Prevention of oculopharyngeal muscular dystrophy by muscular expression of Llama single-chain intrabodies \textit{in vivo}

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Oculopharyngeal muscular dystrophy (OPMD) is a late onset disorder characterized by progressive weakening of specific muscles. It is caused by short expansions of the N-terminal polyalanine tract in the poly(A) binding protein nuclear 1 (PABPN1), and it belongs to the group of protein aggregation diseases, such as Huntington’s, Parkinson’s and Alzheimer diseases. Mutant PABPN1 forms nuclear aggregates in diseased muscles in both patients and animal models. Intrabodies are antibodies that are modified to be expressed intracellularly and target specific antigens in subcellular locations. They are commonly generated by artificially linking the variable domains of antibody heavy and light chains. However, natural single-chain antibodies are produced in Camelids and, when engineered, combined the advantages of being single-chain, small sized and very stable. Here, we determine the \textit{in vivo} efficiency of Llama intrabodies against PABPN1, using the established \textit{Drosophila} model of OPMD. Among six anti-PABPN1 intrabodies expressed in muscle nuclei, we identify one as a strong suppressor of OPMD muscle degeneration in \textit{Drosophila}, leading to nearly complete rescue. Expression of this intrabody affects PABPN1 aggregation and restores muscle gene expression. This approach promotes the identification of intrabodies with high therapeutic value and highlights the potential of natural single-chain intrabodies in treating protein aggregation diseases.

INTRODUCTION

Intrabodies have high therapeutic potential and have been designed for a variety of diseases including protein aggregation diseases such as Huntington’s, Parkinson’s and Alzheimer diseases (1). Intrabodies (or intracellular antibodies) are antibodies that have been designed to be expressed intracellularly. The most common approach to produce an intrabody is to generate a single-chain antibody (scFv) by joining the antigen-binding variable domains of heavy and light chain with an interchain linker. However, cytoplasmic expression of such intrabodies can result in low solubility and stability, and therefore poor efficiency, leading to the requirement of an improvement step in the generation of an efficient intrabody (2,3). Intracellular expression of scFv intrabodies was found to be beneficial in cell or animal models of cancer, human immunodeficiency virus infection and neuropathology, highlighting the interest of intrabodies as therapeutic tools (1). A single-chain scFv intrabody against huntingtin was shown to reduce protein aggregation in a cell model of Huntington’s disease (4). This intrabody is also active in increasing survival to adulthood and adult lifespan in a \textit{Drosophila} model of the disease, although the rescue is not complete (5,6).

In addition to conventional antibodies, the \textit{Camelidae} possess a repertoire of antibodies that is devoid of light chains, the heavy-chain antibodies. These antibodies are thus naturally single-chain. Their variable domain ($V_{HH}$) is small sized and retains the capacity to bind antigens with high affinity, when isolated from the rest of the...
RESULTS

Identification of intrabodies with a beneficial effect for OPMD in vivo

Six intrabodies against PABPN1 have previously been selected (28). Of these, the 3F5 intrabody was mapped precisely in the coiled-coil domain of PABPN1, two others (3A9 and 3E9) were mapped in the 155 N-terminal-most residues, and the last three (#08, #18 and #29) were selected by epitope masking using 3F5 (Fig. 1A) (patent WO 2007/035092 A2). Expression of Llama anti-PABPN1 antibodies in Drosophila muscle nuclei was achieved by cloning their coding sequence downstream of UAS and in frame with a nuclear localization signal (NLS) (Fig. 1B). Expression induced with the muscle specific driver Mhc-Gal4 (29) led to the production in Drosophila adult thoracic muscles of small proteins of 18–20 kDa, targeted to nuclei (Fig. 1C and D). Expression of alanine expanded PABPN1 (PABPN1-17ala) in Drosophila muscles with Mhc-Gal4 results in flies suffering from muscular dystrophy that causes inability of a normal wing posture (20). Such phenotypes are progressive and correlate with muscle degeneration. The capacity of the six PABPN1 intrabodies to reduce OPMD phenotypes in vivo was assayed using the coexpression of PABPN1-17ala with each of the intrabodies in muscles with Mhc-Gal4. In the absence of intrabody, expression of PABPN1-17ala in muscles led to 87% of adults showing an abnormal wing position (at 18°C, day 6) (Fig. 1E). Several transgenic lines expressing the same intrabody were analyzed. For a specific intrabody, the ability of the different lines to decrease OPMD phenotypes fell into the same range (e.g. 60% of flies with abnormal wing posture for the more efficient line expressing intrabody #29, versus 68% for the less efficient line, at day 6, Fig. 1E). On the basis of these results, we classified the six intrabodies from their suppressor activity. The capacity to reduce OPMD phenotypes was different for each of them. Three intrabodies, #08, #18 and #29, had a very weak or null effect on suppressing abnormal wing position (Fig. 1E). Intrabodies 3E9 and 3A9 showed an intermediate effect with the former reducing the percentage of individuals with abnormal wing position down to 31%. Expression of intrabody 3F5 decreased the percentage of flies showing wing-position defects to as little as 3%, thus proving to have a strong suppressor effect (Fig. 1E). The difference in suppressor activities of the six intrabodies could result either from their intrinsic efficiency or from their expression level and stability in muscles. We, therefore, quantified the amounts of the different intrabodies by western blots (Fig. 1C). The suppression efficiency of the six intrabodies did not correlate with their amounts in thoracic muscles (Fig. 1C and E), demonstrating a specific intrinsic efficiency for each intrabody.

The molecular basis to explain the difference in the suppression efficiencies of the six intrabodies remains unknown. However, a possible explanation for the high suppressor activity of the 3F5 intrabody could lie in that, among the six intrabodies, it has the highest affinity for PABPN1 (28).

As intrabody 3F5 showed the strongest suppressor effect, we analyzed its expression level in three independent transgenic lines. The ability to reduce the percentage of individuals with affected wing position depended on this level of...
expression, indicating that the suppressor effect of this specific intrabody is dose-dependent (Fig. 2A and B).

We verified that the suppressor effect of the 3F5 intrabody resulted from the specific binding to its epitope within PABPN1. We took advantage of our previous analysis of domains involved in OPMD phenotypes (20), in which we showed that the coiled-coil domain in PABPN1 (residues 116–147) is not required; PABPN1-17ala deleted for this domain (PABPN1-17ala-DCLD) still induced wing-position defects, as well as muscle degeneration and formation of nuclear inclusions. PABPN1-17ala-DCLD lacks the epitope for 3F5 (Fig. 2C) and we used the 3F5 antibody in western blots to confirm it recognizes PABPN1-17ala, but not PABPN1-17ala-DCLD (Fig. 2C). Accordingly, coexpression of the 3F5 intrabody with PABPN1-17ala-DCLD in muscles did not lead to a decrease in the number of adults with wing-position defects (Fig. 2D). This demonstrates that the suppressor effect of the 3F5 intrabody results from interaction with its epitope in PABPN1.

We next addressed whether the 3F5 intrabody acted in affecting the accumulation of PABPN1-17ala, using western blots to quantify the protein amounts, with or without 3F5 coexpression in muscles (Fig. 2E). The accumulation of PABPN1-17ala was slightly decreased, when the 3F5 intrabody was coexpressed using the transgenic line UAS-3F5 (1) that shows the highest suppressor activity. However, this decrease was not observed with the line UAS-3F5 (2), although this line has a substantial suppressor activity (Fig. 2E). This suggests that PABPN1 destabilization is not the major mechanism of action of the 3F5 or, at least, that additional
mechanisms are involved in its suppressor activity. Consistent with this, prevention of aggregate formation by the 3F5 intrabody in the cell model of OPMD does not correlate with reduced accumulation of PABPN1-17ala (28).

Together, these results identify the 3F5 intrabody as a strong suppressor of OPMD phenotypes in the Drosophila model; its effect is dose-dependent and is achieved through its interaction with an epitope in the coiled-coil domain of PABPN1.

Figure 2. Suppression of OPMD phenotypes with the 3F5 intrabody requires direct binding of the intrabody to PABPN1-17ala. (A) Quantitative analysis of the suppressor effect of the 3F5 intrabody for three independent transgenic stocks, at day 6 and 11, at 18°C. Percentages of individuals with wing-position defects were calculated based on 150 to 250 individuals from two independent crosses. The genotypes are indicated. (B) Western blots showing the expression level of the 3F5 intrabody in UAS-3F5+/Mhc-Gal4+/ adult thoraces, for the three transgenic stocks shown in (A). Protein extracts were from 0.25 thoraces. The 3F5 intrabody was detected using anti-myc. Anti-α-tubulin was used as a loading control. The strength of the effect correlates with the amount of intrabody. The line UAS-3F5(1) was then used throughout. We checked that the expression of UAS-3F5(1) alone with Mhc-Gal4/ does not induce wing-position defects. (C) Top: schematic representation of PABPN1-17ala and PABPN1-17ala-ΔCLD showing that the 3F5 epitope (VREMEE) is lacking in PABPN1-17ala-ΔCLD. Bottom: western blots of adult thoraces showing that PABPN1-17ala-ΔCLD is not recognized by the 3F5 antibody. Protein extracts were from 0.5 thoraces of the indicated genotypes. The blot was first revealed with the purified 3F5-VSV/His6 antibody. 3F5-VSV/His6 was detected using anti-VSV. The blot was then dehybridized and revealed with anti-PABPN1. Anti-α-tubulin was used as a loading control. (D) Quantitative analysis showing that expression of the 3F5 intrabody does not prevent wing-position defects resulting from the expression of PABPN1-17ala-ΔCLD. The genotypes are indicated. The percentages were calculated from 150 individuals scored at day 6, at 18°C. (E) Western blot of adult thoraces showing the amounts of PABPN1-17ala with or without coexpression of the 3F5 intrabody. Protein extracts were from 0.25 thoraces of the indicated genotypes. The blot was revealed with anti-PABPN1. Anti-α-tubulin was used as a loading control.
Suppressor activity of the 3F5 intrabody requires its nuclear localization

Although PABPN1 has a nuclear role in mRNA polyadenylation (13, 14), we have more recently, however, described a cytoplasmic function of the PABPN1 homologue in Drosophila during early development (15). The proposal that rimmed vacuoles in OPMD patients represent a degradation pathway of toxic cytoplasmic PABPN1 (30, 31) led us to suggest that an increased amount of cytoplasmic PABPN1 could contribute to OPMD (20). To further test this hypothesis, we expressed the 3F5 intrabody with Mhc-Gal4, in the absence of an NLS (3F5-DNLS), and analyzed the suppressor capacity of this new transgene. When coexpressed with PABPN1-17ala, 3F5-DNLS showed a weak to intermediate suppressor effect (Fig. 3A), even if its expression level was higher than that of the 3F5 intrabody containing an NLS (Fig. 3B). The transgenic line that had the highest suppression capacity showed some nuclear accumulation of the intrabody in addition to cytoplasmic expression (Fig. 3C), suggesting that the suppressor activity of the 3F5 depended on its nuclear localization. These results indicate that the beneficial effect of the 3F5 intrabody is increased by its nuclear targeting.

Expression of the 3F5 intrabody prevents muscle degeneration and reduces PABPN1-17ala nuclear aggregation

Wing-position defects in the Drosophila model of OPMD correlate with progressive muscle degeneration, as visualized by polarized light or electron microscopy on indirect flight muscles (dorso-longitudinal muscles: DLM or dorso-ventral muscles: DVM) in adult thoraxes (20). Expression of PABPN1-17ala with Mhc-Gal4 led to defects in all indirect flight muscles (DLM and DVM), which appeared thin or were lacking at day 16 (Fig. 4C and E). Coexpression of the 3F5 intrabody with PABPN1-17ala prevented muscle degeneration (Fig. 4D and E). Following expression of PABPN1-17ala, myofibril sarcomeric structure visualized by electron microscopy was strongly disorganized, with broken Z-bands and the absence of M-bands (Fig. 4F). Coexpression of the 3F5 intrabody restored a normal ultrastructural appearance of muscle fibers (Fig. 4G).

Muscular expression of PABPN1-17ala in Drosophila induces the formation of dense PABPN1 nuclear inclusions, composed of filaments, similar to those in OPMD patients (20). Nuclei containing an inclusion were quantified in thoracic muscles expressing PABPN1-17ala and found to represent 10 and 16% of all nuclei at day 6 and 11, respectively (Fig. 5A). Coexpression of 3F5 was not sufficient to reduce the number of nuclei containing inclusions (Fig. 5A). However, strikingly, coexpression of the intrabody had two effects on nuclear inclusions: (i) their size was significantly reduced by a factor of up to 2.5 (Fig. 5B, D and E) and (ii) nuclear inclusions appeared as dispersed small inclusions for 32% of nuclear inclusions (n = 68) (Fig. 5B). When 3F5 was coexpressed, the reduction in size of PABPN1 nuclear inclusions was maintained during the lifespan and was even more marked at day 11 (Fig. 5D). We verified that the 3F5 intrabody was present in the nuclear inclusion (Fig. 5C).

Expression of the 3F5 intrabody restores muscle gene expression

We next analyzed the suppressor activity of the 3F5 intrabody at the level of gene expression using transcriptome analysis.
Using microarrays, thorax gene expression was compared between control flies (Mhc-Gal4+/+), flies expressing PABPN1-17ala, and flies coexpressing PABPN1-17ala and 3F5, at three time points (day 2, 6 and 11). Two-way ANOVA with genotype and time as the first and second variables, respectively, followed by K-means clustering, revealed 795 deregulated genes after expression of PABPN1-17ala (including all time points). Among these genes, 464 (58.4%) showed a partial or total expression rescue when 3F5 was coexpressed with PABPN1-17ala (Fig. 6, Supplementary Material, Tables S1 and 2). The rescue was initiated at day 2 and maintained at a substantial level during the lifespan (Fig. 6A and C, Supplementary Material, Fig. S1). For a large proportion of genes, expression was restored at two

**Figure 4.** Expression of the 3F5 intrabody prevents OPMD muscle degeneration in Drosophila. (A–D) Indirect flight muscles (IFMs) visualized with polarized light. Left panel: dorso-longitudinal muscles (DLMs); right panel: dorso-ventral muscles (DVMs). (A and B) Controls showing normal muscle morphology. (A) Mhc-Gal4/+, (B) UAS-3F5/++; Mhc-Gal4/+. (C) IFMs from UAS-PABPN1-17ala/++; Mhc-Gal4/+ individuals are strongly affected. Arrows indicate regions where muscles are very thin or lacking. (D) IFMs from UAS-PABPN1-17ala/+, UAS-3F5/++; Mhc-Gal4/+ and in UAS-PABPN1-17ala/+, UAS-3F5/++; Mhc-Gal4/+ individuals at day 16, at 18°C are shown. (E) Quantification of affected muscles in UAS-PABPN1-17ala/++; Mhc-Gal4/+ and in UAS-PABPN1-17ala/+, UAS-3F5/++; Mhc-Gal4/+ individuals at day 16, at 18°C. All 6 DLMs and all 7 DVMs were scored per hemi-thorax. The percentages were calculated based on 150 to 200 DLMs and on 150 to 200 DVMs. The genotypes are indicated. Nearly, all IFMs were affected following expression of PABPN1-17ala. In contrast, muscle defects when the 3F5 intrabody was coexpressed with PABPN1-17ala were reduced to background levels. (F and G) Ultrastructure of IFMs visualized by electron microscopy from UAS-PABPN1-17ala/++; Mhc-Gal4/+ (F) and UAS-PABPN1-17ala/+, UAS-3F5/++; Mhc-Gal4/+ (G) at day 11, at 18°C. Expression of PABPN1-17ala led to muscle degeneration characterized by the dissociation of the myosin–actin network in myofibrils and the lack of Z- and M-bands (F). Coexpression of the 3F5 intrabody prevented muscle degeneration as shown by the normal structure of myofibrils (G) (Z: Z-bands, M: M-bands).
consecutive time points, and expression of 100 genes from 464 (21.5%) was restored at all three time points (Fig. 6B). These results show that the expression of the 3F5 intrabody acts in reducing OPMD-induced gene deregulation. This effect in preventing gene deregulation is the strongest in early stages (day 2) and persists during the lifespan.

**DISCUSSION**

Collectively, these results provide strong support that intra-muscular expression of the 3F5 single-chain anti-PABPN1 antibody prevents muscle degeneration in vivo by restoring gene expression and altering the nuclear PABPN1-17ala...
aggregation. The intrabody acts through direct binding to PABPN1-17ala and its efficiency requires nuclear targeting.

We have identified a single-domain Llama antibody that acts as a strong suppressor of OPMD phenotypes in Drosophila. Demonstrating almost complete rescue, 3F5 is in fact the strongest suppressor of the wing-position defect identified to date. Expression and targeting of this antibody in muscle nuclei as an intrabody prevent muscle degeneration associated with PABPN1-17ala expression in Drosophila. We have used the Drosophila OPMD model to identify the intrabody that has the highest efficiency in vivo, from six anti-PABPN1 antibodies. As this model recapitulates the characteristics of OPMD, our results provide strong support to further evaluate the potential of this intrabody in OPMD therapeutic approaches.

A cellular hallmark of the OPMD condition is the presence of mutant-PABPN1 nuclear inclusions in a number of nuclei in muscles of patients (17,30). Nuclear inclusions in affected Drosophila muscles are similar to inclusions in patients both in protein composition (they contain PABPN1-17ala, HSP70, ubiquitin) and at the ultrastructural level (they are composed of unbranched filaments of 8–10 nm outer diameter) (20). Interestingly, 3F5 expression in muscle nuclei does not reduce the occurrence of nuclear inclusions but does reduce their size. In addition, in the presence of the 3F5 intrabody, nuclear inclusions appear as several small dispersed aggregates. In its normal function, PABPN1 accumulates in nuclear speckles, which are nuclear subdomains enriched in mRNA processing factors (32,33). The dispersed aggregates in the presence of the 3F5 could indicate that nuclear inclusions in OPMD would result from several independent aggregate seedings and subsequent assembly in a large inclusion per nucleus. The seedings could occur in nuclear speckles as these sites correspond to the sites of highest PABPN1 concentration.

Coexpression of the 3F5 intrabody with PABPN1-17ala reduces the size of nuclear inclusions and prevents muscle degeneration. This suggests that at least some step of PABPN1 aggregate formation could contribute to OPMD. Transcriptome analysis reveals gene deregulation at day 2 in OPMD flies, prior to formation of visible nuclear inclusions. Rescue of gene expression with the intrabody also starts at day 2. As it has been proposed for several protein aggregation diseases, including OPMD (34,35), this might suggest that early oligomeric forms of PABPN1 are active in the pathology and that the intrabody interferes with these oligomers to prevent their toxicity. Consistent with the idea that oligomeric intermediates may be more deleterious than mature insoluble nuclear inclusions per se, the phenotypic rescue of wing position is fully efficient at day 6, whereas the reduction in size of nuclear inclusions continues after this time.

The 3F5 intrabody is not specific to alanine-expanded PABPN1, as it recognizes an epitope present in the coiled-coil domain of the normal protein (28). Therefore, expression of this intrabody in human cells could potentially affect normal PABPN1 function. We find that the expression of the 3F5 intrabody alone does not induce muscle degeneration. 3F5 expression in mammalian cell models of OPMD does not affect cell viability either (28). Because muscle degeneration in Drosophila can be induced by overexpression of normal PABPN1 (albeit to a lesser extent than expression of
alanine-expanded PABPN1) (20), we think that a higher than normal accumulation of PABPN1 contributes to OPMD. Expression of 3F5 intrabody in diseased tissues could thus counterbalance detrimental amounts of PABPN1 without affecting PABPN1 normal function. Dosage control will be an important point to address in the utilization of the 3F5 intrabody in therapeutic strategies.

Intrabody engineering has emerged as an attractive therapeutic strategy for several diseases, including protein aggregation and neurodegenerative diseases (1,3,5,36,37). Up to now, the beneficial effect in such diseases had been assayed for a single intrabody using the Drosophila model. The single-chain antibody C4 scFv consists in the V\textsubscript{H} and V\textsubscript{L} variable domains of human antibodies bridged by a linker peptide, specific to the N-terminal-most region of huntingtin (4). When expressed as an intrabody in the Drosophila model of Huntington’s disease, C4 scFv decreases neurodegeneration in the compound eye and improves adult survival (5). However, suppression of neuropathology is not complete and this intrabody has been used in combination with other strategies to improve the rescue (6).

Here, we investigate the potential of Llama intrabodies for OPMD therapy. These antibodies are naturally single-chain. This essential feature allows to avoid the difficult step of linking the variable domains of heavy and light chains and facilitates engineering and screening of these antibodies to produce high affinity intrabodies. Moreover, these intrabodies are very stable when expressed intracellularly, making them produce high affinity intrabodies. Moreover, these intrabodies facilitate engineering and screening of these antibodies to linking the variable domains of heavy and light chains and decoding sequence of each single-chain antibody followed by the myc/His\textsubscript{6}-tag was polymerase chain reaction (PCR) amplified using the primers 5’CATGCTAGGCAGCCCGCCAGCC GCCCATGGCC and 5’CATGCTAGCAACAGTAAAGCTC TATGCGGC. This PCR fragment was digested with Nhel and cloned into the puAS-NLS vector digested with XbaI. The UAS-3F5-NLS transgene was constructed by amplifying a PCR fragment corresponding to the 3F5 coding sequence in frame with an ATG, using primers 5’CATGC TAGCCAAAATGGCGCCCACGCGCATGCCG and 5’ CATGCTAGCAACAGTAAAGCTC TATGCGGC. This fragment was digested with Nhel and cloned into the puAST vector digested with XbaI. The sequences of all tagged single-chain antibodies were verified. Transgenic stocks were generated by P-element transformation using the w\textsuperscript{1118} stock and standard methods.

**MATERIALS AND METHODS**

**Drosophila stocks**

The w\textsuperscript{1118} stock was used as a control. The Mhc-Gal4 driver induces expression in muscles (29). The UAS-PABPN1-17ala and UAS-PABPN1-17ala-ΔCLD transgenic stocks have been described earlier (20). Two to five independent transgenic stocks able to express each intrabody were analyzed.

**DNA constructs**

The cDNAs encoding each of the six single-chain antibodies were cloned into the pU8101 vector, in frame with the C-terminal myc- and His\textsubscript{6}-tag (7). The pUAS–intrabody constructs were produced by cloning an EcoRI–XbaI fragment encoding the nuclear localization signal of SV40 (NLS) into the pUAST vector (38), digested with EcoRI and XbaI. The EcoRI–XbaI NLS fragment was obtained by annealing and extending two partially complementary primers: 5’CATGAAATTTCA AATGACTGCTCCAAAGAAAGACGTAAGGC and 5’CATCTAGATTTTCTACGGGCGCCCTTACGCTT.

In order to generate UAS–intrabody constructs, the coding sequence of each single-chain antibody was inserted into a pUAST vector (38), digested with EcoRI and XbaI. The EcoRI–XbaI NLS fragment was obtained by annealing and extending two partially complementary primers: 5’CATGAAATTTCA AATGACTGCTCCAAAGAAAGACGTAAGGC and 5’CATCTAGATTTTCTACGGGCGCCCTTACGCTT.

**Immunostaining and western blots**

Immunostaining and western blots were performed as described previously (20,39). Antibody dilutions were as follows: rabbit anti-PABPN1 (33), 1:1000 for immunostaining, 1:2500 for western blots; monoclonal anti-myc (9E10, Santa Cruz), 1:1000 for immunostaining, 1:5000 for western blots; purified 3F5-VSV/His\textsubscript{6} produced in Escherichia coli (28), 2 µg/ml DAPI. Detection of 3F5-VSV/His\textsubscript{6} antibody was performed with monoclonal anti-VSV, 1:100 (Roche), prior to incubation with the secondary antibody. DNA was labeled with 1 µg/ml DAPI (Sigma-Aldrich). Western blots were performed as described (39). Anti-a-tubulin for western blots was used at dilution 1:5000 (T5168, Sigma-Aldrich).

**Analysis of wing position and adult musculature**

Wing-position defects were scored 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 (20). Visualization of thoracic muscles under polarized light or by electron microscopy was carried out as described (20).

**Microarray analysis**

Total RNA was isolated from 10 adult thoraxes per genotype, per time point, in triplicates, as shown in Supplementary Material, Table S3. RNA was extracted using Trizol (Invitrogen) as recommended by the manufacturer, followed by a RNA cleanup using the RNeasy mini kit (QIagen). Quality and quantity of the RNA were checked using the Bioanalyzer Lab-on-a-Chip. The INDAC oligo set (15K, produced by Illumina) was obtained from Dr O. Sibon (Department of Radiation and Stress Cell Biology, Groningen, The Netherlands) and was printed in duplicate on poly-l-lysine coated slides by Leiden Genome Technology Center. Per sample, 1 µg of total RNA was amplified with the Message Amp kit (Ambion) and the cRNA labeled through incorporation of aminoallyl-uridine-5’-triphosphate and coupling with Amersham monoreactive Cy3 or Cy5 dyes. For all samples,
1.5 μg of labeled cRNA was hybridized to the oligonucleotide arrays against a common reference and dye-swap experiments were performed (in total: six hybridizations per condition, per time point). The common reference is made of a pool of triplicate Mhc-Gal4/+ samples at day 2. Slides were scanned with an Agilent scanner (Model 2565BA) and spot intensities were quantified with the GenePix Pro 5.0 program (Axon). For data analysis, raw intensity files were imported into Rosetta Resolver v7.0 (RosettaBio, Seattle, WA, USA) and normalized with the Axon/Genepix error model. A two-way ANOVA was performed to identify genes that were differentially expressed between any of the three genotypes per time point. Genes were considered differentially expressed when the P-value was <7.1 × 10^{-7} (Bonferroni corrected). Genes with similar expression patterns were clustered using K-means clustering with cosine correlation as a similarity measure that was performed with the Functional Genomics application of the Spotfire Decision Site 7.3 (Spotfire AB, Göteborg, Sweden). The average log[10] ratio and standard errors for the genes within a cluster were calculated in Excel. Clusters with less than five genes were excluded from the analysis. To select gene clusters whose expression is deregulated following PABPN1-17ala expression, and restored or not with coexpression of 3F5, paired Student’s t-tests (Control/OPMD, Control/OPMD + 3F5, OPMD/OPMD + 3F5) were performed for each cluster and at each time point. A cluster was considered differentially expressed in two genotypes when P-value was <0.01. The lists of clusters and the total number of genes in each category are shown in Supplementary Material, Table S2 and Figure 6.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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