

# MBNL1 and CUGBP1 modify expanded CUG-induced toxicity in a *Drosophila* model of myotonic dystrophy type 1

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Myotonic dystrophy type 1 (DM1) is a neuromuscular disorder caused by a CTG expansion in the 3' UTR of the dystrophin myotonia protein kinase (*DMPK*) gene. It has been hypothesized that the pathogenesis in DM1 is triggered by a toxic gain of function of the expanded *DMPK* RNA. This expanded RNA is retained in nuclear foci where it sequesters and induces alterations in the levels of RNA-binding proteins (RNA-BP). To model DM1 and study the implication of RNA-BP in CUG-induced toxicity, we have generated a *Drosophila* DM1 model expressing a non-coding mRNA containing 480 interrupted CUG repeats; i.e. [(CUG)<sub>20</sub>CUCGA]<sub>24</sub>. This (iCUG)<sub>480</sub> transcript accumulates in nuclear foci and its expression leads to muscle wasting and degeneration in *Drosophila*. We also report that altering the levels of two RNA-BP known to be involved in DM1 pathogenesis, MBNL1 and CUGBP1, modify the (iCUG)<sub>480</sub> degenerative phenotypes. Expanded CUG-induced toxicity in *Drosophila* is suppressed when MBNL1 expression levels are increased, and enhanced when MBNL1 levels are reduced. In addition, (iCUG)<sub>480</sub> also causes a decrease in the levels of soluble MBNL1 that is sequestered in the CUG-containing nuclear foci. In contrast, increasing the levels of CUGBP1 worsens (iCUG)<sub>480</sub>-induced degeneration even though CUGBP1 distribution is not altered by the expression of the expanded triplet repeat. Our data supports a mechanism for DM1 pathogenesis in which decreased levels of MBNL and increased levels of CUGBP mediate the RNA-induced toxicity observed in DM1. Perhaps more importantly, they also provide proof of the principle that CUG-induced muscle toxicity can be suppressed.

## INTRODUCTION

Myotonic dystrophy type 1 (DM1) is the most common form of adult onset muscular dystrophy with an incidence of one in 8000 births. Unlike other neuromuscular diseases, DM1 is multisystemic and, in addition to myotonia and muscle degeneration, patients show the presence of cataracts, cardiac conduction defects, insulin resistance, sleeping disorders and testicular atrophy (1).

DM1 is caused by a triple repeat expansion in the 3' UTR of the dystrophin myotonia protein kinase (*DMPK*) gene (2–4).

While normal individuals have as many as 30 CTG repeats, DM1 patients carry a larger number of repeats ranging from 50 to thousands. The severity of the disease and the age of onset correlates with the number of repeats. Patients with adult onsets show milder symptoms and have less than 100 repeats, juvenile onset DM1 patients carry as many as 500 repeats and congenital cases usually have around a thousand CTG repeats (1).

According to one hypothesis, DM1 is a consequence of *DMPK* haploinsufficiency (5–7). Another hypothesis postulated that DM1 is a consequence of alterations in the levels

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of *DMPK* flanking genes (8,9). However, transgenic mice models generated to test these hypotheses failed to reproduce most of the symptoms of the disease (10–14).

Myotonic dystrophy type 2 (DM2), a disease with similar clinical manifestations, maps to a different locus (15) and is also caused by an untranslated repeat expansion (16). These findings pointed towards an RNA gain-of-function mechanism of pathogenesis for the expanded repeats.

Transgenic mice, expressing expanded CTG repeats in muscle tissue under the control of an actin promoter, display myotonia and some of the histopathological abnormalities of the DM1-affected muscle; i.e. central nuclei and variability in fibre size (17). These results demonstrated that the expanded repeats by themselves could account for at least some of the disease features independent of the *DMPK* locus.

The expanded transcripts containing CUG repeats form a secondary structure (18,19), accumulate in the nucleus in the form of nuclear foci (20) and sequester RNA-binding proteins (RNA-BP) (18,21,22). Several RNA-BP have been implicated in the disease including muscleblind-like (MBNL) proteins and CUG-binding protein (CUGBP) (21,23). MBNL proteins are homologous to *Drosophila* muscleblind (Mbl) proteins necessary for photoreceptor and muscle differentiation (24,25). MBNL and CUGBP have been identified as antagonistic splicing regulators of transcripts affected in DM1 such as cardiac troponin T (cTNT), insulin receptor (IR) and muscle-specific chloride channel (CIC-1) (26–30).

MBNL proteins co-localize with the expanded CUG-containing foci (21,22,31–35). A *muscleblind* knockout mouse model in which only the CUG-binding isoforms were eliminated shows myotonia, cataracts, aberrant splicing of *Cln 1* and abnormal muscle histology (26). These data point towards the depletion of muscleblind as an important mechanism in expanded CUG-induced DM1 pathogenesis.

Two different transgenic mouse lines have been generated that overexpress CUGBP1 either in skeletal muscle alone (36), or in both heart and skeletal muscles (27). These mice show abnormal muscle development and muscular dystrophy and, one of them (27) also shows alternative splicing misregulation of transcripts known to be affected in DM1.

Here, we describe a *Drosophila* model of DM1 that shows degenerative phenotypes in muscle and eye tissue as well as key histopathological features of the DM1. These features include accumulation of the expanded transcripts in nuclear foci and their co-localization with muscleblind protein. We used this model to further test the hypothesis that DM1 pathogenesis is caused by the depletion of muscleblind. We show that reduced levels of muscleblind aggravate and, more importantly, overexpression suppresses the muscle and eye phenotypes of DM1 flies.

## RESULTS

### Expanded CUG repeats cause eye and muscle degeneration and accumulate in nuclear foci in *Drosophila*

To test the RNA gain-of-function hypothesis, and to gain insight into the causative mechanisms behind the DM1

pathology, we generated flies expressing a non-coding mRNA containing 480 interrupted CUG repeats [(iCUG)<sub>480</sub>] made of the sequence: [(CUG)<sub>20</sub>CUCGA]<sub>24</sub>. The GAL4/UAS system (37) was used to control the spatial and temporal expression of the (iCUG)<sub>480</sub> mRNA.

Expression of (iCUG)<sub>480</sub> in the eye causes disorganization and fusion of the ommatidia as well as loss and duplication of inter-ommatidial bristles. Eyes expressing the (iCUG)<sub>480</sub> mRNA are also smaller than controls (Fig. 1A–D).

To investigate the effect of (iCUG)<sub>480</sub> in somatic muscles, we expressed (iCUG)<sub>480</sub> using a muscle-specific myosin heavy chain promoter. A hallmark of DM1 pathology is the accumulation of expanded CUG-containing RNA in nuclear foci. We monitored RNA accumulation using a Cy3 labelled anti-CUG probe. We observe the foci formation in the nuclei of third instar larvae muscles (Fig. 1F). No nuclear foci are detected in muscles from wild-type animals (Fig. 1E) or animals expressing (CUG)<sub>20</sub> (data not shown).

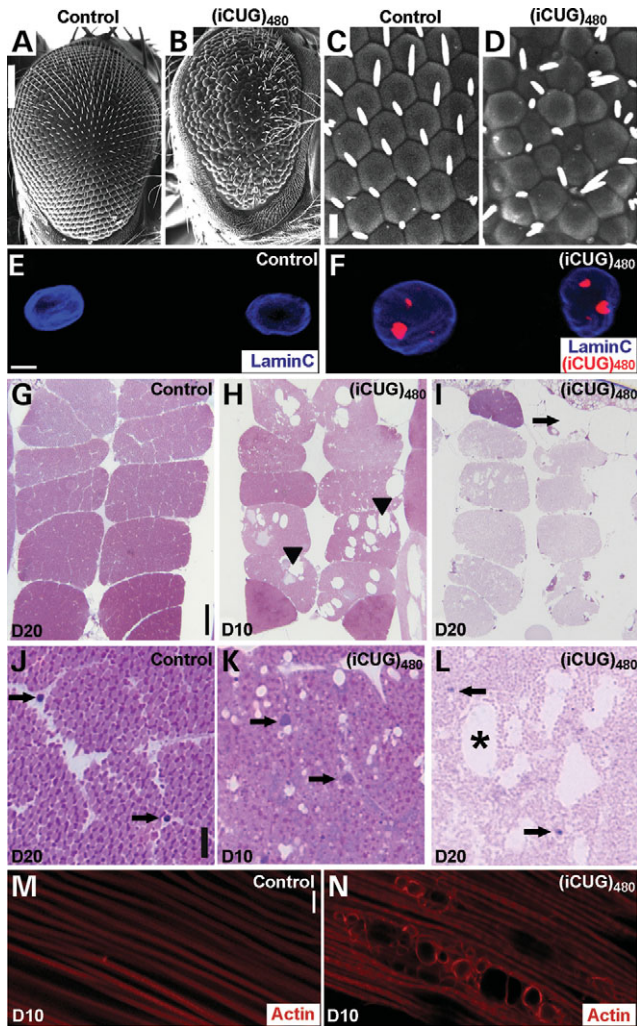
We also investigated whether the expression of (iCUG)<sub>480</sub> leads to toxicity in the *Drosophila* muscles. One-day-old adult animals are unable to fly but present no histological evidence of muscle degeneration (data not shown). Ten-day-old (iCUG)<sub>480</sub> flies show degeneration and vacuolization of the indirect flight muscles (IFM) (Fig. 1H), and loss of the structure of muscle fibres (compare Fig. 1K with J). In 20-day-old (iCUG)<sub>480</sub> flies, the wasting of the IFM is more severe showing a diffuse matrix, the structure of the fibres has been lost and the nuclei appear dispersed (Fig. 1I and L). In contrast, control flies that do not express CUG repeats (Fig. 1G), or expressing (CUG)<sub>20</sub> or (CUG)<sub>200</sub> (Supplementary Material, Fig. S1) show no muscle phenotype.

Therefore, expression of (iCUG)<sub>480</sub> causes IFM dysfunction and progressive muscle degeneration. This degenerative phenotype is also evident by staining dissected *Drosophila* adult IFM with phalloidin that labels actin in the muscle fibres (Fig. 1M and N).

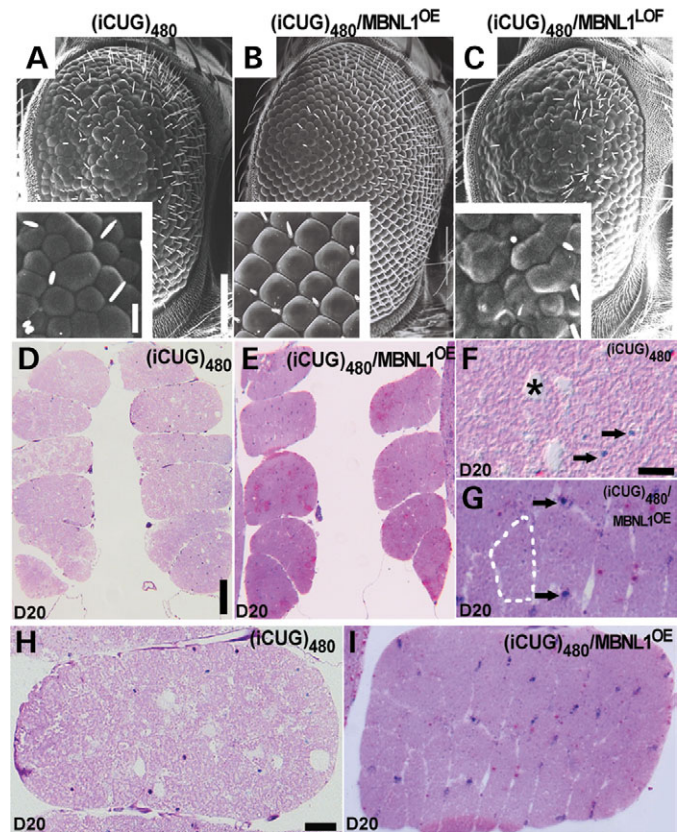
### MBNL1 overexpression suppresses (iCUG)<sub>480</sub>-induced muscle toxicity

Because MBNL proteins have been implicated in DM1 pathogenesis (21,26,31), we investigated whether altering the levels of muscleblind proteins modulates (iCUG)<sub>480</sub>-induced toxicity. Reduction of muscleblind levels by means of a heterozygous loss-of-function mutation in muscleblind (*mbl*) aggravates the (iCUG)<sub>480</sub> eye phenotype increasing ommatidial disorganization/fusion and eye size reduction (compare Fig. 2A and C). Eyes from control flies carrying the same *mbl* mutation but no (iCUG)<sub>480</sub> repeats are normal (Supplementary Material, Fig. S2). These results concur with the muscleblind knockout mouse (26) and support the validity of the *Drosophila* model.

To further test the hypothesis that the (iCUG)<sub>480</sub> phenotypes are caused by titration and loss of function of muscleblind protein, we investigated the consequences of increasing its levels. We generated flies to express a human muscleblind transgene (*MBNL1*) under control of the GAL4/UAS system.



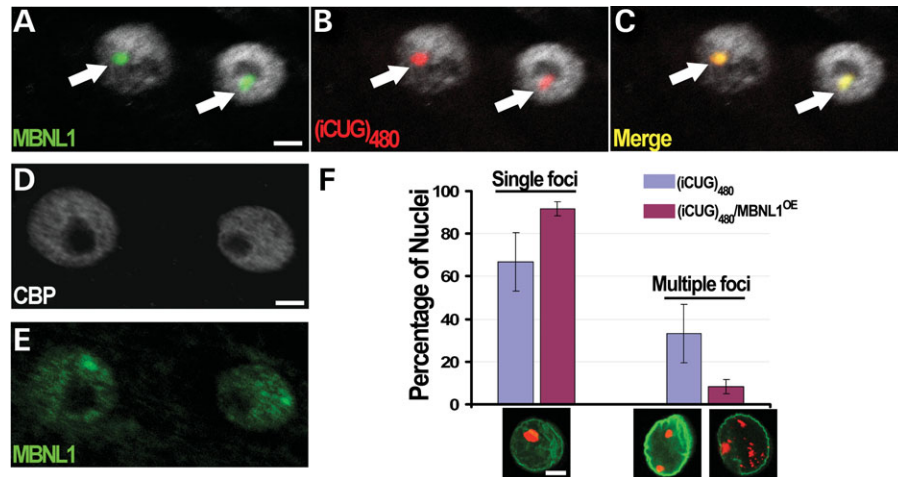
**Figure 1.** Expression of (iCUG)<sub>480</sub> in *Drosophila* causes eye and muscle degeneration and accumulation of expanded transcripts in nuclear foci. (A–D) Scanning electron-microscope (SEM) eye images from control flies or flies expressing (iCUG)<sub>480</sub>. Control eyes show the normal organization of ommatidia and inter-ommatidial bristles (A and C). Expression of (iCUG)<sub>480</sub> causes disorganization of the ommatidia and inter-ommatidial bristles, and reduction of the eye size (B and D). (E and F) *In situ* hybridization in larval muscles using a Cy3-labelled GAC PNA probe of control flies (E) or flies expressing (iCUG)<sub>480</sub> (F). Muscles were stained with anti-laminC (blue) to visualize the nuclei. (iCUG)<sub>480</sub> repeats accumulate in nuclear foci (red) in muscle cells of (iCUG)<sub>480</sub> larvae. (G–L) Transversal resin sections of adult IFM of control flies or flies expressing (iCUG)<sub>480</sub> at different ages. At day 20, control flies show normal organization of the IFM (G) and the muscle fibres are well structured. Note the nuclei located in the periphery of muscle fibres, arrows in (J). Ten days after eclosion, animals expressing (iCUG)<sub>480</sub> in the IFM show vacuolization (arrowheads) and muscle disorganization (H). Higher magnification shows loss of fibre organization (arrows point nuclei, K). At 20 days of age, muscle degeneration and wasting progresses, the density of myofibrils per muscle decreases and some muscles are absent (arrow, I). The loss of muscle fibre organization and vacuolization (asterisk) is enhanced (arrows point nuclei, L). (M and N) Phalloidin staining of IFM in the adult thorax of control flies or flies expressing the (iCUG)<sub>480</sub>. At 10 days of age, control flies show normal actin filaments (M). Flies expressing (iCUG)<sub>480</sub> at day 10 show vacuolization of the tissue (N). Scale bars: (A and B) 100  $\mu$ m, (C and D) 10  $\mu$ m, (E and F) 5  $\mu$ m, (G–I) 50  $\mu$ m, (J–L) 10  $\mu$ m. (M and N) 50  $\mu$ m. Genotypes are A, C: w; *gmr-GAL4/UAS-LacZ*; +; B, D: w; *gmr-GAL4/UAS-LacZ*; UAS-(iCUG)<sub>480</sub>[M5T]/+; E, G, J, M: w; UAS-LacZ/+; *Mhc-GAL4*/+; F, H, I, K, L, N: w; UAS-LacZ/+; *Mhc-GAL4/UAS-(iCUG)<sub>480</sub>[M5T]*.



**Figure 2.** Modification of (iCUG)<sub>480</sub>-induced phenotypes by muscleblind. (A–C) SEM eye images from control flies expressing (iCUG)<sub>480</sub>, (iCUG)<sub>480</sub> and MBNL1, or (iCUG)<sub>480</sub> and also carrying a heterozygous mutation in the *Drosophila muscleblind* gene. Flies expressing (iCUG)<sub>480</sub> and a control transgene show ommatidial disorganization and fusion, and eye size reduction (A). This phenotype is suppressed by MBNL1 overexpression (B). The (iCUG)<sub>480</sub> eye phenotype is enhanced in animals carrying a heterozygous loss-of-function mutation in *muscleblind* (C). This mutation by itself does not cause an abnormal eye phenotype (see Supplementary Material Fig. S2). (D–I) Transversal semi-thin sections of adult IFM (iCUG)<sub>480</sub> control flies, or (iCUG)<sub>480</sub> flies also expressing MBNL1. Control 20-day-old (iCUG)<sub>480</sub> flies show obvious IFM muscle pathology (D, F and H) that is suppressed by expression of an MBNL1 transgene (E, G and I). Note that the fibres are well organized and the density of myofibril per muscle fibre is higher in the MBNL1-rescued flies (dots in G outline a well-structured muscle fibre) as compared with (iCUG)<sub>480</sub> controls where there is vacuolization (asterisk), the structure of the fibres has been lost and the nuclei appear dispersed in the matrix (F). Arrows point to nuclei in F and G. Scale bars: (A–C) 100  $\mu$ m (insets 10  $\mu$ m), (D and E) 50  $\mu$ m, (F–I) 10  $\mu$ m. Genotypes are A: w; *gmr-GAL4/UAS-LacZ*; UAS-(iCUG)<sub>480</sub>[M5T]/+; B: w; *gmr-GAL4/UAS-MBNL1[M10M]*; UAS-(iCUG)<sub>480</sub>[M5T]/+; C: w; *gmr-GAL4/mbi<sup>P1514</sup>* (4); UAS-(iCUG)<sub>480</sub>[M5T]/+ (D–I): D, F, H: w; UAS-LacZ/+; *Mhc-GAL4/(iCUG)<sub>480</sub>[M5T]*/+; E, G, I: w; UAS-MBNL1[M10M]/+; *Mhc-GAL4/UAS-(iCUG)<sub>480</sub>[M5T]*.

As shown in Figure 2B, overexpression of muscleblind dramatically suppresses the (iCUG)<sub>480</sub> eye phenotypes (size and organization of the ommatidia).

Next we investigated whether MBNL1 is able to rescue the (iCUG)<sub>480</sub> somatic muscle phenotype. We examined the muscles of 20-day-old flies co-expressing (iCUG)<sub>480</sub> and human MBNL1. As shown in Figure 2D, F and H, the IFM from (iCUG)<sub>480</sub> flies show severe disorganization of the muscle fibres; they also have randomly arranged myofibrils and a diffuse matrix with vacuolization and dispersed



**Figure 3.** Co-localization of MBNL1 protein with the CUG-containing nuclear foci. (A–C) Images of nuclei from larval somatic muscle co-expressing MBNL1 and (iCUG)<sub>480</sub> after immunofluorescence and *in situ* hybridization. MBNL1 protein is detected in muscle nuclei when stained with an antibody against MBNL1 (A, white arrows, green). The (iCUG)<sub>480</sub> transcripts are also visualized in the form of nuclear foci after *in situ* hybridization (B, white arrows, red). Both MBNL1 and (iCUG)<sub>480</sub> RNA co-localize when the images are merged (C, white arrows, yellow). Muscles are also stained with anti-CBP antibody to visualize the nuclei (white). (D–E) Images of nuclei from larval somatic muscle expressing MBNL1 but no (iCUG)<sub>480</sub> after immunofluorescence. Anti-CBP antibody revealing nuclei outline (D, white), MBNL1 signal shown in green (E). (F) Comparative quantification of nuclei with single versus multiple foci in flies expressing (iCUG)<sub>480</sub> alone, or together with MBNL1. Images below the chart illustrate the types of nuclear foci found; no nucleus of any genotype was found without at least one foci. Notice that co-expression of MBNL1 together with (iCUG)<sub>480</sub> causes an overall decrease in the number of nuclei with multiple foci when compared with the repeats alone. Error bars represent standard deviation. Data was analyzed using Student's *t* ( $P = 0.006$  in both groups). Total muscle nuclei (~200 per larva) from four to five larvae per genotype were analyzed. Scale bar: (A–F) 5  $\mu$ m. Genotypes are A–C, F: w; UAS-MBNL1[M10M]/+; *Mhc-GAL4/UAS-(iCUG)<sub>480</sub>[M5T]*; D–E: w; UAS-MBNL1[M10M]/+; *Mhc-GAL4*/+.

nuclei. In contrast, the muscles from (iCUG)<sub>480</sub> flies over-expressing MBNL1 at appropriate levels show little or no vacuolization (Fig. 2E and I), have a well-defined myofibril matrix (Fig. 2G) and the IFM are well structured in muscle fibres with peripherally located nuclei (Fig. 2G and I). Note also that the rescued muscle in Figure 2E, G and I is more intensely stained than the control muscle in Figure 2D, F and H because of the higher density of the tissue. The muscle phenotype in (iCUG)<sub>480</sub> flies is 100% penetrant, and we also observed rescue in every (iCUG)<sub>480</sub> animal also over-expressing MBNL1. In addition, we quantified the rescuing ability of MBNL1 overexpression by counting the number of normal versus abnormal individual IFM. In 20-day-old (iCUG)<sub>480</sub> flies, only five out of 35 (14.3%) IFM had normal appearance, whereas in (iCUG)<sub>480</sub> flies over-expressing MBNL1 of the same age, 60 out of 67 (89.5%) appeared normal.

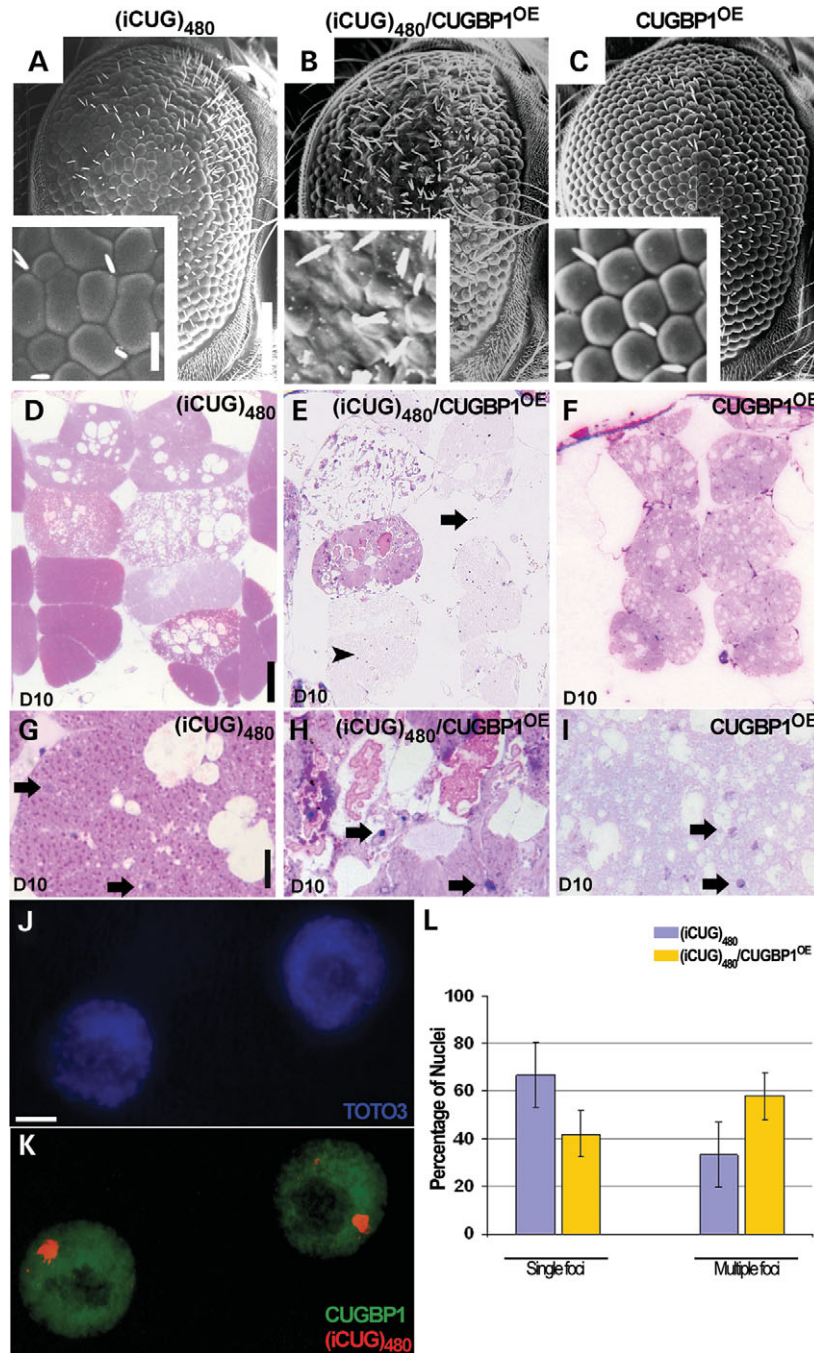
Interestingly, high expressing MBNL1 transgenic lines show a muscle phenotype in the absence of (iCUG)<sub>480</sub> (data not shown) suggesting that steady state levels of MBNL1 are important.

Because MBNL1 localizes to CUG nuclear foci in DM1 cells (21,22,31), we monitored MBNL1 distribution in the somatic muscle of (iCUG)<sub>480</sub> flies. MBNL1 presents a diffuse nuclear distribution in the absence of (iCUG)<sub>480</sub> expression (Fig. 3D and E). In contrast, MBNL1 localizes to nuclear foci in muscles expressing (iCUG)<sub>480</sub> and MBNL1 simultaneously, and no diffuse MBNL1 is detected in these nuclei (Fig. 3A–C). We also found that the average number of foci observed per nucleus is decreased in (iCUG)<sub>480</sub> flies that also overexpress MBNL1 (Fig. 3F).

### Increased levels of CUGBP1 enhance expanded CUG toxicity

In cells expressing the CUG-containing expanded mRNA, the levels of CUGBP are increased (23,38). Higher steady state levels of CUGBP1 in DM1 correlate with alternative splicing defects in specific transcripts (39–41). We generated flies expressing human CUGBP1 and we used the DM1 fly model to investigate whether increased levels of CUGBP1 alter the (iCUG)<sub>480</sub> eye phenotype. Flies co-expressing CUGBP1 and (iCUG)<sub>480</sub> show an aggravated eye phenotype with enhanced ommatidial disorganization in comparison with flies expressing (iCUG)<sub>480</sub> alone (Fig. 4A and B). Flies overexpressing CUGBP1 alone show inter-ommatidial bristle loss, but little if any ommatidial disorganization (Fig. 4C); consequently we conclude that CUGBP1 overexpression enhances the (iCUG)<sub>480</sub> eye phenotype. Increasing the CUGBP1 levels in the *Drosophila* somatic muscles leads to a degenerative phenotype (Fig. 4F) similar to the phenotype caused by (iCUG)<sub>480</sub> with vacuolization and loss of muscle integrity (Fig. 4I). This phenotype complicates interpreting the effect of CUGBP on (iCUG)<sub>480</sub> muscle toxicity. However, co-expressing (iCUG)<sub>480</sub> and CUGBP1 leads to an increased wasting of the muscles compared to animals expressing only (iCUG)<sub>480</sub> (compare Fig. 4E and H with D and G).

CUGBP1 does not localize to expanded CUG-containing nuclear foci in DM1 cells (18,31). We monitored the distribution of CUGBP1 in larval muscles co-expressing (iCUG)<sub>480</sub> and CUGBP1. Consistent with the existing data, we found that CUGBP1 localizes to the nuclei of muscle cells but is not recruited to the (iCUG)<sub>480</sub> nuclear foci (Fig. 4K).



**Figure 4.** CUGBP1 enhances the eye and muscle phenotypes caused by (iCUG)<sub>480</sub>, and does not accumulate in nuclear foci. (A–C) Eye SEM images of flies expressing (iCUG)<sub>480</sub>, (iCUG)<sub>480</sub> and CUGBP1, or CUGBP1 alone. The (iCUG)<sub>480</sub> eye phenotype (A), is enhanced by CUGBP1 overexpression (B). Expression of CUGBP1 by itself in the eye shows inter-ommatidial bristle loss, but little if any ommatidial disorganization (C). (D–I) Transversal semi-thin sections of adult IFM expressing (iCUG)<sub>480</sub>, (iCUG)<sub>480</sub> and CUGBP1, or CUGBP1 alone. Ten-day-old flies expressing (iCUG)<sub>480</sub> show vacuolization of the tissue (D) and loss of muscle fibre structure (arrows point nuclei, G). Co-expression of (iCUG)<sub>480</sub> and CUGBP1 causes a stronger wasting of the muscle in flies of the same age. Notice the absence of some muscle packs (arrow) and the lax distribution of myofibrils in the rest of the tissue (arrowhead) (E). Higher magnification shows a dramatic loss of muscle structure (arrows point nuclei, H). Expression of CUGBP1 alone shows a phenotype similar to the phenotype of (iCUG)<sub>480</sub> flies shown in D with vacuolization (F) and disorganization of fibres (arrows point nuclei, I). (J and K) Images of nuclei from larval somatic muscle co-expressing (iCUG)<sub>480</sub> and CUGBP1 after immunofluorescence and *in situ* hybridization. Muscles are stained with TOTO3 to visualize the nuclei (J, blue). CUGBP1 is distributed throughout the nuclei (K, green) and does not accumulate in (iCUG)<sub>480</sub> nuclear foci (K, red). (L) Comparative quantification of nuclei with single versus multiple foci in flies expressing (iCUG)<sub>480</sub> alone or together with CUGBP1. In nuclei expressing CUGBP1, the average of multiple foci per nucleus is higher than in (iCUG)<sub>480</sub> controls. Error bars represent standard deviation. Data was analyzed using Student's *t* ( $P = 0.006$  in both groups). Scale bars: (A–C) 100  $\mu\text{m}$  (insets 10  $\mu\text{m}$ ), (D–F) 50  $\mu\text{m}$ , (G–I) 10  $\mu\text{m}$ , (J–L) 5  $\mu\text{m}$ . Genotypes are A: *w*; *gmr-GAL4/UAS-LacZ*; *UAS-(iCUG)<sub>480</sub>[M5T]/+*; B: *w*; *gmr-GAL4/UAS-CUGBP1[M2A]*; *UAS-(iCUG)<sub>480</sub>[M5T]/+*; C: *w*; *gmr-GAL4/UAS-CUGBP1[M2A]*;+ D, G: *w*; +; *Mhc-GAL4/UAS-(iCUG)<sub>480</sub>[M5T]*; E, H: *w*; *UAS-CUGBP1[M2A]/+*; *Mhc-GAL4/UAS-(iCUG)<sub>480</sub>[M5T]*; F, I: *w*; *UAS-CUGBP1[M2A]/+*; *Mhc-GAL4/+*; J–L: *w*; *UAS-CUGBP1[M2N]/+*; *Mhc-GAL4/UAS-(iCUG)<sub>480</sub>[M5T]*.

Interestingly, overexpression of CUGBP1 increases the average number of (iCUG)<sub>480</sub> foci observed per nucleus (Fig. 4L).

## DISCUSSION

Several independent lines of evidence support the hypothesis that DM1 pathogenesis is a consequence of RNA gain-of-function toxicity. A second type of myotonic dystrophy (DM2) is caused by a related but distinct mutation (expansion of CCTG repeats) in a locus unrelated to the DM1 gene (16). In addition, a transgenic mouse model expressing expanded CUG in skeletal muscle displays myotonia and myopathy (17), and a *muscleblind* knockout also shows many of the pathological features of DM1 (26), supporting the idea that expanded CUG titrates muscleblind proteins.

To further investigate the RNA gain-of-function hypothesis and the mechanisms of expanded CUG-induced pathogenesis, we have generated a transgenic *Drosophila* DM1 model carrying an interrupted (CTG)<sub>480</sub> repeat. Other models of human disease in *Drosophila* have proved to be a useful tool to investigate the disease mechanisms (42–44). A previously reported DM1 *Drosophila* model generated with (CTG)<sub>162</sub> repeats showed accumulation of the expanded repeats in nuclear foci and co-localization with muscleblind protein, but did not show pathological phenotypes in muscle or other tissues (35). Likewise, flies generated in our laboratory expressing (CUG)<sub>200</sub> did not show abnormal phenotypes in the eye (data not shown) or in the muscle (Supplementary Material Fig. S1). Thus, it appears that fewer than 200 CUG repeats are not enough to trigger obvious pathogenic effects in *Drosophila*.

As reported here, we found that expression of a (iCUG)<sub>480</sub> RNA leads to dysfunction and progressive degeneration of somatic muscle. In addition, we find that the expanded transcripts accumulate in nuclear foci in muscle cells. These foci are an important hallmark of DM1 and their presence in fly muscle nuclei supports the validity of the DM1 *Drosophila* model. We find that overexpression of MBNL1 reduces the percentage of nuclei containing multiple foci (Fig. 3F), whereas, overexpression of CUGBP1 increases this percentage (Fig. 4L). Thus, we find a positive correlation between the number of foci per nuclei and severity of the muscle degenerative phenotype. We also report that expression of (iCUG)<sub>480</sub> in the *Drosophila* eye causes a phenotype that correlates with the muscle phenotype. The eye phenotype provides us with a convenient primary assay for future large-scale genetic screens aimed to identify additional modifiers of (iCUG)<sub>480</sub>-induced toxicity.

Two families of RNA-BP have been shown to be implicated in DM1 pathogenesis: CELF (CUGBP and ETR-3-like factors) proteins and MBNL proteins (21,31,45). CUGBP and MBNL proteins are antagonistic splicing regulators of two transcripts misregulated in DM1 tissues: cTNT and IR (28,39,40). In DM1, the splicing patterns observed for these transcripts are consistent with increased levels and activity of CUGBP1 (39–41,46) and decreased levels of MBNL proteins (30).

Mouse models expressing CUGBP1 in the heart show disrupted splicing of transcripts (27). Overexpression of

CUGBP1 in mice skeletal muscle reproduces some of the muscle histopathology phenotypes observed in DM1 muscle (36). Based on these findings, it could be predicted that increased levels of CUGBP1 would result in an enhancement of the phenotype induced by the expanded RNA. We find that expression of CUGBP1 alone in *Drosophila* somatic muscles leads to a muscle-wasting phenotype similar to the phenotype observed with expression of (iCUG)<sub>480</sub>. This observation is consistent with previous reports describing the effect of CUGBP1 expression in mice (27,36). In agreement with data observed in DM1 cells (18,31), the nuclei of *Drosophila* muscle cells expressing both CUGBP1 and (iCUG)<sub>480</sub> show a diffuse pattern of CUGBP1 accumulation but no aggregation or co-localization with the expanded RNA-containing foci. Co-expression of CUGBP1 and (iCUG)<sub>480</sub> leads to aggravated muscle wasting and degeneration phenotypes. Because of the phenotype caused by CUGBP1 alone, it is difficult to make a strong conclusion from the enhancement of the (iCUG)<sub>480</sub> muscle phenotype; however, these results are consistent with the idea that the increased CUGBP activity in DM1 tissue is relevant for DM1 pathogenesis.

All three mammalian MBNL proteins identified (MBNL1, MBNL2 and MBNL3) have been shown to co-localize with the expanded RNA-containing nuclear foci *in vivo* (21,22,31). We find that MBNL1 shows a diffuse distribution in the nuclei of *Drosophila* muscles. However, in the presence of (iCUG)<sub>480</sub>, MBNL1 accumulates in the nuclear foci and is depleted from the rest of the nucleus.

The phenotype of *muscleblind* knockout mice strongly supports the hypothesis that sequestration and loss of muscleblind normal function is a critical factor in DM1 pathogenesis. This hypothesis predicts that altering the levels of muscleblind proteins should modulate the phenotypes induced by expanded CUG repeats. We have tested this prediction and found that a heterozygous loss-of-function allele of *Drosophila muscleblind* enhances the eye phenotype. More importantly, we find that overexpression of human muscleblind is able to dramatically suppress both the somatic muscle and eye (iCUG)<sub>480</sub> phenotypes. The degree of suppression is dependent on MBNL1 overexpression levels, as too much MBNL1 also causes muscle pathology. Thus, it appears that the steady state levels of MBNL, like those of CUGBP1, are important for normal muscle physiology. These results support the MBNL sequestration model in DM1 pathogenesis and also constitute proof of the principle that expanded CUG-induced toxicity can be suppressed.

## MATERIALS AND METHODS

### Construct generation and transgenic *Drosophila* lines

The UAS-(iCTG)<sub>480</sub> construct was generated by cloning interrupted repeats composed of repeating units of the sequence (CTG)<sub>20</sub>CTCGA (39) into the pUAST *Drosophila* expression vector (37). (CTG)<sub>20</sub> were also cloned into the pUAST vector to generate transgenic lines. The UAS-MBNL1 and UAS-CUGBP1 constructs were generated by cloning the 41 kDa isoform of MBNL1 with an N-terminal Xpress tag and human CUGBP1, respectively, into the pUAST vector. The UAS-(CTG)<sub>200</sub> construct was generated by cloning a 1.4 kb

fragment of the DMPK 3' UTR containing 200 CTG repeats into the pUAST-GFP vector. Transgenic *Drosophila* lines were obtained by injecting these constructs in embryos following standard methods. We generated 13 UAS-(iCTG)<sub>480</sub>, 13 UAS-(CTG)<sub>20</sub>, 10 UAS-MBNL1, 7 UAS-CUGBP1 and 13 UAS-(CTG)<sub>200</sub> transgenic lines.

The UAS-LacZ, *gmr*-GAL4 and *mbl*<sup>P15144</sup> were obtained from Bloomington Stock Center (Indiana, USA). *Mhc*-GAL4 was obtained from G. Davis (UCSF).

### Scanning electron microscopy of *Drosophila* eyes

Flies processing for SEM as well as image acquisition were performed following the previously published procedures (47).

### Semi-thin sectioning of *Drosophila* adult thoraxes

Adult thoraxes were dissected out, fixed overnight in glutaraldehyde–paraformaldehyde–cacodylate buffer, washed, fixed for 2 h at 4°C in 1% osmium tetroxide, dehydrated in ethanol, embedded in EPON for transversal semi-thin sections, and then stained with Multiple Stain Solution (Polysciences). Sectioned muscles of different genotypes to be compared with one another were stained simultaneously to ensure the same amount of staining. At least eight flies per genotype were sectioned for each observation.

### In situ hybridization and immunofluorescence staining of *Drosophila* larval muscles

For immunofluorescence staining, larval or adult muscles were dissected and fixed in 4% formaldehyde. Following standard procedures, the tissue was incubated with anti-CUGBP1 3B1 (1:500, Upstate), anti-Xpress tag (1:200, Invitrogen), anti-CBP (1:1000 provided by Dr Mattias Mannervik), anti-LaminC (1:50, Hybridoma Bank), Phalloidin (1:200, Sigma). Secondary antibodies were obtained from Jackson ImmunoResearch Laboratories and Molecular Probes. For combined *in situ* hybridization and immunofluorescence staining after the fixation, the tissue was fixed again with 40% formamide/1 × SSC following hybridization with Cy3-labelled (CAG)<sub>5</sub> probe (1 ng/μl) and washed to continue with the immunofluorescence staining. We studied eight larvae per genotype and 50 nuclei per larvae. For comparative quantification of the nuclear foci, we studied at least four larvae per genotype and an average of 200 nuclei per larvae.

### SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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