Trehalose reduces aggregate formation and delays pathology in a transgenic mouse model of oculopharyngeal muscular dystrophy

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Oculopharyngeal muscular dystrophy (OPMD) is an autosomal dominant disease that presents in the fifth or sixth decade with dysphagia, ptosis and proximal limb weakness. OPMD is caused by the abnormal expansion of a polyalanine tract within the coding region of poly(A) binding protein nuclear 1 (PABPN1). The resultant mutant PABPN1 forms aggregates within the nuclei of skeletal muscle fibres. We have previously described a transgenic mouse model of OPMD that recapitulates the human disease and develops progressive muscle weakness accompanied by the formation of aggregates in skeletal muscle nuclei. The chemical chaperone trehalose has been used effectively to alleviate symptoms in a mouse model of Huntington’s disease and is thought to elicit its effect by binding and stabilizing partially folded polyglutamine proteins and inhibiting the formation of aggregates. Here, we show that trehalose reduces aggregate formation and toxicity of mutant PABPN1 in cell models. Furthermore, oral administration of trehalose attenuated muscle weakness, reduced aggregate formation and decreased the number of TUNEL-labelled nuclei in skeletal muscle in an OPMD transgenic mouse model. Thus, anti-aggregation therapy may prove effective in the treatment of human OPMD.

INTRODUCTION

Codon reiteration disorders are caused by the abnormal expansion of a homopolymeric stretch of amino acids within the coding region of a protein and can be further categorized into those caused by polyglutamine expansions and those caused by polyalanine expansions. The pathological hallmark of many of these disorders is the formation of aggregates (also known as inclusions), mainly comprising the misfolded mutant protein, in the affected tissue. Huntington’s disease is the most prevalent polyglutamine disorder. Expansions or duplications of polyalanine tracts cause nine known diseases (1) including oculopharyngeal muscular dystrophy (OPMD). OPMD is an autosomal dominant, late-onset, progressive disease that generally presents in the fifth or sixth decade with dysphagia, ptosis and proximal limb weakness. OPMD is caused by the abnormal expansion of a \((\text{GCG})_n\) trinucleotide repeat in the coding region of the poly(A) binding protein nuclear 1 (PABPN1) gene (2). In unaffected individuals, \((\text{GCG})_6\) codes for the first six alanines in a homopolymeric stretch of ten alanines. In most patients, this \((\text{GCG})_6\) repeat is expanded to \((\text{GCG})_{8-13}\) leading to a stretch of 12–17 alanines in mutant PABPN1. PABPN1 with an expanded polyalanine tract forms aggregates consisting of tubular filaments within the nuclei of skeletal muscle fibres (3–5). In addition, polyalanine expansions in four of the nine different disease genes have been recently shown to lead to aggregate formation in cell models (6).

Although there are controversies regarding the role of aggregates in many diseases, a widely accepted view is that aggregates, the aggregation process and/or early oligomeric species are toxic and pivotal to OPMD pathology. Indeed, the reduction of mutant PABPN1 aggregation by chemical or molecular chaperones [including chaperones that do not affect susceptibility to pro-apoptotic insults (7)] correlates with decreased death in cell models (7–9). Furthermore, we have shown that doxycycline attenuates muscle weakness in a transgenic mouse model of OPMD (10). This transgenic model...
OPMD mouse model has exclusively muscle pathology (characteristic of the human disease) and uses the human skeletal actin promoter to express untagged PABPN1 cDNA constructs with either 10 (A10; control) or 17 (A17; expanded) alanines. Attenuation of muscle weakness by doxycycline treatment was accompanied by a decrease in aggregate load in skeletal muscle fibres. Although these data were compatible with the idea that anti-aggregation therapy may be beneficial in OPMD, we could not make this claim conclusively because we found that doxycycline also had distinct anti-apoptotic properties.

The disaccharide trehalose is found in a wide range of non-mammalian species (11,12). In yeast, trehalose is thought to stabilize proteins during heat shock (heat shock is thought to cause protein misfolding and aggregation) and suppresses the aggregation of denatured proteins (12). Trehalose has also been shown to inhibit the formation of amyloid (13). Trehalose is one of many small molecules able to influence protein misfolding/folding and aggregation via direct protein–small molecule interactions and has thus been termed a chemical chaperone (14). More recently, trehalose was shown to inhibit the aggregation of a mutant myoglobin containing an expanded polyglutamine repeat in vitro (15). The authors went on to show that trehalose reduced polyglutamine aggregates and cell death in cell models of Huntington’s disease and furthermore rescued polyglutamine-induced pathology and phenotype in the R6/2 mouse model of Huntington’s disease.

Here, we show that trehalose decreases the aggregation and toxicity of mutant PABPN1 in cell models. Furthermore, treatment of an OPMD mouse model with trehalose resulted in the attenuation of muscle weakness, decreased aggregate formation and a reduced number of TUNEL-positive nuclei in skeletal muscle fibres.

RESULTS

In order to test whether trehalose treatment may have efficacy in OPMD, we initially tested its ability to reduce aggregation and toxicity in a previously described cell model of OPMD (7,9) expressing EGFP-tagged PABPN1 with an expanded polyalanine repeat (17 alanines). The construct forms bright EGFP aggregates in a proportion of cells and increases levels of cell death (abnormal apoptotic nuclei), when compared with cells expressing otherwise identical constructs with wild-type (10 alanines) repeats (7,9). Treatment with 100 mM trehalose reduced the proportion of cells containing aggregates (Fig. 1A) and decreased the number of cells with abnormal apoptotic nuclei (Fig. 1B). Trehalose does not readily cross cell membranes; however, trehalose can be easily and efficiently loaded into mammalian cells using fluid-phase endocytosis and pinocytosis by simply incubating cells in culture medium at 37°C (16–19).

We then tested the therapeutic potential of trehalose in a mouse model of OPMD that develops progressive muscle weakness, intranuclear aggregates and TUNEL-labelling of some myocyte nuclei [A17-1 mice; (10)]. Male A17-1 mice were treated from 6 weeks of age with 2% trehalose in drinking water, the dose and route used to effectively alleviate symptoms in the R6/2 model of Huntington’s disease (15). Trehalose-treated A17-1 mice were stronger than A17-1 mice with normal drinking water and had significantly improved forelimb grip strength at 4–10 months of age (Fig. 2A). There was no difference in forelimb grip strength between the two groups before treatment commenced at 5 weeks of age (data not shown). Although we have found grip strength to be the primary and a specific outcome measure in A17-1 mice, there are some other tasks that have previously shown a difference between A17-1 mice and non-transgenic littermates [wire manoeuvre, vertical gripping (10)]. These are strength dependent but are also influenced by other factors such as weight and age (e.g. a mouse over 30 g is unable to lift its own body weight and thus cannot effectively perform the wire manoeuvre). Trehalose improved the overall performance of A17-1 mice from 5 to 8 months at the wire manoeuvre task (Fig. 2B). A17-1 mice treated with trehalose also performed better than untreated A17-1 mice at the vertical gripping test at 5, 6 and 7 months of age (Fig. 2C). Note there was no difference in weight between untreated A17-1 and trehalose-treated A17-1 mice at any time point (data not shown). Trehalose treatment decreased the severity of late-onset locomotor defects seen in A17-1 mice; at 10 months of age, fewer of the trehalose-treated

![Figure 1. Trehalose reduces aggregate formation and death in a cell model of OPMD.](https://example.com/figure1.png)
mice dragged their pelvis when walking, when compared with untreated mice (Fig. 2D). Note the pelvis dragging phenotype only becomes apparent in A17-1 mice, compared with non-transgenic littermates at 9 months of age (10). Trehalose treatment did not affect the strength of non-transgenic mice (data not shown).

Given the known anti-aggregation action of trehalose, we wanted to see whether it could reduce the formation of polyalanine aggregates in vivo. We looked at aggregate formation in skeletal muscle sections (biceps) from trehalose-treated and untreated A17-1 mice at 6 months of age. Sections were incubated with KCl to remove soluble protein prior to immuno-labelling with a PABPN1 antibody, as previously described (10). There were fewer nuclei containing PABPN1-positive aggregates in biceps sections from trehalose-treated when compared with untreated control A17-1 mice at 6 months (Fig. 3A). We also looked at cell death in biceps sections from 6-month-old A17-1 mice using the wire manoeuvre task.
TUNEL labelling. Trehalose-treated A17-1 mice had a reduced number of TUNEL-positive nuclei when compared with untreated, control A17-1 mice (Fig. 3B). Haematoxylin and eosin staining of 6-month-old control or trehalose-treated (Trehalose) A17-1 mice revealed no obvious abnormalities—these only become apparent at later ages (10).

Western blot showed reduced transgene (PABPN1) levels in gastrocnemius muscle lysates of trehalose-treated when compared with untreated A17-1 mice at 6 months of age (Fig. 4A and B). Interestingly, a similar phenomenon was noted by Sanchez et al. (20) who studied the chemical chaperone Congo red in Huntington’s disease models and showed that Congo red facilitated clearance of their mutant protein. They hypothesized that Congo red made mutant huntingtin a more accessible substrate for the proteasome. Accordingly, we tested whether trehalose enhanced mutant PABPN1 turnover. COS-7 cells were transiently co-transfected with an EGFP-tagged A17 construct and a construct comprising HD exon 1 containing a Q23 repeat fused to EGFP (HDQ23). Note that both A17 and HDQ23 constructs have identical vector backbones and promoters (pEGFP-C1 parent vector). HDQ23 is a wild-type huntingtin exon 1 fragment—mutant Huntington’s disease alleles have 38 or more glutamines and the upper end of the wild-type range is 37 glutamines (21). HDQ23 does not aggregate and does not cause any toxicity when compared with empty green fluorescent protein (GFP). Unlike polyglutamine-expanded huntingtin, which is toxic and aggregate-prone, HDQ23 does not have an overt dependency on macroautophagy for its clearance (22) and is predominantly cleared by the proteasome (Supplementary Material, Fig. S1). Furthermore, HDQ23 clearance is not enhanced by trehalose (Supplementary Material, Fig. S1). Forty-eight hours post-transfection, cells were treated with cyclohexamide alone (to inhibit protein synthesis) or with cyclohexamide and trehalose. At both 24 and 48 h post-treatment (switching off protein synthesis), reduced levels of A17 were seen in cyclohexamide plus trehalose-treated samples when compared with samples treated with cyclohexamide alone when cells were collected 24 h after drug treatment (data not shown). However, at 48 h after treatment, reduced levels of A10 were seen in cyclohexamide plus trehalose-treated samples when compared with samples treated with cyclohexamide alone, and this was reversed by the addition of lactacystin (Supplementary Material, Fig. S2).

We also investigated whether macroautophagy affects mutant PABPN1 aggregate formation. Macroautophagy (which we will call autophagy) is a bulk degradation process in which a portion of the cytosol is enclosed by a double membrane structure to form autophagosome/autophagic vacuole. These then fuse with the lysosomes where their contents are degraded. Autophagy is a key pathway regulating clearance of mutant huntingtin fragments and GFP tagged with polyalanine expansions (22–24). Inducing autophagy with rapamycin (Fig. 5A and B) has a small effect on the proportion of A17-expressing cells with inclusions, whereas blocking autophagy with 3-methyladenine (Fig. 5C and D) or bafilomycin (Fig. 5E and F) has the converse effect. These effects are rather modest, as PABPN1 is almost exclusively nuclear at steady state, although it does shuttle between the nucleus and the

Figure 3. Trehalose attenuates pathology in OPMD mice. (A) Quantification of aggregate-containing nuclei in sections of biceps muscle from 6-month-old control or trehalose-treated (Trehalose) A17-1 mice. Sections were incubated with KCl to remove soluble protein (10) and labelled with a PABPN1-specific antibody to detect inclusions comprising PABPN1. (B) Biceps muscle sections from 6-month-old control or trehalose-treated (Trehalose) A17-1 mice were TUNEL-labelled and the number of positive nuclei scored. **P < 0.001, n = 3, 200 nuclei per sample scored; error bars represent standard error of the mean.
Figure 4. Trehalose promotes the clearance of mutant PABPN1 by the proteasome. (A) Western blot of gastrocnemius muscle lysates from 6-month-old control (C; normal drinking water) or trehalose-treated (T; 2% trehalose in drinking water) A17-1 mice, probed with an antibody to PABPN1; tubulin was used as a loading control. (B) Densitometric quantification of blot from (A). Intensities of PABPN1 bands were normalized to tubulin. C, control A17-1 mice; T, trehalose-treated A17-1 mice. (C) COS-7 cells were co-transfected with an EGFP-tagged A17 construct (A17; EGFP–PABPN1–A17) and EGFP–HDQ23 (HDQ23; this construct has previously been shown not to be cleared by trehalose and serves as a control for transfection efficiency). Forty-eight hours post-transfection (ON), cells were treated with cyclohexamide (10 μg/ml) alone or cyclohexamide and trehalose (100 μM). Cells were harvested at 24 and 48 h and lysates were blotted and probed with an antibody raised against EGFP. (D) Densitometric quantification of three separate experiments as (C). Band intensities for A17 were normalized to Q23 and cyclohexamide alone set to 100%. C, cyclohexamide; C + T, cyclohexamide and trehalose. (E) COS-7 cells were transiently transfected with EGFP–PABPN1–A17 or EGFP–PABPN1–A10 and treated with lactacystin (10 μM) for 24, 48 or 72 h. The number of EGFP-positive cells that contained aggregates was quantified. (F) EGFP-positive cells from (E) were scored for cell death (abnormal nuclei) based on abnormal apoptotic nuclear morphology (n = 6 cover slips, 200 cells per cover slip were scored). As a toxicity control, untransfected cells from EGFP–PABPN1–A17 control and lactacystin-treated slides were also scored for cell death (abnormal apoptotic nuclei). (G) COS-7 cells were co-transfected as (C) but incubated for 24 h before treatment with cyclohexamide (10 μg/ml) alone, cyclohexamide and trehalose (100 μM) or cyclohexamide, trehalose and lactacystin (10 μM). Cells were collected 48 h later and lysates produced were blotted and probed with an antibody raised against EGFP to detect levels of A17 (EGFP–PABPN1–A17) and HDQ23. This experiment was repeated multiple times and a representative blot is shown. (H) Densitometric quantification of four separate experiments as (G). C, cyclohexamide; C + T, cyclohexamide and trehalose; C + T + L, cyclohexamide, trehalose and lactacystin. *P < 0.05, **P < 0.001, ***P < 0.0001; NS, non-significant; error bars represent standard error of the mean.
cystosol (25). The nuclear pool of PABPN1 will be inaccessible to macroautophagy, and this can explain why the autophagy dependence of polyalanine-expanded PABPN1 is so small when compared with GFP-tagged polyalanine expansions (23), where a considerable proportion is cytosolic.

To test whether the effect of trehalose on the aggregation of mutant PABPN1 was independent of possible effects on protein degradation, we treated A17-transfected cells simultaneously with proteasome and autophagy inhibitors (Fig. 6A and B). Trehalose reduced the number of A17-expressing cells

Figure 5. Autophagy influences mutant PABPN1 aggregation. (A) COS-7 cells were transiently transfected with EGFP–PABPN1–A17 and treated with rapamycin (0.2 μg/ml) for 48, 72 or 96 h. The number of EGFP-positive cells that contained aggregates was quantified. (B) EGFP-positive cells from (A) were scored for cell death (abnormal nuclei) based on abnormal apoptotic nuclear morphology (n = 3 cover slips). (C) COS-7 cells transiently transfected with EGFP–PABPN1–A17 or EGFP–PABPN1–A10 were treated with 3-methyladenine (10 mM) for 24 or 48 h and the number of EGFP-positive cells that contained aggregates was quantified. (D) Cell death data (abnormal apoptotic nuclei) of EGFP-positive cells from (C). (E) COS-7 cells were transfected as (C) but treated with bafilomycin (400 nM). The number of EGFP-positive cells containing aggregates was scored. (F) The number of EGFP-positive cells from (E) with abnormal apoptotic nuclei was scored. *P < 0.05; **P < 0.001; ***P < 0.0001; NS, non-significant; error bars represent standard deviation; n = 6 cover slips, 200 cells per cover slip were scored.
containing aggregates when both clearance pathways were compromised, suggesting that the primary effect of this compound on PABPN1 aggregation is not primarily by enhancing the clearance of the protein.

DISCUSSION

The pathological hallmark of OPMD is the formation of tubulo-filamentous inclusions/aggregates comprising misfolded, mutant PABPN1 in nuclei of affected skeletal muscle fibres (3–5). It is likely that these aggregates, the aggregation process and/or early oligomeric species are toxic and therefore one therapeutic strategy for OPMD would be to inhibit aggregation formation. Small molecules such as trehalose have been termed chemical chaperones because of their ability to modulate protein folding and inhibit aggregate formation (12–14). Given that trehalose has been shown to inhibit the formation of polyglutamine aggregates (15), it was possible that it may also inhibit aggregate formation by proteins containing an expanded polyalanine repeat. It is likely that trehalose is acting by reducing the aggregation of mutant PABPN1 in both our cell model and mouse model of OPMD. Polyalanine expansions form amyloid-like fibrils in vitro and in vivo, not dissimilar to what is observed with other proteinopathies-like polyglutamine aggregates (26–28). As trehalose reduces aggregation of polyglutamine and insulin fibrils in vitro and polyglutamine expansions in cell lines and in vivo (13,15), probably the same phenomenon accounts for the effects we have observed with polyalanine-expanded PABPN1. Furthermore, our data suggest that the primary effect of this compound on PABPN1 aggregation is not simply due to enhanced clearance of the protein, as trehalose reduce mutant PABPN1 aggregation in cells treated simultaneously with both proteasome and autophagy inhibitors. However, we cannot exclude the possibility that trehalose may affect mutant PABPN1 aggregation by other effects that are independent of the direct interference of polymerization.

Trehalose delays disease onset, attenuates the phenotype and decreases aggregate formation and cell death in A17-1 mice. This strongly suggests that trehalose and other anti-aggregation therapies may be beneficial in OPMD. Almost all OPMD cases have a positive family history and the mutation can be easily diagnosed by PCR (2). As one can identify almost all cases at risk, this disease is amenable to pre-symptomatic treatment. If one can delay onset of a disease that typically presents around the age of 50 by 40 years, then one has effectively cured the disease. Trehalose would be particularly attractive for this strategy, given its safety and suitability for long-term use. However, the disease course in mice is very constricted when compared with the human situation, and we cannot guarantee that a treatment that is effective in mice will have a similar effect in humans. Also, such a treatment would need to be administered for decades in humans. Although trehalose is appealing in this context, as it appears to be safe at high doses in rodents and rabbits and is well tolerated at high doses in humans (29), further studies will be required to test that it is safe when administered over much longer periods.

MATERIALS AND METHODS

Cell studies

COS-7 (African green monkey kidney) cells were maintained in Dulbecco’s modified medium (Sigma-Aldrich Ltd) supplemented with 10% fetal bovine serum, 100 U ml⁻¹ penicillin/streptomycin, 2 mM L-glutamine and 1 mM sodium pyruvate at 37°C in 5% carbon dioxide. Cells were transiently transfected with plasmids encoding A17 (pEGFPC1–PABPN1–A17) or A10 (pEGFPC1–PABPN1–A10) (7) and/or HD exon 1 containing a Q23 repeat fused to EGFP (pEGFPC1–HDQ23) (30) using lipofectAMINE reagent (Invitrogen), following the manufacturer’s protocol. For counting experiments, A17-transfected cells were treated immediately post-transfection with trehalose (100 mM), lactacystin (10 μM), rapamycin (0.2 μg/ml), bafilomycin (400 nM) or 3-methyl-adenosine (10 mM). Then, 24, 48, 72 or 96 h later, cells were fixed with 4% paraformaldehyde in 0.1 M PBS, pH 7.6, and mounted in Citifluor (Citifluor Ltd) containing 4',6-diamidino-2-phenylindole (DAPI; 3 μg/ml; Sigma-Aldrich Ltd) to visualize nuclei. Transfected cells were scored for abnormal apoptotic nuclei and aggregates. Experiments were carried out in triplicate and 200 cells per cover slip were scored with the investigator blind to the identity of the

![Figure 6. Trehalose reduces the aggregation of mutant PABPN1 when clearance pathways are blocked. (A) COS-7 cells were transfected with EGFP–PABPN1–A17 and 48 h post-transfection, cells were treated with cyclohexamide (10 μg/ml; inhibits protein synthesis), 3-methyladenine (10 mM; inhibits autophagy) and lactacystin (10 μM; proteasome inhibitor) with or without trehalose (10 mM). Cells were fixed 48 h later and the number of EGFP-positive cells that contained aggregates was scored. (B) As (A), but the inhibitor of autophagy bafilomycin (400 nM) was used in place of 3-methyladenine. ***P < 0.0001; error bars represent standard deviation; n = 6 cover slips, 200 cells per cover slip were scored.](https://academic.oup.com/hmg/article-abstract/15/1/23/613091)
sample. Pooled estimates were calculated as odds ratios [OR; the ratios of the proportion of aggregate containing normal (or abnormal apoptotic:normal) nuclei in different experimental conditions] with 95% confidence intervals, as described previously (7). OR and P-values were determined by unconditional logistical regression analysis using the general log linear analysis option of SPSS Version 6.1 (SPSS, Chicago, IL, USA).

For clearance experiments, cells were incubated for 48 h after transfection, and then trehalose (100 mM), cyclohexamide (10 µg/ml) and/or lactacystin (10 µM) were added. Cell pellets were collected at 24 and 48 h.

Stable inducible PC12 cell lines expressing wild-type huntingtin exon 1 fragment with a 23 polyglutamine repeat fused to EGFP (HDQ23) under control of a Tet-ON promoter were maintained as previously described (31). To monitor the clearance of HDQ23, expression was induced by adding doxycycline (1 µg/ml) for 8 h, switched off by removing doxycycline and rapamycin (0.2 µg/ml), trehalose (100 mM) or lactacystin (10 µM) were added.

**OPMD model mice**

The A17-1 line of OPMD transgenic mice has previously been described (10). All studies and procedures were carried out following UK Home Office regulations and under approval of the Cambridge University animal ethics committee. Unless otherwise stated, animals were caged under standard conditions (12 h light, 12 h dark; food and water available *ad libitum*). We treated male A17-1 mice orally from 6 weeks of age with 2% (w/v) trehalose (Sigma-Aldrich Ltd). We dissolved trehalose in drinking water and changed these solutions twice a week.

**Behavioural testing**

Forelimb grip strength was assessed using a grip strength meter (Bioseb, France). Wire manoeuvre and vertical gripping are part of the SHIRPA battery of behavioural tests (32). Mice were assessed with the investigator blind to genotype and treatment, mice were given alphanumeric identities that provided no clue to genotype or treatment. For the wire manoeuvre, we held mice above a horizontal wire by the tail and lowered them to allow the forelimbs to grip the wire. Mice were held in extension, rotated around to the horizontal and released. Mice were scored as follows: 0, active grip with hind legs; 1, difficulty grasping with hind legs; 2, unable to lift hind legs; 3, falls within 30 s; 4, falls immediately. For the vertical gripping test, we placed mice on a horizontal grid that was gripped with both forelimbs and hind limbs. We raised the grid to the vertical and scored mice as follows: 0, grips the grid; 1, falls off the grid. We analysed non-parametric data from the wire manoeuvre test at each time point using Mann–Whitney *U* tests (STATVIEW software, version 4.53; Abacus Concepts). We used χ² tests for vertical gripping data. We analysed grip strength meter data from each treatment time point with unpaired *t*-tests and the overall effect from all treatment time points with repeated-measures ANOVA (STATVIEW software, version 4.53; Abacus Concepts).

**Histology**

Tissue was snap frozen in liquid nitrogen-cooled isopentane and 10 µm sections were cut on a cryostat (Leica Microsystems) to poly-l-lysine coated slides. Sections were fixed in acetone. For immuno-labelling, slides were blocked with 1% normal goat serum in 0.1 M PBS and 0.1% Triton X-100 and then incubated, at 4 °C overnight, in primary antibody (anti-PABPN1; a kind gift from Professor Elmar Wahle, Halle, Germany) diluted (1:500) in 1% normal goat serum in 0.1 M PBS and 0.1% Triton X-100. Slides were washed in 0.1 M PBS, 0.1% Triton X-100 and incubated in fluorophore-conjugated secondary antibody (Alexa Fluor 488 goat anti-rabbit; 1:1000; Molecular Probes) for 2 h at room temperature in the dark. Slides were washed again and sections mounted in Citifluor (Citifluor Ltd) containing DAPI (3 µg/ml; Sigma-Aldrich Ltd) to visualize nuclei. To remove soluble proteins, sections were incubated in 1 M KCl, 30 mM HEPES, 65 mM PIPES, 10 mM EDTA and 2 mM MgCl₂, pH 6.9, for 1 h at room temperature prior to immuno-labelling. Aggregates are resistant to this KCl treatment. Fluorescent DNA fragmentation (TUNEL; terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling) assay was carried out on skeletal muscle sections using a standard kit (ApoAlert DNA fragmentation assay kit; BD Biosciences). Nuclei that contained aggregates and TUNEL-positive nuclei were scored. Three samples per group and 200 nuclei per sample were scored, with the viewer blind to the identity of the slide. Pooled estimates were calculated as odds ratios as described earlier.

**Western blotting**

Protein lysates were prepared by homogenizing tissue or cell pellets in 50 mM Tris–HCl pH 7.4 and 0.5% Triton X-100 with protease inhibitor cocktail (Complete; Roche Diagnostics). Proteins were separated on 10% SDS–polyacrylamide gels and transferred onto nitrocellulose membranes (Hybond ECL membrane; Amersham Biosciences), which were blocked by incubation in 5% dried milk in 0.1 M PBS and 0.1% Tween-20, pH 7.6. Membranes were probed with primary antibodies raised against PABPN1 (a kind gift from Professor Elmar Wahle, Halle, Germany; 1:5000), EGFP (to detect EGFP-linked PABPN1 and HDQ23 constructs; Clontech; 1:10 000) or tubulin (loading control for whole tissue extracts; Sigma-Aldrich Ltd; 1:1000). HRP-conjugated antibodies (Amersham Biosciences; 1:5000) were then added to the blots. Immuno-reactive bands were detected with enhanced chemiluminescence reagent (ECL; Amersham Biosciences) and signal visualized by the exposing membrane to ECL Hyperfilm (Amersham Biosciences). Quantification of western blots was carried out using ImageJ software; for cell studies, band intensities of A17 or A10 were normalized to Q23 and for the *in vivo* data, PABPN1 levels were normalized to tubulin levels. *P*-values were determined using unpaired *t*-tests.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG Online.
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REFERENCES