Investigation of molecular mechanisms underlying Oculopharyngeal Muscular Dystrophy (OPMD)

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Abstract

Oculopharyngeal Muscular Dystrophy (OPMD) is a late-onset dominant/recessive myopathy caused by the expansion of a polyalanine repeat in exon 1 of the *PABPN1* gene. The expression of expanded PABPN1 (expPABPN1) triggers the formation of insoluble nuclear aggregates within muscle fiber nuclei of OPMD patients. These aggregates are enriched in poly(A)RNA and sequester molecular chaperones, ubiquitin and proteasome subunits. In addition to these cellular components, we first identified two novel PABPN1 interacting partners, hnRNPA1 and hnRNPA/B that also localized to the insoluble expPABPN1 aggregates. However, only hnRNPA1 was observed in inclusions of OPMD patients' muscle fiber nuclei. Following this finding, we next established the involvement of the ubiquitin-proteasome pathway in the clearance of misfolded expPABPN1 and provided more insights into the beneficial role of molecular chaperones in OPMD. The inhibition of proteasome correlated with an increase in the aggregation of expPABPN1, suggesting a possible proteasome impairment in OPMD. Conversely, the overexpression of Hsp70 and Hsp40 coincided with a decrease in nuclear aggregates concomitant with a reduced cellular toxicity, suggesting the therapeutic potential of manipulating molecular chaperones levels. Finally, we demonstrated that soluble forms of expPABPN1 are the primary toxic species in OPMD. In the presence of endogenous HSPs, a decrease in expPABPN1 aggregation correlated with an increased cellular toxicity. A defect in polyadenylation or ubiquitination significantly increased expPABPN1 solubility and cell death. Using live-cell imaging, we observed that nuclear aggregates prolonged the survival of expPABPN1-expressing cells, which led us to speculate that protein aggregates are subnuclear structures that preserve cellular

homeostasis by depleting the expPABPN1 from the nuclear soluble pool. We propose that the polyalanine expansion in expPABPN1 could enable aberrant protein-protein interactions that would compromise the cellular function of nuclear factors and the expression of genes essential for muscle integrity and differentiation. For instance, expPABPN1 might compromise the function of hnRNP proteins and lead to altered mRNA processing and nucleocytoplasmic export, which can be detrimental to the cell.

Résumé

La Dystrophie Musculaire Oculopharyngée (DMOP) est une myopathie autosomale dominante/recessive tardive qui est causée par une expansion d'un tractus de polyalanines dans l'exon 1 du gène *PABPN1*. L'expression de la PABPN1 allongée (PABPN1a) induit la formation d'aggrégats nucléaires insolubles dans les noyaux des fibres musculaires chez les patients atteints de la DMOP. Ces aggrégats sont enrichis en ARN poly(A) et séquestrent des chaperons moléculaires, de l'ubiquitine et des sous-unités de protéasomes. En premier lieu, nous avons identifié deux nouveaux partenaires protéiques de la PABPN1: hnRNPA1 et hnRNPA/B. Ceux-ci sont recrutés dans les aggrégats insolubles formés par la PABPN1a. La présence de hnRNPA1 dans les aggrégats a été confirmée dans les fibres musculaires de patients atteints de la DMOP. Par la suite, nous avons établi la contribution du système protéolytique dans la dégradation des protéines PABPN1a anormalement repliées, ainsi que l'effet bénéfique des chaperons moléculaires dans la DMOP. L'inhibition du protéasome a été associée à une augmentation des aggrégats formés par la PABPN1a, suggérant un dysfonctionnement protéolytique dans la DMOP. Toutefois, la surexpression des HSP70 et HSP40 a corrélé avec une diminution des aggrégats et cette diminution a coïncidé avec une réduction de la toxicité cellulaire observée. Nos observations suggèrent que la manipulation de l'expression de chaperons moléculaires pourrait avoir des effets bénéfiques chez les individus souffrant de la DMOP. Finalement, nous avons démontré que la forme soluble de la protéine PABPN1a est la forme la plus toxique dans la DMOP. Une diminution de l'aggrégation de la PABPN1a en présence de niveaux d'HSPs endogènes a été associée avec une augmentation de la mort cellulaire. Une altération du processus de la polyadénylation ou

de l'ubiquitination a entraîné une augmentation significative du niveau de solubilité de la PABPN1a et de la toxicité cellulaire. En utilisant un système d'imagerie de cellules vivantes nous avons observé que les aggrégats nucléaires prolongent la survie des cellules qui expriment la PABPN1a. Ces observations suggèrent que les aggrégats protéiques sont des structures subnucléaires qui maintiennent l'homéostasie cellulaire en séquestrant la forme soluble de la PABPN1a. Nous proposons donc que l'expansion polyalanine de la PABPNla pourrait engendrer des interactions protéines-protéines aberrantes susceptibles de compromettre la fonction de facteurs nucléaires et l'expression de gènes essentiels au maintien de l'intégrité et la différentiation musculaire. Ainsi, la PABPN1a pourrait perturber la fonction des protéines hnRNPs et par conséquent compromettre la maturation et le transport nucléocytoplasmique des ARNm; causant des effets néfastes pour la cellule.

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Contributions of authors

Chapter 2

Xueping Fan[1], **Christiane Messaed**[1], Patrick Dion[1], Janet Laganiere[1], Bernard Brais[2], George Karpati[3] and Guy A. Rouleau[1]. hnRNPA1 and A/B interaction with PABPN1 in Oculopharyngeal Muscular Dystrophy. *Can J Neuro Sci.* 2003; 30(3): 244-51.

Xueping Fan: Conceptual and experimental design. He conducted protein-protein interaction assays (yeast two-hybrid screen, β-galactosidase liquid assay, GST-pull down, cell culture work & transfection) that allowed him to identify PABPN1 interacting partners' hnRNPA1 and A/B. The author contributed to all figures.

Christiane Messaed: Yeast two-hybrid screen and Co-immunoprecipitation assays; Determination of *in vivo* interaction between hnRNPA1 and A/B (Figure 3). Manuscript revision and correction.

Patrick Dion: Immunohistochemistry work; Identification of hnRNPA1 in muscle nuclei of an OPMD patient (Figure 6).

Janet Laganiere: Technical support (Immunocytochemistry and KCl treatment studies); Identification of hnRNPA1 and A/B in insoluble intranuclear inclusions.

Bernard Brais: Collaborator.

George Karpati: Part of the immunohistochemistry study was conducted in his lab.

Guy A. Rouleau: Supervision and manuscript revision.

Chapter 3

Aida Abu-Baker[1], **Christiane Messaed[1]**, Janet Laganiere[1], Claudia Gaspar[1], Bernard Brais[2] and Guy A. Rouleau[1]. Involvement of the ubiquitin-proteasome pathway and molecular chaperones in Oculopharygeal Muscular Dystrophy. *Hum Mol Genet.* 2003;12(20): 2609-23.

Aida Abu Baker: Conceptual and experimental design. She conducted most of the experimental work (cell culture & transfection, immunocytochemistry, immunohistochemistry, solubility and toxicity assays) to study the role of the ubiquitin-proteaosome pathway in the clearance of the misfolded PABPN1 and that of the molecular chaperones in OPMD. The author contributed to all figures.

Christiane Messaed: cell culture & transfection and Western blotting; Study of the induction of HSP70 and ubiquitin conjugates following proteasome inhibition (Figure 5). Data analysis.

Janet Laganiere: Technical support (Immunocytochemistry and immunohistochemistry); Identification of molecular chaperones and proteasome subunits in OPMD intranuclear inclusions.

Claudia Gaspar: Immunohistochemistry work; Identification of PABPN1 cytoplasmic aggregates following proteasome inhibition (Figure 4).

Bernard Brais: Collaborator.

Guy A. Rouleau: Supervision and manuscript revision.

Chapter 4

Christiane Messaed[1], Patrick Dion[1], Aida Abu-Baker[1], Daniel Rochefort[1], Janet Laganiere[1], Bernard Brais[2] and Guy A. Rouleau[1]. Soluble expanded PABPN1 promotes cell death in Oculopharyngeal Muscular Dystrophy. *Neurobiol Dis.* 2007;26(3): 546-57.

Christiane Messaed: Conceptual and experimental design. She conducted most of the experimental work (site-directed mutagenesis, cell culture & transfection, immunocytochemistry, KCl treatment, toxicity assays and live stage microscopy) to study the contribution of nuclear aggregates versus the soluble misfolded PABPN1 in OPMD cellular toxicity. The author contributed to all figures and conducted live-cell imaging studies.

Patrick Dion: Technical advisor, manuscript revision and correction.

Aida Abu Baker: Blind observer for toxicity assays.

Daniel Rochefort: Technical support: Generation of mutant PABPN1 cDNA.

Janet Laganiere: Technical support (immunocytochemistry); Co-localization of ubiquitin in nuclear aggregates (Figure 3).

Bernard Brais: Collaborator.

Guy A. Rouleau: Supervision and manuscript revision.

Claims for originality

1- Identification of novel protein-protein interactions in OPMD

We used a yeast two-hybrid approach to identify two hnRNPs (hnRNPA1 and hnRNPA/B) that bind to the C-terminus of PABPN1. These proteins localize to OPMD's characteristic insoluble nuclear aggregates. Given the abundance of hnRNPs in the nucleus, their sequestration may not perturb their normal cellular function. However, if either hnRNPA1 or A/B need to interact with PABPN1 to carry out their normal role, then aggregation of misfolded expPABPN1 or abnormal interactions between soluble expPABPN1 and hnRNPA1 or hnRNPA/B may compromise the normal activities of these hnRNPs; such situations would interfere with mRNA processing and export and this would likely be detrimental to cells.

2- Determination of the role of the ubiquitin-proteasome pathway in the clearance of the misfolded PABPN1

We reported that an inhibition of the endogenous proteasome system leads to an increase in the formation of expPABPN1 nuclear aggregates, thus suggesting that misfolded expPABPN1 is primarily degraded by the cell's proteolytic machinery. Protein aggregation in OPMD may be the consequence of either an impairment of the proteasome system or a situation where the cell's degradative machinery is overwhelmed by the continuous accumulation of misfolded proteins. A clear understanding of the role of the ubiquitin-proteasome pathway in OPMD represents an attractive avenue for therapeutic intervention.

3- The protective role of molecular chaperones in OPMD

We reported that overexpression of molecular chaperones reduced both the formation of expPABPN1 nuclear aggregates and cellular toxicity. The protective effect of molecular chaperones may reflect their role in protein refolding, degradation and the blocking of apoptotic insults induced by the build-up of non-native proteins. Targeting them directly or indirectly through components involved in regulating their expression may also point toward a new therapeutic approach for OPMD.

4- Identification of the role of polyadenylation and ubiquitination in protein aggregation

We investigated the impact of expPABPN1 polyadenylation role and the consequence of blocking its ubiquitination on protein aggregation in an OPMD cell model. We first confirmed previously reported results and observed that polyadenylation and the binding of the expPABPN1 to poly(A)RNA are indeed essential for the localization of PABPN1 into nuclear aggregates. Second, our speculation concerning the conjugation of multiple ubiquitin molecules to expPABPN1 proved that this process may also enhance the misfolding of the protein and its aggregation.

5- Identification of the soluble expPABPN1 as being the primary toxic species in OPMD

In this work, we demonstrated for the first time that the soluble forms of expPABPN1 are the ones exerting the protein toxic properties and that nuclear aggregates may represent cellular compartments that sequester the misfolded protein to prevent its toxic effect.

This finding is particularly relevant for the design of future therapeutic treatments; given that targeting the dissociation of nuclear aggregates, without necessarily enhancing the clearance of the soluble protein, may in fact be detrimental to the cell. Alternatively, therapeutic approaches that would increase the formation of nuclear aggregates in a controlled manner may prove beneficial in OPMD.

6- Identification of the dynamic properties of OPMD nuclear aggregates

We observed that nuclear aggregates formed by expPABPN1 exhibit a dynamic behavior through the cell cycle. The dissociation of expPABPN1 nuclear aggregates can occur in order for the cells harboring them to undergo mitosis; thus indicating that not all nuclear aggregates are permanent structures.

Abbreviations

ABBP-1	Apobec-1-binding protein
AGP	Alpha-1 acid glycoprotein
Ala	Alanine
Apaf-1	Apoptotic protease activating factor
ApoB	Apolipoprotein
ARE	AU-rich element
ARX	Aristaless-related homeobox
ATP	Adenosine triphosphate
BAX	BCL2-associated X protein
Br	Balbiani ring
CBF-1	C-REPEAT/DRE binding factor 1
CCHS	Congenital central hypoventilation syndrome
C/EBPβ	CCAAT/enhancer binding protein
CFIm	Cleavage factor Im
CFIIm	Cleavage factor IIm
CHIP	Carboxy terminus of HSP70-interacting protein
CNS	Central Nervous System
CPSF	Cleavage and polyadenylation specificity factor
CstF	Cleavage stimulating factor
DM	Myotonic dystrophy
DSE	Downstream element
DMSO	Dimethylsulfoxide
expPABPN1	Expanded PABPN1
GFP	Green fluorescent protein
GST	Glutathione S-transferase
HD	Huntington disease
HDAC	Histone dyacetylase
HCDC34	Human ubiquitin-conjugating enzyme
HnRNP	Heterogeneous nuclear ribonucleoprotein

HOXD13	Homeobox D 13
HPE	Holoprosencepahly
HSP	Heat shock protein
INI	Intranuclear inclusions
KSRP	KH-type splicing regulatory protein
LP	Levator palpebrae
LOX	Lipoxygenase
MIBP	c-Myc-intron binding protein
mRNA	Messenger RNA
mRNP	mRNA ribonucleoprotein
mPABPN1	Mutant PABPN1
MyoD	Myoblast determination protein
NLS	Nuclear localization signal
Nopp140	Nucleolar and coiled-body phosphoprotein 1
OPMD	Oculopharyngeal Muscular Dystrophy
OD	Oligomerization domain
PABP2	Poly(A)binding protein
PABPN1	Poly(A) binding protein nuclear 1
PAP	Poly(A) polymerase
PCBP	Poly(rC) binding protein
PHOX2B	Paired-like homeobox 2B
PNS	Peripheral Nervous System
PTB	Polypyrimidine tract binding-protein
RBD	RNA binding domain
RNP	Ribonucleoprotein
RRM	RNA recognition motif
RNAPII	RNA polymerase II
SBMA	Spino bulbar muscular dystrophy
SCA	Spinocerebellar ataxia
SC35	Splicing component 35
SF2/ASF	Splicing factor, arginine/serine-rich 1 isoform 1

SKIP	Ski-interacting protein
SMRT	Silencing mediator of retinoic acid and thyroid hormone receptor
SnRNP	Small nuclear ribonucleoprotein
SPD	Synpolydactyly
ssDNA	Single stranded DNA
TNR	Trinucleotide repeat
TUNEL	Terminal deoxynucleotide transferase mediated dUTP nick end labeling
UAS	Upstream activation sequences
Ub	Ubiquitin
U2-B	Spliceosomal protein
UES	Upper esophageal sphincter
UPP	Ubiquitin proteasome pathway
wtPABPN1	Wild-type PABPN1
XLMR	X-linked mental retardation
ZIC2	Zing finger protein 2

Chapter 1: Introduction

1.1. OPMD Pathology

1.1.1. Clinical aspects of OPMD

Oculopharyngeal muscular dystrophy (OPMD) was first characterized by Victor *et al.* who coined the present name of the disease and highlighted the myopathic nature of the pathological changes (Victor et al., 1962). OPMD was later classified as a late onset disorder inherited in an autosomal dominant/recessive manner with complete penetrance and remarkable consistancy of signs and symptoms from generation to generation (Little and Perl, 1982). Although, the disease has a world-wide distribution, it is more common in the French-Canadian population (estimated prevalence: 1/1,000). It usually becomes symptomatic after the age of 40 with major complaints such as eyelid drooping (ptosis), swallowing difficulties (dysphagia) and proximal limb weakness (Bouchard et al., 1997; Brais et al., 1999). Disease progression varies widely from one individual to another. Ptosis and dysphagia are the first symptoms of OPMD; proximal limb weakness occurring at later stages of the disease may manifest as moderate to severe but can also be asymptomatic. Some patients in their late fifties and in whom limb muscles are affected will at one point need a wheelchair, while others in their eighties present no significant limb weakness (Bouchard et al., 1997; Little and Perl, 1982). Complications due to dysphagia may include choking, nasal regurgitation, pulmonary aspiration and pneumonia (Young and Durant-Jones, 1997). Interestingly, the severity of ptosis has been shown to correlate with a deterioration of dysphagia in OPMD patients. Often, ptosis is

compensated by the retroflexion of the head which may aggrevate symptoms related to dysphagia (de Swart et al., 2006).

No medical treatment is presently available for OPMD patients. Surgical treatments are used to correct the ptosis (Codere, 1993) and to improve swallowing (Fradet et al., 1997) of moderately to severely affected individuals. Surgical shortening of the levator palpebrae (LP) muscle is performed to alleviate the level of ptosis. A frontal suspension of the lids is considered in case the levator palpebrae function is poor or absent. Both surgeries result in the opening of the eyelids (Rodrigue and Molgat, 1997).

An upper esophageal sphincter (UES) myotomy is the treatment of choice for the relief of swallowing symptoms (Fradet et al., 1997). This operation relieves the obstruction at the level of the UES; this obstruction is caused by a reduced pharyngeal peristalsis (prolonged pharyngeal contractions) that is associated with abnormal relaxation duration of the UES. Postoperative improvement of swallowing reflects a significant decrease in the resting pressures at the UES level and a decrease in the relaxation period of the sphincter (Duranceau, 1997; Fradet et al., 1997). The recurrence of ptosis and dysphagia symptoms post surgery is not uncommon and may be explained by the progression of the myopathic process in the eyelid and pharyngeal muscles.

1.1.2. Muscular phenotype in OPMD patients

OPMD is a myopathy which affects all voluntary muscles but appears to spare smooth and cardiac muscles. The muscle involvement is specific, symmetric and its severity in descending order is: levator palpebrae, pharynx, extraocular muscles, deltoid and hamstrings (Little and Perl, 1982). Muscle biopsies from OPMD patients show typical signs of muscular dystrophy including loss of muscle fibers, increased variability of fiber

size, internalized nuclei, increased fatty connective tissue and autophagic rimmed vacuoles (Tome and Fardeau, 1980). Mitochondrial abnormalities have also been reported in the muscle of OPMD patients (Wong et al., 1996). Electron microscopy analysis of circopharyngeal and deltoid muscles of OPMD patients reveals in rare muscle fiber nuclei (~ 2-5%), clear zones devoid of chromatin containing tubular filaments (~ 8.5 nm in external diameter, 3 nm in inner diameter and up to 0.25 μm in length) that converge to form tangles and palisades (Figure 1). These tubular filaments are referred to as intranuclear inclusions (INIs) or nuclear aggregates in the literature and they are unique to OPMD; as they differ from all other types of inclusions so far described within nuclei of muscle fibers (Brais et al., 1999; Tome and Fardeau, 1986).

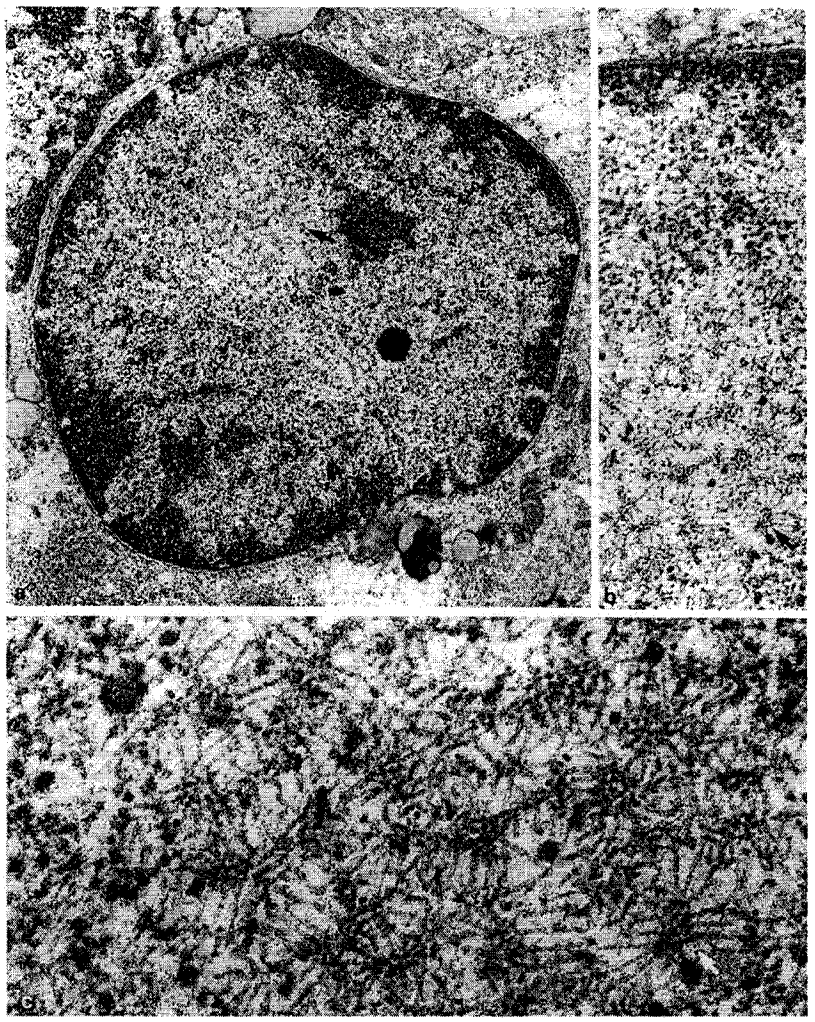

Figure 1: Biopsy of a very severely affected circopharyngeal muscle from a patient with OPMD.

(a) Muscle fiber nucleus with a clear zone area containing a collection of filaments (17,300 X). (b) High magnification of the same area (42,300 X). (c) Higher magnification (94,000 X) shows that the filaments are tubular (~ 8.5 nm) and they converge to form tangles and palisades. The same bunch of filaments is arrowed in the three illustrations. Reproduced from (Tome et al., 1997).

1.1.3. Neurogenic involvement in OPMD

To date the signs and symptoms of OPMD are thought to be of myopathic origins. However, rare cases of central nervous system (CNS) and peripheral nervous system (PNS) involvement have been reported. In 1982, Probst *et al.* reported a severe depletion of myelinated fibers in endomysial nerve twigs of extraocular, pharyngeal and lingual muscles pointing for the first time to neurogenic changes in these muscles and suggesting nerve involvement (Probst et al., 1982). More recently, peroneus muscle and peroneal nerve biopsies respectively indicated the presence of small angulated atrophic fibers and loss of myelinated fibers as well as signs of chronic axonal regeneration, suggesting a lower motor neuron involvement (Boukriche et al., 2002). Interestingly, the loss of myelinated fibers in cranial nerves (especially in oculomotor nerves) (Boukriche et al., 2002) and the post-mortem finding of INIs in cerebellar neurons of an OPMD patient (Dion et al., 2005) suggest a possible CNS involvement in the disease. CNS and PNS alterations may both lead to denervation and contribute to the pathophysiological mechanism of OPMD. Alternatively, the axonal loss in the PNS and CNS as well as the presence of INIs in the CNS may be totally asymptomatic.

1.2. OPMD genetic background

1.2.1. Polyalanine expansions in PABPN1 are associated with OPMD

Prior to the causative gene identification, only the presence of INIs of a specific size (~ 8.5 nm in external diameter) in the muscle fiber nuclei was deemed as the ultimate criterium in making the definite diagnosis of OPMD. Their finding was mandatory to select patients for the linkage studies that mapped OPMD to chromosome 14q11.2-q13

(Brais et al., 1995). The OPMD gene was positionally cloned and identified as the poly(A) binding protein nuclear 1 (PABPN1), formerly known as poly(A) binding protein 2 (PABP2) (Brais et al., 1998). The coding sequence of PABPN1 comprises seven exons. A normal *PABPN1* gene contains 10 alanine residues that are encoded by a $(GCG)_6$ repeat and an adjacent stretch of $(GCA)_3(GCG)$ at the N-terminus of the gene's first exon. The 10 alanines stretch in the normal allele is expanded to 11-17 alanine residues in patients with OPMD. All mutations, including insertions of pure GCG or GCA codon(s) in combination with GCG codons, lead to polyalanine expansions. The inheritance of two $(GCG)_7$ or $(GCG)_9$ alleles causes the recessive form of OPMD. Recently, Robinson D. O *et al.* reported for the first time a point mutation mimicking the effect of polyalanine expansions in one patient from North Western Europe (Robinson et al., 2006). This mutation occurs immediately at the 3' end of the normal polyalanine repeat sequence and results in a glycine to alanine substitution therefore generating a polyalanine expansion (Robinson et al., 2006).

Significant genetic heterogeneity is found in some ethnic populations such as, the German, the Cajun and the British population (UK) (Muller et al., 2006; Robinson et al., 2005; Scacheri et al., 1999). However, a single founder has been identified in other populations such as Bukhara Jewish, Uruguayan families and French Canadian (Blumen et al., 2000; Brais et al., 1998; Muller et al., 2006; Rodriguez et al., 2005). The most frequent mutant allele seen in French Canadian and the British population is the $(GCG)_9$ allele (Brais et al., 1998; Robinson et al., 2005).

1.2.2. Polyalanine expansion length and disease severity

Ptosis and dysphagia are usually the first signs to appear and limb weakness may or may not occur with the disease progression (Muller et al., 2006; Scacheri et al., 1999; van der Sluijs et al., 2003). There is no significant correlation between the repeat length and the age of onset or disease severity. The latter depends on the age of the patients rather than the number of repeats (Muller et al., 2006; Muller et al., 2001). However, Brais *et al.* noticed a more severe phenotype associated with the segregation of the $(GCG)_7$ in combination with the $(GCG)_9$ genotype, suggesting that the polymorphic allele can act as a modifier of disease severity (Brais et al., 1998). Patients homozygous for the $(GCG)_9$ allele also present more severe clinical signs than those who are heterozygous for the same mutation; an observation supporting a gene-dosage effect in OPMD (Brais et al., 1998).

1.2.3. Proposed trinucleotide repeat expansion mechanisms

Trinucleotide repeat (TNR) expansions are common polymorphisms segregating with several human diseases. Polyglutamine tract expansions are associated with many neurodegenerative diseases, including Huntington disease (HD), spinal bulbar muscular atrophy (SBMA) and a series of spinocerebellar ataxias (SCA1-3,6,7,8,12 and 17) (Cleary and Pearson, 2005). Oculopharyngeal muscular dystrophy (Brais et al., 1998) and at least eight other human diseases result from expansions of translated sequences coding for polyalanine tracts (Albrecht and Mundlos, 2005; Amiel et al., 2004). Unlike expanded polyglutamine repeats which are dynamic and continue to mutate within tissues and across generations, polyalanine expansions are stable in somatic and germline tissues and stably transmitted to offspring.

TNR instability depends on the nature of the repeat and its length. Polyglutamine tracts are usually encoded by perfect CAG codons and this makes them more likely to expand than polyalanine tracts which are smaller in size and usually encoded by interrupted triplet repeats. Sequences encoding glutamine amino acids are distinguished by their ability to form unusual DNA structures during DNA metabolism (replication and repair): hairpins, G-quartets and triplexes (Cleary and Pearson, 2005; Pearson et al., 2005). DNA polymerase slippage, simple strand slippage and inappropriate Okazaki fragment processing may account for the formation of these structures, and explain the generation of long polyglutamine expansions underlying conditions such as HD, SBMA, and SCA (Cleary and Pearson, 2005; Pearson et al., 2005). Alanine tract expansion involves the insertion of perfect or imperfect small trinucleotide repeats. The longest polyalanine expansion described to date leads to a 33 alanines stretch in a Paired-like homeobox 2B (PHOX2B) transcription factor (Matera et al., 2004), corresponding to a polyglutamine repeat in the normal range (Scherzinger et al., 1999) and suggesting that the mechanism giving rise to mutations associated with polyalanine diseases is more likely to be unequal crossing-over between two mispaired normal alleles. All mutations resulting in pure GCG expansions or GCA and GCG interspersions in OPMD can be generated by unequal recombination between two normal alleles, except for the $(GCG)_{13}$ allele (Robinson et al., 2005). The insertion of seven pure GCG codons may be explained by slippage, a combination of unequal crossing-over and slippage or unequal crossing-over between the normal $(GCG)_6$ and the $(GCG)_7$ polymorphic allele (Robinson et al., 2005).

1.3. PABPN1 structure

1.3.1. PABPN1 functional domains

PABPN1 is a putative 306 amino acid protein with a predicted molecular mass of 32.8 KDa (Nemeth et al., 1995). Sequence analysis suggests that PABPN1 consists of three distinct domains (Figure 2). The initiating methionine at the beginning of the N-terminus is immediately followed by a sequence of 10 alanines encoded by an imperfect repeat. The latter is adjacent to an acidic region rich in glycine and proline residues followed by a stretch of ~30 amino acids that has a pattern characteristic of α-helical coiled-coil domains (amino acids 113-150) (Figure 2) (Benoit et al., 1999). The central region of PABPN1 corresponds to the RNA recognition motif (RRM) or RNA binding domain (RBD) (amino acids 161-257) (Figure 2) (Kuhn et al., 2003). A basic C-terminal domain highly enriched in dimethylated arginines (amino acids 249-306) contains a potential novel type of nuclear localization signal (NLS) (Figure 2) (Calado et al., 2000a). The general arginine dimethylation and amino acid composition of the C-terminal domain are reminiscent of RGG-type RNA binding domains (Burd and Dreyfuss, 1994; Kuhn et al., 2003). Two potential oligomerization domains, $OD1_{(155-294)}$ and $OD2_{(264-306)}$ overlap respectively with the RBD and the C-terminus of PABPN1 (Figure 2) (Fan et al., 2001).

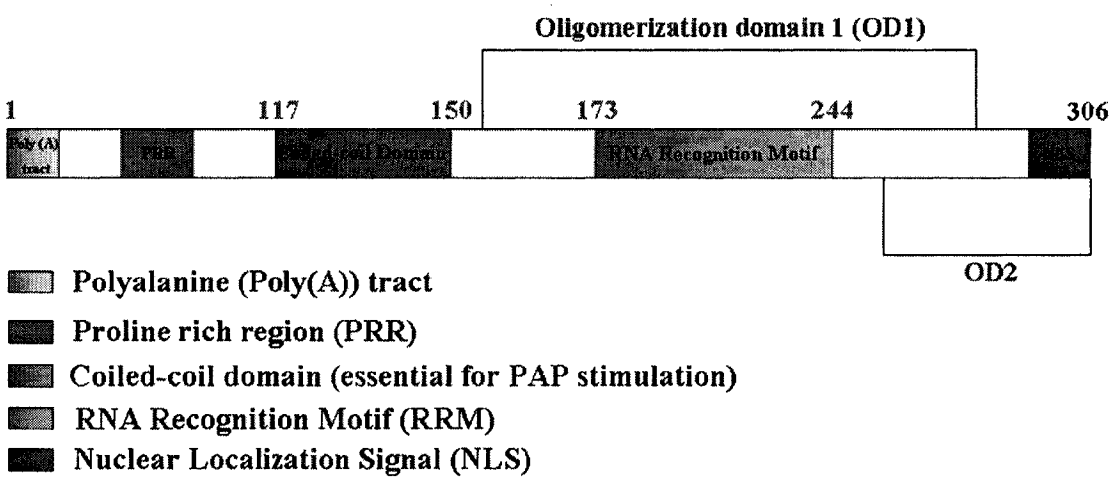

Figure 2: The PABPN1 structure

1.3.2. PABPN1 sequence alignment in between species

The coding sequences are highly conserved between human, bovine and mouse (Brais et al., 1998). Although, the PABPN1 RRM is highly conserved in *H. sapiens*, *M. musculus*, *B. Taurus*, *X. laevis*, *D. melanogaster* and *C. elegans* with 61% identity, the polyalanine domain is only present in vertebrate species (unpublished data).

1.4. PABPN1 cellular function and distribution

The function of RNA-binding proteins depends on motifs that bind to RNA, and auxiliary domains that may be involved in protein-protein interactions as well as the localization of the protein within the cell (Dreyfuss et al., 1993). *In vivo*, PABPN1 is an abundant nuclear protein involved in precursor messenger RNA (pre-mRNA) polyadenylation. It binds to poly(A) tail of mRNA stimulating their extension and controlling their length. It also participates in mRNA nucleocytoplasmic export (Bear et al., 2003; Calado et al., 2000a). In addition to the role of PABPN1 in mRNA processing and export, the protein seems to be involved in general transcription and muscle differentiation (Bear et al., 2003; Kim et al., 2001).

1.4.1. PABPN1 RNA binding activity and PAP stimulation

In vitro, PABPN1 interacts with high affinity to the poly(A) and poly(G) sequences (Wahle, 1991; Wahle et al., 1993). PABPN1 binds RNA as a monomer and tend to form oligomeric structures when complexed to poly(A) covering its length (Keller et al., 2000). The packaging density on the poly(A) tail is approximately 15 adenylate residues per PABPN1 molecule. Both the RBD domain and the C-terminal arginine-rich domain of PABPN1 contribute to RNA binding. The RBD domain binds specifically to poly(A), but

needs the coordination of the RGG-type domain in the C-terminus to reach its full affinity (Kuhn et al., 2003). The RBD motif consists of a sequence of amino acids that can adopt a globular tertiary structure in which RNA binding is primarily supported by β-sheet surface via hydrogen bonds and stacking interactions between bases and amino acid side chains (Allain et al., 1997; Deo et al., 1999). The dimethylation of arginines in the C-terminus domain is a known modification observed in several hnRNP proteins that could serve to regulate the RNA binding activity of RNA binding proteins (Christensen and Fuxa, 1988; Siomi et al., 1993). Arginine side chain makes critical RNA contact suggesting that this amino acid is essential for the RNA-binding activity of the RGG-type domain (Puglisi et al., 1992; Tao and Frankel, 1992). The α-helical coiled-coil domain localized in the N-terminus of PABPN1 (Figure 2) does not contribute to the RNA binding activity. However, it was found to be essential for the stimulation of the poly(A) polymerase (PAP) and may be implicated as well in protein-protein interactions (Kerwitz et al., 2003).

1.4.2. The mechanism of mRNA polyadenylation

All eukaryotic mRNA are post-transcriptionally modified at their 3' ends by addition of a poly(A) tail. It has been proposed that the poly(A) tail confers mRNA stability, promoting mRNA translation efficiency and playing a role in transport of processed mRNA from the nucleus to the cytoplasm (Lewis et al., 1995; Sachs et al., 1997; Wickens et al., 1997). The formation of poly(A) tails in the nucleus involves a number of trans-acting protein factors, most of them hetero-oligomers including the cleavage and polyadenylation specifity factor (CPSF), cleavage stimulation factor (CstF), cleavage factors Im and IIm (CFIm and CFIIm), PAP and PABPN1. The initiation of the

polyadenylation reaction requires an endonucleolytic cleavage of the pre-mRNA at the 3' end, at a particular phosphodiester bond ~20 nucleotides downstream from the polyadenylation signal AAUAAA which is recognized by the CPSF, a hetero-oligomeric factor essential for both cleavage and polyadenylation. CstF, CFIm and CFIIm participate only to the cleavage reaction by binding to GU-rich or U-rich downstream elements (DSE) within 30 nucleotides of the cleavage site (Barabino and Keller, 1999). Following the cleavage event, the CPSF factor interacts with PAP and stimulates the polymerase activity, thus providing it with specificity for RNA sequence carrying the CPSF binding site. However, poly(A) synthesis in the presence of PAP and CPSF is slow and inefficient. Processive and efficient polymerization requires PABPN1 (Wahle and Kuhn, 1997). After PAP, with the help of CPSF, has added 10-11 adenylate residues to the 3' end of pre-mRNA, PABPN1 binds to the growing poly(A) tail (Keller et al., 2000). Together, CPSF and PABPN1 act synergistically to increase the processivity of polyadenylation by tethering the polymerase to the RNA primer. In the presence of both proteins, PAP can add without dissociating from the RNA up to 250 nucleotides, corresponding to the average size of a newly synthesized poly(A) tail in a mammalian cell (Figure 3). Although the mechanism by which the poly(A) tail length is regulated is unknown, it has been proposed that PABPN1 could function as a "molecular ruler" to measure the length of the tail as it is synthesized (Keller et al., 2000).

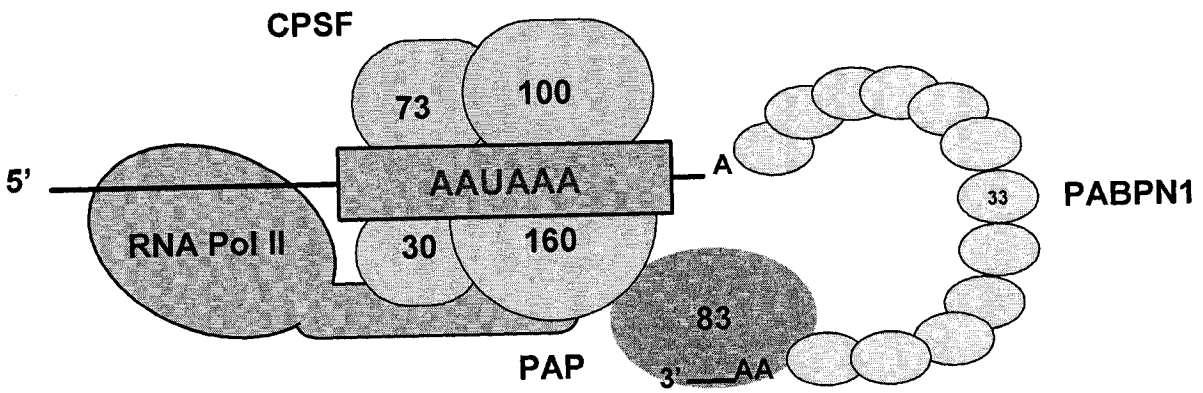

Figure 3: The polyadenylation reaction

CPSF (four subunits of 160, 100, 73 and 30 KDa) binds to the polyadenylation signal AAUAAA and PABPN1 (33 KDa) binds to the growing poly(A) tail, forming oligomeric structures that cover its length. Together, CPSF and PABPN1, they tether PAP (83 KDa) to the RNA and stimulate polyadenylation.

1.4.3. The transcriptional activity of PABPN1, a role in muscle differentiation

Despite the ubiquitous expression pattern of PABPN1, the clinical and pathological phenotypes are restricted to the skeletal muscle in all patients with OPMD, although in rare cases neurogenic involvement has been suggested. Characterizing the functional roles of PABPN1 in muscle fibers provided the first evidence of PABPN1's involvement in transcription in skeletal muscle (Kim et al., 2001). Overexpression of PABPN1 in a mouse muscle cell line enhanced myotube formation accompanied by increased expression of the myogenic factors, myoD and myogenin. PABPN1 was found to co-operate with ski-interacting protein (SKIP) to stimulate MyoD-dependent transcription (Kim et al., 2001). SKIP is a transcription cofactor present in all eukaryotes, originally identified as a protein that interacts with the viral form of the oncoprotein SKi. SKIP like SKi is expressed in most, if not all human tissues (Dahl et al., 1998). Several papers reported interactions between SKIP and other proteins (e.g. the DNA binding protein CBF1, the cell surface receptor NotchIC, the *c-myc*-intron binding protein1 (MIBP) and members of histone dyacetylase (HDAC) such as the SMRT protein), involved in the repression and/or activation of transcription (Fukuda et al., 2002; Kostrouchova et al., 2002; Zhou et al., 2000a; Zhou et al., 2000b).

A recent study provides more evidence for the role of PABPN1 in transcription. A high level of mRNA biosynthesis takes place on the Balbiani ring (BR) genes in the salivary gland of the insect *Chironomus tentans* (*C. tentans*) enabling easy visualization of transcription complexes and ribonucleoprotein (RNP) assemblies along these active regions of chromatin (Daneholt, 2001). Exploiting the BR system Bear *et al.* reported that PABPN1 is primarily located along the chromatin axis and is associated with the

elongating transcription complex. PABPN1 interacts with the RNA polymerase II (RNAPII) *in vivo* and may assemble onto RNAPII before, at, or shortly after the start of transcription (Bear et al., 2003). It has been proposed that the transfer of PABPN1 from RNAPII to the newly formed poly(A) tail might initiate the rapid processive poly(A) synthesis as well as serve as a signal for RNAPII to terminate transcription (Figure 3) (Bear et al., 2003).

1.4.4. The nucleocytoplasmic export of mRNA

Although PABPN1 can shuttle back and forth between the nucleus and the cytoplasm, under electron and fluorescence microscopy it is mainly present in the nucleus. PABPN1 binds via its NLS a nuclear transport receptor (transportin) in a Ran GTP-sensitive manner, suggesting the involvement of this transport receptor in mediating import of the protein into the nucleus (Calado et al., 2000a). Transportin has previously been shown to mediate nuclear import of shuttling hnRNP proteins (Siomi et al., 1997). Similarly to hnRNPs, PABPN1 is exported to the cytoplasm by a carrier-mediated mechanism that is independent of ongoing transcription, suggesting that the protein may play a role in mRNA transport (Calado et al., 2000a). In agreement with the role of PABPN1 in mRNA trafficking, immunoelectron microscopy analysis shows that PABPN1 remains associated with the 3' end of the salivary gland BR mRNA ribonucleoprotein (BR mRNP) complex until the mRNA is translocated through the nuclear pore. It seems likely that PABPN1 dissociates from the mRNA during or shortly after the translocation of the particle through the pore and rapidly returns to the nucleus (Bear et al., 2003). It has been suggested that PABPN1 exchanges its cargo with a cytoplasmic PABPC1, formerly called PABP or PABP1, before or soon after nucleocytoplasmic translocation of the mRNA

through the nuclear pore (Hall, 2002; Hosoda et al., 2006). While PABPC1 regulates the stability and translation of mRNA in the cytoplasm, PABPN1 may be responsible for the protection of the poly(A) tail from degradation throughout the lifetime of the mRNA transcript in the nucleus (Feral et al., 1999; Hall, 2002).

1.4.5. Cellular distribution of PABPN1

PAPBN1 is normally concentrated in 20-40 discrete nuclear domains that are referred to as nuclear speckles or SC35 domains (Krause et al., 1994). These nuclear substructures rich in poly(A)RNA correspond to clusters of interchromatin granules and perichromatin fibrils (Krause et al., 1994; Spector et al., 1991). Perichromatin fibrils found at the periphery of regions of condensed chromatin are dispersed throughout the interchromatin space which contains particles with a mean diameter of 200-250 Å which are linked together by thin fibrils (Fakan and Puvion, 1980; Spector et al., 1991). Several lines of evidence suggest that most splicing occurs co-transcriptionally on perichromatine fibrils which contain nascent RNA and factors known to be involved in pre-mRNA processing such as small nuclear ribonucleoprotein (snRNP), heterogeneous nuclear ribonucleoproteins (hnRNP), PABPN1 and numerous splicing factors such as SC35, SF2/ASF and U2-B (Krause et al., 1994; Spector et al., 1991). Clusters of interchromatin granules contain only low levels of rapidly labeled RNA and hnRNP proteins (Fakan and Bernhard, 1971; Fakan et al., 1984; Fakan and Nobis, 1978). Their true function in nuclear RNA metabolism remains unknown. Spector *et al.* proposed that interchromatin granules may be sites of spliceosome assembly and/or storage from which splicing factors are recruited to the perichromatin fibrils where nascent transcripts are located (Spector et al., 1991). The recruitment of PABPN1 to the speckles is a consequence of its binding to

poly(A)RNA (Calado and Carmo-Fonseca, 2000) while the assembly of splicing factors into these structures involves protein-protein interactions (Spector et al., 1991). The nuclear localization of PABPN1 and its association with perichromatin fibrils as well as its co-localization with factors known to be involved in pre-mRNA processing are in agreement with the protein's role in polyadenylation and transcription.

1.5. Misfolded expanded PABPN1 nuclear localization and dysfunction

Hydrophobic polyalanine stretches in the normal range have been described as flexible domains that confer stability and flexibility to the three dimensional conformation of the native protein. However, the extension of these homopeptide sequences may compromise the proper folding of the protein and lead to aberrant conformation (protein misfolding) that may result in inappropriate DNA-protein or protein-protein interactions, protein nuclear mislocalization, aggregation or degradation (Karlin et al., 2002).

1.5.1. Expanded PABPN1 is sequestered to OPMD intranuclear inclusions

The extension of polyalanine tracts leads to protein aggregation in OPMD and several other polyalanine disease models (Albrecht et al., 2004; Bachetti et al., 2005; Brown et al., 2005; Caburet et al., 2004; Nasrallah et al., 2004). Immunohistochemical analysis revealed that filamentous INIs of OPMD muscle nuclei contain PABPN1, poly(A) RNA, ubiquitin (Ub) and proteasome subunits (Becher et al., 2000; Calado et al., 2000b; Uyama et al., 2000). The overexpression of expPABPN1 in cellular and mouse models induces the formation of nuclear aggregates reminiscent of the typical OPMD INIs found in patients. However, the INIs observed in cells and mouse muscle nuclei are less condensed and organized than those seen in OPMD muscle biopsies (Corbeil-Girard et

al., 2005; Hino et al., 2004; Tavanez et al., 2005). Immunocytochemistry shows that OPMD INIs are different from nuclear speckles, considering their insoluble status and the fact they occupy mutually exclusive nuclear areas that do not overlap with SC35 domains (Abu-Baker et al., 2005).

1.5.2. Potential effect of polyalanine expansion on PABPN1 dysfunction

The expansion of polyalanine tracts has already been reported to result in a gain or complete to partial loss-of-function (Brown et al., 2005; Bruneau et al., 2001). It is conceivable that when expPABPN1 is aggregated, it becomes dysfunctional as it is trapped in INIs. However, a toxic gain-of-function or a loss-of-function is considered if the misfolded PABPN1 interacts aberrantly with critical nuclear factors and compromises their function. Aberrant DNA-protein or protein-protein interactions have been associated with transcriptional dysregulation in synpolydactyly type II (SPD) (Bruneau et al., 2001), congenital central hypoventilation syndrome (CCHS) (Bachetti et al., 2005) and holoprosencephaly type 5 (HPE) (Brown et al., 2005).

1.6. The aggregation process in OPMD

1.6.1. Polyalanine tract length and β-sheet formation

Protein aggregation appears to be specific to polyalanine expansions in polyalanine diseases. In addition to polyalanine expansion, missense mutations and deletions are associated with X-linked mental retardation (XLMR) (Nasrallah et al., 2004) and CCHS (Trochet et al., 2005). However, only polyalanine expansions induce protein aggregation in cellular models of these diseases (Nasrallah et al., 2004; Trochet et al., 2005). This

may imply that the polyalanine tract is an entity by itself responsible for protein aggregation regardless of the protein conformational change induced by the expansion. *In vitro*, polyalanine peptides containing 7 to 15 alanines undergo variable levels of conformational transition from a monomeric α-helix to a predominant macromolecular β-sheet. Those peptides harboring a number of alanines greater than 15 are completely converted from monomer to β-sheet. *In vivo*, expanded polyalanine tracts may exhibit a pronounced propensity to adapt β-sheet complexes that may promote stronger protein-protein interactions leading to amyloid-like fibrillar assemblies (or aggregates) (Blondelle et al., 1997; Scheuermann et al., 2003; Shinchuk et al., 2005a). Longer polyalanine tracts in the N-terminus of PABPN1 drastically reduce the lag phase of fibril formation. Therefore, the onset of protein aggregation seems to correlate with the length of the polyalanine sequence (Scheuermann et al., 2003).

1.6.2. PABPN1 oligomerization and polyadenylation contribute to INIs formation in OPMD

In cellular models, the overexpression of the wtPABPN1 and a mutant counterpart that lacks the entire polyalanine tract in the N-terminus leads to the formation of aggregate-like structures also referred to as nuclear speckles, given their co-localization with SC35 domains (Abu-Baker et al., 2005; Calado and Carmo-Fonseca, 2000; Tavanez et al., 2005). These observations suggest an intrinsic capacity for the protein to aggregate as well as the implication of other auxiliary domains than the polyalanine tract in the aggregation process. The first two potential domains described to act in concert with polyalanine stretches and facilitate aggregation are the oligomerization domains (OD1 and OD2) described above (section 1.3.1). Deletions (6 to 8 amino acids) in either of the

ODs prevent the formation of expPABPN1-induced nuclear aggregation (Fan et al., 2001). The other domains to be implicated in expPABPN1 aggregation are the RBD and the α-helical coiled-coil domain. Mutations in the expPABPN1's RBD or α-helical domain affecting respectively the RNA binding activity of the protein or its ability to stimulate PAP, significantly compromise the formation of aggregates (Tavanez et al., 2005). The ability of PABPN1 to form compact oligomeric particles *in vitro* (Keller et al., 2000), in the presence or absence of mRNA, suggests that both oligomerization and polyadenylation can work independently.

1.6.3. The involvement of molecular chaperones and proteasome activity in protein aggregation

The aggregation process may also be dictated by the cell type or the cellular compartment, as the efficacy and rate of the misfolded protein clearance may vary from a cell type to another and even in between the nuclear and cytoplasmic compartments (Bachetti et al., 2005; Rusmini et al., 2006; Trochet et al., 2005). This may be explained by differences in the molecular chaperones and proteasome activity that exist between different cell types and even between different nucleocytoplasmic compartments (O'Neill et al., 2006; Rusmini et al., 2006; Scott et al., 2003). For instance, even though expPABPN1 is ubiquitously expressed *in vivo*, it usually only forms nuclear inclusions in the affected skeletal muscles of OPMD patients (Brais et al., 1999).

1.7. The general role of molecular chaperones and the ubiquitin-proteasome pathway

In addition to poly(A)RNA, Ub and proteasome subunits, OPMD nuclear aggregates have been reported to recruit molecular chaperones such as heat shock proteins 70 and 40 (HSP70 and HSP40) (Bao et al., 2002; Calado et al., 2000b). The presence of these cellular components in aggregates suggests that expPABPN1 is recognized as an unfolded conformer and targeted by the proteasome degradation pathway. The activation of these cellular quality-control mechanisms that implicate the upregulation of heat shock proteins and protein degradation is important in protecting cells against harsh conditions such as heat shock, oxidative stress (Jaattela et al., 1998; Sakahira et al., 2002; Sherman and Goldberg, 2001) and in the case of OPMD the accumulation of potential toxic products that may interfere with cellular function and homeostasis. The formation of protein aggregates occurs when the cell's proteolytic systems and molecular chaperones, which normally prevent aggregation (HSP70 and HSP40) (Frydman, 2001; Hartl and Hayer-Hartl, 2002) or resolubilize microaggregates (HSP104) (Glover and Lindquist, 1998), cannot keep up with the rate of production of unfolded molecules.

1.7.1. The ubiquitin-proteasome degradation pathway

In all tissues, the majority of intracellular proteins are degraded by the ubiquitin-proteasome pathway (UPP). However, extracellular, cell surface and cytoplasmic proteins are taken up by endocytosis and degraded within lysosomes (Lecker et al., 2006). During the past two decades, the UPP has taken a center stage in the understanding of the control of protein turnover. The start point in a proteasome pathway is the conjugation of

Ub (a 76 amino acid protein) onto misfolded proteins. This ATP-dependent process first requires the activation of Ub by the Ub-activating enzyme (E1). Once activated the Ub that is bound to E1 is transferred to a Ub-conjugating enzyme (E2). Subsequently a Ub-ligase (E3) catalyses the transfer of the activated Ub from an E2 initially to a lysine in the target protein and later to lysines that are present in Ub yielding a substrate anchored chain of Ub molecules. The selectivity of the process is ensured by the E3 molecule that acts as a specificity factor (Goldberg, 2003; Lecker et al., 2006). Polyubiquitinated protein substrates are recognized and degraded by a large molecular complex, the 26S proteasome, which consists of a barrel-shaped proteolytic core of 20S capped at both ends by 19S regulatory complexes. The protein to be degraded is first recognized by the 19S regulatory subunits which cleaves the polyubiquitin chain off the substrate and uses ATP hydrolysis to unfold and translocate a linearized protein through the gate entry channel into the 20S central proteolytic chamber. After the substrate enters the 20S's central chamber, the polypeptide is cleaved by its proteolytic sites into small peptides that exit the particle (Lecker et al., 2006).

1.7.2. Molecular chaperones functional activities

1.7.2.1. A role for molecular chaperones in protein folding and degradation

In addition to their role in the proper folding of nonnative proteins, molecular chaperones participate in the degradation process of certain proteins. HSP70 chaperones cooperate with HSP40 and together they associate with exposed hydrophobic domains to promote the refolding of misfolded proteins. Most HSP40s recognize nonnative polypeptide segments and target them to HSP70 by a direct interaction. In addition, HSP40 activates

the HSP70 ATPase thereby catalyzing the formation of the ADP-state of HSP70 which binds protein substrate tightly (Hartl and Hayer-Hartl, 2002). Alternatively, unsuccessful folding and consequent prolonged association of the substrate with the chaperones can facilitate the ubiquitination and the recognition of abnormal proteins by the proteolytic machinery. The role of HSPs in protein degradation may be attributed to their activity in preventing the formation of intramolecular β-sheets by hydrophobic polypeptides and maintaining proteins in a degradation-competent state (Huang et al., 2001; Murata et al., 2001; Wickner et al., 1999). A novel ubiquitin ligase CHIP (carboxy terminus of HSP70-interacting protein) has been shown to cooperate with molecular chaperone HSP70 and HSP90 to facilitate substrate recognition by the 26S proteasome (Figure 4) (Frydman, 2001; Meacham et al., 2001; Murata et al., 2001). HSP70 and HSP40 were observed to enhance protein degradation in a SBMA cellular model (Bailey et al., 2002). Several other reports point to a role of the HSP70/40 chaperone system in the rapid degradation of proteins (Bercovich et al., 1997; Lee et al., 1996; Ohba, 1997).

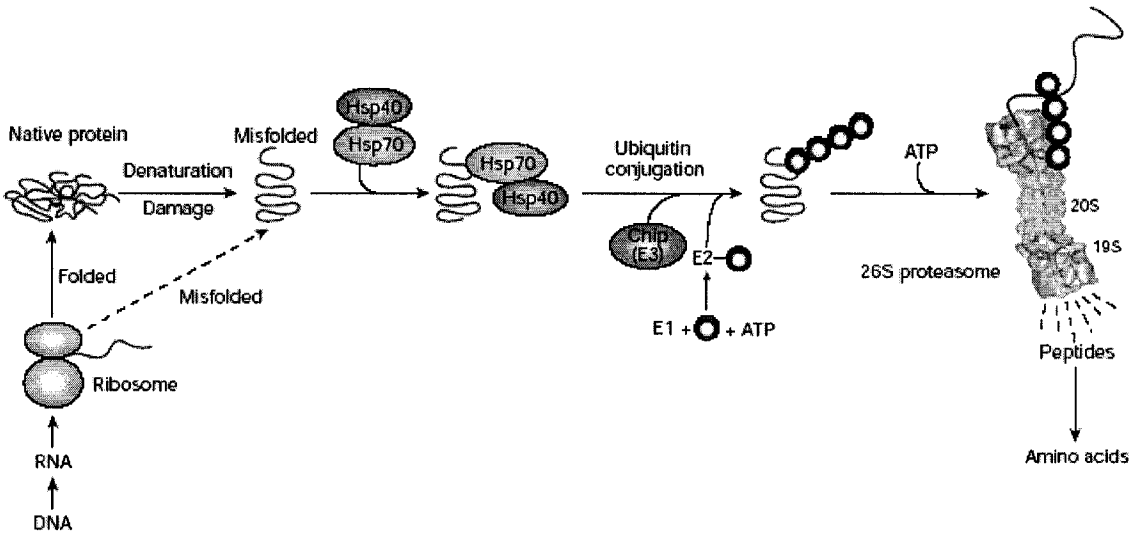

Figure 4: The ubiquitin proteasome pathway.

Molecular chaperones (HSP70 and HSP40) associate with exposed hydrophobic domains of misfolded proteins and promote their refolding. Alternatively, they can facilitate their recognition and degradation by the 26S proteosome. Reproduced from (Goldberg, 2003).

1.7.2.2. Molecular chaperones protect against apoptotic insults

The role of molecular chaperones in cellular resistance against stress was first suggested based on the correlation between HSP70 synthesis and the development of resistance to elevated temperatures (Li and Werb, 1982). More direct evidence supporting the role of HSP70 in thermotolerance came from studies showing a correlation between the inhibition of HSP70 expression or suppression of its function and a significant increase in cellular sensitivity to heat (Johnston and Kucey, 1988; Riabowol et al., 1988). The protective effect of HSP70 in response to adverse stress is mediated by their anti-apoptotic properties (Beere et al., 2000; Li et al., 2000). HSP70 protects cells from a number of apoptotic stimuli, including heat shock, tumor necrosis factor, growth factor withdrawal, oxidative stress, chemotherapeutic agents, ceramide, radiation and misfolded proteins build-up (Li et al., 2000). Apoptotic cell death is orchestrated by the activation of caspases, a family of cysteine proteases with specificity for aspartic acid residues, which cleave specific intracellular substrates to activate them and produce the characteristic features of apoptosis including membrane bledding, nuclear condensation and cell shrinkage (Jaattela et al., 1998; Wolf and Green, 1999). The initial step in caspase activation requires the release of cytochrome *c* from mitochondria which then in the presence of dATP/ATP triggers the oligomerization of the apoptotic protease activating factor 1 (Apaf-1), resulting in the formation of a so-called "apoptosome" (Zou et al., 1999). The apoptosome complex recruits and activates procaspase-9, which in turn recruits, cleaves and activates caspase-3 and caspase-7 (Cain et al., 1999; Hu et al., 1999). Recent evidence suggests that HSP70 inhibits apoptosis-associated nuclear changes, chromatin condensation and fragmentation by acting downstream of cytochrome *c* release

and thereby preventing the recruitment of procaspase-9 to the Apaf-1 apoptosome (Beere et al., 2000).

1.8 OPMD cellular toxicity: cellular and mouse models

When unfolded proteins build-up and exceed the cell's degradative capacity, the continued accumulation of misfolded proteins eventually triggers the activation of JNK kinases and apoptosis (Meriin et al., 1999; Sherman and Goldberg, 2001). It is now well established that damage to cell proteins just like damage to DNA or chromosomal organization can activate the cell-death programme. In OPMD the overexpression of an expPABPN1 coincides with protein aggregation and an increase in cellular toxicity in cellular and mouse models (Bao et al., 2002; Davies et al., 2005; Hino et al., 2004). An increase in the proportion of TUNEL-positive myocyte nuclei has been reported in two transgenic mouse models expressing the mutated protein in skeletal muscles (Davies et al., 2005; Hino et al., 2004). TUNEL-positive nuclei have been shown to be more concentrated in muscle fibers showing more severe degeneration (Hino et al., 2004). Bax upregulation and cytochrome c release have been observed respectively in OPMD mouse muscle and COS7 cells expressing the expPABPN1 and are consistent with apoptotic processes. The Bax protein permeabilizes mitochondria, leading to cytochrome *c* release and subsequent activation of caspase 9 and 3, after various proapoptotic insults. Thus, the increase in Bax levels and the cytochrome *c* release are relevant to mutant PABPN1-induced programmed cell death (Davies et al., 2005). Although degeneration has generally been accepted to occur by necrotic pathways, apoptosis seems to precede necrotic cell death during the phase of acute muscle degeneration (Tidball et al., 1995).

In human Duchenne muscular dystrophy, apoptotic nuclei were detected mainly in the interstitium. Apoptotic cells in the interstitium were identified as inflammatory cells and activated satellite cells, supporting the involvement of apoptosis in muscle degeneration (Sandri et al., 1998).

1.9. hnRNPs, the other RNA binding proteins

hnRNPs are the most abundant hnRNA-binding proteins in the nucleus. More than 20 hnRNP proteins named hnRNPA1 (34 KDa) through hnRNPU (120 KDa) have been identified by two-dimensional gel electrophoresis of human hnRNP complexes immunopurified with monoclonal antibodies initially raised against authentic hnRNA-binding proteins purified by *in vivo* UV cross-linking (Dreyfuss et al., 1993; Krecic and Swanson, 1999). The ability of hnRNPs to interact with themselves, other factors, as well as DNA and RNA strands allow them to accomplish many cellular functions in mammalian cells.

1.9.1. hnRNPs structure and RNA binding activity

Most of the hnRNP proteins appear to be highly conserved among vertebrates, both immunologically and structurally. The most prominent structural feature of hnRNPs is that they contain one or more RBDs and adjacent auxiliary domains that probably mediate protein-protein interactions. For instance, all of the hnRNPA/B proteins contain two RBDs and a glycine-rich auxiliary domain at the carboxyl terminus. Diversity among the hnRNPA/B proteins is generated by posttranslational modifications, including methylation of arginines and phosphorylation. hnRNPs are characterized by their RNA

sequence binding specificity, meaning they have different binding affinities to different RNA binding sites. Interestingly, high-affinity sites described so far correspond to sequences important for pre-mRNA processing. It is thus conceivable that hnRNA-hnRNP interactions at these sites may expose the RNA primer to different nuclear factors and ensure the formation of specialized complexes to perform essential functions in the processing pathways of hnRNAs. Cooperative protein-protein interactions between hnRNP proteins may contribute to the complete coating of the hnRNA with hnRNPs so that almost all of it become exposed (Dreyfuss et al., 1993).

1.9.2. Multiple functions for hnRNPs in the nucleus and the cytoplasm

Similar to PABPN1, hnRNP proteins are distributed throughout the nucleus and predominantly localized to perichromatin fibrils, rich in nascent RNA. This staining may represent hnRNPs bound to nascent RNAPII transcripts or to RNA that are at different stages of processing and suggests a role for hnRNPs in mRNA processing and transcription (Fakan et al., 1984; Fakan et al., 1986). Although mainly nuclear, some hnRNPs such as those in the A and B groups, shuttle between the nucleus and the cytoplasm. The presence of hnRNPs in the cytoplasm is transient and they rapidly reaccumulate in the nucleus (Borer et al., 1989). The shuttling of some hnRNPs suggests that these proteins may participate in mRNA nucleocytoplasmic export as well as cytoplasmic functions and highlights the dynamic nature of hnRNP complexes.

1.9.2.1. Role of hnRNPs in pre-mRNA splicing

Alternative pre-mRNA splicing is a daunting regulatory task that requires the coordination of many cellular components. The preferential use of some exons over

others in a particular gene allows for the production of distinct protein isoforms during development and in different cell type. Splice-site selection is strongly influenced by pre-mRNA structure including exon size, RNA secondary structure as well as enhancer and repressor elements in both exons and introns. Several intronic splicing enhancers and repressors associate with hnRNPs. For instance, hnRNPA1 can function as an exonic splicing repressor and it may influence alternative splicing of its own pre-mRNA by binding to a conserved intronic element involved in downstream exon skipping (Chabot et al., 1997; Del Gatto-Konczak et al., 1999). In neuronal cells, alternative splicing of the c-*src* N1 exon is regulated by an intronic enhancer which binds a complex of hnRNPs F and H, the KH-type splicing regulatory protein (KSRP), and a CU-rich repressor element that binds PTB/hnRNPI (Krecic and Swanson, 1999). The PTB/hnRNPI protein is an important regulator of alternative splicing for several other genes (Krecic and Swanson, 1999). Splice-site selection is also hnRNP type concentration-dependent. Interestingly, the higher amount of hnRNPA1 relative to the ASF/SF2 splicing factor, favors the use of distal 5' splicing sites over the 3' proximal sites of a pre-mRNA. Therefore, the activities of hnRNPA1 and ASF/SF2 may directly compete to determine splice site selection (Mayeda and Krainer, 1992).

1.9.2.2. hnRNPs transcriptional and telomerase activity

hnRNPs may regulate transcription either via direct DNA binding or protein-protein interactions. A role for hnRNPs in transcription was first reported for the hnRNPK protein which binds preferentially to single stranded DNA (ssDNA) *in vitro*. The hnRNPK protein activates transcription by binding to pyrimidine-rich elements located upstream of the regulatory region (Krecic and Swanson, 1999). It has been proposed that

DNA-hnRNPK interaction may promote remodeling of chromatin structure facilitating the interaction between promoter bound general transcription factors and factors bound to upstream activation sequences (UAS) (Krecic and Swanson, 1999). hnRNPK can also mediate transcriptional repression without prior ssDNA binding. It has been shown that hnRNPK forms a heterodimer with a CCAAT/enhancer binding protein (C/EBPβ) that inhibits transactivation of the *alpha-1 acid glycoprotein (agp)* gene, possibly by preventing the formation of an activator complex between C/EBPβ and a nucleolar RNA-binding protein, Nopp140 (Miau et al., 1998).

Given that many of hnRNP proteins can bind both ssDNA and RNA, they are also implicated in telomere length maintenance. *In vitro*, hnRNPA1, D and E proteins form a complex with oligonucleotides containing telomere repeats (Ishikawa et al., 1993). *In vivo*, it has been shown that transformed mouse erythroleukaemic cells deficient in hnRNPA1 possess substantially shorter telomeres than similar cells expressing hnRNPA1 and that restoring hnRNPA1 expression increases the length of telomeres (LaBranche et al., 1998). hnRNPA1 has two RRMs, RRM1 and RRM2, that respectively bind to telomeric DNA sequences and the RNA component of telomerase. This simultaneous interaction may help to position the telomerase in preparation for the extension of 3' overhangs (Fiset and Chabot, 2001). Previous studies have also suggested that the binding of hnRNPA1 may help shield the end of the chromosomes from nucleolytic attack and from surveillance mechanisms that detect double-stranded DNA breaks (Dallaire et al., 2000).

1.9.2.3. The involvement of hnRNPs in mRNA nucleocytoplasmic export, translation and turnover

Electron microscope observations of BR mRNAs in *C. tentans* have shown that hnRNP proteins are transported to the cytoplasm as a ribonucleoprotein particle (Kiseleva et al., 1997; Mehlin et al., 1992; Mehlin et al., 1991). Their exit from the nucleus bound to mRNA suggests their role in nucleocytoplasmic export of RNA.

In the cytoplasm hnRNPs have been shown to be implicated in translational repression. The hnRNPK and PCBP1/hnRNPE1 and PCBP2/hnRNPE2 proteins bind CU-rich repeat motif in the 3' UTR of mammalian erythroid 15-lipoxygenase (LOX) poly(A) RNA. The formation of the hnRNP-RNA complex subsequently blocks 80S ribosome complex assembly on LOX mRNA (Ostareck et al., 1997).

Several major hnRNPs, including the C, D, L and PCBP/hnRNPE proteins also bind to RNA elements, such as AU-rich element (ARE), that regulate mRNA turnover (Kiledjian et al., 1997; Rajagopalan et al., 1998). For instance, the hnRNPD and the PCBP/αCP proteins are part of the α-globin mRNA stability complex which binds to a C-rich 3' UTR RNA element required for the exceptional stability of α-globin mRNA (Kiledjian et al., 1997).

1.10. Objectives of proposed thesis project

The expression of PABPN1 with a polyalanine expansion induces the formation of filamentous nuclear aggregates in cellular and mouse models reminiscent of those observed in OPMD patients (Corbeil-Girard et al., 2005; Hino et al., 2004; Tavanez et al., 2005). Whether these INIs are pathological or simply the cellular response against

misfolded proteins build-up is still not clear in OPMD. Regardless of protein aggregation, the polyalanine extention may either confer to PABPN1 a toxic gain-of-function or result in a partial or complete loss-of-function. In order to explore mechanisms underlying OPMD pathogenesis, we designed many experimental procedures to attain three fundamental objectives:

Objective 1: Identification of PABPN1 interacting partners in OPMD

We hypothesized that the identification of proteins that interact with PABPN1 might not only provide us with more information concerning normal PABPN1 cellular function but it might also sheld light on the molecular pathogenesis of OPMD. We therefore used a readily available human fetal brain cDNA library to look for PABPN1 interacting proteins in a yeast two-hybrid screen. Any direct interaction between PABPN1 and unreported cellular partners would subsequently be confirmed by other protein-protein interaction assays (GST-pull down and co-immunoprecipitation). Given that PABPN1 interacting proteins may be sequestered to INIs; immunocytochemistry and immunohistochemistry were expected to be used to observe if this is indeed the case.

Objective 2: Investigation of the role of molecular chaperones and the ubiquitin-proteasome degradative pathway in OPMD

OPMD nuclear aggregates recruit molecular chaperones, Ub and proteasome subunits (Bao et al., 2002; Calado et al., 2000b). We hypothesized that alterations of the molecular chaperones level and the proteasome activity may be involved in OPMD pathogenesis. If that is indeed the case, protein aggregation and cellular toxicity in OPMD may end up being accentuated by a build-up of misfolded PABPN1 that would overwhelm the cell's

defense mechanisms. To demonstrate the role of molecular chaperones and proteasome components in OPMD, we planned to 1) inhibit the proteasome and 2) overexpress HSP70 and HSP40 (independently or simultaneously). We suspected that proteasome inhibition would lead to an increase in protein aggregation and altered expression levels of HSPs and ubiquitin conjugates. Protein aggregation and protein expression level would be assessed using immunocytochemistry, immunohistochemistry, cell count analysis and Western blotting.

We also expected that the induction of molecular chaperones would decrease the formation of nuclear aggregates and consequently increase expPABPN1 solubility which would be analyzed using solubility analysis by filter retardation assays. As molecular chaperones are known to have multiple protective properties (Beere et al., 2000; Li et al., 2000), we were also expecting them to protect the cells against expPABPN1-induced cellular toxicity. The latter would be determined using a cell viability assay.

Objective 3: Investigation of the role of nuclear aggregates versus the soluble misfolded PABPN1 in OPMD cellular toxicity

The expansion of the polyalanine tract in PABPN1 could produce aberrant protein-protein interactions that may lead to a gain or a loss-of-funtion phenotype. For instance, expPABPN1 aggregates or soluble expPABPN1 may abnormally bind to critical nuclear factors involved in muscle differentiation and compromise their function. To evaluate the contribution of both soluble expPABPN1 and expPABPN1 aggregates in cellular toxicity, we first planned to target three independent mechanisms that are known to modulate expPABPN1 aggregation: (1) poly(A) RNA binding (Khun et al., 2003), (2) polyadenylation (Tavanez et al., 2005) and (3) ubiquitination (Abu-Baker et al., 2003).

We therefore planned to generate PABPN1 mutant proteins that have reduced poly(A) RNA binding activity and capacity to stimulate polyadenylation, using site-directed mutagenesis. To decrease protein ubiquitination we used a dominant negative form of the human ubiquitin-conjugating enzyme hCDC34(CL→S) (Saudou et al., 1998). The expression of PABPN1 mutant proteins with a decreased RNA binding activity or a polyadenylation defect, as well as the co-expression of the hCDC34(CL→S) with expPABPN1 was expected to increase the solubility of expPABPN1. We therefore planned to assess the protein solubility and the degree of aggregation using immunocytochemistry, KCl treatment and cell count analysis. Cellular toxicity will be determined using a cell viability assay.

To futher investigate the role of nuclear aggregates in OPMD without necessarily interfering with any cellular pathway, we decided to use a live-cell imaging system.

Chapter 2: *Identification of PABPN1 interacting partners in OPMD*

Xueping Fan[1], Christiane Messaed[1], Patrick Dion[1], Janet Laganiere[1], Bernard Brais[2], George Karpati[3] and Guy A. Rouleau[1]. hnRNPA1 and A/B interaction with PABPN1 in Oculopharyngeal Muscular Dystrophy. *Can J Neuro Sci.* 2003; 30(3): 244-51.

2.1 Rationale

OPMD is a myopathy that affects all voluntary muscles (Little and Perl, 1982). Electron microscopy analysis of circopharyngeal and deltoid muscles of OPMD patients reveals the presence of INIs within muscle fiber nuclei (Brais et al., 1999; Tome and Fardeau, 1986). The overexpression of expPABPN1 in cellular and mouse models induces the formation of nuclear aggregates reminiscent of the typical OPMD INIs found in patients (Corbeil-Girard et al., 2005; Hino et al., 2004; Tavanez et al., 2005). A gain-of-function may be considered if nuclear aggregates sequester cellular factors and alter their function. Alternatively, the soluble expPABPN1 may lead to aberrant protein-protein interactions or lose its ability to bind crucial cellular components. Identifying proteins that interact with PABPN1 may therefore shed light on new cellular pathways that PABPN1 may be involved in and therefore help us identify molecular mechanisms underlying OPMD.

2.2 Abstract

Background: Oculopharyngeal muscular dystrophy (OPMD) is an adult-onset disorder characterized by progressive ptosis, dysphagia and proximal limb weakness. The autosomal dominant form of this disease is caused by short expansions of a $(GCG)_6$ repeat to $(GCG)_{8-13}$ in the PABPN1 gene. The mutations lead to the expansion of a polyalanine stretch from 10 to 12-17 alanines in the N-terminus of PABPN1. The mutated PABPN1 (mPABPN1) induces the formation of intranuclear filamentous inclusions that sequester poly(A) RNA and are associated with cell death. ***Methods:*** Human fetal brain cDNA library was used to look for PABPN1 binding proteins using yeast two-hybrid screen. The protein interaction was confirmed by GST pull-down and co-immunoprecipitation assays. Oculopharyngeal muscular dystrophy cellular model and OPMD patient muscle tissue were used to check whether the PABPN1 binding proteins were involved in the formation of OPMD intranuclear inclusions. ***Results:*** We identify two PABPN1 interacting proteins, hnRNPA1 and hnRNPA/B. When co-expressed with mPABPN1 in COS-7 cells, predominantly nuclear protein hnRNPA1 and A/B co-localize with mPABPN1 in the insoluble intranuclear aggregates. Patient studies showed that hnRNPA1 is sequestered in OPMD nuclear inclusions. ***Conclusions:*** The hnRNP proteins are involved in mRNA processing and mRNA nucleocytoplasmic export, sequestering of hnRNPs in OPMD intranuclear aggregates supports the view that OPMD intranuclear inclusions are "poly(A) RNA traps", which would interfere with RNA export, and cause muscle cell death.

Résumé

Interaction de hnRNPA1 et A/B avec PABPN1 dans la dystrophie musculaire oculopharyngée. ***Introduction*****:** La dystrophie musculaire oculopharyngée (DMOP) est une maladie de l'âge adulte caractérisée par une ptose progressive des paupières, une dysphagie et une faiblesse musculaire proximale. La forme autosomique dominante est causée par de courtes expansions d'une répétition $(GCG)_6$ à $(GCG)_{8-13}$ dans le gène PABPN1. Les mutations donnent lieu à une expansion d'un tractus de polyalanine de 10 à 12-17 alanines dans la partie N-terminale de PABPN1. Le gène PABPN1 muté (PABPN1m) induit la formation d'inclusions filamenteuses intranucléaires qui séquestrent l'ARN poly(A) et entraînent la mort cellulaire. ***Méthodes***: Une librairie d'ADNc provenant de cerveau foetal humain a été utilisée pour chercher la protéine liant PABPN1 au moyen du système à double-hybrides dans la levure. L'interaction protéine-protéine a été confirmée par GST pull-down et co-immunoprécipitation. Le modèle cellulaire de DMOP et le tissu musculaire provenant de patients atteints DMOP ont été utilisés pour vérifier si les protéines liant PABPN1 étaient impliquées dans la formation des inclusions intranucléaires dans la DMOP. ***Résultats***: Nous avons identifié deux protéines interagissant avec PABPN1, hnRNPA1 et hnRNPA/B. En co-expression avec PABPN1m dans des cellules COS-7, les protéines hnRNPA1 et A/B à prédominance nucléaire se retrouvent avec PABPN1m dans les agrégats intranucléaires insolubles. Des études chez les patients atteints de DMOP ont montré que hnRNPA1 est séquestré dans les inclusions nucléaires. ***Conclusions***: Les protéines hnRNP sont impliquées dans la maturation de l'ARNm et le transport nucléocytoplasmique de l'ARNm. La séquestration de hnRNPs dans les agrégats intranucléaires appuie l'hypothèse selon laquelle les

inclusions intranucléaires de la DMOP sont des "piètes à ARN poly(A)" qui interfèrent avec le transport de l'ARN et causent la mort des cellules musculaires.

2.3 Introduction

Autosomal dominant oculopharyngeal muscular dystrophy (OPMD) is an adult-onset disease that presents in the fifth or sixth decade. The disease is characterized by progressive eyelid drooping (ptosis), swallowing difficulties (dysphagia) and proximal limb weakness (Blumen et al., 2000; Brais et al., 1999; Grewal et al., 1999; Mirabella et al., 2000; Muller et al., 2001; Nagashima et al., 2000). Pathological studies showed the presence of unique intranuclear filamentous inclusions in skeletal muscle fibers of OPMD patients (Tome et al., 1997; Tome and Fardeau, 1980). The OPMD locus was mapped by linkage analysis to chromosome 14q11.1 (Brais et al., 1997; Brais et al., 1995; Xie et al., 1998) and the gene was identified as PABPN1, encoding the poly(A) binding protein nuclear 1 (PABPN1, PABP2, PAB II) (Brais et al., 1998). Dominant OPMD is caused by expansion of a short GCG trinucleotide repeat in the PABPN1 gene. The normal PABPN1 gene has a $(GCG)_6$ trinucleotide repeat coding for a polyalanine stretch at the 5' end, while in OPMD patients this $(GCG)_6$ repeat is expanded to $(GCG)_{8\text{-}13}$. Due to the presence of GCA GCA GCA GCG coding sequences adjacent to the (GCG)6 repeat, the wild-type PABPN1 has a 10 alanine stretch in its N-terminus, while the mutant proteins have 12 to 17 alanines. In addition to OPMD, at least five other diseases are associated with alanine stretch expansions in the disease gene products. Synpolydactily is caused by an alanine stretch expansion from 15 to 22-25 in the HOXD13 gene (Muragaki et al., 1996), while cleidocranial dysplasia is associated with an expansion from 17 to 27 alanines in CBFA1 protein (Mundlos et al., 1997). The alanine stretch lengthening from 15 to 25 in ZIC2 protein results in holoprosencephaly (HPE) (Brown et al., 1998), whereas the ones expanded from 15 to 22-33 in HOXA13 protein and from 14 to 24 in

FOXL2 protein cause hand-foot-genital syndrome (Goodman et al., 2000) and type II blepharophimosis/ptosis/epicanthus (Crisponi et al., 2001) respectively. Among these alanine-expanded proteins, mutated PABPN1 (mPABPN1) is the only one that is reported to induce the formation of intranuclear inclusions. Oculopharyngeal muscular dystrophy intranuclear inclusions are similar to those found in a number of inherited neurodegenerative diseases caused by mutated proteins with an expanded polyglutamine (polyQ) stretch encoded by a CAG repeat. The mutant form of each protein typically has a polyQ tract of greater than 40 glutamine residues, whereas the wild-type protein may contain around 20 glutamines (Ferrigno and Silver, 2000). This group of disorders includes Huntington's disease (1993), spinobulbar muscular atrophy (La Spada et al., 1991), dentatorubral pallidoluysian atrophy (Koide et al., 1994; Nagafuchi et al., 1994), and the spinocerebellar ataxias type 1, 2, 3, 6 and 7 (David et al., 1997; Imbert et al., 1996; Kawaguchi et al., 1994; Koob et al., 1998; Orr et al., 1993; Pulst et al., 1996; Sanpei et al., 1996).

PABPN1 has 306 amino acids and is comprised of an alanine stretch and a proline-rich region in the N-terminus, a ribonucleoprotein (RNP)-type RNA binding domain in the central region, and an arginine-rich C-terminus. PABPN1 is an abundant nuclear protein that binds with high affinity to the poly(A) tail of mRNA and is involved in mRNA polyadenylation (Wahle, 1991), a two-step reaction whereby endonucleolytic cleavage of the nascent mRNA transcript is followed by an addition of ~250 adenylate residues to the up-stream cleavage product (Barabino and Keller, 1999; Colgan and Manley, 1997; Minvielle-Sebastia and Keller, 1999). Poly(A) tail synthesis is catalyzed by poly(A) polymerase through interaction with CPSF, the cleavage and polyadenylation specificity

factor. However, this process is slow and inefficient, and the length of poly(A) tail is poorly controlled. Adding PABPN1 to this reaction will stimulate poly(A) synthesis and control the size of the tail to be ~250 nt in length (Bienroth et al., 1993; Wahle, 1991; Wahle, 1995).

PABPN1 has been identified as a component of the filamentous inclusions present in the nuclei of OPMD muscle fibers (Becher et al., 2000; Calado et al., 2000b; Uyama et al., 2000). Expression of mPABPN1 in COS-7 cells induces the formation of intranuclear protein aggregation that is associated with cell death (Bao et al., 2002; Fan et al., 2001; Kim et al., 2001; Shanmugam et al., 2000). PABPN1 contains two oligomerization domains that make the protein form oligomers (Fan et al., 2001). Oligomerization of mPABPN1 facilitates the formation of OPMD nuclear inclusions (Fan et al., 2001). These inclusions contain ubiquitin, the subunits of the proteasome, poly(A) RNA (Calado et al., 2000b), and PABPN1 interacting protein SKIP (Kim et al., 2001). A recent study showed that overexpression of chaperone proteins can reduce aggregation of mPABPN1 and cell death (Bao et al., 2002). To further investigate how OPMD intranuclear protein aggregates cause cell death, we searched for PABPN1 interacting proteins using yeast two-hybrid library screen and found that both heterogeneous nuclear ribonucleoprotein (hnRNP) A1 and A/B interact with the C-terminus of PABPN1. These hnRNP proteins are sequestered in the OPMD nuclear inclusions. The hnRNPs are mRNA binding proteins and involved in mRNA export from the nucleus to the cytoplasm (Nakielny and Dreyfuss, 1999). The fact that OPMD intranuclear aggregates sequester hnRNP proteins supports the hypothesis that the OPMD aggregates impair mRNA nucleocytoplasmic export and cause cell death.

2.4 Materials and methods

Yeast two-hybrid screening and cloning of hnRNPA1 and A/B

The DupLEX-A^{TM} yeast two-hybrid system (OriGene Tech, Rockville, MD) was used to identify PABPN1 interacting proteins. To generate bait for the yeast two-hybrid screening, the cDNA encoding the C-terminus of PABPN1 from amino acids 249 to 306 was cloned with *Eco*R1/*Ba*mH1 into vector pEG202NLS that allows the fusion of the bait to the DNA binding domain of LexA. A human fetal brain cDNA library (OriGene Tech, Rockville, MD) was constructed in vector pJG4-5 that fuses proteins to the transcription activation domain of B42. The *lacZ* gene in construct pSH18-34 and *LEU2* gene in the genome of yeast strain EGY48 were used as reporter genes. The pEG202NLS-AA249-306, pSH18-34 and library construct pJG4-5 were co-transformed into EGY48. The clones were characterized as positive if the yeast cells turn blue in the presence of X-gal and grow in the absence of leucine. EST clones 549503 and 610850 encoding the full-length cDNA of hnRNPA1 and hnRNPA/B, respectively, were obtained from IMAGE. The full-length human PABPN1 cDNA and the cDNA encoding AA287-306 of PABPN1 were cloned into vector pEG202 with *Eco*R1/*Ba*mH1. The full-length cDNAs of hnRNPA1 and A/B were cloned into vector pJG4-5 with *Eco*R1/*Xho*1. All constructs were verified by DNA sequencing. β –galactosidase liquid assays was performed exactly as described (Fan et al., 2001). The β-galactosidase unit is calculated using the formula $[A_{420} \times 1000] / A_{600} \times$ time (in min) x volume (in ml)].

In vitro binding assay

To make GST-PABPN1 fusion protein, the coding cDNA of wtPABPN1 was cloned into the GST vector pGEX-5X-1 (Amersham Pharmacia Biotech, Piscataway, NJ) allowing the fusion of GST to the N-terminus of PABPN1. The construct was verified by DNA sequencing and transformed into *E. Coli XL1-Blue*. The Bulk and RediPack GST purification modules (Amersham Pharmacia Biotech, Piscataway, NJ) were used for expression and purification of GST-PABPN1 according to manufacturer's instructions. Purified proteins were analyzed by Western blotting using anti-GST antibody. The cDNAs of hnRNPA1 and A/B were cloned with *Eco*R1/*Xho*1 into the vector pJG4-6 that allows the fusion of the HA tag to the N-termini of A1 and A/B. HA tagged hnRNPA1 and A/B were expressed in the yeast strain EGY48 and confirmed by Western blotting using the antibody against the HA epitope. GST pull-down assay was performed exactly as described (Fan et al., 2001).

Immunofluorescence

The cDNAs encoding the wild-type PABPN1 (wtPABPN1) and polyalanine-expanded PABPN1 with 17 alanines (mPABPN1) were cloned with *Eco*R1/*Ba*mH1 into the GFP (green fluorescent protein) vector pEGFP-C2 (Clontech, Palo Alto, CA) which allows the fusion of GFP to the N-terminus of PABPN1 proteins. The cDNAs of hnRNPA1 and A/B were cloned into pEGFP-C2 (with the GFP gene removed) using *Eco*R1-*Xho*1. The HA tag sequence was inserted into the construct at the *Eco*R1 site. The constructs were verified by DNA sequencing. COS-7 cells were transfected or co-transfected using Lipofectamine reagent (Gibco BRL, MD) according to the manufacturer's instructions. In 72 hours, the transfected cells were fixed with 4% paraformaldehyde and

immunodetection was performed using an anti-HA antibody (Santa Cruz Biotech, CA) (1:500). Rhodamine conjugated secondary antibody (1:500) was used, and the signals were visualized using a fluorescent microscope. To remove the soluble proteins, the co-transfected cells were treated with 0.5M potassium chloride (KCl) in HPEM (30 mM HEPES, 65 mM Pipes, 10 mM EDTA, 2 mM MgCl 2 , pH 6.9) for five minutes at room temperature. Then the cells were fixed and immunodetected using an anti-HA antibody. The microtome sections of paraffin-embedded deltoid muscle from an OPMD patient and a control subject were used. Sections were deparaffinized, permeabilized and immunostained with polyclonal anti-PABPN1 antibody or monoclonal antibody against hnRNPA1. Rhodamine conjugated secondary antibody was used and the signal was visualized using a fluorescent microscope. To remove the soluble proteins, the deparaffinized sections were treated with 1 M KCl in HPEM for five minutes at room temperature before the immunostaining was performed.

Immunoprecipitation

In 48 hours post-transfection, the COS-7 cells co-expressing GFP-wtPABPN1 and HA-hnRNPA1 or A/B were washed twice with ice-cold phosphate buffer, lysed using Nonidet-P-40 lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 10% glycerol, 0.5% NP-40, protease inhibitors) and further disrupted by homogenizing. The lysate was briefly spined. The supernatant was added with DNase 1 and RNase A to remove DNA and RNA, and incubated overnight at 4°C with 5 μg of polyclonal GFP antibody (Clontech, Palo Alto, CA) and 50 μl of Protein A Sepharose 4 Fast Flow suspension (Amersham Pharmacia Biotech, Piscataway, NJ). The beads were washed six times with washing

buffer (50 mM Tris pH 7.5, 150 mM NaCl, 10% glycerol, 0.1% NP-40) and suspended in 40 µl of protein sample buffer and heated to 95°C for five minutes. The immuno-precipitates were resolved on 12% SDS-PAGE, probed with anti-HA antibody. The same membrane was stripped and re-probed with anti-GFP antibody.

2.5 Results

hnRNPA1 and A/B interact with PABPN1

The yeast two-hybrid system was used to search for proteins interacting with the C-terminal domain of PABPN1 (AA249-306) that excludes the putative RNA binding domain. $3x10^6$clones were screened, and 20 positive clones were isolated from a human fetal brain cDNA library, seven of which encoded hnRNPA1, and five encoded hnRNPA/B. The hnRNPA/B was previously named C-type hnRNP protein (Kumar et al., 1987), and subsequently renamed to type A/B hnRNP protein (Khan et al., 1991). The full-length cDNA for hnRNPA1 and A/B were obtained from Integrated Molecular Analysis of the Human Genome and its Expression (I.M.A.G.E.) (ID A1: 549503; A/B: 610850), and the full-length hnRNPA1 and A/B proteins were tested for interacting to PABPN1. Both full-length PABPN1 and its C-terminus strongly interact with hnRNPA1 or A/B, while the last 20 amino acid segment (AA287-306) does not interact with either hnRNPA1 or A/B (Figure 1).

Interaction between PABPN1 and hnRNPA1 or A/B was confirmed by GST pull-down and immunoprecipitation assays

To confirm the observed interaction, we performed a GST pull-down assay. PABPN1 was fused to GST, while hnRNPA1 and A/B were HA tagged. The GST tagged proteins were incubated with HA tagged proteins and pulled down by glutathione sepharose 4B beads. The pulled-down proteins were resolved on SDS-PAGE, and blotted with anti-HA antibody. GST-PABPN1 is able to pull-down HA tagged hnRNPA1 and A/B while GST alone is not (Figure 2). In co-immunoprecipitation assay, the wild-type PABPN1

(wtPABPN1) was fused to the green fluorescent protein (GFP), while hnRNPA1 and A/B were HA-tagged. GFP and HA fusion proteins were co-expressed in COS-7 cells. The cells were homogenized and the lysates were treated with DNase 1 and RNase A to digest the DNA and RNA. The GFP fusion proteins were immunoprecipitated using GFP polyclonal antibody, and the immunoprecipitates were resolved on SDS-PAGE and blotted with anti-HA antibody. Both HA tagged hnRNPA1 and A/B are co-immunoprecipitated with GFP-wtPABPN1, but cannot be precipitated using polyclonal anti-histidine antibody (Figure 3).

Polyalanine-expanded PABPN1 induces insoluble intranuclear aggregates

It was reported that the expression of polyalanine-expanded PABPN1 (mPABPN1) in COS-7 cells induces large insoluble intranuclear protein aggregates (Bao et al., 2002; Fan et al., 2001; Kim et al., 2001; Shanmugam et al., 2000). We expressed GFP-wtPABPN1, GFP-mPABPN1 (with 17 alanines), HA-hnRNPA1 and HA-hnRNPA/B in COS-7 cells. Three days post-transfection, the transfected cells were either fixed or treated with 0.5 M KCl in HPEM before fixation. Potassium chloride treatment can remove soluble proteins that are not associated with insoluble pathological protein aggregates (Calado et al., 2000b). The HA tagged proteins were immunodetected using the anti-HA antibody and a rhodamine conjugated secondary antibody. All the expressed proteins are predominantly localized to the nucleus (Figure 4). GFP-wtPABPN1, HA-hnRNPA1 and A/B are soluble proteins and are therefore not resistant to KCl treatment. However, the aggregates induced by mPABPN1 are resistant to KCl treatment.

hnRNPA1 and A/B co-localize with mPABPN1 to the intranuclear insoluble aggregates

The hnRNP proteins are predominantly nuclear RNA binding proteins and distribute throughout the nucleoplasm (Figure 4). In order to examine whether hnRNPA1 and A/B are involved in intranuclear protein aggregation induced by mPABPN1, the HA tagged hnRNP proteins were co-expressed with GFP-mPABPN1, and detected by immunocytochemistry using anti-HA antibody and a rhodamine conjugated secondary antibody. Without KCl treatment, A1 and A/B are predominantly localized to the nucleus (Figures 4 and 5). After KCl treatment, the soluble A1 and A/B are removed, but the KCl resistant aggregates still contain hnRNPA1 and A/B signals (Figure 5). These signals co-localize completely with mPABPN1 aggregates (see the gold aggregates in Figure 5).

Insoluble OPMD nuclear inclusions contain hnRNPA1

PABPN1 in nuclear inclusions of the muscle from OPMD patients is insoluble and resistant to KCl treatment, whereas the protein localized in the nucleoplasm is solubilized (Calado et al., 2000b; Fan et al., 2001). In order to confirm whether hnRNPA1 is part of the insoluble nuclear inclusions from the OPMD patient, we detected the presence of PABPN1 and hnRNPA1 in muscle nuclei from an OPMD patient and a control subject using immunohistochemistry (Figure 6). In the control subject, both PABPN1 and hnRNPA1 are soluble and not resistant to KCl treatment (Figures 6B and 6D), whereas in the OPMD patient, both PABPN1 and hnRNPA1 localize to the insoluble nuclear inclusions that are resistant to KCl treatment (Figures 6F and 6H). We also performed co-immunostaining using polyclonal anti-PABPN1 and monoclonal anti-A1 antibodies. Unfortunately, these two antibodies were not compatible and failed to pick up PABPN1

and A1 on the same section. The presence of hnRNPA/B in OPMD nuclear inclusions could not be detected due to the lack of efficient anti-hnRNPA/B antibody.

2.6 Discussion

The yeast two-hybrid library screen is an efficient and sensitive method to look for protein interacting partners but, with RNA binding proteins, false positive results may occur since RNA can bridge two RNA binding proteins together. It was previously shown that a double point mutant of PABPN1 in the putative RNA binding domain (Y to A at position 175, F to A at position 215) loses its RNA activity (Calado et al., 2000a), indicating that the C-terminus AA249-306 does not have RNA binding activity. We therefore used the C-terminus of PABPN1 from AA249-306, excluding the putative RNA binding domain, as bait to screen the cDNA library, and identified PABPN1 interacting proteins hnRNPA1 and A/B. The observations were further confirmed by GST pull-down and immunoprecipitation assays, indicating the interactions between PABPN1 and these hnRNP proteins are real, and not false positives caused by their RNA binding activities. PABPN1 coats the RNA poly(A) tail, while hnRNP proteins bind to the rest of the RNA molecule. The binding domain in PABPN1 for hnRNPA1 or A/B is located in the C-terminus that does not overlap the putative RNA binding domain, suggesting that PABPN1 is able to interact with these hnRNP proteins while binding to the poly(A) tail. We have recently found that the last 18 residues of PABPN1 from AA289-306 serve as a nuclear localization signal (NLS) for this protein (unpublished observation). The segment of PABPN1 from AA287-306 does not interact with hnRNPA1 and A/B, implying that the NLS of PABPN1 is not the binding domain for these two hnRNP proteins.

PABPN1 is an abundant nuclear protein that normally forms nuclear speckles (Krause et al., 1994), while expression of mPABPN1 with expanded polyalanines in cells induces the formation of large intranuclear aggregates (Bao et al., 2002; Fan et al., 2001; Kim et

al., 2001; Shanmugam et al., 2000). hnRNPA1 and A/B are also predominantly localized to the nucleus, but do not form speckles (Figure 4). PABPN1 present in the OPMD inclusions is resistant to KCl treatment, like other pathological aggregates that are typically insoluble in KCl (Calado et al., 2000b). The wtPABPN1, hnRNPA1 or A/B expressed in COS-7 cells are not resistant to KCl treatment, while the large intranuclear aggregates induced by mPABPN1 are. These observations suggest that intranuclear aggregates induced by mPABPN1 have the same insoluble property as the ones found in OPMD patients. When hnRNPA1 and A/B are co-expressed with mPABPN1 in COS-7 cells, the hnRNP proteins co-localize to the aggregates that are resistant to KCl treatment. Studies on muscle tissue from OPMD patients also showed that hnRNPA1 is localized to the insoluble OPMD nuclear inclusions. Thus, we conclude that OPMD intranuclear inclusions sequester hnRNP proteins that interact with PABPN1.

Oculopharyngeal muscular dystrophy is caused by the expansion of a polyalanine stretch in PABPN1 from 10 to 12-17 residues (Brais et al., 1998), which leads to intranuclear protein aggregation and cellular toxicity (Bao et al., 2002; Fan et al., 2001; Kim et al., 2001; Shanmugam et al., 2000). In addition to genetic evidence, PABPN1 is detected in OPMD nuclear inclusions using immunohistochemistry (Becher et al., 2000; Calado et al., 2000b), suggesting a direct role in protein aggregation. We have recently found two oligomerization domains that are located in the C-terminus of PABPN1 far from the polyalanine stretch. Inactivating oligomerization by deletions in either of the oligomerization domains prevents intranuclear protein aggregation and reduces death rate of the cells expressing the non-oligomerizing mPABPN1 (Fan et al., 2001). Those observations suggest that OPMD intranuclear aggregates might be toxic and critical in

initiating OPMD pathogenesis. That raises the question of why OPMD intranuclear aggregates are toxic. Patient studies showed that OPMD nuclear inclusions sequester poly(A) RNA, suggesting that these inclusions might be "mRNA traps" that interfere with mRNA export (Calado et al., 2000b). Using the N-terminus of PABPN1 as bait in yeast two-hybrid screen, Kim *et al* (Kim et al., 2001) recently identified a PABPN1 interaction with SKIP, a potential co-transcription factor working with PABPN1 in muscle cells to stimulate muscle-specific gene expressions. SKIP might be sequestered in the OPMD intranuclear inclusions. Identification of hnRNP proteins in OPMD intranuclear inclusions suggests that: 1) OPMD intranuclear inclusions sequester PABPN1 interacting proteins; 2) OPMD intranuclear inclusions interfere with mRNA export as hnRNP proteins are involved in this function.

The hnRNP proteins are predominantly nuclear RNA-binding proteins associated specifically with pre-mRNA and mRNA molecules (Dreyfuss et al., 1993). The nucleocytoplasmic shuttling protein, hnRNPA1 has been reported to play a direct role in mRNA export (Nakielny and Dreyfuss, 1997; Pinol-Roma and Dreyfuss, 1992; Visa et al., 1996). PABPN1 is also a shuttling protein, coating the poly(A) tail of mRNA in the nucleus, and is suggested to involve in mRNA export (Calado et al., 2000a). hnRNPA1 and A/B were identified using the C-terminus of PABPN1, suggesting that the hnRNPs can interact with the wild-type PABPN1. The interaction between hnRNPA1 and PABPN1 is probably required for packaging mRNA for export. Identifying hnRNPA1 in OPMD intranuclear aggregates provides evidence that OPMD aggregates may interfere with mRNA export.

The hnRNPA1 protein is also involved in pre-mRNA splicing, telomere length maintenance, transcription regulation and pre-mRNA 3' end processing (Krecic and Swanson, 1999). Sequestering some hnRNPA1 molecules in the OPMD aggregates probably reduce the normal concentration of this protein in the nucleus and affect its functions. It could also be detrimental to the cell if, to carry out its normal function, hnRNPA1 needs its interacting partner PABPN1 which is largely aggregated in the OPMD inclusions. Therefore, it is possible that the intranuclear inclusion might interfere with hnRNP functions. The hnRNPA/B is homologous to hnRNPA1 (Dreyfuss et al., 1993; Khan et al., 1991). The binding of hnRNPA/B to RNA disrupts the residual secondary structure of RNAs (Dreyfuss et al., 1993). Its isoform protein was cloned in 1997 and named ABBP-1 (Lau et al., 1997). ABBP-1 binds apolipoprotein B (apoB) mRNA and is involved in apoB pre-mRNA editing (Lau et al., 1997). ApoB mRNA editing is a post-transcriptional regulation, consists of a C to U conversion of the codon CAA, encoding glutamine 2153, to UAA, an in-frame stop codon in apoB mRNA (Innerarity et al., 1996). The process requires the presence of the poly(A) tail of apoB mRNA (Lau et al., 1991). Because the poly(A) tail of mRNA is always coated by PABPN1 molecules in the nucleus, the interaction between ABBP-1 and PABPN1 implies that PABPN1 might be involved in apoB mRNA editing. Sequestering hnRNPA/B protein in OPMD intranuclear inclusions suggests that the inclusions might interfere with mRNA processing and trap pre-mRNA. The presence of abnormal protein aggregates is a common finding in a number of neuronal degenerative diseases (Ferrigno and Silver, 2000; Sherman and Goldberg, 2001). Expansions of the polyglutamine stretch in the disease gene products lead to protein aggregation and abnormal association with

various cell proteins (Orr, 2001; Sherman and Goldberg, 2001). Pathogenesis studies showed that, although polyglutamine tracts themselves are very toxic, residues outside of the polyglutamine tract in each disease-causing protein have an important role in defining the course and specificity of disease (Orr, 2001). These residues participate in important cellular processes such as the subcellular localization of the polyglutamine protein and its interaction with other cellular molecules important in disease progression (Orr, 2001). These observations also apply to OPMD, since OPMD aggregates sequester PABPN1 interacting proteins such as hnRNPs, SKIP (Kim et al., 2001), and mRNAs (Calado et al., 2000b). Interestingly there is no evidence indicating that polyalanine expanded PABPN1 loses any of its known functions.

In vitro polyadenylation assay showed that polyalanine expanded PABPN1 is still able to stimulate RNA poly(A) tail synthesis and control its length (unpublished observation). The poly(A) length of mRNA isolated from muscle of OPMD patients is not affected (Calado et al., 2000b). However, polyalanine expanded PABPN1 has a gain-of-function that induces the formation of large intranuclear protein aggregates (Bao et al., 2002; Fan et al., 2001; Kim et al., 2001; Shanmugam et al., 2000), and these aggregates are toxic and associated with cell death (Fan et al., 2001). We suggest that OPMD intranuclear inclusions that trap mRNA and sequester PABPN1 interacting proteins play critical roles on this disease progression.

2.7 Acknowledgements

We thank Dr G. Dreyfuss for providing monoclonal antibody against hnRNPA1; Drs. P. Lau and L. Chen for antibody against hnRNPA/B and discussing with us about the roles of ABBP-1 and poly(A) tail in apoB pre-RNA editing; to Dr M. Jannatipour for showing us the yeast two-hybrid screen techniques; Mr. D. Rochefort for his invaluable suggestions on construct making. We thank Drs. C. Gaspar, and A. Toulouse for careful reading and comments on the manuscript. This work was supported by the Muscular Dystrophy Association (USA) and the Federation Foundation of Greater Philadelphia. B.B. is a chercheur-boursier of the FRSQ. G.A.R. is supported by the CIHR.

2.8 Figures

Figure 1

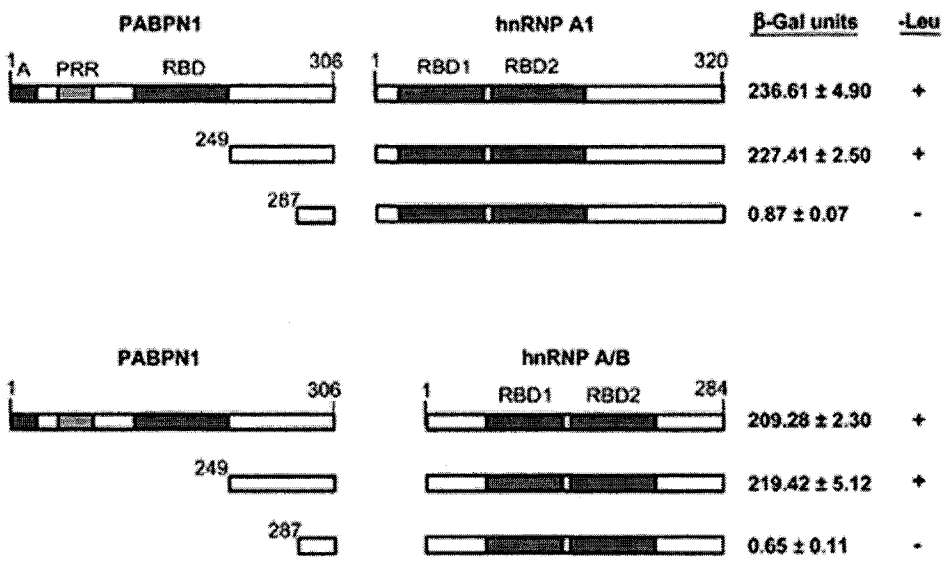

Figure 1

Yeast two-hybrid analysis of the interactions between PABPN1 and hnRNPA1 or A/B

AA249-306 of PABPN1 was used as bait to screen a human fetal brain cDNA library. The LacZ and LEU2 were used as reporter genes. The interaction between two co-expressed proteins was characterized as positive if the yeast cells express β-galactosidase (LacZ gene activated) and grew in the absence of leucine (LEU2 gene activated). β-galactosidase activity was measured using β-gal liquid assay. In the –leucine column, (+): yeast cells grow in the absence of leucine; (-): not grow. A: alanine stretch; PRR: proline rich region; RBD: putative RNA binding domain.

Figure 2

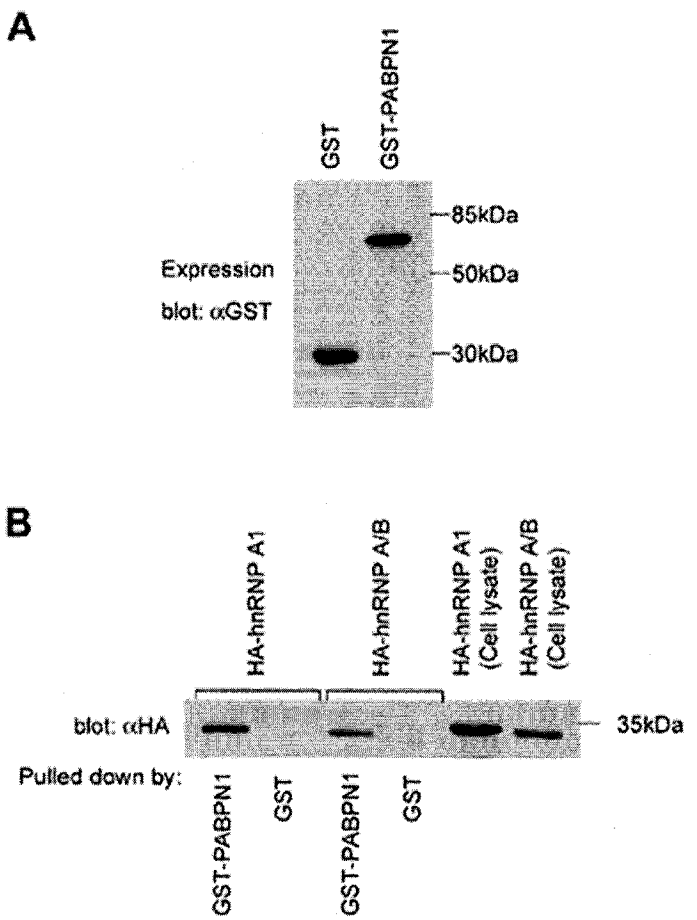
A
GST
GST-PABPN1
85kDa
50kDa
30kDa
Expression
blot: αGST
B
HA-hnRNP A1
HA-hnRNP A/B
HA-hnRNP A1 (Cell lysate)
HA-hnRNP A/B (Cell lysate)
blot: αHA
35kDa
Pulled down by:
GST-PABPN1
GST
GST-PABPN1
GST

Figure 2

GST pull-down analysis of the interaction between PABPN1 and hnRNPA1 or A/B

(A) Expression of GST and GST-PABPN1. The proteins were expressed in bacteria, purified using Bulk and RediPack GST Purification Modules, and detected using anti-GST polyclonal antibody. **(B)** The hnRNPA1 and A/B were HA tagged, expressed in yeast cells and confirmed by Western blotting using anti-HA polyclonal antibody (the right two lanes). Yeast cells were lysed and the lysate was incubated with 5 μg purified GST or GST-PABPN1 obtained from A, pulled-down by Glutathione Sepharose 4B beads, and assayed by Western blotting using the HA antibody.

Figure 3

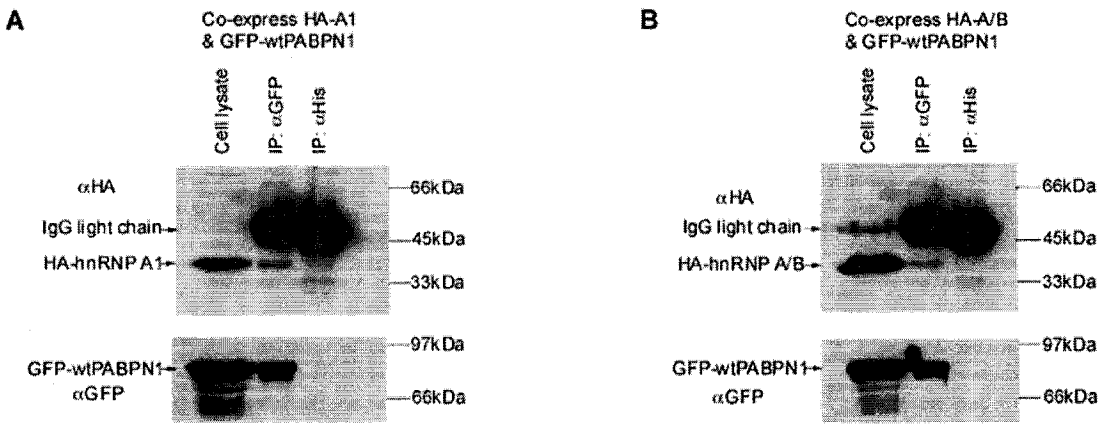
A
Co-express HA-A1
& GFP-wtPABPN1
Cell lysate
IP: αGFP
IP: αHis
αHA
IgG light chain
HA-hnRNP A1
66kDa
45kDa
33kDa
GFP-wtPABPN1
αGFP
97kDa
66kDa
B
Co-express HA-A/B
& GFP-wtPABPN1
Cell lysate
IP: αGFP
IP: αHis
αHA
IgG light chain
HA-hnRNP A/B
66kDa
45kDa
33kDa
GFP-wtPABPN1
αGFP
97kDa
66kDa

Figure 3

Co-immunoprecipitaion of hnRNPA1 and A/B with wtPABPN1

(A) GFP-wtPABPN1 and HA-hnRNPA1 were co-expressed in COS-7 cells. The GFP-wtPABPN1 was immunoprecipitated using polyclonal GFP antibody and the immunoprecipitates were resolved on SDS-PAGE and blotted using anti-HA polyclonal antibody to check the presence of HA-hnRNPA1 (middle lane). The polyclonal anti-Histidine antibody was used as a negative control (right lane). The cell lysate without immunoprecipitation was used to check the expression of HA-hnRNPA1 (left lane). The same Western blot was stripped and re-detected with anti-GFP antibody to confirm the presence of GFP-wtPABPN1 in the immunoprecipitates (lower panel). **(B)** The same experiment as **(A)** was performed except using hnRNPA/B instead of A1.

Figure 4

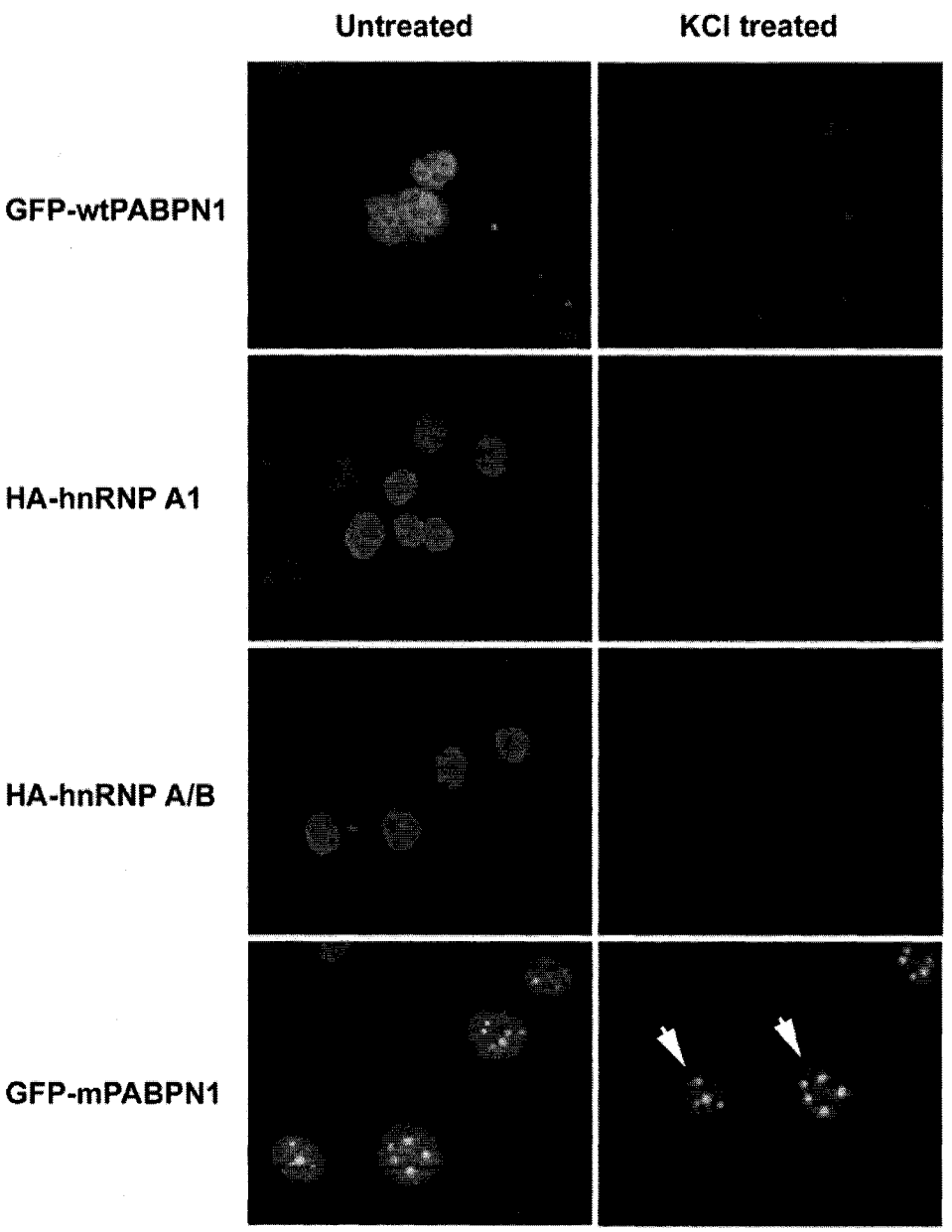
Untreated
KCl treated
GFP-wtPABPN1
HA-hnRNP A1
HA-hnRNP A/B
GFP-mPABPN1

Figure 4

Expression of mPABPN1 induces insoluble intranuclear aggregates

GFP-wtPABPN1, HA-hnRNPA1, HA-hnRNPA/B, and GFP-mPABPN1 were transiently expressed in COS-7 cells. Three days post-transfection, the transfected cells were either fixed (left panels) or treated with 0.5 M KCl in HPEM buffer to remove the soluble proteins (right panel). The HA-hnRNPs expressed cells were immunostained using anti-HA antibody. Arrowheads indicate the nuclei containing insoluble aggregates induced by mPABPN1.

Figure 5

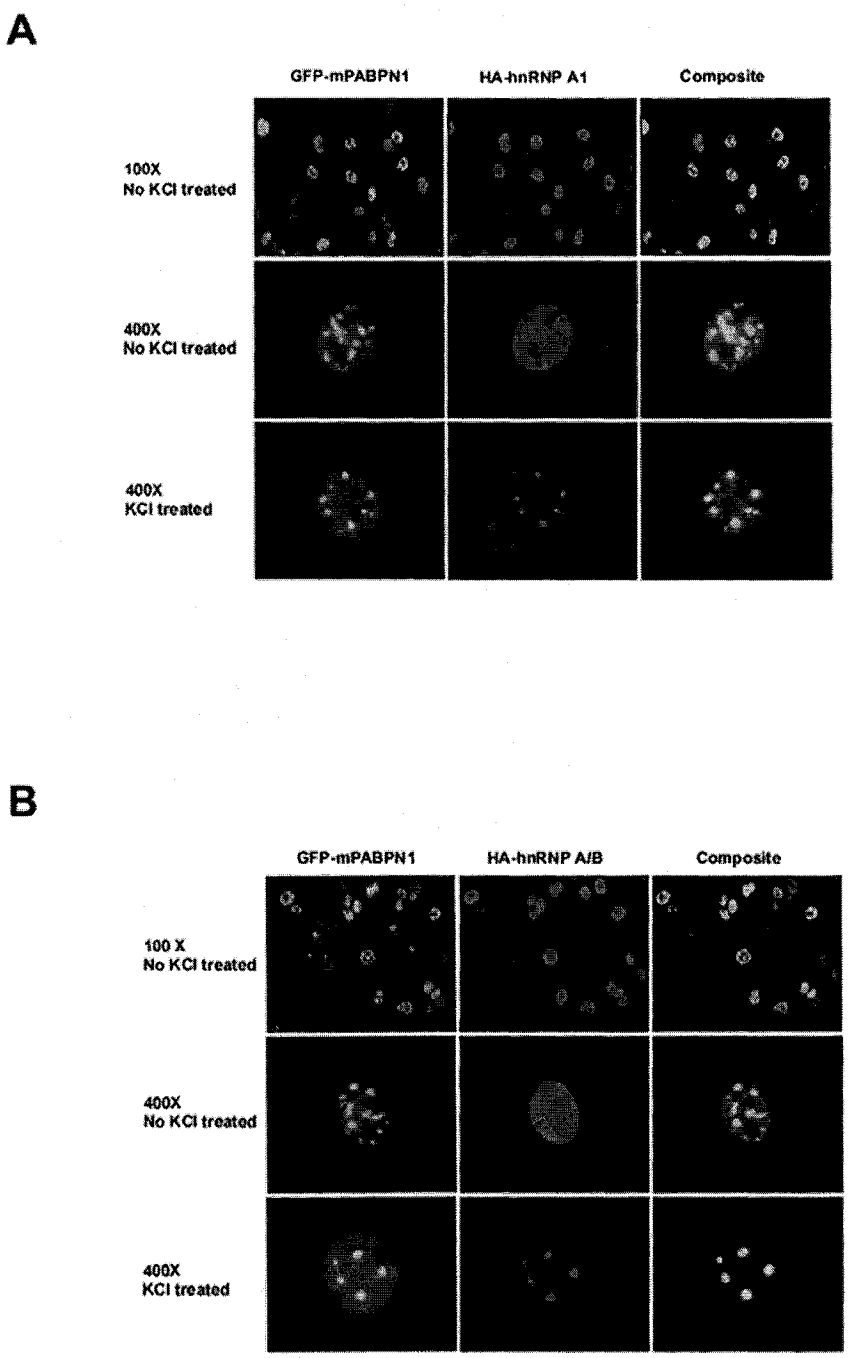
A
GFP-mPABPN1
HA-hnRNP A1
Composite
100X
No KCl treated
400X
No KCl treated
400X
KCl treated
B
GFP-mPABPN1
HA-hnRNP A/B
Composite
100 X
No KCl treated
400X
No KCl treated
400X
KCl treated

Figure 5

The hnRNPA1 and A/B co-localize with mPABPN1 to the insoluble intranuclear aggregates

(A) HA-hnRNPA1 and GFP-mPABPN1 were co-expressed in COS-7 cells. In 72 hrs, the cells were either fixed or treated with 0.5M KCl in HPEM buffer before fixation. The HA-hnRNPA1 was immunostained using an anti-HA polyclonal antibody and rhodamine conjugated secondary antibody (in red). Pictures were taken under low magnification to show the efficiency of transfection, high magnification to enable to see the aggregates, and composition to show the co-localization (left). **(B)** The same experiment as **(A)** was performed except using hnRNPA/B instead of A1.

Figure 6

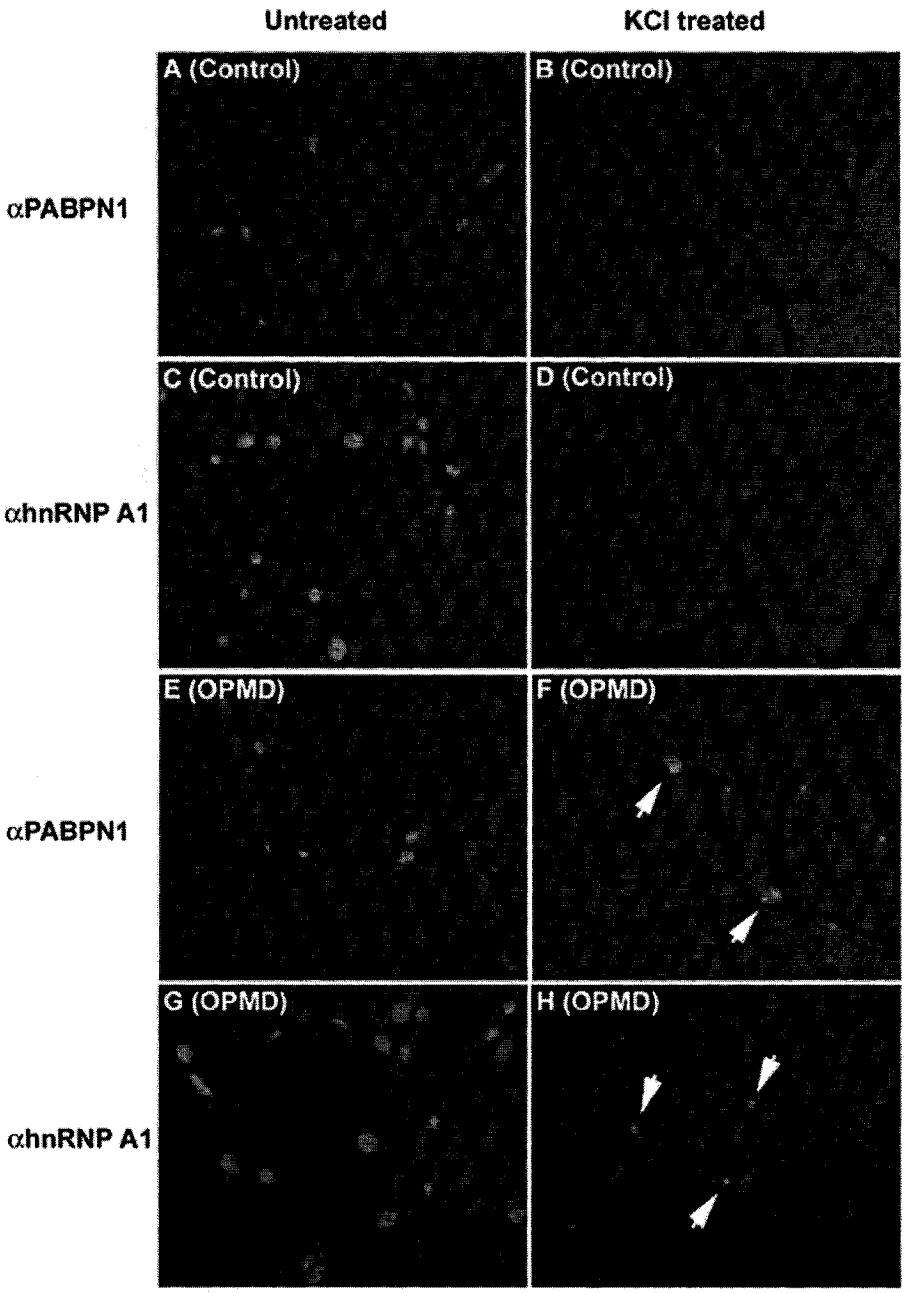
Untreated
KCl treated
A (Control)
B (Control)
αPABPN1
C (Control)
D (Control)
αhnRNP A1
E (OPMD)
F (OPMD)
αPABPN1
G (OPMD)
H (OPMD)
αhnRNP A1

Figure 6

The intranuclear inclusions in OPMD muscle nuclei sequester PABPN1 and hnRNPA1

Immunohistochemistry was performed on cross microtome sections of the deltoid muscle from a control subject (**A**, **B**, **C**, and **D**) and an OPMD patient (**E**, **F**, **G** and **H**). Sections were either immunostained without KCl treatment (**A**, **C**, **E**, and **G**) or treated with 1 M KCl in HPEM buffer for 5 min. at room temperature before immunustaining to remove soluble proteins (**B**, **D**, **F** and **H**). Immunohistochemistry was done using polyclonal anti-PABPN1 antibody (**A**, **B**, **E** and **F**) or monoclonal anti-hnRNPA1 antibody (**C**, **D**, **G** and **H**), and rhodamine conjugated secondary antibody. Arrowheads indicate the positively stained insoluble intranuclear inclusions.

Chapter 3: *Investigation of the role of molecular chaperones and the ubiquitin-proteasome degradative pathway in OPMD*

Aida Abu-Baker[1], Christiane Messaed[1], Janet Laganiere[1], Claudia Gaspar[1], Bernard Brais[2] and Guy A. Rouleau[1]. Involvement of the ubiquitin-proteasome pathway and molecular chaperones in Oculopharygeal Muscular Dystrophy. *Hum Mol Genet.* 2003;12(20): 2609-23.

3.1 Rationale

OPMD nuclear aggregates sequester molecular chaperones, Ub and proteasome subunits (Bao et al., 2002; Calado et al., 2000b; Uyama et al., 2000). The recruitment of these cellular components has been previously described in many polyglutamine disease models (Chan et al., 2000; Cummings et al., 1998; Kobayashi et al., 2000). The formation of protein aggregates occurs when the cell's proteolytic systems and molecular chaperones, which normally prevent aggregation (HSP70 and HSP40) (Frydman, 2001; Hartl and Hayer-Hartl, 2002) are overwhelmed by the build-up of misfolded proteins; alternatively the proteasome components may become completely impaired. Furthermore, age-associated impairment of the proteasome and reduction of molecular chaperones were reported in several cellular models and humans (Bence et al., 2001; Bregegere et al., 2006; Keller et al., 2002; Merker et al., 2000) and such circumstances may occur in the affected skeletal muscles of OPMD patients and lead to the formation of INIs. To further investigate the process of protein aggregation in OPMD, we decided to study the role of molecular chaperones and the UPP. If the clearance of expPABPN1 is mediated by the UPP, the inhibition of the proteasome may most likely lead to an increase in protein aggregation. Also, in cells expressing expPABPN1, the endogenous level of molecular chaperones may not be sufficient to prevent expPABPN1 aggregation or cellular toxicity. However, the overexpression of HSP70 and HSP40 (independently or simultaneously) along with expPABPN1 may reduce protein aggregation and expPABPN1 induced-cell death.

3.2 Abstract

Oculopharyngeal muscular dystrophy (OPMD) is a late onset autosomal dominant muscular dystrophy that results from small expansions of a polyalanine tract in the PABPN1 gene. Intranuclear inclusions are the pathological hallmark of (OPMD). The mechanism by which protein aggregation in OPMD might relate to a toxic gain-of-function has so far remained elusive. Whether protein aggregates themselves are pathogenic or are the consequence of an unidentified underlying molecular mechanism is still unclear. Here, we report that protein aggregation in a cell model of OPMD directly impaires the function of the ubiquitin-proteasome pathway (UPP) as well as molecular chaperone functions. The proteasome inhibitor lactacystin causes significant increase of protein aggregation and toxicity. Moreover, overexpression of molecular chaperones (HSP40 and HSP70) suppressed protein aggregation and toxicity. We also provide evidence that mPABPN1-ala17 protein aggregation proportionally correlates with toxicity. Furthermore, we show that co-expression of chaperones in our OPMD cell model increases the solubility of mPABPN1-ala17 and transfected cell survival rate. Our studies suggest that molecular regulators of polyalanine protein solubility and degradation may provide insights into new mechanisms in OPMD pathogenesis. Further analysis of the cellular and molecular mechanisms by which UPP and molecular chaperones influence the degradation of misfolded proteins could provide novel concepts and targets for the treatment and understanding of the pathogenesis of OPMD and neurodegenerative diseases.

3.3 Introduction

Oculopharyngeal muscular dystrophy (OPMD) is an adult-onset autosomal dominant disease that affects skeletal muscles, especially those for eyelid elevation and swallowing. The OPMD locus was mapped by linkage analysis to chromosome 14q11.1 (Brais et al., 1995; Xie et al., 1998) and the gene was identified as PABPN1, which encodes for the poly (A) binding protein nuclear 1 (PABPN1, PABII, PABP2) (Brais et al., 1998). The wild-type PABPN1 gene has a $(GCG)_6$ repeat encoding for alanine in the N-terminus of the protein, while OPMD patients show a short expansion of $(GCG)_{8-13}$ (Brais et al., 1998), which in turn leads to an expanded alanine tract. In PABPN1 $(GCG)_6$ codes for the first six alanines in a homopolymeric stretch of 10 alanines. OPMD is therefore associated with an expansion of a 12-17 uninterrupted alanine tract in the PABPN1 protein. In addition to OPMD, at least eight other diseases are associated with alanine stretch expansions in the disease gene products (Brown et al., 1998; Crisponi et al., 2001; Goodman et al., 2000; Mundlos et al., 1997; Muragaki et al., 1996).

The mechanism leading polyalanine-expanded PABPN1 to form aggregates associated with the disease is still unknown. One hypothesis is that the polyalanine stretches are able to adapt β-sheet structures, which leads to the formation of strong fibres extremely resistant to chemical denaturation and enzymatic degradation (Forood et al., 1995). Also, the expanded polyalanine tract in mPABPN1 may destabilize the native conformation of the protein, thereby causing it to misfold and aggregate. We previously observed that transient expression of the polyalanine expanded PABPN1 (mPABPN1-ala17) in COS-7 cells induces the formation of intranuclear inclusions (INIs) (Shanmugam et al., 2000). It was reported that expression of 19 or 37 alanine repeats fused to GFP leads to INIs

formation and increases cell death (Rankin et al., 2000). In addition, our group showed that oligomerization of polyalanine expanded PABPN1 facilitates INIs formation (Fan et al., 2001). We also demonstrated that preventing these INIs by inactivating oligomerization of mPABPN1-ala17 significantly reduces cell death. There is also the possibility that OPMD INIs sequester mRNA or other nuclear components and interfere with mRNA production, export and/or processing. The INIs found in the OPMD muscle fibers (Becher et al., 2000; Calado et al., 2000b; Uyama et al., 2000) were shown to sequester poly (A) RNA (Calado et al., 2000b) and other nuclear proteins. Potential molecular mechanisms for OPMD pathogenesis were recently reviewed by our group (Fan and Rouleau, 2003).

The INIs formation in mPABPN1-ala17 may result from an imbalance between protein refolding and aggregation. Different observations converge to suggest that a gain-of-function of PABPN1 may cause the accumulation of nuclear filaments observed in OPMD (Tome and Fardeau, 1980). OPMD intranuclear inclusions (INIs) are similar to those found in a number of inherited neurodegenerative diseases caused by mutated proteins with an expanded polyglutamine (polyQ) stretch. Many observations suggest that various types of inclusions arise through common mechanisms and elicit similar host responses. For example, all these inclusions contain components of the UPP and also molecular chaperones, which represent the two main systems that protect cells against the buildup of unfolded polypeptides. The presence of these components in nuclear inclusions implies that the misfolded and aggregated protein is targeted for degradation (Chai et al., 1999b; Cummings et al., 1998; Stenoien et al., 1999; Wyttenbach et al.,

2000). However, the presence of INIs suggests that mutant proteins are not adequately cleared by the UPP, and accumulate as a result.

Cells respond to toxic conditions by induction of a set of highly conserved genes that encode heat shock proteins (HSPs). Among the HSPs in eukaryotic cells are many molecular chaperones, which function to retard protein denaturation and aggregation, several antioxidant enzymes, which reduce oxidative damage to cell proteins, and components of the UPP (Seufert and Jentsch, 1990; Sommer and Seufert, 1992; Watt and Piper, 1997). The UPP catalyzes the selective degradation of misfolded, unassembled or damaged proteins in the nucleus and cytosol that could otherwise form toxic aggregates (Ciechanover et al., 2000; Hershko and Ciechanover, 1998; Schwartz and Ciechanover, 1999). The relative importance of different protective mechanisms may depend on the nature of the mutated protein or the specific environmental stress.

Blocking the expression or accelerating the degradation of the toxic mPABPN1 protein may be an effective therapy of OPMD. It has been shown that reducing expression of the mutant polyglutamine containing protein in transgenic mice can reverse the phenotype (Yamamoto et al., 2000). Other therapeutic strategies include inhibiting the tendency of the protein to aggregate (either with itself or with other proteins), up-regulating heat shock proteins that protect against toxic effects of misfolded protein, enhancing proteasome activity and blocking downstream effects, such as triggers of apoptosis.

Here we hypothesize that inclusion formation in OPMD is partly a consequence of insufficient proteasome and/or molecular chaperone function. We have found evidence that links the UPP and chaperone components in this disease; namely, we demonstrate that proteasome function is closely linked to aggregate formation, since treatment with

the specific proteasome inhibitor lactacystin resulted in an enhancement of both nuclear and cytoplasmic protein aggregation. Furthermore, in the absence of lactacystin there seems to be no significant difference in expression levels of HSP70 between cells expressing wtPABPN1-ala10 and cells expressing mPABPN1-ala17, whereas upon treatment with the proteasome inhibitor we find a clear induction of HSP70 expression levels, as well as an increase in ubiquitin conjugates, exclusively in cells transfected with mPABPN1-ala17. Finally, we show that HSP40 and HSP70 may be involved in suppressing aggregation and toxicity in OPMD through modification of the solubility of the mutant protein. These results represent the first report of the direct involvement of the UPP pathway in modulation of aggregate formation and cell survival in OPMD.

3.4 Materials and methods

Plasmid construction

The cDNAs encoding wtPABPN1 with 10 alanines (wtPABPN1-ala10) and mPABPN1 with 17 alanines (mPABPN1-ala17, as seen in OPMD patients) were cloned into the pEGFP-C2 vector (Clontech, Palo Alto, CA, USA), resulting in a fusion of GFP to the N-termini of PABPN1 proteins. pFlag-CMV-2-HSP40 and pcDNA3.1/HisA-HSP70 expression vectors were kindly provided by Dr Huda Zoghbi (Baylor College of Medicine, Houston, TX, USA) (Tome and Fardeau, 1980). All constructs were validated by DNA sequencing.

Cell culture and transfection

Twenty-four hours before transfection, COS-7 or Hela cells were seeded in Dulbeco's modified Eagle's medium (DMEM) (Gibco BRL, MD, USA) containing 10% fetal calf serum (Gibco BRL) at a concentration of 2 x 10^5 cells per well in six-well plates containing sterile cover slips. The cells were transfected with plasmid DNA (2.0 μg) using Lipofectamine reagent (Gibco BRL) according to the manufacturer's instructions. For co-transfection experiments, pFlag-CMV-2 expressing full-length human HSP40 and/or pcDNA3.1/HisA expressing full-length human HSP70 were co-transfected with GFP-wtPABPN1-ala10 or GPF-mPABPN1-ala17 constructs at 4:1 ratio. Cells were transfected with 0.4 μg of GFP construct and 1.6 μg of the appropriate chaperone or control (empty) vector DNA per well, to ensure that all cells expressing the GFP fusion proteins also expressed the appropriate chaperone proteins. Different ratios of co-transfection (chaperone: mPABPN1-ala17; 3:1, 2:1) were examined and fixation was

performed 48 h post transfection with 4% paraformaldehyde; cells were then visualized using a fluorescence microscope with a filter appropriate for GFP in three independent experiments.

Proteasome inhibitor treatment

Twenty-four hours after transfection, cells were incubated with the proteasome inhibitor lactacystin (lac.; Calbiochem, La Jolla, CA, USA) for a further 24 h. The results shown in Figure 3B are from three representative independent experiments. The concentration used was 10 μM lactacystin (unless otherwise stated). Control cells were incubated with equivalent amounts of the carrier agent DMSO.

Immunocytochemistry and Immunohistochemistry

Fourty-eight hours after transfection, cells were washed with PBS, fixed for 15 min. with 4% paraformaldehyde, permeabilized for 5 minutes with 0.05%Triton-X/PBS, then blocked with 10% normal goat serum (NGS)/PBS. Cells were incubated overnight in primary antibody in the following dilutions: α GFP=1:500; α PABN1=1:100; α ubiquitin= 1:1000; α HSP70= 1:1000. For colorimetric detection, cells were washed three times in PBS, and then incubated for 1 h in appropriate biotinylated secondary antibody (1:500). After three PBS washes, amplification was carried out using the ABC Elite kit (Vector). Cells were incubated for 1 h, and the reaction product was visualized using the VIP (Vector) kit. Coverslips were then mounted on slides. For fluorescent detection experiments, cells were incubated in the appropriate secondary fluorescent antibody (CY3, 1:300), washed, and then mounted in Slow-Fade Mount (Molecular

Probes, Eugene, Oregon, USA). Cells were visualized using appropriate filters on a Leica Polyvar microscope.

The microtome sections of paraffin-embedded deltoid muscle from OPMD patients and a control subject were used (kindly provided by Dr G. Karpati, MNI). Sections were deparaffinized, permeabilized and immunostained with monoclonal antibody against HSP70. Rhodamine conjugated secondary antibody was used and the signal was visualized using a fluorescent microscope. The concentration of anti-HSP70 antibody (Chemicon) was 1:300.

Western blotting

Fourty-eight hours after transfection cells were harvested and protein was extracted in lysis buffer. Equal amounts of protein were electrophoresed on 12% SDS-PAGE and transferred to a nitrocellulose membrane. The membranes were probed with a monoclonal anti-GFP antibody (Clontech, 1:1000), polyclonal anti-PABPN1 antibody (1:100), anti-HSP70 antibody (1:1000), and anti-ubiquitin antibody (1:100) and detected using the Western blot chemiluminescence reagent *Plus* kit (NEN Life Science Products, Boston, MA, USA). Parallel samples were probed with anti-actin antibody to verify equal loading of lysates.

Quantitation of aggregates and cell viability

Quantitation of aggregates in transfected cells was carried out by an observer blind to the treatment conditions. All transfected cells were scored by their staining pattern as having the nucleus diffusely labeled, or as containing nuclear aggregates. The percentage of cells with INIs was obtained by dividing the number of cells with INIs by the total

number of transfected cells. This was repeated three times for three different fields, and then the average of the three ratios was computed and presented as a percentage.

For cell toxicity assay, the number of living cells was measured every 24 h post-transfection, for a total of 7 days. Briefly, each well was washed with DMEM to remove detached cells and the cells expressing GFP were counted in a 1 mm^2 area under low magnification (25X). Three different areas were counted for each well. All wells were in duplicate and the experiments were repeated three times (n=6). All values were expressed as means $\pm$ SEM. Statistical analysis was performed using the Anova single factor, with $P< 0.05$ considered statistically significant.

Solubility analysis by filter retardation assay

Since insoluble mPABPN1-ala17 cannot be adequately quantified by Western blotting (Fan et al., 2001), we used a filter trap assay (Scherzinger et al., 1997; Wanker et al., 1999) for quantitative analysis of insoluble form of the mutant PABPN1.

For solubility studies, transfected cells were harvested and resuspended for 20 min on ice in SDS lysis buffer (10 mM Tris pH8.0, 150 mM NaCl, 2% SDS, supplemented with protease inhibitors PMSF and pepstatin). Samples were fractionated by centrifugation at 16 000 g for 10 min. The supernatant (soluble fraction) was then transferred to a separate tube. The insoluble pellet was resuspended in an equal volume of lysis buffer for 10 min, and sonicated briefly. The supernatant and the pellet fractions represent the soluble and insoluble fractions of the extract, respectively. Equal amounts of supernatant and pellet fractions were then analyzed by filter trap assay. The filter trap assay (Scherzinger et al., 1997; Wanker et al., 1999), was performed with 0.45 μM nitrocellulose (Schleicher & Schuell, Dassel, Germany), and two pieces of Whatman filter paper to support the

membrane, using a dot-blot apparatus. The membrane was washed twice with washing buffer (0.1% SDS, 10 mM Tris, pH 8.0, 150 mM NaCl). Samples were prepared in a final volume of 100 µl in lysis buffer, boiled for 3 min, and equal amounts of protein extracts were loaded. The membrane was washed twice with wash buffer, and then removed from the apparatus. Dot-blots were probed as described above for Western blots.

3.5 Results

Expression of mPABPN1-ala17 is sufficient to induce the formation of INIs

PABPN1 is an abundant nuclear protein that binds with high affinity to the poly (A) tail of mRNA, and is involved in mRNA polyadenylation (Wahle, 1991). PABPN1 has 306 amino acids and comprises an alanine stretch and a proline-rich region in the N-terminus, an RNA binding domain in the central region, and a nuclear localization domain in the C-terminus (Wahle, 1991).

We have established an *in vitro* model of OPMD using transfected Hela and COS-7 cells. In our model, mPABPN1-ala17 forms INIs in both cell lines (Fig. 1A and B).

Both HSP70 and ubiquitin are recruited into INIs of OPMD cell-based model and human tissue

Calado *et al.* (Calado et al., 2000b) found that ubiquitin and the 20S proteasome co-localize to INIs in OPMD patient muscle (Calado et al., 2000b). Recently, it was also demonstrated that components of the proteasome, HSP70, and ubiquitin co-localize to aggregates formed by mutated bovine PABPN1 in transfected COS-7 cells (Bao et al., 2002). To confirm the previous results, and to get a better comparison between HSP70 and ubiquitin redistribution into aggregates, we performed immunocytochemical staining on cells transfected with wtPABPN1-ala10 as well as mPABPN1-ala17. Figure 2A and C shows the uniform distribution of HSP70 and ubiquitin in cells transfected with wtPABPN1-ala10. A diffuse staining of both HSP70 and ubiquitin throughout the cytoplasm and nucleus was observed in these cells (Fig. 2A and C). Immunostaining was negative for the transfected green fluorescent protein (GFP) vector alone. In contrast,

INIs of COS-7 and Hela cells transfected with mPABPN1-ala17 immunostained positively for both HSP70 and ubiquitin, (Fig. 2B and D). These results confirm that HSP70 and ubiquitin have been redistributed to the INIs in our cell culture model. The presence of ubiquitin and HSP70 in the INIs supports the hypothesis that misfolded and aggregated proteins are targeted for proteolysis by the UPP. Together with the previous studies, our results provide evidence that polyalanine containing mPABPN1-ala17 elicits a stress response in cells.

To confirm the relevance of our findings in our cell model, we performed immunohistochemical staining on muscle sections of OPMD patients using the monoclonal HSP70 antibody. As shown in Figure 2E, INIs immunostained positively for the HSP70 in OPMD muscle cells. Muscles in control subject did not show immunostaining of subnuclear structures.

Proteasome inhibition by lactacystin increases mPABPN1-ala17 INI formation in a dose dependent manner

The redistribution of ubiquitin and HSP70 into mPABPN1-ala17 positive INIs seems to suggest that INI formation may partly be a consequence of insufficient ubiquitin-proteasome and/or molecular chaperone function. To determine if this might be the case, we tested whether inhibition of the proteasome by lactacystin would promote polyalanine aggregation. We used the construct of mPABPN1-ala17 which leads to aggregation in our cell model, and we proceeded to quantify INI formation in the presence or absence of lactacystin. In untreated cells, mPABPN1-ala17 formed INIs in 40% of the total transfected cells. Treatment of transfected Hela or COS-7 cells with the specific proteasome inhibitor lactacystin, at a dose of 10 μM, led to a marked statistically

significant increase in INIs formation (80%) ($P < 0.05$). This increased INIs formation occurred in a polyalanine size-dependent manner, since INIs number did not vary in cells expressing wtPABPN1-ala10 (Fig. 5C). The increased number of INIs was associated with increased cell toxicity (data not shown). We also determined that INI formation was promoted by lactacystin in a dose-dependent manner, from 2.5-10 μM (Fig. 3A and B). Higher doses of lactacystin were lethal to cells. There was no difference in the transfection efficiency between cells incubated with or without lactacystin (Fig. 3A). We observed no difference in INI formation in control cells which were incubated with equivalent amounts of the carrier agent, dimethyl sulfoxide (DMSO), compared to cells transfected with mPABPN1-ala17 alone (Fig. 3B). We performed this experiment three independent times and calculated the number of cells containing INIs. The percentage of cells with INIs was obtained by dividing the number of cells with INIs by the total number of transfected cells. This was repeated three times for three different fields, then the average of the 3 ratios was computed and presented as a percentage (Fig. 3B).

Cytoplasmic inclusion formation is enhanced after lactacystin treatment of mPABPN1-ala17 transfected cells

Expression of mPABPN1-ala17 induces INI formation (Fig. 4A1 and A2). However, we were also able to detect a few cytoplasmic inclusions in cells transfected with mPABPN1-ala17 (Fig. 4A1). Moreover, inhibition of the proteasome by lactacystin at a dose of 10 μM seems to lead to an increase in cytoplasmic inclusion formation in COS-7 cells, as shown in Figure 4B, suggesting that these structures develop when a threshold of misfolded protein is exceeded. This is the first report showing the presence of cytoplasmic inclusions in cells transfected with mPABPN1-ala-17. Based on our model,

enhancing proteasome activity would thus be expected to reduce protein aggregation that is associated with cell death. A very recent report suggested that the proteasome activator REGg may be an attractive therapeutic target for neurodegerative diseases (Goellner and Rechsteiner, 2003).

Lactacystin treatment increases the insoluble fraction of mPABPN1-ala17 protein

We previously demonstrated that the INIs induced by expression of mPABPN1-ala17 in COS-7 cells, as seen in OPMD patients, are insoluble and resistant to KCl treatment (Fan et al., 2001). To understand the mechanism by which protein aggregation is associated with increased cell death in OPMD (Fan et al., 2001), we sought to determine if lactacystin treatment alters the solubility of mPABPN1-ala17.

Protein extracts from cells transfected with mPABPN1-ala17 showed significant increase in insoluble fraction of mPABPN1-ala17 compared to cells transfected with wtPABPN1-ala10 (data not shown), when blotted with anti-PABPN1 antibody using a filter trap assay. The addition of lactacystin increased the insoluble fraction of mPABPN1-ala17 protein (Fig. 4D) and decreased the soluble fraction of mPABPN1-ala17 (data not shown): when cells transfected with mPABPN1-ala17 were incubated with 10 μM lactacystin for 24 h, the extracted proteins showed a distinct insolubility pattern, compared to the control cells transfected with mPABPN1-ala17 that were incubated with DMSO. This is consistent with previous studies reporting an increase in the aggregation of polyglutamine containing proteins in the presence of proteasome inhibitors (Chai et al., 1999b; Wyttenbach et al., 2000).

Lactacystin treatment leads to HSP70 and ubiquitin conjugate induction in cells transfected with mPABPN1-ala17

The inhibition of proteasomal function has been reported to induce the expression of HSP70 due to the accumulation of misfolded protein; therefore, we tested the possibility that the HSP70 is induced in our system. As shown in Figure 5A, lactacystin treatment increases both the constitutive HSP73 (upper band) and inducible HSP72 (lower band) forms of HSP70 in a dose dependent manner in cells transfected with mPABPN1-ala17. Equal amounts of proteins were loaded. A recent study reported that expression of HSP40 or HSP70 chaperones is induced under various conditions of cell stress, which results in unfolding and aggregation of certain proteins (Sakahira et al., 2002). To further confirm that lactacystin inhibition of proteasome activity leads to the increased aggregation that is associated with toxicity, anti-ubiquitin immunoblots were performed on cell lysates. Hela cells expressing mPABPN1-ala17 and incubated with lactacystin (at different doses) exhibited an increased level in total ubiquitinated conjugates. Bao *et.al* (Bao et al., 2002) demonstrated that incubation of cells which were transfected with PABPN1-ala17 with 0.4% DMSO for 24 h significantly reduced the proportion of cells with inclusions. In our study, we did observe that ubiquitin levels in cells transfected with mPAPBN1-ala17 and treated with DMSO were lower than in cells transfected with mPABPN1-ala17 and treated with lactacystin. However, when we examined the effect of DMSO alone, at different doses, on the total ubiquitin conjugates, we did not observe any increase in these bands compared to the cells expressing mPABPN1-ala17 alone or control nontransfected cells (Fig. 5B). Perhaps the difference in DMSO dose explains the discrepancies observed between the two studies. As shown in Fig. 5B, there was a

dramatic accumulation of ubiquitin immunoreactive bands only in cells transfected with mPABPN1-ala17 and treated with lactacystin. To address the issue that mPABPN1-ala17 is ubiquitinated and degraded by the proteasome, we examined whether there were alterations in the overall mPABPN1-ala17 protein level following treatment with lactacystin. As shown in Fig. 5B, the level of mPABPN1-ala17 protein did not change with proteasomal inhibition. In the control nontransfected cells, there is a faint band corresponding to the endogenous PABPN1. To demonstrate equal protein loading, the blot was probed with anti-actin antibody.

Lactacystin has no effect on cells transfected with wtPABPN1-ala10

In our cellular model, wtPABPN1-ala10 can aggregate at high expression levels. However, the number and the size of the protein aggregates in mPABPN1-ala17 are significantly larger than in wtPABPN1-ala10. At lower expression levels, mPABPN1-ala17 formed significantly more aggregates compared to wtPABPN1-ala10 (Fig. 3A left panel, and Fig. 5C left panel); therefore we used low DNA concentrations in our transfection experiments for both wt-PABPN1-ala10 and mPABPN1-ala17. Another group previously reported that both wtPABPN1-ala10 and mPABPN1-ala17 proteins can aggregate at high expression levels (Bao et al., 2002). However, it is worth mentioning that cells transfected with mPABPN1-ala17 in our model tend to die after 3 days, while cells transfected with wtPABPN1-ala10 die after 8 days ((Fan et al., 2001), see also Fig. 6D). PABPN1 forms both linear filaments and discrete-sized, compact oligomeric particles *in vitro* (Keller et al., 2000) called speckles. We were able to detect speckles in cells transfected with both wtPABPN1-ala10 and mPABPN1-ala17. PABPN1 present in the intranuclear inclusions from OPMD patients is insoluble and resistant to KCl

treatment, which is known to be able to dissolve soluble protein aggregates (Calado et al., 2000b). Our group reported also that the aggregates induced by GFP-mPABPN1-ala17 are insoluble and remained after KCl treatment, whereas the GFP-wtPABPN1–ala10 signal is soluble and not resistant to KCl treatment (Fan et al., 2001).

Parallel sets of lactacystin treatment experiments were performed on cells transfected with wtPABPN1-ala10. No aggregation increase was observed after lactacystin treatment of cells transfected with wtPABPN1-ala10. Figure 5C shows the fluorescent micrographs of COS-7 cells transfected with wtPABPN1-ala10 and incubated with 10 μM lactacystin or 10 μM DMSO. There was no significant difference in aggregation formation in cells expressing wtPABPN1-ala10 following 10 μM lactacystin treatment compared with the control cells (Fig. 5C). Since aggregation was not increased in cells transfected with wtPABPN1-ala10, we conclude that the increased aggregate formation occurred only in cells transfected with mPABPN1-ala17. The effect of lactacystin treatment on cells transfected with wtPABPN1-ala10 was confirmed by measuring both HSP70 and ubiquitin levels. There was a slight change in HSP70 levels in cells expressing wtPABPN1-ala10 and treated with 10μM lactacystin as compared to cells treated with DMSO (Fig. 5D). Equal amounts of protein were loaded.

It was also important to determine whether treating cells with lactacystin alone would induce HSP70. Therefore, we included the lysates from nontransfected cells, cells expressing wtPABPN1-ala10 and mPABPN1-ala17 in one blot and probed it with anti-HSP70 antibody. As shown in Figure 5D, nontransfected cells treated with 10μM lactacystin did not show any increase in HSP70 level. HSP70 induction was highly exclusively detected in cells expressing mPABPN1-ala17 and treated with 10 μM

lactacystin (Fig. 5D). Anti-actin antibody was used to confirm that equal amounts of protein were loaded. Thus, we conclude that proteasome inhibition leads to ubiquitin conjugate and HSP70 induction only in cells transfected with mPABPN1-ala17.

HSP70 expression levels similar in both cells expressing wtPABPN1-ala10 and cells expressing mPABPN1-ala17

The recruitments of HSP70 to the INIs of OPMD prompted us to look for HSP70 chaperone induction by the expanded polyalanine mPABPN1-ala17. As shown in Figure 5D (lanes 3 and 5), in the absence of lactacystin there is no significant change of chaperone HSP70 induction level between cells expressing mPABPN1-ala17 and cells expressing wtPABPN1-ala10 (treated with control DMSO). One possible explanation is that the HSP70 chaperone is trapped in the insoluble inclusions of mPABPN1-ala17 preventing its activity. Another reason could be the aggregate burden: there is marked induction of HSP70 only when the aggregate burden is high, as it is in the cells transfected with mPABPN1-ala17 and treated with lactacystin (Fig. 5D).

Molecular chaperones (HSP40 and HSP70) suppress protein aggregation and cell toxicity of mPABPN1-ala17

Sittler *et al.* (Sittler et al., 2001) demonstrated that huntingtin protein aggregation in cells can be suppressed by chemical compounds (geldanamycin) activating a specific heat shock response. Recently, several studies in fruit fly and mouse models in neurodegenerative disorders have provided direct evidence that molecular chaperones can suppress neurotoxicity (Cummings et al., 2001; Fernandez-Funez et al., 2000; Kazemi-Esfarjani and Benzer, 2000; Warrick et al., 1999). Based on polyglutamine disease

literature (Cummings et al., 2001; Fernandez-Funez et al., 2000; Kazemi-Esfarjani and Benzer, 2000; Warrick et al., 1999), it seems plausible that HSPs might also have a role in OPMD pathogenesis.

To determine if molecular chaperones could modulate the frequency of aggregation in our cellular model, we co-expressed HSP40 and HSP70 with full-length mPABPN1-ala17, and determined the percentage of mPABPN1-ala17-expressing cells that contained INIs. As shown in Figure 6A, molecular chaperone HSP40 coexpression significantly decreased the aggregation frequency in our cell culture model. It was important to determine if the chaperones are altering total mPABPN1-ala17 protein expression levels, which may explain reduced INI formation. The level of mPABPN1-ala17 protein expression remained constant after co-expression with the chaperones: as shown in Figure 6B, HSP40 and HSP70 overexpression does not change the protein level of mPABPN1-ala17 when co-expressed in of COS-7 cells. Anti-PABPN1 antibody was used as primary antibody. Equal amounts of protein were loaded in each lane.

Quantification of protein aggregates was carried out (see Materials and Methods). HSP40 expression suppressed INI formation of mPABPN1-ala17 by ~54%, while HSP70 expression showed a more modest effect of ~30% reduction. The co-expression of both HSP40 and HSP70 with mPABPN1-ala17 seemed similar to the HSP40 alone (Fig. 6C). This may suggest that there is no significant synergistic effect of chaperones on aggregate suppression. Protein aggregation suppression, in our cell model, by chaperone overexpression is consistent with other studies of chaperone modulation of aggregation in polyglutamine expansion disorders (Chai et al., 1999a; Chan et al., 2000; Cummings et al., 1998), and seems to indicate functional effects of these chaperones in this cell culture

system. The capacity of molecular chaperones to associate selectively with unfolded polypeptides facilitates substrate recognition by ubiquitin enzymes and perhaps degradation by the proteasome, reviewed by Sherman and Goldberg (Sherman and Goldberg, 2001).

To determine whether chaperone overexpression could mitigate the toxicity of mPABPN1-ala17, we assessed the percentage of living cells every 24 h after transfection for a total of 7 days (Fig. 6D). The cells were counted every 24 h post-transfection. Each well was counted three times in different areas at one time point, and the mean was used for statistics. The percentage of living cells transfected represents the variation of the amount of living transfected cells at different time points compared with the number of transfected cells obtained on day 1. [Mean $\pm$ SEM; $P < 0.05$ compared with any other groups (ANOVA analysis)]. Coexpression of HSP40 and HSP70 reduced mPABPN1-ala17 induced cell death at all time points tested. The cells transfected with mPABPN1-ala17 alone tend to die after 3 days. In the presence of either HSP40 or HSP70, the rate of cell survival was almost 40% higher ($P < 0.05$). The effect of both chaperones was similar to the HSP40 expression alone on cell survival (Fig. 6D). No significant synergistic effect of both chaperones was observed on mitigating toxicity. We therefore conclude that chaperone co-expression significantly reduces the cell toxicity of mPABPN1-ala17.

Modulation of mPABPN1-ala17 solubility by HSP40 and HSP70 chaperones

Increase in the expression levels of molecular chaperones offers new perspectives in the solubilization of proteins (Mogk et al., 2002). The bulk of the available evidence supports an indirect role of chaperones in proteolysis, reflecting the ability of chaperones

to maintain abnormal proteins in a soluble state (Bailey et al., 2002; Mogk et al., 2002; Muchowski et al., 2000; Sherman and Goldberg, 2001). Since both HSP40 and HSP70 significantly decreased INIs in our OPMD cell model, and cell survival increased was without altering the total mPABPN1-ala17 protein expression levels, we further analyzed the effects of these chaperones on the biochemical properties of mPABPN1-ala17. To understand the mechanism by which the chaperones suppress aggregation, we sought to determine if these chaperones alter the solubility of mPABPN1-ala17 protein. First, we analyzed the solubility of both wtPABPN1-ala10 and mPABPN1ala17 using a filter trap assay. As shown in Figure 7A, the expression of wtPABPN1-ala10 shows a significant higher level of soluble protein compared to mPABPN1-ala17 expression. The dot blot was probed with anti-PABPN1 antibody. Probing with an anti-actin antibody confirmed that equivalent amounts of protein were loaded. Second, we studied the effect of chaperone coexpression on modulation of mPABPN1-ala17 solubility. Soluble and insoluble mPABPN1-ala17 levels in cells where chaperones are coexpressed (filter trap assay) are shown in Figure 7B. The expression of HSP40 or HSP70 resulted in a significant increase in soluble mPABPN1-ala17 compared with mPABPN1-ala17 coexpressed with control vector. To further confirm these results, we also showed a significant decrease in insoluble mPABPN1-ala17 when coexpressed with the chaperones, compared to mPABPN1-ala17 coexpressed with control vector (Fig. 7B). Equal amounts of the lysates were loaded. This may suggest proteasome degradation involvement after chaperone overexpression. It was necessary to confirm that chaperone overexpression did not change the mPABPN1-ala17 levels in the transfected Hela cells, using the filter trap assay. As shown in Figure 7C, the level of mPABPN1-ala17

remained constant after HSP40 and/or HSP70 overexpression. The same results were obtained using different co-transfection ratios (mPABPN1-ala17: chaperone; 1:4 and 1:3) using anti-PABPN1 antibody. The dot blot assay in Figure 7C confirms the previous results shown in Figure 6B. We therefore conclude that the effect of chaperones on reducing mPABPN1-ala17 INIs and toxicity acts through modulation of mPABPN1-ala17 solubility, and conformation stabilization, and not by reducing mPABPN1-ala17 expression level.

3.6 Discussion

We have presented evidence that the UPP and molecular chaperones are part of the cellular response to mutant polyalanine-containing PABPN1.

The recruitment of UPP and chaperone molecules into INIs may represent an effort by the cell to clear the misfolded mPABPN1-ala17. Intracellular aggregation of polyglutamine proteins has been suggested to impair the UP system (Bence et al., 2001; Verhoef et al., 2002). Once the β-sheet oligomers of mPABPN1-ala17 are initiated, ubiquitin, molecular chaperones and proteasome regulatory complexes may become unable to refold or degrade these oligomers, and hence trapped in the INIs. This trapping of UP particles may result in a partial inhibition of proteasomal activity and eventually cellular dysfunction. Both HSP40 and HSP70 have been reported to be localized predominantly in the cytoplasm before heat shock and relocalize to the nucleus after heat shock (Hattori et al., 1993; Welch and Feramisco, 1984). The presence of HSP70 in mPABPN1-ala17 INIs may reflect a stress response. However, a recent study showed that polyglutamine protein aggregates are dynamic structures and that molecular chaperones are not sequestered into aggregates, but are instead transiently associated (Kim et al., 2002).

The proteasome inhibition data presented here are the first evidence showing a direct link between the role of proteasome function and OPMD. Our findings that proteasome inhibition increases protein inclusion formation imply that proteasome activity may play an important role in modulating aggregation in OPMD. Ravikumar *et al.* (Ravikumar et al., 2002) also demonstrated that epoxomicin (another proteasome inhibitor) increased the proportion of COS-7 cells expressing GFP-ala19 aggregates. They also noted a marked increase in HSP70 on epoxomicin treatment, consistent with inhibition of the proteasome.

These and our results are consistent with those obtained for polyglutamine diseases, where treatment of transfected cells with proteasome inhibitors resulted in greater accumulation of polyglutamine polypeptides, increase in the number of inclusion bodies, and enhanced apoptosis (Chai et al., 1999b; Cummings et al., 1998; Wyttenbach et al., 2000). We examined whether there were alterations in the levels of mPABPN1-ala17, following proteasome inhibition, as would be expected if it is normally degraded through the proteasome. We did not detect any changes in mPABPN1-ala17 protein levels. It is possible that aggregated mPABPN1-ala17 is not soluble even in SDS buffer. Alternatively, mPABPN1 may not be degraded exclusively by the proteasome. We can not exclude the possibility that mPABPN1-ala17 could also be targeted to the lysosomal pathway. A recent study showed that antifungal antibiotic (rapamycin) enhanced the clearance of GFP-ala19-associated aggregates (Ravikumar et al., 2002) suggesting the autophagy-lysosomal pathway as an alternative degradation system.

Lactacystin promoted aggregation of full-length mPABPN1-ala17. In the presence of lactacystin, mPABPN1-ala17 formed more cytoplasmic, perinuclear aggregates as well as INIs, whereas under control conditions it formed exclusively INIs. The formation of cytoplasmic inclusions, despite the presence of nuclear localization signal (NLS) in mPABPN1-ala17 (Fig. 4B), suggests that lactacystin caused mPABPN1-ala17 to aggregate before it could be transported to the nucleus, essentially trapping the protein in the cytoplasm.

We have recently shown that oligomerization of mPABPN1-ala17 facilitates nuclear protein aggregation of OPMD (Fan et al., 2001). When proteasome activity is blocked with lactacystin or other inhibitors, the concentration of misfolded polyalanine protein

would increase, favoring oligomerization and aggregation. In order to be degraded by the proteasome, a protein must first be unfolded to be able enter the central proteolytic chamber. mPABPN1-ala17 may form a highly insoluble stable β-sheet that resists unfolding and thus blocks entrance to the chamber. Alternatively, proteasome inhibitors could increase polyalanine aggregation indirectly through effects on other cellular processes, such as induction of the apoptosis pathway. The higher the concentration of hydrophobic amino acid chains in the protein, the more likely it is that protein aggregation occurs (Mogk et al., 2002). The fate of the misfolded mPABPN1-ala17 would reflect the relative affinities of non-native protein for proteases or chaperones and the relative rates of degradation, aggregation and folding.

The cellular defenses against unfolded proteins, including UPP and molecular chaperones, are highly inter-linked. We found that lactacystin treatment led to an increase in ubiquitin and HSP70 levels in cells transfected with mPABPN1-ala17. Our results suggest that polyalanine aggregation may be necessary, but not sufficient, to elicit a stress response. There is marked HSP induction only when the aggregate burden is high, as it is after lactacystin treatment. Various observations indicate that aged organisms and senescent cultures of mammalian cells are less able to induce HSPs in response to protein-damaging conditions (Heydari et al., 1994; Rattan and Derventzi, 1991). The late onset of OPMD implies that the affected muscles can deal successfully with the mutant PABPN1 for many years. We suggest that one possible factor contributing to the development of symptomatic OPMD is a reduced capacity of cells from older individuals to cope with abnormal expanded PABPN1. As a consequence of decreased HSP inducibility, the expanded polyalanine-containing PABPN1 builds up in

cells of aged individuals to higher levels and results in greater tendency to form insoluble INIs, and may lead to cell death.

According to the literature, most chaperones bind to peptide segments that are enriched in hydrophobic amino acids (Mogk et al., 2002), which are found in the core of native proteins. One possible explanation for the impairment of chaperone function in OPMD is that in mPABPN1-ala17 the expanded polyalanine tracts form β-sheet structures whose stability exceeds the capacity of chaperones to disaggregate and refold the protein to the active conformation. Molecular chaperone overexpression may prevent protein aggregation directly by shielding the interactive surfaces of nonnative polyalanine and indirectly by inhibiting intramolecular β-sheet conformation and thus block ordered oligomerization. Another possibility is that overexpression of chaperones enhances the function of the UPP for mPABPN1-ala17 degradation because the function of the UPP is related to the expression level of chaperones (Bukau and Horwich, 1998).

The finding that molecular chaperones suppress the aggregation and toxicity associated with polyglutamine disease models and enhance the protein solubility (Adachi et al., 2003; Bailey et al., 2002; Chai et al., 1999b; Cummings et al., 1998; Jana et al., 2000; Kazemi-Esfarjani and Benzer, 2000; Kobayashi et al., 2000; Mogk et al., 2002; Muchowski et al., 2000; Stenoien et al., 1999; Warrick et al., 1999) formed the basis for our studies aimed at better understanding their effects. First, we have confirmed previous data (Bao et al., 2002) showing that both HSP40 and HSP70 suppress protein aggregation and toxicity associated with mutated bovine PABPN1-ala17. Second, we examined the effect of chaperones on the solubility of mPABPN1-ala17. Our data reveal that the overexpression of either HSP40 or HSP70 enhances the solubility of mPABPN1-ala17,

while not affecting mPABPN1-ala17 expression levels. Therefore, suppression of OPMD protein aggregation by molecular chaperones may occur through alteration of mPABPN1-ala17 solubility and conformational correction, rather than reducing levels of mPABPN1-ala17. Furthermore, our results suggest that enhanced solubility of mPABPN1-ala17 by molecular chaperones may increase the cell survival in OPMD. The increase in soluble mPABPN1-ala17 protein was accompanied by a concomitant decrease in the insoluble mPABPN1-ala17 protein when coexpressed with the chaperone. These observations suggested that overexpression of HSP40 or HSP70 enhanced the function of the UPP and subsequently accelerated the degradation of mPABPN1-ala17 protein (Bukau and Horwich, 1998). The UPP, particularly its activity, is related to chaperone expression levels (Bukau and Horwich, 1998). A very recent paper demonstrated that HSP70 overexpression ameliorates spinal and bulbar muscular atrophy phenotypes in mice by reducing nuclear-localized mutant androgen receptor, probably caused by enhanced mutant androgen receptor degradation (Jana et al., 2000). Another recent report showed that increased levels of HSP70 and HSP90 promote tau solubility and tau binding to microtubules in tau transgenic mouse and Alzheimer's disease brains, suggesting that HSP70 may help to recruit misfolded proteins as substrates for parkin E3 ubiquitin ligase activity (Dou et al., 2003). A Recent finding indicated that HSP70 enhances parkin binding and ubiquitination of expanded polyglutamine protein *in vitro* (Tsai et al., 2003). Based on the chaperone solubility results, we tested the effect of lactacystin, a proteasome inhibitor, on mPABPN1-ala17 solubility. We found that significant increase in protein aggregation after lactacystin treatment is accompanied with an increased level of the insoluble form of mPABPN1-ala17. These results demonstrate that insolubility of

mPABPN1-ala17 renders the protein to be more toxic to the cells. Our observations may be the basis for a possible strategy for OPMD treatment. Also, the finding that protein aggregation is not a dead-end in the life cycle of a protein could be used in the design of therapies for this and similar diseases: use of the bichaperone system may avoid the treatment of inclusion bodies with strong denaturing agents and may promote folding into the biologically active protein conformation.

3.7 Acknowledgments

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3.8 Figures

Figure 1

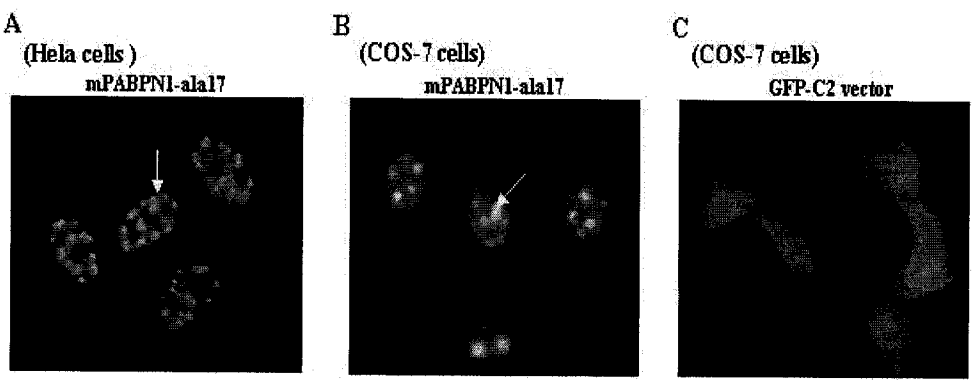

Figure 1

Expression of mPABPN1-ala17 in Hela and COS-7 cells induces insoluble intranuclear inclusions

Hela (**A**) and COS-7 (**B**) cells transiently transfected with mPABPN1-ala17. Fourty eight hours after transfection cells were fixed with 4% paraformaldehyde and then visualized using a fluorescence microscope. Arrows indicate the nuclei containing INIs induced by expression of mPABPN1-ala17. Cells transfected with control GFP-C2 vector alone are shown (**C**).

Figure 2

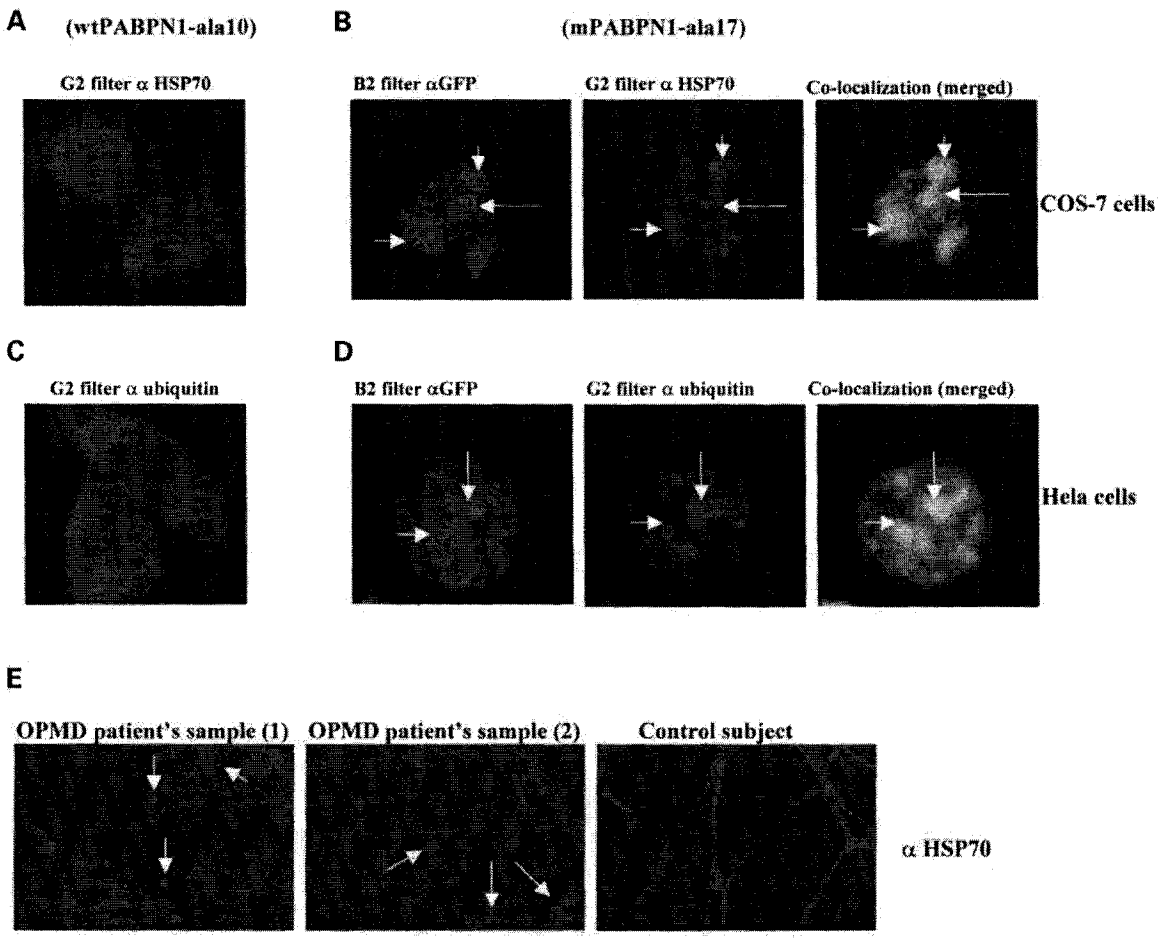
A
(wtPABPN1-ala10)
B
(mPABPN1-ala17)
G2 filter α HSP70
B2 filter αGFP
G2 filter α HSP70
Co-localization (merged)
COS-7 cells
C
D
G2 filter α ubiquitin
B2 filter αGFP
G2 filter α ubiquitin
Co-localization (merged)
Hela cells
E
OPMD patient's sample (1)
OPMD patient's sample (2)
Control subject
α HSP70

Figure 2

HSP70 and ubiquitin co-localize with INIs of mPABPN1-ala17 in cell culture model. HSP70 is recruited into INIs of OPMD muscle cells

Immunocytochemical detection on COS-7 and Hela cells transfected with GFP-wtPABPN1-ala10 and GFP-mPABPN1-ala17, 48 h post-transfection. CY3 conjugated secondary antibody (red) was used to label either HSP70 or ubiquitin. HSP70 and ubiquitin localize diffusely to the nucleus and cytoplasm in cells transfected with wtPABPN1-ala10 with no specific protein redistribution (**A** and **C**, respectively). In contrast, both HSP70 and ubiquitin co-localize to INIs of mPABPN1-ala17 (**B** and **D,** respectively). Merging (yellow) of the two signals (red and green) illustrates co-localization. Arrows indicate the INIs. (**E**) Immunohistochemical detection was performed on cross sections of the deltoid muscle from OPMD patients, using monoclonal anti-HSP70 antibody and CY3 conjugated secondary antibody. Nuclear inclusions stain positively for HSP70. Muscles in control subject did not show immunostaining of subcellular structures. Arrows indicate positively stained aggregates.

Figure 3

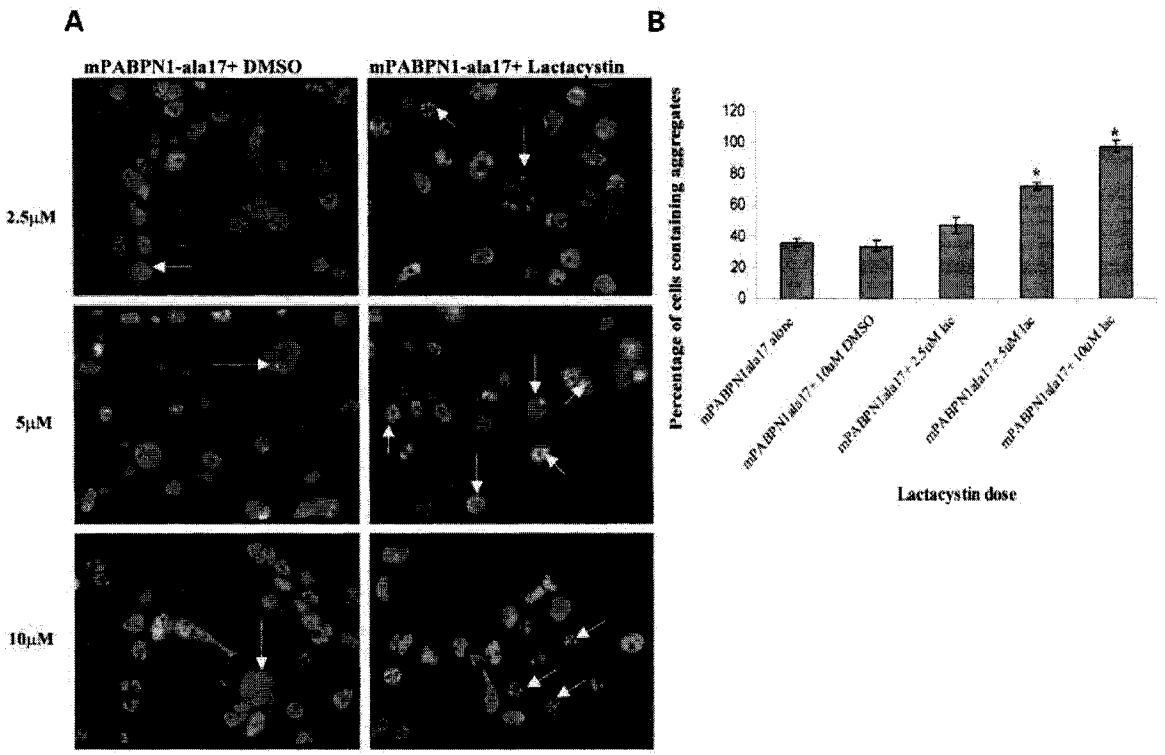
A
B
mPABPN1-ala17+ DMSO
mPABPN1-ala17+ Lactacystin
2.5μM
5μM
10μM
Percentage of cells containing aggregates
120
100
80
60
40
20
0
mPABPN1ala17 alone
mPABPN1ala17+ 10uM DMSO
mPABPN1ala17+ 2.5uM lac
mPABPN1ala17+ 5uM lac
mPABPN1ala17+ 10uM lac
Lactacystin dose

Figure 3

Lactacystin treatment increases INIs in cells transfected with mPABPN1-ala17 in a dose-dependent rate

(**A**) Immunofluorescence of COS-7 cells transfected with mPABPN1-ala17 and incubated with or without lactacystin (controls were treated with the carrier, DMSO). Lactacystin causes a marked increase in INIs. The arrows identify cells containing INIs or strong nuclear fluorescence signal. (**B**) Quantitation of lactacystin-induced mPABPN1-ala17 aggregation in COS-7 cells (2.5, 5 and 10 μM lactacystin) shows dose-dependence of lactacystin-induced aggregation. Cells were incubated for 24 h with the indicated concentrations of lactacystin and scored for INI formation. All transfected cells were scored by their staining pattern as having the nucleus diffusely labeled, or as containing nuclear aggregates. All experiments were repeated three times. All values were expressed as means $\pm$ SEM. Statistical analysis was performed using the Anova single factor, with $P < 0.05$ considered statistically significant.

Figure 4

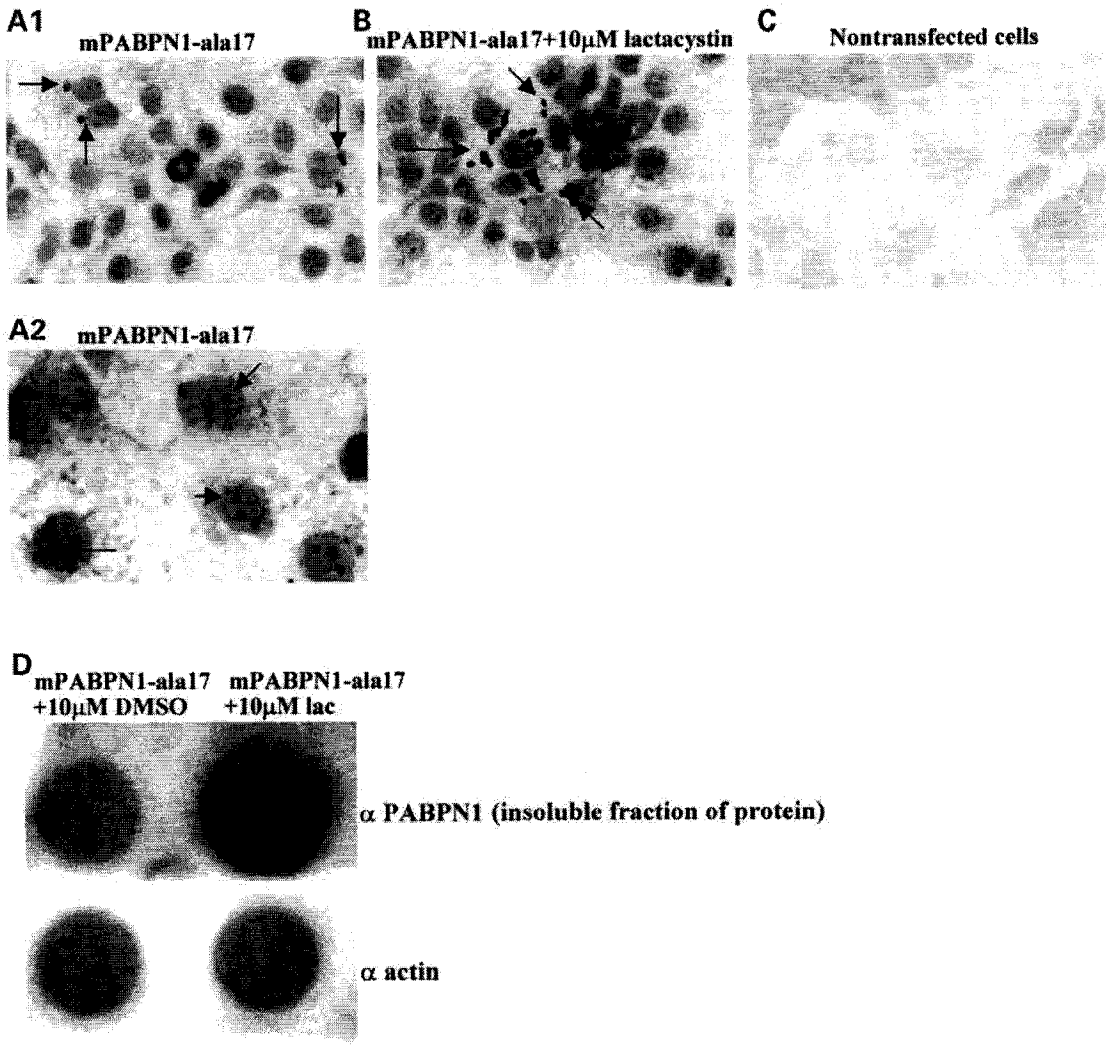
A1
mPABPN1-ala17
B
mPABPN1-ala17+10μM lactacystin
C
Nontransfected cells
A2
mPABPN1-ala17
D
mPABPN1-ala17
+10μM DMSO
mPABPN1-ala17
+10μM lac
α PABPN1 (insoluble fraction of protein)
α actin

Figure 4

Lactacystin treatment enhances cytoplasmic inclusion formation in cells transfected with mPABPN1-ala17, and increases insoluble fraction of mPABPN1-ala17 protein

Immunocytochemistry of COS-7 cells transfected with mPABPN1-ala17 and incubated without or with lactacystin (**A** and **B**) and control cells (**C**). (**A1**) Expression of mPABPN1-ala17 induces INIs and some cytoplasmic inclusions. Arrows indicate cytoplasmic inclusions. (**A2**) Higher magnification of cell nuclei transfected with mPABPN1-ala17; arrows indicate nuclei containing INIs. (**B**) Lactacystin treatment of mPABPN1-ala17 promotes increased cytoplasmic inclusion formation, as well as nuclear inclusions. Arrows indicate nuclei flanked by cytoplasmic inclusions. (**C**) Control nontransfected cells. (**D**) Lactacystin treatment increases the insoluble fraction of mPABPN1-ala17 protein as shown by dot blot assay. Fractionation of lysates into insoluble fraction was carried before immunoblot analysis with anti-PABPN1 antibody. Equal loading of lysates was confirmed using anti-actin antibody.

Figure 5

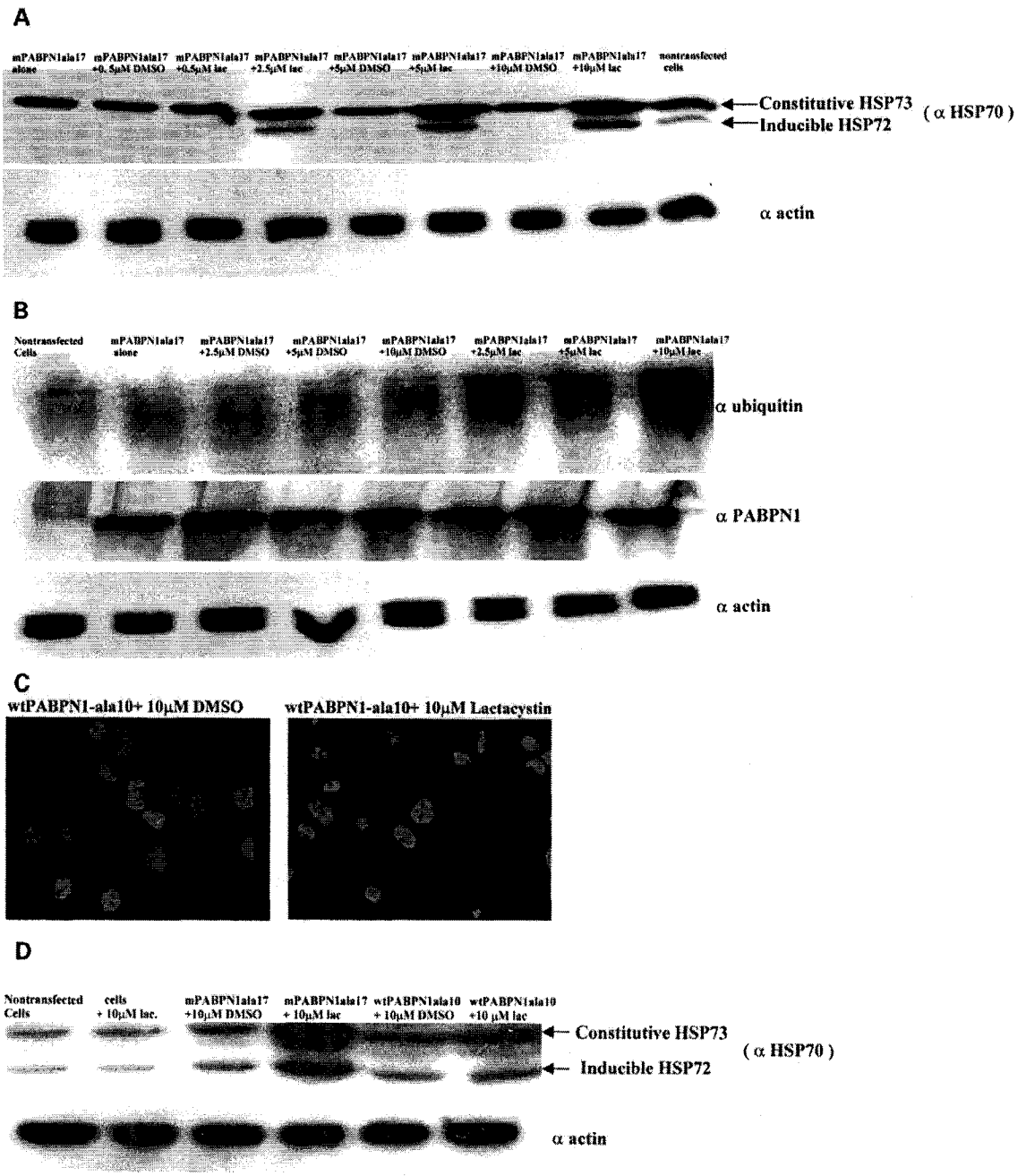
A
mPABPN1ala17 alone
mPABPN1ala17 +0. 5μM DMSO
mPABPN1ala17 +0.5μM lac
mPABPN1ala17 +2.5μM lac
mPABPN1ala17 +5μM DMSO
mPABPN1ala17 +5μM lac
mPABPN1ala17 +10μM DMSO
mPABPN1ala17 +10μM lac
nontransfected cells
Constitutive HSP73
Inducible HSP72
(α HSP70)
α actin
B
Nontransfected Cells
mPABPN1ala17 alone
mPABPN1ala17 +2.5μM DMSO
mPABPN1ala17 +5μM DMSO
mPABPN1ala17 +10μM DMSO
mPABPN1ala17 +2.5μM lac
mPABPN1ala17 +5μM lac
mPABPN1ala17 +10μM lac
α ubiquitin
α PABPN1
α actin
C
wtPABPN1-ala10+ 10μM DMSO
wtPABPN1-ala10+ 10μM Lactacystin
D
Nontransfected Cells
cells + 10μM lac.
mPABPN1ala17 +10μM DMSO
mPABPN1ala17 + 10μM lac
wtPABPN1ala10 + 10μM DMSO
wtPABPN1ala10 +10 μM lac
Constitutive HSP73
Inducible HSP72
(α HSP70)
α actin

Figure 5

Proteasome inhibitor (lactacystin) treatment leads to ubiquitin conjugate and HSP70 induction only in cells transfected with mPABPN1-ala17

(**A**) Lysates from control cells and cells expressing mPABPN1-ala17 in the absence or presence of lactacystin (0.5, 2.5, 5, and 10 μM) were examined; immunoblotting with anti-HSP70 antibody showed increase in both the constitutive HSP73 (upper band) and inducible HSP72 (lower band) forms of HSP70 after lactacystin treatment. Equal loading of proteins was confirmed using anti-actin antibody. (**B**) Hela cells expressing mPABPN1-ala17 and incubated with different doses of lactacystin (2.5, 5, and 10 μM) exhibited an increased level of total ubiquitinated conjugates, compared with the controls, as shown by Western blot using anti-ubiquitin antibody. Control cells were transfected with mPABPN1-ala17 and treated with the carrier, DMSO, at similar doses of lactacystin. Cells expressing mPABPN1-ala17 alone as well as nontransfected cells are also shown. Total ubiquitin conjugate levels remained constant in cells expressing mPABPN1-ala17 and treated with DMSO at different doses. Similar ubiquitin conjugate levels were observed in cells expressing mPABPN1-ala17 alone or nontransfected cells. The blot was re-probed with anti–PABPN1 antibody to measure PABPN1 protein levels: PABPN1 expression level did not change after lactacystin treatment in cells expressing mPABPN1-ala17. Note the faint band of the nontransfected cells that corresponds to the endogenous PABPN1 protein. Equal loading of proteins was confirmed using anti-actin antibody. There was no clear HSP70 induction in cells expressing mPABPN1-ala17 compared with cells expressing wtPABPN1-ala10. (**C**) Green fluorescent micrographs of COS-7 cells transfected with wt-PABPN1-ala10 and incubated with 10 μM DMSO or 10 μM

lactacystin. There was no difference in aggregation formation in cells expressing wtPABPN1-ala10 following lactacystin treatment compared to the control cells. **(D)** Lysates from nontransfected cells, cells expressing wtPABPN1-ala10 and mPABPN1-ala17 were examined by immunoblot with anti-HSP70 antibody. The lysates from cells were treated with 10 μM DMSO or 10 μM lactacystin. Lactacystin did not induce HSP70 in nontransfected cells. HSP70 induction was highly detected in cells expressing mPABPN1-ala17 and treated with 10 μM lactacystin. There was a slight change in HSP70 level in cells expressing wtPABPN1-ala10 and treated with 10 μM lactacystin. Equal loading of proteins was confirmed using anti-actin antibody.

Figure 6

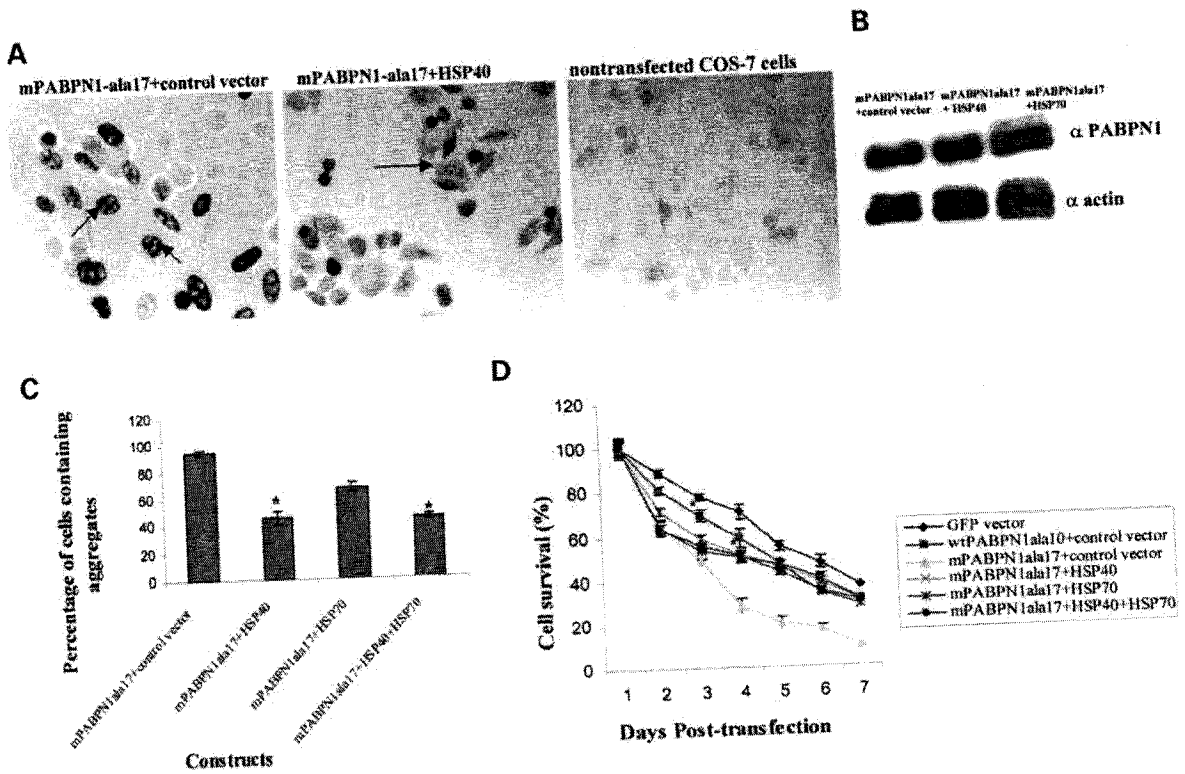
A
mPABPN1-ala17+control vector
mPABPN1-ala17+HSP40
nontransfected COS-7 cells
B
mPABPN1ala17 +control vector
mPABPN1ala17 +HSP40
mPABPN1ala17 +HSP70
α PABPN1
α actin
C
Percentage of cells containing aggregates
120
100
80
60
40
20
0
*
*
mPABPN1ala17+control vector
mPABPN1ala17+HSP40
mPABPN1ala17+HSP70
mPABPN1ala17+HSP40+HSP70
Constructs
D
Cell survival (%)
120
100
80
60
40
20
0
1 2 3 4 5 6 7
Days Post-transfection
GFP vector
wtPABPN1ala10+control vector
mPABPN1ala17+control vector
mPABPN1ala17+HSP40
mPABPN1ala17+HSP70
mPABPN1ala17+HSP40+HSP70

Figure 6

Molecular chaperone overexpression reduces INIs and cell toxicity of mPABPN1-ala17 without affecting protein expression levels

(**A**) Immunocytochemistry of COS-7 cells co-transfected with mPABPN1-ala17 and control vector shows cells containing INIs. We found significant reduction of INIs in cells co-transfected with mPABPN1-ala17 and HSP40. Nontransfected cells were used as control. Arrows indicate the transfected cells containing insoluble INIs. (**B**) Molecular chaperone HSP40 overexpression does not change the protein level of mPABPN1-ala17 when co-transfection in COS-7 cells was carried out as shown by Western blot. Anti-PABPN1 antibody was used as primary antibody. Equal amounts of protein were loaded in each lane. Experiment was repeated three times, all showing that the chaperones (HSP40 or HSP70) do not alter total mPABPN1-ala17 protein level. (**C**) COS-7 cells co-transfected with mPABPN1-ala17 along with control vector or HSP40, HSP70, HSP40 and HSP70 expressing vectors. The proportions of GFP-positive cells with INIs were determined at 48 h post-transfection. The results come from three representative independent experiments. Mean $\pm$ SEM; $P < 0.05$ (ANOVA analysis). (**D**) Percentage of living cells expressing co-transfected constructs at different times post-transfection. COS-7 cells co-transfected with wtPABPN1-ala10 or mPABPN1-ala17 and control vector or HSP40, HSP70, HSP40 and HSP70 expression vectors. The cells were counted every 24 h post-transfection. Each well was counted three times in different areas at one time point, and the mean was used for statistics. The percentage of living cells transfected represents the variation of the amount of living transfected cells at different

time points compared with the number of transfected cells obtained on day 1. Mean $\pm$ SEM; $P < 0.05$ compared with any other groups (ANOVA analysis).

Figure 7

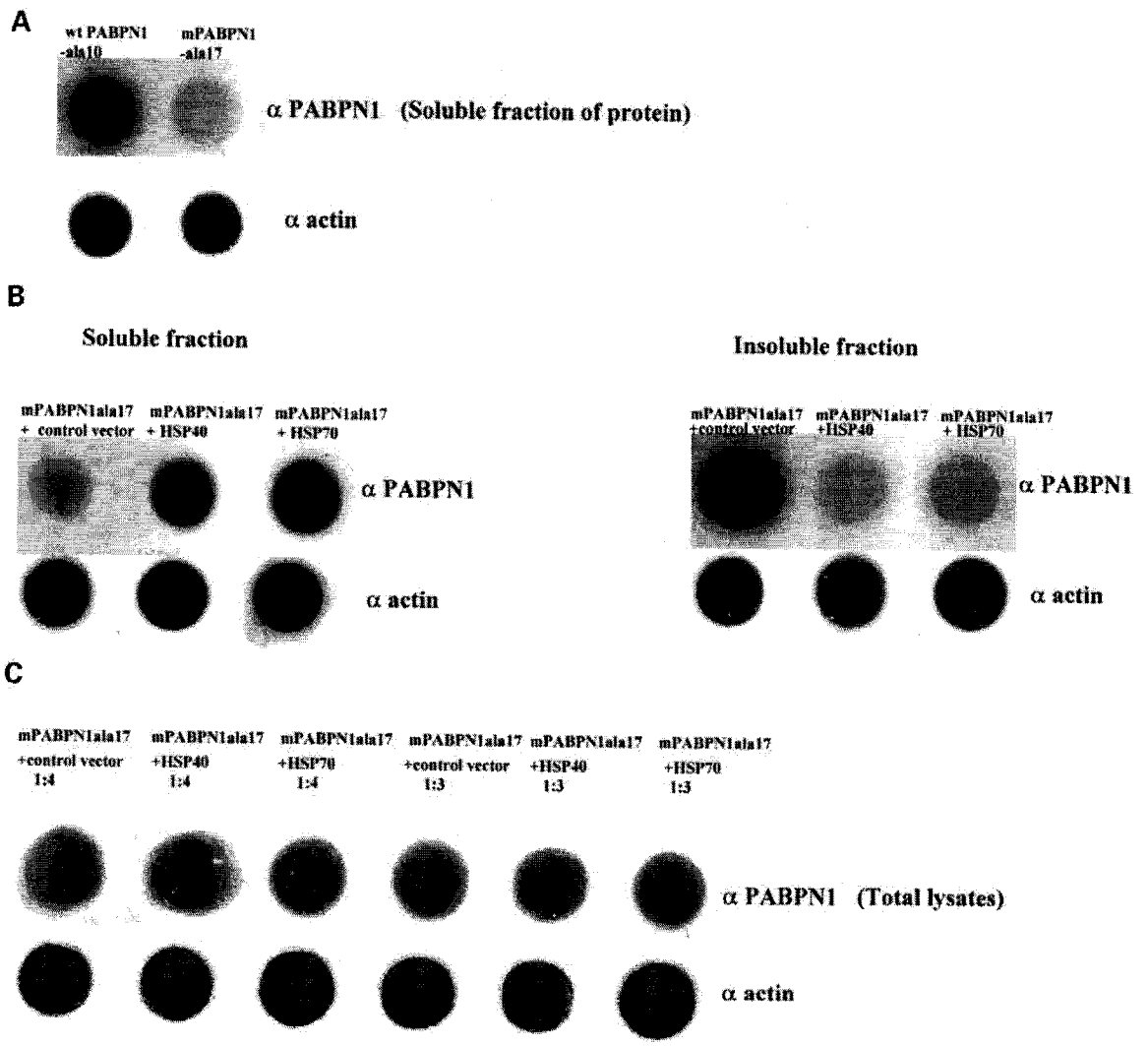
A
wt PABPN1 -ala10
mPABPN1 -ala17
α PABPN1 (Soluble fraction of protein)
α actin
B
Soluble fraction
Insoluble fraction
mPABPN1ala17 + control vector
mPABPN1ala17 + HSP40
mPABPN1ala17 + HSP70
α PABPN1
α actin
C
mPABPN1ala17 +control vector 1:4
mPABPN1ala17 +HSP40 1:4
mPABPN1ala17 +HSP70 1:4
mPABPN1ala17 +control vector 1:3
mPABPN1ala17 +HSP40 1:3
mPABPN1ala17 +HSP70 1:3
α PABPN1 (Total lysates)
α actin

Figure 7

Molecular chaperone overexpression increases soluble fraction of mPABPN1-ala17 protein without affecting the level of mPABPN1-ala17 protein

(**A**) The expression of wtPABPN1-ala10 shows a significant more soluble protein level when compared to mPABPN1-ala17 expression. Nitrocellulose dot-blot was used to detect soluble fraction of the protein. Immunoblotting was performed using anti-PABPN1 antibody. Probing with an anti-actin antibody confirmed that equivalent amounts of the protein were loaded. (**B**) Detection of soluble and insoluble fractions of mPABPN1-ala17 after chaperone co-transfection. Overexpression of HSP40, or HSP70 with mPABPN1-ala17 increases the soluble fraction of mPABPN1-ala17 protein as shown in dot blot assay. The increase in soluble protein was accompanied by a concomitant decrease in the insoluble mPABPN1-ala17 protein. Transfected Hela cells were harvested and fractionation of lysates into soluble and insoluble fractions was carried out before immunoblot analysis with anti-PABPN1 antibody. Equal loading of lysates was confirmed using anti-actin antibody. (**C**) Molecular chaperone overexpression does not change the protein level of mPABPN1-ala17 when co-transfection of Hela cells was carried out at different ratios (mPABPN1-ala17: chaperone, 1:4 or 1:3). The filter membrane was blotted with anti-PABPN1 antibody. Equal loading of lysates was confirmed using anti-actin antibody. The filter trap assay experiments were performed three times.

Chapter 4: *Investigation of the role of nuclear aggregates versus the soluble expanded PABPN1 in OPMD cellular toxicity*

Christiane Messaed[1], Patrick Dion[1], Aida Abu-Baker[1], Daniel Rochefort[1], Janet Laganiere[1], Bernard Brais[2] and Guy A. Rouleau[1]. Soluble expanded PABPN1 promotes cell death in Oculopharyngeal Muscular Dystrophy. *Neurobiol Dis*. 2007; 26(3): 546-57.

4.1 Rationale

While we observed a direct correlation between the reduction of expPABPN1 aggregation and the suppression of cellular toxicity in our previous cellular model, both events may nonetheless have occured independently. In that, a decrease in cellular toxicity may have been the outcome of the protective properties of molecular chaperones, inducing the refolding and the clearance of expPABPN1 (Huang et al., 2001; Murata et al., 2001; Wickner et al., 1999) or inhibiting the activation of apoptotic pathways (Beere et al., 2000; Li et al., 2000); rather than a consequence of the reduction of aggregate formation. To evaluate the relative contribution of aggregated and soluble expPABPN1 we decided to manipulate the level of protein aggregation and increase the solubility of expPABPN1 before cell survival was assessed. We also planned to use live-cell imaging to follow the evolution of cells expressing expPABPN1. Considering that the soluble expPABPN1 may result in aberrant interactions with crucial cellular components, it was predicted that an increase of soluble expPABPN1 in the presence of endogenous HSPs level would significantly increase cell death. Nuclear aggregates may therefore represent cellular compartments that recruit soluble toxic expPABPN1. The protective role of nuclear aggregates has been elucidated in many polyglutamine disease models (Arrasate et al., 2004; Bodner et al., 2006).

4.2 Abstract

Oculopharyngeal muscular dystrophy (OPMD) is an autosomal dominant disease caused by the expansion of a polyalanine repeat $(GCG)_{8-13}$ in exon 1 of the *PABPN1* gene. Skeletal muscle fibers nuclei from OPMD patients contain insoluble polyalanine expanded PABPN1 (expPABPN1) nuclear aggregates that sequester different cellular components. Whether these aggregates are pathogenic, or the consequence of a molecular defense mechanism, remains controversial in the field of neurodegenerative disorders and OPMD. Our cellular model shows that interfering with the formation of expPABPN1-induced large nuclear aggregates increases the availability of nuclear expPABPN1 and significantly exacerbates cell death. Live microscopy reveals that cells harboring an increased amount of the soluble forms of expPABPN1 are significantly more prone to toxicity than those with nuclear aggregates. This is the first report directly indicating that nuclear aggregation in OPMD may reflect an active process by which cells sequester and inactivate the soluble toxic form of expPABPN1.

4.3 Introduction

Oculopharyngeal muscular dystrophy (OPMD) is a late-onset degenerative disorder inherited in an autosomal dominant manner (Little and Perl, 1982). Symptoms usually begin between the fourth and sixth decade of life and primarily involve eyelid drooping (ptosis), difficulty swallowing (dysphagia) with proximal limb weakness (Bouchard et al., 1997; Brais et al., 1999). This myopathy was shown to result from the short expansion of a polyalanine stretch that is encoded by a $(GCG)_{8\rightarrow13}$ repeat in the poly(A) binding protein nuclear 1 (*PABPN1*) gene. The polyalanine domain lies at the beginning of the gene's first exon (Brais et al., 1998). Wild-type PABPN1 (wtPABPN1) is an abundant nuclear protein primarily involved in pre-mRNA polyadenylation processes, though a role in transcription has also been suggested (Bear et al., 2003; Kim et al., 2001). PABPN1 proteins stimulate polyadenylation of poly(A)RNA by tethering the poly(A) polymerase (PAP) to the RNA primer (Kerwitz et al., 2003; Kuhn and Wahle, 2004).

Light and electron microscopy (EM) analysis of muscle biopsies obtained from OPMD patients typically show the presence of nuclear aggregates within nuclei of skeletal muscles (Tome and Fardeau, 1980). Under EM the nuclear aggregates, which are formed by the polyalanine expanded PABPN1 proteins (expPABPN1), appear as tubular filaments that converge to form tangles and palisade structures (only distinguishable under EM). The observation of these structures is deemed as one of the major criteria in a definitive OPMD diagnosis (Brais et al., 1995). In OPMD cases, these visible aggregates always contain expanded PABPN1 proteins (expPABPN1) but they also contain other proteins such as molecular chaperones, ubiquitin and proteasome

components (Abu-Baker et al., 2003). This parallel recruitment of additional non-pathological proteins to the aggregates is also a well documented observation among polyglutamine disorders (Waelter et al., 2001). Additionally, OPMD nuclear aggregates are also found to be tightly associated with poly(A)RNA as expPABPN1 proteins still contribute to the polyadenylation of cellular RNA, at least until the expPABPN1 proteins physical status begins to shift toward a visibly aggregated form (Calado et al., 2000b; Tavanez et al., 2005).

In the past, reports provided data suggesting that expPABPN1 nuclear aggregates may promote cell death in OPMD (Bao et al., 2004). Despite these data, the role of expPABPN1 polyalanine aggregates as the primary pathogenic species remains ambiguous; moreover recently published reports have also shown that, in polyglutamine disorders, aggregates seem to have a protective role (Bodner et al., 2006; Bowman et al., 2005). In both cellular and animal models, the overexpression of soluble forms, which includes monomers and functional oligomers, of expPABPN1 is the necessary precursor event that leads to the appearance of aggregate, thus making it difficult to evaluate separately the relative toxicities of early soluble monomers, functional oligomers, misfolded monomers and large visible insoluble aggregates. Nevertheless, the presence of large aggregates remains a useful pathological marker and it additionally provides valuable clues to the pathogenesis.

To evaluate the toxic contribution of both the expPABPN1 soluble forms and the aggregated expPABPN1, we targeted three independent cellular processes previously reported to interfere with expPABPN1 aggregation. Previous reports suggested that interfering with the binding of expPABPN1 to poly(A) RNA, mRNA polyadenylation by

the poly(A)polymerase or the ubiquitination processes are three different strategies for modulating the formation of expPABPN1 nuclear aggregates (Abu-Baker et al., 2003; Tavanez et al., 2005). To investigate the role of expPABPN1 aggregates in cell toxicity, we separately manipulated each one of these three processes to decrease the formation of aggregates and conversely increase the amount of soluble expPABPN1 protein available inside the cells. Here we report that both interfering with PABPN1 polyA(RNA) binding or recruitment of PAP to the polyadenylation sites lead to a reduction of nuclear aggregates and an increase in cell death. In parallel, we show that blocking protein ubiquitination also reduces expPABPN1 aggregate formation and induces cell death. Finally, using an automated microscopy system we followed individual cells at different time intervals and observed that cells containing nuclear aggregates are more resistant to cell death than cells that harbor only the soluble form of expPABPN1. Our results suggest that the expression of soluble forms of expPABPN1 increases the risk of cell death.

4.4 Materials and methods

Cloning of wild-type and expanded PABPN1 fused to GFP

Human cDNA clones encoding either wtPABPN1 with a (GCG_6) repeat or expPABPN1 with a (GCG_{14}) repeat were subcloned in a pEGFP-C2 vector (Clontech, USA). The wild-type and expanded PABPN1 mutated variants (F215A and Δ131-132) were generated by site-directed mutagenesis. We used the Quick change™ site directed mutagenesis kit (Stratagene, USA) to generate the mutations and all procedures were performed according to manufacturer's instructions. The (F215A) missense mutation was generated using primer 5'-215ala (GGC CAT CCC AAA GGG GCA GCG TAT ATA GAG) in conjunction with primer 3'-215ala (CTC TAT ATA CGC TGC CCC TTT GGG ATG GCC). The two amino acids deletion (Δ131-132) was generated using primer 5'-Δ131-132 (GTC AGG GAG ATG GAG GCT GAG AAG CTA AAG) in conjunction with primer 3'-Δ131-132 (CTT TAG CTT CTC AGC CTC CAT CTC CCT GAC). The integrity of all clones was confirmed by direct sequencing.

Cell culture and transfection

One day prior to transfection HeLa cells were seeded at a density of ~2.5×10^5 cells per well using 6-well plates in 2 ml of Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL USA) supplemented with 10% of fetal bovine serum (FBS) (Gibco, BRL). Previous reports on the formation of aggregates by either polyalanine or polyglutamine protein showed that HeLa cells are an adequate model of the cellular events involved in various diseases (Kim et al., 2002; Tavanez et al., 2005). The cells were transfected with 1 μg of plasmid DNA pre-complexed with the Plus Reagent and diluted in Lipofectamine

Reagent according to manufacturer's instructions (Gibco BRL, USA). In co-transfection experiments, the $pCS2^{+}$-hCDC34(CL→S) vector encoding the human Dominant Negative Ubiquitin-Conjugating Enzyme or the corresponding empty vector $pCS2^{+}$ was co-transfected with either pEGFP, pEGFP containing wtPABPN1 or pEGFP containing expPABPN1 at 2:1 ratio.

KCl treatment and immunocytochemistry

HeLa cells were grown on glass cover slips placed in a 35 mm tissue culture dish prior to the cells' transfection that was performed as described in the section above. Forty-eight hours post-transfection, cells expressing the GFP-PABPN1 fusion proteins were washed once with 1X phosphate buffered saline (PBS) and fixed in 4% paraformaldehyde for 15 minutes. The cover slips were mounted on slides and the cells were visualized using a fluorescent microscope with the appropriate GFP filter (GFP fluorescence is excited at 488 nm and its emission examined at 516 nm). For KCl treatment experiments, cells expressing GFP-wtPABPN1, GFP-expPABPN1 and the GFP-expPABPN1 mutated forms were treated with 1.5 M KCl in HPEM buffer (30 mM HEPES, 65 mM PIPES, 10 mM EDTA, 2 mM MgCl2, pH 6.9) for 20 minutes prior to fixation. Immunodetection were done using a JL-8 monoclonal antibody against GFP (Clontech, #632680) at 1:10,000, a polyclonal antibody against ubiquitin (DakoCytomation, #Z0458) at 1:300, and a monoclonal antibody against SC35 (Sigma-Aldrich, #S4045) at 1:2,000.

Western blot analysis

Twenty-four hours post-transfection, cells were harvested and the proteins extracted in 3X Laemmli buffer. Equal amounts of protein were separated by electrophoresis on 10%

SDS-PAGE before transferring them to nitrocellulose membranes. The membranes immunodetected using a monoclonal antibody directed against GFP (1:2,000) (Clontech, USA) and monoclonal antibody directed against human actin (1:5,000) (Chemicon International, USA), and revealed them using the Western blot chemiluminescence reagent *Plus* Kit (NEN Life Science Products, Boston, MA, USA).

Assessment of aggregation and cell survival assay

The number of cells presenting nuclear aggregates versus the number of cells with a diffusely distributed nuclear signal was assessed over ten different fields. At each time point, the percentage of cells with nuclear aggregates was calculated by dividing the number of cells with aggregates over the total number of transfected cells. The average of the ten separate ratios obtained was calculated and used as the percentage for each time point. For cell survival assay, the number of transfected living cells was measured every 24 hours post-transfection, for a total of 7 days. Each well was gently and briefly washed two times with DMEM to remove unattached cells and counted the GFP-positive cells occupying a 1-mm2 area under low magnification (20X). Three different areas within each well were counted. All wells were in triplicate and the experiments were repeated three times (n=9). All values were expressed as means ± SEM. Statistical analysis were done using Anova single factor analysis given it is the appropriate comparison test to deal with a single independent variable between all the compared values (P<0.05 was considered as statistically significant).

Live cell imaging

The day before transfection HeLa cells were seeded ~1.25×10^5 cells in nine separate wells of a twelve-well plate. Cells were transfected with 0.7 μg of plasmid DNA encoding the GFP-expPABPN1 protein using the procedure described before. A twelve-well plate was mounted on a Leica automated microscope stage, connected to the *Openlab* software (Improvision). The *Openlab* system was programmed to memorize nine distinct fields of cells (one in each separate well) and to control the stage to return to the same position at pre-fixed intervals. Cell survival was monitored from 24 hours post-transfection at 60 minutes interval for a period of ten hours. Each field presented three classes of cells: (1) cells with diffusely distributed soluble GFP-expPABPN1, (2) cells with diffusely distributed GFP-expPABPN1 and nuclear aggregates and (3) cells with only aggregated GFP-expPABPN1. The percentage of cells dying from each class was determined by dividing the number of cells that died from each group by the total number of dead cells. The percentage of cell death of each group of cells was scored for nine different fields. The average of the nine ratios was calculated and the results presented as a percentage ± SEM. All values were expressed as means ± SEM. Statistical analysis were done using Anova single factor analysis given it is the appropriate comparison test to deal with a single independent variable between all the compared values ($P<0.05$ was considered as statistically significant).

4.5 Results

A mutation in the RNA binding domain of expPABPN1 diminishes nuclear aggregation

PABPN1's high affinity for poly(A)RNA (Calado et al., 2000b; Kerwitz et al., 2003; Krause et al., 1994; Kuhn et al., 2003) partly explains the propensity of wtPABPN1 and expPABPN1 to oligomerize, a property which is thought to favor inclusion formation (Calado and Carmo-Fonseca, 2000; Fan et al., 2001; Tavanez et al., 2005). A point mutation (F215A) within the RNA binding domain of wtPABPN1 was previously reported to strongly impair the RNA binding activity of PABPN1 (Kuhn et al., 2003). We therefore hypothesized that an F215A mutation in expPABPN1 would decrease the formation of large aggregates, conversely increasing the amount of soluble expPABPN1 in cells expressing this mutant protein. All expression vectors, including wtPABPN1(F215A), expPABPN1(F215A) and their non mutated forms wtPABPN1 (10 alanines) and expPABPN1 (18 alanines), were made using a PABPN1 cDNA that was fused to a green fluorescence protein (GFP) tag. These vectors were transfected in HeLa cells to express the fusion proteins transiently before observing the cells using a fluorescent microscope. Despite the fact that OPMD is a disorder primarily affecting muscles cells, HeLa cells are commonly used to study aggregate formation as the regulators of this process are likely to be well conserved across cell type (Kim et al., 2002; Tavanez et al., 2005). The expression of GFP-wtPABPN1 confirmed the appearance of the previously described "nuclear speckles" pattern (Bear et al., 2003; Calado and Carmo-Fonseca, 2000; Kim et al., 2001) in ~70% of transfected cells. In contrast, the expression of GFP-expPABPN1 resulted predominantly in nuclear

aggregates, which are structures that appear more irregular, larger and clumped together, in ~70% of transfected cells (Fig. 1A and B). The majority of cells (~70%) transfected with GFP-wtPABPN1(F215A), revealed a diffusely distributed nuclear signal rather than the speckle pattern of GFP-wtPABPN1. In contrast ~70% of cells expressing GFP-expPABPN1(F215A) showed small nuclear aggregates within a diffuse signal that was stronger than the one observed with expression of GFP-expPABPN1 (Fig. 1A). We interpreted these data represent a fraction shift of the expPABPN1(F215) proteins from aggregates to soluble throughout the nucleoplasm. The aggregates formed by GFP-expPABPN1(F215A) appeared noticeably smaller than those observed in cells expressing GFP-expPABPN1. Also some cells expressing GFP-expPABPN1(F215A) protein showed a complete absence of nuclear aggregates and presented only a diffusely distributed signal. To show that protein expression of the different vectors was similar, Western blot immunodetections were done using an antibody directed against GFP. All the different vectors were found to give similar amount of protein, as shown in Fig. 1C. Based on these results we concluded that this approach increased the amount of soluble PABPN1 proteins within cells. Furthermore, we concluded that despite a strong diminution of their RNA binding activity and an intensification of the soluble protein signal, the GFP-expPABPN1(F215A) proteins remained nonetheless prone to form aggregates, though these were smaller in size. The recruitment of GFP-wtPABPN1(F215A) proteins to the nuclear speckles was strongly impaired and these proteins were mostly observed in soluble forms (Fig. 1A), though some cells (~30%) presented structures that were considered to be aggregates (Fig. 1B). The status of these

GFP-wtPABPN1(F215) proteins structures was resolved by a KCl treatment which will be detailed later on (Fig. 3).

A mutation in the putative α-helical domain of the expPABPN1 diminishes nuclear aggregation

In vitro, PABPN1 has been shown to bind to poly(A)RNA and increase the affinity of the poly(A) polymerase (PAP) for these molecules (Kerwitz et al., 2003). Defective stimulation of the PAP enzyme by PABPN1 has been predicted to compromise the formation of nuclear aggregates by expPABPN1 (Tavanez et al., 2005). Protein structure analysis of PABPN1 identified a highly conserved putative α-helical region near the N-terminus of PABPN1, which is essential for the stimulation of PAP (Benoit et al., 1999; Kerwitz et al., 2003). Missense mutations of charged amino acids in this α-helical region were shown to cause a defect in the stimulation of polyadenylation (Kerwitz et al., 2003). As in the experiment described above, our hypothesis was that the expression of this mutant would not only affect polyadenylation and reduce the formation of large expPABPN1 aggregates, but that it would also lead to an increase in soluble expPABPN1 in HeLa cells expressing it. In order to achieve this, two of the glutamate residues (Δ131-132) located within the α-helical domain of GFP-wtPABPN1 and GFP-expPABPN (Fig. 1A), were deleted. Cells transiently expressing GFP-expPABPN1(Δ131-132) protein formed few aggregates and ~70% of them displayed a strong diffusely distributed signal, which was more intense than the one observed when using GFP-expPABPN1(F215A). A fraction of cells (~30%) expressing GFP-expPABPN1(Δ131-132) protein formed some aggregates (Fig. 1A and B). The GFP-wtPABPN1(Δ131-132) proteins (Fig. 1A and B)

failed to assemble into speckles. It is important to point out that even though it affects the poly(A) polymerase, the expression of PABPN1 harboring the (Δ131-132) mutation does not appear to have a dominant negative effect on its own expression. This could be deduced from the examination of Western blots, which showed no change in the amount of the protein presenting this mutation (Fig. 1C), and from the observation that the number of GFP-positive cells per field was always identical for all the expression vectors (data not shown). We concluded that this second mutation (Δ131-132) also resulted in an increased proportion of soluble expPABPN1 protein. When we expressed expPABPN1 with either of the described mutations, GFP-expPABPN1(Δ131-132) or GFP-expPABPN1 (F215A), the diffusely distributed signal observed in the cell nuclei intensified, though substantially more with the (Δ131-132) mutation. This difference in the intensity of the two diffusely distributed signals is likely due to the small remaining aggregates observed with the (F215A) mutation, which presumably retain a fraction of the protein. In conclusion, the Δ131-132 mutation was more effective than the F215A mutation in increasing the soluble fraction of expPABPN1.

Aggregates of expPABPN1 partially localize to splicing factor compartments

In mammalian nuclei, most splicing factors (e.g. SC35, SF2/ASF and U2-B) are concentrated in 20-40 discrete domains referred to as "nuclear speckles". These domains also contain poly(A)RNA and consequently PABPN1 proteins since they are bound to poly(A)RNA (Calado and Carmo-Fonseca, 2000). Though most of these nuclear speckles are stationary, with respect to their overall position within the nucleus, some (less than 20%) exhibit dynamic behaviors such as peripheral extensions or the dis(association) of

particles, which are coupled to ongoing transcription (Misteli et al., 1997). It has been proposed that this dynamic movement enables these speckles to supply pre-mRNA splicing factors and transcription components (e.g. CBP/p300 and RNA polymerase II) to nearby activated sites of transcription. In order to establish the distribution of expPABPN1 and analyze the effect of mutations (F215A and Δ131-132) in the RBD and α-helical domains of both wtPABPN1 and expPABPN1, an antibody directed against the spliceosome assembly factor SC35 was used. This antibody has repeatedly been used to visualize nuclear speckles in the past (Fu and Maniatis, 1990; Hall et al., 2006). While GFP-wtPABPN1 protein co-localizes to the domains containing SC35 (Fig. 2A), the GFP-expPABPN1 and GFP-expPABPN1(F215A) proteins only partially overlap with nuclear speckles (Fig. 2B and D). Additionally, overlaid exposure images show numerous areas and punctate structures of GFP-expPABPN1 and GFP-expPABPN1(F215A) that did not co-localize with these splicing sites, suggesting the existence of nuclear areas of exclusive localization for either GFP-expPABPN1/GFP-expPABPN1(F215A) and SC35. All GFP-wtPABPN1(F215A) (Fig. 2C), GFP-wtPABPN1(Δ131-132) (Fig. 2E) and GFP-expPABPN1(Δ131-132) (Fig. 2F) proteins failed to localize with nuclear speckles, presumably a consequence of the decreased RNA binding activity or polyadenylation.

Nuclear aggregates formed by the mutated forms of expPABPN1 are KCl resistant

The conformational shift in expPABPN1 protein structure, which results from the polyalanine tract expansion, is generally believed to increase the protein's tendency to oligomerize and form stable β-sheet structures (Scheuermann et al., 2003). This

particular property of the mutated protein is thought to directly contribute to the formation of aggregates, in both patient's muscle and cellular models (Shinchuk et al., 2005b). Furthermore this property was shown to render expPABPN1 and other disease associated protein aggregates (e.g. neuroserpin) resistant to a potassium chloride (KCl) treatment (Calado and Carmo-Fonseca, 2000; Fan et al., 2001; Tavanez et al., 2005). In order to identify the aggregates that were KCl-resistant, a KCl treatment was performed on GFP-wtPABPN1 and GFP-expPABPN1 expressing cells. KCl treatment should remove both the transcriptional speckles and the diffusely distributed nuclear signals, while the aggregates structure should remain unaffected. All vectors including GFP-wtPABPN1, GFP-expPABPN1 and their mutated forms (F215A and Δ131-132) were transiently expressed for 48 hours in HeLa cells and then treated with 1.5 M KCl and fixed for microscopy. As expected all transcriptional speckles from GFP-wtPABPN1 completely dissociated upon KCl treatments (Fig. 3A and B), but the aggregates formed by GFP-expPABPN1 did not (Fig. 3C and D). The remaining smaller aggregates formed by GFP-expPABPN1(F215A) resisted the treatment, but the diffusely distributed nuclear signal it produced was lost (Fig. 3E and F). The diffusely distributed nuclear signal from GFP-expPABPN1(Δ131-132) was also completely removed (Fig. 3G and H). As expected with the two mutated forms of wtPABPN1, GFP-wtPABPN1(F215A) and GFP-wtPABPN1(Δ131-132), the diffusely distributed signal was always entirely washed away by the treatment (data not shown). Consequently it can be concluded that the expPABN1 aggregates contain insoluble forms of expPABPN1, while the diffusely signal produced by expPABPN1 consisted of soluble forms of the mutated protein.

In order to complement these KCl treatments, immunodetection with an antibody directed against ubiquitin showed that this cellular degradation pathway component only co-localized with the large nuclear aggregates formed by GFP-expPABPN1 (Fig. 3I). In comparison, cells expressing GFP-wtPABPN1, its mutated forms GFP-wtPABPN1(F215A) and GFP-wtPABPN1(Δ131-132), as well as cells expressing GFP-expPABPN1(F215A) and GFP-expPABPN1(Δ131-132), all showed a uniformly distributed ubiquitin signal (supplemental data 3). Neither the small aggregates observed in the absence of a KCl treatment following the expression of either GFP-expPABPN1(F215A) nor the aggregates formed by GFP-expPABPN1(Δ131-132) (Fig. 1A) showed co-localization with ubiquitin. The fact that only large expPABPN1 nuclear aggregates appeared to be both insoluble and ubiquitinated suggests that the ubiquitination of these aggregates occurs late in the process of their formation.

PABPN1-induced cellular toxicity inversely correlates with the degree of nuclear aggregate formation

In order to establish the contribution to cellular toxicity of the soluble mutant forms of GFP-expPABPN1, cells were independently transfected with GFP-expPABPN1(F215A) and GFP-expPABPN1(Δ131-132) as well as with GFP alone, GFP-wtPABPN1 and wtPABN1 mutated counterparts (GFP-wtPABPN1(F215A) and GFP-wtPABPN1(Δ131-132)). The transfection of a vector expressing GFP alone is a necessary control to assess any background cellular toxicity that would occur in the presence of the fluorescent tag used to generate the fusion proteins. Using a fluorescent microscope, the transfection efficiencies were estimated to be ~80%, independently of the plasmid used. In parallel,

the exogenous PABPN1 proteins levels were evaluated by Western blot in order to confirm that their level of expression was equivalent between all transfections (Fig. 4A). Cell survival was monitored for seven consecutive days (day 1 corresponding to 24 hours post-transfection) and the number of GFP-positive living cells was counted every 24 h (Fig. 4B). The results show that the expression of GFP-expPABPN1(Δ131-132) protein, which is diffusely distributed in the nuclear compartment, appeared to be the most toxic to cells. Beginning at 48 hours (day 2) post-transfection, the number of living cells expressing GFP-expPABPN1(Δ131-132) was significantly lower than the number of living cells expressing GFP-expPABPN1 ($P<0.001$, Anova single factor analysis). This difference between GFP-expPABPN1 and GFP-expPABPN1(Δ131-132) remained significant throughout the experiment. When comparing cells that expressed GFP-expPABPN1 with cells that expressed GFP-expPABPN1(F215A), no difference in cell survival was observed during the first four days of the experiment ($P>0.05$, Anova single factor analysis). No significant difference was observed when the survival rate of cells expressing GFP-wtPABPN1 protein was compared to that of cells expressing the mutated variants GFP-wtPABPN1(Δ131-132) or GFP-wtPABPN1(F215A) or GFP alone. These data suggest that the soluble forms of expPABPN1 are significantly toxic.

Dominant Negative hCDC34(CL→S) prevents expPABPN1 aggregation and accelerates cell death

The nuclear aggregates that are formed by misfolded proteins in OPMD, Huntington's disease and other neurodegenerative disorders were reported to contain ubiquitin and components of the proteasome pathway (Abu-Baker et al., 2003; Cummings et al., 1999;

Schmidt et al., 2002; Waelter et al., 2001). Ubiquitin selectively binds to misfolded proteins and targets them for rapid degradation by the 26S proteasome (Ciechanover, 1998; Coux et al., 1996). In a previous report, a neuronal cell model where a huntingtin protein with polyglutamine expansion was expressed, Saudou *et al.* reported that the coexpression of a dominant negative form of the human ubiquitin-conjugating enzyme hCDC34(CL→S) could both prevent the ubiquitination of nuclear aggregates and reduce their formation (Saudou et al., 1998). Two mutations (C88S and L97S) within hCDC34(CL→S) were shown to produce a dominant negative effect in the ubiquitination processes (Banerjee et al., 1995; Pagano et al., 1995; Yew and Kirschner, 1997). To further investigate the impact of soluble expPABPN1 on cell survival, the strategy used by Saudou *et al* was applied to the OPMD cellular model used here. HeLa cells were co-transfected with plasmids GFP-wtPABPN1 or GFP-expPABPN1 together with hCDC34(CL→S) and cell survival was monitored for seven days, at 24 hour intervals. Since hCDC34(CL→S) was cloned in $pCS2^+$ we also cotransfected all PABPN1-encoding plasmids with an empty $pCS2^+$. As anticipated, the co-expression of hCDC34(CL→S) with GFP-expPABPN1 significantly suppressed the formation of aggregates in cells (Fig. 5A and B) ($P<0.001$, Anova single factor analysis). It also significantly accelerated cell death (Fig. 4C) by comparison to what was seen in cells expressing either GFP-expPABPN1 or GFP-wtPABPN1 with an empty $pCS2^+$ vector ($P<0.001$, Anova single factor analysis) (Fig. 5C). While this result was of interest, it was however critical to evaluate the portion of the reduction in cell survival that was attributable to the expression of hCDC34(CL→S) alone (Butz et al., 2005; Chauhan et al., 2004). In order to establish the specific impact of hCDC34(CL→S) on cell survival,

co-transfection of a vector encoding simply GFP along with either a $pCS2^+$ vector or a $pCS2^+$ with hCDC34(CL→S) vector (Fig. 5C) were performed. The expression of hCDC34(CL→S) alone decreased cell survival by ~10%. This indicates the cell survival difference attributable to the presence of the soluble form of expPABPN1, as determined from the comparison of the co-transfection of GFP-expPABPN1/pCS2+ and GFP-expPABPN1/hCDC34(CL→S) fluctuated between 15 and 10% (between day 2 and 4). The preparation of two parallel sets of co-transfections, GFP-expPABPN1 with pCS2+ and GFP-expPABPN1 with hCDC34(CL→S), also controls for the possibility that cells with aggregates might simply have died more rapidly, as in both situations the expression of GFP-expPABPN1 will have prompted aggregation in the same way. However, in the presence of hCDC34 the aggregation process of expPABPN1 is impaired, leaving the expPABPN1 protein to accumulate in a diffusely distributed signal across the nuclei. When observing cells in the course of the two co-transfections situations, the number of cells with GFP signal (with or without aggregates) appeared undistinguishable at the onset of the experiment (day 1), however as time passed the fraction of cells with only a diffusely distributed signal decreased. It therefore appears that cells presenting aggregates survived better. Altogether, this experiment indicated once again that an increased pool of soluble GFP-expPABPN1 significantly decreased cell survival.

Live microscopy shows that cells with expPABPN1 aggregates survive longer and more frequently undergo cell divisions

In order to gain additional insights on the impact of expPABPN1 aggregates on cell survival, we used an automated microscopy system to monitor, every 60 min for a period

of 10 h (0 h corresponding to 24 h post-transfection), nine fields of cells transiently expressing GFP-expPABPN1. The *Openlab* software (Improvision) precisely realigned the microscope objective with the live stage plate and periodically brought it back over the same field of cells. During this observation period, the majority (~62%) of cells dying had exclusively a diffusely distributed signal (Fig. 6A). In contrast, the presence of GFP-expPABPN1 aggregates appeared to significantly improve cell survival ($P<0.001$, Anova single factor analysis), as only a small fraction (~10%) of these cells died (Fig. 6B, Supplemental data 1).

At 48h post-transfection, the majority of cells that escaped cell death contained GFP-expPABPN1 nuclear aggregates. In order to precisely evaluate the number of cells expressing expPABPN1 that escaped cell death we observed the cells for a shorter period (4 h). It was estimated that ~46% of cells that had aggregates underwent mitosis, while almost all cells which had only a diffusely distributed signal did not. During mitosis events, nuclear aggregates disintegrated, the cell divided and the nuclear aggregates reappeared (Supplemental data 2). These observations suggest that nuclear aggregates prolonged cell survival, presumably by sequestering the soluble toxic forms of expPABPN1. Another group recently reported that 47% of cells with expPABPN1 aggregates underwent mitosis, which is similar to our results (Marie-Josee Sasseville et al., 2006).

4.6 Discussion

We used a cellular model to assess the relative toxicity conferred by expPABPN1 nuclear aggregates versus the toxicity conferred by the soluble forms of the same protein. We report here that nuclear aggregation seems to be part of a strategy used by cells to maintain homeostasis, essentially to protect them from the deleterious effects of soluble expPABPN1. In order to increase the fraction of expPABPN1 that is soluble, we targeted RNA binding, polyadenylation and ubiquitination, three processes involved in expPABPN1 polyalanine-associated aggregation. When we manipulated the PAP stimulation activity of GFP-expPABPN1(Δ131-132), the decrease in nuclear aggregate formation coincided with an increase of the diffuse form of the protein and a significant increase in cell death. In parallel, the F215A mutation within the RNA binding region of GFP-expPABPN1 also increased cell death, albeit at a later time-point and not to the same extent as the Δ131-132 mutation. The relative resistance of cells expressing GFP-expPABPN1(F215A) to cell death might possibly be due to the small remaining aggregates observed. Finally, co-expressing hCDC34(CL→S) with GFP-expPABPN1, which also increased the amount of the soluble form of expPABPN1, led to more cell death than the expression of GFP-expPABPN1 alone or the co-expressing hCDC34(CL→S) with GFP-wtPABPN1. These results lead us to disagree partially with previous reports suggesting a toxic role for expPABPN1 nuclear aggregates in cells. These reports described that (1) the formation of expPABPN1 aggregates was concomitant with the development of pathological symptoms (muscle weakness, loss of coordination) in transgenic animals (Davies et al., 2005; Dion et al., 2005; Hino et al., 2004); (2) doxycycline treatments *in vitro*, but not *in vivo,* decreased the number of

aggregates and increased cell viability (Bao et al., 2004; Davies et al., 2005); (3) overexpression of molecular chaperones (e.g. HSP70 and HSP40) *in vitro* decreased the number of aggregates and increased cell viability (Abu-Baker et al., 2003; Bao et al., 2004); and (4) deletion in the oligomerization domain decreased the number or aggregates and increased cell viability (Fan et al., 2001). In connection with the first argument, which suggests that expPABPN1 aggregates are toxic, it could alternatively be interpreted that cells with aggregates are those that survived a more extended period, henceforth supporting their protective role. One explanation for *in vitro/in vivo* disparity in the doxycycline experiment may be due to the fact that this drug presents both anti-amyloidogenic and anti-apoptotic protection properties (Bao et al., 2004; Chen et al., 2000). Davies *et al.* recently demonstrated that doxycycline offered therapeutic potential as they observed that its effect on expPABPN1 aggregation seemed distinct from its protection from the proapoptotic insult brought about by expPABPN1 (Davies et al., 2005). Davies *et al.* concluded that doxycycline-related reduction of proapoptotic Bax levels appears to be critical in the increase of myofiber survival. These observations support our findings, as the toxic (or proapoptotic) insult is most likely due to the unaggregated form of expPABPN1. As for the effects on cell viability that were observed following HSP overexpression, their role in preventing apoptosis (Beere et al., 2000; Mosser et al., 2004; Zhang et al., 2004) may explain their effects on cell survival (Yew et al., 2005). Finally, the mutation affecting the oligomerization of expPABPN1 had other effects, such as preventing nuclear localization, which may have contributed to a reduction in toxicity.

The automated microscopy method has been previously used to provide direct evidence for the protective role of inclusion formation in a neuronal cell model for Huntington's disease (Arrasate et al., 2004). Using the same approach, we observed that the soluble form of expPABPN1 was the most toxic, as cells failing to form aggregates were more at risk for cell death. Thus, we believe that the visible OPMD polyalanine-dependent aggregates share protective properties demonstrated in huntingtin polyglutamine-associated inclusion bodies (Arrasate et al., 2004), and also in alpha-synuclein amyloid associated beta-aggregation (Parkinson's disease) (Matsuzaki et al., 2004). In the case of polyglutamine expansions, recent reports demonstrated that neuronal dysfunction occurs in the absence of ubiquitin-proteosome system impairment and inversely correlates with the degree of nuclear aggregate formation (Bowman et al., 2005). Latouche *et al.* demonstrated that toxicity is determined by the type of aggregate rather than the cellular misfolded protein response, which includes the ubiquitin-proteosome system, and observed that large aggregates of polyglutamine-containing protein are protective (Latouche et al., 2006). Latouche *et al.* also compared the effect of both polyalanine and polyglutamine, but the length of polyalanine used (90 alanines) was far beyond naturally observed polyalanine expansions, which in this case generated small insoluble micro-aggregates, instead of the large visible aggregates we observe with an expPABPN1 that harbors a pathological polyalanine expansions. Furthermore, the promotion of large aggregates by the chemical compound B2 was recently shown to lessen cellular pathology in both Huntington's and Parkinson's diseases (Bodner et al., 2006). Finally, aggregate formation by expanded polyglutamine-containing protein ataxin-2 was reported to be independent of SCA2 pathogenesis in mouse or human models (Huynh et al., 2000).

These results, together with the data presented here, strongly suggest a protective role for large aggregates containing the mutant protein in several diseases. Nuclear aggregates may therefore represent the final step of a molecular cascade where the upstream soluble form of expPABPN1 would generate most of the cellular toxicity. The composition of the GFP-expPABPN1 large nuclear aggregates, which eventually sequester components of the ubiquitin-proteasome pathway and molecular chaperones, suggest their possible role in accelerating the degradation of misfolded expPABPN1 proteins when cells are overloaded with unaggregated species.

Our observations lead us to hypothesize that the increased cell toxicity which follows the *in vitro* reduction of expPABPN1 aggregation may have resulted from new and yet unidentified protein-protein interactions that would occur with the soluble form of expPABPN1. The structural change in expPABPN1, induced by the pathogenic alanine expansion, may lead to a gain of aberrant protein interactions, or alternatively prevent normally occurring interactions to take place. The interaction between soluble expPABPN1 and wtPABPN1 may also have compromised wtPABPN1's normal functions, and indirectly altered the expression of a number of genes early during OPMD disease progression. Candidate PABPN1-protein interactions which may be involved are those with transcription factors like MYO-D (Kim et al., 2001). A recent study showed that overexpression of expPABPN1 in a different cellular model led to a down-regulation and up-regulation of multiple genes, including endogenous PABPN1 itself (Corbeil-Girard et al., 2005). Identification of new expPABPN1 interacting proteins may shed new light on the molecular mechanisms underlying OPMD.

Our study demonstrates that a complex balance exists between expPABPN1 expression, nuclear aggregate formation and cell viability. Determining if a particular change within the protein is pathogenic or beneficial will have implications for a better understanding of OPMD and for the development of therapeutical strategies for this and related diseases.

4.7 Acknowledgements

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4.8 Figures

Figure 1

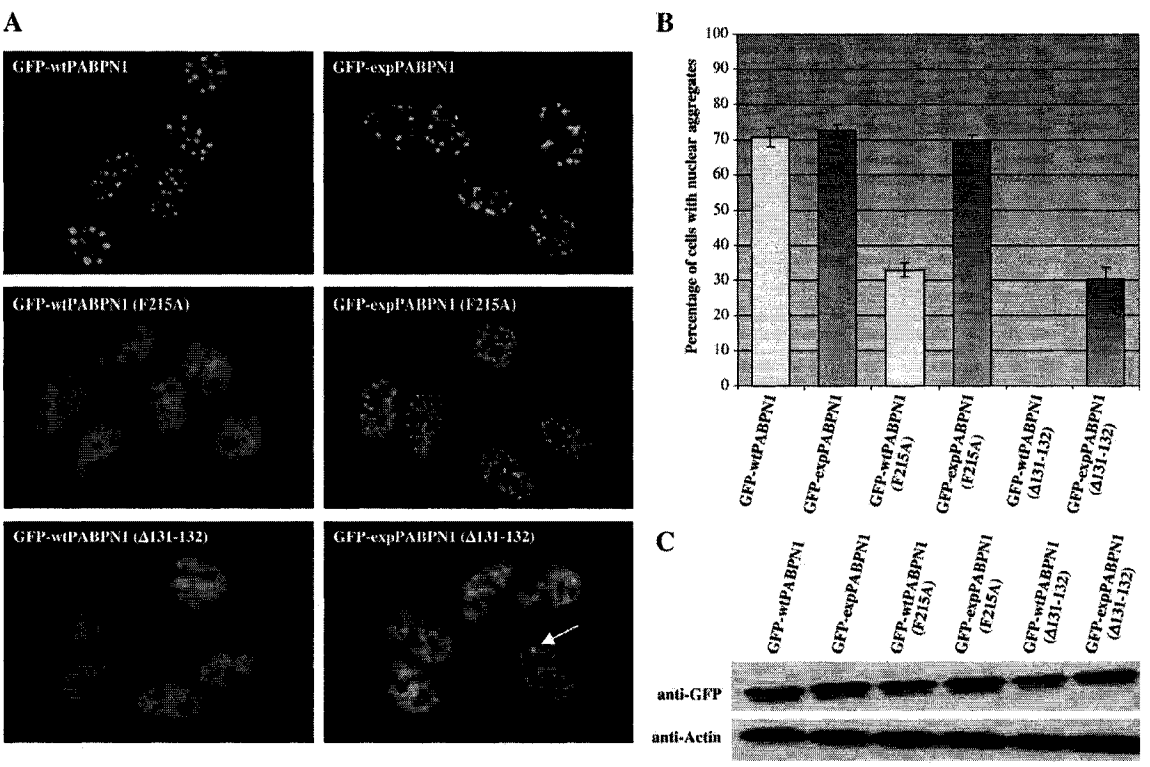


Figure 1

Formation of nuclear aggregates is dependent on polyadenylation

HeLa cells transfected with plasmids coding for different PABPN1 forms fused to GFP. GFP-wtPABPN1 and GFP-expPABPN1 respectively led to the apparition of nuclear speckle structures and nuclear aggregates in ~70% of transfected cells (**A** and **B**). Expression of the GFP-expPABPN1(F215A) (mutated in RNA binding domain) remained more prone to aggregate in the majority of cells (~70%) in comparison to its wild-type counterpart (~30%) (**A** and **B**). Expression of expPABPN1(Δ131-132) significantly compromised nuclear aggregate formation in ~70% of transfected cells, while GFP-wtPABPN1(Δ131-132) was diffused uniformly throughout the nucleus of all cells (**A** and **B**). Some cells (~30%) expressing GFP-expPABPN1(Δ131-132) were still able to form aggregates (**A**, arrow head). Following transfections, equal amounts of the exogenous transiently expressed PABPN1 proteins were immunodetected with an anti-GFP antibody on a Western blot; a control immunodetection, with an anti-actin antibody, was done in parallel (**C**).

Figure 2

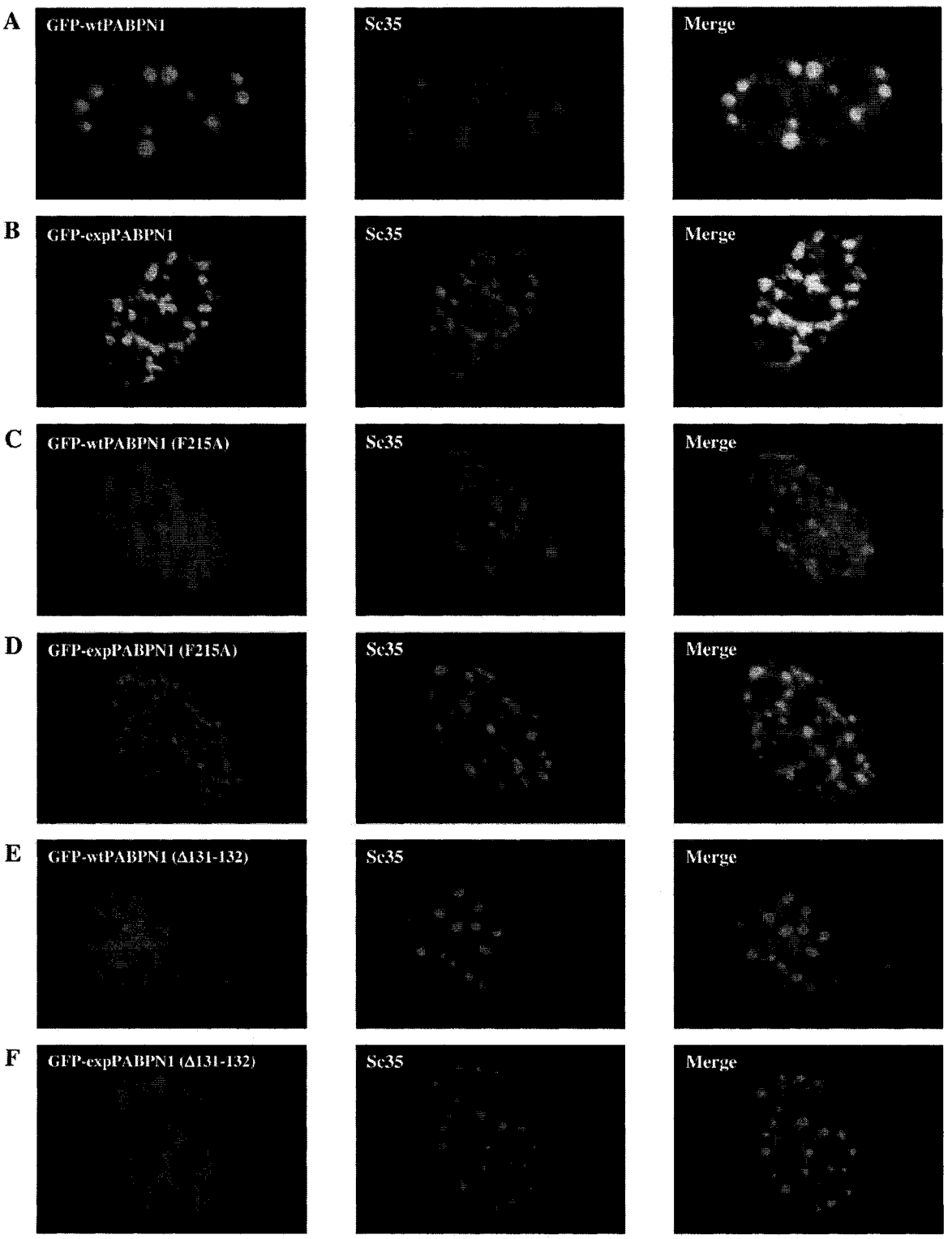

A
GFP-wtPABPN1
Sc35
Merge
B
GFP-expPABPN1
Sc35
Merge
C
GFP-wtPABPN1 (F215A)
Sc35
Merge
D
GFP-expPABPN1 (F215A)
Sc35
Merge
E
GFP-wtPABPN1 (Δ131-132)
Sc35
Merge
F
GFP-expPABPN1 (Δ131-132)
Sc35
Merge

Figure 2

Localization of both wild-type and expanded PABPN1 into transcriptional speckles depend on polyadenylation

Immunocytochemistry detections were prepared using anti-GFP and anti-SC35 antibodies. Characteristic results showed that both GFP-wtPABPN1 (**A**) and GFP-expPABPN1 (**B**) co-localized with the splicing factor SC35 in nuclear speckles. However, co-localization of the latter was not complete. Some, but not all GFP-expPABPN1(F215A) aggregates co-localized with SC35 domains (**D**). The soluble form of GFP-wtPABPN1(F215A) (**C**),GFP-wtPABPN1(Δ131-132) (**E**) and GFP-expPABPN1(Δ131-132) (**F**) did not show co-localization with to these nuclear speckles.

Figure 3

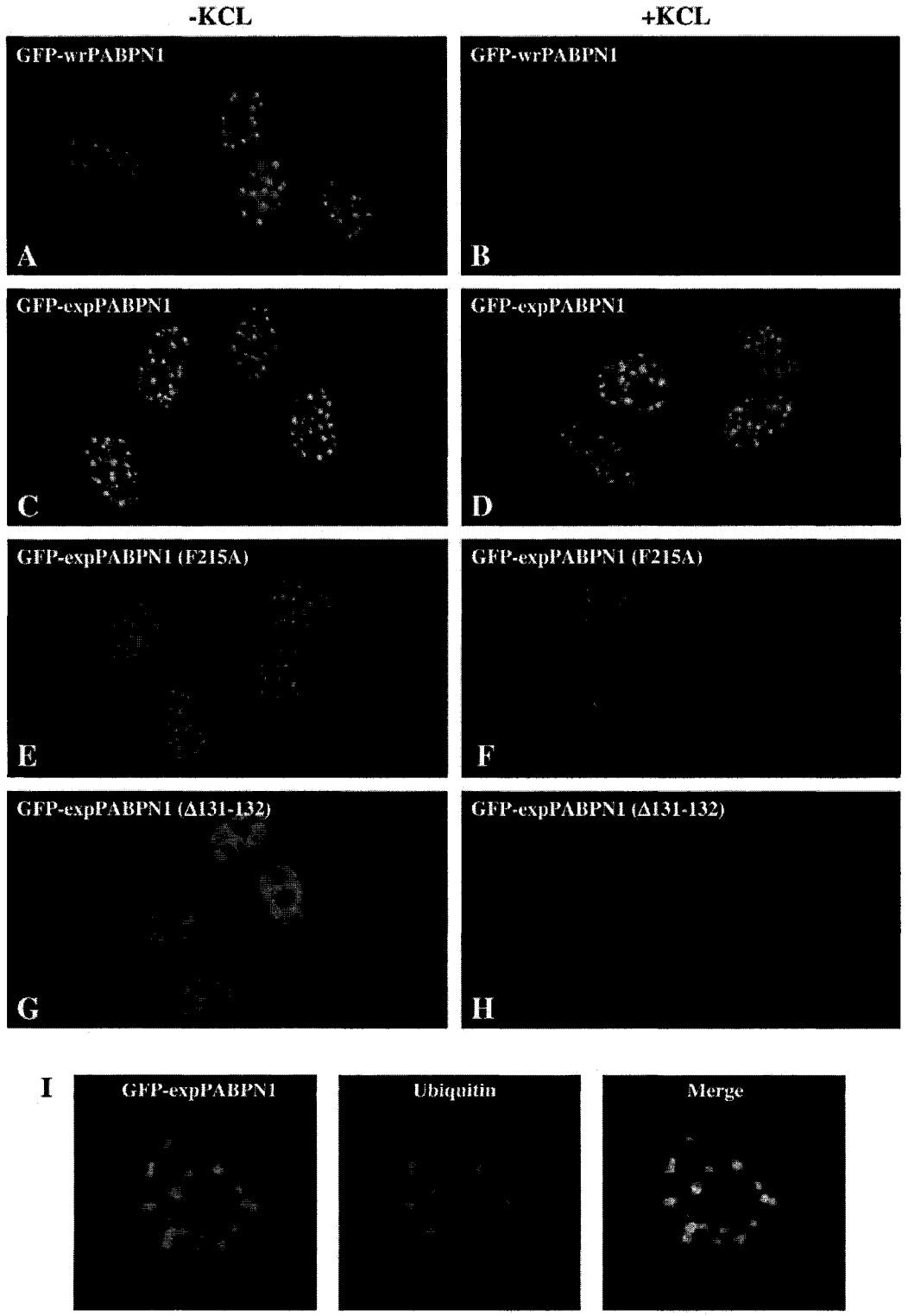

-KCL
+KCL
GFP-wrPABPN1
A
GFP-wrPABPN1
B
GFP-expPABPN1
C
GFP-expPABPN1
D
GFP-expPABPN1 (F215A)
E
GFP-expPABPN1 (F215A)
F
GFP-expPABPN1 (Δ131-132)
G
GFP-expPABPN1 (Δ131-132)
H
I
GFP-expPABPN1
Ubiquitin
Merge

Figure 3

Expression of GFP-expPABPN1 or mutated GFP-expPABPN1 induced formation of insoluble nuclear aggregates

HeLa cells transfected with different PABPN1 forms were untreated (**A**, **C**, **E** and **G**) or treated with 1.5 M KCl in HPEM buffer for 20 minutes prior to fixation; this procedure extracts soluble proteins *in situ* (**B**, **D**, **F**, **H** and **I**). Nuclear speckle structures generated by GFP-wtPABPN1 completely disappeared following KCl extraction (**B**). Nuclear aggregates formed by GFP-expPABPN1 and GFP-expPABPN1(F215A) resisted the KCl treatment and remained visible (**D** and **F**). Signal from GFP-expPABPN1(Δ131-132), which failed to localize into aggregates in most cells, was completely solubilized by this treatment (**G** and **H**). Ubiquitin and GFP-expPABPN1 proteins appeared sequestered together in KCl resistant aggregates (**I**). .

Figure 4

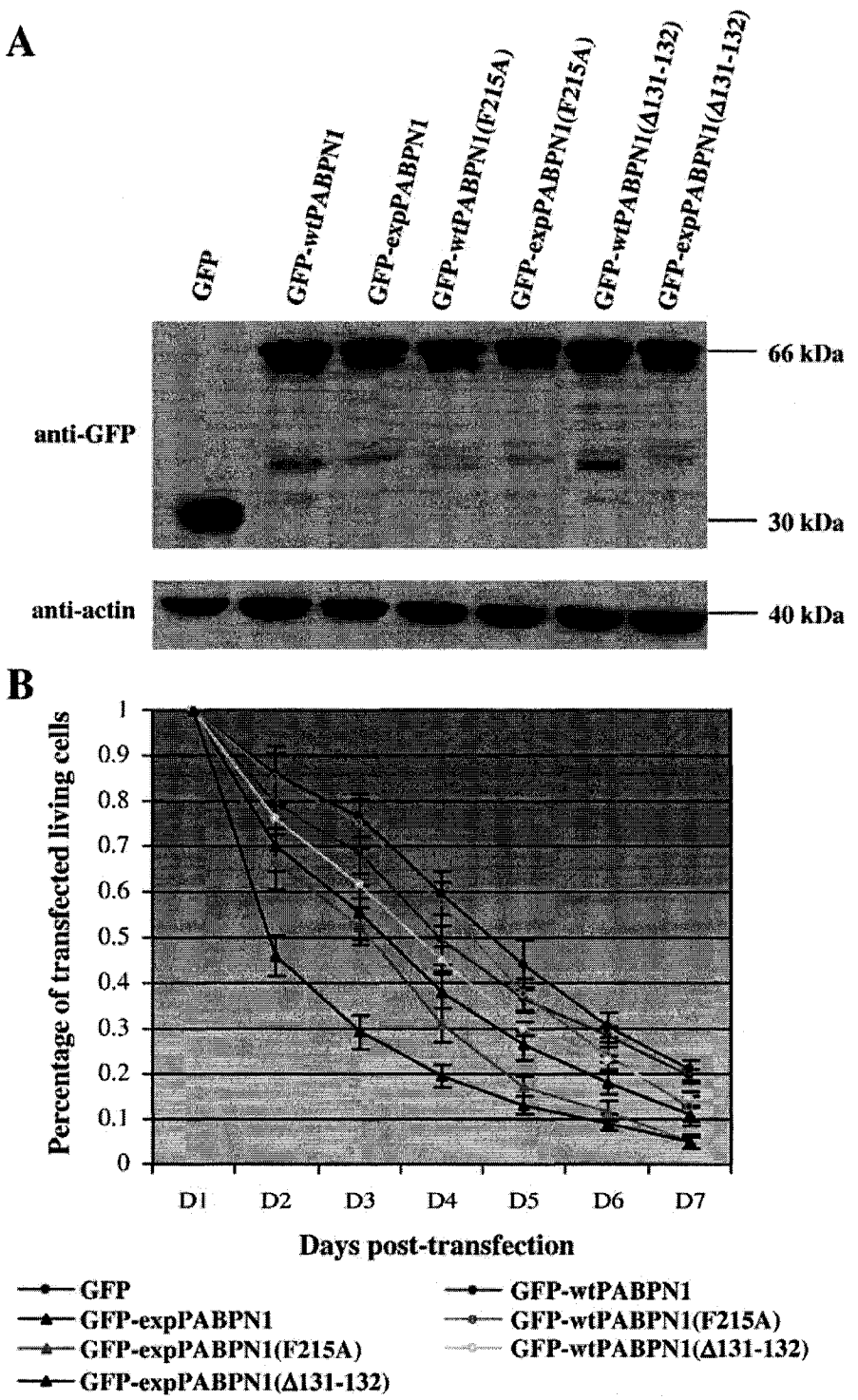

A
GFP
GFP-wtPABPN1
GFP-expPABPN1
GFP-wtPABPN1(F215A)
GFP-expPABPN1(F215A)
GFP-wtPABPN1(Δ131-132)
GFP-expPABPN1(Δ131-132)
anti-GFP
66 kDa
30 kDa
anti-actin
40 kDa
B
Percentage of transfected living cells
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
D1
D2
D3
D4
D5
D6
D7
Days post-transfection
GFP
GFP-wtPABPN1
GFP-expPABPN1
GFP-wtPABPN1(F215A)
GFP-expPABPN1(F215A)
GFP-wtPABPN1(Δ131-132)
GFP-expPABPN1(Δ131-132)

Figure 4

Nuclear aggregation levels directly correlate with cell survival

Following transfections, equal amounts of the exogenous transiently expressed PABPN1 proteins were immunodetected with an anti-GFP antibody on a Western blot; a control immunodetection with an anti-actin antibody was done in parallel (**A**). The percentage of living cells expressing GFP and the different GFP-PABPN1 forms was measured for seven days starting at 24 hours post-transfection (**B**). The survival rates of cells were estimated by determining the number of living GFP-positive cells at each time point divided by the number observed at day 1 (24 hours post-transfection). A significant increase in cell death was observed when GFP-expPABPN1(Δ131-132) protein was expressed, in comparison to GFP-expPABPN1 or GFP-expPABPN1(F215A) expression ($P<0.001$, Anova single factor analysis).

Figure 5

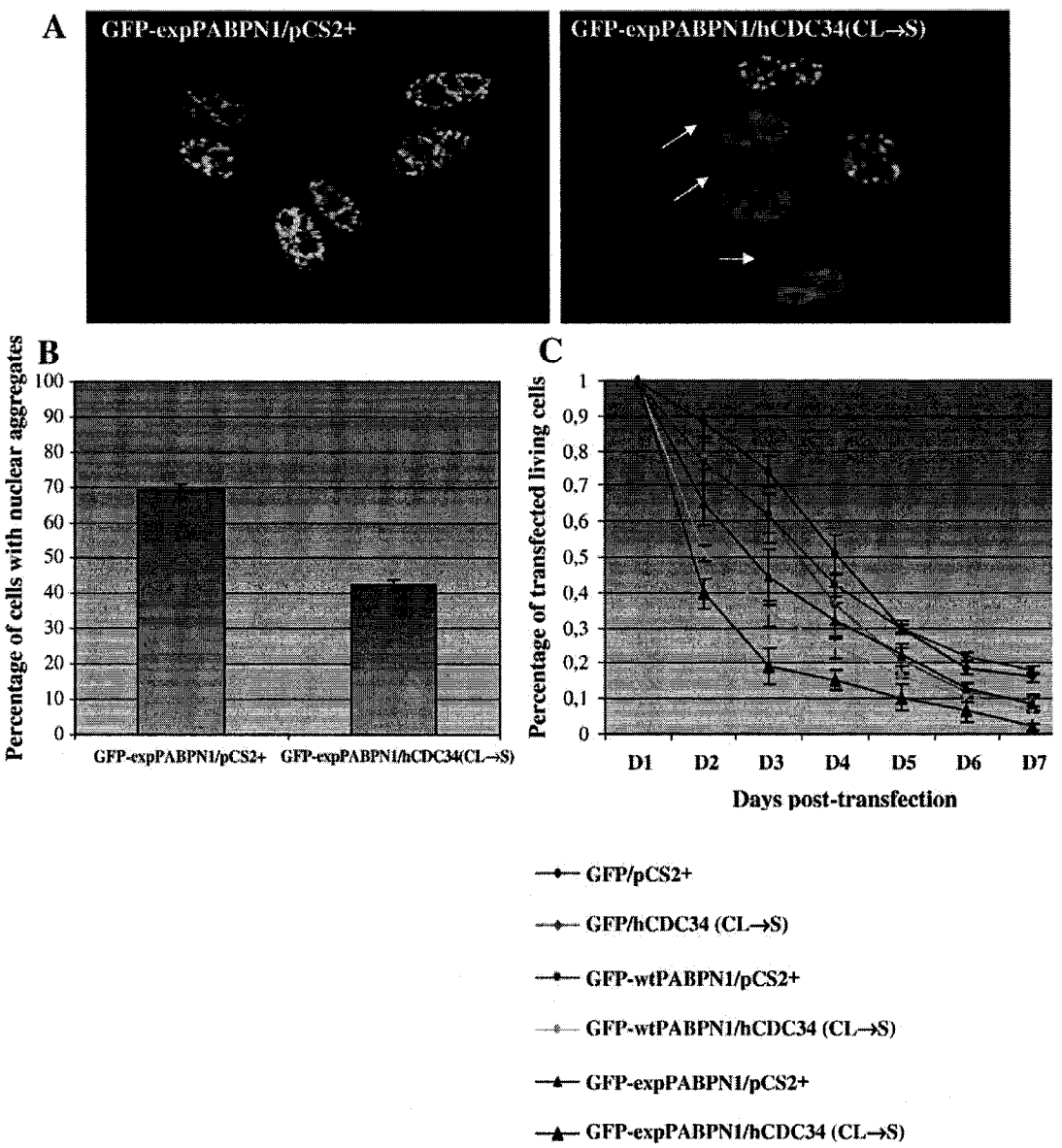
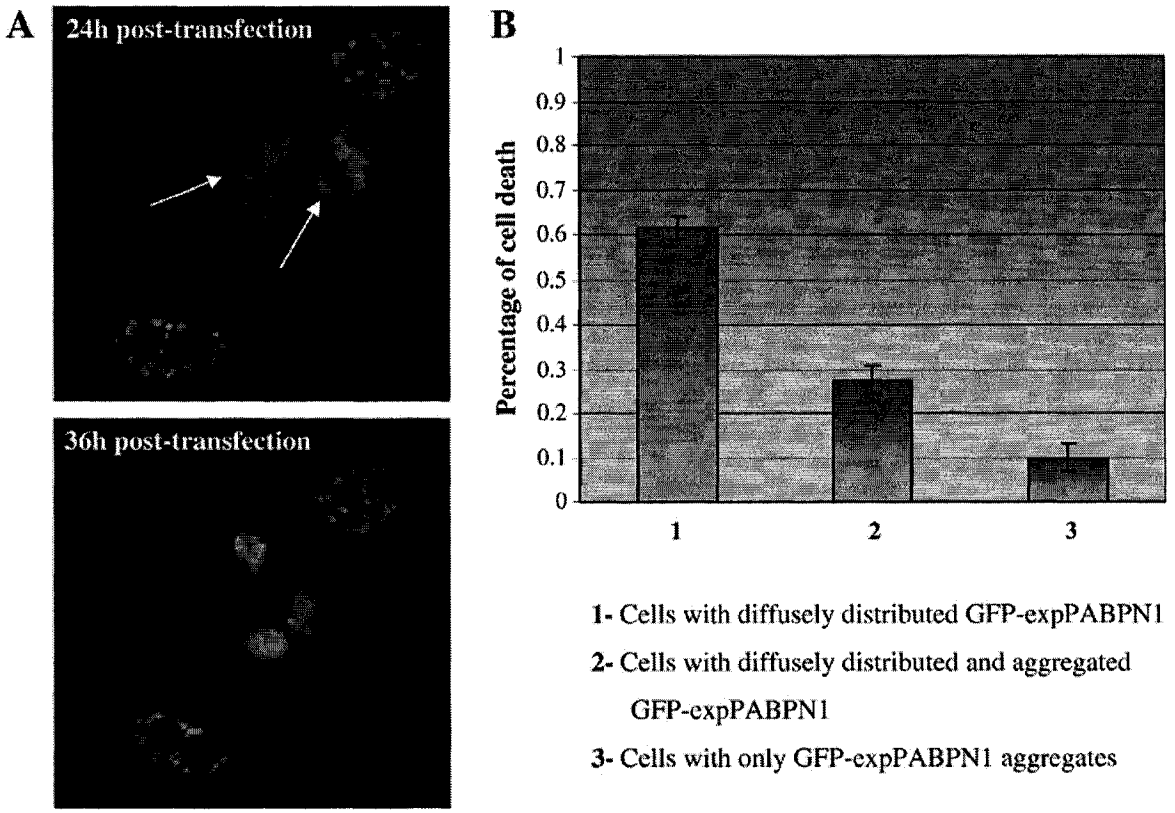

A
GFP-expPABPN1/pCS2+
GFP-expPABPN1/hCDC34(CL→S)
B
Percentage of cells with nuclear aggregates
100
90
80
70
60
50
40
30
20
10
0
GFP-expPABPN1/pCS2+
GFP-expPABPN1/hCDC34(CL→S)
C
Percentage of transfected living cells
1
0,9
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
D1
D2
D3
D4
D5
D6
D7
Days post-transfection
GFP/pCS2+
GFP/hCDC34 (CL→S)
GFP-wtPABPN1/pCS2+
GFP-wtPABPN1/hCDC34 (CL→S)
GFP-expPABPN1/pCS2+
GFP-expPABPN1/hCDC34 (CL→S)

Figure 5

Dominant negative ubiquitin-conjugating enzyme hCDC34(CL→S) compromises aggregation and exacerbates expPABPN1-induced cell toxicity

HeLa cells were cotransfected with GFP-expPABPN1 and the expression vector $pCS2^+$ containing hCDC34(CL→S) or with a control, the corresponding empty vector $pCS2^+$ (**A**). Many cells presented a diffusely distributed signal of GFP-expPABPN1 when co-expressing hCDC34(CL→S). In the majority of cells, the number of aggregates was significantly reduced, which coincided with an increase of a diffusely distributed staining (**A,** arrows). The number of cells with nuclear aggregates was significantly decreased with co-expression of hCDC34(CL→S) (**B**). The percentage of living cells was determined at 24 h intervals for 7 days. GFP-wtPABPN1, GFP-expPABPN1 and GFP alone were cotransfected along with $pCS2^+$ hCDC34(CL→S), or the empty $pCS2^+$ control vector. The reduction of nuclear aggregates formed by expPABPN1 protein correlated with a significant increase of the rate of cell death in the presence of hCDC34(CL→S) ($P<0.001$, Anova single factor analysis) (**C**).

Figure 6



1- Cells with diffusely distributed GFP-expPABPN1

2- Cells with diffusely distributed and aggregated GFP-expPABPN1

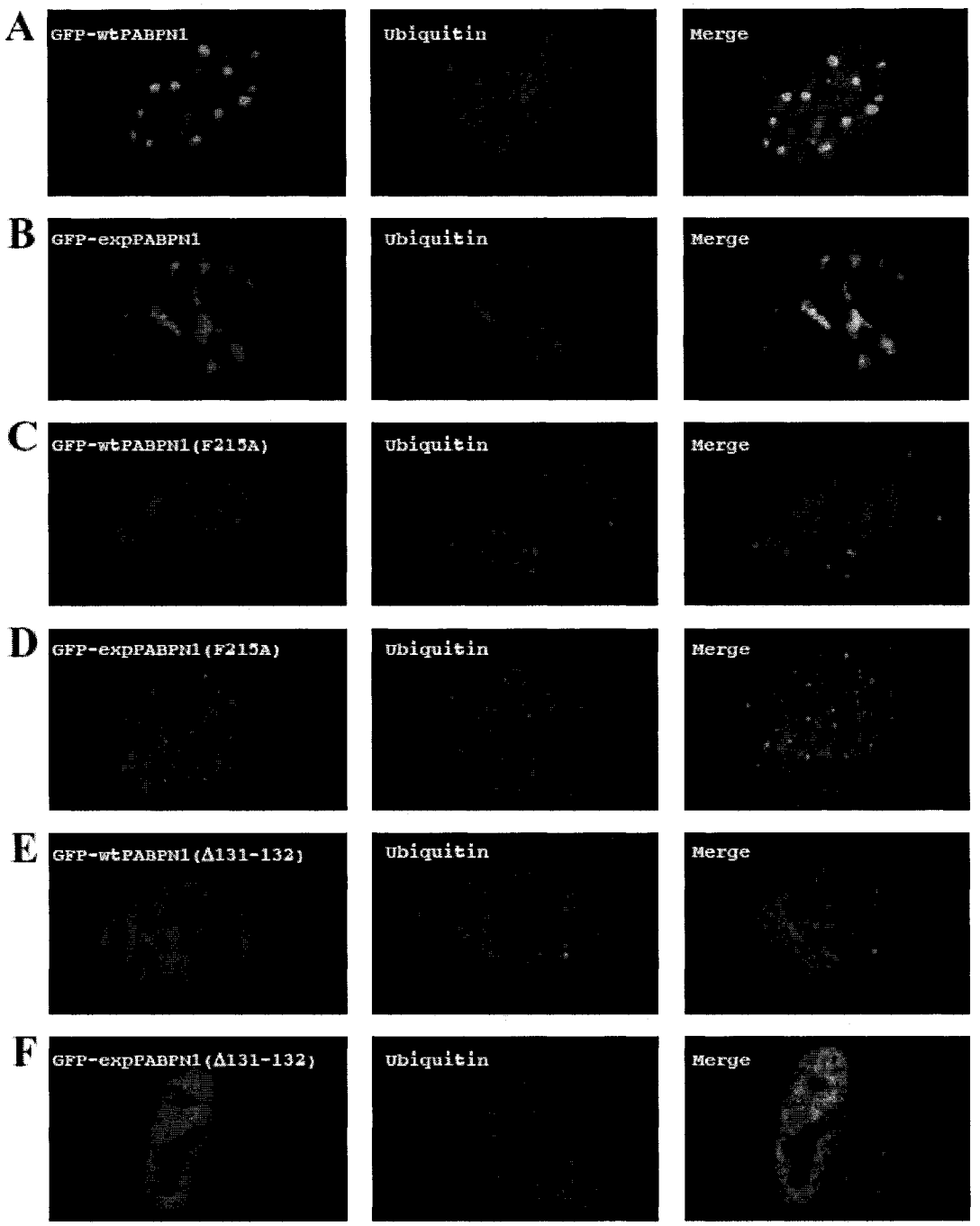
3- Cells with only GFP-expPABPN1 aggregates

Figure 6

The formation of nuclear aggregates is associated with slowing of cell death

HeLa cells were transfected to express GFP-expPABPN1 transiently and cell survival was monitored every 60 minutes for a period of 10 h that began 24 h post-transfection. Cells that presented GFP-expPABPN1 aggregates resisted cell death longer than those harboring the soluble GFP-expPABPN1 (**A** arrows and **B**). Some cells which harbored both aggregated and uniformly distributed GFP-expPABPN1 were found to present an intermediate level of resistance (**B**) The differences of cell survival rates observed were highly significant ($P < 0.001$, Anova single factor analysis).

Supplemental data 3



Supplemental data 3

Ubiquitin is recruited to the large nuclear aggregates formed by GFP-expPABPN1

Immunocytochemistry using anti-GFP and anti-ubiquitin shows that the aggregates formed by GFP-expPABPN1 contained ubiquitin (**B**). The expression of GFP-wtPABPN1 (**A**), GFP-wtPABPN1(F215) (**C**), GFP-expPABPN1(F215) (**D**), GFP-wtPABPN1(Δ131–132) (**E**) and GFP-expPABPN1(Δ131–132) (**F**) did not show the presence of aggregates containing both the PABPN1 proteins and ubiquitin.

Chapter 5: Discussion and Conclusion

5.1. Discussion

5.1.1. Protein aggregation, a common feature in polyalanine diseases

In addition to OPMD there are at least eight other developmental disorders associated with polyalanine expansions including synpolydactyly type II (HOXD13) (Goodman et al., 1997), cleidocranial dysplasia (RUNX2) (Mundlos et al., 1997), holoprosencephaly type 5 (ZIC2) (Brown et al., 2001), hand-foot-genital syndrome (HOXA13) (Goodman et al., 2000; Innis et al., 2004), blepharophimosis, ptosis and epicanthus inversus (FOXL2) (De Baere et al., 2003; De Baere et al., 2001), congenital central hypoventilation syndrome (PHOX2B) (Matera et al., 2004), X-linked mental retardation (syndromic and non syndromic) (ARX) (Kato et al., 2003; Stromme et al., 2002) and X-linked hypopituitarism (SOX3) (Laumonnier et al., 2002; Woods et al., 2005). The presence of nuclear and /or cytoplasmic aggregates, often referred to as inclusions in literature has been reported in cellular models of all polyalanine diseases (Albrecht et al., 2004; Bachetti et al., 2007; Bao et al., 2002; Caburet et al., 2004; Nasrallah et al., 2004; Woods et al., 2005) with the exception of holoprosencephaly type 5 (ZIC2) (Brown et al., 2005). However, at the moment only the aggregation of expPABPN1 has been reported in the affected tissues of OPMD patients; expPABPN1 aggregates are observed in ~2-5 % of the muscle fiber nuclei of OPMD patients (Brais et al., 1999). In cellular models of SPD (Albrecht et al., 2004) and CCHS (Trochet et al., 2005) the degree of protein aggregation has been reported to correlate with the size of polyalanine expansion. In OPMD, protein

aggregation not only depends on the polyalanine tract length, it is also dependent on the cell type and the protein context. For instance, even though expPABPN1 is ubiquitously expressed *in vivo*, it usually only forms nuclear inclusions in the affected skeletal muscles of OPMD patients (Brais et al., 1999). Also, in our cellular model (section 3.4 and 4.4) the overexpression of the wtPABPN1 leads to the formation of aggregates. The aggregation of wtPABPN1 occured albeit to a lesser extent than the expPABPN1. However, wtPABPN1 fails to aggregate in the muscle fiber nuclei of OPMD transgenic mice models (Davies et al., 2005; Hino et al., 2004). While the aggregation of wtPABPN1 and expPABPN1 is modulated by a number of intrinsic and extrinsic factors (Calado and Carmo-Fonseca, 2000; Hino et al., 2004; Tavanez et al., 2005), the biochemical properties of nuclear aggregates observed *in vivo* depend only on the polyalanine tract length. This observation was confirmed by our use of KCl treatment; the wtPABPN1 and the expPABPN1 respectively form KCl-soluble aggregates and KCl-insoluble aggregates (section 2.4 and 4.4). The expansion of the alanine tract most probably stabilizes protein-protein interactions (Shinchuk et al., 2005a).

The expPABPN1 nuclear aggregates are enriched in poly(A) RNA and recruit molecular chaperones, Ub and proteasome subunits. The sequestration of these cellular components into inclusions has been reported in several other polyglutamine and polyalanine disease models (Bachetti et al., 2007; Nasrallah et al., 2004; Stenoien et al., 1999). It is a clear indication that misfolded proteins are being recognized and that the activation of cellular defense mechanisms has been triggered.

5.1.2. The sequestration of hnRNPA1 and hnRNPA/B into OPMD aggregates

In addition to poly(A) RNA, molecular chaperones, Ub and proteasome subunits, we determined that hnRNPA1 and A/B were also recruited into the insoluble OPMD nuclear aggregates in our cellular model (Section 2.4). The recruitment of critical nuclear factors into aggregates has been reported to induce cellular toxicity in huntingtin disease models (Nucifora et al., 2001). However, hnRNPs are considered to be the most abundant proteins in the nucleus. Thus, the sequestration of a fraction of hnRNPs into OPMD aggregates may not necessarily alter their cellular functions. Moreover, hnRNPs are known for their functional redundancy so that specifically depleting a particular hnRNP protein from the nuclear pool may not inescapably produce a measurable effect (Dreyfuss et al., 1993). Nevertheless, the function of hnRNPs may be compromised if to carry out their multiple roles, they need PABPN1 protein levels normally produced by two functional copies of the gene; when expPABPN1 is trapped in insoluble aggregates it becomes dysfunctional and unavailable. However, irrespective of protein aggregation, the soluble expPABPN1 may have gained an abnormal function or completely lost the ability to interact with crucial cellular components. It is known that misfolded proteins, notably those with polyalanine expansions, result in aberrant DNA-protein and protein-protein interactions (section 1.5.2). Given that hnRNPA1 is required for mRNA splicing, packaging and export (Krecic and Swanson, 1999) and that hnRNPA/B is required for mRNA editing (Lau et al., 1997); aberrant interactions between expPABPN1 and hnRNPA1 or hnRNPA/B, may indeed interfere with mRNA processing and nucleocytoplasmic transport which could be detrimental to the cell. The available wtPABPN1 may compensate for the gain or loss-of-function of expPABPN1, but the total

PABPN1 activity may reach a plateau which is below what is necessary to maintain cellular homeostasis in the muscle.

5.1.3. The clearance of the expPABPN1 is mediated by the ubiquitin-proteasome pathway

Cells possess proteolytic systems to carry out protein turn over and they also have complex regulatory mechanisms to ensure that the continual proteolytic processes are highly selective. In all tissues, the majority of intracellular proteins are degraded via the UPP. However, extracellular, cell surface and cytosolic proteins are degraded by autophagy (Lecker et al., 2006). Although PABPN1 shuttles between the nucleus and the cytoplasm, it is almost exclusively nuclear at steady state and so it is more likely to be degraded by the UPP rather than the macroautophagy pathway. Our finding that lactacystin, a proteasome inhibitor, increased the formation of expPABPN1 nuclear aggregates indicated that the UPP is indeed a key pathway in the clearance of expPABPN1 (Section 3.4). In agreement with this, lactacystin also inhibits the *in vivo* clearance of expPABPN1 by trehalose, a disachariride with HSPs properties that stabilizes protein conformation and inhibits the formation of amyloid structures (Davies et al., 2006).

An increase of protein aggregation following lactacystin treatment in our cellular model may also suggest a possible proteasome impairment. Proteasome dysfunction has been associated with protein aggregation and neuronal dysfunction in many cellular and mouse models of polyglutamine diseases. Investigators using a GFP-based reporter of the UPP activity observed near-complete impairment of the UPP by polyglutamine inclusions in a

cell culture model (Bence et al., 2001). Impairment of UPP activity was further confirmed in other cell culture models by examination of endogenous proteasome targets and *in vitro* proteasome activity assays (Jana et al., 2001; Nishitoh et al., 2002). Also, *in vitro* experiments demonstrated that long polyglutamine tracts are not efficiently degraded by the active sites present in the eukaryotic proteasome (Venkatraman et al., 2004). However, contradicting data have been presented, showing that polyglutamine-containing proteins are efficiently degraded by the UPP in cultured cells regardless of glutamine tract length (Michalik and Van Broeckhoven, 2004). More interestingly, protein aggregation has been associated with higher proteasome activity in a SCA7 knock-in mouse model (Bowman et al., 2005) and a SBMA cellular model (Rusmini et al., 2006). A greater understanding of this quality-control process is therefore not only of interest at the cellular level, it might also lead toward new therapies for OPMD.

5.1.4. A role for molecular chaperones in OPMD

5.1.4.1. Inhibition of proteasome induces molecular chaperones and ubiquitin conjugates

When unfolded proteins accumulate and eventually exceed the capacity of the cell's degradative machinery (e.g. in the presence of proteasome inhibitors), cells activate the heat shock response (Kisselev and Goldberg, 2001; Lee and Goldberg, 1998) and induce the synthesis of more proteasome (Meiners et al., 2003). Indeed, the inhibition of proteasome by lactacystin in our Hela cell model resulted in an increase of the insoluble fraction of expPABPN1 and the induction of HSP70 and ubiquitin conjugates (Section 3.4). It is possible that the joint induction of both molecular chaperones and ubiquitin

occurred alongside an induction of proteasome expression to compensate for proteasome inhibition. However, the induction of proteasome expression has not been investigated in our cellular model.

Molecular chaperones and ubiquitin ligases work in concert to facilitate the targeting of non-native proteins for degradation (Goldberg, 2003). However, the continuous accumulation of misfolded PABPN1 in the presence of lactacystin may overwhelm the cell's defense mechanisms and eventually trigger the activation of apoptotic pathways. A prominent induction of HSP70 has been noticed with higher doses of lactacystin (Section 3.4). In addition to their involvement in protein refolding and degradation (Huang et al., 2001; Murata et al., 2001; Wickner et al., 1999), molecular chaperones have been reported to protect cells from apoptotic insults (section 1.7.2.2) (Beere et al., 2000). In that, a marked induction of HSP70 may reflect the cells response against an activation of apoptotic pathways which is associated with the build-up of misfolded PABPN1.

5.1.4.2. Molecular chaperones (HSP70 and HSP40) suppress expPABPN1 protein aggregation and cellular toxicity

When present at sufficient levels, HSP70 in concert with HSP40 binds to extended hydrophobic polypeptides and inhibits the formation of intramolecular β-sheet conformation; thus preventing fibrils and aggregates formation (Sakahira et al., 2002). In our cellular model, the endogenous level of molecular chaperones in cells expressing expPABPN1 did not prove to be sufficient to prevent expPABPN1 aggregation or cellular toxicity. Alternatively, the overexpression of HSP70 and HSP40 (independently or simultaneously) along with expPABPN1 reduced significantly protein aggregation and

expPABPN1 induced-cell death (Section 3.4). The suppression of toxicity observed when HSP40 is overexpressed alone is likely due to the ability of this co-chaperone to activate the endogenous HSP70. The decrease in protein aggregation coincided with an increase in the protein solubility, thus reflecting a successful refolding process which may explain in part the beneficial effect of molecular chaperones. HSPs have been confirmed to suppress aggregate formation and cellular toxicity in many polyglutamine disease models (Carmichael et al., 2000; Chan et al., 2000; Cummings et al., 1998; Kobayashi et al., 2000). However, although the reduction of aggregate formation correlated with the suppression of cellular toxicity in our experimental model, both events may occur independently. Thus, HSPs could reduce aggregate formation as a molecular chaperone, and independently suppress cellular toxicity by simultaneously or independently enhancing the clearance of the misfolded proteins or inhibiting the activation of apoptotic pathways. In agreement with this, trehalose promotes the clearance of expPABPN1 by the proteasome and reduces TUNEL-labeled nuclei in the tissue muscle from OPMD mouse (Davies et al., 2006). Similar to trehalose, the chemical chaperone Congo red facilitates the clearance of the mutant huntingtin in a Huntington's disease model (Sanchez et al., 2003). Also, in a CCHS cellular model, Geldanamycin (GA) a naturally occurring antibiotic, significantly increases HSP70 expression level and proteasome-mediated degradation, thereby decreasing the amount of misfolded proteins and reducing the fraction of cells in advanced apoptotic stages expressing the expPHOX2B (+13Alanine) (Bachetti et al., 2007). Finally, the expression of a dominant negative mutant form of HSP70 increases polyglutamine toxicity (Warrick et al., 1999). These observations support the role of molecular chaperones in regulating the clearance of

misfolded proteins and cellular toxicity in polyalanine and polyglutamine diseases. Therefore, targeting them and components involved in regulating their expression represents promising and interesting therapeutic approaches.

5.1.5. Polyadenylation and ubiquitination are coupled to protein aggregation

5.1.5.1. A decrease in polyadenylation efficiency and the RNA binding activity of expPABPN1 suppresses protein aggregation

OPMD nuclear aggregates accumulate a higher concentration of poly(A) RNA than is normally distributed throughout the nucleoplasm (Calado et al., 2000b). The binding of expPABPN1 to poly(A) RNA has been reported to enhance the assembly of nuclear aggregates in OPMD (Tavanez et al., 2005). In agreement with these findings, a missense mutation (F215A) in the RBD or a deletion of two glutamic amino acids (Δ131-132) in the α-helical domain of PABPN1, known to alter respectively the RNA binding activity and the capacity of the protein to stimulate polyadenylation, reduced considerably the ability of the expPABPN1 to aggregate (Section 4.4). However, the deletion in the α-helical domain affected to a greater extent protein aggregation than the missense mutation. In that, the GFP-expPABPN1(Δ131-132) was found to aggregate to a much lesser extent than the GFP-expPABPN1(F215A) (Section 4.4). In contrast to the deletion mutation, the missense mutation did not significantly decrease the percentage of cells containing nuclear aggregates. However, it significantly reduced the size of aggregates formed by the expPABPN1 at the single-cell level. These observations suggest that polyadenylation and the consequent binding to poly(A) RNA contribute to protein

aggregation. The sequestration of RNA into nuclear aggregates has previously been described in a myotonic dystrophy type 1 (DM1) model (Jiang et al., 2004), and other neurodegenerative diseases (Ginsberg et al., 1997; Ginsberg et al., 1998). The assembly of aggregates into neurofibrillary tangles in Alzheimer's disease is strongly enhanced by RNA (Kampers et al., 1996).

5.1.5.2. Mutations in hCDC34, an E2-type ubiquitin-conjugating enzyme, prevents expPABPN1 aggregation

The fact that the inhibition of proteasome activity with lactacystin is associated with an increase in nuclear aggregates in cells expressing expPABPN1 (Section 3.4) suggests that preventing the turnover of ubiquitinated forms of expPABPN1 may contribute to protein aggregation. It is conceivable that the conjugation of multiple ubiquitin molecules to expPABPN1, which already has a native conformation destabilized by an expanded polyalanine tract, may contribute to its misfolding and aggregation. In agreement with this, the co-expression of the dominant-negative ubiquitin-conjugating enzyme hCDC34(CL→S) with expPABPN1 significantly reduced the formation of nuclear aggregates in our cellular model (Section 4.4). Assuming that expPABPN1 is a CDC34 substrate, then the loss of this E2 activity would alter the misfolded PABPN1 ubiquitination and consequent aggregation. The involvement of ubiquitination in the protein aggregation process has been highlighted in a huntingtin and SCA1 cellular and mouse model respectively (Cummings et al., 1999; Saudou et al., 1998). The expression of hCDC34(CL→S) has been shown to inhibit the formation of intranuclear inclusions in neuronal cells expressing the mutant huntingtin protein (Saudou et al., 1998). Also, a

decrease in the frequency of mutant ataxin-1 aggregation has been reported in animals deficient in E6-AP (an E3 ligase) (Cummings et al., 1999).

5.1.6. Soluble expPABPN1 is the primary toxic species in OPMD

Despite our observation of a correlation between the reduction of aggregate formation and the suppression of cellular toxicity, concomitant with the overexpression of heat shock proteins (Section 3.4), the role of expPABPN1 nuclear aggregates was still ambiguous. A decrease in cellular toxicity may have been the outcome of the protective properties of molecular chaperones (Section 1.7.2.2) neutralizing the toxic effect of the soluble pool of misfolded PABPN1, rather than a consequence of a decrease in nuclear aggregates. Indeed, a defect in polyadenylation or the capacity of PABPN1 to bind poly(A)RNA increased significantly the degree of solubility of wtPABPN1 and expPABPN1 but only exacerbated the process of cell death in cells expressing higher levels of the soluble expPABPN1. Considering the intensity of diffuse fluorescence at the single-cell level, GFP-expPABPN1(Δ131-132)-expressing cells had a higher level of soluble protein than GFP-expPABPN1(F215A)-expressing cells and were consequently significantly more prone to cell death. When compared to GFP-expPABPN1-expressing cells, those expressing the GFP-expPABPN1(F215A) showed a moderate level of cellular toxicity at later time points (Section 4.4). Moreover, the co-expression of hCDC34 (CL→ S) along with expPABPN1 significantly decreased the formation of nuclear aggregates while increasing cellular toxicity (Section 4.4). In this context, the accelerated cell death may be associated with an increase level of soluble expPABPN1 which is even more toxic if not properly ubiquitinated and turned-over. In all cases, an increase in the expPABPN1

solubility coincided with an increase in cellular toxicity. In agreement with these findings, our live-cell imaging system showed that GFP-expPABPN1-expressing cells most vulnerable to toxic insults were those expressing high soluble levels of the misfolded expPABPN1 and devoid of nuclear aggregates. Alternatively, cells with bright nuclear aggregates were distinguished by higher survival rate. Cells that formed small aggregates within a diffuse signal had a moderate level of toxicity. Moderate cellular toxicity has been also observed with GFP-expPABPN1(F215A)-expressing cells with small aggregates within a uniformely distributed staining (Section 4.4). The soluble expPABPN1 in our experimental model seems to exert cellular toxicity in a dose-dependent manner, suggesting that soluble expPABPN1 is the primary toxic species in OPMD. In agreement with our findings, Berger Z *et al.* reported that proteins with long polyalanine tracts may exert toxic properties when expressed at higher levels (Berger et al., 2006). They also reported that irrespective of their expression level, polyalanine peptides can exhibit protective properties by inducing a heat shock response. They speculated that the emergence of an overall toxicity at higher polyalanine expression levels may be due to a dose-dependent increase in the toxic properties overwhelming the protective responses.

5.1.6.1. Mechanisms underlying the soluble expPABPN1 cellular toxicity

PABPN1 is a multifunctional protein ubiquitously expressed in all tissues. However, OPMD phenotype is restricted to skeletal muscles. Muscle biopsies from OPMD patients show typical signs of muscle degeneration (Tome and Fardeau, 1980). Recently, Perie *et al.* reported a premature proliferative arrest of satellite cells isolated from affected

circopharyngeal OPMD muscle. They speculated that the molecular mechanism involved in muscle degeneration and premature senescence of myogenic cells in OPMD may be induced by a premature senescence of dividing satellite cells caused by the expanded repeat (Perie et al., 2006). This is in agreement with our observation where cells expressing high levels of soluble expPABPN1 lost their ability to divide (Section 4.4). The most probable explanation is that the polyalanine expansion in PABPN1 may result in aberrant protein-protein interactions that may perturb the function of critical factors involved in muscular integrity, proliferation and regeneration. A misfolded PABPN1 may interfere with poly(A)RNA processing or nucleocytoplasmic export by impairing the normal function of mRNA processing proteins such as hnRNPA1 or hnRNPA/B, and such a mechanism has been hypothesized in cultures derived from congenital myotonic dystrophy (Furling et al., 2001). Alternatively, the polyalanine expansion may antagonize the normal function of factors implicated in transcription. It has been reported that normal PABPN1 interacts with RNAPII during transcription (Bear et al., 2003). Also, PABPN1 binds as a co-transcription factor to SKIP and MyoD to regulate muscle cell differentiation (Kim et al., 2001). Abnormal binding of expPABPN1 with RNAPII or the wild-type counterpart target proteins (SKIP or MyoD) may alter the assembly of the transcription complex. Therefore, the OPMD PABPN1 mutation may compromise the expression of genes essential for muscle cell survival, proliferation and differentiation. It is also conceivable that the expPABPN1 may aberrantly interact with its wild-type counterpart and alter its function, therefore exerting a partial dominant negative effect. Polyalanine expansions have been previously reported to exert a dominant negative effect in a SPD mouse model (Bruneau et al., 2001). The expanded Homeobox D13 (HOXD13)

protein may antagonize the function of other HOXD proteins (HOXD11 and HOXD12) including its wild-type counterpart, probably by competing with the latter for DNA binding sites or by sequestering other cofactors that might be important for their normal activity (Bruneau et al., 2001).

Bear DG *et al.* showed that PABPN1 is primarily located along the chromatin axis (Bear et al., 2003). Whether PABPN1 is capable of directly binding to DNA and regulate transcriptional activity is still an open question. Considering the possibility of a direct DNA-PABPN1 interaction, a polyalanine expansion in OPMD is expected to affect the DNA-protein binding leading to transcriptional dysregulation. The role of expanded polyalanine tracts in altering DNA-protein and /or protein-protein binding has been reported in many polyalanine diseases. Polyalanine expansions in both ZIC2 (HPE) and PHOX2B (CCHS) are associated with transcriptional repression, a consequence of the proteins reduced DNA binding activity (Bachetti et al., 2005; Brown et al., 2005; Trochet et al., 2005). Longer repeats in PHOX2B have a more drastic effect on the formation of the DNA-protein complex and transcription activation (Trochet et al., 2005).

5.1.7. Nuclear aggregates prolong cell survival in OPMD

In the light of our experimental procedures, nuclear aggregates have proven to be rather protective nuclear substructures that delay cell death most probably by depleting the misfolded expPABPN1 from the soluble pool. Their sequestration of components of the UPP and molecular chaperones (Section 3.4 and 4.4), suggests their possible role in accelerating the degradation of misfolded PABPN1 proteins. Alternatively, the observed association of HSP70 with OPMD aggregates (Section 3.4) may result in the coating of

potentially dangerous surfaces. The protective role of nuclear aggregates has been elucidated in many polyglutamine disease models. First, Arrasate *et al.* demonstrates that nuclear inclusions prolong cell survival in neurons expressing mutant huntingtin (Arrasate et al., 2004). Second, the chemical compound B2 promotes the formation of large inclusions by mutant huntingtin and α-synuclein in Huntington's and Parkinson's disease respectively, thereby lessening cellular pathology (Bodner et al., 2006). It has been suggested that B2 rescues cells from the toxic effect of mutant huntingtin by preventing huntingtin-mediated proteasome dysfunction, while increasing the number of nuclear inclusions (Bodner et al., 2006). Inclusions have been previously described as "sinks" that sequester misfolded proteins to de-saturate the proteasome machinery and enhance protein degradation in a SBMA disease model (Rusmini et al., 2006). Alternatively, inclusions have been reported as sites of highly concentrated proteasome activity, protecting the cell in the setting of excess misfolded proteins in a polyglutamine disease model (Taylor et al., 2003).

5.1.8. Dynamism of expPABPN1 nuclear aggregates

Nuclear aggregates appeared to improve cell survival and preserve cellular homeostasis in our OPMD cellular model. Our live-cell imaging system revealed that unlike cells with high soluble levels of GFP-expPABPN1, cells with nuclear aggregates maintained their ability to divide and proliferate. Although biochemically insoluble, nuclear aggregates were highly dynamic during mitosis. These subnuclear structures disintegrated to allow cell division and associated rapidly after cytokenisis (Section 4.4). The mobility of expPABPN1 in nuclear aggregates during the cell cycle has been shown in a parallel

study conducted by Marie-Josee Sasseville *et al.* (Marie-Josee Sasseville et al., 2006). Interestingly, OPMD-like nuclear aggregates formed by the normal PABPN1 protein in oxytocin-neurosecretory neurons from rat hypothalamus have been also reported to show dynamic behaviours in parallel with changes in cellular activity (Berciano et al., 2004). These aggregates disassemble when the synthesis and release of oxytocin is induced by neurons during parturation and lactation and assemble after weaning, a process that results in the reversal of these effects (Berciano et al., 2004). These observations, in addition to our data, suggest that the dynamism of nuclear aggregates may occur under specific physiological conditions. The dynamism of protein aggregates has been reported in a variety of neurodegenerative diseases. Live analysis of cytoplasmic aggregates revealed that smaller aggregates can move and fuse to form larger aggregates in a SBMA cellular model (Chai et al., 2002). *In vivo,* protein aggregates in OPMD may grow irreversibly into insoluble/static bodies in neurons and muscle because these cells cannot divide. Yet, even Huntington's aggregates in neurons can be dismantled (Yamamoto et al., 2000). It is also conceivable that in a muscle cell nuclei and under the same physiological conditions, protein aggregates in OPMD can be either static/insoluble or dynamic/soluble structures depending on their level of maturation.

5.2. Conclusion

5.2.1. Proposed mechanisms underlying OPMD

Expanded polyalanines can exhibit both protective and toxic properties irrespective of their expression level (Berger et al., 2006). The emergence of toxicity in OPMD may be due to a dose-dependent increase in the toxic properties overwhelming the protective

response. At sub-toxic doses, proteins with long polyalanines activate HSF-1-driven transcription and induce a heat shock response (Berger et al., 2006). The induction of HSP70 has also been observed *in vivo* in OPMD mouse muscle (Berger et al., 2006). A polyalanine-induced heat shock response may neutralize the toxic effect of the soluble pool of misfolded expPABPN1 in the early stages of the disease, thereby delaying its progression. However, with aging, the continuous expression of expPABPN1 may saturate the heat shock responses as they are physiologically subject to feedback control (Morimoto, 2002). Moreover, stress-induced expression of the ATP-dependent chaperone HSP70 and HSP90 decreases with age in a variety of systems (Bregegere et al., 2006). Deficits in chaperones may therefore limit protein turnover and lead to expPABPN1 aggregation. Polyalanine aggregation reactions are kinetically driven by a nucleation process which can deplete soluble pools of misfolded PABPN1, providing further protection to the nuclear compartment. However, nuclear inclusion formation *in vivo* may prove to be insufficient to halt the disease, as the accumulation of the soluble toxic forms of expPABPN1 overwhelms the cellular defense mechanisms with aging leading eventually to muscle cell degeneration. Unfortunately, satellite cells expressing expPABPN1 have a reduced capacity to proliferate and fuse into myotubes (Perie et al., 2006) to regenerate and repair muscle fibers, leading to the observed late onset OPMD phenotype.

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Appendix

Ethical approval

Copyright waivers

anormale de la muqueuse se terminant en cul-de-sac (à la manière d'un doigt de gant) et communiquant avec l'œsophage. La présence du diverticule de Zenker traduit l'existence d'une faiblesse des muscles cricopharyngiens. Ces muscles créent ainsi une pathologie constrictive du sphincter oesophagien supérieur et favorisent l'apparition du diverticule. Quand il est de taille trop importante, la présence du diverticule peut induire entre autre des difficultés à avaler ou à respirer. La thérapie pour ces deux maladie est essentiellement chirurgicale et consiste en une myotomie des muscles cricopharyngiens, c'est à dire à retirer les muscles qui causent la pathologie.

2.1.2. Objectifs du projet

Les objectif de ce projet sont d'étudier les muscles retirés lors de la chirurgie thérapeutique afin de 1) mieux comprendre les caractéristiques propres à chaque maladie (DMOP ou diverticule de Zenker) et de 2), de manière plus générale, mieux comprendre les pathologies qui touchent spécifiquement la région oeso-pharyngienne.

Afin de répondre à ces objectifs nous allons étudier les muscles retirés lors de la chirurgie pour regarder la structure et le profil des fibre musculaires pour chacune des deux maladies. Nous allons établir des lignées de cellules musculaires (ou myoblastes) qui nous permettront d'avoir une quantité importante de matériel biologique afin de mener à bien notre projet de recherche. Les lignées cellulaires sont des cellules provenant des muscles qui ont été traitées de manière à se multiplier en laboratoire.

La comparaison entre les deux pathologies nous permettra de mettre en évidence leurs points communs et leurs différences afin mieux définir le rôle des muscles cricopharyngiens.

Finalement, nous utiliserons l'ADN extrait de votre sang pour confirmer les études moléculaires et génétiques.

2.2. Nature de la participation du sujet

Vous êtes invité à participer à ce projet parce que vous avez ou devez être opéré(e) par le Dr Duranceau (ou un autre chirurgien) dans le cadre d'une chirurgie thérapeutique (ou myotomie des muscles pharyngaux) pour vos problèmes à avaler. Vous pouvez nous aider à répondre aux objectifs de la recherche comme sujet atteint de l'une ou l'autre des maladies.
Prenez connaissance, avec soin, des différents points concernant la nature de votre participation au projet de recherche. Vous devrez donner votre accord pour chacun de ces points dans la section CONSENTEMENT à la fin du présent formulaire.

1. On vous demande de répondre aux questions des chercheurs sur vos antécédents médicaux afin d'établir qu'il n'y pas d'autres maladies qui pourraient influencer l'étude.

2. On vous demande de donner 50mL (environ 3 cuillères à table) de sang. L'ADN isolé de votre sang sera préservé pour utilisation pendant toute l'étude et sera ensuite détruit quand l'étude sera complétée.

3. On vous demande d'autoriser les chercheurs à utiliser une partie des muscles obligatoirement enlevés lors de la chirurgie thérapeutique (à faire ou déjà effectuée) pour des fins de recherches sur la DMOP et le diverticule de Zenker.

4. On vous demande d'accepter qu'un petit échantillon de muscle omo-hyoïdien soit prélevé au cours de la chirurgie en tant que muscle témoin moins atteint pour ce projet de recherche. Ce muscle est un muscle peu atteint dans la DMOP, et non-atteint dans le diverticule de Zenker. Cette biopsie musculaire n'aurait aucun impact sur les résultats de la chirurgie et n'entraînerait aucune complication.

5. On vous demande d'accepter à ce que, dans le cadre d'une collaboration scientifique, une petite quantité de muscle, de myoblastes en culture ou d'ADN puisse être partagée avec d'autres laboratoires de recherche universitaire travaillant sur ces maladies avec l'approbation des comités d'éthique des différentes institutions.

3. Bénéfices, risques et inconvénients

3.1 Bénéfices

Vous ne retirerez aucun bénéfice direct en participant à cette étude. Toutefois, les résultats de votre participation à cette étude contribueront grandement à l'avancement des connaissances dans le domaine des problèmes de déglutition causés par des pathologies de la région oeso-pharyngienne.

3.2 Risques et inconvénients

3.2.1. Risques physiques

Toute personne donnant de son sang encoure de faibles risques de complications tels que douleur, hématome et ecchymose au site du prélèvement.

Lors de l'opération, si vous acceptez d'avoir un biopsie du muscle omo-hyoïdien, ceci n'entraînera pas d'augmentation des risques opératoires, il n'y a aucun risque supplémentaire sur le plan chirurgical puisque votre participation ne modifiera pas de façon significative l'opération pratiquée. De plus, la durée de la chirurgie ne sera prolongée que de 2 à 3 minutes. Le chirurgien ne prélèvera pas plus de muscle pendant la cricomyotomie proprement dite que ce qui est strictement requis pour corriger votre dysphagie. La taille de la cicatrice ne sera aucunement modifiée par l'ajout de la biopsie de l'omo-hyoïdien car l'ouverture chirurgicale habituelle expose l'omo-hyoïdien.

3.2.2. Risques socio-économiques

A l'heure actuelle, il est difficile d'évaluer les risques socio-économiques que comporte le fait de participer à un projet de recherche. Un des risques associés à des projets comme le nôtre pourraient venir de la divulgation éventuelle à des tiers (employeur, assureur) de votre participation ou d'informations vous concernant.
Dans le cas présent, nous, responsable du projet de recherche, avons pris des mesures strictes en matière de confidentialité et de sécurité des données recueillies.

4. Financement du projet

Le projet est subventionné par une subvention des instituts canadiens de recherche en santé (ICRS). Le montant reçu couvre les dépenses du chercheur durant la durée du projet.

5. Confidentialité

L'ADN extrait de votre sang, les muscles et les cultures de myoblastes seront conservés au laboratoire du Dr Bernard Brais du Centre de recherche du CHUM. Les échantillons auront un numéro codé afin de conserver la confidentialité. Le Dr Bernard Brais est responsable de protéger la correspondance entre le nom du participant et le numéro des échantillons. La banque de correspondance ne sera pas conservée sur le disque dur d'aucun ordinateur du laboratoire. Les échantillons fournis ne seront employés qu'à l'étude de la dystrophie musculaire oculopharyngée (DMOP) et du diverticule de Zenker.

Les données de cette étude seront traitées de façon strictement confidentielle par tous les chercheurs impliqués. En particulier, on ne mentionnera ni votre nom, ni celui d'autres membres de votre famille au cours de présentations publiques ou scientifiques, ou dans des articles scientifiques. Les résultats de cette étude ainsi que les échantillons ne seront jamais partagés avec des organismes gouvernementaux, des compagnies d'assurance et ou des organismes enquêtant sur les risques individuels. Seul le comité d'éthique de la recherche pourrait examiner votre dossier dans le but de s'assurer que le projet respecte les règles d'éthique.

6. Liberté de consentement et liberté de se retirer

Votre participation est entièrement volontaire. Toutefois, vous gardez le droit de vous en retirer à n'importe quel moment ou pour n'importe quel motif sans que ceci ne modifie en rien la qualité des soins que vous recevrez.

En signant ce formulaire, vous ne renoncez cependant à aucun de vos droits, ni ne libérez le chercheur ou l'hôpital de leur responsabilité civile et professionnelle.

Ce consentement respecte tous les principes de participation informée, confidentielle et sans préjudices énoncés en 2000 par le Comité de direction du Réseau de médecine génétique appliquée (RMGA) du Fonds de recherche en santé du Québec (FRSQ). Si vous retirez votre consentement en cours d'étude, les données recueillies ainsi que les échantillons biologiques (muscles, myoblastes et ADN) seront détruits et vous ne subirez aucun préjudice dans la suite de vos soins. Vous permettez que l'ADN et autre échantillons biologiques soient conservés au laboratoire du D^{r} Bernard Brais jusqu'au 11 janvier 2027. Après cette date, ils devront être détruits.

7. Personnes ressources:

Si vous désirez des renseignements supplémentaires au sujet du déroulement de ce projet de recherche ou pour nous communiquer tout changement d'adresse, vous pouvez contacter Bernard Brais, Chercheur, au (514)890-8000, poste 28929. Si vous désirez discuter de votre participation avec une personne qui n'est pas impliquée directement dans le projet ou pour tout autre problème éthique concernant les conditions dans lesquelles se déroule votre participation en tant que sujet de recherche, vous pouvez communiquer avec M^{me} Louise Brunelle, commissaire locale adjointe à la qualité des services du CHUM au (514)890-8000, poste 26047.

8. CONSENTEMENT

Je reconnais par la présente avoir été informé(e), à ma satisfaction, des objectifs et du déroulement de cette étude à laquelle on me demande de participer. On a répondu à toutes mes questions ayant trait à ma participation à cette étude. Je consens à participer à ce projet de recherche.

1. Je consens à répondre aux questions des chercheurs sur mes antécédents médicaux.

Oui: ☐ **Non:** ☐

2. Je consens à donner 50mL de sang pour extraire de l'ADN.

Oui: ☐ **Non:** ☐

3. J'autorise les chercheurs à utiliser les muscles retirés lors de la chirurgie thérapeutique et l'ADN extrait de mon sang pour des fins de recherches.

Oui: ☐ **Non:** ☐

4. Je consens à ce qu'un échantillon de muscle omo-hyoïdien soit prélevé au cours de la chirurgie en tant que muscle témoin.

Oui: ☐ **Non:** ☐

5. Je consens à ce qu'une petite quantité de matériel biologique (muscle, myoblastes ou ADN) puisse être partagée avec d'autres laboratoires de recherche universitaire travaillant sur ces maladies.

Oui: ☐ **Non:** ☐

8. CONSENTEMENT (suite)

Nom du participant	Signature du participant	Date
Nom du témoin	Signature du témoin	Date

ENGAGEMENT DU CHERCHEUR:

Je certifie qu'on a expliqué la nature du projet de recherche ainsi que le contenu du présent formulaire, qu'on a répondu à toutes ses questions et qu'on lui a indiqué qu'il reste à tout moment libre de mettre un terme à sa participation sans nuire à ses soins . Une copie signée du présent formulaire de consentement lui est remise.

Nom du chercheur	Signature du chercheur	Date
Nom de la personne désignée par le chercheur	Signature	Date

Author

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