Reduction of toxic RNAs in myotonic dystrophies type 1 and type 2 by the RNA helicase p68/DDX5

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Myotonic dystrophies type 1 (DM1) and type 2 (DM2) are neuromuscular diseases, caused by accumulation of CUG and CCUG RNAs in toxic aggregates. Here we report that the increased stability of the mutant RNAs in both types of DM is caused by deficiency of RNA helicase p68. We have identified p68 by studying CCUG-binding proteins associated with degradation of the mutant CCUG repeats. Protein levels of p68 are reduced in DM1 and DM2 biopsied skeletal muscle. Delivery of p68 in DM1/2 cells causes degradation of the mutant RNAs, whereas delivery of p68 in skeletal muscle of DM1 mouse model reduces skeletal muscle myopathy and atrophy. Our study shows that correction of p68 may reduce toxicity of the mutant RNAs in DM1 and in DM2.

myotonic dystrophy | RNA foci | p68 RNA helicase | CUG repeats

Myotonic dystrophy type 1 (DM1) is a neuromuscular disease characterized by myotonia, distal muscle weakness, heart conduction defects, and, in the congenital form, a delay in myogenesis and severe cognitive abnormalities (1). DM1 is caused by expanded CTG repeats within the 3′ untranslated region of the *DMPK* gene (2). Myotonic dystrophy type 2 (DM2) is a late-onset disease that is caused by expanded CCTG repeats in intron 1 of the *ZNF9*/*CNBP* gene (3). Development of therapeutic approaches for DM1 or DM2 is an urgent need. Numerous data suggest that DM1 and DM2 are caused by RNA gain-of-function mechanisms (4–6). Initial studies showed that mutant RNAs mainly affect two RNA-binding proteins, CUG-binding protein 1 (CUGBP1) and muscleblind-like protein 1 (MBNL1) (7–9). CUG repeats elevate protein levels of CUGBP1 by increasing its stability (5). In addition, CUG repeats change signal transduction pathways, such as the glycogen synthase kinase 3β (GSK3β)–cyclin D3 pathway, regulating CUGBP1 activity (5, 10). CUG and CCUG repeats form double-stranded hairpin structures and sequester MBNL1 (9, 11, 12). Several other RNA-binding proteins, such as Staufen1 and two members of the DEAD-box RNA helicases family, DDX5/p68 and DDX6, are also involved in DM1 (13–15).

We showed that the mutant CUG and CCUG RNAs are very stable (16), suggesting that the activity of RNA-binding proteins regulating RNA decay is reduced in DM1 and in DM2. In this study, we tested this hypothesis by isolation and analysis of several CCUG-binding proteins. We found that the levels of one of these proteins, p68, are reduced in DM1 and DM2 biopsied muscle and that correction of p68 leads to degradation of the mutant CUG and CCUG RNAs, disintegration of RNA foci, and reduction of DM muscle pathology.

Results and Discussion

Identification of p68 Helicase as CUG/CCUG-Binding Protein, Which is Reduced During Degradation of CCUG Repeats. Given the critical role of RNA CUG and CCUG repeats in DM1/2, we performed a careful analysis of CCUG-binding proteins with altered activities upon accumulation and degradation of the mutant CCUG repeats in tetracycline (tet)-regulated CHO cell model of DM2. In this model, $CCUG_{100}$ repeats mainly increased activities of two RNA-binding proteins with the approximate molecular weights 50 and 100 kDa (Fig. 1*A*). Previous analysis of tet-regulated cell models expressing CUG and CCUG repeats showed that a single dose of Dox causes maximal accumulation of CUG or CCUG repeats at 17–24 h after Dox addition, whereas approximately half of the mutant RNAs is degraded at 36–48 h after Dox addition (16). Analysis of CCUG-binding proteins in tet-HeLa-$CCUG_{100}$ cells at 7–24 h after Dox addition showed that CCUG repeats increase activities of two proteins with molecular weights 50 and 100 kDa similar to those observed in inducible CHO cells (Fig. 1*B*), whereas the activity of CCUG-binding protein with molecular weight 75 kDa is increased at 24 h. The activities of these three proteins were reduced during degradation of the mutant CCUG repeats (48 h after Dox addition) (Fig. 1*B*). UV cross-linking analysis of proteins from normal and DM2 myoblasts showed that activity of the 75-kDa CCUG-binding protein is also reduced in DM2 (Fig. 1*C*). Normal human myoblasts contain several other CCUG-binding proteins migrating in the positions 40, 50, 55, and 100 kDa. The activities of CCUG-binding proteins with molecular weights 50 and 100 kDa were increased in DM2 myoblasts, whereas the activities of proteins with approximate molecular weights 40, 55, and 75 kDa are reduced (Fig. 1*C*).

Because the identified proteins have altered CCUG-binding activity in DM2 myoblasts, we suggested that these proteins might be involved in the degradation of the mutant CCUG repeats. Therefore, we performed purification of the CCUG repeat binding proteins from HeLa cells expressing $CCUG_{100}$ RNA, using a series of HPLC-based chromatographies. First separation of proteins in the

Significance

Inherited multisystemic diseases, myotonic dystrophies type 1 (DM1) and type 2 (DM2), are caused by long CUG and CCUG RNA repeats. The mutant RNA CCUG repeats should be degraded after intron excision; however, this RNA accumulates in cells, leading to pathology. Although mutant RNAs may be degraded with synthetic oligonucleotides, the identification of a cause of the increased stability of CUG and CCUG RNAs would help to improve the efficiency of their degradation. We found that the reduction of RNA helicase p68 in skeletal muscle biopsies of DM1 and DM2 patients contributes to the delay of degradation of the mutant RNAs. Our work suggests RNA helicase p68 as a therapeutic target in DM, correction of which improves degradation of the mutant RNAs, reducing DM pathology.

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The authors declare no conflict of interest.

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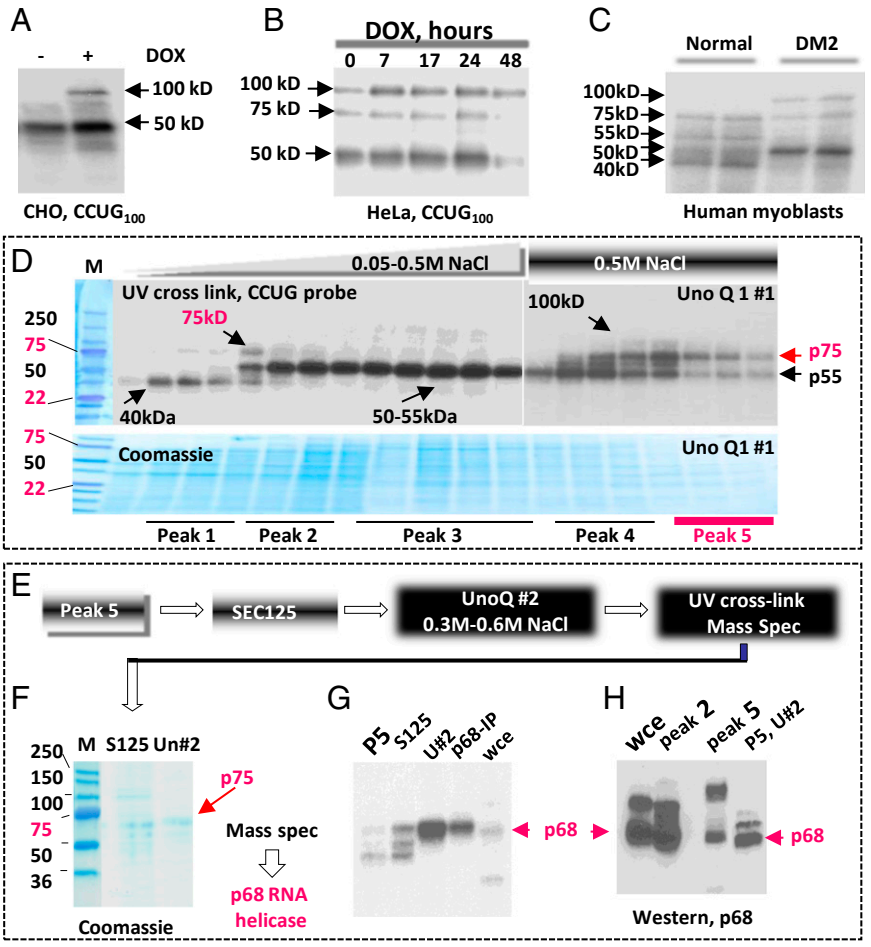

Fig. 1. P68 is a CCUG-binding protein with the reduced activity in DM2. (*A*) Mutant CCUG repeats increase activities of 50- and 100-kDa RNA-binding proteins. UV cross-link analysis of cytoplasmic proteins with ^{32}P-CCUG$_{100}$ probe. (*B*) UV cross-link analysis identifies three CCUG-binding proteins with approximate molecular weights 50, 75, and 100 kDa increased by CCUG$_{100}$ repeats in tet-regulated HeLa cells. (*C*) UV cross-link analysis of cytoplasmic proteins from normal and DM2 myoblasts with ^{32}P-CCUG$_{100}$ RNA; arrows: proteins with increased and reduced activities in DM2 myoblasts. (*D*) Identification of CCUG-binding proteins (shown by arrows) by UV cross-link assay in the chromatography fractions (UnoQ column; run 1) using the CCUG$_{100}$ probe. The protein fractions were stained with Coomassie Blue. Five peaks with different protein composition are underlined. (*E*) A diagram of purification procedure for a CCUG-binding protein with molecular weight 75 kDa (see text). (*F*) Coomassie staining of the purified protein with molecular weight 75 kDa after SEC125 and UnoQ (run 2). Mass spectrometry showed that a CCUG-binding protein with molecular weight 75 kDa is a p68 RNA helicase. (*G*) UV cross-link analysis of proteins at different steps of the purification of p68—peak 5 (first step), S125 column (second step), and UnoQ (run 2; third step)—using CCUG$_{100}$ probe; arrow: p68 bound to CCUG probe. HeLa whole-cell extract (WCE) and immunoprecipitated p68 (p68-IP) from the HeLa cells were examined as controls. (*H*) Western blot analysis with anti-p68 confirms that the purified protein with molecular weight 75 kDa is p68 RNA helicase. Antibodies to p68 recognize p68 in the HeLa WCE in peaks 2 and 5 at the first steps of purification and in peak 5 after two rounds of purification.

ion exchange UnoQ column and UV cross-link analysis identified CCUG-binding proteins with molecular weights 40, 50–55, 75, and 100 kDa (Fig. 1*D*). The 75-kDa CCUG-binding protein was detected in peaks 1, 4, and 5. Because peak 5 contains a much smaller number of proteins than do other peaks, the protein fractions from peak 5 were subjected to further separation by chromatography on the size exclusion column, SEC125, and by the second ion exchange chromatography on the UnoQ column (run 2) (Fig. 1*E*). This separation yielded a significant enrichment of the CCUG-binding protein with the approximate molecular weight 75 kDa (Fig. 1 *F* and *G*). Mass spectrometry analysis revealed that this protein is the p68 RNA helicase. The identity of this protein was confirmed in western blot assay with antibodies to p68 (Fig. 1*H*). Taken together, we conclude that p68 RNA helicase binds to RNA CCUG repeats and that its activity is reduced in DM2 myoblasts.

Correction of p68 Levels in DM1 and DM2 Cells Dissociates CUG and CCUG Foci in Nucleus and in Cytoplasm. The reduction of RNA-binding activity of p68 in DM2 myoblasts suggested that p68 might also be reduced in skeletal muscle of patients with DM2. Western blot analysis showed that the levels of p68 are reduced in biopsied skeletal muscle from patients with DM2 (Fig. 2*A*). Recent analysis of the proteins bound to CUG repeats showed that p68 binds to CUG foci and modulates splicing activity of MBNL1 (14). It appears that p68 levels are increased in immortalized cultured DM1 myoblasts (17). However, p68 levels were reduced in DM1 biopsied muscle (Fig. 2*B*). CUGBP1 (used as a marker) was increased in the same muscle biopsies from patients with DM1 and DM2. It is likely that the reduction of p68 in DM1 muscle is caused by the mutant CUG repeats because p68 was also reduced in muscle of DM1 mice expressing 250 CUG repeats in the 3′ UTR of human skeletal actin (*HSA^{LR}* model) (18) (Fig. 2*C*).

The RNA helicases are proteins involved in dsRNA unwinding (19), allowing ribonucleases to access RNA-binding sites to facilitate RNA degradation. Given these activities of helicases, we considered p68 as a reasonable candidate to unwind ds-CUG and CCUG hairpins during decay of the mutant RNAs. Because p68 is

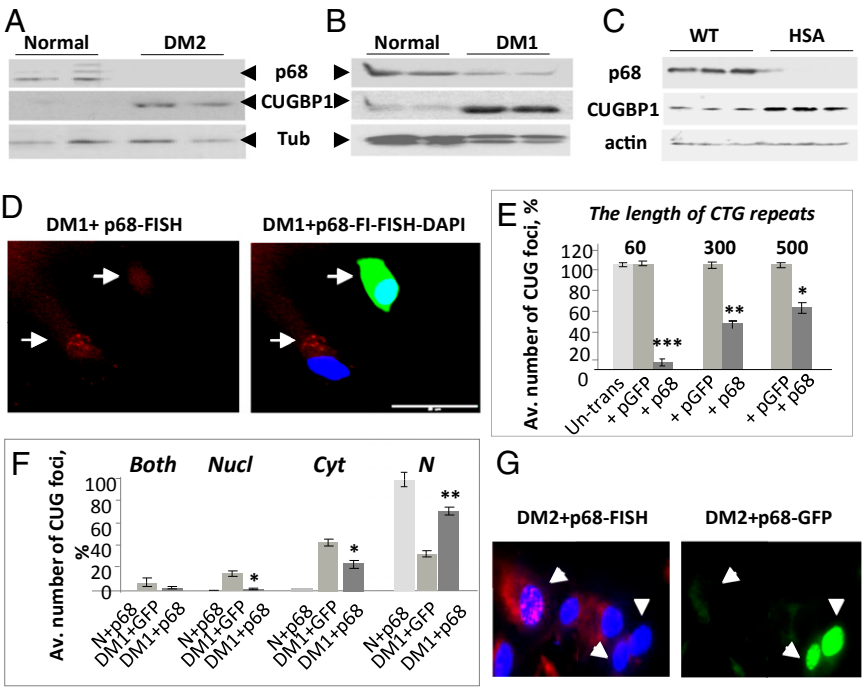

Fig. 2. Correction of p68 activity in DM1 and DM2 causes disintegration of CUG and CCUG foci. Western blot analysis of proteins from normal, DM2 (*A*), and DM1 (*B*) biopsied muscles with antibodies to p68, CUGBP1, and α-tubulin. (*C*) Western blot analysis of skeletal muscle proteins from WT and HSA^{LR} mice with antibodies to p68, CUGBP1, and β-actin. (*D*) Ectopic expression of p68 reduces the number of cells with CUG foci in DM1 myoblasts. FISH analysis of DM1 myoblasts transfected with p68-GFP using CAG probe (red). DM1 cells transfected with p68-GFP were detected by fluorescent analysis (green). Nuclei were stained with DAPI (blue). (*E*) p68-dependent dissociation of CUG foci depends on the length of CTG expansions. Bar graphs show the average percentage of cells with CUG foci in DM1 myoblasts from patients with 60, 300, and 500 CTG repeats transfected with GFP or p68-GFP. (*F*) Delivery of p68 in DM1 myoblasts with 300 CTG repeats causes dissociation of foci in both the nucleus and cytoplasm. CUG foci were counted in both the nucleus and cytoplasm (300 cells per set). (*G*) Ectopic expression of p68 reduces the number of CCUG foci in DM2 fibroblasts. CCUG foci (red) were detected by FISH analysis in DM2 fibroblasts transfected with p68-GFP (green). Nuclei were stained with DAPI. Original magnification is 60×. Data are presented as mean ± SEM. $*P < 0.05$, $**P < 0.005$, $***P < 0.0005$.

reduced in DM1 and DM2 biopsied skeletal muscle, this deficiency of p68 might prevent unwinding of CUG and CCUG hairpins and might reduce degradation of the mutant RNAs causing their aggregation in foci. To examine this hypothesis, DM1 myoblasts with 300 CTG repeats were transfected with p68-GFP, and CUG foci were examined by FISH assay. We found that CUG foci are disintegrated in 55% of cells transfected with p68 (Fig. 2 *D* and *E*). In DM1 myoblasts with 60 CTG repeats, ectopic p68 almost completely dissociated CUG aggregates (*SI Appendix*, Fig. S1 *A* and *B*). However, in DM1 myoblasts with 500 CTG repeats, ectopic p68 reduced CUG foci in 40% of cells (Fig. 2*E*), suggesting that p68 needs additional proteins to unwind long CUG expansions in the 3′ UTR of *DMPK*.

The UV cross-link analysis suggested that the mechanism by which p68 reduces CUG foci might be associated with the direct binding of p68 to CUG repeats. We found that both endogenous p68 and ectopic p68, expressed in HeLa cells, bind to the expanded CUG repeats in vitro (*SI Appendix*, Fig. S1 *C* and *D*).

It has been suggested that the mutant *DMPK* mRNA form foci in the nuclei only and that after RNA foci dissociation the mutant *DMPK* migrates to the cytoplasm. We observed CUG-positive foci in both the nucleus and cytoplasm of DM1 myoblasts (Fig. 2*D*), whereas ∼30% of DM1 myoblasts did not have CUG aggregates in both compartments. Quantification of foci in DM1 myoblasts transfected with p68 showed that p68 causes the dissociation of RNA foci in the nucleus and cytoplasm of DM1 myoblasts to an equal extent (Fig. 2*F*). This indicates that p68 has an equally beneficial effect on CUG foci dissociation in the nucleus and cytoplasm and that foci formation in the cytoplasm is not due solely to foci dissociation in the nucleus and migration of the mutant RNA to cytoplasm. Similar to DM1 cells, ectopic expression of p68 causes disintegration of CCUG foci in DM2 fibroblasts (Fig. 2*G* and *SI Appendix*, Fig. S2) and in the Dox-inducible HeLa model of DM2 (Fig. S3).

Ectopic Expression of p68 Causes Degradation of the Mutant CUG and CCUG RNAs in DM1 and DM2 Cells. Foci dissociation in DM1 and DM2 cells by p68 suggested that RNA foci might disintegrate due to degradation of mutant RNAs. Therefore, we compared levels of the mutant *DMPK* mRNA in DM1 fibroblasts after transfection with p68 using northern blot assay. We found that ectopic expression of p68 causes significant reduction of the mutant *DMPK* mRNA (Fig. 3*A* and *SI Appendix*, Fig. S4*A*). Similar reduction of the mutant CUG RNA was observed in Dox-inducible CHO cells expressing CUG_{914} RNA (Fig. 3*B* and *SI Appendix*, Fig. S4*B*). It appears that the effect of p68 on the reduction of the mutant *DMPK* mRNA or CUG RNA was specific because no degradation of *GAPDH* mRNA was observed (Fig. 3 *A* and *B*).

We also analyzed the effects of ectopic expression of p68 on the degradation of the mutant CCUG repeats in DM2 fibroblasts. We found that mutant CCUG repeats migrate as a smear band on the gel (Fig. 3*C*). After transfection with p68, the CCUG-positive signal was significantly reduced (Fig. 3*C* and *SI Appendix*, Fig. S4*C*). Degradation of the mutant CCUG repeats is specific because *ZNF9* and *GAPDH* mRNAs were not degraded (Fig. 3*C* and *SI Appendix*, Fig. S4*D*). p68 also degrades the mutant $CCUG_{100}$ RNA in tet-regulated HeLa cells. The intensity of the $CCUG_{100}$ signal was reduced in HeLa cells transfected with p68 (Fig. 3*D*). Respectively, transfected cells accumulated low–molecular-weight CCUG-positive RNA that likely represents products of degradation of $CCUG_{100}$ RNA (Fig. 3*D* and *SI Appendix*, Fig. S4*E*). Based on these data, we

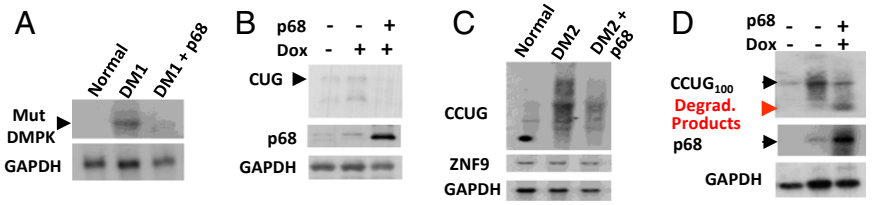

Fig. 3. Ectopic expression of p68 degrades mutant RNAs in DM1 and in DM2 cells. (*A*) Northern blot analysis of DM1 fibroblasts transfected with p68-GFP using CAG and GAPDH probes. The levels of the mutant *DMPK* mRNA were quantified as ratios to the *GAPDH* signal (see *SI Appendix*, Fig. S4*A*). (*B*) Northern blot analysis of tet-inducible CHO cells expressing CUG_{914}, transfected with p68-GFP using CAG, p68, and GAPDH probes. The CUG signal was quantified as ratio to *GAPDH* (*SI Appendix*, Fig. S4*B*). (*C*) Northern blot analysis of DM2 fibroblasts transfected with p68-GFP using CAGG, ZNF9, and GAPDH probes. CCUG signal is diffused in DM2 fibroblasts. Quantifications of the CCUG and normal *ZNF9* mRNA signals are shown as ratio to *GAPDH* in *SI Appendix*, Fig. S4 *C* and *D*. (*D*) Northern blot analysis of inducible HeLa cells expressing $CCUG_{100}$ RNA transfected with p68 using CAGG, p68, and GAPDH probes. Positions of degraded (red arrow) and undegraded (black arrow) $CCUG_{100}$ RNAs are shown. Quantification of degraded $CCUG_{100}$ signals as ratios to *GAPDH* is shown in *SI Appendix*, Fig. S4*E*.

conclude that normalization of p68 is beneficial for degradation of the mutant RNAs in DM1 and DM2.

Normalization of p68 in Skeletal Muscle of HSA^{LR} Mice Reduces Muscle Pathology. To determine if correction of p68 might reduce DM1 muscle pathology, recombinant adeno-associated vector expressing p68-GFP (*SI Appendix*, Fig. S5*A*) was injected in *Gastrocnemius* (gastroc) muscle of 2-mo-old HSA^{LR} mice. At this age, the HSA^{LR} mice do not show significant muscle phenotype. By 3 mo, however, skeletal muscle in these mice is characterized by myopathy including a variability of myofiber size with central nuclei and atrophy. Muscles were analyzed 2–4 wk after injection, and expression of p68-GFP was confirmed by fluorescent analysis (*SI Appendix*, Fig. S5*B*). The UV cross-link analysis indicated that p68-GFP binds to CUG RNA in vitro (*SI Appendix*, Fig. S5 *C* and *D*). Recombinant p68 caused the reduction of CUG foci in muscle of HSA^{LR} mice (*SI Appendix*, Fig. S6). H&E staining showed significant improvement of muscle histology in HSA^{LR} mice treated with p68 (Fig. 4 and *SI Appendix*, Fig. S7). In control HSA^{LR} muscle treated with PBS, fibers were variable in size. However, there was a recovery of myofiber uniformity within muscle tissue treated with p68. In HSA^{LR} mice treated with PBS, at 3 mo of age the average cross-sectional area of myofibers is increased due to presence of hypertrophic fibers similar to untreated HSA^{LR} mice (10). We found that p68 treatment normalized the average area of myofibers (Fig. 4 and *SI Appendix*, Fig. S7*A*). To confirm the recovery of the fiber size in the p68-treated HSA^{LR} mice, the mean minimal Feret's diameter was examined. We found that the minimal myofiber diameter was increased in the PBS-treated muscle of HSA^{LR} mice relatively WT muscle; however, the mean fiber diameter was normalized in the p68-treated muscle (*SI Appendix*, Fig. S7*B*). Muscles treated with p68 showed improved myofiber bundle bunching compared with muscle treated with PBS (Fig. 4). Quantification of myofiber number in similar regions of gastroc muscles from PBS- and p68-treated mice showed that p68 treatment significantly increases the total number of myofibers with reduced central nuclei (Fig. 4 and *SI Appendix*, Fig. S7 *C* and *D*). EM analysis showed the improvement of myofibrillar organization in the p68-treated HSA^{LR} muscle (*SI Appendix*, Fig. S8). These data show that normalization of p68 causes reduction of myopathy and muscle atrophy associated with DM1.

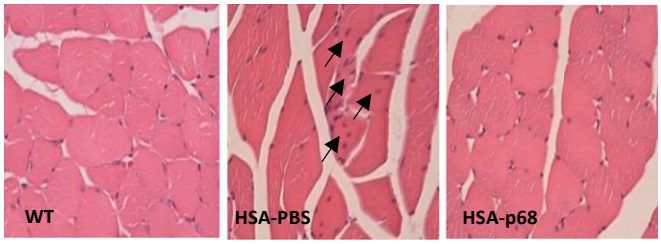

Fig. 4. Intramuscular delivery of p68 corrects myopathy and atrophy in HSA^{LR} mice. H&E analysis of gastroc muscles of WT and HSA^{LR} mice injected with p68-GFP or with PBS. Arrows show fibers with central nuclei. Quantification of the average myofiber size, myofiber number, and central nuclei is shown in *SI Appendix*, Fig. S7.

Hypothetical Role of p68 in DM1/2 Pathology. Whereas many studies are focused on the correction of toxic effects caused by the mutant CUG and CCUG repeats, the mechanisms which prevent degradation of these repeats are not well understood. Recent reports showed that RNA helicases p68/DDX5 and DDX6 bind to and remodel the mutant CUG RNA (14, 15). It has been proposed that these RNA helicases have a different effect on the mutant CUG repeats. Ectopic expression of DDX6 decreased CUG foci in DM1 cells (15). In contrast, p68 might increase the stability of CUG repeats because inhibition of p68 in tet-regulated HeLa cells by siRNA to p68/p72 reduces the number of CUG foci (14). The findings described in this paper show that the correction of p68 levels in DM1 cells reduces the number of CUG foci (Fig. 2 *D* and *E*). Therefore, we suggest that p68 and DDX6 might unwind the ds-CUG repeats within the mutant *DMPK* mRNA and improve its processing.

It appears that the beneficial effect of p68 in DM1 is stronger than that of DDX6 because the disintegration of CUG foci by p68 promotes degradation of the mutant *DMPK* mRNA (Fig. 3*A* and *SI Appendix*, Fig. S4*A*), whereas DDX6 disassembles CUG foci without degradation of the mutant *DMPK* mRNA (15). In addition, DDX6 does not reduce the stability of CCUG foci (15), whereas correction of p68 improves disintegration and degradation of both CUG and CCUG mutant RNAs (Figs. 2 and 3). The reason for the reduction of CUG foci by the inhibition of p68/p72 in the Laurent et al. paper (14) remains to be determined. It is possible that the inhibition of both p68 and p72 might have a different effect on the stability of CUG foci than has p68 alone.

Examination of the role of p68 in vivo showed that intramuscular injection of p68 in HSA^{LR} mice reduces DM1 muscle histopathology. This result is in agreement with the reduction of CUG foci in the HSA^{LR} muscle, treated with p68, but not with GFP (*SI Appendix*, Fig. S6).

It has been shown that the bridging integrator-1 (BIN1) protein contributes to muscle weakness in DM1 (20). The splicing of *BIN1* mRNA is misregulated in DM1, leading to an increase of the isoform lacking exon 11 (20). We examined the expression of this isoform in HSA^{LR} muscle and found that the *BIN1* isoform lacking exon 11 is increased in untreated HSA^{LR} mice, whereas the isoform retaining exon 11 is reduced (*SI Appendix*, Fig. S9). In contrast, HSA^{LR} muscle treated with p68 shows improvement of the splicing of *BIN1* by reduction of the isoform lacking exon 11 and increase of

the isoform retaining exon 11 (*SI Appendix*, Fig. S9). Because this splicing event is regulated by MBNL1, the improvement of the splicing of *BIN1* in the p68-treated muscle suggests that p68 is beneficial for the correction of at least some splicing events, misregulated by MBNL1 in DM1. We hypothesize that this effect might be due to disintegration of CUG foci, which releases MBNL1 and possibly other RNA-binding proteins associated with the mutant CUG repeats. We previously suggested that several RNA-binding proteins might bind to the mutant CUG foci, including p68 and MBNL1 (21). Both p68 and DDX6 might reduce stability of CUG foci releasing sequestered RNA-binding proteins such as MBNL1. This hypothesis is supported by the findings in this study, which show that the correction of p68 is sufficient to reduce CUG foci by ∼50% in DM1 cells with 300–500 CTG repeats (Fig. 2*E*). Based on these data, we suggest that a synergistic effect of p68, DDX6, and MBNL1 might be needed to disintegrate the majority of CUG foci in DM1.

Further studies are necessary to determine the relationship of p68 and MBNL1 in RNA processing in DM1. It is possible that during regulation of splicing p68 promotes MBNL1 binding to its targets, as was shown by Laurent et al. (14).

The mechanism by which p68 protein is reduced in DM1 and in DM2 is unknown. Our recent study showed that the inhibitors of GSK3, correcting CUGBP1 activity in DM1 mice, significantly reduce DM1 pathology (10). We found that GSK3 inhibitor TDZD-8 restores p68 levels in HSA^{LR} mice (*SI Appendix*, Fig. S10). Because CUGBP1 regulates many mRNAs, we hypothesize that p68 is one of the targets of CUGBP1 and that the reduction of the repressive form of CUGBP1 by GSK3 inhibitors leads to the normalization of p68 and degradation of the mutant CUG RNA. Regardless of whether p68 is a direct or indirect target of CUGBP1, data presented in this study show that the correction of p68 helicase leads to the reduction of toxic RNAs and to correction of DM pathology.

Materials and Methods

Purification of RNA-Binding Proteins Using HPLC Techniques. Cytoplasmic proteins were collected from the induced HeLa cells expressing $CCUG_{100}$ RNA. Twenty milligrams of proteins were loaded on ion exchange column UnoQ and eluted with a gradient 0–0.5 M NaCl. Chromatography fractions were used for the electrophoretic analysis, UV cross-linking with $CCUG_{100}$ probe, and for western blot assay. Fractions containing proteins of interest were combined and further separated by size exclusion and ion exchange chromatographies, as shown in Fig. 1*E*. The purified p75 protein was subjected to mass spectroscopy at the Baylor College of Medicine Protein Core Facility. FISH assay, Western and Northern blot analyses were performed as described (16). Detailed description of these methods is also provided in *SI Appendix, Experimental Procedures*.

UV cross-linking assay. Proteins were incubated with ^{32}P-$CCUG_{100}$ or ^{32}P-CUG_{123} for 30 min at room temperature, subjected to UV light, and then treated with RNase A. The RNA–protein complexes were separated by SDS/PAGE, and proteins were transferred to nitrocellulose membrane. The membranes were exposed to X-ray film and stained with Coomassie blue to verify protein loading.

Animal experiments. The experiments with animals were approved by the Institutional Care and Use Committee at Baylor College of Medicine and Cincinnati Children's Hospital. AAV8-GFP-p68 (2.55×10^{10} GC) was administered to the right gastroc muscle of the 2-mo-old HSA^{LR} mice while the left muscle of the same mouse was injected with the same amount of AAV8-GFP or PBS. Gastroc muscles were collected 2–4 wk after injections and examined by H&E staining and by electron transmission microscopy. WT mice of the matching sex and age were used as controls. The analysis of the number of total fibers and the average fiber size in the largest area of the cross sections were determined as previously described (10). Some 5-mo-old HSA^{LR} mice were treated with TDZD-8 (at a dose of 10 mg/kg) three times a week for 1 wk.

For other methods, see *SI Appendix, Experimental Procedures*.

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