A CTG repeat-selective chemical screen identifies microtubule inhibitors as selective modulators of toxic CUG RNA levels

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A CTG repeat expansion in the DMPK gene is the causative mutation of myotonic dystrophy type 1 (DM1). Transcription of the expanded CTG repeat produces toxic gain-of-function CUG RNA, leading to disease symptoms. A screening platform that targets production or stability of the toxic CUG RNA in a selective manner has the potential to provide new biological and therapeutic insights. A DM1 HeLa cell model was generated that stably expresses a toxic r(CUG)480 and an analogous r(CUG)0 control from DMPK and was used to measure the ratio-metric level of r(CUG)480 versus r(CUG)0. This DM1 HeLa model recapitulates pathogenic hallmarks of DM1, including CUG ribonuclear foci and missplicing of pre-mRNA targets of the muscleblind (MBNL) alternative splicing factors. Repeat-selective screening using this cell line led to the unexpected identification of multiple microtubule inhibitors as hits that selectively reduce r(CUG)480 levels and partially rescue MBNL-dependent missplicing. These results were validated by using the Food and Drug Administration-approved clinical microtubule inhibitor colchicine in DM1 mouse and primary patient cell models. The mechanism of action was found to involve selective reduced transcription of the CTG expansion that we hypothesize to involve the LINC (linker of nucleoskeleton and cytoskeleton) complex. The unanticipated identification of microtubule inhibitors as selective modulators of toxic CUG RNA opens research directions for this form of muscular dystrophy and may shed light on the biology of CTG repeat expansion and inform therapeutic avenues. This approach has the potential to identify modulators of expanded repeat-containing gene expression for over 30 microsatellite expansion disorders.

Significance

Toxic RNA molecules containing hundreds to thousands of CUG repeats cause myotonic dystrophy type 1 (DM1). Selectively blocking expression of this toxic CUG RNA has the potential to alleviate all downstream pathogenic consequences to the cell. To identify new hits and cellular targets that selectively regulate toxic CUG RNA levels, we developed a cell-based screening assay that permits the ratio-metric measurement of toxic CUG RNA abundance relative to an analogous control RNA. A pilot screen identified multiple microtubule inhibitors as hits that selectively regulate toxic CUG RNA levels in multiple DM1 models. These findings open avenues of research and therapy in DM1 and provide proof of concept for repeat-selective screening to identify unanticipated hits in other microsatellite expansion diseases.


The authors declare no competing interest.

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levels, unbiased screens expedite the discovery of new compounds and can identify unanticipated cellular targets. Small-molecule and genetic screens have been employed in the search for therapeutics as well as to inform interesting new biology associated with the CTG repeat expansion (13, 31–36). However, a CTG repeat-selective screen targeting r(CUG)EXP production and/or stability in cells has not been reported. Selectivity for the expanded CTG repeat is important for the development of effective therapy, but can also reveal new biology of CTG and other repeat expansions. To address this gap in the field, we generated a DM1 HeLa cell model permitting the sensitive ratio-metric evaluation of r(CUG)480 levels relative to an r(CUG)0 control, enabling the identification of small molecules or cellular targets that selectively modulate r(CUG)EXP levels. We applied this cell line to a pilot screen of the LOPAC1280 (Sigma-Aldrich) chemical library targeting diverse cellular pathways, leading to the identification of multiple microtubule inhibitors as regulators of toxic r(CUG)EXP levels. We validated microtubule inhibition using the FDA-approved natural compound colchicine in the HSA1LR DM1 mouse model and in DM1 patient-derived myotubes showing a selective reduction of toxic r(CUG)EXP levels and partially correcting several DM1-associated missplicing events. This assay is a repeat-selective screen targeting r(CUG)EXP production/stability in cells and has yielded FDA-approved compounds, providing biological and therapeutic insight into DM1.

Results

Generation of a HeLa DM1 Cell Line for Repeat-Selective Screening.

To screen for novel compounds and cellular targets that reduce expanded CUG RNA levels in a selective manner, we generated stable dual-construct HeLa cell lines. These cell lines contain an interrupted (CTG)480 repeat tract consisting of 24 modules of [(CTG)20(CTCGA)] and a (CTG)0 control tract; both contain exons 11–15 of human DMPK (37), each with a unique qPCR probe binding sequence 3′ of the repeat tract to distinguish the 2 transcripts produced (Fig. L4). These constructs permit ratio-metric evaluation of repeat selectivity following treatments by measuring the abundance of r(CUG)480 relative to r(CUG)0. Multiplex qRT-PCR was performed where the same primer was used to reverse transcribe both RNA species, and the same primers and probes were used in the qPCR reaction but with different fluorescent probes against the unique sequences to distinguish r(CUG)480 from r(CUG)0 in the same reaction (Fig. L4 and SI Appendix, Fig. S1). Both constructs were randomly integrated into the HeLa genome by using the PiggyBAC Transposon system. Several puromycin-resistant clones were expanded following single cell sorting, and the expression of r(CUG)480 and r(CUG)0 was measured in these lines (SI Appendix, Fig. S1). The HeLa (CTG)480 clonal lines robustly expressed both r(CUG)0 and r(CUG)480, while only marginal signal was detected in the HeLa parental cell line (SI Appendix, Figs. S1 and S2). All of the clones exhibited reduced cell viability to varying degrees relative to the HeLa parental line, which may be attributed to a combination of PiggyBAC cloning, single cell sorting, and transgene expression (SI Appendix, Fig. S3).

To test the potential use of the developed HeLa (CTG)480 clones in CTG repeat-selective screens, each clone was treated with actinomycin D (ActD), a natural drug that was previously shown to selectively bind to and reduce transcription from expanded CTG repeats in the low nanomolar range (25, 38–40) (SI Appendix, Fig. S4). Treatment with ActD resulted in a selective reduction of r(CUG)480 levels as expected in each of the clonal lines (SI Appendix, Fig. S4). Further testing of clones 1 and 19, which expressed the highest levels of r(CUG)480, and comparable expression of r(CUG)0 confirmed a highly reproducible response to ActD in clone 19 (Fig. 1B; Z = 0.64 from the method of Zhang et al. (41)), whereas clone 1 displayed greater variability, yielding a poor Z′ value (Fig. 1B).

Based on the combination of robust expression of r(CUG)0 and r(CUG)480 at comparable levels, a highly reproducible response to ActD and favorable cell viability, clone 19 was a strong candidate for use in screening. Further characterization of clone 19 confirmed the presence of important cellular and molecular hallmarks of DM1, including ribonuclear foci and MBNL-dependent missplicing (Fig. 1 C–E). We also evaluated clones 1 and 11 and confirmed the presence of ribonuclear foci and MBNL-dependent missplicing in these additional HeLa DM1 model cell lines (SI Appendix, Figs. S5 and S6). RNA-sequencing (RNA-seq) analysis comparing HeLa (CTG)480 clone 19 to its parental HeLa cell line (mean, n = 4 biological replicates). *P < 0.05; **P < 0.01; ***P < 0.001.

![Fig. 1. Establishing a DM1 HeLa cell line for repeat-selective screening.](image-url)
Small-Molecule Screen for Selective Reduction of Expanded CUG RNA Identifies Multiple Microtubule Inhibitors. We carried out a pilot screen to identify compounds that selectively reduce r(CUG)480 levels in HeLa (CTG)480 clone 19 using the LOPAC1280 library (Sigma-Aldrich) that targets diverse cellular processes (Fig. 2A). Three dimethyl sulfoxide (DMSO) treatments and 3 ActD treatments (at 20 nM, where optimal CTG repeat selectivity was observed) were included on each plate of the LOPAC1280 (16 plates) as controls. The mean of the relative r(CUG)480 levels from the entire LOPAC1280 library treatments was ~1.0, while treatment with ActD across the screen (~n = 48) revealed that r(CUG)480 abundance was decreased ~2-fold relative to r(CUG)0 following treatment (Fig. 2B). Based on ActD activity, a threshold of <0.5 r(CUG)480 relative to r(CUG)0 was set. Application of this cutoff revealed 20 primary hits that were rescreened and ranked relative to ActD (Fig. 2C and SI Appendix, Fig. S7). The top 5 ranked hits included 3 microtubule inhibitors (colchicine, thiocolchicine, and suprafenacine), a DNA intercalator (amsacrine), and a purine nucleoside analog (azathioprine) (Fig. 2D). These candidates were taken forward for further characterization.

We evaluated rescue of MBNL-dependent missplicing in clone 19 following treatment with the top 5 hits at the screening dose of 1 μM (Fig. 2E). Treatment with all 3 microtubule inhibitors revealed a partial rescue of missplicing in 3 MBNL-dependent events, while amsacrine and azathioprine treatments did not lead to any significant reversal (Fig. 2E). Furthermore, rescreening all 3 microtubule inhibitors in additional HeLa DM1 (CTG)480 clones produced a comparable selective reduction in r(CUG)480 levels (SI Appendix, Fig. S8).

Microtubule inhibitors are categorized as either destabilizing or stabilizing agents, depending on their binding site of tubulin and subsequent mode of action in deregulating microtubule polymers composed of αβ-tubulin heterodimers (44). Microtubule polymers are dynamic, and their activity is coordinated through both active polymerization and depolymerization of αβ-tubulin heterodimers. For example, it was shown that the involvement of microtubule dynamics in coordinating chromosome positioning during DNA repair is sensitive to both microtubule-stabilizing and -destabilizing drugs (45). All 3 microtubule inhibitors identified from the primary screen (colchicine, thiocolchicine, and suprafenacine) destabilize microtubules via the colchicine binding site of β-tubulin (44, 46). To test if both classes of microtubule drugs had a similar effect, we treated HeLa (CTG)480 clone 19 with paclitaxel (Taxol) and epothilone D, microtubule-stabilizing agents that function through binding to the taxane site of β-tubulin (44, 47) (SI Appendix, Fig. S9). There was a dose-dependent reduction in relative r(CUG)480 levels upon treatment with paclitaxel and epothilone D, supporting the role of both microtubule-stabilizing and -destabilizing agents in selectively reducing r(CUG)480 levels (SI Appendix, Fig. S9).

Fig. 2. Chemical screen identifies multiple microtubule inhibitors that selectively reduce r(CUG)480 levels. (A) Schematic of the CTG repeat-selective chemical screen. Following drug treatment (structure of ActD is shown as an example), cells are permeabilized, and RNA is directly taken forward from 96-well plates into RT-qPCR steps without the need for an RNA-extraction step. (B) Summary of LOPAC1280 screen on relative r(CUG)480 levels [normalized to r(CUG)0]. Mean of ActD treatments (20 nM, n = 48 ± SD) and mean of entire LOPAC1280 treatments (1 μM, n = 1,280 ± SD). (C) LOPAC1280 screen results of each individual drug treatment (1 μM) on relative r(CUG)480 levels [normalized to r(CUG)0]. The dashed red line indicates the 0.5 cutoff to identify primary hits falling below threshold. (D) Ranked list of top 5 primary hits from the LOPAC1280 screen. (E) RT-PCR isoform analysis of the indicated alternative cassette exon events in HeLa (CTG)480 clone 19 following treatment with each of the indicated compounds (1 μM for 48 h) (mean, n = 3 biological replicates). *P < 0.05; **P < 0.01.
By developing and implementing a screen for compounds that selectively reduce abundance of transcripts containing expanded CUG repeats, we identified microtubule inhibitors as compounds that selectively reduce r(CUG)480 to levels sufficient to partially reverse several DM1-relevant missplicing events. Because a role for microtubule inhibitors in the selective regulation of r(CUG)EXP levels had not been described, we further evaluated microtubule inhibition in multiple DM1 models. We primarily focused on colchicine because it is an inexpensive, FDA-approved, natural therapeutic that is generally well-tolerated and is currently used in the clinic to treat gout and familial Mediterranean fever (FMF) (44, 48).

Validation of Colchicine In Vivo Using the HSA<sup>LR</sup> DM1 Transgenic Mouse Model. To determine whether microtubule perturbations have a similar effect on expanded CUG RNA levels in vivo, we tested the effects of colchicine treatment in a transgenic DM1 mouse model. The HSA<sup>LR</sup> mouse model expresses ~220 CUG repeats under the human skeletal actin promoter in the absence of DMPK sequence context (49). HSA<sup>LR</sup> mice were treated with 0.4 mg/kg colchicine or phosphate-buffered saline (PBS) (control) daily for 14 d through i.p. injection, followed by RNA extraction from quadriceps muscle for analyses of gene expression and alternative splicing (Fig. 3). Treatment with colchicine resulted in a significant reduction in the relative abundance of HSA transgene mRNA containing the CUG expansion without affecting the levels of endogenous Dmpk transcript (Fig. 3A and B). Importantly, RNA-seq analysis revealed very little global transcriptome change following colchicine treatment (Fig. 3C and Dataset S3). Approximately 1.3% of the transcriptome was altered, with only 1 transcript showing a greater than 2-fold change (Fig. 3C). In total, there were 181 genes up-regulated.

Fig. 3. Colchicine treatment selectively reduces HSA transgene mRNA levels and partially rescues missplicing in the HSA<sup>LR</sup> DM1 mouse model. (A and B) Effect of colchicine treatment (0.4 mg/kg) or PBS (control) for 14 d by i.p. injection on HSA transgene mRNA levels (A) or endogenous Dmpk levels (normalized to Gtf2b) (B) (n = 6 age- and gender-matched littermates in each treatment group, mean ± SD). ****P < 0.0001. (C) MA plot displaying gene-expression differences comparing colchicine-treated to PBS control-treated mice. Gene-expression change (log<sub>2</sub> fold) is plotted against the mean of normalized counts. Red points represent changes with adjusted P < 0.1. (D) Heat map displaying the rescue of alternative cassette exon inclusion by mean normalized PSI identified from the top 100 missplicing events (top 50 cassette inclusion and top 50 exclusion events) in HSA<sup>LR</sup> mice treated with colchicine compared to HSA<sup>LR</sup> mice treated with PBS (control) and wild-type mice (P < 0.0005, FDR < 0.05). (E) RT-PCR isoform analysis of the indicated alternative cassette exon events following treatment with PBS-control or colchicine (mean, n = 6). ***P < 0.001. (F) Immunofluorescence against Clcn1 protein in colchicine-treated and PBS control-treated HSA<sup>LR</sup> mouse quadriceps muscle sections. DAPI staining for nuclei is shown in blue.
This number is an overestimate because the toxic CUG repeats cause transcriptional dysregulation (50). Comparing the transcriptional changes in the treated mice to mice without repeats reduced the total off-target events to 218 and showed that 113 transcripts reverted to expression levels comparable to mice without CUG repeats (SI Appendix, Fig. S10). Collectively, these data demonstrate that microtubule inhibition in vivo leads to a selective reduction in expanded CUG RNA levels without broadly affecting the transcriptome.

A selective reduction in HSA transgene mRNA containing the CUG expansion is expected to reverse MBNL-dependent mis-splicing pertinent to DM1. To evaluate potential rescue of mis-splicing from colchicine treatment, we analyzed RNA-seq data from wild-type and HSA LR mice to identify a panel of the top 100 cassette exon mis-splicing events (top 50 cassette exon inclusion and top 50 exclusion events showing the greatest change in normalized exon percent spliced in [PSI] between wild-type and HSA LR mice) (Dataset S4). Of these, we identified multiple mis-splicing events showing varying levels of rescue from 14 d of colchicine treatment (Fig. 3D). Several of these include well-characterized targets in DM1 patients directly contributing to muscle phenotypes, such as Atp2a1, Tnnt3, and Clasp1 (10, 11) (Fig. 3D). We then validated rescue of MBNL-dependent mis-splicing using RT-PCR for Clasp1 and Atp2a1 events and the Clcn1 event known to cause myotonia through reduced muscle...
chloride channel protein levels when misspliced in DM (9, 12) (Fig. 3E). Staining for Clcn1 protein in muscle sections of the colchicine-treated HSA4ΔK mice confirmed increased Clcn1 protein in muscle membranes compared to control HSA4ΔK mice (Fig. 3F). Thus, colchicine treatment selectively reduces toxic expanded CUG RNA levels in vivo, leading to a partial correction of missplicing in a DM1 transgenic mouse model.

**Validation of Colchicine in Primary DM1 Patient Cells.** Having validated the effect of colchicine in 2 independent transgenic systems in vitro and in vivo, we next sought to test its effect in primary patient cells containing the repeat expansion within the endogenous genomic context. We used myoblasts derived from a DM1 patient containing an expansion of ~1,900-3,000 CTG repeats in the 3′ UTR of DMPK (51). These primary myoblasts were differentiated into postmitotic myotubes, then treated with colchicine, and the effects on r(CUG)EXP foci and rescue of the myoblasts (51) did not reverse missplicing of the nuclear envelope, forming a cellular relay system to transmit cytoplasmic stimuli to the nucleus (56). Notably, we observed a significant increase in the inclusion of SYNE1 (encoding NESPRI N1) exon 137 in control myotubes treated with colchicine (SI Appendix, Fig. S13).

To directly test if LIN C complex activity played a role in the selective reduction of r(CUG)480 levels resulting from microtubule perturbations, which could be through direct interaction with the CTG expansion, for example, at the nuclear lamina or potentially through an unknown factor (Fig. 5E), we treated HeLa (CTG)480 clone 19 with small interfering RNAs (siRNAs) against the LIN C complex core components SUN1/2 from this reduction in r(CUG)480 levels (Fig. 5D). Consistent with the established sensitivity of cancer cells to microtubule inhibitors, an FDA-approved, natural microtubule inhibitor currently used in the clinic. Our results provide proof of
principle for the identification of compounds and cellular targets that selectively modulate r(CUG)$_{\text{exp}}$ levels in DM1 using cell-based screening.

Our observations of a partial rescue in DM1-relevant mis-splicing in multiple models (Figs. 2E, 3D and E, and 4B) warrants further evaluation of colchicine. This study is not sufficient to address the therapeutic efficacy of colchicine or of general microtubule inhibition for the treatment of DM1. It is important to determine if there is a positive trade-off between therapeutic potential, the discovery of microtubule dynamics, which we postulate may be at least in part driven by interactions with the LINC complex. Independent of therapeutic potential, the discovery of microtubule involvement in the selective reduction of r(CUG)$_{\text{exp}}$ levels is interesting. By taking an approach that selectively measures toxic CUG RNA levels, we were able to identify a potential role of microtubule dynamics, which we postulate may be at least in part driven by interactions with the LINC complex. Compared to previous screens which have not reported these targets, our approach may provide an advantage by selectively measuring the toxic CUG RNA levels directly, rather than downstream consequences, such as ribonuclear foci or missplicing. Several recent studies have highlighted the importance of microtubules in regulating chromosome functions such as transcription, DNA replication, and repair (45, 57, 60–62). Many of these studies implicate an interaction between cytoplasmic microtubules and chromosomes via the LINC complex. Furthermore, telomere repeats are specifically targeted to the nuclear envelope through interactions with SUN1 protein to promote homolog pairing during meiosis in normal gametogenesis (63). Thus, one hypothesis is that microtubule perturbations may directly interfere with LINC complex-mediated transcriptional positioning of the expanded CUG repeat tract within the nucleus. Consistent with this hypothesis, we observed an effect of knocking down LINC complex components SUN1/SUN2 (Fig. 5 E–G). It was recently demonstrated that disease-associated tandem repeats, including CTG repeats, associate with boundaries of chromatin domains, with repeat expansions disrupting this nuclear organization (64). It is not known if perturbing this organization potentially through a LINC complex–microtubule axis may impact transcription of these repeat sequences. However, we cannot at this stage rule out the effect to be secondary. For example, de Lange and colleagues (45) identified a 53BP1, LINC complex, and microtubule dynamics-mediated activity facilitating chromatin mobility in double-strand-break repair of DNA, including telomere repeats. Intriguingly, another recent study found evidence for microtubule filaments present in the nucleus that function in DNA double-strand-break repair (62). Although the exact nature by which microtubules may interact
with chromatin is not yet clear, in light of these results, one plausible hypothesis is that expanded CTG repeats undergo a similar DNA-repair process relying on microtubule dynamics. We may then observe reduced r(CUG)EXP levels as a consequence of microtubule perturbations, leading to transcriptional interference during failed repair of the expanded CTG repeat tract. However, microtubules are important for many functions within the cell, and, thus, to separate direct and secondary effects, future studies are necessary.

Although the precise mechanism through which microtubule inhibition selectively reduces r(CUG)EXP levels remains cryptic, the identification of hits that function in a CTG repeat-selective manner from a modest library of 1,280 compounds validates our approach and is encouraging for future studies involving larger compound libraries. Given the recent success of CRISPR/Cas9 knockout screens (65), we have established an amenable platform for such screens to probe genome-wide targets that selectively reduce r(CUG)EXP levels. Finally, as there are over 30 neurological and neuromuscular disorders involving microsatellite expansions, our approach to repeat-selective screening may be applicable to a wide variety of repeat expansion disorders.

**Methods**

**Generation of Stable DM1 HeLa (CTG)480 Cell Lines.** Stable barcoded HeLa cell lines expressing r(CUG)0 and r(CUG)480 were generated as described (26). Briefly, (CTG)480 and (CTG)480 DMPK (exons 11–15) expression cassettes (37) containing a 22-bp unique sequence plus an additional 8-bp unique barcode for pooled RNA-seq were inserted into pac156 (gift from Albert Cheng, The Jackson Laboratory, Farmington, CT), a plasmid with PiggyBac transposon terminal repeats as well as a puromycin selection cassette. Both plasmids were transiently transfected together with the PiggyBac mPB transposase into HeLa cells (ATCC) and selected by puromycin. Single cells were isolated by flow cytometry, and colonies were cultured in 96-well plates. Colonies were expanded and subjected to measurement of barcoded transcripts by amplicon sequencing to screen for integration and expression from both plasmids as described (26).

**LOPAC1280 Screen.** Approximately 1 × 10^4 cells per well were plated and cultured overnight in 200 μL of medium containing Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin under standard conditions of 37 °C and 5% CO2. The next day, medium was removed and replaced with fresh medium (100 μL) containing drug at a final concentration of 1 μM or DMSO as a control. Following ~24 h of treatment, cells were washed with PBS, and the plates were stored at ~80 °C. The following day, plates were thawed, and each well was incubated with 20 μL of 0.25% (wt/vol) IgG per well containing 10 mM Tris·HCl (pH 7.5) and 150 mM NaCl on an orbital shaker for 3 h at room temperature. Two microliters of “IgGase” was then directly taken forward to cDNA generation by using HT_RT_primer (IDT) (SI Appendix, Table S1) and SuperScript IV RT (Thermo Fisher). qPCR was carried out on a Bio-Rad C1000 Thermal Cycler by using Hot Start Taq 2× Master Mix (NEB) with HT_Forward and HT_Reverse primers (IDT) (SI Appendix, Table S1) and custom HT_Prbe1 and HT_Prbe2 fluorescent probes (IDT) (SI Appendix, Table S1). Data were analyzed by using the comparative ΔΔCt method. The levels of r(CUG)480 from drug treatments were normalized to r(CUG)0 and presented as relative mRNA levels by comparing to DMSO control treatments.

**Cell Culture and Treatment of DM1 Myoblasts.** Primary patient and control myoblast cell lines were derived from muscle biopsy samples under a University of Florida-approved Institutional Review Board (IRB) protocol with informed consent from all subjects. For cell culture of myoblasts, ~1 × 10^5 cells were plated per well in 12-well plates in DMEM-2 BulletKit growth medium (Lonza). Cells were allowed to reach >90% confluence and then differentiated for 7 d to myotubes in DMEM-F12 50:50 medium (Corning) supplemented with 2% (vol/vol) donor equine serum (HyClone). After 7 d, the differentiation medium was replaced with SKGM-2 growth medium, and the indicated concentrations of drug were added. Myoblasts were harvested at 72 h of treatment. Quantitative real-time PCR was performed by using SYBR Green Supermix (Bio-Rad) according to the package insert using primers (IDT) listed in SI Appendix, Table S2. Samples were run on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad) and analyzed by using the comparative ΔΔCt method.

**Fluorescence in Situ Hybridization Microscopy.** Cells were cultured in 8-chamber slides with or without drug treatments as indicated. Cells were fixed with 4% paraformaldehyde (PFA), permeabilized by using 70% ethanol, and prehybridized in the presence of rRNA (Thermo Fisher catalog no. AM7119) for 30 min at 37 °C. Cells were probed for 4 h at 50 °C with a Cy3-labeled r(CUG)_0 probe (IDT). Slides were washed with 42 °C prewarmed 40% (vol/vol) formamide in 2× saline sodium citrate and mounted by using ProLong Diamond Antifade mountant with DAPI (Life Technologies). Nuclei were imaged on a Zeiss LSM 840 confocal scanning microscope with a 40× oil objective.

**Immunofluorescence Microscopy.** The protocol was carried out as described with minor modifications (66). For myoblasts, r(CUG)480 mice treated with either PBS (control) or 0.4 mg/kg colchicine as a positive control were used. Incubation with Cy3-CAG)0 probe (IDT) was then directly taken forward to cDNA generation by using HT_RT_primer (IDT) (SI Appendix, Table S1) and SuperScript IV RT (Thermo Fisher). qPCR was carried out on a Bio-Rad C1000 Thermal Cycler by using Hot Start Taq 2× Master Mix (NEB) with HT_Forward and HT_Reverse primers (IDT) (SI Appendix, Table S1) and custom HT_Prbe1 and HT_Prbe2 fluorescent probes (IDT) (SI Appendix, Table S1). Data were analyzed by using the comparative (ΔΔCt) method. The levels of r(CUG)480 from drug treatments were normalized to r(CUG)0 and presented as relative mRNA levels by comparing to DMSO control treatments.

**Cell Culture and Treatment of DM1 Fibroblasts.** Primary patient and control fibroblast cell lines were derived from skin biopsies under a University of Florida-approved IRB protocol with informed consent from all subjects. Approximately 3.5 × 10^4 cells were plated per well in 24-well plates and cultured to confluence in DMEM supplemented with 15% FBS and 1% penicillin and streptomycin under standard conditions of 37 °C and 5% CO2. Once at confluence, medium was replaced with DMEM supplemented with 0.15% FBS and 1% penicillin and streptomycin with colchicine dissolved in DMSO added to the indicated concentrations, and cells were cultured under standard conditions of 37 °C and 5% CO2 for ~48 h.

**Colchicine Treatment in Mice.** Mouse handling and experimental procedures were conducted in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care. All of the procedures for animal experiments were approved by the institutional animal care and use committee of Osaka University. HSA<sup>4</sup> transgenic mice in line 20b (FVB inbred background) were described (49). Xenogene-matched homozygous HSA<sup>4</sup> mice of 6–7 wk of age were treated with colchicine at a concentration of 0.4 mg/kg as a PBS solution by daily i.p. injection for 14 d. The control group received PBS. Mice were killed 1 d after the final injection, and vastus lateralis muscles (quadriceps) were obtained for splicing analysis. RNA extraction and cDNA preparation were carried out as described (13). Quantitative real-time PCR of human skeletal actin (HSA) transgene RNA and mouse G3PDH and Dmpk RNA was performed by using the TaqMan Gene Expression Assay (Thermo Fisher) assay on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) and analyzed by using the comparative (ΔΔCt) method.
well in 96-well plates in SkGM-2 BulletKit growth medium (Lonza). Cells were allowed to reach >90% confluency and then differentiated for 7 d to myotubes in DMEM/F-12 50/50 medium (Corning) supplemented with 2% vol/vol donor equine serum (HyClone). After 7 d of differentiation and 72 h of drug/DMSO treatment, medium was replaced with fresh SkGM-2 growth medium, and PrestoBlue cell viability reagent (Thermo Fisher) was added to the cells according to the package insert and incubated at 37 °C and 5% CO₂ protected from light for 3 h. Absorbance at 570 and 600 nm was read on a BioTek Cytation 3 plate reader. The 570-nm/600-nm absorbance ratios were calculated for all samples with a background subtraction of the average 570-nm/600-nm values of no-cell plus drug/DMSO control wells.

For HEK293 cells, 5 × 10⁵ cells were plated per well in 96-well plates in DMEM with 10% FBS and 1% penicillin/streptomycin overnight. The next day, medium was replaced with fresh medium containing drug or DMSO (control) and incubated for 48 h, following which the medium with drug/DMSO was replaced with fresh medium and PrestoBlue cell viability reagent (Thermo Fisher) was added according to the package insert, and the cells were subsequently treated as described above.

RNA-Seq Library Preparation. The protocol was carried out as described with minor modifications (66). RNA was isolated from cells by using an Aurum Total RNA mini kit (Bio-Rad) according to the package insert with on-column DNase1 treatment. For mouse experiments, RNA was TRIZol-extracted from total RNA mini kit (Bio-Rad) according to the package insert with on-column DNase1 treatment. 

RMATs with colchicine treatment; 4% of all input RNA from each sample. The manufacturer’s protocols were followed, with the following exceptions: 40X adaptor dilutions were used; all bead incubations were done at RT; we used 4X lower concentrations of index primers; and 10 cycles of library amplification were performed. The resulting libraries were pooled in equimolar amounts, quantified by using the KAPA Library Quant Kit for Illumina, quality checked via capillary electrophoresis on Fragment Analyzer using the RNA 6000 NR Kit (Agilent), and sequenced using paired-end, 75-base-pair sequencing on the Illumina NextSeq 500 massively parallelly sequenced at the University of Florida Center for NeuroGenetics.

Transcriptome Analysis from RNA-Seq Data. The protocol was carried out as described with minor modifications (66). Raw reads were checked for quality and aligned to GRCm38.p5 mouse genome by using STAR (Version 2.5.1b) (67) and a .gtf file generated from Version M16 GENCODE gene models. Uniquely aligned pairing sequences were input to Stringtie (Version 1.3.4d), and the prepDE.py script (offered with Stringtie package) was used to generate gene counts. Differential expression analysis was performed with DESeq2 (Version 1.16.1) (68). Differential expression was considered significant with P < 0.1. Of those events, a percent rescue of ≥10% was considered "Rescue" with colchicine on a Fragment Analyzer using the NGS Analysis DNF-474 kit (Advanced Analytical), and sequenced using paired-end, 75-base-pair sequencing on the Illumina NextSeq 500 massively parallelly sequenced at the University of Florida Center for NeuroGenetics.

% rescue = [100 - (WT_EXP + drug_EXP)] / (WT_EXP)] * 100. [2]

Splicing Analysis from RNA-Seq Data. The protocol was carried out as described with minor modifications (66). Raw reads were checked for quality and aligned to GRCm38.p5 mouse genome by using STAR (Version 2.5.1b). After reads were aligned, rMATs (Version 3.2.5) (69) was used to analyze isoform abundances and compared to 3 wild-type sample sets. ES events were considered significant with a false discovery rate (FDR) ≤ 0.1 and P < 0.01. Events were considered misspliced in the wild-type vs. HSA LR datasets if the PSI change was ≥15% for a given ES event. To determine the percent rescue of a given ES event, Eq. 3 was used, where HSA PSI = PSI of PBS control-treated HSA LR mice, WT PSI = PSI of wild-type mice, and drug PSI = PSI of HSA LR mice treated with colchicine.

% rescue = (HSA PSI - drug PSI) / (HSA PSI - WT PSI) * 100. [3]
et al