A *Drosophila* model of oculopharyngeal muscular dystrophy reveals intrinsic toxicity of PABPN1

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Oculopharyngeal muscular dystrophy (OPMD) is an adult-onset syndrome characterized by progressive degeneration of particular muscles. OPMD is caused by short GCG repeat expansions within the gene encoding the nuclear poly(A)-binding protein 1 (PABPN1) that extend an N-terminal polyalanine tract in the protein. Mutant PABPN1 aggregates as nuclear inclusions in OPMD patient muscles. We have created a *Drosophila* model of OPMD that recapitulates the features of the human disorder: progressive muscle degeneration, with muscle defects proportional to the number of alanines in the tract, and formation of PABPN1 nuclear inclusions. Strikingly, the polyalanine tract is not absolutely required for muscle degeneration, whereas another domain of PABPN1, the RNA-binding domain and its function in RNA binding are required. This demonstrates that OPMD does not result from polyalanine toxicity, but from an intrinsic property of PABPN1. We also identify several suppressors of the OPMD phenotype. This establishes our OPMD *Drosophila* model as a powerful *in vivo* test to understand the disease process and develop novel therapeutic strategies.

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Introduction

Polyalanine tract expansions in various proteins are the cause of several human diseases or malformations (Brown and Brown, 2004). Among these, oculopharyngeal muscular dystrophy (OPMD), an autosomal dominant muscular dystrophy characterized by progressive eyelid drooping, swallowing difficulties and proximal limb muscle weakness, is particular in that the alanine-expanded protein forms nuclear inclusions (Brais, 2003). OPMD is caused by expansions of an N-terminal polyalanine tract in the nuclear poly(A)-binding protein 1 (PABPN1, previously called PABP2) (Brais *et al*,

1998). The primary function of PABPN1 is during nuclear polyadenylation (Zhao *et al*, 1999; Kühn and Wahle, 2004). PABPN1 binds to nascent poly(A) tails during this reaction and it has two distinct roles: stimulation of poly(A) polymerase, and control of poly(A) tail length (Wahle, 1991; Kühn and Wahle, 2004). Stimulation of poly(A) polymerase depends on a coiled-coil N-terminal domain in the protein (Kerwitz *et al*, 2003), whereas a central RNP-type RNA-binding domain, as well as an arginine-rich C-terminal domain are involved in binding to poly(A) tails (Kühn *et al*, 2003). In addition to its well-described role in nuclear polyadenylation, PABPN1 appears to have other functions in mRNA metabolism. Although PABPN1 is nuclear at steady-state levels, it shuttles from nuclear to cytoplasmic compartments (Calado *et al*, 2000a). A role for PABPN1 in mRNA export has not been investigated directly; however, PABPN1 has been found to be associated with an mRNA during its docking at the nuclear pore, and was present on the cytoplasmic side of the nuclear envelope (Bear *et al*, 2003). This suggests possible roles of PABPN1 in mRNA export and/or cytoplasmic metabolism. More recently, we described a new cytoplasmic function of the *Drosophila* homolog of PABPN1 in shortening poly(A) tails during early development (Benoit *et al*, 2005).

The molecular mechanisms leading from expansion of the polyalanine tract in PABPN1 to OPMD are unknown. Because intranuclear inclusions containing PABPN1 represent a pathological hallmark of muscle cells from OPMD patients, it has been proposed that alanine expansion in PABPN1 induces the formation of inclusions (Brais, 2003). It should be noted, however, that PABPN1 oligomerization occurs during the wild-type function of PABPN1 in nuclear polyadenylation (Kühn *et al*, 2003). In addition to PABPN1, OPMD nuclear inclusions contain poly(A) RNA, as well as ubiquitin, proteasomes and HSP70 (Calado *et al*, 2000b; Abu-Baker *et al*, 2003). Molecular chaperones and factors of the protein degradation pathways are also present in intracellular aggregates containing polyglutamine-expanded proteins found in several neurodegenerative disorders, including Huntington's disease (Bossy-Wetzel *et al*, 2004; Forman *et al*, 2004). Polyglutamine diseases differ from OPMD in that the alanine expansion in OPMD is very short (2–7 alanines) (Brais *et al*, 1998).

Several cell models of OPMD have been generated, in which nuclear or cytoplasmic aggregates, containing PABPN1 or polyalanine tracts, are monitored (Fan *et al*, 2001; Bao *et al*, 2002; Abu-Baker *et al*, 2003; Tavanez *et al*, 2005). More recently, mice models of OPMD have also been reported (Hino *et al*, 2004; Davies *et al*, 2005; Dion *et al*, 2005). Expression in mice of alanine-expanded PABPN1, either ubiquitously or in muscles, leads to progressive muscle weakness and formation of PABPN1 nuclear inclusions (Hino *et al*, 2004; Davies *et al*, 2005). With the aim of providing a genetically tractable *in vivo* test, we generated a *Drosophila* model of OPMD. We first demonstrated that the *Drosophila* model reproduces faithfully the disease characteristics. Next,

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using this model we showed that although muscle defects are proportional to the length of the polyalanine tract in PABPN1, the polyalanine tract is not absolutely required to induce muscle degeneration, whereas the RNA binding function of PABPN1 is required. This *Drosophila* model represents a powerful genetic complement to PABPN1 transgenic mice, and highlights *Drosophila* as an excellent model to study muscular dystrophies.

Results

Expression of PABPN1 in *Drosophila* muscles induces progressive wing position phenotypes

Wild-type human PABPN1 contains a stretch of 10 alanines following the initial methionine, which is expanded to 12–17 alanines in OPMD patients. In *Drosophila*, the PABPN1 homolog is the poly(A)-binding protein 2 (PABP2), which has the same function as PABPN1 in nuclear polyadenylation but lacks a polyalanine tract at the N-terminus (Benoit *et al*, 1999). We used the *UAS/Gal4* system to express various versions of mammalian PABPN1 in *Drosophila*. A wild-type PABPN1 cDNA (encoding the 10 alanine tract) was cloned downstream of *UAS* sequences (*UAS-PABPN1*), as were cDNAs in which either the alanine-encoding repeat was expanded to encode seven additional alanines (*UAS-PABPN1-17ala*) or in which the repeats were missing (*UAS-PABPN1-Δala*). Transgenic lines containing these constructs were crossed to three *Gal4* drivers, leading to ubiquitous expression (*daughterless-Gal4* (*da-Gal4*)), expression in the mesoderm (*24B-Gal4*) or muscle-specific expression (*Mhc-Gal4*). Expression of each construct using *da-Gal4* or *24B-Gal4* was lethal from embryonic to pupal stages. When the PABPN1 versions were expressed using the *Mhc-Gal4* driver, adults were viable and two wing position phenotypes (wings up and wings down) were obtained (Figure 1A). To quantify the phenotypes, three transgenic lines expressing similar levels of PABPN1, PABPN1-17ala or PABPN1-Δala when crossed to *Mhc-Gal4* were selected by Western blot (Figure 1B). The specificity of the antibody for PABPN1 and its lack of reactivity against *Drosophila* PABP2 was verified (Supplementary Figure 1). The wing position phenotype appeared progressively: at 25°C, all flies had normal wings on the day of birth (day 1), with 40–70% showing abnormal wing position at day 2, and more than 90% at day 3 (Figure 1C). The percentage of abnormal-winged flies at day 2 depended on the PABPN1 construct used, with PABPN1-17ala producing the strongest phenotype. At 18°C, *Gal4* is less active, and the phenotypic progression was slower, with abnormal wing position first observed at day 3 (15%) and reaching 90% at day 5 with PABPN1-17ala. At this temperature, the phenotype was proportional to the length of the alanine tract (Figure 1C). These results show that the phenotype strength reflects the number of alanines in the tract, consistent with a role of the polyalanine expansion in the disease. However, the alanine tract is not absolutely required to induce the wing position phenotypes.

Expression of PABPN1 in *Drosophila* muscles induces progressive muscle degeneration

Much of the muscle in the fly thorax corresponds to indirect flight muscles (IFMs), which are involved in flight and wing posture. IFMs are composed of dorso-longitudinal muscles

Figure 1 Muscular expression of mammalian PABPN1 induces a progressive wing position phenotype in adults. (A) Abnormal wing position phenotypes (wings down or up, right panel) observed when PABPN1, PABPN1-17ala or PABPN1-Δala is expressed in muscles with the *Mhc-Gal4* driver; wild-type wing position in the control *Mhc-Gal4/+* (left panel). (B) Quantitation of PABPN1 levels by Western blot. Protein extracts are from 0.25 thoraxes of *UAS-PABPN1/Mhc-Gal4*, *UAS-PABPN1-17ala/+; Mhc-Gal4/+*, *UAS-PABPN1-Δala/+; Mhc-Gal4/+* adult males at day 1 raised at 25°C (left panel) or 18°C (right panel). α -Tubulin was used as a loading control. Comparable levels of PABPN1 are produced in these three selected transgenic lines. (C) Quantitative analysis of the abnormal wing position phenotype of *UAS-PABPN1/Mhc-Gal4*, *UAS-PABPN1-17ala/+; Mhc-Gal4/+* and *UAS-PABPN1-Δala/+; Mhc-Gal4/+* individuals raised at 25 or 18°C. The percentages of adults with abnormal wing position were calculated based on 200 flies from two independent crosses. Control *Mhc-Gal4/+* flies did not show abnormal wing position at 25 or 18°C (0% abnormal wing position, $n > 200$).

(DLM) and dorso-ventral muscles (DVM) (Figure 2A and B), both of which were analyzed by polarized light. No musculature defects were observed following expression of PABPN1, PABPN1-17ala or PABPN1-Δala using *Mhc-Gal4* at 25°C at day 2, indicating that PABPN1 expression does not interfere with IFM development and that the day 2 wing position defects were not caused by defects in IFM structure. In contrast, muscle defects were clear by day 6 and were increasing, with 50% ($n=26$) and 100% ($n=35$) of the thoraxes showing muscle degeneration at day 6 and day 16, respectively. Muscle fibers became thin and irregular (Figure 2C and D), most strongly at day 16 (Figure 2E and F). Muscle

Figure 2 Muscular expression of PABPN1-17ala induces progressive muscle degeneration. (A–F) Indirect flight muscles (IFMs) in the adult thorax observed under polarized light. (A, B) Control fly with focus on dorso-longitudinal muscles (DLMs, six muscles 1–6) (A) and dorso-ventral muscles (DVMs, seven muscles II 1, II 2, II 3 out of focus, III 1, III 2, III 3) (B). Anterior is to the left and dorsal is up. (C–F) *UAS-PABPN1-17ala/+; Mhc-Gal4/+* adult raised at 25°C, at day 6 (C, D) and day 16 (E, F). Muscle defects are clearly visible at day 6, with thinner muscles (white arrows, C, D). At day 16, these defects are strongly enhanced, with very thin or missing muscles (white arrows, E, F). (G, H) Ultrastructure of IFMs visualized by electron microscopy. (G) Longitudinal section through IFM in a control fly showing sarcomeric structure of myofibrils. Z-bands (Z) separate adjacent sarcomeres showing central M-bands (M). mt: mitochondria. (H–K) *UAS-PABPN1-17ala/+; Mhc-Gal4/+* adult at day 6, raised at 25°C. (H) Muscle degeneration is characterized by disintegration of mitochondria and their replacement by vacuoles (black arrowheads), and disruption of myofibril integrity with the dissociation of the myosin–actin network and broken Z-bands (white arrows). Similar defects are observed when PABPN1 or PABPN1-Δala is expressed in muscles at 25°C. (I, J) Examples of vacuoles observed in degenerating muscles. (J) Rimmed vacuole surrounded by a ring of membranous structures that closely resembles rimmed vacuoles characteristic of OPMD patient biopsies. (K) Apoptotic nucleus showing a disintegrating nuclear membrane. Scale bars : 1 μm.

fibers were also analyzed at the ultrastructural level by electron microscopy. Consistent with the polarized light results, myofibrils from affected flies at day 2 appeared normal, although occasional vacuoles were found in the area containing mitochondria (Supplementary Figure 2). At day 6, however, the muscle fibers of flies expressing the different PABPN1 versions were strongly affected (Figure 2G and H), having thin and disorganized myofibrils with broken Z bands and no M bands. Mitochondria were lacking, and many vacuoles were present (Figure 2I and J). Some of these were rimmed vacuoles (Figure 2J), similar to those in the muscles of OPMD patients (Brais, 2003). Apoptotic nuclei were also observed (Figure 2K).

Wing position defects at day 2 were not caused by altered myofibril structure; they could therefore result from defective IFM function. Consistent with this, the flight capacity of flies expressing PABPN1-17ala or PABPN1-Δala was analyzed at day 2, and 100% of these flies were flightless (Supplementary Figure 2).

Together, these results show that expression of the PABPN1 constructs in *Drosophila* muscles induces a progressive muscle degeneration involving apoptosis. The progressive wing posture phenotype starts before defects in myofibril structure are visible and probably result at this early stage from altered muscle function.

Expression of PABPN1-17ala leads to the formation of nuclear inclusions

Muscle fibers from OPMD patients display salt extraction-resistant nuclear inclusions containing PABPN1, HSP70, ubiquitin, proteasomes and poly(A) RNA (Calado *et al*, 2000b; Abu-Baker *et al*, 2003). At the ultrastructural level, these inclusions comprise unbranched tubular filaments having an 8.5 nm outer diameter (Tome *et al*, 1997). Anti-PABPN1 immunostaining of *Drosophila* adult IFM expressing PABPN1-17ala revealed that nuclear inclusions were present from day 2, and appeared as dense, variably sized structures in many nuclei by day 6 (Figure 3A–C). These inclusions recruit the

chaperone protein HSP70 (Figure 3B) and contain conjugated ubiquitin (Figure 3C). Nuclei containing inclusions were dramatically affected: they were larger, with their DNA excluded from the inclusion and often condensed at the nuclear periphery (Figure 3B and C). Nuclear inclusions were analyzed at the ultrastructural level and appeared as tangled tubular filaments 8.3–10 nm in diameter, similar to those in OPMD nuclear inclusions (Figure 3D–F).

In affected IFM expressing PABPN1- Δ ala, very dense nuclear inclusions did not form, although uncondensed aggregates could be visualized in a low number of nuclei. In addition, substantial amounts of PABPN1 were present in the cytoplasm after expression of PABPN1-17ala or PABPN1-

Δ ala (Figure 3A). This suggested that the nuclear import machinery could not relocate elevated levels of PABPN1 to the nucleus. Consistent with this, we also found a certain amount of cytoplasmic PABPN1, when PABPN1 constructs were expressed at a lower level at 18°C, although this cytoplasmic amount was lower than at 25°C (data not shown).

These data suggested that the formation of dense PABPN1-containing nuclear inclusions depended on the polyalanine tract. We confirmed this by monitoring the formation of nuclear inclusions in different muscles, in third instar larvae. Locomotor larval muscles were strongly affected and appeared thin with an irregular pattern of nuclei, when

Figure 3 Characterization of nuclear inclusions induced by expression of PABPN1-17ala in muscles. (A) Immunostaining of wild-type IFMs with anti-PABP2 (control), and of IFMs from *UAS-PABPN1-17ala/+; Mhc-Gal4/+* (PABPN1-17ala) and *UAS-PABPN1- Δ ala/+; Mhc-Gal4/+* (PABPN1- Δ ala) adults raised at 25°C, at day 6, with anti-PABPN1. DNA is revealed with DAPI staining. The control shows the distribution of PABP2 throughout nuclei, whereas dense nuclear inclusions of PABPN1 are observed when PABPN1-17ala is expressed. A diffuse nuclear staining is visible when PABPN1- Δ ala is expressed. A substantial level of PABPN1 is also present in the cytoplasm when PABPN1-17ala and PABPN1- Δ ala are expressed using *Mhc-Gal4*. (B, C) Presence of HSP70 and conjugated ubiquitin in PABPN1 nuclear inclusions. (B) Immunostaining of wild-type IFMs (control) with anti-PABP2 and anti-HSP70, and immunostaining of *UAS-PABPN1-17ala/+; Mhc-Gal4/+* (PABPN1-17ala) IFMs with anti-PABPN1 and anti-HSP70. (C) Immunostaining of wild-type IFMs (control) with anti-PABP2 and anticonjugated ubiquitin (Ubi), and immunostaining of *UAS-PABPN1-17ala/+; Mhc-Gal4/+* (PABPN1-17ala) IFMs with anti-PABPN1 and anticonjugated ubiquitin. DNA is visualized by DAPI staining. DNA is excluded from the dense PABPN1 nuclear inclusions that show anti-HSP70 and anticonjugated ubiquitin staining. Scale bar is identical for B–C: 4 μ m. (D–F) Ultrastructure of nuclei in *UAS-PABPN1-17ala/+; Mhc-Gal4/+* IFMs at day 6 and 25°C showing a nuclear inclusion. In this example (D), the nuclear inclusion almost fills the entire nucleus (clear zone); it is surrounded by chromatin (black) close to the nuclear membrane. (E) Higher magnification of a central region of the inclusion shown in (D). Tangled filaments are visible. (F) Higher magnification of the region within the rectangle in (E). Arrows indicate tubular filaments disposed in various directions. Scale bars: 1 μ m in (D), 0.2 μ m in (E) and 0.1 μ m in (F).

Figure 4 The formation of nuclear inclusions depends on the presence of the alanine tract in PABPN1. (A) Immunostaining of third instar larval muscles 6 (top) and 7 (bottom) from wild-type larvae with anti-PABP2 (control) and from *UAS-PABPN1-17ala/+; Mhc-Gal4/+* (PABPN1-17ala) and *UAS-PABPN1-Δala/+; Mhc-Gal4/+* (PABPN1-Δala) larvae raised at 29°C, with anti-PABPN1. (B) Immunostaining of third instar larval muscles as in (A) showing higher magnification of nuclei. DNA is visualized by DAPI staining. After expression of PABPN1-17ala, dense nuclear inclusions are visible (arrowhead) and the nuclear structure is affected, with DNA at the periphery and excluded from the inclusions. Inclusions do not form, however, after expression of PABPN1-Δala and the nuclear structure does not appear affected. (C) PABPN1-17ala nuclear inclusions are resistant to KCl. Immunostaining of third instar larval muscles from wild-type larvae with anti-PABP2 (control) and from *UAS-PABPN1-17ala/+; Mhc-Gal4/+* (PABPN1-17ala) larvae raised at 25°C, with anti-PABPN1. After 1 M KCl treatment, PABP2 or PABPN1-17ala that is diffuse in nuclei are soluble, but PABPN1-17ala within nuclear inclusions is not (bottom panels). Arrowheads indicate nuclear inclusions. Nuclei are visualized by DAPI staining.

PABPN1-17ala was expressed in muscles with *Mhc-Gal4* at 25 or 29°C (Figure 4A). The nuclear structure was affected and nuclear inclusions containing PABPN1-17ala and resistant to 1 M KCl extraction were present in many nuclei (Figure 4B and C). In contrast, larval muscle defects induced by expression of PABPN1-Δala with *Mhc-Gal4* were weaker and nuclear inclusions did not form (Figure 4A and B).

These results show that PABPN1-containing nuclear inclusions assemble when PABPN1-17ala is expressed in *Drosophila* muscles. These nuclear inclusions have the characteristics of PABPN1 inclusions found in OPMD patient muscles, even at the ultrastructural level. Expression of PABPN1-Δala, however, does not lead to the presence of such dense nuclear inclusions. This indicates that the alanine tract has a role in the formation of OPMD-like nuclear inclusions.

The RNP-type RNA-binding domain of PABPN1 and its function in RNA binding are required for OPMD-like phenotypes

PABPN1 has several domains, including a coiled-coil domain involved in poly(A) polymerase stimulation, an RNP-type RNA-binding domain (RRM) required for poly(A) binding and an arginine rich C-terminal domain that is also involved in RNA binding (Kerwitz *et al*, 2003; Kuhn *et al*, 2003). The C-terminal 50 residues contain a nuclear localization signal,

and the final 20 are required for protein self-association (Calado *et al*, 2000a,b; Fan *et al*, 2001; Kuhn *et al*, 2003). We generated PABPN1-17ala deletions to determine whether domains outside the alanine tract could contribute to the OPMD-like phenotypes. Three deletions were constructed, removing the coiled-coil domain, the RRM or a proximal region of the C-terminal domain (Figure 5A). As a major feature of OPMD is the accumulation of PABPN1 as insoluble nuclear inclusions, we did not alter the extreme C-terminus of the protein, which is involved in nuclear import (Calado *et al*, 2000a). Transgenic lines were obtained containing these deleted forms inserted downstream of *UAS*, and expression of the truncated proteins was induced in muscles using *Mhc-Gal4*. PABPN1-17ala lacking either the coiled-coil domain or the C-terminal segment induced wing position phenotypes, muscle defects and altered nuclear morphology with the formation of nuclear inclusions (Figure 5B, D and E). Strikingly, PABPN1-17ala with a deleted RRM (PABPN1-17ala-ΔRRM) was unable to induce any wing position phenotypes or muscle degeneration, even when expressed at high levels with four copies of the transgene (Figure 5B and D, Supplementary Figure 3). Immunostaining of muscles showed that PABPN1-17ala-ΔRRM had a nuclear distribution similar to that of endogenous *Drosophila* PABP2. The presence of this truncated protein in nuclei did not induce affected nuclear morphology, nor the formation of nuclear

inclusions (Supplementary Figure 3). These results demonstrated that the region in PABPN1 between residues 164 and 273 that contains the RRM was required to induce the OPMD-like phenotypes; this suggested that the RRM and potentially its function in RNA binding were involved. To determine whether the RNA-binding function of the RRM was indeed

required, we generated an additional PABPN1-17ala construct containing a double point mutation within the RRM (PABPN1-17ala-dm) (tyrosine to alanine at position 175 and phenylalanine to alanine at position 215 in PABPN1) (Figure 5A). These point mutations were shown to strongly reduce the binding of PABPN1 to poly(A) *in vitro* (Kuhn *et al*,

2003). When PABPN1-17ala-dm was expressed in muscles using *Mhc-Gal4*, no or a very low number of flies with abnormal wing position were obtained (Figure 5C). The musculature of these flies appeared unaffected, as was the nuclear morphology. In addition, the nuclear distribution of PABPN1-17ala-dm was similar to that of endogenous PABP2 (Figure 5D and E).

These results demonstrate that the RNA-binding function of PABPN1 is required to generate OPMD-like phenotypes. Therefore, these phenotypes are not due to toxicity of the alanine tract, but rather depends directly on PABPN1 itself and its ability to bind RNA.

Molecular chaperones and an antiapoptotic protein are suppressors of the OPMD-like phenotypes

As our results indicate that PABPN1 itself, and not the polyaniline tract, is an intrinsic cause of OPMD, the pathways involved in OPMD and in polyglutamine diseases are likely to be different. Nevertheless, both types of disease involve nuclear inclusions that recruit molecular chaperones and protein degradation components. We tested candidate genes that have been identified as suppressors of polyglutamine diseases in *Drosophila* for their ability to suppress PABPN1-associated phenotypes. Overexpression of molecular chaperones, in particular HSP70 and genes involved in the HSP70 pathway, efficiently suppressed the wing posture phenotype induced by PABPN1-17ala expression in muscles (Table I). The viral antiapoptotic protein P35 was also an efficient suppressor, confirming that apoptosis is involved in PABPN1-17ala-induced muscle degeneration.

Discussion

We present here a model of OPMD in *Drosophila*. Expression of PABPN1 in *Drosophila* muscles induces muscular defects that closely resemble those of OPMD patients at the structural, ultrastructural and molecular levels. Affected muscles contain rimmed vacuoles and altered mitochondria. Insoluble inclusions that include PABPN1, recruit HSP70 and ubiquitin, and are composed of tubular filaments similar to those seen in OPMD patients, form in many nuclei. All of these phenotypes develop progressively. This *in vivo* model provides important insights into the molecular mechanisms

of OPMD. In contrast with polyglutamine diseases, where the polyglutamine tract is neurotoxic by itself in *Drosophila* models (Kazemi-Esfarjani and Benzer, 2000; Marsh et al, 2000), the alanine tract in PABPN1 is not absolutely required to induce muscle defects, but rather acts to enhance the phenotypes. Most importantly, another domain of the protein, the RRM, is essential. This strongly suggests that OPMD does not result from toxicity of the polyaniline tract alone,

Table I Suppressors of the wing position phenotype induced by expression of PABPN1-17ala in muscles

	Day 6 95%	Day 11 96%	n	Suppression
Control			84	
<i>HSP70 pathway</i>				
<i>UAS-HSPA1L</i> (human HSP70)	46%	60%	140	Yes
<i>Hsp70Ab</i> ^{EY01148}	26%	43%	95	Yes
<i>UAS-Hsc70-4</i>	25%	32%	90	Yes
<i>HSP83</i> ^{e6A}	50%	64%	105	Yes
<i>Co-chaperone</i>				
<i>UAS-dhdj1</i>	76%	86%	50	Weak
<i>Antiapoptosis</i>				
<i>UAS-P35</i>	36%	49%	160	Yes

UAS-PABPN1-17ala/+; Mhc-Gal4/+ individuals also bearing one copy of the indicated transgene or mutation were raised at 18°C and analyzed at days 6 and 11. The percentages of abnormal wing position, calculated from the indicated numbers of flies (n) from two independent crosses are shown. *Mhc-Gal4* drives expression in muscles of the transgenes *UAS-HSPA1L* (Warrick et al, 1999) (human HSP70), *UAS-Hsc70-4* (Elefant and Palter, 1999) (constitutive HSP70), *UAS-dhdj1* (Kazemi-Esfarjani and Benzer, 2000) (human HSP40 homologue), *UAS-dtrp2* (Kazemi-Esfarjani and Benzer, 2000) (human TRP2 homologue) and *UAS-P35* (Warrick et al, 1998) (viral antiapoptotic). *Mhc-Gal4* also drives expression in muscles of *Hsp70* from the *P-UAS*-element insertion in *Hsp70Ab*^{EY01148} (Bellen et al, 2004). Expression in muscles of these genes or transgenes with *Mhc-Gal4* in the absence of *UAS-PABPN1-17ala* did not induce an abnormal wing position phenotype, except for the *UAS-dtrp2* transgene, which induces a phenotype in 100% of flies (n=200). This transgene was therefore not tested as a suppressor of the phenotype produced by PABPN1-17ala expression in muscles. *Hsp83*^{e6A} is a point mutant, homozygous lethal (Cutforth and Rubin, 1994; Yue et al, 1999). Hsp83 is the *Drosophila* molecular chaperone Hsp90; it negatively regulates heat-shock transcription factor, leading to repression of *Hsp70* expression (Zou et al, 1998).

Figure 5 The PABPN1 RNA-binding domain is required for muscle defects. (A) Schematic representation of the truncated and mutant versions of PABPN1-17ala. A, alanine tract; CLD, coiled-coil domain; RRM, RNP-type RNA-binding domain; R rich C-term, arginine-rich C-terminal domain. (B) Expression of the PABPN1-17ala-truncated forms, analyzed by Western blots, and quantitative analysis of the abnormal wing position phenotype. Protein extracts were from 0.25 thoraxes of *UAS-PABPN1-17ala/+; Mhc-Gal4/+* (PABPN1-17ala), *UAS-PABPN1-17ala-ΔCLD/+; Mhc-Gal4/+* (PABPN1-17ala-ΔCLD), *UAS-PABPN1-ΔC-term/+; Mhc-Gal4/+* (PABPN1-17ala-ΔC-term) and *UAS-PABPN1-17ala-ΔRRM/+; Mhc-Gal4/+* (PABPN1-17ala-ΔRRM) adult males at day 1. α-Tubulin was used as a loading control. Note that PABPN1-17ala-ΔRRM protein is substantially shorter and might be less reactive to the antibody than other PABPN1-17ala versions. Thus, expression level of *UAS-PABPN1-17ala-ΔRRM* transgene could not be assayed by Western blots and was analyzed by RT-PCR (Supplementary Figure 3). The percentages of adults with abnormal wing position were calculated based on 200 flies from two independent crosses at 18°C. *Indicates that 0% of flies shows an abnormal wing position phenotype. Expression of PABPN1-17ala-ΔRRM from four copies of the transgene in the presence of two copies of *Mhc-Gal4* did not produce any abnormal wing position phenotype either. (C) Expression of PABPN1-17ala bearing a double point mutation (PABPN1-17ala-dm) in *UAS-PABPN1-17ala-dm/+; Mhc-Gal4/+* individuals, analyzed by Western blots, and quantitative analysis of the abnormal wing position phenotype. Legend is as in (B). Results of two independent transgenic lines (1 and 2) are shown. (D) IFMs visualized under polarized light in (from left to right) *UAS-PABPN1-17ala-ΔCLD/+; Mhc-Gal4/+*, *UAS-PABPN1-17ala-ΔC-term/+; Mhc-Gal4/+*, *UAS-PABPN1-17ala-ΔRRM/+; Mhc-Gal4/+*, *UAS-PABPN1-17ala-dm/+; Mhc-Gal4/+* individuals raised at 18°C, at day 16. Defects were visible after expression of PABPN1-17ala-ΔCLD or PABPN1-17ala-ΔC-term, whereas muscles appeared normal after expression of PABPN1-17ala-ΔRRM or PABPN1-17ala-dm. White arrows indicate regions where muscle fibers are affected. (E) Immunostaining of adult IFMs with anti-PABP2 for the wild-type control (left panels), or anti-PABPN1. DNA was revealed with DAPI. Expression of PABPN1-17ala or PABPN1-17ala-ΔCLD led to the formation of nuclear inclusions. In contrast, expression of PABPN1-17ala-dm did not induce the formation of nuclear inclusions, nor did it affect nuclear structure. The distribution of PABPN1-17ala-dm in nuclei is similar to that of PABP2 in wild-type nuclei.

but rather depends on properties of PABPN1. Consistent with this, a 27-residue alanine tract within a truncated form of the human MJD protein is nontoxic when expressed in *Drosophila* mesoderm or neurons (S Gaumer, N Bonini, personal communication). In addition, the fact that OPMD results from very short expansion of the polyalanine tract, starting from an expansion of two alanines, also indicates that toxicity of the polyalanine tract alone is unlikely. We propose that extending the alanine tract may stabilize PABPN1, gradually increasing its concentration and thereby causing toxicity. We showed, using a double point mutation in the RRM, known to cause defective poly(A) binding (Kuhn *et al*, 2003), that the toxicity of PABPN1 is linked to its ability to bind RNA poly(A). Thus, increased PABPN1 concentration would affect some aspect of mRNA metabolism. Data on OPMD patient biopsies also suggest that mRNA metabolism might be affected in OPMD as poly(A)- and RNA-binding proteins are found in PABPN1 nuclear inclusions (Calado *et al*, 2000b; Fan *et al*, 2003; Corbeil-Girard *et al*, 2005). More recent studies on polyglutamine diseases led to the new concept that although the polyglutamine expansion is essential for the pathogenesis, the host protein context is also important as modifications outside the polyglutamine tract can be determinant for the toxicity. The conclusion from this set of data was that toxicity would involve an altered function of the host protein caused by the polyglutamine tract, rather than properties of the polyglutamine tract alone (La Spada and Taylor, 2003; Gatchel and Zoghbi, 2005 and references therein).

An attractive possibility for OPMD would be that the potential stabilization of alanine-expanded PABPN1 would lead eventually to increased cytoplasmic amounts of PABPN1, as it is visible in *Drosophila* affected muscles (Figure 3A). Higher levels of cytoplasmic PABPN1 might disrupt essential cytoplasmic events on mRNAs. In particular, binding of mRNA poly(A) tails by cytoplasmic poly(A)-binding protein (PABP) is an important step for the activation of translation initiation (Wakiyama *et al*, 2000; Kahvejian *et al*, 2005). We have shown that the homolog of PABPN1 in *Drosophila* binds poly(A) tails together with PABP in the cytoplasm, during early development (Benoit *et al*, 2005). Therefore, increased amounts of cytoplasmic PABPN1 could affect translation through its binding to poly(A). Consistent with a possible toxicity of cytoplasmic PABPN1, rimmed vacuoles in OPMD have been proposed earlier to represent a degradation pathway of toxic cytoplasmic PABPN1 (Tome and Fardeau, 1980; Tome *et al*, 1997).

We also show that muscle defects and the presence of nuclear inclusions can be uncoupled. Expression of PABPN1- Δ ala in *Drosophila* muscles, in contrast to that of PABPN1-17ala, does not induce the formation of dense nuclear inclusions, although it induces wing position phenotypes and muscle defects. When quantified, the phenotypes of wing position caused by expression of PABPN1- Δ ala were weaker than that caused by expression of PABPN1-17ala. These data corroborate the earlier proposed model in which alanine-tract expansion in PABPN1 contributes to the formation of nuclear inclusions in OPMD patient muscles (Brais, 2003). However, strikingly, these data also suggest that nuclear inclusions are not always the cause of the disease and that PABPN1-induced muscle degeneration could result from at least two different pathways. For Huntington's

disease, competing models have described intracellular inclusions as pathogenic or incidental, and more recent studies have revealed a protective role of the inclusions, the formation of which decreases the amounts of mutant huntingtin outside the inclusions (Arrasate *et al*, 2004). Two sets of data are consistent with the proposition that OPMD does not result primarily from the formation of PABPN1 nuclear inclusions. First, PABPN1 filamentous nuclear inclusions, which contain ubiquitin and proteasomes, are present in wild-type rat hypothalamus neurons (Berciano *et al*, 2004). This shows that PABPN1 nuclear inclusions can exist under physiological conditions. Second, in a cell model of OPMD, nuclear inclusions were found to be dynamic structures in and out of which alanine-expanded PABPN1 move rapidly (Tavanez *et al*, 2005). This is very different from the view that all PABPN1 inclusions are static structures, which contribute to pathogenesis by irreversibly trapping other nuclear components. These results could be reconciled in a model where during early steps of the disease, muscle degeneration could be caused by increasing amounts of PABPN1, through its function in RNA binding, and alanine-expanded PABPN1 would progressively accumulate as nuclear inclusions which might not be detrimental when they first appear. After these inclusions have reached a significant portion of nuclei, nuclear function would be affected, leading to cell death through a different pathway.

We find that overexpression of HSP70 and HDJ1 alleviates OPMD-like phenotypes in the *Drosophila* model. This highlights a protective role *in vivo* for molecular chaperones in OPMD. In addition, expression of the antiapoptotic protein P35 also substantially suppresses alanine-expanded PABPN1-induced phenotypes, consistent with a role of apoptosis at some step of OPMD pathogenesis (Hino *et al*, 2004; Davies *et al*, 2005).

Our *Drosophila* model should prove to be extremely useful as an *in vivo* test for identifying suppressor genes or molecules for OPMD therapy, which could also be relevant to polyglutamine diseases.

Materials and methods

Fly stocks

Gal4 drivers were *Mhc-Gal4* (Schuster *et al*, 1996), *24B-Gal4* (Brand and Perrimon, 1993) and *daughterless-Gal4* (*da-Gal4*), which induce expression in muscles, in the mesoderm, and ubiquitously, respectively. *UAS* transgenes were *UAS-HSPA1L* (Warrick *et al*, 1999) (human *HSP70*), *UAS-dhdJ1* and *UAS-dtrpr2* (Kazemi-Esfarjani and Benzer, 2000), *UAS-Hsc70-4* (Elefant and Palter, 1999) and *UAS-P35* (Warrick *et al*, 1998). Other *Drosophila* stocks were *Hsp70Ab^{EY01148}* (Bellen *et al*, 2004) and *Hsp83^{66A}* (Cutforth and Rubin, 1994).

DNA constructs

PABPN1, *PABPN1-17ala* and *PABPN1- Δ ala* cDNAs cloned into the pGM10 vector were provided by Pr E Wahle (Smith *et al*, 1999; Scheuermann *et al*, 2003). The cDNAs were digested from the vector with *NdeI*, filled with Klenow and digested with *BamHI*. The resulting fragments were cloned into pBS-SK+ (Stratagene) digested with *EcoRV* and *BamHI*. These constructs were then digested with *EcoRI* and *XbaI* and the cDNAs were cloned into the transformation vector pUAST (Brand and Perrimon, 1993) digested with *EcoRI* and *XbaI* to produce the *UAS* transgenes. Truncated and mutant versions of PABPN1-17ala were constructed using the *PABPN1-17ala* cDNA cloned into pBS-SK+ (pBS-PABPN1-17ala). The double point mutation in the RRM was generated by replacing a *PpuMI*-*BamHI* wild-type fragment within pBS-PABPN1-17ala by

the corresponding fragment containing the two point mutations (Y175A, F215A), isolated from pEGFP-dmPABPN1 (Calado *et al*, 2000a) digested with *Ppu*MI and *Bam*HI. For the coiled-coil domain deletion (residues 116–147 in PABPN1), pBS-PABPN1-17ala was digested with *Ppu*MI, filled with Klenow and digested with *Bam*HI. The resulting plasmid was ligated to a PCR fragment amplified with primers 5'-AATATGAGTCCACCTCCGGGC and T3 from pBS-PABPN1-17ala and digested with *Bam*HI. For the RRM deletion (residues 165–272 in PABPN1), pBS-PABPN1-17ala was digested with *Bcl*I, filled with Klenow and digested with *Bam*HI. The resulting plasmid was ligated to a PCR fragment amplified with primers 5'-TACAACAGTTCCTCGTCTCG and T3 from pBS-PABPN1-17ala and digested with *Bam*HI. For the C-terminal domain deletion (residues 256–272 in PABPN1), a plasmid containing PCR fragment was generated from pBS-PABPN1-17ala using primers 5'-GCTGATCGCTGCTGTGGT and 5'-TACAACAGTTCCTCGTCTCG and religated. The truncated and mutant cDNAs were then digested with *Eco*RI and *Xba*I and cloned into the pUAST vector digested with *Eco*RI and *Xba*I. The complete sequence of all the constructed cDNAs was verified. *P*-element transformation was performed with the *w*¹¹¹⁸ stock using standard methods. Several independent lines were analyzed for each construct. For *UAS-PABPN1-17ala-ΔRRM*, eight independent lines gave identical results.

Analysis of wing position, adult musculature and electron microscopy

Abnormal wing position was determined by collecting adult males at birth, pooling five males per vial and scoring abnormal wing position at different days, by direct observation of the flies through the vial, without anesthesia. Visualization of thorax muscles under polarized light was carried out as follows: adults were immobilized on a slide with a drop of water, frozen in liquid nitrogen and cut sagittally with a razor blade. They were then dehydrated in ethanol/H₂O baths containing 50, 70 and 100% ethanol. Heads, wings and abdomens were removed and the thoraxes were cleared in methyl salicylate (Sigma) overnight at room temperature and mounted in Gary's Magic Mountant (2 g of Canada balsam (Fluka) in 1 ml methyl salicylate), air dried and observed under a light microscope. For electron microscopy, thoraxes were cut in two halves as above and fixed in 2.5% glutaraldehyde in NaH₂PO₄ 0.1 M pH 7.4 (phosphate buffer), overnight at 4°C. They were then washed in phosphate buffer and postfixed in 2% osmic acid in phosphate buffer for 1 h at room temperature. After three washes of 15 min in phosphate buffer, the thoraxes were dehydrated by two 10 min incubations in each of a series of ethanol/H₂O baths containing 30, 50, 70, 80, 90 and 100% ethanol, followed by three 15 min incubations in 100% ethanol. The thoraxes were embedded in Spurr. Ultrathin sections (85 nm) were collected at various levels of

each block and stained with 1.5% uranyl acetate, 70% ethanol and lead nitrate/Na citrate. Observations were made with a Hitachi 7100 transmission electron microscope.

Immunohistochemistry and Western blots

For immunostaining of adult IFMs, thoraxes of etherized adults were opened dorsally with a tungsten needle, cut into halves with a surgical scissor and transferred to 1 × PBS. Thoraxes were fixed for 15 min in 4% paraformaldehyde, 1 × PBS and washed in 1 × PBS. IFMs were dissected using a tungsten hook, incubated four times for 15 min in 1 × PBS, 0.3% Triton X-100, and blocked in 1 × PBS, 0.3% Triton X-100, 1% BSA for 1 h at room temperature. Incubations with primary antibodies were performed overnight at 4°C with 1:1000 rabbit anti-PABPN1 (Krause *et al*, 1994), 1:1000 rabbit anti-*Drosophila* PABP2 (Benoit *et al*, 1999), 1:2000 monoclonal anticonjugated Ubiquitin (FK2, Affiniti Research, UK) or 1:100 monoclonal anti-HSP70 (MA3-007, Affinity BioReagents, USA). IFMs were then washed four times for 15 min with 1 × PBS, 0.3% Triton X-100 and incubated with fluorescent secondary antibodies for 1 h at room temperature. After four washes of 15 min in 1 × PBS, 0.3% Triton X-100, DNA was labeled with 2 μg/ml DAPI in 1 × PBS, 0.3% Triton X-100 for 15 min at room temperature. IFMs were rinsed three times and washed for 1 h in 1 × PBS, 0.3% Triton X-100, and then mounted in Vectashield. For immunostaining of larval muscles, third instar larvae were opened dorsally, dissected and fixed opened with needles. These larvae were then treated as for adult IFM immunostaining. KCl treatment was with 1 M KCl in 1 × PBS for 30 min at room temperature, before fixation. Western blots were performed as described (Benoit *et al*, 1999).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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