Goti et al., 2004), but not from unaffected individuals (Berke et al., 2004), and were enriched in the nuclear fractions of disease vulnerable brain areas

(Colomer Gould et al., 2007; Goti et al., 2004). *In vitro* studies have further shown that ataxin-3 C-terminal fragments containing the expanded polyglutamine tracts induce a stronger aggregation and toxicity than the full-length expanded ataxin-3 protein (Breuer et al., 2010; Haacke et al., 2006; Ikeda et al., 1996; Paulson et al., 1997b).

Although there has been debate on whether calcium-dependent calpains are involved in normal ataxin-3 proteolysis (Section 1.2.8), there is increasing evidence for their involvement in the cleavage of expanded ataxin-3 and the resulting SCA3 pathogenesis (Goti et al., 2004; Haacke et al., 2007; Hubener et al., 2013; Simoes et al., 2012). Calpain-2-mediated cleavage of expanded ataxin-3 was found to produce C-terminal fragments that were prone to aggregation (Hubener et al., 2013). Furthermore, *in vivo* studies where calpain activity was inhibited reduced expanded ataxin-3 cleavage, aggregation, nuclear localisation, and toxicity (Haacke et al., 2007; Simoes et al., 2012). In contrast, an SCA3 transgenic mouse with its endogenous calpain inhibitor calpastatin knocked-out showed an increase in INIs and accelerated cerebellar degeneration (Hubener et al., 2013). The involvement of calpains in expanded ataxin-3 proteolysis may also explain the neuronal specificity of SCA3 pathology – calpains are calcium-dependent and require the excitation-mediated influx of calcium (Koch et al., 2011).

1.2.10.3 Localisation of expanded ataxin-3 fragments

Numerous studies have demonstrated the importance of the nucleus in the pathogenesis of SCA3 and other polyglutamine diseases, with the nuclear localisation of the expanded protein essential for disease (Schols et al., 2004; Shao and Diamond, 2007). Bichelmeier and colleagues (2007) demonstrated that C-terminal ataxin-3 fragments containing only the expanded

polyglutamine tract could aggregate in the nucleus or cytoplasm when coupled to a respective synthetic NLS or NES, *in vitro*. The INIs were shown to accumulate, whereas the ICIs were targeted for degradation (Bichelmeier et al., 2007). In transgenic SCA3 mice, artificially targeting expanded ataxin-3 to the nucleus increased levels of INIs and promoted earlier death, while forcing the nuclear export of expanded ataxin-3 reduced INIs and lessened disease symptoms (Bichelmeier et al., 2007). Although INIs are the pathological hallmark of SCA3, whether they are directly toxic or formed as a protective cellular response to cope with the toxicity of the expanded disease-proteins is still uncertain.

MITOL, a mitochondrial ubiquitin ligase, and parkin may be involved in the proteasomal degradation of expanded ataxin-3 C-terminal fragments (Sugiura et al., 2011; Tsai et al., 2003). As for full-length expanded ataxin-3, it was shown to be degraded *via* both the UPP and autophagy (Berger et al., 2006; Jana et al., 2005; Matsumoto et al., 2004; Mishra et al., 2008; Ying et al., 2009).

1.2.10.4 Impaired protein degradation in SCA3

As described previously (Sections 1.2.5.1 and 1.2.5.3), ataxin-3 has been shown to participate in the UPP and other protein homeostasis systems. Expansion of the polyglutamine tract in ataxin-3 could alter its normal function within these mechanisms through aberrant protein interactions and aggregation, leading to toxicity. In fact, even though there is no significant difference in the deubiquitinase activity between normal and expanded ataxin-3 (Berke et al., 2004; Burnett and Pittman, 2005), an *in vitro* study reported a global reduction in deubiquitinated protein in the expanded ataxin-3 model (Winborn et al., 2008). Furthermore,

the INIs described in SCA3 patients have contained many important proteins including ubiquitin, proteasomal components, chaperones, transcription factors, and normal ataxin-3 (Chai et al., 1999a; Chai et al., 1999b; Doss-Pepe et al., 2003; Mori et al., 2005; Paulson et al., 1997b; Schmidt et al., 1998; Takahashi et al., 2001).

Expanded ataxin-3 shows a more efficient binding of VCP, prolonging its interaction with the E4B/VCP complex and thus delaying its own degradation in the proteasome (Boeddrich et al., 2006; Matsumoto et al., 2004). Other consequences of this prolonged interaction with VCP may be the impairment of ERAD (Wang et al., 2006; Zhong and Pittman, 2006), inducing ER proteotoxic stress and subsequent degeneration, and interference with the down-regulation of neddylation (Yang et al., 2014). The CHIP/ataxin-3 interaction is also affected by expanded ataxin-3, with the expanded protein showing a six-fold increase in affinity which may target CHIP for degradation (Scaglione et al., 2011). Furthermore, despite normal and expanded ataxin-3 having similar binding affinities for polyubiquitinated parkin, expanded ataxin-3 is more efficient at cleaving its polyubiquitin chains, promoting the degradation of parkin *via* autophagy (Durcan et al., 2011). The resulting decrease in parkin levels may represent the Parkinson-like symptoms observed in some SCA3 patients (Buhmann et al., 2003; Gwinn-Hardy et al., 2001; Tuite et al., 1995). Additionally, aggregates in SCA3 patient brain samples were found to trap beclin-1 (Nascimento-Ferreira et al., 2011), a protein with a central function in autophagy, and whose dysfunction has been implicated in neurodegeneration (Wong and Cuervo, 2010).

1.2.10.5 Transcription dysregulation in SCA3

Expansion of the polyglutamine tract in ataxin-3 may also affect its proposed involvement in transcription regulation (Section 1.2.5.2). Its observed aberrant protein interactions with transcription factors and co-activators in SCA3, along with the sequestering of transcription factors to expanded ataxin-3 aggregates, suggest a role of transcriptional dysregulation in the disease pathogenesis (Evert et al., 2006; Riley and Orr, 2006). In fact, the altered transcription of several genes has been identified through analyses of brain tissue from SCA3 patients and transgenic mice, and an SCA3 neuronal cell model (Chou et al., 2008; Evert et al., 2001; Evert et al., 2003). Expanded ataxin-3 was found to down-regulate messenger RNA (mRNA) expressions of proteins involved in glutamatergic neurotransmission, intracellular calcium signaling/mobilisation or MAP kinase pathways, $GABA_{A/B}$ receptor subunits, Hsps, and transcription factors regulating neuronal survival and differentiation (Chou et al., 2008). Conversely, mRNA expressions were upregulated for proteins involved in inflammation and neuronal cell death (Chou et al., 2008; Evert et al., 2001; Evert et al., 2003).

The down-regulation of mRNA expressions for proteins involved in intracellular calcium signaling/mobilisation and MAP kinase pathways is consistent with the aberrant interaction of expanded ataxin-3 with the type 1 inositol (1,4,5)-trisphosphate receptor (InsP3R1) reducing intracellular calcium levels in neurons (Chen et al., 2008; Chou et al., 2008). InsP3R1 is an intracellular calcium release channel with an important role in calcium signalling (Berridge, 1993). Interestingly, expanded ataxin-3 was also reported to alter the kinetics of voltage-gated potassium channels in neuronal cell culture and the Purkinje cells of SCA3 transgenic mice (Jeub et al., 2006; Shakkottai et al., 2011). Changes in neuronal physiology

may underlie the observed motor symptoms in SCA3, and likely contribute to the disease pathogenesis (Shakkottai et al., 2011).

More recently, expanded ataxin-3 was found to have a reduced ability to promote FOXO4-mediated SOD2 expression and to also interfere with the binding of FOXO4 to the *SOD2* promotor in response to oxidative stress (Araujo et al., 2011). There is also evidence for an overall decrease in antioxidant enzyme ability in cellular models of SCA3 (Yu et al., 2009). Taken together, the resulting accumulation of reactive oxygen species and free radicals could lead to the observed mitochondrial dysfunction and eventual cell damage in SCA3 (Kazachkova et al., 2013; Laco et al., 2012; Yu et al., 2009), as has been suggested for other polyglutamine diseases (Ajayi et al., 2012; Goswami et al., 2006; Kim et al., 2003; Miyata et al., 2008).

1.3 Oculopharyngeal muscular dystrophy

Oculopharyngeal muscular dystrophy (OPMD) was originally described in a family of French-Canadian descent (Taylor, 1915), and now has a world-wide distribution with cases reported in at least 33 countries. OPMD is an autosomal-dominant muscle disease with late-onset selective progressive ptosis, dysphagia, and proximal limb weakness (Victor et al., 1962). Although rare, some cases of autosomal-recessive inheritance have been reported (Blumen et al., 1999; Fried et al., 1975). The age of onset for OPMD is often the fifth or sixth decade of life (Bouchard et al., 1997; Brais et al., 1999), with a life expectancy for patients close to normal (Becher et al., 2001). The leading causes of death are starvation and aspiration pneumonia.

1.3.1 Clinical features

OPMD is a myopathy shown to affect all skeletal muscles, yet appears to spare both smooth and cardiac muscle. Muscle involvement is specific, symmetric, and its severity has been documented in the following descending order: levator palpebrae, tongue, pharynx, extraocular muscles, iliopsoas, adductor femoris, gluteus maximus, deltoids, and hamstrings (Little and Perl, 1982). Aside from the main symptoms (ptosis, dysphagia, and proximal limb weakness), affected individuals may present with facial muscle weakness, upgaze limitations, dysphonia, and tongue weakness/atrophy (Bouchard et al., 1997). Certain patients may also develop mild to severe ophthalmoparesis, occasionally causing diplopia (Tomé and Fardeau, 1994). Complete external ophthalmoplegia, however, is rare (Tomé and Fardeau, 1994). Currently, no treatment for OPMD is available.

1.3.2 Myopathological and neuropathological features

Histological studies of biopsied skeletal muscle from OPMD patients typically show changes common to most muscular dystrophies, including loss of muscle fiber, abnormal variation in fiber size, an increased number of internalised nuclei, expanded interstitial fibrous and fatty connective tissue, and autophagic rimmed vacuoles (Tome and Fardeau, 1980). Non-specific mitochondrial abnormalities have also been reported (Wong et al., 1996). The most significant ultrastructural change in OPMD is the presence of INIs in patient skeletal muscle (Brais et al., 1999; Tome and Fardeau, 1980, 1986). Electron microscopy reveals chromatin-surrounded clear zones containing tubular filaments with 8.5 nm outer and 3 nm inner diameters

(Tome and Fardeau, 1980). These filaments are up to 250 nm in length, unbranched, and converge to form tangles and palisades (Tome and Fardeau, 1980).

At present, the primary etiology of OPMD is considered to be myopathic, although there is mounting evidence for involvement of the peripheral and central nervous systems in the disease. Probst and colleagues were the first to indicate neurogenic changes in the peripheral nervous system (PNS) with their report of severe depletions of myelinated fiber in the endomysial nerve twigs of extraocular, pharyngeal, and lingual muscles in an OPMD patient (Probst et al., 1982). In accordance, the findings by Boukriche *et al*. suggest that lower motor neurons may also be involved in OPMD after biopsies performed on peroneus muscle revealed the presence of small angulated atrophic fibers and the loss of myelination, while those performed on the peroneal nerve showed signs of chronic axonal regeneration (Boukriche et al., 2002). Probst and colleagues were also the first to detail the potential involvement of the central nervous system (CNS) in OPMD with the observed loss of myelinated fibers in the cranial nerves, particularly cranial nerve III, in post mortem patient tissue (Probst et al., 1982). Additionally, Dion *et al.* described the presence of INIs in cerebellar neurons of an OPMD patient (Dion et al., 2005). These neurogenic changes in the PNS and CNS may lead to denervation, and ultimately contribute to the pathophysiology of OPMD.

1.3.3 Molecular genetics

The dominant OPMD locus has been mapped to chromosome 14q11.2-q13 (Brais et al., 1995), and the gene identified as polyadenylate-binding protein nuclear 1 (*PABPN1*); previously referred to as polyadenylate-binding protein 2 (*PABP2*) (Brais et al., 1998). *PABPN1* consists

of seven exons within a 2,001 bp coding region. The causative mutation was shown to be an expansion of a GCG repeat tract within the first exon, encoding for polyalanine in the PABPN1 protein (Brais et al., 1998). The normal *PABPN1* allele has a $(GCG)_6$ repeat that encodes for the first six alanine residues in a homopolymeric stretch of 10 alanines $[(GCG)_6(GCA)_3GCG]$, whereas the expanded allele has a $(GCG)_{8\text{-}13}$ repeat in a stretch of 12 to 17 alanines $[(GCG)_{8\text{-}13}(GCA)_3GCG]$ (Brais et al., 1998). The GCG repeat in *PABPN1* is meiotically and mitotically stable, thus its expansion during meiosis is uncommon (Nakamoto et al., 2002). Furthermore, anticipation is not observed (Nakamoto et al., 2002).

No significant correlations between the length of the GCG repeat within the expanded *PABPN1* allele and the age of onset for the disease, or the severity of disease, have been reported. Instead, disease severity appears to relate to the patient's age (Muller et al., 2006; Muller et al., 2001). The decade-specific penetrance for the most commonly found dominant mutation in OPMD, $[(GCG)_{13}(GCA)_3GCG]$, were the following: age < 40 years, 1%; 40-49 years, 6%; 50-59 years, 31%; 60-69 years, 63%; and age > 69 years, 99% (Brais et al., 1997).

Interestingly, Brais *et al.* observed a $[(CGC)_7(GCA)_3GCG]$ polymorphism that acted as a modifier of disease severity in dominant OPMD, with its inheritance increasing the number of symptoms in comparison to the normal *PABPN1* allele (Brais et al., 1998). Furthermore, homozygosity for this polymorphism was found to produce a later onset and less severe autosomal-recessive form of OPMD (Brais et al., 1998; Hebbar et al., 2007). Conversely, patients homozygous for the dominant mutation present with an average age of onset 18 years earlier than $[(CGC)_{13}(GCA)_3GCG]$ heterozygotes, and a more severe phenotype including an

increase in the number of muscle nuclei containing INIs (9.4% *versus* 4.9%) (Blumen et al., 1996; Brais et al., 1998). In addition, homozygotes for the dominant mutation experience mental changes such as paranoid behaviour or subcortical dementia, as well as a reduced life-span (Blumen et al., 2009). These findings suggest a gene dosage effect in OPMD (Brais et al., 1998; Brais et al., 1999).

1.3.4 Polyadenylate-binding protein nuclear 1

PABPN1 is a ubiquitous protein, with domain structures and amino acid identities highly conserved between humans, bovines, and mice (Brais et al., 1998). In unaffected humans PABPN1 has a molecular weight of 32.8 kDa; however, due to posttranslational modifications is closer to 49 kDa (Nemeth et al., 1995). The normal PABPN1 protein consists of three distinct domains: an acidic N-terminal; a central ribonucleoprotein-type RNA binding motif (RRM); and a basic arginine-rich C-terminal. Within the N-terminal domain, the initiating methionine is immediately followed by a stretch of 10 consecutive alanines encoded by an imperfect repeat tract located adjacent to an acidic region rich in glycine and proline residues (Kerwitz et al., 2003). These residues are followed by an α-helical coiled-coil region essential for the protein's interaction with polyadenylate polymerase (PAP) (Kerwitz et al., 2003). The central domain RRM mediates high affinity binding to polyadenylate RNA (Kuhn et al., 2003). The basic C-terminal domain is enriched with arginine residues that are asymmetrically dimethylated, and contains the NLS (Calado et al., 2000; Smith et al., 1999). Two potential oligomerisation domains (ODs) may also exist within the normal PABPN1 protein: OD1, overlaps with the RRM

of the central domain; and OD2, overlaps with the NLS of the C-terminal domain (Fan et al., 2001).

1.3.5 Normal cellular and physiological role of PABPN1

1.3.5.1 Involvement in mRNA polyadenylation

In the nuclei of eukaryotic cells, mRNA is posttranscriptionally modified at its 3'-end by the addition of a polyadenylate tail *via* a two-step reaction. This tail is thought to confer stability to the mRNA transcript, increase the efficiency of its translation, and assist its nuclear export (Lewis et al., 1995; Sachs et al., 1997; Wickens et al., 1997).

Polyadenylation is initiated by the endonucleolytic cleavage of a precursor mRNA transcript at its 3'-end by the cleavage factors (CF) Im and CFIIm (Bienroth et al., 1993). This process requires the assistance of two additional factors - the cleavage/polyadenylation specificity factor (CPSF), and the cleavage stimulation factor (CstF) (Bienroth et al., 1993). CPSF binds to the polyadenylation consensus sequence AAUAAA, catalysing the cleavage of a phosphodiester bond located 10 to 30 nucleotides downstream (Bienroth et al., 1993), whereas CstF adds further specificity by binding to GU- or U-rich elements downstream of the cleavage site (Barabino and Keller, 1999). Following the cleavage event, approximately 250 adenylate residues are added to the upstream product to form the polyadenylate tail. Synthesis of the tail is catalysed by the enzyme PAP through its interaction with CPSF (Bienroth et al., 1993). Although slow and inefficient, the PAP/CPSF complex is responsible for the initial addition of 10 to 11 adenylate residues to the 3'-end, allowing PABPN1 to bind (Keller et al., 2000; Wahle, 1991). CPSF and PABPNI act synergistically to increase the processivity of polyadenylation

by tethering PAP to the RNA transcript, permitting PAP to complete its addition of ~250 adenylate residues without dissociating (Bienroth et al., 1993; Kerwitz et al., 2003; Kuhn et al., 2009; Wahle, 1991, 1995). Interestingly, the binding of PABPN1 to the PAP/CPSF complex may also indirectly regulate polyadenylate tail length. This "molecular ruler" hypothesis proposes that PABPN1 effectively counts the number of adenylate residues incorporated into the mRNA tail and terminates processive polyadenylation once a threshold size is met (Keller et al., 2000; Kuhn et al., 2009).

In addition to polyadenylation, PABPN1-dependent promotion of PAP activity was shown to stimulate nuclear RNA decay through the generation of hyperadenylated decay substrates (Bresson and Conrad, 2013). These substrates were recognised by the exosome, and ultimately degraded (Bresson and Conrad, 2013). Only export-deficient mRNAs were targeted, supporting an mRNA quality control function for this pathway (Bresson and Conrad, 2013).

A recent study has shown that PABPN1 may also be involved in the choice of alternative polyadenylation sites (Jenal et al., 2012). Alternative polyadenylation functions to regulate gene expression, as any change to the length of the 3'-UTR of a transcript could alter its interactions with RNA binding proteins or microRNAs, significantly impacting transcript stability and translation (Di Giammartino et al., 2011). It has been suggested that PABPN1 binds to and masks proximal "weak" polyadenylation sites, enhancing the use of distal canonical polyadenylation sites (Jenal et al., 2012).

Surprisingly, PABPN1 was found to promote the decay of long noncoding RNAs (lncRNAs) *via* a polyadenylation-dependent pathway (Beaulieu et al., 2012). These PABPN1-sensitive lncRNAs were targeted by the exosome and the RNA helicase MTR4/SKIV2L2 (Beaulieu et al., 2012).

1.3.5.2 Involvement in the export of polyadenylated RNA

Despite being an abundant nuclear protein, PABPN1 has been shown to shuttle between the nucleus and cytoplasm *via* a carrier-mediated mechanism (Calado et al., 2000; Chen et al., 1999). Through its NLS on the C-terminal domain, PABPN1 directly binds the nuclear transport receptor, transportin, in a Ran GTP-sensitive manner, suggesting an active nuclear import pathway (Calado et al., 2000). Interestingly, transportin also mediates the nuclear import of heterogeneous nuclear ribonucleoproteins (hnRNPs) (Siomi et al., 1997), proteins with known involvement in mRNA processing and mRNA nuclear export (Izaurralde et al., 1997; Pollard et al., 1996). Furthermore, shuttling hnRNPs and PABPN1 were found to be exported to the cytoplasm by a facilitated transport pathway acting independent of mRNA synthesis (Calado et al., 2000; Pinol-Roma and Dreyfuss, 1992). The similarities among these proteins suggest that PABPN1 may also be involved in mRNA transport (Calado et al., 2000). In fact, PABPN1 was observed to remain associated with the 3'-end of the salivary gland Balbiani ring (BR) mRNA ribonucleoprotein (mRNP) complex until it was translocated through the nuclear pore (Bear et al., 2003). In the same study, low levels of PABPN1 were detected on the cytoplasmic side of the nuclear envelope, suggesting PABPN1 was displaced from the mRNPs during or shortly after passage through the nuclear pore, and rapidly returned the nucleus (Bear et al., 2003).

Further studies on the export of polyadenylated RNA propose that PABPNI exchanges its cargo with polyadenylate-binding protein cytoplasmic 1 (PABPC1) following the first or "pioneer" round of translation (Hall, 2002; Hosoda et al., 2006; Ishigaki et al., 2001). While PABPC1 regulates the stability and translation of mRNA in the cytoplasm, PABPN1 may be responsible for RNA quality control in the cytoplasm and the protection of the mRNA polyadenylated tail from degradation while in the nucleus (Feral et al., 1999; Hall, 2002; Ishigaki et al., 2001).

1.3.5.3 Involvement in transcription regulation

An *in vitro* investigation into the functional role of PABPN1 in skeletal muscle uncovered its potential involvement in the expression of muscle-specific genes and skeletal myogenesis (Kim et al., 2001). PABPN1 overexpression was observed to enhance myotube formation, as well as increase expression of the myogenic factors, MyoD and myogenin (Kim et al., 2001). Under normal conditions, the expressions of these myogenic factors are induced as an early event in myogenic differentiation (Olson and Klein, 1994), and their actions as transcriptional regulators are required for terminal myoblast differentiation (Cusella-De Angelis et al., 1992; Hasty et al., 1993; Nabeshima et al., 1993). Furthermore, PABPN1 was found to cooperate with ski-interacting protein (SKIP) to stimulate MyoD-dependent transcription of myogenin, and to accelerate the morphological differentiation of myotubules (Kim et al., 2001). Interestingly, SKIP is a transcription cofactor present in all eukaryotes, and known to interact with proteins involved in the activation and/or repression of transcription (Kostrouchova et al.,

2002; Zhou et al., 2000a; Zhou et al., 2000b). These findings support a potential role for PABPN1 as a transcription cofactor (Kim et al., 2001).

In vivo, PABPN1 was shown to interact with RNA polymerase II (RNAPII; an enzyme required for the initiation and synthesis of RNA), forming a PABPN1/RNAPII complex before, at, or shortly after the start of transcription (Bear et al., 2003). Additionally, the transfer of PABPN1 from this complex to the growing polyadenylated tail is thought to signal RNAPII to terminate transcription (Bear et al., 2003).

1.3.5.4 Intracellular localisation

PABPN1 is primarily localised to discrete nuclear substructures referred to as nuclear speckles or SC35 domains (Krause et al., 1994). Nuclear speckles are several micrometers in diameter, and composed of 20 to 25 nm interchromatin granule clusters connected by thin perichromatin fibrils resulting in a beads-on-a-string appearance (Fakan et al., 1984; Perraud et al., 1979; Puvion et al., 1984; Spector et al., 1991). These substructures are also rich in polyadenylated RNA (Carter et al., 1993; Carter et al., 1991; Visa et al., 1993). Several studies have identified perichromatin fibrils as the site of cotranscriptional splicing due to their association with nascent RNA and factors with known involvement in pre-mRNA processing, including PABPN1, hnRNPs, splicesomal small nuclear ribonucleoproteins (snRNPs), and other non-snRNP splicing factors (Fakan et al., 1984, 1986; Huang and Spector, 1991; Krause et al., 1994; Xing et al., 1993; Xing et al., 1995). More recently, cleavage factors were also found to be cotranscriptionally associated (Cardinale et al., 2007). Conversely, interchromatin granule clusters contain low levels of nascent RNA and hnRNPs (Fakan and Bernhard, 1971; Fakan et

al., 1984; Fakan and Nobis, 1978), suggesting a role as sites of splicing factor storage and/or spliceosome reassembly (Spector et al., 1991). In fact, splicing factors were observed to shuttle between storage and/or reassembly sites (interchromatin granule clusters) and sites of active transcription (perichromatin fibrils) (Misteli et al., 1997). The association of PABPNl with perichromatin fibrils in the nucleus further supports its involvement in mRNA polyadenylation and transcription regulation.

1.3.6 Expanded PABPN1 and disease pathogenesis

Under normal conditions, hydrophobic homopolymeric stretches of alanines have been described as flexible spacer elements, conferring stability to the three-dimensional shape of the native protein (Karlin et al., 2002). In OPMD, expansion of the polyalanine tract in the N-terminal of PABPN1 may compromise proper protein folding (Scheuermann et al., 2003). As a result, the expanded PABPN1 protein would experience alterations in its stability and degradation, aggregation, subcellular localisation, DNA binding and/or protein-protein interactions (Karlin et al., 2002). Furthermore, these affected properties would lead to a loss and/or gain of function, resulting in cellular dysfunction and the observed pathogenesis.

1.3.6.1 Protein aggregates

In vitro studies performed under physiological conditions have shown that alanine stretches of 7 to 15 amino acids experience variable degrees of conformational transition from a monomeric α-helix to a predominant macromolecular β-sheet (Blondelle et al., 1997; Forood et al., 1995). Above 15 alanines, peptides are completely converted to β-sheet fibrillar

molecules that are extremely resistant to chemical denaturation and enzymatic degradation (Blondelle et al., 1997; Forood et al., 1995).

In terms of disease, it seems likely that an expansion of the alanine repeat tract above 12 to 22 amino acids results in misfolding and/or aggregation of the protein due to biophysical limitations – PABPN1 only requires an expansion of two alanine residues to reach this threshold (Brais et al., 1998; Perutz et al., 2002). Perutz and colleagues interpreted this small expansion leading to aggregation as a result of changes in free energy between the correctly folded and denatured state (Perutz et al., 2002). Due to their hydrophobic property, alanine would occupy internal positions in the folded protein (Perutz et al., 2002). The additional alanine residues would be misfits that lower the free energy barrier to unfolding of the protein (Perutz et al., 2002).

Presently, the role of the additional alanine residues on PABPN1 aggregation is still unclear. A series of studies using truncated versions of the PABPN1 protein lacking either the N-terminal or C-terminal domain showed successful formation of fibrils with each variant; however, the conditions and the properties of the fibrils differed (Scheuermann et al., 2003; Winter et al., 2012). The version lacking the C-terminal domain formed fibrils with classical amyloid-like characteristics, but required an elevated protein concentration for formation to occur (Scheuermann et al., 2003). In contrast, the variant lacking the N-terminal could form fibrils at a low protein concentration, but these fibrils lacked the typical amyloid-like structure (Winter et al., 2012). The alanine-dependent fibril formation of the PABPN1 N-terminal domain at high protein concentrations is in agreement with the reported strong concentration

dependence of oligo-alanine peptides to form fibrils (Shinchuk et al., 2005). Contrarily, fibril formation of the PABPN1 C-terminal domain is in agreement with *in vivo* studies indicating an alanine-independent aggregation (Tavanez et al., 2005), and the identification of several oligomerisation sites outside of the N-terminal domain (Fan et al., 2001; Ge et al., 2008; Song et al., 2008; Tavanez et al., 2005). These finding suggest that PABPN1 may have an intrinsic capacity to aggregate, and that domains not containing the polyalanine tract may also promote fibril formation – just not of an amyloid-like nature.

1.3.6.2 Intranuclear inclusions

The expansion of polyalanine tracts leads to protein aggregation in OPMD and several other polyalanine diseases (Albrecht et al., 2004; Bachetti et al., 2005; Brown et al., 2005; Caburet et al., 2004; Nasrallah et al., 2004). There is confusion, however, as to whether aggregates are pathogenic, or the consequence of a molecular defense mechanism. Nonetheless, the filamentous INIs in OPMD patient muscle nuclei are considered pathological hallmarks of the disease. Furthermore, these INIs not only contain expanded PABPN1, but have been shown to sequester the normal PABPN1 protein, polyadenylated RNA, hnRNPs, Hsps, and components of the UPP (Abu-Baker et al., 2003; Bao et al., 2002; Calado et al., 2000; Fan et al., 2003). Thus, the toxicity of INIs in OPMD could be the result of a direct toxic gain-of-function in which expanded PABPN1 leads to apoptosis, or of the sequestration of RNAs and/or proteins essential for proper cellular functions, including PABPN1.

1.3.6.3 Toxic gain-of-function hypothesis in OPMD

In OPMD patients homozygous for the dominant mutation, INI formation is enhanced (Blumen et al., 1999). The earlier age of onset in these individuals strengthened the idea of INI toxicity (Blumen et al., 1999). *In vitro*, the increased formation of INIs in cells transfected with expanded *PAPBPN1* constructs was also shown to correlate with an earlier cell death (Abu-Baker et al., 2003; Bao et al., 2002). Overexpression of the molecular chaperones Hsp40 and Hsp70 (Abu-Baker et al., 2003), or addition of the anti-amyloid compound Congo red or doxycycline (Bao et al., 2002), increased the solubility of the expanded PABPN1 protein in this model, reducing INI formation and cell toxicity. Similarly, treating OPMD transgenic mice with doxycycline, or the chemical chaperone trehalose led to reduced INI formation, and attenuation of the toxic phenotype (Davies et al., 2006; Davies et al., 2005).

Despite the correlation between expanded PABPN1, the enhancement of INI formation, and the increase of cellular toxicity in cell culture and transgenic mice, no correlation was found between these parameters in a transgenic *Drosophila* model of OPMD (Chartier et al., 2006). Furthermore, a series of experiments using cell culture and transgenic *C. elegans* models have demonstrated a greater cellular toxicity in cells lacking INIs and with the expanded PABPN1 in soluble form (Catoire et al., 2008; Messaed et al., 2007). The soluble expanded PABPN1 protein exerted cellular toxicity in a dose-dependent manner in these experiments, suggesting that the soluble form may be the primary toxic species in OPMD (Catoire et al., 2008; Messaed et al., 2007). In addition, both normal and expanded PABPN1 proteins were found not to be irreversibly sequestered into INIs, but rather able to diffuse rapidly in and out (Berciano et al.,

2004; Tavanez et al., 2005). A later study confirmed the dynamism of expanded PABPN1 INIs, and revealed their ability to disassemble during mitosis (Marie-Josee Sasseville et al., 2006).

1.3.6.4 Transcription dysregulation in OPMD

As previously mentioned (Section 1.3.6.2), both polyadenylated RNAs and normal PABPN1 are sequestered into expanded PABPN1 INIs (Calado et al., 2000). Recent analyses of OPMD patient muscle fibers and expanded PABPN1 overexpression in primary human myoblast cultures revealed that INIs develop in close proximity to nuclear speckles, and gradually deplete the nuclear speckles of polyadenylated RNA and normal PABPN1 (Bengoechea et al., 2012). This event could have an adverse effect on nascent mRNA processing, and lead to dysregulation of gene expression in OPMD (Bengoechea et al., 2012). In fact, the ectopic expression of expanded PABPN1 in mouse myoblast cultures reduced the mRNA expressions of muscle-specific proteins including α-actin, slow troponin C, creatine kinase, and the myogenic factors, MyoD and myogenin (Wang and Bag, 2006). Furthermore, microarray analysis in affected skeletal muscle of transgenic OPMD mice revealed significant changes in the transcription level of 2,336 genes – the majority encoding for proteins with roles in mRNA processing, protein transport, and the UPP (Trollet et al., 2010). These findings were later corroborated by an integrated high-throughput transcriptome study in affected muscles of OPMD patients, transgenic OPMD mice, and transgenic OPMD *Drosophila* (Anvar et al., 2011). Interestingly, the UPP was found to be the most predominantly dysregulated cellular pathway across species (Anvar et al., 2011).

Moreover, the transcription factors CBP and p300 were found to be sequestered into expanded PABPN1 INIs in cell culture models of OPMD (Abu-Baker and Rouleau, 2007). Although sequestration in itself is not proof of dysregulation, it reinforces the premise of altered transcription in the pathophysiology of the disease.

1.3.6.5 Involvement of the ubiquitin-proteasome pathway and molecular chaperones in OPMD

The involvement of the UPP and molecular chaperones in OPMD is supported by the findings that ubiquitin (Abu-Baker et al., 2003), proteasomal subunits (Calado et al., 2000), and Hsps (Abu-Baker et al., 2003; Bao et al., 2002) are sequestered into expanded PABPN1 INIs. Generally, cells rely on molecular chaperones to prevent the aggregation and promote the refolding of misfolded proteins (Wickner et al., 1999). If the native state is unachievable, misfolded proteins are then targeted for degradation *via* the UPP (Huang et al., 2001; Murata et al., 2001; Wickner et al., 1999). The loss of this protective response could compromise the ability of cells to cope with the accumulation of expanded protein. This is evidenced by several studies that have shown enhanced levels of molecular chaperones reduce INI formation and cell toxicity (Abu-Baker et al., 2003; Bao et al., 2002; Davies et al., 2006; Davies et al., 2005). Additionally, inhibition of the proteasome with lactacystin was shown to increase the formation of expanded PABPN1 INIs and cell toxicity (Abu-Baker et al., 2003). Thus, the formation of INIs in OPMD suggests an underlying incapacitation of the cellular chaperones and proteasome machinery by the expanded PABPN1 protein (Abu-Baker et al., 2003).

1.3.6.6 Impairment of mRNA transport and/or processing in OPMD

Given that polyadenylated RNAs are sequestered into expanded PABPN1 INIs (Calado et al., 2000), and the transcription of genes encoding proteins necessary for mRNA processing and transport is dysregulated in OPMD (Anvar et al., 2011; Trollet et al., 2010), levels of these proteins may become insufficient and contribute to cell death. In support of this hypothesis, Fan and colleagues identified two proteins that colocalised with expanded PABPN1 INIs – hnRNP A1 and hnRNP A/B (Fan et al., 2003). These hnRNPs bind to mRNA and are involved in its maturation and export from the nucleus to the cytoplasm (Nakielny and Dreyfuss, 1997; Pinol-Roma and Dreyfuss, 1992; Visa et al., 1996). Subsequently, expanded PABPN1 INIs were found to sequester the polyadenylation enzyme PAP (Tavanez et al., 2005).

1.3.6.7 Apoptosis

Another potential mechanism contributing to the observed pathology in OPMD is apoptosis. This is evidenced by a series of recent experiments in which the treatment of transgenic OPMD mice with the antiapoptotic drug doxycycline (Davies et al., 2005), trehalose (Davies et al., 2006), or cystamine (Davies et al., 2010) was shown to decrease the toxicity of the expanded *PABPN1* transgene, attenuating the OPMD disease phenotype. Similar results were obtained by genetically blocking apoptosis by the overexpression of B-cell lymphoma 2 (BCL2) (Davies and Rubinsztein, 2011). Furthermore, the viral anti-apoptotic protein p35 was shown to ameliorate the disease phenotype in a transgenic *Drosophila* model of OPMD (Chartier et al., 2006).

1.4 Huntington's disease

Huntington's disease is an autosomal-dominant neurogenetic disorder affecting populations worldwide (De Souza and Leavitt, 2014), with the highest incidence amongst individuals of European descent (Pringsheim et al., 2012). It is a highly penetrant disease which affects both sexes equally (Gendelman et al., 2008). Individuals that inherit the causal mutation for HD in the huntingtin (*HTT*) gene can develop symptoms at any time between the ages of 1 and 80 years old, with the average age of onset being 40 years old (Myers, 2004). Only 5% to 7% of the patient population develop HD before the age of 20, and in these cases the disorder is termed juvenile HD (Nance and Myers, 2001). HD patients can live with the disease from 10 to 30 years following diagnosis, and often succumb to complications associated with the disease, including aspiration pneumonia, dysphagia, or injuries through fall (Folstein, 1989).

1.4.1 Clinical features

Prior to their diagnosis, patients undergo what is called the prediagnostic phase in which they will experience minute changes in motor control, personality and cognition. These changes can be subtle enough that the patients themselves are unaware (Snowden et al., 1998). Common changes include: fidgeting; restlessness; slower intellectual processes; difficulty multitasking; anxiety; disinhibition; diminished mental flexibility; and irritability (Craufurd and Snowden, 2002; Folstein, 1989). In juvenile cases, early indicators of HD can be progressively delayed motor milestones, as well as deteriorating school performance (Walker, 2007). Diagnosis is usually made when symptoms progress to recognisable signs of HD: the inability to maintain motor movements; chorea; incoordination; and slow saccadic eye movements (Watts and Koller, 1997; Weiner and Lang, 1989). It should be noted that diagnosis before symptom onset

is possible through predictive testing for disease *HTT*. Due to the penetrance of the disease, at-risk patients may learn through predictive testing whether or not they will develop the disease at some point in their lives with complete certainty, which could be a heavy burden on the individual (Gendelman et al., 2008).

The movement deficits in HD involve both voluntary and involuntary movements, and tend to accumulate sequentially as the disease progresses (Mahant et al., 2003). In the early stages of the disease, involuntary movements are affected from the occurrence of hyper-reflexia, hypotonia and chorea. In later stages of the disease, the addition of compromised voluntary motor movements, due to bradykinesia and rigidity, render HD patients functionally disabled (De Souza and Leavitt, 2014). While choreiform movements typify the classic case of HD, they are not always used as disease milestones since certain patients with early-onset HD have been reported not to develop this symptom. Furthermore, certain HD patients will only transiently experience chorea, while most will gradually have their chorea masked or replaced by dystonia and rigidity as the disease progresses (Mahant et al., 2003; Young et al., 1986). Motor impersistence on the other hand, being the inability to sustain a voluntary muscle contraction, is extremely common in HD and invariably declines over the course of the disease, providing a more reliable measure of disease severity (Reilmann et al., 2001).

Similar to the movement deficits, the cognitive changes exhibited in the prediagnostic phase worsen over time as HD advances. Patients gradually develop subcortical dementia as their executive functions and the learning of new motor skills are affected. Speech typically

deteriorates at a faster rate than comprehension, whereas long-term memory is often spared (Craufurd and Snowden, 2002; De Souza and Leavitt, 2014).

Historically, the neuropsychiatric symptoms of HD have received less attention than the cognitive and motor symptoms as they are not used in the diagnosis of HD, even though they significantly impact the quality of life for patients. Symptoms in this category include: anxiety; apathy; depression; mania; psychosis; and suicidal ideation (Craufurd and Snowden, 2002; Folstein, 1989). Depression and contemplation of suicide are particularly common in individuals that are at-risk in the presymptomatic phase, or in the late stages of the disease (Paulsen et al., 2005). This is likely due to the fact that 92% of HD patients are aware of HD in their family history and have firsthand experience with how the disease progresses from watching a family member struggle with it (Almqvist et al., 2001; Siesling et al., 2000). Certain patients also opt for predictive genetic testing and live with the burden of their fate for years before developing any symptoms. It is estimated that suicide is 5 to 10 times more frequent in HD patients than in the general population (Baliko et al., 2004; Craufurd and Snowden, 2002; Di Maio et al., 1993; Robins Wahlin et al., 2000). Unlike the cognitive and motor symptoms, the behavioural symptoms of HD do not degenerate with time.

In addition to these three large categories of symptoms, HD patients may also experience problems with metabolism, sleep disorders, and testicular degeneration (Craufurd and Snowden, 2002; Van Raamsdonk et al., 2007). In cases of juvenile HD, seizures are common, along with cerebellar dysfunction (Kremer, 2002; The Huntington's Disease Collaborative Research Group, 1993). Due to the variability in disease presentation, it is understandable how some patients

with no known family history of HD were misdiagnosed prior to the discovery of the causal gene, and the development of a diagnostic test.

1.4.2 Imaging and neuropathological features

The examination of affected individuals has revealed that the pathology of HD is not only restricted to the brain, but almost exclusively to the caudate and the putamen (Reiner et al., 1988; Vonsattel and DiFiglia, 1998). These structures undergo progressive atrophy and cell death due to the preferential degeneration of GABAergic medium-sized spiny neurons (Vonsattel, 2008). Jointly, the caudate and the putamen form the striatum, and in 1985 J-P Vonsattel established a classification system for HD severity based on the degree and form of striatal degradation using post mortem tissue from clinically diagnosed patients. In grade 0, no neuropathological abnormalities are detected after gross examination; however, 30% to 40% neuronal loss is often detected in the head of the caudate nucleus through histological techniques. Grade 1 striatal degradation is characterised by astrogliosis, a 50% neuronal loss, and atrophy in the tail and body of the caudate nucleus. Grades 2 to 4 feature progressive increases in the number of astrocytes, and progressive reductions in neuron counts. Grade 4 comprises the most advanced cases of HD, with striatal atrophy and up to 95% neuronal loss (Vonsattel et al., 1985).

Brain imaging techniques have extrapolated these findings to living patients, and have not only helped in the diagnosis of HD, but have helped further our understanding of disease pathogenesis. Confirmation of HD diagnosis is made through routine computerised axial tomography and MRI sessions in moderate to severe cases, as these techniques are able to detect

decreases in striatal volume as well as increased in the size of the frontal horns of the lateral ventricles (Stober et al., 1984). While these techniques are inadequate to assist in the diagnosis during the early stages of the disease, specialised MRI techniques have been able to show atrophy in the putamen and caudate of individuals carrying the expanded *HTT* allele as early as 9 and 11 years before symptom onset, respectively (Aylward et al., 2004).

Further investigation using immunohistochemistry revealed that specific populations of striatal projection neurons are affected in the different stages of HD. In the early to moderate stages, the medium spiny neurons containing encephalin and projecting to the external globus pallidum are more vulnerable to degradation than the substance P-containing medium spiny neurons that project to the internal globus pallidum (Gutekunst et al., 2002; Rubinsztein, 2003). Of the substance P-containing neurons that are depleted, it is those that specifically project to the substantia nigra pars reticulata rather than the substantia nigra pars compacta that are particularly susceptible (Gutekunst et al., 2002; Rubinsztein, 2003). Interneurons are generally unaffected (Gutekunst et al., 2002; Rubinsztein, 2003). The selective degradation of these neurons early in HD supports the predominance of chorea over other motor dysfunctions, as the indirect pathway of the basal ganglia-thalamocortical circuit is compromised (Paulsen et al., 2005). Thus, the termination of motor movements is affected (Paulsen et al., 2005). In the most advanced stages of the disease, the majority of projections to the striatum will have been lost, and the population of aspiny neurons will have been depleted (Reiner et al., 1988).

While no other brain structure is affected to the same degree as the striatum in HD pathology, certain other brain structures are impacted in grades 3 and 4. These include: the

substantia nigra; cortical layers III, V and VI; the globus pallidus; the centromedial-parafascicular complex of the thalamus; the subthalamic nucleus; the CA1 region of the hippocampus; the angular gyrus in the parietal lobe; the lateral tuberal nuclei of the hypothalamus; white matter; and Purkinje cells of the cerebellum (Heinsen et al., 1999; Jeste et al., 1984; Kassubek et al., 2004; Kremer, 1992; Kremer et al., 1990; Kremer et al., 1991; Macdonald and Halliday, 2002; Macdonald et al., 1997; Politis et al., 2008; Spargo et al., 1993; Vonsattel and DiFiglia, 1998).

Neuropathological findings also indicate that neuronal dysfunction exists before the onset of neurodegeneration, which clarifies why the early symptoms of HD are present before any detection of neuronal cell loss or atrophy (Gomez-Tortosa et al., 2001; Mizuno et al., 2000; Myers et al., 1991). Cytoskeletal integrity, axonal transport, and synaptic function are altered in asymptomatic individuals carrying expanded *HTT* alleles, as well as in patients in the early stages of the disease. Evidence of the implication of these processes is demonstrated through the reduced levels of complexin 2 concentrations and the decreased staining of neurofilaments, tubulin, nerve fibers and MAP2 in cortical neurons (Di Maio et al., 1993; Modregger et al., 2002). One of the hallmarks of HD is the presence of nuclear and cytoplasmic inclusions containing expanded polyglutamine huntingtin proteins which appear before symptom onset (Davies et al., 1997). While the occurrence of these inclusions invariably denotes HD pathology, it does not necessarily indicate cellular dysfunction and has even been shown to improve cell survival (Arrasate et al., 2004).

1.4.3 Molecular genetics

The search for the genetic mutation responsible for HD began in the early 1980's in remote fishing villages around Venezuela's Lake Maracaibo. The world's largest HD family live in this community, and the analysis of blood samples collected from them permitted a US-Venezuelan collaborative research team to localise the HD mutation to the short arm of chromosome 4 (Gusella et al., 1983). Ten years later, the Huntington Disease Collaborative Research Group found that the Interesting Transcript 15 (*IT15)* gene was linked to HD, and that the causative mutation was due to a polyglutamine-encoding CAG repeat tract expansion in exon 1 at 4p16.3 (The Huntington's Disease Collaborative Research Group, 1993). Following the discovery of *IT15*'s role in HD, it was renamed huntingtin (*HTT*).

HTT is a large gene composed of 67 exons, and found in both vertebrates and invertebrates (Baxendale et al., 1995; Gissi et al., 2006; Margolis and Ross, 2001; The Huntington's Disease Collaborative Research Group, 1993). After comparing exon 1 of *HTT* in HD patient populations with controls, it was found that while CAG repeat tracts are normally present in *HTT*, expansions of 35 or more CAG repeats are often causative of the disease (Margolis and Ross, 2001; Ranen et al., 1995; Rubinsztein et al., 1996). Penetrance is dependent on repeat length, as expansions of 40 CAG repeats or greater have been shown to have complete penetrance by the age of 65 years old (Langbehn et al., 2004). Repeat tract lengths of 35 to 40, on the other hand, have an incomplete penetrance (Langbehn et al., 2004). Most cases of adult-onset HD express alleles with 40 to 50 CAG repeats, whereas the presence of 60 or more corresponds to juvenile HD (Fahn, 2005; Rubinsztein, 2002). In rare cases of homozygosity, patients will have a similar age of onset as a heterozygote with the same repeat length, but may

experience an enhanced rate of disease progression (Squitieri et al., 2003; Wexler et al., 1987). As a whole, increases in CAG repeat tract length correlate to earlier ages of onset; however, 40% of the influence is attributed to genetic modifiers and environment (Chattopadhyay et al., 2005; Djousse et al., 2004; Rosenblatt et al., 2001; Wexler et al., 2004).

CAG repeat tract expansions greater than 28 repeats are unstable during replication. They may lengthen (73% of the time) or contract (23% of the time) as they are passed from parent to child, and also from one generation of cells to another within the same individual (Chattopadhyay et al., 2005; Djousse et al., 2004; Gonitel et al., 2008; MacDonald et al., 1999). Somatic instability of CAG repeat tracts has been identified in the striatum, and the resulting mosaicism may help explain the susceptibility of this brain region to neurodegeneration (Kennedy and Shelbourne, 2000). In gametogenesis, this repeat instability is higher in spermatogenesis than it is in oogenesis, and the generation of large expansions during replication occurs almost exclusively in males causing anticipation in successive generations of paternally inherited HD (Andrew et al., 1993; Duyao et al., 1993; Fahn, 2005; Harper, 1996; Kremer et al., 1995; Margolis and Ross, 2001; Ranen et al., 1995; Trottier et al., 1994). Thus, it is unsurprising that juvenile HD patients were shown to typically inherit the disease from an affected father, and that they had long CAG repeat tract expansions (Duyao et al., 1993; Fahn, 2005; Harper, 1996; Riley and Lang, 1991). Likewise, patients with no prior family history tend to inherit from an unaffected father with an *HTT* allele containing a CAG tract of 28 to 35 repeats that underwent an expansion to become a disease allele (Harper, 2002).

1.4.4 Huntingtin

The product of *HTT*, huntingtin, is a 348 kDa soluble protein (Cattaneo et al., 2005; De Souza and Leavitt, 2014). Normal huntingtin is largely found in the cytoplasm; however, it has also been traced to the nucleus (Kegel et al., 2002). Determining the protein's function has been difficult as huntingtin bears no homology with other proteins (De Souza and Leavitt, 2014). The protein is highly conserved in vertebrates, and orthologues of huntingtin have been found in many species, including *Drosophila* and *Danio rerio* (Jones, 2002). Huntingtin is ubiquitously expressed in all human and mammalian cells, with the highest concentrations found in the brain and testes. Within the brain, *HTT* mRNA is predominantly expressed in neurons (DiFiglia et al., 1995). An alternate *HTT* mRNA species is also produced through the differential polyadenylation of *HTT* mRNA, and while the functional distinction is not yet clear it has been shown that the larger transcript is primarily expressed in the brain, whereas the shorter transcript is expressed in a broad range of tissue types (Lin et al., 1993). The regulation of huntingtin expression patterns is partially attributed to the cell survival regulator, transcription factor p53, implying that huntingtin may have a role in this process (Feng et al., 2006).

In addition to the CAG repeat tract, several other motifs have been identified in huntingtin. The protein contains 37 consensus motifs called HEAT for their presence in huntingtin, elongation factor 3, protein phosphatase 2A and the rapamycin 1 target, TOR1. Each HEAT repeat is approximately 50 amino acids in length and contains two anti-parallel α-helices resulting in a hairpin configuration (Andrade and Bork, 1995). The presence of these repeat domains is thought to be important for protein-protein interactions (Takano and Gusella, 2002). A short repeat of proline amino acids, called the polyproline stretch, is located upstream of the

HEAT repeats and is thought to be implicated in the folding of huntingtin and the maintenance of its soluble state (Steffan et al., 2004). In addition to the polygutamine tract at the N-terminal, an amphipathic α-helical membrane-binding domain is present in the first 17 amino acids of huntingtin permitting its association with the plasma membrane, endosomal/autophagic vesicles, mitochondria, the ER and the Golgi apparatus (Kegel et al., 2005; Rockabrand et al., 2007). Furthermore, a functionally active NES and NLS are present in the C-terminal, suggesting that the protein may be involved with trafficking molecules from the nucleus (Xia et al., 2003).

Scientists have long struggled to discover the structure of huntingtin, as thus far all crystallography and mass spectrometry studies have been hindered due to the large size of the protein. This is an important step in not only providing functional information on the protein, but is also necessary for the development of effective therapeutics. In fact, researchers are at the point of desperation that there is a current collaboration with the Center for the Advancement of Science in Space to see if they are able to finally elucidate its structure through the use of the zero gravity environment of the International Space Station (NASA, 2014).

1.4.4.1 Posttranslational modification of huntingtin

Huntingtin undergoes many kinds of posttranslational modifications, and the investigation into the impact of these changes has given many insights into the function of the protein. The huntingtin-interacting protein 14 (HIP14) is responsible for palmitoylating the cysteine 214 residue of huntingtin (Huang et al., 2004). As with many other proteins, the palmitoylation of huntingtin permits an interaction with vesicles. Furthermore, it has been

shown that huntingtin is required for vesicle trafficking and fusion with the plasma membrane (Brandstaetter et al., 2014). In addition to palmitoylation, huntingtin is also phosphorylated at serines 421 and 434. The phosphorylation of these residues impacts the cleavage, function and cellular localisation of the protein, and seem to predominantly confer cell protection (Aiken et al., 2009; Schilling et al., 2006; Thompson et al., 2009; Wang et al., 2010; Warby et al., 2009). Acetylation of lysine 444 targets huntingtin for autophagy (Jeong et al., 2009). The N-terminal lysines (6, 9, and 15), may be ubiquitinated or sumoylated (Kalchman et al., 1996; Steffan et al., 2004). Ubiquitination targets huntingtin for degradation through the UPP. In contrast, sumoylation will not only prevent the ubiquitin-mediated degradation of huntingtin, but also stabilises it, increases its capacity to repress transcription, and decreases its ability to aggregate (Steffan et al., 2004). Finally, huntingtin is also subject to proteolytic cleavage. Various caspases, calpains and an aspartic protease recognise an assortment of cleavage consensus sites and are responsible for producing fragments of huntingtin that vary in length, cellular localisation, and function, both in normal and expanded full-length huntingtin proteins (Goldberg et al., 1996; Wellington et al., 1998). The various huntingtin fragments may even have specific functions, as brain region-specific cleavage has been reported (Mende-Mueller et al., 2001).

1.4.5 Normal cellular and physiological role of huntingtin

Huntingtin has been studied extensively for many years now, yet the normal functions of this protein are still poorly understood. The main factors contributing to the slow progress are the large size of the protein, the ubiquitous expression pattern, and the identification of over 200 protein partners (Borrell-Pages et al., 2006; Harjes and Wanker, 2003; Kaltenbach et al.,

2007; Li and Li, 2004). Together, this provides huntingtin with seemingly limitless possibilities for function.

1.4.5.1 Involvement in embryonic development

Huntingtin was identified as an important protein for embryonic development through the use of a *HTT* knockout mouse. This mouse proved to be lethal before embryonic day 8.5, which precedes the stages of gastrulation and neurulation, indicating that huntingtin has an important role outside of the nervous system (Duyao et al., 1995; Nasir et al., 1995; Zeitlin et al., 1995). The cause of this lethality has been pinned to increased apoptotic activity in the ectoderm soon after gastrulation is initiated due to defective tissue organisation (Leavitt et al., 2001; Van Raamsdonk et al., 2005).

Subsequent stages of development are also dependant on huntingtin. The creation of a mouse line in which its expression is reduced by 50% permitted the observation of dysregulated developmental stages, and overcame the embryonic lethality caused by the complete knockout of *HTT* (White et al., 1997). Analysis of these mouse pups showed widespread CNS malformation: misshapen fore and midbrain; displaced ventricles; ectopic masses in the subventricular zone and ventricles; as well as structural irregularities in midline structures such as the thalamus and striatum (White et al., 1997). These findings demonstrate the importance of huntingtin for the normal development of brain structure.

The use of a chimeric mouse in which embryonic stem cells that were null for *Hdh*, the mouse orthologue of *HTT,* were injected into a normal blastocyst identified a role for huntingtin

in region-specific brain maturation (Reiner et al., 2001). In particular, few neurons and glia derived from the *Hdh (-/-)* donor cells were found in the striatum, basal ganglia, cerebral cortex, thalamus, or the Purkinje cell layer of the cerebellum (Reiner et al., 2001). Furthermore, the absence of neurons in these areas was due to a deficit in neuronal maturation (Reiner et al., 2003). Taken together, it has been well established that huntingtin has a crucial role in several stages of embryonic development, and is of particular importance in brain maturation.

1.4.5.2 Involvement in cellular survival

Several reports have evidenced the role for huntingtin in cellular survival. *In vitro*, overexpression protected against lethal stresses, including the mitochondrial toxin 3-nitropropionic acid, and serum deprivation (Rigamonti et al., 2000). It was subsequently demonstrated that the neuroprotection conferred by huntingtin was through its ability to block the cleavage of procaspase-9 into the active apoptotic effector, caspase-9 (Rigamonti et al., 2001). Moreover, huntingtin binds and sequesters HIP1, a proapoptotic protein involved in the recruitment and activation of caspase-8, indicating that normal huntingtin has a range of anti-apoptotic properties (Gervais et al., 2002; Hackam et al., 2000; Kalchman et al., 1997).

In addition to its ability to disrupt apoptotic processes, huntingtin has been further implicated in cell survival through its impact on brain derived neurotrophic factor (BDNF). This interaction is of particular interest to HD pathology as BDNF is critical for the survival of striatal neurons and for corticostriatal synapse activity (Zuccato and Cattaneo, 2007). Although BDNF is necessary for striatal cells, it is not produced in the striatum, and instead these cells are dependent on the delivery of this molecule from the cerebral cortex *via* corticostriatal afferents

(Altar et al., 1997; Baquet et al., 2004; Fusco et al., 1999). Overexpression of huntingtin both *in vitro* and *in vivo* results in the increase of BDNF expression (Zuccato et al., 2001). Interestingly, BDNF application alone is able to significantly rescue the abnormal development caused by the knockdown of huntingtin expression in *Danio rerio* (Diekmann et al., 2009). The influence of huntingtin on BDNF was discovered through its sequestration of RE1-silencing transcription factor (REST). This transcription factor typically binds to a response element in the BDNF promoter responsible for generating the BDNF species that is transported to the striatum, thereby silencing its transcription (Zuccato et al., 2010). Its sequestration by huntingtin thus allows for the unhindered transcription and subsequent translation of BDNF, promoting striatal neuron survival.

1.4.5.3 Involvement in axonal and vesicle transport

Huntingtin has an inherent capacity to associate with many cell structures due to its motifs and posttranslational modifications. Thus, it is not surprising that it has been shown to be involved with axonal and vesicle transport. Its role in this process was first proposed due to its association with the plasma membrane and clathrin-coated vesicles (Velier et al., 1998). This study also made mention of interactions with HIP1, which binds to the actin cytoskeleton and the dynactin-binding protein huntingtin-associated protein 1 (HAP1). The interaction with these protein partners suggested that huntingtin could possibly be serving as an intermediate between vesicles and the cytoskeleton-binding protein complexes during vesicle transport. In addition to trafficking vesicles, huntingtin has also been implicated in the fast axonal transport of mitochondria (Trushina et al., 2004).

A link between huntingtin's involvement with vesicle transport and HD was made when full-length normal huntingtin was shown to stimulate BDNF vesicle transport in cultured neurons (Gauthier et al., 2004). This was mediated through huntingtin's interaction with the p150 subunit of dynactin *via* HAP1. In addition, the phosphorylation of serine 421 in huntingtin acts as a molecular switch for the directionality of BDNF vesicle transport (Colin et al., 2008). Phosphorylation of the residue recruits the molecular motor kinesin-1 and facilitates anterograde transport, while dephosphorylation leads to the detachment of kinesin-1 and causes BDNF to preferentially undergo retrograde transport (Colin et al., 2008). The role of huntingtin in regulating the transport of BDNF was further established when it was found to also modulate the transport of its receptor, TrkB in striatal neurons (Liot et al., 2013). As both the substrate and receptor involved in striatal neuron survival are regulated by huntingtin, it is clear why this brain region is particularly compromised in HD pathology.

1.4.5.4 Involvement in synaptic activity

In addition to its role in trafficking synaptic vesicles, huntingtin seems to also be involved in their transmission. Several proteins involved in synaptic endo- and exocytosis, such as syntaxin, HIP1, clathrin, HAP1, dynamin, and protein kinase C and casein kinase substrate in neurons 1 (PACSIN1), directly interact with huntingtin (Smith et al., 2005). Of particular interest is the interaction of huntingtin with the essential postsynaptic density scaffolding protein, PSD95 (Sun et al., 2001). Huntingtin may also associate with the presynaptic terminal through its protein interaction with HIP1 (Parker et al., 2007). Finally, huntingtin has also been shown to regulate the expression of certain synaptic proteins such as rabphilin 3A and complexin II (Morton and Edwardson, 2001; Smith et al., 2005).

1.4.6 Expanded huntingtin and disease pathogenesis

In HD, the conformational change caused by the expansion of the polyglutamine tract of huntingtin has variable impact on the several different roles of the normal protein. In many instances, the expanded huntingtin protein inherits a novel gain-of-function, rather than a loss-of-function (De Souza and Leavitt, 2014). The mutant protein also seems to retain much of the essential function of the normal huntingtin protein in many of the cellular mechanisms (Leavitt et al., 2001). This is especially true in early development.

1.4.6.1 Protein aggregation and intranuclear inclusions

The formation of inclusions is a pathological feature in HD, like in all other polyglutamine diseases (Imarisio et al., 2008). In HD, these insoluble inclusions are both nuclear and cytoplasmic and are characterised by the presence of a self-aggregating expanded polyglutamine huntingtin protein (Davies et al., 1997). The basis of aggregate formation involves the production of fibrils from oligomeric precursors of expanded huntingtin protein (Poirier et al., 2002). Isolated aggregates are β-sheet-enriched, but the exact molecular organisation is not fully understood (Rothlein et al., 2014). While the presence of these aggregates is clearly a signature of HD pathogenesis, considerable debate has ensued over whether inclusions are protective, neutral, or toxic to the cell.

In cell culture models of the disease, there is a strong association between the formation of aggregates and cell death (Hackam et al., 1998; Wyttenbach et al., 2000). In addition, the lengthening of the polyglutamine tract was shown to increase the kinetics of polyglutamine self-aggregation *in vitro* (Scherzinger et al., 1997). While this parallels the observation that HD

patients with longer polyglutamine expansions have a higher frequency of neuronal inclusions (Becher et al., 1998), no clear association has been found between the density of inclusions and the degree to which a brain area is affected (Kuemmerle et al., 1999). Furthermore, a transgenic mouse model of HD failed to form aggregates, despite obvious neurodegeneration and motor deficits (Hodgson et al., 1999).

The presence of various proteins important for normal cellular function have also been identified within expanded huntingtin aggregates, indicating in this case that the aggregates may have a negative role through protein sequestration (Soto, 2003). Members of the UPP and several molecular chaperones have been found in particular abundance within expanded huntingtin aggregates, suggesting that the degradation of misfolded proteins and protein quality control may be compromised in HD (Sherman and Goldberg, 2001). In this regard, the aggregates would contribute to a loss-of-function.

More recently, two lines of evidence have pointed toward the formation of expanded huntingtin inclusions as being protective to cells. In one report, it was found that cells were more susceptible to neurotoxicity when they did not contain inclusions (Gauthier et al., 2004). In the other, it was shown that diffuse intracellular huntingtin was a better indicator for vulnerability to cell death than the presence of inclusions (Arrasate et al., 2004). Furthermore, the presence of inclusions leads to a reduction of the diffuse expanded huntingtin, improving cell survival (Arrasate et al., 2004). Together, these findings suggest that inclusions in HD may be nothing more than a natural coping mechanism for toxic intracellular protein species, and thus protective.

1.4.6.2 Cleavage of expanded huntingtin

HD pathogenesis has also been suggested to involve the cleavage of expanded huntingtin. As seen with normal huntingtin, expanded huntingtin is cleaved by proteases into protein fragments of various lengths. However, expanded huntingtin has a greater number of cleavage sites than normal huntingtin, and the generation of N-terminal fragments containing the polyglutamine stretch seems to be toxic. Alone, these N-terminal fragments are capable of producing HD-like disorders in nonhuman primates and mice (Davies et al., 1997; Palfi et al., 2007; Schilling et al., 1999).

The generation of these toxic fragments is mediated through caspase-2, -3, and -6 (Gafni et al., 2004; Wellington et al., 1998). The phosphorylation of expanded huntingtin has been shown to modulate the proteolysis of the protein into toxic fragments (Humbert et al., 2002; Luo et al., 2005; Schilling et al., 2006). Interestingly, the inhibition of calpain and caspase activity *in vitro* was able to reduce the toxicity of expanded huntingtin in a cell culture model of HD (Gafni et al., 2004). More importantly, the pathological and behavioural HD phenotypes of mice expressing full-length expanded huntingtin proteins were rescued through the inhibition of caspase-6 cleavage (Graham et al., 2006). Therefore, the toxic fragment hypothesis is not only a plausible pathogenic mechanism for HD, but also a promising pathway for therapeutic intervention.

1.4.6.3 Expanded huntingtin and BDNF

The polyglutamine expansion in huntingtin causes the protein to lose its ability to promote the transcription of BDNF in cortical neurons. This is specifically due to the expanded

protein's inability to sequester REST in the cytoplasm, thus permitting nuclear translocation and its binding to the response element on the BDNF gene (Zuccato et al., 2001; Zuccato et al., 2003). Expanded huntingtin also impairs the trafficking of BDNF and its receptor, TrkB, resulting in a reduction in the amount of BDNF transported to the striatum from the cortex, as well as the decreased quantity of TrkB receptors at postsynaptic densities (Gauthier et al., 2004; Liot et al., 2013). As a consequence, the survival signalling necessary for striatal neurons is compromised, and the striatum is vulnerable to neurodegeneration (Liot et al., 2013).

1.4.6.4 Transcription dysregulation in HD

The altered expression of genes in HD, as well as the interaction of several transcription factors with the expanded huntingtin protein, suggests that transcriptional dysregulation is involved in HD pathogenesis. In addition to the effect on BDNF transcription, the inability of expanded huntingtin to bind REST/NRSF influences a number of other genes. There are over 1300 copies of the RE1/NRSE site that is recognised by REST/NRSF in the human genome, and most are located on genes important for neuronal differentiation and development (Bruce et al., 2004; Johnson et al., 2006). The increased binding of REST/NRSF to RE1/NRSE response elements results in the downregulation of 958 genes in the motor cortex (Zuccato and Cattaneo, 2007). Genes of interest to HD include *OAT*, *OSBP2*, and *B3GAT1*. The ornithine aminotransferase protein (OAT) is involved in the synthesis of glutamate and is found to have reduced activity in HD patient brains, which is in accordance with the evidence of an impaired corticostriatal glutamatergic pathway in HD (Wong et al., 1982; Zeron et al., 2004; Zeron et al., 2002). Oxysterol-binding protein 2 (OSBP2) is important for signal vesicle transport, among

other processes (Wang et al., 2005), while β-1,3 glucuronyltransferase-1 (B3GAT1) is associated with schizophrenia-like psychosis (Jeffries et al., 2003).

The conformational change of the huntingtin protein due to the expansion of the polyglutamine stretch causes the expanded protein to interact with additional transcription factors. These include: specificity protein 1 (SP1); p53; CBP; mSin3A, nuclear receptor corepressor (NCoR); TBP; and TBP-associated factor 130 kDa (TAFII130) (Boutell et al., 1999; Dunah et al., 2002; Shimohata et al., 2000; Steffan et al., 2000). The downstream consequences of expanded huntingtin associating with these transcription factors is extremely variable. SP1 has been shown to have an increased interaction with *DRD-2* (encodes the dopamine receptor D2), *PPE*, and *REST/NRSF*, while its interaction with *NR1* [encodes the *N*-methyl-*D*-aspartate (NMDA) receptor subunit 1] is unchanged (Chen-Plotkin et al., 2006; Ravache et al., 2010). Increased SP1 activity was also found in a transgenic HD mouse model, and HD pathology was shown to improve with SP1 suppression (Qiu et al., 2006). Another report indicated that the binding of SP1 to the promoter of *NGFR* (encodes the nerve growth factor receptor) was inhibited by expanded huntingtin (Li et al., 2002b). Similar to SP1, the potential for transcription dysregulation of p53's downstream targets is vast. Genes involved in transcription, cell signalling, vesicle trafficking, and lipid metabolism were shown to be affected through microarray analyses (Sipione et al., 2002). The likelihood of p53 transcription abnormalities is further increased due to the fact that expanded huntingtin has been shown to interact not only with p53, but also with the p53 coactivator, CBP, and the p53 corepressor, mSin3A (Steffan et al., 2000). Expanded huntingtin has also been shown to structurally destabilise TBP, and inhibit its deactivation through aberrant interactions with HSPs (Schaffar et al., 2004). Finally, the

binding of TAFII130 to polyglutamine stretches was found to decrease CREB-dependant transcriptional activation (Shimohata et al., 2000).

Polyglutamine aggregates may also cause transcriptional dysregulation through the sequestration of transcription factors. The transcription factors CBP, TBP, SP1, and TAFII130 all contain polyglutamine or glutamine-rich sequences which are sufficient to permit interaction with huntingtin (Escher et al., 2000; Kazantsev et al., 1999). In addition, TBP and SP1 have been shown to contain C-terminal domains, which allow for a stronger interaction with huntingtin (Dunah et al., 2002). While CBP and TBP have been shown to be incorporated into expanded huntingtin aggregates (Matsumoto et al., 2006; Steffan et al., 2000; Suhr et al., 2001; van Roon-Mom et al., 2002), SP1 and TAFII130 bind to a soluble form of the expanded protein (Dunah et al., 2002; Li et al., 2002b). In all four cases, the transcriptional activity of these proteins was shown to be suppressed. The expression of soluble CBP in HD patient brain samples, as well as its nuclear availability in a neuronal cell model of HD, was found to be greatly reduced as a consequence of CBP sequestration (Nucifora et al., 2001). This reduction has been found to impact CBP-associated histone acetyltransferase activity, as well as the expression of encephalin and Jun (Dunah et al., 2002; Luthi-Carter et al., 2000; Nucifora et al., 2001; Richfield et al., 1995). Interestingly, the depletion of CBP in an HD mouse model was found to have no effect on striatal degeneration, inclusion formation, the severity of motor deficits, or the global levels of histone acetylation (Klevytska et al., 2010). In contrast, the overexpression of CBP, or the co-overexpression of SP1 and TAFII130, has been shown to be sufficient in the prevention of neuronal cell toxicity in *in vitro* models of HD (Dunah et al., 2002; Nucifora et al., 2001). The sequestration of SP1 was shown to lead to the downstream

downregulation of NGFR and/or DRD-2 (Li et al., 2002b). In addition, p53, CBP, and mSin3A have also been found in huntingtin aggregates (Boutell et al., 1999). While the impact of sequestration on the activities of these transcription factors is clear, and several target genes have been identified as dysregulated, the pathological consequence of these events has yet to be clarified. This is likely due to the vast number of impacted genes and cellular pathways.

1.4.6.5 Striatal excitotoxicity in HD

One possible explanation for the restricted neurodegeneration of the striatal medium spiny neurons in HD is the occurrence of excitotoxicity. Excitotoxicity is a process in which neuronal cell death is caused by the overstimulation of neurons by excitatory neurotransmitters (Lipton, 2008). The cortical projections to the medium spiny neurons are primarily glutamatergic and thus, excitatory. The release of glutamate onto the dendrites of medium spiny neurons activates glutamate receptors, (e.g. NMDA receptors), which enables the influx of calcium into the neuron (Raymond et al., 2011). The link between HD and excitotoxicity comes from the compromised calcium homeostasis that is present in neural cells due to the expression of expanded huntingtin.

Inositol (1,4,5)-trisphosphate (IP_3)-linked agonists, and components of the phosphatidylinositol cycle, are subject to transcriptional changes in striatal cells expressing expanded huntingtin (Lim et al., 2008). Consequently, the basal level of calcium is reduced, IP_3 production is hindered, and an increased sensitivity of the mitochondrial permeability transition pore is incurred. This renders the mitochondria of these striatal cells incapable of handling large

quantities of calcium, and, therefore, particularly vulnerable to calcium influx from NMDA receptor activation (Lim et al., 2008).

Additionally, expanded huntingtin has been shown to modulate the localisation and activity of NMDA receptors. The stimulation of NMDA receptor subunit NR2, subtype B will cause a greater influx of calcium when expanded huntingtin is present (Li et al., 2003; Li et al., 2004). Furthermore, the enzymes responsible for the posttranslational modifications of NMDA that control its presence at the synapse, striatal-enriched protein tyrosine phosphatase (STEP) and calpain, are upregulated by expanded huntingtin (Cowan et al., 2008; Graham et al., 2009). STEP dephosphorylation of NMDA receptor subunits, as well as the C-terminal cleavage of the receptors by calpain, result in a reduction of NMDA receptors at the synapse (Gladding et al., 2012).

1.4.6.6 Mitochondrial dysfunction in HD

In addition to the above-mentioned damaging consequences to mitochondrial calcium capacity and sensitivity, expanded huntingtin has been shown to provoke other forms of mitochondrial dysfunction. Interference in the production and trafficking of mitochondria, increases in mitochondrial fragmentation, and reduced membrane potential have been associated with the presence of expanded huntingtin (Chang et al., 2006; Milakovic et al., 2006; Panov et al., 2003; Wang et al., 2009). Inefficient mitochondrial respiration is also caused through the decreased expression of oxidative phosphorylation enzymes and the resultant aberrant production of adenosine triphosphate (ATP) (Benchoua et al., 2006; Gu et al., 1996). The expanded protein also reduces the enzymatic function of mitochondrial phosphorylation

pathway complexes II, III and IV in the striatum of HD patients (Browne et al., 1997; Gu et al., 1996). Various studies involving the imaging of patient brains have also corroborated the theory that energy metabolism is deficient in this disease. Positron emission tomography scans of HD patients have revealed decreased cerebral glucose metabolism (Stoessl et al., 1986), and striatal lactate levels were shown to be elevated with magnetic resonance spectroscopy (Harms et al., 1997).

1.5 Programmed ribosomal frameshifting – from virus to mammals

Programmed ribosomal frameshifting (PRF) was first described in 1979 in the RNA of certain viruses (Atkins et al., 1979). This mechanism allows for the translation of two new proteins from the same RNA molecule by making use of alternative reading frames, and thus allowing a more efficient use of the size-limited viral genome (Farabaugh, 1996). One of the particular ways through which this is achieved is *via* ribosomal frameshifting events that bring the reading frame one base upstream (i.e. in the 5'-direction) from the primary reading frame to a -1 frame (-1 PRF); this mechanism was first noticed to be used by viruses to bypass a termination codon and translate the RNA into a longer protein (Dinman, 2006). It is noteworthy that in viruses the main frame and frameshifted proteins have different functions, thus implying that PRF also acts as a regulator of stoichiometric ratios between structural and enzymatic proteins (Brierley, 1995; Farabaugh, 1996). For these reasons, PRF has been regarded as a target of choice for the design of antiviral drugs (Dinman et al., 1998). Over the years, a vast amount of information has emerged to explain PRF in detail, strongly suggesting that it is more frequent in, but not exclusive to, viral molecules (Atkins et al., 1990; Farabaugh, 1996; Gesteland and Atkins, 1996), as PRF has now been identified in several prokaryotic and

eukaryotic chromosomally encoded genes (Advani et al., 2013; Belcourt and Farabaugh, 1990; Blinkowa and Walker, 1990; Clark et al., 2007; Craigen et al., 1985; Ivanov et al., 1998; Lux et al., 2005; Manktelow et al., 2005; Namy et al., 2004; Shigemoto et al., 2001; Sulima et al., 2014). Furthermore, -1 PRF events were demonstrated to have an impact on mRNA half-life, indicating that this mechanism may act to posttranscriptionally regulate gene expression *via* a nonsense-mediated decay pathway (Plant et al., 2004).

Three major factors seem to affect -1 PRF: a heptameric nucleotide sequence known as the slippery site (N_NNW_WWH) (Jacks et al., 1988; Plant and Dinman, 2006), a downstream stimulatory mRNA secondary structure (often a pseudoknot) (Endoh et al., 2013; Giedroc et al., 2000; Yu et al., 2011), and a spacer sequence between the slippery site and the stimulatory structure (Bekaert et al., 2003; Napthine et al., 1999). The slippery site facilitates the slippage of transfer RNAs (tRNAs) within two ribosomal sites (P and A) on the mRNA, while the stimulatory structure provides an energetic barrier that causes an elongating ribosome to pause on the slippery sequence. In addition, the specific tRNAs present at the slippery site and the type of organism may also impact PRF type and frequency (Napthine et al., 2003; Sung and Kang, 2003). Currently, several groups are engaged in the development of predictive software to identify and characterise chromosomally encoded PRF signals in genomes from all three kingdoms (Belew et al., 2008; Hammell et al., 1999; Jacobs et al., 2007; Theis et al., 2008). At present, it remains to be determined how extensive a role the -1 PRF mechanism plays in human regulation of gene expression, and whether these frameshifting modulating factors are functionally present in the translation events of human genes. In support of future work to be done in humans, a recent study reported a -1 PRF signal in the human mRNA encoding CCR5,

the HIV-1 co-receptor (Belew et al., 2014). Specifically, the -1 PRF event on the *CCR5* mRNA directs translating ribosomes to a premature termination codon, destabilising it through the nonsense-mediated mRNA decay pathway.

1.6 Hypothesis and objectives

Based on our initial work on -1 frameshifting in SCA3 (Gaspar et al., 2000; Toulouse et al., 2005), Wills and Atkins proposed the existence of potential slippery sequences in the *ATXN3* (A_AAA or A_AAG) and *HTT* (A_AAG or G_AAG) transcripts (Wills and Atkins, 2006). Furthermore, the group led by W.J. Kryzosiak published a series of reports describing the formation of stable CAG repeat hairpins within the context of mRNAs associated with neurodegenerative disorders, where they show that hairpin architecture and stability depend on the nature of flanking sequences and repeat length (Busan and Weeks, 2013; de Mezer et al., 2011; Michlewski and Krzyzosiak, 2004; Sobczak et al., 2003; Wills and Atkins, 2006). Taking all these factors into consideration, we could speculate that the required conditions are in place for a -1 translational frameshifting event to occur in expanded CAG repeat tracts in *ATXN3*, even with the relevance of the putative slippery sequence in *ATXN3* yet to be experimentally determined. Hypothetically, the ribosome would encounter the *ATXN3* slippery site and the expanded CAG mRNA hairpin and pause, leading to the -1 translational frameshifting event. This model proposes that frameshifting in the *ATXN3* gene would occur near the beginning of

the CAG repeat tract, resulting in the decoding of GCA repeats in the -1 frame, and the production of a hybrid ataxin-3 protein containing both glutamine and alanine residues.

The purpose of the following experimental undertakings was to gain further insight into the potential role of -1 ribosomal frameshifting as a mechanism for pathogenesis in polyglutamine repeat expansion diseases *via* the production of polyalanine-containing peptides. Our first objective was to supplement our previous *in vitro* findings that -1 frameshifting events occur in the *ATXN3* transcript, and that these events lead to increased toxicity (Chapter 2). We proposed to achieve this with the generation of a transgenic SCA3 animal model and organotypic nervous tissue cultures. Our second objective was to develop a screening tool that would allow for the selective detection of polyalanine-containing peptides in disease, in the form of a polyalanine antibody (Chapter 3).

Chapter 2 : Expanded *ATXN3* frameshifting events are toxic in *Drosophila* and mammalian neuron models

Reference:

S.J. Stochmanski, M. Therrien, J. Laganiere, D. Rochefort, S. Laurent, L. Karemera, R. Gaudet, K. Vyboh, D.J. Van Meyel, G. Di Cristo, P.A. Dion, C. Gaspar and G.A. Rouleau. Expanded *ATXN3* frameshifting events are toxic in *Drosophila* and mammalian neuron models. *Human Molecular Genetics*. 2012;21:2211-2218.

2.1 Rationale

Coding CAG trinucleotide repeat expansions cause at least nine neurodegenerative disorders (see Table 1.1). The presence of INIs containing expanded protein in the majority appears to be the unifying link between these traits. Although it remains unclear how these aggregates affect disease progression (to the extent that it is not certain whether they are protective or harmful), several pathological mechanisms spanning a variety of cellular functions have emerged over the past 20 years to explain these conditions. A common mechanism is therefore likely to exist that explains the features shared by these disorders, whereas cell-specific factors/pathways may explain the phenotypic characteristics that render each disease a separate clinical entity. Despite the recent advances in the field, there remains a pressing need to identify new and potent therapeutic targets for polyglutamine repeat expansion diseases, as no treatment for these patients is currently available.

The observation of similar INIs in OPMD and expanded CAG repeat diseases led our group to predict that the mechanisms of toxicity in polyglutamine and polyalanine repeat expansion disorders could be related. This idea brought us to the realisation that a -1 translational frameshift error occurring within an expanded CAG repeat tract would lead to a GCA alanine-encoding frame, resulting in proteins with long stretches of alanine residues - perhaps much larger than the 12 to 17 alanines observed in OPMD. The presence of INIs in both expanded CAG and expanded GCG repeat tract disorders, the relatively short alanine polymers needed for toxicity in OPMD, and the physical properties of these homopolymers led us to propose that the production of expanded polyalanine stretches may contribute to the disease phenotype in both groups of diseases. We therefore hypothesised that (i) translational

frameshifts in large CAG repeat tracts result in a new reading frame with the formation of a hybrid protein containing a mixed polyglutamine/polyalanine tract, (ii) the resultant polyalanine polymers aggregate, and (iii) polyalanine-containing peptides are toxic to cells.

To test these hypotheses, our group performed a series of exploratory experiments using SCA3 as a model. We initially demonstrated the presence of frameshifted ataxin-3 protein species in lymphoblastoid cell lines from SCA3 patients, and in INIs in pontine neurons of SCA3 patient brain autopsy material (Gaspar et al., 2000). The subsequent development of an *in vitro* transfection model using truncated *ATXN3* cDNAs epitope-tagged in each of the three possible reading frames allowed us to demonstrate that (i) the frameshifting events lead to increased toxicity, (ii) the frameshifts seem to happen *via* ribosomal frameshifting in the *ATXN3* transcript (to produce an alanine-containing ataxin-3 protein), and (iii) the frameshifts are repeat-length and -type dependent (Toulouse et al., 2005).

Although versatile, our *in vitro* model was not neurologically representative of the disease. In contrast, transgenic animals and organotypic nervous tissue cultures are more biologically significant models. Thus, to evaluate -1 frameshifting events in an *in vivo* or *ex vivo* context, we proposed to generate a transgenic *Drosophila* model and mouse organotypic cortical and cerebellar culture models of *ATXN3* -1 frameshifting.

2.2 Abstract

Spinocerebellar ataxia type-3 (SCA3) is caused by the expansion of the coding CAG repeat tract in the *ATXN3* gene. Interestingly, a -1 base frameshift occurring within an expanded CAG repeat tract would henceforth lead to translation from a GCA frame, generating polyalanine stretches instead of polyglutamine. Our results show that transgenic expression of expanded CAG *ATXN3* led to -1 frameshifting events which are deleterious in *Drosophila* and mammalian neurons. Conversely, transgenic expression of polyglutamine-encoding expanded CAA *ATXN3* was not toxic. Furthermore, expanded CAG *ATXN3* mRNA does not contribute *per se* to the toxicity observed in our models. Our observations indicate that expanded polyglutamine tracts in *Drosophila* and mouse neurons are insufficient for the development of a phenotype. Hence, we propose that -1 ribosomal frameshifting contributes to the toxicity associated with expanded CAG repeat tracts.

2.3 Introduction

Nine neurodegenerative disorders are caused by expansion of a coding CAG repeat tract, among which is SCA3 (Kawaguchi et al., 1994). Previous investigations established a number of shared clinical, genetic and molecular features among these disorders; the most intriguing being mutant protein aggregation (often as intranuclear inclusions, INIs) which is deemed to be their hallmark trait. Fibrillary INIs are also observed in oculopharyngeal muscular dystrophy (OPMD), caused by the expansion of a short polyalanine repeat in the polyadenylate binding protein nuclear 1 (*PABPN1*) gene (Brais et al., 1998). A -1 base frameshift occurring within an expanded CAG repeat tract would lead to translation from a GCA frame, generating polyalanine

stretches instead of polyglutamine. Using cell culture models of SCA3 or HD, we and others have previously shown that -1 frameshifting occurs *in vitro* (Davies and Rubinsztein, 2006; Gaspar et al., 2000), that frameshifts seem to happen at the ribosomal level (Toulouse et al., 2005), and that they lead to the production and aggregation of proteins containing polyalanine stretches (Gaspar et al., 2000; Toulouse et al., 2005); nonetheless, the biological relevance of this phenomenon remains unclear.

2.4 Results

2.4.1 -1 frameshifting events are deleterious in *Drosophila*

We developed and characterised *Drosophila* transgenic lines expressing *ATXN3* with polyglutamine expansions (transgenes are schematised in Figure 2.lA) to examine frameshifting in the context of a model more complex than cultured cells. Each transgene construct contained full-length *ATXN3* and bore epitope tags in the three reading frames to allow the monitoring of any frameshifting events; several transgenic lines were obtained for each construct (Table 2.S.1). For phenotypic characterisation, flies were examined upon their eclosion and compared to isogenic control fly crosses. Direct visualisation of the external eye (Figure 2.1B, i-iii) revealed that two of the three expCAG$_{92}$ lines obtained had an overt eye phenotype from eclosion, while the third line developed a phenotype five days post-eclosion. This eye phenotype was characterised by visible disruption of both morphology ("rough eye") and pigmentation, and it was progressive as it worsened over time; at 20 days post-eclosion, the pigmentation was completely absent and the morphology severely disrupted. In contrast, none of the *Drosophila* lines expressing expCAA$_{96}$ transgenes presented overt phenotypic anomalies (Figure 2.1B, iii), either at the time of eclosion or later adult life. To determine if the difference

in phenotypic presentation between expCAG$_{92}$ and expCAA$_{96}$ flies could be due to differential expression of the transgenes, we prepared Western blots using lysates from these flies and used antibodies against ataxin-3. These detections revealed comparable levels of ataxin-3 (~72 kDa) in all lines (expCAG$_{92}$ or expCAA$_{96}$) (Figure 2.1C). The comparison of these lines suggested that *in vivo* expression of polyglutamine *per se* was not responsible for the fly eye phenotype we observed; rather it appears that it is the expression of an expanded CAG repeat tract that is toxic.

To examine the cellular alterations leading to the phenotype described above, sections of the various transgenic fly lines were prepared in three different ways. First, epon-embedded three-day-old adult fly heads were prepared and stained using toluidine blue to observe the eye tissue structure underlying the external eye phenotype (Figure 2.1B, iv-vi). Tangential sections showed intact ommatidia with preservation of photoreceptor cells in isogenic control and expCAA$_{96}$ flies (Figure 2.1B, iv and vi), whereas expCAG$_{92}$ flies exhibited a marked degeneration of cells in the retina and severely disrupted morphology (Figure 2.lB, v). Second, cryosections of the same fly lines were immunostained for the ataxin-3 epitope to confirm the adequate and exclusive transgene expression in the eye of every expCAG$_{92}$ and expCAA$_{96}$ fly line (Figure 2.1B, viii and ix). Lastly, transversal sectioning was performed on all expCAG$_{92}$ and expCAA$_{96}$ fly lines, and revealed degeneration of the eye, signified by a thinning of the retina, in only expCAG$_{92}$ flies (shown by the double-ended arrows in Figure 2.1B, x-xii and measured in Figure 2.1D).

To elucidate the mechanisms underlying the phenotypic discrepancies observed between the expCAG$_{92}$ and expCAA$_{96}$ flies, we next monitored the production of main-frame and frameshifted (in both -1 and +1 frames) ataxin-3 proteins in our *ATXN3* fly models. Immunohistochemical detections were made using an antibody against human influenza hemagglutinin (HA), and the exclusive presence of -1 frameshifted proteins in expCAG$_{92}$ flies were revealed to be in a ring-like perinuclear pattern (Figure 2.S.1); whereas the Myc antibody against main-frame ataxin-3 showed that the protein was localised normally to the nucleus of these flies. Visualisation of HA and Myc laser-scanning signals through the whole z-stack confocal revealed that -1 frameshifted protein structures surrounded the entire nucleus (Figure 2.1E, i) and that main-frame-ataxin-3 was intranuclear in all flies tested (expCAG$_{92}$ and expCAA$_{96}$, Figure 2.1E, i and ii). Interestingly, the occurrence of +1 frameshifting was tested using an anti-His antibody, but never detected (data not shown). These observations made using a model organism are altogether in agreement with previous observations from cultured cell model experiments (Toulouse et al., 2005), as they further validate our original hypothesis about -1 frameshifting within expanded CAG repeat tracts.

To confirm that our observation of -1 frameshifted peptides was genuinely due to ribosomal frameshifting and not a transcriptional error that could have generated these, cDNAs were derived from three different expCAG$_{96}$ lines, cloned into a TOPO vector, and sequenced. This generated a total of 70 clones, none of which suggested the presence of -1 frameshifted products could be attributed to an altered reading frame; nonetheless, 19 clones had a 15 to 20 amino acid deletion upstream from the CAG repeat tract that did not alter the reading frame.

2.4.2 RNA does not confer toxicity in *Drosophila*

In lieu of frameshifting, the increased toxicity associated with expCAG$_{92}$ *versus* expCAA$_{96}$ in our flies could also be due to the distinct mRNAs transcribed by the two DNA sequences. RNA-mediated pathogenesis associated with expansion of trinucleotide repeats has been implicated in a number of degenerative diseases (Ranum and Day, 2004), among which myotonic dystrophy (DM1) (Jiang et al., 2004), fragile X-associated tremor ataxia syndrome (FXTAS) (Jin et al., 2003), and SCA3 (Li et al., 2008). To assess the contribution of RNA toxicity to the *Drosophila* phenotype described above, a new set of fly lines for which a STOP codon was introduced just upstream of the repeat (expCAG or expCAA) was created (Table 2.S.1 and Figure 2.2A). As a result, the expanded repeat tract of these transgenes will not be translated, while the entire encoding mRNAs of the transgenes will nonetheless have been transcribed; in the end the only proteins that will come from either of these STOP modified transgenes (expCAG or expCAA) will be identical ataxin-3 truncated protein lacking the polyglutamine stretches. Comparison of these two sets of fly lines will enable us to determine whether the expCAG$_{94}$ is indeed toxic at the RNA level. The comparative analysis of the STOP -CAG$_{94}$ and STOP -CAA$_{94}$ fly lines revealed a complete absence of eye phenotype for either one of the two constructs (Figure 2.2B), despite the observed adequate expression of the two proteins and their messenger RNAs; as verified by Western blotting (Figure 2.2C), RT-PCR (Figure 2.2D), and quantitative real-time PCR using two separate probes (Figure 2.S.2). Finally, retinal thicknesses (shown by the double-ended arrows in Figure 2.2B, xiii-xvi and measured in Figure 2.2E) did not show significant differences among the STOP -CAG$_{94}$, STOP -CAA$_{94}$ fly lines, and the isogenic control lines (+ / *gmr*-GAL4). These results argue against a contribution

of RNA toxicity to the differential phenotypes observed in our expCAG and expCAA fly models (Figure 2.1).

2.4.3 -1 frameshifting events are deleterious in mammalian neurons

Next, we used a biolistic approach to transfect mouse cortical and cerebellar organotypic slice cultures with bicistronic full-length *ATXN3* cDNA containing various sized CAG repeat tracts (DsRed in the main-frame, at the N terminal; EGFP in the -1 frame, at the C terminal; Figure 2.3A). This approach should allow the *ex vivo* evaluation of expanded CAG -1 frameshifting events in a disease relevant mammalian tissue environment. These transgenes were engineered for direct visualisation of main-frame ataxin-3 in red, and frameshifted ataxin-3 in green, without the use of antibodies for their detection. Transfection of postnatal mouse pup (8 to 9 days) cerebellar slices with the wtCAG$_{14}$ construct resulted in expression of ataxin-3 throughout the Purkinje cell layer and the formation of aggregates, mainly in their nucleus (Figure 2.3B, i). A post live-imaging examination performed using an antibody against calbindin further revealed that across all slices cells of the Purkinje layer retained a normal morphology up to 72 hours post transfection. In contrast, expression of expCAG$_{92}$ led to an improper development of the Purkinje cell layer (Figure 2.3B, ii). This phenotype, which was evident as early as 24 hours post transfection, progressed rapidly to severe degeneration and cell death at 72 hours post transfection. Purkinje cells exhibiting expression of frameshifted ataxin-3 (Figure 2.3B, ii) appeared dysmorphic with aberrantly shaped nuclei, severely shortened arborisations, and the presence of aggregates in both their nucleus and dendrites. Interestingly, in these same cerebellar slice cultures, any Purkinje cells expressing only main-frame ataxin-3 and no -1 frameshifted ataxin-3 proteins retained their normal morphology and survived

similarly to those transfected with wtCAG$_{14}$ (Figure 2.3B, iii). By comparison, Purkinje cells from organotypic slices transfected with expCAA$_{96}$ never showed the presence of frameshifted ataxin-3 (Figure 2.3B, iv); despite a high proportion of protein aggregation, which in this case can only be due to polyglutamine and not frameshifted polyalanine, these cells survived over time just like those transfected with wtCAG$_{14}$. Similar results were obtained for the cortical organotypic slice transfection experiments. In the case of expCAG$_{92}$, transfected pyramidal cells expressed -1 frameshifted ataxin-3 protein as early as 24 hours post transfection, and also rapidly progressed to severe degeneration and cell death by 72 hours post transfection (Figure 2.3C, i). The incomplete colocalisation of frameshifted ataxin-3 protein with non-frameshifted (main-frame) protein in the nucleus (Figure 2.3C, ii) suggests the two proteins are perinuclear and nuclear, respectively.

2.5 Discussion

Using a *Drosophila* developing eye transgenic expression model, we tested the impact of full-length *ATXN3* constructs with disease-relevant expanded CAG repeat tracts, and epitope tags in every one of the three possible translation frames to demonstrate the presence of -1 frameshifting exclusively in expCAG$_{92}$ flies. Our results showed that the occurrence of -1 frameshifted ataxin-3 proteins correlated with the development of the eye phenotype of these animals. Indeed, our results indicate that the *in vivo* expression of polyglutamine-containing ataxin-3 alone is not sufficient to cause a degenerative phenotype in the fly, and that -1 frameshifting events and their concomitant production of polyalanine-containing ataxin-3, are key contributing factors for the development of the toxic phenotype observed in this model. Furthermore, biolistic transfection of mouse cerebellar and cortical organotypic cultures

validated these observations in a mammalian neuronal context. Moreover, expression of the expCAG *ATXN3* mRNA *per se* did not produce the phenotype, which differs from results reported earlier by Li and colleagues (Li et al., 2008) who also used an ataxin-3 *Drosophila* model; albeit transgenes used by this group were not designed to observe translational frameshifting events. This discrepancy between phenotypes could simply be due to the fact that truncated *ATXN3* cDNA transgenes rather than full-length were used, as it was previously reported that artificially truncated constructs bearing expanded CAG repeat tracts are in fact associated with increased toxicity of the transgenes (Haacke et al., 2006). Recent evidence led us to consider the possibility that the stretch of polyalanine we observed may not be due to -1 frameshifting, but rather to a hypothesised property of CAG repeat tracts that allows the initiation of translation in the three reading frames (RAN translation) (Zu et al., 2011). Our observations of flies expressing STOP modified transgenes do not support such events as proteins with polyglutamine, polyserine, or polyalanine could not be detected. Hence, we concluded RAN translation events do not occur in *Drosophila*.

Programmed ribosomal frameshifting (PRF) was originally described in viruses (Atkins et al., 1979). It allows the translation of more than one protein from the same RNA molecule through the use of the different possible alternative reading frames; thus yielding a more efficient use of the limited sized viral genome (Farabaugh, 1996). Frameshifting to the -l frame (-1 PRF), in particular, is used in viral mRNAs mainly to bypass the STOP codon to produce a longer frameshifted protein (Dinman, 2006). Following reports which established that main-frame and frameshifted proteins have different functions, one of the known consequences of PRF is now deemed to be the regulation of stoichiometric ratios between structural and

enzymatic proteins (Brierley, 1995; Farabaugh, 1996), so PRF is considered a target of choice for the design of some antiviral drugs (Dinman et al., 1998). Over the years, a vast amount of information has emerged to explain PRF in detail, strongly suggesting that it is more frequent in, but not exclusive to, viral molecules, as PRF has been identified in several prokaryotic and eukaryotic (Plant and Dinman, 2006) chromosomally encoded genes, including mammalian genes (Manktelow et al., 2005). It; however, remains to be determined if -1 PRF plays a major role in human regulation of gene expression. Several groups are engaged in the development of predictive software to identify and characterise chromosomally encoded PRF signals in genomes from all kingdoms (Bekaert et al., 2006; Gao et al., 2003; Gurvich et al., 2003; Hammell et al., 1999; Shah et al., 2002), which will help determine if frameshifting-modulating factors are also functionally present in translation events of human genes. The results described herein represent the experimental confirmation of the occurrence of -1 frameshifting in *Drosophila* and in mammalian neuronal cells in the context of a human DNA sequence, with pathological consequences.

Expansion of polyalanine repeat tracts leads to an increasing number of human diseases, most of them involving severe malformations (Abu-Baker and Rouleau, 2007; Albrecht and Mundlos, 2005). Here, we provided *in vivo* and *ex vivo* evidence that suggests these alanine homopolymers may also be involved in expanded CAG repeat tract disorders, implying that long polyalanine tracts could, directly or indirectly, underlie the pathology of close to 20 severe human phenotypes, with potentially more to be discovered. Our results suggest that preventing -1 frameshifting may help to alleviate symptoms of SCA3 patients, and possibly other expanded CAG disorders. According to the results presented here, polyglutamine diseases may have a

polyalanine component, or at least stem from the combined effects of both types of molecules; assessing the contribution of -1 frameshifting in expanded CAG repeat tract toxicity may therefore be important for our understanding of these diseases, as this mechanism offers a novel therapeutic target.

2.6 Materials and methods

2.6.1 Transgenic *Drosophila* lines

Constructs are depicted in Figures 2.1A and 2.2A. Full-length *ATXN3* cDNAs bearing wtCAG$_{14}$, expCAG$_{92}$, expCAA$_{96}$, STOP-CAG$_{94}$ or STOP-CAA$_{94}$- repeats were subcloned in pUAST (some vectors have a STOP codon upstream of the repeat). Epitope tags were added to each reading frame: Myc for main frame, HA for -1 frame and His for +1 frame. Vectors sequenced before injection into w^{1118}*Drosophila* eggs; a step followed by selection of positive transformants, mapping and balancing (Genetic Services, Inc.). Flies bearing transgenic constructs in a homozygous state were maintained at 25°C. Adult males were crossed to virgin *gmr*-GAL4 flies to obtain lines expressing transgenes in developing eyes (wtCAG$_{14}$/*gmr*-GAL4, expCAG$_{92}$/*gmr*-GAL4, expCAA$_{96}$/*gmr*-GAL4, STOP-CAG$_{94}$/*gmr*-GAL4 and STOP-CAA$_{94}$/*gmr*-GAL4 genotypes). To obtain isogenic control flies, w^{1118}male flies were crossed with virgin *gmr*-GAL4.

2.6.2 Epon embedding and microtome preparation of sections

Heads from adult flies were fixed (4 hours, 2% glutaraldehyde, on ice) and dehydrated by ethanol immersions (10 min of successive 50%, 70%, 80%, 95% and 100%) before their

transfer in phosphate buffer with 2% osmium tetroxide (1 hour) and finally in propylene oxide (30 min). For embedding, heads were successively placed in 1:1 propylene oxide/Epon (overnight, 4°C), 100% Epon (first overnight, room temperature and another overnight incubation, 60°C). Embedded heads were sectioned (1 μm) on a microtome and stained with toluidine blue.

2.6.3 Western blot analysis

30 fly heads were collected in RIPA with protease and phosphatase inhibitors (Boehringer), homogenised, sonicated 2x10 sec, and spun (10,000 rpm, 5 min, 4°C). Protein concentrations of supernatants were measured by Bradford and 5 μg of each were boiled (10 min) in Laemmli buffer, separated by SDS-PAGE and transblotted on nitrocellulose membranes (Bio-Rad). Immunodetection was performed as described previously (Toulouse et al., 2005) using mouse anti-ataxin-3 monoclonal antibody (1:50,000; Chemicon) and mouse anti-actin monoclonal antibody (1:50,000; Chemicon), and anti-mouse IgG horseradish peroxidase (HRP) conjugated secondary antibody (1:10,000; Cell Signaling).

Densitometry measures of the ataxin-3 and actin bands were obtained from Western blots, and the ratio of these two bands was calculated for each protein extract that was loaded. Densitometry was carried out using the Image J software, and repeated three times on two different protein extracts.

2.6.4 *Drosophila* immunohistochemistry

For transversal and coronal sectioning: heads of adult flies (3 days) were embedded in Tissue-Tek (Sakura Finetek) and placed on dry ice. 10 µm sections were prepared, dried (30 min, room temperature) and fixed (4% paraformaldehyde, 15 min). Detections were carried out after permeabilisation (0.2% Triton X-100) and blocking (10% normal goat serum, NGS). Primary antibodies were used overnight: mouse anti-Myc (1:1,000, Invitrogen), mouse anti-HA (1:100, Sigma-Aldrich) or rabbit anti-His (1:500, Invitrogen). Appropriate fluorescent secondary antibodies were used (anti-mouse or anti-rabbit Alexa Fluor-tagged secondary antibodies, 1:500; Invitrogen). DAPI (blue) was used to reveal the localisation of the nuclei. Visualisation was carried out on a Leica CTR6000 fluorescence microscope or a Leica SP5 Laser Scanning confocal microscope. Measurement of the retinal thickness was performed using Volocity software (PerkinElmer). Four measurements per eye were made, and an average calculated per eye. A minimum of 20 eyes were measured per line.

2.6.5 RT-PCR and sequencing

RNA from 20 heads was extracted using Trizol. After homogenisation, chloroform was added and the tubes centrifuged (12,000 rpm, 15 min, 4°C). Isopropanol precipitation was done on the aqueous phase, pellets washed with 75% ethanol and resuspended in RNase-free water. Reverse transcription was performed using the QuantiTect kit (Qiagen), preceded by a genomic DNA wipeout step. 5 µl of the 1/10 dilution of the RT product was used for *ATXN3* amplification [1 cycle 98°C/30 sec; followed by 10 cycles (98°C/10 sec, 63°C→58°C/30 sec, 72°C/1 min); followed by 30 cycles (98°C/10 sec, 58°C/30 sec, 72°C/1 min); and finally 1 cycle 72°C/10 min].

For sequencing, the resulting PCR products were introduced into pCR-Blunt II TOPO vectors using the Zero Blunt PCR Cloning kit (Invitrogen), and One Shot *E. coli* cells (Invitrogen) were transformed. Colonies were then isolated from lysogeny broth agar plates containing kanamycin, and the DNA extracted was sent for sequencing.

2.6.6 Quantitative real-time PCR

RNA was extracted as described above. cDNA synthesis was performed using the Superscript Vilo cDNA Synthesis kit (Invitrogen) from 1 μm of RNA. Quantitative RT-PCR was performed using the TaqMan method (Applied Biosystems) with two probes against *ATXN3* [(HS01026447_n1 and HS00245259_n1)], and against *Drosophila RPL32* (ribosomal protein L32; Dn02151827_g1). Fluorescent signal was captured using ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). The level of expression was determined by converting the threshold cycle (Ct) values using the 2-Δ ΔCt method (Livak and Schmittgen, 2001). Expression of *ATXN3* was normalised with the *Drosophila RPL32* probe and was calculated in comparison of the mean of expCAG lines. Three experiments were carried out using two different RNA extractions.

2.6.7 Constructs for organotypic slice culture

Previously produced full-length *ATXN3* fly constructs were digested to excise full-length *ATXN3* cDNA with various repeat tract lengths (14 or 92 CAG; 92 CAA). Fragments were cloned into pDsRed-Express-C1 at BglII-EcoRI, upstream of EGFP (keeping intact expression in the main or -1 frames). Plasmid DNA was transformed in DH5α *E. coli*, colonies isolated for plasmid direct sequencing; large-scale purification of plasmid DNA was performed and

products resequenced. The resulting bicistronic constructs contain DsRed-encoding sequences at the N-terminal and EGFP-encoding sequences at the C-terminal.

2.6.8 Cerebellar-slice organotypic culture

C57B16 cerebellar organotypic slices were prepared following the Feneli and De Boni procedure (Fenili and De Boni, 2003). Brains of postnatal day 8 or 9 pups were immersed in Hank's Balanced Salt Solution (HBSS; Invitrogen). Parasagittal slices of cerebellum (200 μm) were cut using a Tissue Chopper (Stoelting), and placed on Millicell six-well plate transparent inserts (Millipore) with 1 ml of culture medium [v/v; 50% minimum essential medium (MEM), 22% HBSS, 15% heat-inactivated horse serum, 10% heat-inactivated FBS, 1% insulin-transferrin-selenium, 1% penicillin-streptomycin solution and 1% of 0.5g/ml D-glucose (all from Invitrogen)]. Final concentrations of glucose, penicillin and streptomycin were 0.6% (w/v), 100 units/ml and 100 μg/ml, respectively). Slices were kept at 34°C with 5% CO_2; media was changed every three days. At equivalent postnatal day (EP) 18, slices were transfected using the Helios Gene Gun (Bio-Rad).

2.6.9 Cortical-slice organotypic culture

Brains from C57B16 postnatal pups (8 or 9 days) were removed and immersed in ice-cold artificial low-sodium cerebral spinal fluid containing 4mM KCl, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 26 mM $NaHCO_3$, 10 mM glucose and 8% sucrose, saturated with 95% O_2 and 5% CO_2). 400 μm coronal slices of cortex were cut and placed on Millicell six-well plate transparent inserts (Millipore) with 1 ml of medium [Dulbecco's Modified Eagle Medium (DMEM) with 20% horse serum, 1 mM glutamine, 13 mM glucose, 1 mM $CaCl_2$, 2 mM $MgSO_4$, 0.5 μM/ml

insulin, 30 mM HEPES buffer, 5 mM $NaHCO_3$, and 0.001% ascorbic acid] and kept at 34°C with 5% CO_2; media was changed every three days. At EP 18, slices were transfected using the Helios Gene Gun (BioRad).

2.6.10 Organotypic culture immunohistochemistry

Post transfection (48 to 72 hours), cerebellar and cortical slices were prepared. Slices were fixed (4% PFA overnight, 4°C), transferred in PBS with 30% sucrose (10 min) and placed at -20°C (20 min). Immunohistochemical detections were carried out after permeabilisation (1% Triton X-100) and blocking (10% NGS, 2 hours). Slices were incubated in PBS with 5% NGS, 0.1% Triton X-100 and anti-calbindin (1:400, Abcam; overnight, 4°C), and then in PBS with 5% NGS, 0.1% Triton X-100 and Alexa Fluor 647 $F(ab')_2$ fragment antibody (1:400, Invitrogen). Visualisation was carried out using a TCS SP5 Laser Scanning confocal microscope (Leica).

2.7 Funding

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2.8 Acknowledgements

The authors would like to thank Linh-An Tuong, Jennie Yang, and Graham Thomas for technical assistance.

2.9 Figures

Figure 2.1: Characterisation of the *ATXN3* transgenic fly lines

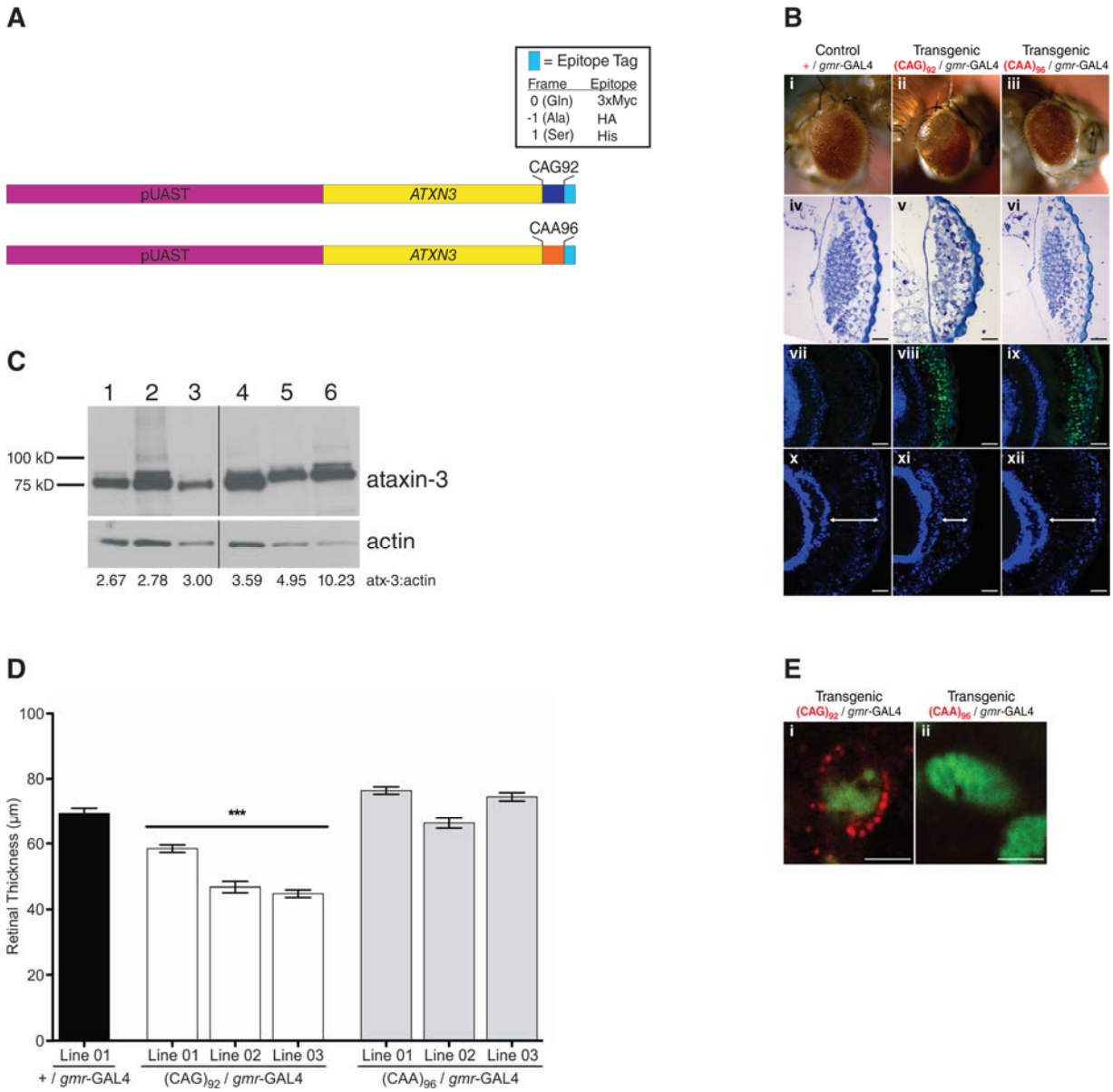

Figure 2.1: Characterisation of the *ATXN3* transgenic fly lines

(A) Full-length *ATXN3* constructs used to generate transgenic *Drosophila* lines. **(B)** Phenotypic presentation of the *ATXN3* transgenic *Drosophila* lines. **i-iii**: external visualisation of the eyes; **iv-vi**: epon sections of eyes showing ommatidia and photoreceptor organisation; **vii-ix**: immunohistochemistry showing patterns of expression of ataxin-3 (green) in the retina, and DAPI-stained nuclei (blue); **x-xii**: transversal sections stained with DAPI (blue) - arrows denote retinal thickness. Only expCAG$_{92}$ flies showed external **(ii)** and internal **(v, viii, and xi)** degeneration, which was characterised by cell death and irregular ommatidia and photoreceptor distribution. Scale, 25 μm. **(C)** Western blot analysis of expCAG$_{92}$ and expCAA$_{96}$ *Drosophila* lines. Lanes 1-3: expression of ataxin-3 in expCAG$_{92}$ fly lines; Lanes 4-6: expression of ataxin-3 in expCAA$_{96}$ fly lines. An anti-actin antibody was used as a loading control. Densitometry of the ataxin-3 / actin ratio was measured, and all lines expressed similar levels of ataxin-3 protein as indicated below each lane. **(D)** Retinal thickness was measured for all expCAG$_{92}$ and expCAA$_{96}$ transgenic lines, and a significant thinning of the retina can be observed in the expCAG flies ($P < 0.0001$). **(E)** Immunohistochemical detection of -1 frameshifting in adult expCAG$_{92}$ **(i)** and expCAA$_{96}$ **(ii)** flies. Frameshifted species were detected with anti-HA antibody (red), while main frame species were detected with an anti-Myc antibody (green). Frameshifted ataxin-3 aggregated in a perinuclear fashion and was present only in expCAG fly lines **(i)**. Scale, 2.5 μm.

Figure 2.2: Analysis of the STOP transgenic *Drosophila* lines

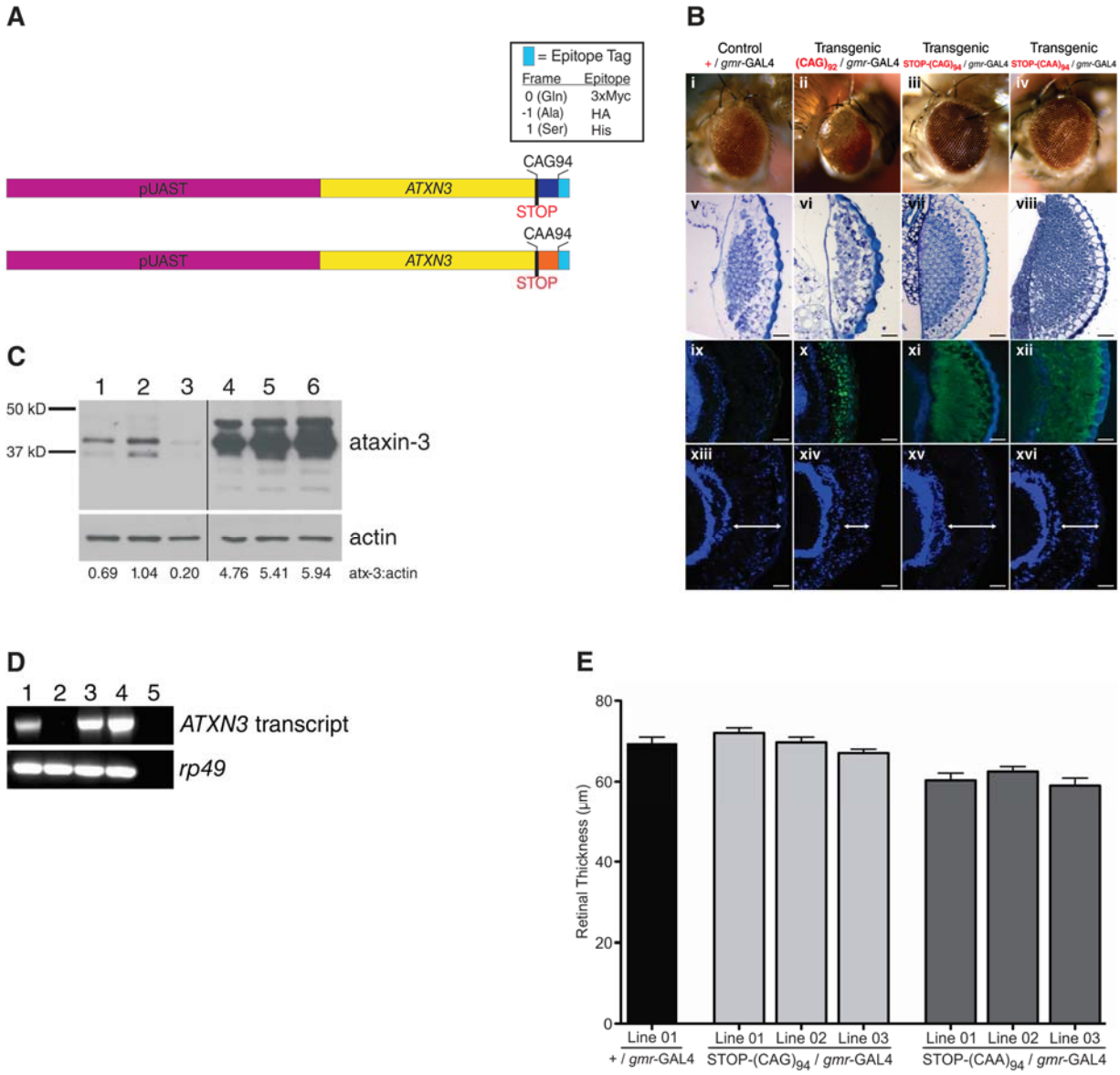

Figure 2.2: Analysis of the STOP transgenic *Drosophila* lines

(A) Schematic representation of the STOP-*ATXN3* constructs. **(B)** Phenotypic representation of the *ATXN3* transgenic *Drosophila* lines. **i-iv**: external visualisation of the eye; **v-viii**: epon sections of eyes showing ommatidia and photoreceptor organisation; **ix-xii**: pattern of expression of ataxin-3 (green) in the retina, and DAPI-stained nuclei (blue). Only expCAG flies showed external **(ii)** and internal **(vi, x, and xiv)** degeneration characterised by cell death and irregular ommatidia and photoreceptor distribution. Ataxin-3 formed aggregates localised in the nucleus of expCAG **(x),** but not in the STOP-CAG_{94} **(xi)** and STOP-CAA_{94} **(xii)** flies where ataxin-3 was expressed in a diffused manner. **xiii-xvi**: transversal sections stained with DAPI (blue) - arrows denote retinal thickness. Scale, 25 μm. **(C)** Western blot analysis of ataxin-3 in STOP *Drosophila* lines. Lanes 1-3: expression of ataxin-3 in STOP-CAG_{94} fly lines; Lanes 4-6: expression of ataxin-3 in STOP-CAA_{94} fly lines. An anti-actin antibody was used as a loading control. All constructs have similar expression levels as shown by the ataxin-3 / actin protein ratio located below each lane. **(D)** RT-PCR analysis of *ATXN3* mRNA expression in the transgenic *Drosophila* lines (*rp49* was used as an internal control). Lane 1: expCAG$_{92}$/*gmr*-GAL4; lanes 2: +/*gmr*-GAL4 (negative control); lane 3: STOP-CAG_{94}/*gmr*-GAL4; lane 4: STOP-CAA_{94}/*gmr*-GAL4; lane 5: water control. The *ATXN3* mRNA is present in similar amounts in all transgenic flies. **(E)** Retinal thickness was measured for all STOP-CAG_{94} and STOP-CAA_{94} transgenic lines, and no significant thinning of the retina among these lines was observed.

Figure 2.3: Mouse organotypic culture model of *ATXN3* -1 frameshifting

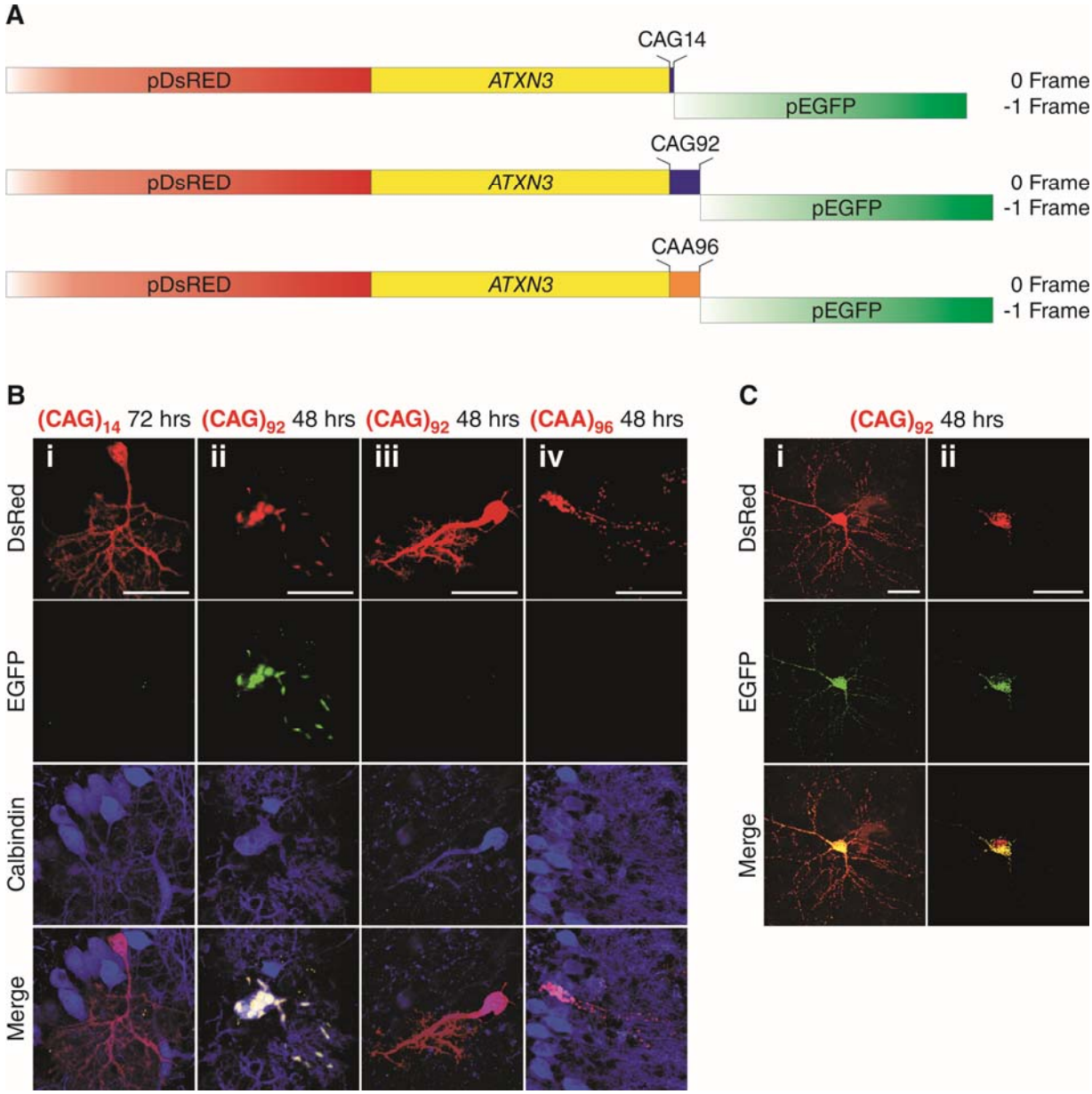

Figure 2.3: Mouse organotypic culture model of *ATXN3* -1 frameshifting

(A) Schematic representation of the dual-fluorescence *ATXN3* constructs used. DsRed and EGFP reporters were added bicistronically to express in the main frame ataxin-3 and frameshifted ataxin-3, respectively. **(B)** Mouse cerebellar Purkinje cells and **(C)** cortical pyramidal cells were transfected biolistically, fixed 48 hours post transfection, and imaged using a Leica TCS SP5 inverted laser-scanning confocal microscope. Both (B) and (C) images show expression of main-frame ataxin-3 in red, and frameshifted ataxin-3 in green; in (B) calbindin, a Purkinje cell specific marker in the cerebellum, is shown in blue. Overlay of the three (B) or two (C) signals is represented by white and yellow, respectively. C-**i,** Z-stacking images (750X) show the expCAG$_{92}$ expression throughout the cortical pyramidal cells, while higher magnification images (1,200X**,** C-**ii**) show incomplete nuclear colocalisation of -1 frameshifted ataxin-3 protein with main-frame ataxin-3 protein. Scale, 25 μm.

2.10 Supplementary data

Table 2.S.1: Transgenic *Drosophila* lines

Construct	Line	Eye phenotype* 1 day post-eclosion	Eye phenotype* 5 days post-eclosion	Eye phenotype* 20 days post-eclosion	Western blot reference
$(CAG)_{92}$/*gmr*-GAL4	Line 01	No	Yes	Yes	Fig. 1C – Lane 1
	Line 02	Yes	Yes	Yes	Fig. 1C – Lane 2
	Line 03	Yes	Yes	Yes	Fig. 1C – Lane 3
$(CAA)_{96}$/*gmr*-GAL4	Line 01	No	No	No	Fig. 1C – Lane 4
	Line 02	No	No	No	Fig. 1C – Lane 5
	Line 03	No	No	No	Fig. 1C – Lane 6
STOP-$(CAG)_{94}$/*gmr*-GAL4	Line 01	No	No	No	Fig. 2C – Lane 1
	Line 02	No	No	No	Fig. 2C – Lane 2
	Line 03	No	No	No	Fig. 2C – Lane 3
STOP-$(CAA)_{94}$/*gmr*-GAL4	Line 01	No	No	No	Fig. 2C – Lane 4
	Line 02	No	No	No	Fig. 2C – Lane 5
	Line 03	No	No	No	Fig. 2C – Lane 6
+/*gmr*-GAL4	Line 01	No	No	No	–

Fly lines generated using full length *ATXN3* constructs containing either an expCAA or expCAG repeat tract. *Eye phenotype = rough eye and loss of pigmentation upon crossing with the *gmr*-GAL4 driver line.

Figure 2.S.1: Immunohistochemical detection of -1 frameshifting in adult expCAG$_{92}$ fly heads

Adult flies were decapitated and heads were fixed, cryosectioned and immunostained with anti-Myc (green) and anti-HA (orange) antibodies. Nuclei were counterstained with DAPI (blue). Myc signal corresponding to main-frame ataxin-3 signal was confined to intranuclear structures in expCAG$_{92}$ flies, whereas HA signal corresponding to -1 frameshifted ataxin-3 protein manifested itself as smaller perinuclear inclusions. HA signal was absent in expCAA$_{96}$ and isogenic control flies (not shown), and +1 frameshifting (His tag) was never detected. Scale, 5 μm.

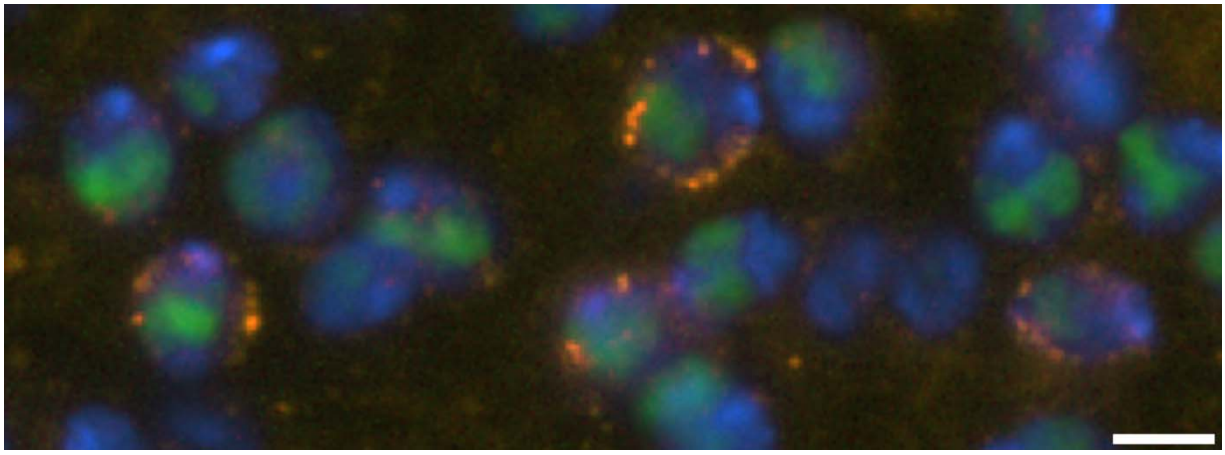

Figure 2.S.2: Quantitative real-time PCR analysis of transgenic *Drosophila* lines.

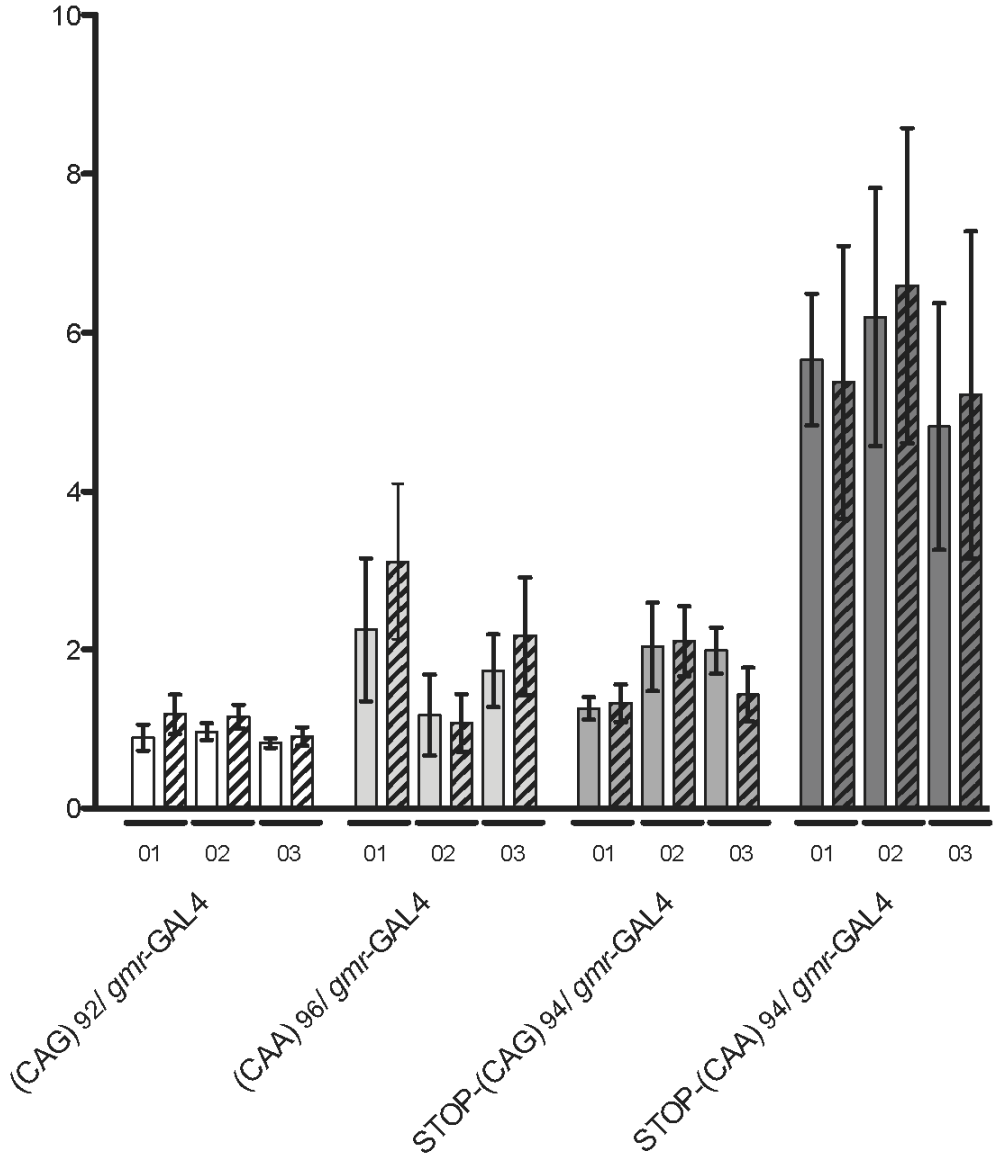

Figure 2.S.2: Quantitative real-time PCR analysis of transgenic *Drosophila* lines.

RNA was extracted from adult fly heads, and used to produce single-stranded cDNA. The cDNA was then quantified using two separate probes against *ATXN3* (probe HS01026447_n1 filled columns; probe HS00245259_n1 striped columns), and normalised to the *Drosophila RPL32* (Dn02151827_g1) probe. All lines show mRNA expression at similar or higher levels than the expCAG$_{92}$ fly lines.

Chapter 3 : A polyalanine antibody for the diagnosis of oculopharyngeal muscular dystrophy and polyalanine-related diseases

Reference:

S.J. Stochmanski, F. Blondeau, M. Girard, P. Hince, D. Rochefort, C. Gaspar, P.A. Dion, P.S. McPherson and G.A. Rouleau. A polyalanine antibody for the diagnosis of oculopharyngeal muscular dystrophy and polyalanine-related diseases. *Manuscript in preparation*.

3.1 Rationale

As stated earlier, polyalanine toxicity may underlie a large number of severe human disorders. It would therefore be useful to develop a screening tool that would allow the selective detection of alanine polymers in the proteins implicated in these diseases. A similar tool was developed for the expanded CAG repeat tract diseases in the form of a polyglutamine antibody, and led to the identification of SCA2 (Trottier et al., 1995), SCA6 (Ishikawa et al., 2001), SCA7 (Stevanin et al., 1996) , and SCA17 (Nakamura et al., 2001) as polyglutamine expansion diseases prior to the identification of their causative gene. Furthermore, this antibody has been used to characterise the subcellular localisation of the polyglutamine-containing proteins involved in expanded CAG repeat tract diseases, providing insight into their mechanisms of toxicity. More recently, antibodies generated against putative RAN-translated products across the *C9orf72* GGGGCC repeat tract led to the identification of glycine-proline dipeptide repeat proteins as a toxic species in *C9orf72* ALS/FTD (Ash et al., 2013). We believe that these discoveries highlight the usefulness of antibodies developed against expanded repeat tract proteins, and that there exists the need for such a tool to investigate and identify the involvement of polyalanine-containing proteins in disease.

3.2 Abstract

Eighteen severe human diseases have so far been associated with trinucleotide repeat expansions coding for either polyalanine (encoded by a GCN repeat tract) or polyglutamine (encoded by a CAG repeat tract). Among them, oculopharyngeal muscular dystrophy (OPMD), spinocerebellar ataxia type-3 (SCA3), and Huntington's disease (HD) are late-onset autosomal-

dominant disorders characterised by the presence of intranuclear inclusions (INIs). We have previously identified the OPMD causative mutation as a small expansion (from 6 in normal to 8-13 in disease) of a GCG repeat tract in the *PABPN1* gene. In addition, -1 ribosomal frameshifting has been reported to occur in expanded CAG repeat tracts in the *ATXN3* (SCA3) and *HTT* (HD) genes, resulting in the translation of a hybrid CAG/GCA repeat tract and the production of a polyalanine-containing peptide. Previous studies on OPMD suggest that polyalanine-induced toxicity is very sensitive to the dosage and length of the alanine stretch. Here we report the characterisation of a polyclonal antibody that selectively recognises pathological expansions of polyalanine in PABPN1. Furthermore, our antibody also detects the presence of alanine proteins in INIs of SCA3 and HD patient samples.

3.3 Introduction

Expansion of trinucleotide repeated sequences within the coding regions of distinct genes has been established to cause a number of severe human phenotypes [for reviews see (Albrecht and Mundlos, 2005; La Spada and Taylor, 2010; Messaed and Rouleau, 2009; Orr and Zoghbi, 2007)]. The expanded coding triplet sequences so far implicated in disease are either CAG repeats, which translate into polyglutamine tracts, or GCN repeats, which encode for proteins containing polyalanine stretches. The former were shown to cause at least nine distinct adult-onset neurodegenerative conditions such as Huntington's disease (HD), spinal bulbar muscular atrophy (SBMA), spinocerebellar ataxia (SCA) types 1, 2, 3, 6, 7 and 17 and dentatorubral-pallidoluysian atrophy (DRPLA) (La Spada and Taylor, 2010; Orr and Zoghbi, 2007); whereas polyalanine expansions have been implicated in oculopharyngeal muscular

dystrophy (OPMD) and in numerous developmental disorders (Albrecht and Mundlos, 2005; Messaed and Rouleau, 2009).

The so-called "polyglutamine" diseases share a number of genetic and molecular events/features; among which are their mutation process (dynamic expansion of their respective CAG repeat), intergenerational repeat instability, anticipation, and a disease course that is progressive following a late onset (10 to 20 years) (Zoghbi and Orr, 2000). For these reasons, it has been proposed that expanded CAG repeat tract diseases also share, to some extent, a common pathogenic mechanism, whereas the phenotypic variability of each disease would reflect the intrinsic properties of the cellular environment where the affected protein is expressed.

Mutant protein aggregation, often in the form of intranuclear inclusions (INIs), is a hallmark of these disorders and INIs were at first believed to be key contributors of the toxicity leading to the neurodegeneration associated with pathological repeat expansions. However, some evidence now suggests that the soluble form of these mutant proteins may be more toxic than their insoluble counterparts found in INIs (Arrasate et al., 2004), and aggregation might actually protect cells from the toxic insults inherent to misfolded soluble forms of the mutant proteins (Kayed et al., 2003; Klement et al., 1998; Saudou et al., 1998). Finally, for each of the polyglutamine diseases, the repeat tract expansion mutation affects specific populations of neuronal cells, despite ubiquitous expression of the mutant proteins [for a review see (Orr and Zoghbi, 2007)]. This could either be due to native properties of each protein, or could be

explained by novel interactions of the mutant species with other cellular factors, specific for each cell type.

Fibrillar INIs have also been described in oculopharyngeal muscular dystrophy (OPMD). OPMD is mainly a disease of the skeletal muscle cell, with some reports suggesting partial neurological involvement (Boukriche et al., 2002; Schober et al., 2001; Tome and Fardeau, 1980). The disease is caused by the expansion of a short polyalanine repeat in the polyadenylate binding protein nuclear 1 (*PABPN1*) gene (Brais et al., 1998). The INIs can be typically found in the nuclei of affected muscle fibers, but an OPMD transgenic mouse model developed by our group presents with INIs in muscle cells as well as neuronal cells of the spinal cord and cerebellum, which implies that the polyalanine expansion within *PABPN1* can also be toxic to nervous tissues (Dion et al., 2005). This finding was confirmed in postmortem cerebellar samples of an OPMD patient (Dion et al., 2005).

A -1 base shift in reading frame within an expanded CAG repeat tract would lead to translation of the protein from the GCA reading frame, which codes for polyalanine. Using (SCA3) as a model, we have previously postulated that (i) translational frameshifts in large CAG stretches result in a new reading frame with formation of a hybrid protein containing a mixed polyglutamine/polyalanine tract, (ii) the resultant polyalanine polymers aggregate, and (iii) polyalanine-containing peptides are toxic to cells. We have demonstrated the presence of -1 frameshifting events in cells cultured *in vitro*, in transgenic *Drosophila* lines, in mouse organotypic cultures, as well as in pontine neurons from SCA3 human brain autopsy material (Gaspar et al., 2000; Stochmanski et al., 2012; Toulouse et al., 2005). In cell culture, -1

translational frameshifts seems to be CAG length-dependent and to occur during translation (Toulouse et al., 2005). More importantly, we have established a direct correlation between the -1 translational frameshifts events (which we will henceforth refer to as frameshifting) and cellular toxicity using a stably transfected cell model. In addition, treating cells with specific antibiotics that are known to either enhance (e.g. sparsomycin) or inhibit (e.g. anisomycin) frameshifting can modulates the frequency of frameshifting events and the toxicity associated with these. Sparsomycin favours frameshifting by slowing the peptidyl transfer, allowing time for transfer RNA (tRNA) realignment, whereas anisomycin inhibits the accommodation of the frameshifted tRNA to the codon in the -1 frame (Dinman et al., 1997; Toulouse et al., 2005). Finally, the substitution of the expanded CAG repeat in the *ATXN3* cDNA by an expanded CAA repeat of similar length (which also encodes a polyglutamine stretch in the main frame but will not produce polyalanine-containing peptides if a -1 translational frameshift occurs) abolishes the toxicity of the transgene (Stochmanski et al., 2012; Toulouse et al., 2005). These findings suggest a major pathogenic role for the -1 frameshifted protein species in SCA3, and possibly in other expanded CAG repeat tract diseases. Frameshifting has recently been shown to occur within the CAG repeats of the huntingtin gene (*HTT*) (Davies and Rubinsztein, 2006; Girstmair et al., 2013), but a clear link has not been established between these events and toxic outcomes *in vivo*. The question thus remains as to the biological relevance of -1 ribosomal frameshifting within large CAG repeats of HD patients.

Polyalanine toxicity may underlie a number of severe human disorders. It would therefore be useful to develop a screening tool that would allow the detection of alanine polymers at a size above pathological threshold. A similar tool was developed for the

polyglutamine expansion diseases in the form of an antibody directed against polyglutamine (Trottier et al., 1995), as well as for the expanded GGGGCC repeat in *C9orf72* (amyotrophic lateral sclerosis and frontotemporal dementia; ALS/FTD) in the form of antibodies generated against the dipeptide products which were observed to arise from the pathological expansion of the GGGGCC hexonucleotide, antiC9RANT (Ash et al., 2013). Here we report the characterisation of a polyclonal polyalanine-targeting antibody, antibody 4340 (Ab4340), that selectively recognises pathological expansions of the protein PABPN1 implicated in OPMD, as well as alanine-containing INIs in SCA3 and HD patient samples.

3.4 Results

3.4.1 Generation of a polyclonal antibody sensitive to polyalanine at the pathological threshold in OPMD

We generated an antibody (4340) against a 19-mer peptide composed of 18 alanines followed by a glycine. In order to evaluate the usefulness of this antibody, it was critical to determine the number of alanine repeats it could detect. Using OPMD as the disease model and Western blot immunodetection as a first assay, our analyses revealed that the antibody was able to produce a strong signal from whole protein lysates prepared from HeLa cells that transiently expressed a vector encoding a GFP-tagged *hPABPN1* cDNA bearing alanine repeat lengths of 13, 17, 30, and 40 (Figure 3.1A). In contrast, only a weak signal was observed from lysates prepared from cells expressing the same cDNA if it encoded a 10-alanine repeat, and no signal could be observed from lysates prepared from cells that were either expressing a cDNA with no polyalanine tract (0-alanine) or that were untransfected (Figure 3.1A). To test whether the

signals detected were the putative GFP-hPABPN1- alanine proteins, we probed the same samples with an antibody against GFP, and observed corresponding bands at ~75 kDa (Figure 3.1B). This suggests that the ~75 kDa bands detected by both antibodies correspond to the same protein, whereas the ~55 kDa bands detected by our antibody alone appear to be an unspecific contaminating signal.

HeLa cells that were transfected with the same expression vectors which were used for the Western blot analyses were also used to test the sensitivity of Ab4340 to polyalanine tracts through an *in vitro* immunofluorescence assay. The fusion of an N-terminal GFP-tag to each construct made it possible to visualise protein expression using fluorescence microscopy. Intranuclear expression with a strong GFP signal was observed across all constructs (Figure 3.1C, i-vi). Using our 4340 antibody, we were able to specifically target the alanine-containing proteins and detect their expression in cells transfected with the expression vectors that encoded repeat lengths of 10-, 13-, 17-, 30-, and 40-alanines (Figure 3.1C, ii-vi). No alanine signal was detected following the expression of the 0-alanine construct (Figure 3.1C, i). The alanine-containing protein appears in aggregates, colocalising with the GFP-expressing INIs (Figure 3.1C, ii-vi). These findings indicate that Ab4340 is more sensitive in detecting alanine expansions using an immunofluorescence assay (immunocytochemistry) than Western blot immunodetection.

3.4.2 Differentiation can be made between OPMD and control patient samples

The results from our Western blot immunodetections based on HeLa cells transiently expressing *hPABPN1* cDNA with different polyalanine tracts demonstrate that the sensitivity of

Ab4340 coincides with the pathological threshold known to cause OPMD. To determine whether or not it could be used to discriminate between samples obtained from OPMD patients and control individuals, we performed another series of Western blot immunodetections for which the protein lysates were prepared from lymphoblastoid cell lines (LCLs). Furthermore, we used our 4340 antibody in immunohistochemistry assays of cerebellar sections from OPMD patients and controls.

Western blots probed with Ab4340 reveal a strong signal at ~60 kDa in nuclear lysates prepared from OPMD patient material (Figure 3.2A, lanes 3-6), whereas no bands were detected in nuclear lysates prepared from unaffected individuals (Figure 3.2A, lanes 1 and 2). These same lysates were probed with an antibody directed against PABPN1, and a corresponding band at ~60 kDa was observed (Figure 3.2B). This indicates that the ~60 kDa bands detected by the two antibodies are the same predicted PABPN1-alanine protein. In contrast to the results obtained from HeLa cells, no unspecific contaminant signal was observed from patient lymphoblastoid cell lines.

Immunohistochemistry detections made using Ab4340 and an antibody directed against ubiquitin revealed strongly stained intranuclear structures in cerebellar neurons of the OPMD patient (Figure 3.2C). The ubiquitin-detecting antibody also revealed intranuclear signals in sections prepared using tissue sections of a control individual (Figure 3.2D, i-iii); however, when Ab4340 was used on similar sections no intranuclear signal was observed (Figure 3.2D, iv-vi).

3.4.3 Alanine-containing proteins are detected in a transgenic *Drosophila* model of SCA3, and lymphoblastoid cells of SCA3 and HD patients

To test whether our antibody could detect polyalanine-containing proteins in polyglutamine diseases that have a propensity to present -1 frameshifting, we investigated SCA3 and HD. Using expCAG$_{92}$ and isogenic control flies from our previously reported transgenic *ATXN3 Drosophila* model (Stochmanski et al., 2012), we made immunohistochemical detections with both our 4340 antibody and one directed against ataxin-3. Alanine-containing proteins (red) were observed exclusively within the eyes of expCAG$_{92}$ flies (Figure 3.3A). In these same flies, ataxin-3 containing aggregates (green) were present throughout the eye, confirming transgene expression (Figure 3.3A, i). No ataxin-3 containing proteins were detected in the isogenic control flies (Figure 3.3A, ii).

Immunocytochemical detections were also made with LCLs derived from SCA3 patients, HD patients, and control individuals. Ab4340 detected alanine-containing protein aggregates in LCLs from both SCA3 (arrows in Figure 3.3B, i-ii) and HD (arrows in Figure 3.3B, iii-iv) patients, whereas no aggregates were observed in the control individual's LCLs (Figure 3.3B, v-vi). When comparing the number of cells presenting aggregates among the SCA3 and HD patients, their occurrence were observed more frequently in HD patients LCLs.

3.5 Discussion

Ab4340 was assessed for its ability to selectively detect alanine-containing proteins in disease models of OPMD, SCA3, and HD, while confirming that unaffected control individuals

would not present significant levels of these same polyalanine peptides. We chose to test the antibody's sensitivity using OPMD as a model since the protein underlying this pathology contained an expanded polyalanine tract, and this disease shared a number of similarities with polyglutamine expansion diseases: late-onset, autosomal-dominant, repeat expansion effects age of onset and severity, and the presence of aggregated proteins (INIs) (Brais et al., 1998; Tome and Fardeau, 1980). Importantly, of the nine severe human diseases that have been associated with expansions of the polyalanine tract, *PABPN1* is the only gene that does not encode for a transcription factor fundamental during early development phases (Albrecht and Mundlos, 2005). The results of Western blots prepared using lysates of HeLa cells expressing GFP-tagged *hPABPN1* showed that the signal generated by Ab4340 was substantially stronger in lysates of cells where the length of the polyalanine tract was longer than what is found in the unaffected population (10 alanines) and within the pathological threshold (11 to 17 alanines). While fluorescent immunohistochemistry detections of these same HeLa cells did not show a corresponding profile (increased signal in cells expressing a pathological length polyalanine tract) the antibody could discriminate between biological materials of OPMD patients and control individuals; the antibody did so by both Western blots and immunohistochemistry detections. The discrepancy seen with the transient expression assays made using HeLa cells may be due to the combination of the strong cytomegalovirus promoter used and the high sensitivity of the confocal microscopy which could detect lower amounts of fluorescence-tagged proteins (Semwogerere and Weeks). Another explanation for this discrepancy may be the structural conformation of complexes formed during the aggregation of polyalanine expanded proteins. *In vitro* studies have shown that polyalanine proteins transition from α-helical monomers to macromolecular β-sheets as the number of alanine residues increase (7 to 15),

whereas *in vivo* these same polyalanine proteins adopt mainly β-sheet confirmations (Blondelle et al., 1997; Scheuermann et al., 2003; Shinchuk et al., 2005). Thus, the affinity of our antibody could be directed toward the α-helical/β-sheet transition complex of 10-alanine repeats found predominantly in HeLa cells transiently expressing them.

In support of earlier reports where we established the occurrences of -1 frameshifting in SCA3 using cell culture, cerebellar and cortical organotypic slice culture, transgenic *Drosophila*, and patient tissue samples (Gaspar et al., 2000; Stochmanski et al., 2012; Toulouse et al., 2005), we detected the expression of alanine-containing proteins in the expCAG$_{92}$ *Drosophila* line, as well as alanine-containing protein aggregates in the LCLs of SCA3 patients. Moreover, the antibody could also detect alanine-positive aggregates in LCLs of HD patients; the morphology of these aggregates was similar to what was observed with SCA3. This result with HD LCLs is in agreement with the detection of -1 frameshifted products in human *huntingtin* (*HTT*) stable transfectant cells, an *HTT* transgenic mouse model, and HD patient tissue samples (Davies and Rubinsztein, 2006; Girstmair et al., 2013).

It is important to observe that Ab4340 did not detect alanine-containing proteins in any form of samples obtained from control individuals, and this is noteworthy as there are currently over 100 known human proteins to comprise a polyalanine tract of seven alanines or greater (Lavoie et al., 2003). Since the majority of these polyalanine-containing proteins are DNA binding transcription regulators, which often bind transcription factors, it is likely that they share a similar low level of expression that is below the detection threshold level of our antibody (Lavoie et al., 2003; Vaquerizas et al., 2009).

In summary, our experiments with Ab4340 demonstrate that it is a valuable tool for the detection of alanine-containing proteins in OPMD, SCA3, and HD. This antibody could be used to screen other "orphan" neurodegenerative or developmental diseases for the presence of expanded alanine tracts which may help uncover new polyalanine diseases. It could also help to further characterise the subcellular localisation of proteins containing such polyalanine tracts.

3.6 Materials and methods

All the methods used for the work described herein were carried out in accordance with approved guidelines. The experimental protocols for the use of animals were approved by Montreal Neurological Institute Animal Care Committee at McGill.

3.6.1 Production of polyalanine antibody

A 19-mer peptide comprising 18 alanine residues followed by one glycine was generated. Two rabbits were immunised with the fusion protein and the resulting serum (final bleed after 3 boost injections) was affinity purified.

3.6.2 Transgenic *Drosophila* lines

Stocks used in this study were previously described (Stochmanski et al., 2012). Adult males bearing the expCAG$_{92}$ transgenic construct were crossed to virgin *gmr-GAL4* females to obtain lines expressing the transgenic protein in the developing eye. To produce isogenic controls, adult males of the w^{1118} background were crossed with virgin *gmr-GAL4* females.

3.6.3 Cell culture and transfections

All cell lines were cultured at 37°C in a humid atmosphere enriched with 5% CO_2. HeLa cells were grown in Dulbecco's Modified Eagle Medium (Gibco), supplemented with 10% fetal bovine serum (Gibco) and 1% Penicillin/Streptomycin/Glutamine (Gibco), while the lymphoblastoid cells were grown in Iscove's Modified Dulbecco's Medium (Gibco) supplemented with 10% fetal bovine serum (Gibco), 1% Penicillin/Streptomycin/Glutamine (Gibco) and Fungizone antimycotic (Gibco).

For transient transfections, HeLa cells were transfected at 70% confluency for 48 hours with GFP-tagged *hPABPN1* plasmid DNA containing various length alanine expansions (0, 10, 13, 17, 30, and 40) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. These constructs were graciously provided by Dr. Bernard Brais (McGill University), and previously described (Klein et al., 2008).

3.6.4 Western blots

48 hours post transfection, HeLa cells were collected in ice-cold phosphate buffered saline (PBS), and lysed in radioimmunoprecipitation (RIPA) buffer [50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS)] supplemented with protease inhibitors (Roche), and sonicated for five 1 sec pulses. For OPMD patient and control individual lymphoblastoid cell lines, protein extractions were performed using the NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Thermo Scientific) according to the manufacturer's instructions.

Protein concentration was measured by Bradford assay using the Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad), and plotting O.D. values against a BSA (New England BioLabs) standard curve. Four-hundred micrograms of each protein extract were aliquoted, mixed with 5XLSB sample loading buffer (2 M Tris-HCl pH 6.8, 30% SDS, 2 M sucrose, β-mercaptoethanol, bromophenol blue), electrophoresed on a 12% polyacrylamide gel (SDS-PAGE), and transblotted to a nitrocellulose membrane (Bio-Rad). Membranes were blocked for 24 hours at 4°C in a PBS-T (0.1% Tween-20 in PBS) solution containing 5% milk (instant skim milk powder) and 5% bovine serum albumin (BSA; Fisher), and incubated overnight at 4°C in PBS-T (5% milk and 5% BSA) with one of the following primary antibodies: rabbit monoclonal anti-PABPN1 antibody (1:1,000; Abcam); mouse monoclonal anti-GFP antibody (1:5,000; Clontech); or rabbit polyclonal antibody 4340 (1:500-3,000). Membranes were then washed three times for 10 min in PBS-T, incubated for 2 hours at room temperature with the appropriate horseradish peroxidise (HRP) conjugated secondary antibody [donkey anti-mouse IgG antibody (1:5,000; Jackson ImmunoResearch), or donkey anti-rabbit IgG antibody (1:2,500; Jackson ImmunoResearch)], followed by three 10 min washes in PBS-T. Immunodetection was performed using the enhanced chemiluminescence (ECL) system (Perkin Elmer), and membranes were exposed to HyBlot CL autoradiography film (Denville Scientific Inc.).

3.6.5 Human immunohistochemistry

Formalin-fixed paraffin-embedded OPMD patient and control individual cerebellum samples were sectioned (5 μm) and placed on glass slides. The sections were deparaffinised, rehydrated, and incubated in an antigen retrieval solution (DAKO) at 85 °C for 1 hour. Sections were cooled to room temperature, and washed three times in PBS. Immunohistochemical

detection was carried out by permeabilising sections in 0.2% Triton-X100 in PBS for 30 min, followed by blocking in PBS containing 10% normal goat serum (NGS; Gibco) for 1 hour, and incubating with primary antibodies overnight at room temperature [mouse monoclonal anti-ubiquitin antibody (1:1000, Millipore), rabbit polyclonal antibody 4340 (1:500)]. Biotinylated secondary antibodies were used at a 1:500 dilution, and amplified using the ABC Elite kit (Vector). Reaction product was revealed using the DAB Substrate kit (Vector), mounted with VectaMount (Vector), and visualised on a Leica CTR6000 fluorescence microscope.

3.6.6 *Drosophila* immunohistochemistry

Adult flies were decapitated (3 days post eclosion), with heads immediately placed in Tissue-Tek (Sakura) and on dry ice to freeze. Ten micron sections were obtained by cryosectioning on a Leica CM3050S cryostat, dried for 30 min at room temperature, and then fixed in 4% paraformaldehyde (PFA) for 15 min. Permeabilisation, blocking, and incubation in primary antibodies [mouse monoclonal anti-SCA3 antibody (1:1,000, Chemicon), rabbit polyclonal antibody 4340 (1:500)] was performed as described above. Sections were then incubated with the appropriate fluorescent secondary antibodies for 1 hour (anti-mouse or anti-rabbit fluorescent tagged secondary antibodies, 1:500, Alexafluor) and mounted with Mowiol. Visualisation of immunofluorescence stainings was carried out on a Leica CTR6000 fluorescence microscope.

3.6.7 Human immunocytochemistry

Cells from HD and SCA3 patients, and control individuals were washed in PBS and deposited onto glass slides using a StatSpin Cytofuge 2 (Beckman Coulter) at 7,000 rpm for 4

min. Slides were dried for 30 min at room temperature, and fixed in 4% PFA for 20 min. Permeabilisation, blocking, and incubation in primary antibody [rabbit polyclonal antibody 4340 (1:500)] was performed as described above. Cells were then incubated for 2 hours at room temperature in HRP-conjugated secondary antibody [donkey anti-rabbit IgG antibody (1:500; Jackson ImmunoResearch)]. Reaction product was revealed using the Vector VIP Substrate kit, mounted with VectaMount (Vector), and visualised on a Leica CTR6000 fluorescence microscope.

3.7 Funding

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3.8 Acknowledgements

The authors would like to thank all patients and their families for the generous donation of cell and tissue samples, B. Brais for the gift of *hPABPN1* cDNA, D. Van Meyel for the use of his *Drosophila* facilities, and S. Cameron for his technical assistance. P.S.M. is a James McGill Professor. G.A.R. holds a Canada Research Chair in Genetics of the Nervous System and the Wilder Penfield Chair in Neurosciences.

3.9 Figures

Figure 3.1: Testing of Ab4340 sensitivity in *hPABPN1* transfected cells

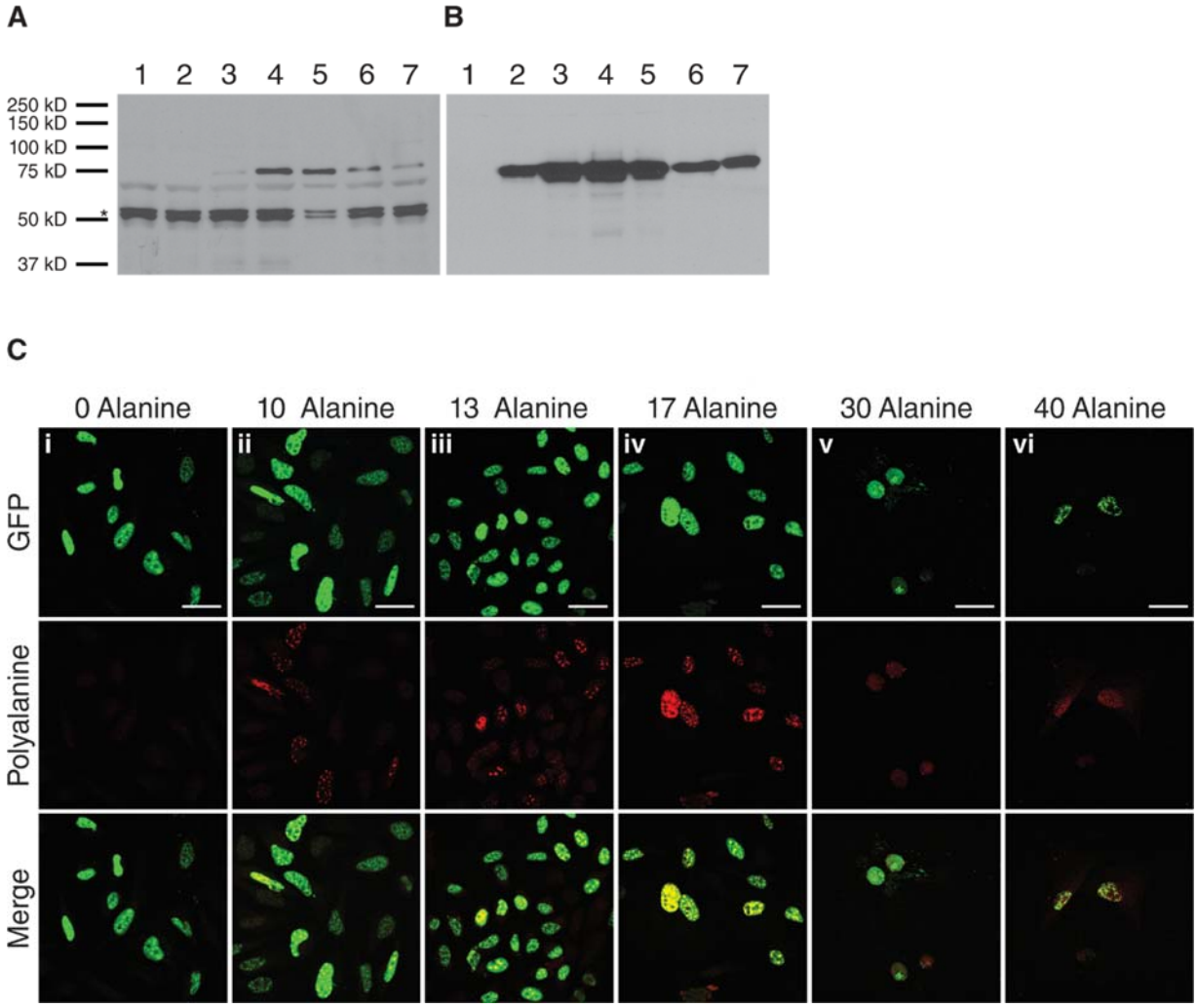

Figure 3.1: Testing of Ab4340 sensitivity in *hPABPN1* transfected cells

Western blot immunodetections of polyalanine **(A)** and GFP **(B)** from HeLa cells transiently expressing GFP-*hPABPN1* vectors where the cDNA contained various lengths of alanine repeats. Lane 1: untransfected cells; Lane 2: expression of GFP-hPABPN1-0Ala; Lane 3: expression of GFP-hPABPN1-10Ala; Lane 4: expression of GFP-hPABPN1-13Ala; Lane 5: expression of GFP-hPABPN1-17Ala; Lane 6: expression of GFP-hPABPN1-30Ala; and Lane 7: expression of GFP-hPABPN1-40Ala. (*) refers to an unspecific contaminant signal. Ab4340 strongly detected GFP-hPABPN1 protein containing 13 or more alanine repeats [(A), Lanes 4-6)], but showed a weaker ability to detect an alanine repeat length of 10 [(A), Lane 3] despite adequate GFP expression [(B), Lanes 2-7]. **(C)** Double-labelling immunofluorescence detection of alanine (red) and GFP (green) in HeLa cells fixed 48 hours post transfection with the same constructs used for the Western blot analysis. Strong detection of alanine-containing aggregates was achieved with repeat lengths of 10-alanine and greater **(ii-vi)**, whereas no detection was made in cells not expressing alanine **(i)**. Scale bar, 25 μm.

Figure 3.2: Testing the ability of Ab4340 to differentiate between OPMD patient and control individual samples

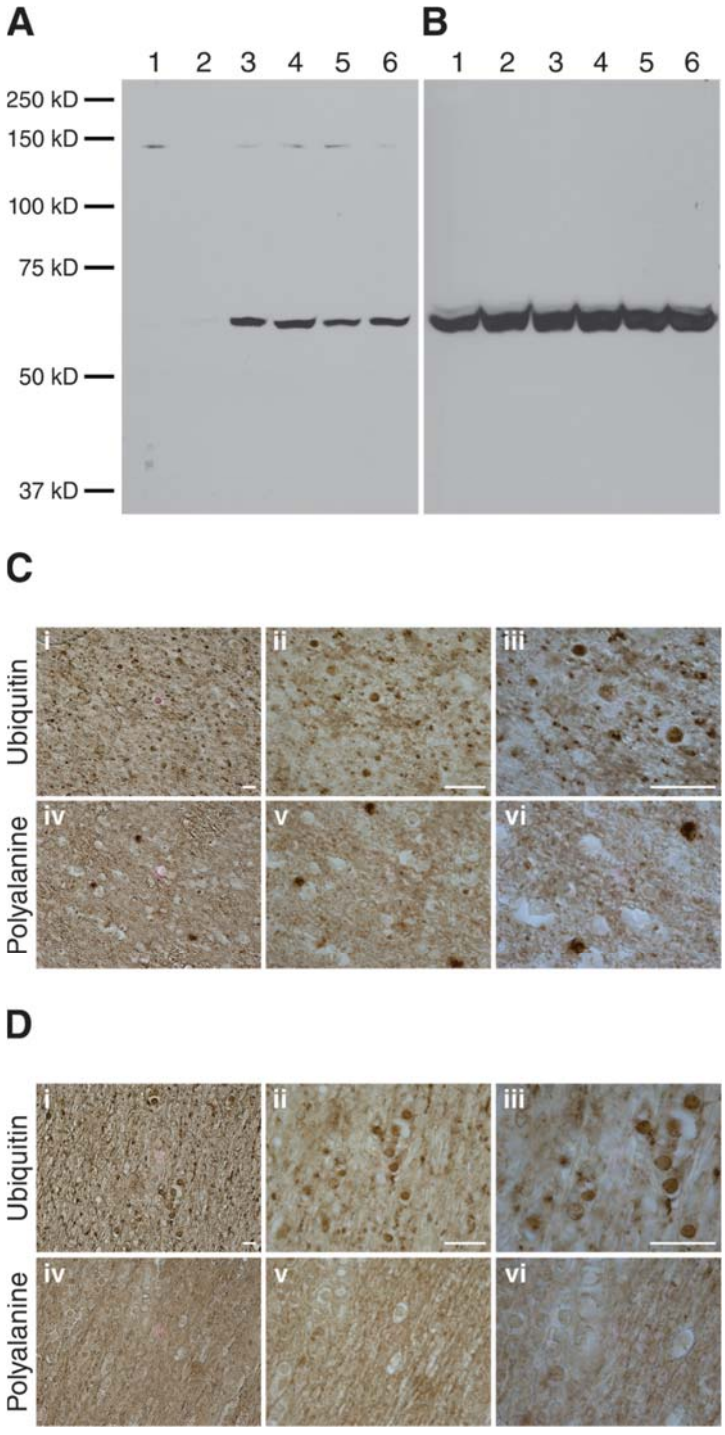

Figure 3.2: Testing the ability of Ab4340 to differentiate between OPMD patient and control individual samples

Western blot immunodetections of alanine **(A)** and PABPN1 **(B)** from nuclear extracts prepared from LCLs. Lanes 1 and 2: extracts from control individuals; and Lanes 3-6: extracts from OPMD patients. The 4340 antibody cleanly detected alanine-containing proteins exclusively from OPMD patient extracts [(A), Lanes 3-6], despite strong detection of PABPN1 in all patient extracts (B). Immunohistochemical detection of ubiquitin **(i-iii)** and polyalanine **(iv-vi)** containing proteins in cerebellar neurons of an OPMD patient **(C)** and control individual **(D)**. Both antibodies immunostained intranuclear structures in the OPMD patient's sample (C), whereas only ubiquitin immunostaining was achieved in the control patient's sample (D, i-iii). Scale bar, 2.5 μm.

Figure 3.3: Detection of polyalanine in a transgenic *Drosophila* model of SCA3, and lymphoblastoid cells of an SCA3 and HD patient

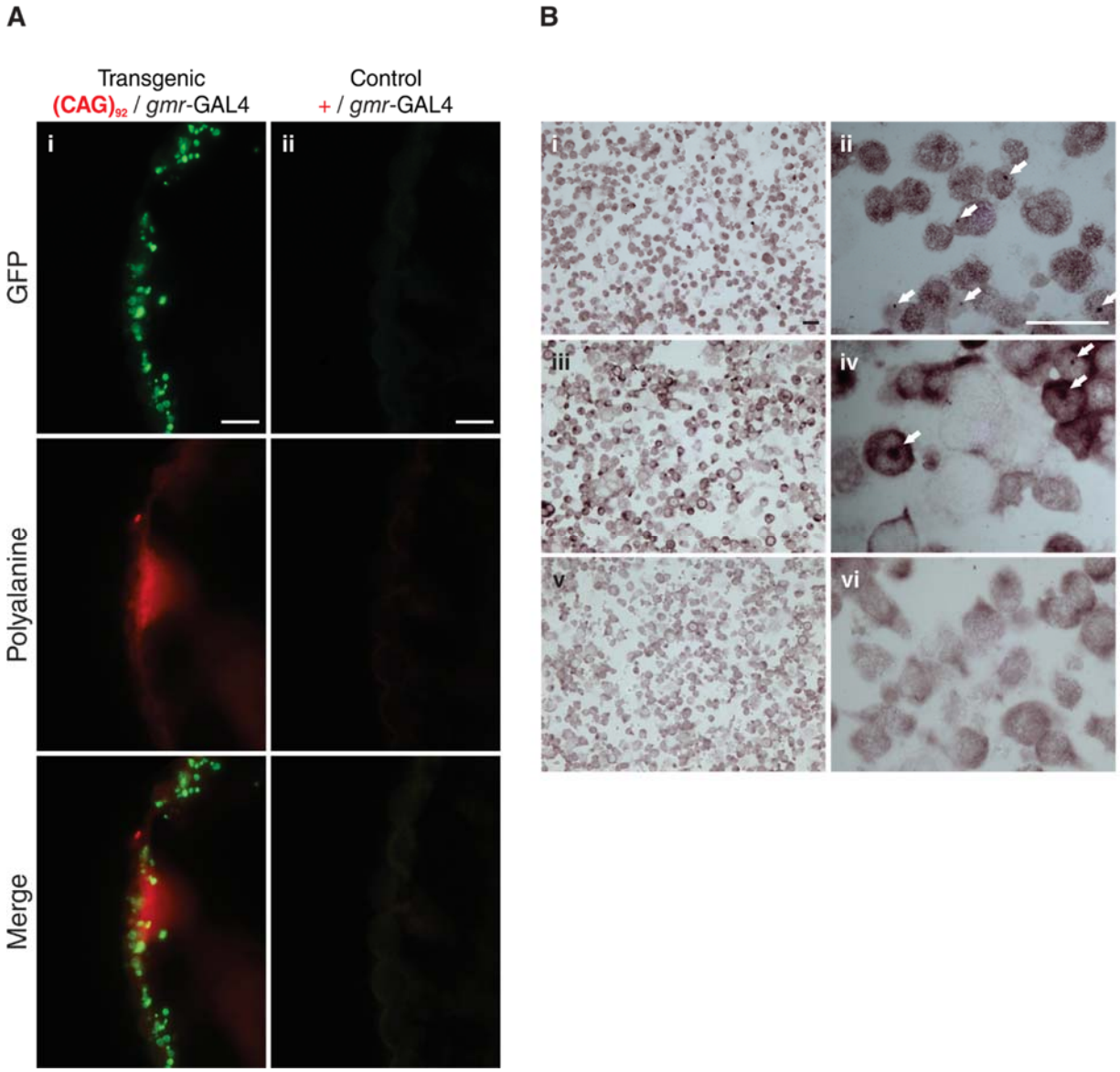

Figure 3.3: Detection of polyalanine in a transgenic *Drosophila* model of SCA3, and lymphoblastoid cells of an SCA3 and HD patient

(A) Double-labelling immunofluorescence detection of alanine (red) and GFP (green) in an expCAG$_{92}$ transgenic fly **(i)** and an isogenic control **(ii),** showing polyalanine- and ubiquitin-labeled aggregates exclusive to the transgenic line (i). Scale bar, 25 μm. **(B)** Immunocytochemical detection of polyalanine containing proteins in lymphoblastoid cells of an SCA3 patient **(i and ii)**, HD patient **(iii and iv)**, and control individual **(v and vi)**. The 4340 antibody immunostained intranuclear inclusions in both the SCA3 (i and ii, arrows) and HD (iii and iv, arrows) patient cells, whereas no intranuclear staining was present in the control patient lymphoblast cell line (v and vi). Magnification in (B): Scale bar, 2.5 μm.

Chapter 4 : Discussion

Since our group first identified the causative mutation leading to OPMD 17 years ago, the hypothesis that polyalanine may also represent a toxic protein species that results from coding expanded CAG repeat tract diseases has been the focus of several studies conducted by our laboratory. OPMD was the first description of a human disease caused by a short expansion of a trinucleotide repeat, where the addition of a single repeat produces a recessive phenotype when homozygous in an individual, and the addition of two or more heterozygous repeats lead to a dominant phenotype. This is different from what was observed across polyglutamine disorders where expansions typically represented 20 to 40 additional repeats; which suggests that polyalanine-induced toxicity is very sensitive to the length of the alanine tract. Thus, if a -1 translational frameshifting error was to occur within an expanded CAG repeat tract the ribosome would be reading the mRNA sequence in a GCA alanine-encoding frame. This would result in the decoding of a potentially toxic alanine-containing protein in a "polyglutamine" disease. It is noteworthy that translational frameshifting has now been shown to occur in both SCA3 and HD (Davies and Rubinsztein, 2006; Gaspar et al., 2000; Girstmair et al., 2013; Toulouse et al., 2005).

4.1 Ribosomal frameshifting occurs both *in vitro* and *in vivo*

Our group has previously demonstrated the presence of -1 frameshifting events in cell culture models of SCA3, as well as in the lymphoblastoid cells and pontine neurons of SCA3 patients (Gaspar et al., 2000; Toulouse et al., 2005). We have now validated our earlier observations using *in vivo* models: a *Drosophila* developing eye transgenic expression model

of SCA3; and mouse cortical and cerebellar organotypic slice cultures biolistically transfected with full-length *ATXN3* cDNAs (Chapter 2).

Consistent with our findings in SCA3, the products of ribosomal frameshifting events were also observed in cell culture models of HD (Girstmair et al., 2013), autopsy brain material from HD patients, and a transgenic HD mouse model (Davies and Rubinsztein, 2006). Only -1 frameshifted products (alanine) were consistently detected in cultured cells (Girstmair et al., 2013), whereas both -1 (alanine) and +1 (serine) frameshift products were found in HD patient and transgenic mouse samples (Davies and Rubinsztein, 2006).

4.2 Factors that may contribute to frameshifting

Among the current frameshifting models for SCA3 and HD, the occurrence of -1 translational frameshifts appears to be CAG repeat tract length-dependent. In SCA3, for example, CAG repeat tract lengths of 75 or greater were required for the detection of frameshifted products in patients (Gaspar et al., 2000), while in cell culture detection required the expression of *ATXN3* cDNAs with CAG repeat tract lengths close to or above pathological threshold (approximately 60) (Toulouse et al., 2005). Although not all cells expressing *ATXN3* or *HTT* transcripts with expanded CAG repeat tracts contained frameshifted proteins, the number of such detections was shown to increase with increases in the CAG repeat tract length (Girstmair et al., 2013; Toulouse et al., 2005). These findings are in agreement with clinical observations that the length of the CAG repeat tract expansion is correlated with disease severity and age of onset (1993; Duyao et al., 1993; Maciel et al., 1995; Maruyama et al., 1995; Matsumura et al., 1996). Furthermore, somatic CAG repeat tract mosaicism may contribute to

the observed cell-selective disease pathogenesis as the longest tract expansions occur in the brain and were found to vary among brain cell types (Hashida et al., 2001; Kennedy et al., 2003; Telenius et al., 1994; Watanabe et al., 2000).

Evidence from our SCA3 models also suggests that frameshifting is specific to CAG-encoded glutamine stretches. *In vitro*, replacing the expanded CAG repeat tract in the *ATXN3* cDNA by an expanded CAA repeat tract of similar length, which also encodes a polyglutamine stretch in the main frame but is unable to form a hairpin structure, prevented the detection of frameshifted products (Toulouse et al., 2005). Consistent with these results, frameshifted proteins were not detected in either our *Drosophila* or organotypic slice culture models following the substitution of the expCAG$_{92}$ *ATXN3* transgene with an expCAA$_{96}$ *ATXN3* transgene (Figures 2.1B, D and 2.3B, C), despite similar levels of glutamine expression (Figure 2.1C).

4.3 -1 frameshifted products are toxic to cells

Alanine-containing proteins resulting from -1 translational frameshifting events in expanded CAG repeat tract *ATXN* and *HTT* transcripts appear to enhance polyglutamine-associated toxicity. In both SCA3 and HD, frameshifted products were detected in the INIs formed by the expanded polyglutamine disease-proteins, and shown to alter the nuclear morphology of the cell and induce death. We have previously shown that cultured cells transfected with expanded CAG repeat tract *ATXN3* cDNA in which the tract preceding the CAG repeat was mutated to code for GCA (alanine-encoding) stretches resulted in an earlier and more rapid accumulation of alanine-containing proteins and a more severe phenotype than

those transfected without the GCA mutation (Gaspar et al., 2000). Additionally, the expression of an almost exclusive polyalanine tract was sufficient for the formation of perinuclear and cytoplasmic aggregates and an abnormal nuclear morphology, independent of the protein context (Gaspar et al., 2000). Finally, by modifying the *ATXN3* construct by replacing the longest CAG repeat tract with a glutamine-encoding CAA repeat tract of similar size (which also encodes a glutamine stretch in the main frame but will not produce alanine-containing peptides if a -1 translational frameshift occurs) we were able to abolish the toxicity of the transgene (Toulouse et al., 2005).

Results from our transgenic SCA3 *Drosophila* models also indicate that the *in vivo* expression of polyglutamine-containing ataxin-3 alone is not sufficient to cause a degenerative phenotype in the fly, and that -1 frameshifting events and their concomitant production of alanine-containing ataxin-3 are essential factors for the development of the observed toxic phenotype. Direct visualisation of the external eyes of our expCAG$_{92}$ lines revealed visible disruptions in both morphology and pigmentation that worsened over time. In contrast, none of the lines expressing expCAA$_{96}$ transgenes presented overt phenotypic anomalies (Figure 2.1B).

Biolistic transfection of mouse cerebellar and cortical organotypic cultures with expCAG$_{92}$ and expCAA$_{96}$ *ATXN3* transgenes validated our *Drosophila* observations in a mammalian neuronal context. Purkinje cells expressing frameshifted ataxin-3 proteins appeared dysmorphic with aberrantly shaped nuclei, severely shortened arborisations, and the presence of aggregates in both their nucleus and dendrites (Figure 2.3B, ii). Furthermore, these neurons progressed rapidly to severe degeneration and cell death. In these same cultures, Purkinje cells

expressing only main-frame ataxin-3 proteins retained their normal morphology and survival time despite a high proportion of protein aggregation.

Translational frameshifting events (-1) were also shown to occur in cultured cells transfected with expanded CAG repeat tract *HTT* exon 1 cDNAs, producing alanine-containing huntingtin proteins (Girstmair et al., 2013). The presence of these frameshifted products altered the normal aggregation properties of the main-frame expanded polyglutamine huntingtin protein, resulting in the formation of two distinct inclusion morphologies depending on their glutamine to alanine ratio: ring-shaped structures (longer glutamine stretch); or small, dense puncta (longer alanine stretch) (Girstmair et al., 2013). Despite their morphology, these inclusions were found in the vicinity of cytoplasmic or nuclear membranes, with certain perinuclear inclusions forming local indentations in the nuclear membrane and disrupting the nuclear envelope (Girstmair et al., 2013).

4.4 Mechanisms of translational frameshifting

The proposed existence of potential slippery sequences in the *ATXN3* transcript (Wills and Atkins, 2006), combined with the *in silico* prediction of ribosome-stalling mRNA hairpin structures formed by expanded CAG repeat tracts (Michlewski and Krzyzosiak, 2004) and our experiments with anisomycin and sparsomycin (Toulouse et al., 2005), provide strong evidence that frameshifting in SCA3 occurs during translation and may involve ribosome pausing with slippage into the -1 frame. The "simultaneous-slippage model" proposes that peptidyl- and aminoacyl-tRNAs slip simultaneously by one base in the 5'-direction and re-pair with the -1 frame codons in the slippery sequence (Jacks and Varmus, 1985). This shift is thought to occur

after delivery of the aminoacyl-tRNA to the A-site, but prior to peptidyl transfer (Harger et al., 2002). The mRNA secondary structure resists the 5'-movement of the ribosome, causing it to pause over the slippery sequence. The resulting strain along the mRNA is relieved by unpairing the tRNAs from the mRNA, thus allowing the mRNA to shift one base forward relative to the tRNA/ribosome complex and re-pairing of the tRNAs in the -1 frame (Plant et al., 2003). A second and third model proposes that slippage occurs during translocation (Leger et al., 2007; Namy et al., 2006; Weiss et al., 1989).

Recently, *in vitro* work on HD has provided evidence in support of a new feature facilitating -1 frameshifting in expanded CAG repeat tracts – hungry codons (Girstmair et al., 2013). The "hungry codon" hypothesis suggests that ribosomes tend to shift at "hungry" A-site codons calling for aminoacyl-tRNA in short supply (Weiss et al., 1988). Girstmair and colleagues have proposed that the frameshifted alanine-containing huntingtin proteins result from the depletion of charged glutaminyl-transfer RNA ($tRNA^{Gln\text{-}CUG}$) that pairs exclusively to the CAG codon (Girstmair et al., 2013). In support, they have shown that levels of $tRNA^{Gln\text{-}CUG}$ decreased with increasing lengths of encoded glutamine stretches, and that this decrease correlated with a higher frameshifting frequency. Furthermore, the intrinsic $tRNA^{Gln\text{-}CUG}$ concentration was found to be lower in mouse striatal and hippocampal tissues than in the cortical and cerebellar regions (Girstmair et al., 2013). If these same concentration differences are present in humans, hungry codons may help to explain the cell-selective disease pathology in HD, and present a new therapeutic target (Girstmair et al., 2013).

4.5 RNA does not confer toxicity in our *Drosophila* model of SCA3

RNA-mediated pathogenesis associated with the expansion of trinucleotide repeat tracts has been implicated in a number of degenerative diseases as a result of the similar molecular architecture between CAG and CUG repeat tract RNAs (Kiliszek et al., 2009, 2010; Sobczak et al., 2003; Sobczak et al., 2010), and the ability of muscleblind-like 1 (MBNL1) alternative splicing factor to bind them (Yuan et al., 2007). The hallmark of expanded CUG repeat tract toxicity is the formation of nuclear RNA foci that sequester MBNL1, resulting in the dysregulated alternative splicing of MBNL1-regulated genes (Miller et al., 2000; Taneja et al., 1995). More recent studies have now shown that expanded CAG repeat tracts form similar nuclear RNA foci, and that these foci also colocalise with MBNL1 (Ho et al., 2005; Li et al., 2008).

We were in the early stages of our transgenic *Drosophila* frameshifting experiments when Li and colleagues demonstrated that the CAG repeat tract in *ATXN3* RNA conferred toxicity in their *Drosophila* model of SCA3 (Li et al., 2008), permitting us to assess the contribution of RNA toxicity to the observed phenotype in our model (Section 2.4.2). To do so, we created a new set of *Drosophila* lines in which a termination codon was introduced just upstream of the expanded repeat tract (expCAG or expCAA; Table 2.S.1 and Figure 2.2A). As a result, the expanded repeat tracts would not be translated, but the entire encoding mRNAs of the transgenes will nonetheless have been transcribed. The comparative analysis of these new lines revealed a complete absence of phenotype for either one of the two transgenes (Figure 2.2B), despite the adequate expression of the two proteins and their mRNAs. The discrepancy in phenotypes between models could be attributed to the use of truncated *ATXN3* cDNA

transgenes by Li *et al.* rather than the full-length transgenes used by our group, as it was previously reported that artificially truncated constructs bearing expanded CAG repeat tracts are in fact associated with increased toxicity of the transgenes (Haacke et al., 2006). Nonetheless, our results argue against a contribution of RNA toxicity to the differential phenotypes observed in our expCAG and expCAA *Drosophila* lines (Figure 2.1).

4.6 RAN translation does not occur in our *Drosophila* model of SCA3

Repeat-associated non-ATG (RAN) translation has recently been proposed as a novel class of protein toxicity in which RNA transcripts with expanded CAG, CGG, and GGGGCC repeat tracts can be translated in the absence of an ATG start codon (Zu et al., 2011). In addition, this noncanonical translation can initiate in all reading frames of the sense and antisense strands of disease-relevant transcripts to produce a series of homopolymeric or dipeptide repeat proteins (Zu et al., 2011).

RAN translation events were originally described across SCA8 and myotonic dystrophy type 1 (DM1) expanded CAG repeat tract transcripts, which resulted in the expression of polyglutamine proteins in the CAG frame, polyserine in the AGC frame, and polyalanine in the GCA frame (Zu et al., 2011). In SCA8, polyalanine was the most expressed RAN-translated protein, whereas polyglutamine had the highest detection level in DM1 (Zu et al., 2011). These findings led us to consider the possibility that the observed alanine-containing ataxin-3 proteins in our studies may not be due to -1 frameshifting events, but rather RAN translation. Analyses of our *Drosophila* lines expressing STOP-modified transgenes did not support such events; however, as we were unable to detect polyglutamine, polyserine, or polyalanine proteins. Thus,

we concluded that RAN translation events do not occur in our *Drosophila* model of SCA3. In support of our findings, the generation of RAN-translated proteins have currently only been observed at expanded repeat tracts located in the noncoding regions of a few genes, including *ATXN8* (SCA8) (Zu et al., 2011), the 3'-UTR of *DMPK* (DM1) (Zu et al., 2011), the 5'-UTR of *FMR1* (Fragile X-associated tremor ataxia syndrome; FXTAS) (Todd et al., 2013), and the 5'-UTR of *C9orf72* (ALS/FTD) (Almeida et al., 2013; Ash et al., 2013; Donnelly et al., 2013; Gendron et al., 2013; Mackenzie et al., 2013; Mann et al., 2013; Mori et al., 2013; Zu et al., 2013).

4.7 Intrabodies for therapeutic intervention

To abrogate the pathogenic effect of the expanded polyalanine protein, the development of an intrabody directly targeting the polyalanine tract would not only neutralise the effects of the frameshifted proteins in SCA3 and HD (and possibly other expanded CAG repeat tract disorders), but could also be applicable to all disorders associated with expansions of polyalanine. It is important to stress that the use of intrabodies is of heightened relevance in the context of ribosomal frameshifting, as this is a posttranscriptional mechanism; thus, a therapeutic agent that targets the mutant protein is the only approach that will directly silence the pathogenic effect.

Intrabodies (or intracellular antibodies) were first described in 1998 by J.R. Carlson, who designed an intrabody against alcohol dehydrogenase I (ADHI) in *Saccharomyces cerevisiae* (Carlson, 1988). Intrabodies are genetically engineered single-chain/single-domain antibodies that can be expressed intracellularly in eukaryotic cells. Single-chain Fv (scFv)

antibodies are composed of the antigen-binding domains of the variable Ig heavy (VH) and light (VL) chain regions, connected by a flexible peptide linker, all encoded by a single gene. This single-gene construction allows intracellular expression in eukaryotic cells, where intrabodies bind to, neutralise, or modify the function or localisation of their target protein, thus achieving specific phenotypic knockdown of antigen function and the manipulation of biological processes [for reviews (Cardinale et al., 2014; Lo et al., 2008; Messer and Joshi, 2013)]. A growing number of reports describe the application of this technology to treat viral infection (Aires da Silva et al., 2004; Doorbar and Griffin, 2007; Marasco et al., 1998; Mukhtar et al., 2009), organ transplantation (Busch et al., 2004; Mhashilkar et al., 2002), cancer (Groot et al., 2008; Lo et al., 2008; Tanaka et al., 2007), and autoimmune disease (Heng et al., 2005; Richardson et al., 1998). It is noteworthy that this technology has been previously used to target molecules implicated in neurodegenerative disorders (Messer and Joshi, 2013), which include Parkinson's disease (Lynch et al., 2008; Messer and McLear, 2006; Zhou and Przedborski, 2008), HD (Messer and Joshi, 2013; Miller et al., 2003), Alzheimer's disease (Liu et al., 2004; Paganetti et al., 2005; Rangan et al., 2003; Sudol et al., 2009), tauopathies (Visintin et al., 2002), prion diseases (Fujita et al., 2011; Heppner et al., 2001; Leclerc et al., 2000; Shimizu et al., 2010), and amyloidogenic disorders (Kayed et al., 2003; O'Nuallain and Wetzel, 2002). Furthermore, a recent report describes the use of intrabodies against PABPN1 [developed in (Verheesen et al., 2006)] to rescue the OPMD-like phenotype in a *Drosophila* model of this disease (Chartier et al., 2009). Taken together, these reports constitute a good indication of the applicability of this technology to trinucleotide repeat expansion diseases.

The intrabody technology capitalises on the high specificity of the interaction between an antibody and its antigen, while allowing the production of the therapeutic agent directly inside the cell. Intrabodies are designed to modify or abrogate the impaired function of mutant proteins by altering several properties inherent to these molecules (e.g., folding, protein–protein interactions, and localisation). Although RNA interference can also reduce target protein levels, there is a great possibly that the level of the normal protein would be affected as well. Furthermore, the conformational selectivity of intrabodies allows a broader, proteomic approach that is particularly applicable in trinucleotide repeat expansion diseases, in which misfolded and modified versions of otherwise normal proteins are the toxic species.

As detailed above, intrabodies combine the advantage of being highly specific for their targets with the ability to be expressed intracellularly in various eukaryotic systems. In addition, they represent the ideal therapeutic tool for posttranscriptional pathogenic mechanisms such as ribosomal frameshifting.

Chapter 5 : Conclusion

The expansion of polyalanine tracts leads to an increasing number of human diseases, most of them involving severe malformations. Here, I also propose and provide preliminary supporting evidence that these very same homopolymers might also be involved across the expanded CAG repeat tract disorders, implying that long polyalanine tracts could, directly or indirectly, be the cause of close to 20 severe human phenotypes, with potentially many more to be discovered. Should these hypotheses be correct, preventing -1 translational frameshifting in the context of expanded CAG repeat tracts would likely contribute to the alleviation of symptoms of patients affected by SCA3 and other expanded CAG repeat tract disorders, underscoring the importance of engaging the focus of research in the field towards this possibility. The so-called "polyglutamine" diseases might very well turn out to be "polyalanine" diseases, or at least stem from a combined action of both types of molecules. The assessment of the extent of the contribution of -1 translational frameshifting to expanded CAG repeat tract toxicity therefore becomes crucial for the improvement of the understanding of these diseases and the development of effective therapies.

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  2. Shawn J. Stochmanski, Martine Therrien, Janet Laganière, Daniel Rochefort, Liliane Karemera, Patrick A. Dion, Don J. Van Meyel, Claudia Gaspar and Guy A. Rouleau. -1 frameshifting events in *ATXN3* expanded CAG tracts are toxic in *Drosophila* and mammalian neurons. *Hum Mol Genet.* 2012 May 15; 21(10):2211-8.

- Manuscrits en préparation
  1. Shawn Stochmanski, Francois Blondeau, Martine Girard, Claudia Gaspar, Patrick Dion, Peter McPherson and Guy Rouleau. Characterisation of a polyalanine antibody for the diagnosis of oculopharyngeal muscular dystrophy and other polyalanine-related diseases.

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1. S.J. Stochmanski, L. Ma, C.T. Dickson. Slow Wave (<1 Hz) Oscillations: Their Presence and Generation in Medial Entorhinal Cortex Slices. Joseph R. Royce Research Conference, 2005. Edmonton, Alberta.

2. S.J. Stochmanski, C. Gaspar, D. Rochefort, J. Laganiere, P. Hince, G. Di Cristo, G. A. Rouleau. In depth investigation of -1 frameshifting in expanded SCA3 using time-lapse live cell and confocal imaging. 6th International Conference on Unstable Microsatellites and Disease, 2009. Guanacaste, Costa Rica.

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1. S.J. Stochmanski, B.N. Hamam, C.T. Dickson. Theta Stimulation and Its Effects on the Postsynaptic Potentials in Layer II of the Rat Medial Entorhinal Cortex. Joseph R. Royce Research Conference, 2006. Edmonton, Alberta.

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