Elimination of Toxic Microsatellite Repeat Expansion RNA by RNA-Targeting Cas9

Highlights

- RNA-targeting Cas9 (RCas9) supports efficient targeting of repetitive RNAs
- An RNA endonuclease fused to nuclease-null Cas9 enables an RNA-specific CRISPR system
- An RCas9 system with truncated Cas9 can be packaged in adeno-associated virus
- RCas9 reverses splicing defects in myotonic dystrophy type 1 patient cells

Authors

Ranjan Batra, David A. Nelles, Elaine Pirie, ..., Kevin D. Corbett, Maurice S. Swanson, Gene W. Yeo

Correspondence
geneeyo@ucsd.edu

In Brief

An RNA-targeting Cas9 system induces degradation of microsatellite repeat expansion RNAs, highlighting the potential of RNA-targeting CRISPR systems for therapeutic purposes.
Elimination of Toxic Microsatellite Repeat Expansion RNA by RNA-Targeting Cas9

Ranjan Batra,1,2,3,10 David A. Nelles,1,2,3,10 Elaine Pirie,1,2,3 Steven M. Blue,1,2,3 Ryan J. Marina,1,2,3 Harrison Wang,1,2,3 Isaac A. Chaim,1,2,3 James D. Thomas,4 Nigel Zhang,1,2,3 Vu Nguyen,1,2,3 Steven M. Blue,1,2,3 Ryan J. Marina,1,2,3 Harrison Wang,1,2,3 Isaac A. Chaim,1,2,3 James D. Thomas,4 Nigel Zhang,1,2,3 Vu Nguyen,1,2,3 Steven M. Blue,1,2,3 Ryan J. Marina,1,2,3 Harrison Wang,1,2,3 Isaac A. Chaim,1,2,3 James D. Thomas,4 Nigel Zhang,1,2,3 Vu Nguyen,1,2,3 Steven M. Blue,1,2,3 Ryan J. Marina,1,2,3 Harrison Wang,1,2,3 Isaac A. Chaim,1,2,3 James D. Thomas,4 Nigel Zhang,1,2,3 Vu Nguyen,1,2,3 Steven M. Blue,1,2,3 Ryan J. Marina,1,2,3 Harrison Wang,1,2,3 Isaac A. Chaim,1,2,3 James D. Thomas,4 Nigel Zhang,1,2,3 Vu Nguyen,1,2,3 Steven M. Blue,1,2,3 Ryan J. Marina,1,2,3 Harrison Wang,1,2,3 Isaac A. Chaim,1,2,3 James D. Thomas,4 Nigel Zhang,1,2,3 Vu Nguyen,1,2,3 Steven M. Blue,1,2,3 Ryan J. Marina,1,2,3 Harrison Wang,1,2,3 Isaac A. Chaim,1,2,3 James D. Thomas,4 Nigel Zhang,1,2,3 Vu Nguyen,1,2,3 Steven M. Blue,1,2,3 Ryan J. Marina,1,2,3 Harrison Wang,1,2,3 Isaac A. Chaim,1,2,3 James D. Thomas,4 Nigel Zhang,1,2,3 Vu Nguyen,1,2,3 Steven M. Blue,1,2,3 Ryan J. Marina,1,2,3 Harrison Wang,1,2,3 Isaac A. Chaim,1,2,3 James D. Thomas,4 Nigel Zhang,1,2,3 Vu Nguyen,1,2,3 Steven M. Blue,1,2,3 Ryan J. Marina,1,2,3 Harrison Wang,1,2,3 Isaac A. Chaim,1,2,3 James D. Thomas,4 Nigel Zhang,1,2,3 Vu Nguyen,1,2,3 Steven M. Blue,1,2,3 Ryan J. Marina,1,2,3 Harrison Wang,1,2,3 Isaac A. Chaim,1,2,3 James D. Thomas,4 Nigel Zhang,1,2,3 Vu Nguyen,1,2,3 Steven M. Blue,1,2,3 Ryan J. Marina,1,2,3 Harrison Wang,1,2,3 Isaac A. Chaim,1,2,3 James D. Thomas,4 Nigel Zhang,1,2,3 Vu Nguyen,1,2,3 Steven M. Blue,1,2,3 Ryan J. Marina,1,2,3 Harrison Wang,1,2,3 Isaac A. Chaim,1,2,3 James D. Thomas,4 Nigel Zhang,1,2,3 Vu Nguyen,1,2,3 Steven M. Blue,1,2,3 Ryan J. Marina,1,2,3 Harrison Wang,1,2,3 Isaac A. Chaim,1,2,3 James D. Thomas,4 Nigel Zhang,1,2,3 Vu Nguyen,1,2,3 Steven M. Blue,1,2,3 Ryan J. Marina,1,2,3 Harrison Wang,1,2,3 Isaac A. Chaim,1,2,3 James D. Thomas,4 Nigel Zhang,1,2,3 Vu Nguyen,1,2,3 Steven M. Blue,1,2,3 Ryan J. Marina,1,2,3 Harrison Wang,1,2,3 Isaac A. Chaim,1,2,3 James D. Thomas,4 Nigel Zhang,1,2,3 Vu Nguyen,1,2,3 Steven M. Blue,1,2,3 Ryan J. Marina,1,2,3 Harrison Wang,1,2,3 Isaac A. Chaim,1,2,3 James D. Thomas,4 Nigel Zhang,1,2,3 Vu Nguyen,1,2,3 Steven M. Blue,1,2,3 Ryan J. Marina,1,2,3 Harrison Wang,1,2,3 Isaac A. Chaim,1,2,3 James D. Thomas,4 Nigel Zhang,1,2,3 Vu Nguyen,1,2,3 Steven M. Blue,1,2,3 Ryan J. Marina,1,2,3 Harrison Wang,1,2,3 Isaac A. Chaim,1,2,3 James D. Thomas,4 Nigel Zhang,1,2,3 Vu Nguyen,1,2,3 Steven M. Blue,1,2,3 Ryan J. Marina,1,2,3 Harrison Wang,1,2,3 Isaac A. Chaim,1,2,3 James D. Thomas,4 Nigel Zhang,1,2,3 Vu Nguyen,1,2,3 Steven M. Blue,1,2,3 Ryan J. Marina,1,2,3 Harrison Wang,1,2,3 Is...
Overexpression of MRE RNA-bound proteins can compensate for some features of DM1 (Kanadia et al., 2006), but the tissue specificity of sequestered proteins and the toxicity of translated products from MRE RNAs reduces the therapeutic potential of this approach. Thus, an RNA-directed platform encoded in AAV may provide long-term therapeutic value for MRE diseases.

CRISPRs form the bases of bacterial immune systems that recognize and destroy invading genetic material. Reconstitution of these DNA-targeting systems in eukaryotic cells has supported powerful applications in genome editing and other DNA-level manipulations (Sheridan, 2017; Yu et al., 2017). A recent study demonstrated that MREs can be excised from DNA using genome engineering techniques (van Agtmaal et al., 2017), although simultaneous induction of a pair of double-stranded breaks flanking the MRE followed by DNA repair in the absence of the repeat has not been realized with sufficient efficiency. In contrast to RNA-directed systems, genome editing features the risk of permanent, off-target edits to DNA (Schaefer et al., 2017).

We recently established the ability of a repurposed type II CRISPR system to target and track RNA in human cells (Nelles et al., 2016) indicating the potential of other manipulations to RNA using RNA-targeting Cas9 (RCas9). Here, we describe the ability of CRISPR/Cas9 to both visualize and eliminate MRE RNAs in human cells. We observe efficient elimination of all MRE RNAs studied (CUG exp, CCUG exp, CAG exp, GGGGCC exp) and describe a reduced version of the RNA-targeting CRISPR/Cas9 system optimized for AAV packaging. We also observe reversal of disease-associated molecular defects including reduction of polyglutamine proteins and near-complete reversal of DM1-associated splicing defects to resemble healthy patient cells (93% reversal in patient myotubes).

As the most prominent symptoms (myotonia, muscle weakness, and cardiac conduction defects) associated with DM1 are caused by dysfunctional RNA splicing, this efficient splicing reversal indicates the broad efficacy of this approach.

RESULTS

RNA-Targeting Cas9 Supports Imaging of Microsatellite Repeat Expansion RNAs that Cause DM1, DM2, and CAG exp Diseases

We recently demonstrated that GFP-tagged, nuclease-dead Cas9 (dCas9) with an antisense oligonucleotide carrying a protospacer adjacent motif (PAMmer) and a single guide RNA (sgRNA) enables visualization of mRNAs in human cells (Nelles et al., 2016). To target repeat expansion RNAs for visualization (Figure 1), we expressed MRE RNA in COS-M6 cells with plasmids encoding 105 copies of CTG (CTG exp) found in DM1, 300 copies of CCTG (CCTG exp) from DM2, and 80 copies of CAG (CAG exp).
from HD (Bañez-Coronel et al., 2015) and various SCAs. RNA fluorescence in situ hybridization (FISH) for these repeats revealed formation of RNA foci in a manner consistent with typical manifestation of these diseases (Figure 1B) (see probe sequences in Table S1) (Wojciechowska and Krzyzosiak, 2011). We next constructed plasmids encoding sgRNAs utilizing optimized scaffold sequences (Chen et al., 2013) targeting CUG, CCUG, and CAG MRE RNAs. Co-transfection with plasmids expressing repeat RNAs, their cognate sgRNAs, and dCas9-GFP resulted in colocalization of RCas9 signal and RNA foci (Figure 1B) that was absent in the presence of a non-targeting sgRNA (sequences in Table S2). RCas9 localization was dependent on repetitive RNA and not the plasmids, as demonstrated by treatment with DNase (did not alter FISH signal) and RNase (abolished the nuclear foci) (Figures S1A and S1B). Co-staining for nucleolin and FISH against CUG repeat RNA showed little overlap between nucleoli and CUG signal (Figure S1C), suggesting co-localization is not coincidental (Chen et al., 2013). In these experiments, we found that the PAMmer oligonucleotide was unnecessary to promote co-localization of RNA FISH and RCas9 signal that is consistent with previous studies that indicate RNA recognition does not require, but is enhanced by, the PAMmer (Nelles et al., 2016; O’Connell et al., 2014). We conclude that RCas9 enables the visualization of microsatellite repeat expansion RNA in live cells.

RNA-Targeting Cas9 Eliminates CUG\textsuperscript{exp}, CCUG\textsuperscript{exp}, GGGGCC\textsuperscript{exp}, and CAG\textsuperscript{exp} RNA Foci

Next, we investigated whether the PIN domain enhances cleavage of repetitive RNA. We combined increasing concentrations (10–40 μg) of COS-M6 total cell extract (ranging from 0–40 μg of total protein) from cells co-transfected with dCas9-GFP and either a CUG-targeting sgRNA or a non-targeting control sgRNA to evaluate dCas9-GFP binding to 10 ng of \textsuperscript{32P}-labeled (CUG)\textsuperscript{12} RNA. Protein–RNA ternary complex formation in the presence of non-targeting (NT) sgRNA, extract containing dCas9-GFP and a CUG-targeting sgRNA resulted in retarded (CUG)\textsuperscript{12} migration that is dependent on the concentration of the extract (Figure 3A). All measurements were conducted in the absence of a PAMmer. Addition of an anti-GFP antibody prevented the (CUG)\textsuperscript{12} RNA from entering the gel (supershift), indicating that dCas9-GFP is indeed bound to (CUG)\textsuperscript{12} RNA. Further, immunoprecipitation of dCas9-GFP:CUG-targeting sgRNA (and not non-targeting -sgRNA) using an anti-GFP antibody yielded in vitro transcribed \textsuperscript{32P}-labeled (CUG)\textsuperscript{54} RNA (Figure 3B). These results indicate that RCas9 can directly interact with CUG MRE RNA in the absence of a cognate PAMmer.

We next investigated whether the PIN domain enhances cleavage of repetitive RNA. We combined increasing concentrations (10–40 μg) of COS-M6 cellular extract containing CUG-targeting sgRNA and either dCas9-GFP or PIN-dCas9 with radiolabeled (CUG)\textsuperscript{12} RNA and observed that PIN-dCas9 with cognate sgRNA, but not with non-targeting -sgRNA, promoted cleavage of (CUG)\textsuperscript{12} RNA while dCas9-GFP did not (Figure 3C). We next assessed the dose-dependence of this RNA cleavage activity in living cells via transfection of increasing amounts of plasmids encoding dCas9-GFP or PIN-dCas9 with the cognate sgRNA and a plasmid expressing (CUG)\textsuperscript{54} RNA. We observed that CUG repeat RNA was more efficiently eliminated by lower levels of PIN-dCas9 compared to dCas9-GFP (Figures 3D and 3E). Therefore, subsequent repeat RNA elimination experiments were conducted with PIN-dCas9.

We next conducted transcriptional arrest experiments utilizing a drug-inducible reporter to evaluate whether RCas9-based RNA elimination occurs post-transcriptionally (Figures 3F–3I). We utilized a plasmid containing a tetracycline-inducible promoter (tetracycline responsive element or TRE) driving the expression of GFP and exons 11–15 of DMPK, where exon 15 carries 960 CTG repeats (Lee et al., 2012) (Figure 3F). We transfected COS-M6 cells with this plasmid along with a separate plasmid containing the tetracycline transactivator (tTA) and...
Figure 2. Degradation of Microsatellite Repeat Expansion RNA with RNA-Targeting Cas9

(A) Schematic description of elimination of microsatellite repeat expansion RNA with RNA-targeting Cas9 (RCas9) fused to EGFP or PIN domain.

(B) CUG RNA foci measured by RNA-FISH in COS-M6 cells transfected with (CTG)\textsuperscript{105}, either non-targeting sgRNA (NT), CUG-targeting sgRNA (+), or no sgRNA (−), and with (+) or without (−) HA-tagged PIN-dCas9. Scale bars in (B)–(E) are 10 μm.

(C) CUG RNA foci measured by RNA-FISH in COS-M6 cells transfected with (CTG)\textsuperscript{105} and PIN-dCas9, with either non-targeting sgRNA (NT) or CUG-targeting sgRNA (+), and with (+) or without (−) cognate PAMmer.

(D) CCUG RNA foci measured by RNA-FISH in COS-M6 cells transfected with (CCTG)\textsuperscript{300} and PIN-dCas9 or dCas9, with either non-targeting sgRNA (NT) or CCUG-targeting sgRNA (+).

(E) GGGGCC RNA foci measured by RNA-FISH in COS-M6 cells transfected with (GGGGCC)\textsuperscript{120} and PIN-dCas9 or dCas9, with either non-targeting sgRNA (NT) or GGGGCC-targeting sgRNA (+).

(F) Quantification of RNA-FISH signal in COS-M6 cells transfected with various MREs and PIN-dCas9 or dCas9 with MRE-targeting (+) or non-targeting (NT) sgRNA. Cells containing at least 1 RNA focus are considered positive for MRE RNA. Measurements are normalized to the condition with the MRE-targeting sgRNA and MRE RNA but lacking dCas9. Error bars denote SDs determined from 3 biological replicates enumerating 100 transfected cells each.

(G) RNA dot blot of (CUG)\textsuperscript{exp} levels in COS-M6 cells transfected with (CTG)\textsuperscript{105}, CTG-targeting or non-targeting (NT) sgRNA, and various forms of Cas9 (PIN-dCas9, dCas9-GFP, and wtCas9). U6 snRNA served as a loading control in (G)–(I).

(H) RNA dot blot of (CCUG)\textsuperscript{exp} levels in COS-M6 cells transfected with (CCTG)\textsuperscript{105}, CCTG-targeting or non-targeting (NT) sgRNA, and various forms of Cas9 (PIN-dCas9, dCas9-GFP, and wtCas9).

(I) RNA dot blot of (GGGGCC)\textsuperscript{exp} levels in COS-M6 cells transfected with (GGGGCC)\textsuperscript{105}, GGGGCC-targeting or non-targeting (NT) sgRNA, and various forms of Cas9 (PIN-dCas9, dCas9-GFP, and wtCas9).

See also Figures S2 and S3 and Tables S1 and S2.
Figure 3. RCas9 Binds and Targets CUG Repeat Expansion RNA for Degradation
(A) Gel shift assay of in vitro transcribed radiolabeled (CUG)$^{12}$ RNA and increasing doses of COS-M6 cellular extract (0, 1.25, 2.5, 5, 10, 20, or 40 μg of total cellular protein) containing dCas9-GFP and sgRNAs (1.5 μg transfected plasmid per 1.5 million cells) targeting this repetitive RNA (CUG sgRNA) or a non-targeting control (NT-sgRNA) with 40 μg cell extract. The rightmost lane includes a GFP antibody (40 μg cell extract).

(B) Immunoprecipitation and dot blot quantitation of radiolabeled in vitro transcribed (CUG)$^{54}$ after incubation with extract from cells expressing dCas9-GFP and sgRNAs targeting this repetitive RNA (CUG) or a non-targeting control (NT).

(C) In vitro cleavage assay of radiolabeled (CUG)$^{12}$ RNA combined with increasing concentrations of extract (10, 20, 40 μg) from COS-M6 cells expressing dCas9-GFP or PIN-dCas9 and a CUG-targeting sgRNA or nontargeting (NT) sgRNA, as indicated.

(D) RNA dot blot assay of CUGexp RNA levels in COS-M6 cells transfected with plasmids encoding (CTG)$^{105}$, and the indicated amounts of dCas9-GFP (top) or PIN-dCas9 (middle). U6 snRNA served as a loading control. Bottom: densitometric quantification.

(E) Scheme describing the relative activity of dCas9-GFP and PIN-dCas9 in the context of CUG microsatellite repeat expansion (MRE) RNA elimination as observed in (D).

(F) Scheme describing a tetracycline-inducible expression system for (CTG)$^{960}$ repeat expansion in DMPK to assess whether RCas9-mediated repeat expansion RNA can be eliminated in the absence of transcription. TRE is the tetracycline responsive element.

(G) Detection of CUGexp RNA foci by RNA-FISH in COS-M6 cells transfected and treated with doxycycline according to (E). Scale bars are 20 μm.

(H) Quantification of CUGexp-FISH signal in COS-M6 cells transfected and treated with doxycycline according to (E). Cells containing at least 1 RNA focus were considered positive for CUG repeat RNA. Measurements were normalized to the total number of GFP-positive cells. Error bars denote SDs determined from 3 biological replicates enumerating 100 GFP-positive cells each.

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added doxycycline to induce expression of GFP and the repeat-harboring DMPK transcript. 24 hr later, we removed doxycycline to halt transcription of GFP and CUG repetitive RNA. We then transfected dCas9 (PIN-dCas9 or dCas9-GFP) and sgRNA (CUG-targeting or non-targeting). We assessed the amount of CUG repeat expansion RNA by RT-qPCR after 24 hr. We observed that CUG repeat RNA foci appeared upon addition of doxycycline, and after transcription of the repeat was halted, the foci were efficiently eliminated by PIN-dCas9 in the presence of the CUG-targeting but not the non-targeting sgRNA (Figures 3G and 3H). After transcriptional arrest, using PCR primers that interrogate a portion of DMPK exon 15 just upstream of the repeat (Lee et al., 2012), we observed that both dCas9-GFP and PIN-dCas9 efficiently attenuated levels of the repeat-harboring transcript in the presence of CUG-targeting sgRNA (Figures 3I and 3J). This result shows that RCas9 eliminates CUG repeat RNA foci post-transcriptionally. We used a similar approach that involves doxycycline-inducible expression of the hexanucleotide repeat (GGGGCC)90 to also demonstrate a contribution of post-transcriptional elimination of RNA foci (Figure S2A).

RNA-Targeting Cas9 Eliminates Pathogenic Repeat RNA Foci in Patient Cells

We next assessed the ability of RCas9 to eliminate endogenous MRE RNA in myoblasts and fibroblasts prepared from DM1 and DM2 patient biopsies (Gao et al., 2016; Xia et al., 2013), respectively. For purposes of ensuring homogeneity of expression, we constructed lentiviral vectors based upon the LentCRISPRV2 construct (Sanjana et al., 2014), which express U6 promoter-driven sgRNAs targeting CUGexp or CCGUGexp RNAs along with elongation factor-1 short (EFS)-driven PIN-dCas9 and a puromycin-resistant gene that enabled cell selection. Patient cells were infected with lentivirus carrying an sgRNA targeting the cognate repeat RNA or a non-targeting control (Table S2) and subjected to puromycin selection (4 days with 2 μg/mL). Unlike cells that are subject to exogenous repeat RNA expression, ~99% of untreated patient myoblasts and fibroblasts exhibited characteristic nuclear RNA foci of DM1 and DM2. Importantly, while the presence of PIN-dCas9 and non-targeting sgRNA did not reduce RNA foci, PIN-dCas9 with sgRNAs targeting the respective repeat RNAs resulted in near-complete elimination of both DM1 and DM2 RNA foci (Figures 5A–5C) and reduction of CUGexp RNA levels to levels observed in healthy patient cells (Figure 5D).

To assess whether the observed loss of repeat expansion RNA in DM1 patient cells could be attributed to interactions between dCas9 and genomic (DNA) repeat expansions, we constructed lentivirus carrying PIN-dCas9 and two distinct sgRNAs targeting the template DNA strand of the CTG repeat expansion, i.e., guides targeting CAG repeats (“CAG2” and “CAG3”). Both of these guides did not affect CUG repeat RNA levels by dot blot analysis (Figure 5E). To assess whether RCas9 interacted directly with DMPK mRNA harboring the CUG repeat expansion, we conducted RNA immunoprecipitation (RIP) PCR of dCas9 in the presence of sgRNA targeting CUG repeat RNA and normalized to pull-down with non-targeting sgRNA. We observed significant enrichment of DMPK RNA (p < 0.0037 versus an intergenic region) but not of other genes with short CTG or CAG repeats (TCF4 and AR, respectively) (Figure 5F).

We also explored whether RCas9 could reduce endogenous CUG repeat RNA levels via mechanisms at the DNA level: alteration of transcription of the DMPK 3’UTR that harbors the expansion and destabilization of the repeat expansion itself. To investigate the first mechanism, we conducted chromatin immunoprecipitation for RNA polymerase II (pol II) and measured its association at one site upstream and two sites downstream of the repeat expansion in DM1 patient myotubes. We observed no RCas9-induced alterations in pol II occupancy at these sites (Figure S2B and S2C), indicating that progression of elongating pol II through repeat site is not impaired in the presence of the CUG-targeting RCas9 system. In contrast, we observed that the reduction of pol II occupancy at MYOG, encoding the muscle-specific transcription factor myogenin, in DM1 patient cells was rescued

(i) Quantification of CUGexp-containing DMPK transcript levels in COS-M6 cells transfected and treated with doxycycline according to (E) using RT-qPCR. DMPK mRNA levels were normalized to GAPDH and expressed as fold change over no-doxycycline condition. Error bars denote SDs determined from 3 biological replicates.

(J) RNA blot analysis quantifying CUGexp-containing DMPK transcript levels in COS-M6 cells transfected with the tetracycline-inducible expression system for (CTG)960 and treated with doxycycline according to (E). The legend from (i) also applies to this panel. See also Figure S2 and Tables S1 and S2.
with RCas9, suggesting reversal of the impairment of muscle differentiation in DM1 myotubes (Timchenko et al., 2001). We also observed no alteration in transcript levels of the SIX5 gene, whose transcriptional start site is located just 400 bp downstream of DMPK (Figure S2 D). To investigate whether RCas9 leads to contraction of the genomic repeat expansion, we conducted repeat-primed PCR. We detected no difference in the length of the expansion in patient cells between cells treated with targeting and non-targeting RCas9 systems (Figure S2 E). We conclude that CUG-targeting RCas9 reduces endogenous RNA foci in patient cells independent of affecting pol II occupancy.

RNA-Targeting Cas9 Restores MBNL1 Localization and Known Splicing Defects of DM1

DM1 is characterized by the presence of CUG exp RNA foci that sequester splicing factor MBNL1, resulting in widespread dysregulation of AS (alternative splicing) in muscle (Nakamori et al., 2013). We investigated whether CUG-targeting RCas9 reverses these molecular hallmarks of the disease. We first evaluated the localization of GFP-tagged MBNL1 in the presence of (CUG) exp RNA and the RCas9 system. We co-transfected COS-M6 cells with plasmids carrying GFP-tagged MBNL1, PIN-dCas9, and an sgRNA targeting (CUG) exp RNA or a non-targeting sgRNA and visualized (CUG) exp RNA by RNA FISH. We observed reversal of MBNL1 co-localization with RNA foci to a diffuse nuclear pattern upon application of the RCas9 system (Figure 6A).

We observed similar redistribution of endogenous MBNL1 localization evaluated by immunofluorescence with an anti-MBNL1 antibody in primary patient myoblasts infected with lentivirus encoding the RCas9 system. Our result indicates the ability of RCas9 to reverse this key disease biomarker when the repeat is present in its native context (Figures 6 B and 6C).

We next set out to determine the status of established splicing biomarkers (Nakamori et al., 2013) in DM1 patient and healthy control myoblasts and myotubes in the presence of the RCas9 system. Semiquantitative RT-PCR-based splicing assays were carried out in samples subjected to RCas9 with sgRNAs targeting CUG exp RNA or non-targeting controls with primers flanking known alternative exons of the Ca2+-ATPase ATP2A1 (exon 22) also known as SERCA1, insulin receptor IR (exon 11), muscle-blind-like proteins MBNL2 (exon 6) and MBNL1 (exon 6; see Table S5 for primer sequences and targets). In all cases, we observed that the RCas9 system targeting CUG exp RNA resulted in reversal of DM1-specific AS patterns to those of healthy control cells (Figures 6 D).

RCas9 Reduces the Levels of Disease-Associated Polyglutamine Protein

A host of neurodegenerative conditions are linked to the production of polyglutamine (polyQ)-containing proteins (Paulson et al., 2001). We therefore next investigated whether CUG-targeting RCas9 reduces polyglutamine protein levels. We found that RCas9 targeting CUG exp RNA results in a reduction of polyQ protein levels in patient cells (Figure 6 B).
2000) from RNAs harboring expansions of the trinucleotide repeat CAG. We therefore also assessed whether polyQ protein levels could be attenuated by RCas9 targeting CAGexp. We transfected a plasmid carrying 80 CAG repeats along with various versions of Cas9 and CAG-targeting or non-targeting sgRNAs (Figure 6E) into COS-M6 cells. We observed that the levels of polyQ protein produced from this plasmid were dramatically reduced in cells co-transfected with CAG-targeting sgRNA, and either wtCas9, PIN-dCas9, or dCas9, but not dCas9 ΔHNH construct, while no reduction was seen in the absence of the sgRNA or a non-targeting sgRNA. We conclude that RCas9 can target CAGexp RNAs to reduce disease-associated polyQ protein production.

**RNA-Targeting Cas9 Corrects Transcriptome-wide Splicing Defects in DM1 Patient Cells**

Previous reports describe that hundreds of developmentally regulated splicing events are misregulated in DM1 tissues (Batra et al., 2014; Charizanis et al., 2012; Poulos et al., 2011). To study the genome-wide changes in AS, we used RNA sequencing (RNA-seq) to profile global splicing patterns in DM1 and control primary patient myoblasts and myotubes. We performed RNA-seq data analysis using Olego and Quants software suites (Charizanis et al., 2012; Wu et al., 2013) and found 350 high-confidence misregulated AS events ($p < 10^{-4}$, false discovery rate [FDR] < 0.0015, differential index of splicing [dI] > 0.15) in DM1 myotubes compared to control myotubes (Figure 7A; Table S3). The differential splicing index (SI or percent-spliced in [PSI] value) of a specific exon between two compared samples is the dI value. Hierarchical clustering of these top 350 AS changes showed that the control samples are expectedly distinct from the DM1 samples, but strikingly, DM1 patient myoblasts treated with lentivirus encoding the RCas9 system that target the CUG MRE RNA (CTG-DM1) grouped with the control group (Figure 7A). Similar clustering with the control group was also observed for CTG-DM1 myotubes (Figure 7B). 211 significant AS changes were found ($p < 10^{-4}$, FDR < 0.002, dI > 0.15) between control and DM1 myoblasts (Table S3). Remarkably, treatment with lentivirus encoding the CUG-targeting RCas9 system reversed 327 of 350 (93%) DM1-specific AS differences in myotubes and 157 of 211 (74%) AS changes in myoblasts (Figures 7C–7E). The genome browser tracks show RNA-seq reversal of congenital myotonic dystrophy...
related AS defects in CTG-DM1 myoblasts and myotubes (Figure 7F). RT-PCR-based assays validated reversal of aberrant AS detected by our RNA-seq experiments in dystonin (DST), alpha-spectrin 1 (SPTAN1), TEA domain family member 1 (TEAD1), Troponin 2 (TNNT2), and neuron navigator 3 (NAV3) exons (Figure 7G). Quantification of splicing changes is shown in Figure S4. We also determined changes in gene expression after treatment with the CUG-targeting RCas9 system in DM1 myoblasts (Table S3A). Expression of 9 genes was altered (adjusted p < 0.05; Table S3B) and no changes in genes containing non-pathological CTG or CAG repeats were observed in the RNA-seq data (Figure S5B; Table S3B). In COS-M6 cells transfected with (CTG)105 and the CUG-targeting RCas9 system, we observed a drop in DMPK transcript levels but not in TCF4 (CTG) or AR (CAG) transcript levels (Figure S5A). Finally, we observed an increase in the expression of muscle differentiation markers in CTG-DM1 myotubes when compared to NT-DM1 myotubes (Figure 7H). This efficient reversal of DM1-associated splicing defects in myoblasts and increase in myotube differentiation markers (Timchenko et al., 2001) demonstrates that RCas9 can reverse hallmark molecular features of this disease (Figure 7I, uncropped splicing electropherograms depicted in Figure S6).

DISCUSSION

Pathogenic expansions in both coding and non-coding regions of DNA that produce RNAs with toxic gains-of-function are linked to a host of human diseases (La Spada and Taylor, 2010). The mechanisms of MRE-linked toxicity depend on their sequence context, size, protein binding partners, and the properties of translated products. DM-linked C(C)UGexp expansions (DM1-linked CTG exp in the 30 UTR of DMPK and DM2-linked (CCTG)exp in the first intron of CNBP) form nuclear RNA foci and sequester MBNL proteins leading to the loss of MBNL-dependent RNA processing functions. Loss of MBNL function through sequestration is directly responsible for most DM-associated phenotypes that are recapitulated in mouse models of (CTG)exp and Mbnl knockouts (Lee et al., 2013). Hexanucleotide repeats (GGGGCC exp) associated with familial ALS are present in the first intron of the C9orf72 gene (DeJesus-Hernandez et al., 2011), RNA binding protein (RBP) sequestration (Goodwin and Swanson, 2014), RNA splicing alterations (Cooper-Knock et al., 2015; Prudencio et al., 2015), translation of repetitive polypeptides (Ash et al., 2013; Zu et al., 2013), and alteration of nuclear-cytoplasmic transport (Zhang et al., 2015). (CAG)exp is associated with Huntington’s disease, SBMA, and various SCAs in the coding regions of HTT, AR, and ATXN1, ATXN2, ATXN3, and other loci, respectively. The resulting poly-glutamine proteins are toxic in the CNS. Importantly, elimination of the MRE RNAs associated

See also Figures S2 and S6 and Tables S1, S2, and S5.
with these conditions provides a common therapeutic principle that could address gain-of-function toxicity on both RNA and protein levels.

Motivated by the lack of a common and efficient means to engage MRE RNAs, we repurposed the CRISPR/Cas9 system to visualize and target these toxic RNA transcripts. Recent work established the ability of nuclease-null Streptococcus pyogenes Cas9 (spyCas9) fused to GFP (dCas9-GFP) to bind and track RNA in living cells (Nelles et al., 2016). As MRE RNAs frequently form highly localized RNA foci, we initiated our efforts by evaluating the ability of dCas9-GFP to reveal the localization of DM1-associated (CUG)exp RNA (Figure 1B). We observed that only one sgRNA among the three tested sgRNAs was capable of promoting co-localization among dCas9-GFP and RNA-FISH for the repeat expansion RNA, indicating that the structure of (CUG)exp RNA may restrict access of sgRNAs depending on the targeted frame.
Using appropriate sgRNAs, this approach also allowed imaging of CUG\textsuperscript{exp} (DM2) and CAG\textsuperscript{exp} (HD, SBMA) RNA foci (Figure 1B) that indicates the flexibility of this approach in the context of MRE RNA targeting.

We observed that higher doses of dCas9-GFP resulted in elimination of repetitive RNA foci (Figure 2). Consistent with this result, other reports involving RNA-targeting ASOs (that do not form a substrate for RNase H) and programmable RNA binding proteins (lacking nuclease activity) indicate that simple binding to CUG repeats is sufficient to promote their elimination (Wheeler et al., 2009; Zhang et al., 2014). This widely observed phenomenon is likely due to displacement of RNA binding proteins that stabilize these repetitive RNAs. We observed that dCas9-GFP binds directly to CUG repeat RNA both by gel shift and pull-down assays (Figures 3A and 3B), supporting binding-mediated displacement of sequestered RBPs and subsequent destabilization of CUG repeat RNA.

Previous work involving RNA-targeting using CRISPR/Cas9 indicated that an antisense oligonucleotide that carries a short DNA motif (the protospacer adjacent motif or PAM of the form 5’-NGG-3’) can increase the affinity of Cas9 for RNA when hybridized to a target transcript. While the PAMmer increased the efficiency of RNA pull-down by dCas9 (O’Connell et al., 2014) and improved colocalization of dCas9 and FISH signal in the context of targeting non-repetitive RNA (Nelles et al., 2016), the PAMmer was not an obligate requirement for RNA recognition in both these previous studies. Consistent with these reports, we observe RNA recognition in the absence of the PAMmer (Figure 3A). In contrast to previous reports that indicate improved RNA recognition with the PAMmer, we observed no further increase in repeat RNA elimination (Figure 2C) or pull-down efficiency (Figure 3B) in the presence of the PAMmer. This dispensability of the PAMmer in the context of repetitive RNAs may be due to the presence of multiple sgRNA binding sites in target RNAs that increase the on-rate of target recognition. As the PAMmer must be chemically synthesized and cannot be encoded for viral delivery, this system lacking a PAMmer provides permutation of the RCas9 system compatible with AAV-packaged therapeutics.

In addition to supporting AAV-mediated delivery, an ideal therapeutic embodiment of RCas9 in the context of repeat expansion disease is the efficient elimination of repetitive RNAs even at low doses of protein-RNA complexes. We find that a PIN RNA endonuclease fused to dCas9 promotes more efficient repeat RNA elimination at lower doses than the non-nucleolytic dCas9-GFP. Motivated by this efficient elimination of pathogenic RNA, we utilized this PIN-dCas9 fusion in all subsequent studies of RCas9 in patient primary cells. The importance of the PIN domain for efficient RNA elimination supports an RNA-level therapeutic mechanism. Indeed, delivery of the RCas9 system after transcriptional arrest of the CUG\textsuperscript{960} RNA strongly suggests that the elimination of repeat RNA is independent of transcription of the repeat expansion (Figures 3G–3I). Similar albeit less efficient elimination of (GGGGCC)\textsuperscript{90} repeat RNA after transcriptional arrest indicates the importance of an RNA-level mechanism for this repeat as well (Figure S2A). This reduced efficiency of (GGGGCC)\textsuperscript{90} RNA elimination compared to CUG\textsuperscript{960} RNA could be due to slower kinetics of repeat engagement due to its high GC content and highly structured conformation (Fratta et al., 2012) or a contribution of a DNA-level mechanism to hexanucleotide repeat RNA elimination due to its high density of PAM motifs (Sternberg et al., 2014). Cumulatively, these data indicate the predominance of an RNA-level mechanism for elimination of CUG repeat expansion RNA and suggest its importance for other repeat expansions, although a DNA level mechanism cannot be completely discounted for every MRE disease.

RNA-targeting therapeutics must continuously engage pathogenic RNAs as they are transcribed, and AAV provides a promising means to generate therapeutic transgenes such as the RCas9 system for long periods (Naldini, 2015) but are limited to a packaging capacity of ~4.7 kb. Using truncated dCas9 proteins (Sternberg et al., 2015; Nishimasu et al., 2014) fused to the PIN domain, we report versions of the RCas9 system that are capable of reducing repetitive RNA levels that fall within the packaging capacity of AAV. The truncations fused to PIN termed PIN-dCas9(\Delta HNH) and PIN-dCas9(\Delta HNH,\Delta REC2) are ~4.3 and 3.9 kb long, respectively.

Having established that RCas9 engages repeat expansion RNAs in overexpression models, we also assessed the ability of RCas9 to engage repeat expansion RNAs in patient cells. After observing efficient elimination of CUG and CCG repeat expansion RNAs in DM1 and DM2 patient cells, respectively (Figures 5A–5D), we investigated whether this effect could occur at the level of DNA. Cumulatively, our results indicate that sgRNAs targeting both DNA strands do not alter transcriptional dynamics of CTG repeats in patient cells both in terms of CUG repeat RNA levels (Figure 5E) and ChIP-PCR for elongating pol II flanking the repeat expansion (Figures S2B and S2C). These results, as well as assessments of repeat expansion integrity (Figure S2E), indicate that RNA foci elimination in DM1 patient cells is likely not due to DNA-level transcriptional disruption or destabilization of the DNA repeats themselves.

While the elimination of repetitive RNAs provides a common recourse for many MRE-linked diseases, the downstream manifestations of these diseases are diverse. DM1 is a well-known spliceopathy and loss of MBNL proteins through sequestration by (CUG)\textsuperscript{exp} leads to hundreds of AS changes to resemble fetal splicing patterns. As a result, colocalization of MBNL protein with CUG repeat RNA foci is a hallmark of this disease that is efficiently reversed to resemble patterns in healthy patient cells by (CUG)\textsuperscript{960} leads to hundreds of AS changes to resemble fetal splicing patterns. As a result, colocalization of MBNL protein with CUG repeat RNA foci is a hallmark of this disease that is efficiently reversed to resemble patterns in healthy patient cells by RCas9 (Figures 6B and 6C). This result correlates with reversal of DM1-associated splicing defects that occur in an sgRNA-dependent manner (Figures 6C and 6D). Polyglutamine diseases are linked by the presence of CAG repeat expansion translation products that are similarly reduced by the RCas9 system (Figure 6E). Overall, these data indicate the potential of RCas9 to reverse the hallmark molecular defects associated with microsatellite repeat expansion diseases.

RNA-targeting therapeutics must specifically alter target RNAs and avoid unintended alterations to the transcriptome. Transcriptome-wide measurements of RNA splicing revealed highly efficient (>93%) reversal of DM1-associated splicing pathology in patient myotubes (Figure 7E). As myotubes cultured in vitro constitute an approximation of developing muscle tissue, this result indicates the therapeutic potential of RCas9 to broadly reverse DM1 molecular pathology. We also assessed off-target...
effects in the transcriptome and observed <10 expression changes in patient myotubes among treated and untreated cells. These changes could be due to the presence of the RCas9 system or variations in the stage of differentiation among the treated and untreated cells. Indeed, expression of myotube markers were different among these groups (Figure 7H). Surprisingly the expression of TCF4, a gene containing small CTG repeat expansions (~20 repeats) (Du et al., 2015) and CAG repeat-containing gene AR (8–31 repeats) (Edwards et al., 1992) were not affected by the CUG-targeting RCas9 system by both RNA-seq and qPCR. These results were also corroborated by our RIP experiment showing that RCas9 specifically binds DMPPK RNA (>2,700 repeats) in a sgRNA-dependent manner but not TCF4 (off-target with ~20 repeats, Figure 5F). This highlights the specific targeting achieved with RCas9 as off-target genes with small CTG repeat expansions on both the coding (CTG) and template (CAG) DNA strands are not affected.

A major concern among DNA-mediated CRISPR-based therapeutics is the potential for permanent off-target genetic lesions. As the PIN domain fused to nuclease null Cas9 utilized here is RNA-specific, we obviate the risk of permanent off-target DNA alterations as RNA-level effects are limited to the typically short lifetime of RNA (hours to days). Further, the sgRNAs utilized to target CUG repeat expansion RNA cannot efficiently bind DNA as the encoding DNA sequence lacks a canonical PAM.

Overall, our findings indicate the potential of a new therapeutic mechanism for the CRISPR/Cas9 system among a class of neurological and neuromuscular conditions linked to microsatellite repeat expansions. While the precise pathological mechanisms among these diseases are highly diverse, they are linked by the potential of therapeutics that target the RNA products of these repetitive DNA tracts. Beyond these in vitro demonstrations of highly efficient elimination of repeat expansion RNAs, further work is required to evaluate their behavior in vivo utilizing these rationally truncated Cas9 proteins.

STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

- **KEY RESOURCES TABLE**
- **CONTACT FOR REAGENT AND RESOURCE SHARING**
- **EXPERIMENTAL MODEL AND SUBJECT DETAILS**
- **METHOD DETAILS**
  - Constructs and materials
  - Lentivirus production
  - Cell culture and lentiviral infections
  - Transfections
  - RNA fluorescence in situ hybridization
  - RNA isolation and RNA dot blots
  - Electrophoretic mobility shift assay
  - PIN-mediated RNA cleavage assay
  - RNA immunoprecipitation
  - In vitro transcription and immunoprecipitation
  - RNA-seq library preparation and data processing
- **QUANTIFICATION AND STATISTICAL ANALYSIS**
  - Splicing quantification and statistical analysis
  - RNA dot blot quantification

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and five tables and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2017.07.010.

AUTHOR CONTRIBUTIONS


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REFERENCES


STAR METHODS

KEY RESOURCES TABLE

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CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Gene W. Yeo (geneyeo@ucsd.edu). Important plasmids described in this study will be deposited in the Addgene plasmid repository and available under a standard MTA.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Human cell lines and primary cells and an African green monkey kidney cell line (COS-M6) were utilized in this study. Patient myoblast cell lines were derived from muscle biopsies according to a University of Florida-approved IRB protocol involving informed consent of all patients. The genders of the patient cell lines are not available. The Lenti-X HEK293T cell line is derived from human female tissue and COS-M6 from African green monkey male tissue.

METHOD DETAILS

Constructs and materials

Transient transfections in the presence of overexpressed repeat expansion RNAs were conducted with a pair of vectors encoding Cas9 and sgRNA. The dCas9-2xNLS sequence was derived from pHR-SFFV-dCas9-BFPKRAB (a gift from Stanley Qi and Jonathan Weissman; Addgene plasmid no. 46911), tagged with two SV40 NLS’s on the C terminus, and fused to EGFP or PIN or placed without fusion in pCDNA 3.1 (Life Technologies) using Gibson assembly. The sgRNA plasmid carries a human U6 promoter and a modified
sgRNA (Chen et al., 2013) with BbsI sites for insertion of spacer (Nelles et al., 2016). Lentivirus encoding the RCas9 system was created via replacement of wtCas9 in LentiCRISPR v2 (Sanjana et al., 2014) with PIN-dCas9 in-frame with downstream 2A-puromycin present in the parent vector using Gibson assembly. We also replaced the sgRNA scaffold with the modified scaffold mentioned above. All amplifications were conducted with ClonAmp HiFi PCR premix (Clontech).

**Lentivirus production**

LentiCRISPRv2-derived constructs encoding both PIN-dCas9 and a U6 promoter-driven sgRNA were co-transfected with psPAX2 (packaging plasmid) (gift from Didier Trono, Addgene plasmid #12260) and VSV-G (viral envelope) (Stewart et al., 2003) into Lenti-X HEK293T cells (Clontech) at a mass ratio of 5:4:3 using polyethyleneimine (PEI) (4:1 mass ratio PEI:DNA). Viral supernatants were collected and concentrated with Lenti-X Concentrator reagent (Clontech) according to the manufacturer’s protocol (100X concentration) and aliquoted for storage.

**Cell culture and lentiviral infections**

COS-M6 cells were cultured in DMEM with 10% FBS and 1% penicillin/streptomycin (GIBCO) and passaged at 90%–100% confluency. Primary myoblasts were maintained in complete SKGM-2 media (Lonza) and passaged at 70% confluency. Myotube differentiation was carried out by in DMEM with 2% horse serum (GIBCO) on collagen (GIBCO) at 90% confluency.

Myoblasts and myotubes were transduced with 100X concentrated lentivirus 24 hr after plating in growth media. Puromycin selection (2 μg/ml) was initiated at 48 hr and harvested after 3 days of drug selection.

**Transfections**

COS-M6 cells were seeded into 4-chambered glass slides at density of 5x10^4 cells/well for FISH and 3x10^5 cells per well of a 6-well plate for RNA isolation. For FISH, 50ng MRE plasmid was added to 100 μL optiMEM along with 250ng of the appropriate sgRNA and 250ng of the appropriate Cas9 plasmid. For RNA isolation, 150ng of the MRE plasmid was used with 500ng each of sgRNA and Cas9 plasmids. Transfection was carried out using Lipofectamine 3000 according to the manufacturer’s instructions. Cells were incubated for 24 hr at 37°C and 5% CO2. For GGGGCC repeats, 50ng of the MRE plasmid was used along with 500ng each of the sgRNA and Cas9 plasmids (1:10) for slides.

**RNA fluorescence in situ hybridization**

Fixation was carried out for 10 min with 4% PFA after media aspiration and PBS rinse. Next, cells were rinsed 3X with PBS and incubated overnight in the 70% ethanol at 4°C. Next, ethanol was removed and cells rehydrated for 10 min with wash buffer 40% formaldehyde and 2X SSC buffer. Cells were then incubated with pre-hybridization buffer (10% Dextran sulfate, 2mM ribonucleoside vanadyl complex, 2X SSC pH = 5, 50% fresh & deionized RNase-free formamide, 200 μg/mL BSA, 1 mg/ml yeast RNA, DEPC-treated water to attain final target volume) for 15 min at 37°C (65°C for GGGGCC) in a hybridization oven. Probe was denatured at 100°C for 10 min and 500pg/μL (1 μg/μL for G4C2; final concentration) of the probe was added to cold pre-hyb buffer and immediately added to the cells. Cells were hybridized for 2 hr at 37°C (65°C for GGGGCC) in a hybridization oven. Next, cells were washed 3X for 20 min each with wash buffer. Cells were then washed 1X with PBS and slides were mounted with DAPI containing-mounting medium (ProLong Diamond Antifade Mountant with DAPI). Image quantification (Figure 4C) was conducted by counting the number of cells containing at least one RNA focus in the presence of the CRISPR/Cas9 and either NT- or C(C)TG-targeting sgRNA.

**RNA isolation and RNA dot blots**

RNA isolations were carried out with Trizol according to the manufacturer’s protocol. RNA quality and concentrations were estimated using the Nanodrop spectrophotometer or Agilent 2200 Tapestation using the manufacturers’ protocol. 2-5 μg of total RNA was re-suspended in 1mM EDTA, pH 8.0 to a final volume of 50 μL followed by addition of 30 μL 20X SSC and 20 μL 37% formaldehyde to denature the RNA. The RNA was incubated for 30 min at 60°C followed by incubation on ice. The Biorad Bio-Dot apparatus was assembled according to the manufacturer’s protocol with Hybond N+ nylon membrane (GE Healthcare) on the top. The membrane was equilibrated by passing 100 μL of 20X SSC through the slots using vacuum. The denatured RNA was passed through the slots and the membrane was washed with 20X SSC. The membrane was crosslinked at the auto setting in the UV Stratalinker (Stratagene). Expressshyb (Clontech) pre-warmed at 50°C was used to pretreat the membrane for 60 min at 55°C. The probe was end-labeled with α-32P ATP (Perkin Elmer) and T4-PNK (NEB) for 30 min at 37°C, filtered through a G-50 Illustra spin column (GE Healthcare), denatured at 100°C for 10 min and placed on ice. The probe was added to the prehybridization solution (1 μg/mL) being careful to not add it to the membrane directly. Hybridization was conducted overnight and membrane was washed 1X with 1X SSC, 0.1% SDS at 55°C and 2X with 0.5X SSC, 0.1% SDS at 55°C. The membrane was exposed to autoradiography film (Thermo Fisher) with an intensifying screen (Kodak) at ~80°C overnight (~16 hr).

**Electrophoretic mobility shift assay**

1 μg of (CUG)12 RNA (IDT) was 5’ end-labeled using γ-32P-ATP (Perkin-Elmer) and T4-PNK (NEB) in a 100 μL reaction (10 μL 10X PNK buffer, 1 μL of 1 μg/μL RNA, 8 μL T4-PNK, 2 μL or 20uCi γ-32P-ATP, 79 μL RNase free water) at 37°C for 1 hr. The probe was cleaned with G-25 probe-quant micro columns (GE) according to the manufacturer’s protocol. Cellular extracts were prepared 48h
after transfection of COS-M6 cells with sgRNA and Cas9 plasmids (1.5 μg each/1.5 million cells in a 10 cm cell culture dish) using NE-PER kit (Thermo) and combining nuclear and cytoplasmic extracts and supplemented with MgCl₂ to a final concentration of 10mM. Protein concentration was measured using the BCA assay (Biorad). For electrophoretic mobility shift assay (EMSA), increasing concentrations (0, 1.25, 2.5, 5, 10, 20, or 40 μg of total protein) of cold cellular extract was mixed with 10 ng (1 μL of labeled reaction or ~100,000 cpm) of RNA diluted 1:10 with folding buffer (20mM Tris pH 7.5, 150mM NaCl, and 10mM MgCl₂) per reaction on ice. Samples were incubated at 37°C for 45 min for complex formation and run on a pre-ran (10 min) native 6% TBE gel (Novex) with 0.5X TBE buffer supplemented with MgCl₂ to a final concentration of 10mM in cold conditions. The gel was exposed to X-ray film for 30 min.

**PIN-mediated RNA cleavage assay**

32P-labeled (CUG)₁² RNA and cellular extracts were prepared as described for EMSA. Increasing concentrations (10, 20, or 40 μg of total protein) of cold cellular extract was mixed with 10 ng (1 μL of labeled reaction or ~100,000 cpm) of RNA and the reaction was incubated at 37°C for 1h. 2X RNA loading dye (95% deionized formamide, 5% RNase-free water, xylene cyanol, bromophenol blue) was added and the reaction was denatured at 65°C for 5 min and immediately transferred to ice. The samples were run on a 6% TBE-urea gel and gel was exposed to an X-ray film for 30 min and developed as described above.

**RNA immunoprecipitation**

RNA immunoprecipitation (RIP) was carried out as described previously (Niranjanakumari et al., 2002) with modifications. DM1 patient myoblasts were transduced with lentivirus containing either NT or CTG-targeting sgRNA and PIN-dCas9-HA followed by puromycin selection as described above. Cells were fixed with 0.5% formaldehyde (v/v) in PBS. The cross-linking was stopped with 0.25M glycine (in PBS) for 5 min at RT, and cells were washed with PBS 3X for 2 min each. Fixed cells were lysed in 500 μL of RIPA buffer (50 mM Tris–Cl, pH 7.5, 1% Igepal, 0.5% sodium deoxycholate, 0.05% SDS, 1mM EDTA, 150mM NaCl) containing protease inhibitor cocktail (Roche) and DNA digestion was carried out using 10U of TurboDNase (Thermo) for 10 min at 37°C. The samples were spun at 4°C in a microcentrifuge at full speed for 20 min and supernatant was used for IP. Dynabeads protein A (Thermo) coated with anti-HA antibody were used for IP with lysate at 4°C for 2 hr. The beads with Protein-RNA complexes were washed 3 times with RIPA buffer. DNAse digestion was repeated and complexes were washed with RIPA buffer again. 1 mg/ml Proteinase K (NEB) was used to digest proteins and RNA was extracted using RNA phenol (Thermo) and chloroform. cDNA preparation was done using Superscript III (Thermo) with random primers according to the manufacturer’s protocol. qPCR was carried out with primers for DMPK, TCF4, AR and an intergenic region.

**In vitro transcription and immunoprecipitation**

CUG repeats were in vitro transcribed using the MAXIscript SP6 in vitro transcription kit (Thermo) and α-32P UTP (Perkin Elmer). Cellular extracts were prepared 48 hr post transfection with either NT-sgRNA and Cas9-GFP or CUG-targeting sgRNA and Cas9-GFP with 1×TBS (50mM Tris-HCl pH 7.0, 150mM NaCl) and 0.1% Igepal followed by sonication in the bioruptor. Equal CPM amounts of RNA was mixed with 500 μg (protein content) of cellular extract and 1mg/ml yeast tRNA for 60 min at 37°C. Dynabeads M-280 sheep anti-rabbit (Thermo) were coated with anti-GFP antibody (Abcam Ab290) and immunoprecipitation was carried out for 2 hr at RT. The beads were washed with Tris-HCl, 0.1% Igepal 5X or until the IgG control sample did not have any detectable radiation as measured by a Geiger counter. The RNA was eluted using Proteinase K in PK buffer for 30 min at 37°C and was blotted on Hybond N+ membrane using the Bio-Dot apparatus as described above. The membrane was washed 1X with 1X SSC and developed with autoradiography film (Kodak) with an intensifying screen at –80C for 2-4 hr.

**RNA-seq library preparation and data processing**

Library preparation and sequencing was done as described previously (Batra et al., 2016, NSMB). Total RNA was isolated using TRIzol reagent (Life Technologies) and treated with Turbo DNase (Life Technologies). Libraries were prepared with Illumina TruSeq polyA mRNA Sample Preparation reagents according to the manufacturer’s protocol. All samples were sequenced on the Illumina HiSeq 4000 platform. Demultiplexed Fastq files were checked for quality and aligned to the hg19 human genome platform. Cluster 3.0 software and Java Treeview were used in combination to perform and visualize results from hierarchical gene expression clustering results. For AS analysis, we used Olego and Quantas software suite as previously described (Batra et al., 2016; Wu et al., 2013).

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Splicing quantification and statistical analysis

RNA-seq data processing involved alignment with the Olego de-novo splice junction aligner and were quantified using Quantas. Quantas calculates exon inclusion indices (SI or splicing index equivalent to PSI values) and differential splicing measures between
two groups (di values). Fisher exact test in Quantas is used to call FDR adjusted P values. We use FDR < 0.05 and di > 0.15 as significant AS events.

**RNA dot blot quantification**
RNA dot blots and western blots were quantified using the gel analysis function in FIJI (Schindelin et al., 2012).

**DATA AND SOFTWARE AVAILABILITY**

The accession number for the data reported in this paper is GEO: GSE100943.
**Figure S1. Colocalization of RCas9 and FISH-Targeting CUG Repeat RNA Is Not Due to Recognition of Encoding Plasmid or Nonspecific Nucleolar Association, Related to Figure 1**

(A) RNA-FISH for (CUG)\textsuperscript{exp} RNA using the (CAG)\textsuperscript{10} probe in COS-M6 cells transfected with a plasmid encoding (CTG)\textsuperscript{105}, with (top) and without (bottom) DNase treatment of cells prior to FISH. Scale bars represent 20 \( \mu \text{m} \).

(B) RNA-FISH using the (CAG)\textsuperscript{10} probe for (CUG)\textsuperscript{exp} RNA in COS-M6 cells transfected with a plasmid encoding (CTG)\textsuperscript{105} with (top) and without (bottom) RNase treatment of cells prior to FISH. Scale bars represent 20 \( \mu \text{m} \).

(C) RNA-FISH for (CUG)\textsuperscript{exp} RNA using the (CAG)\textsuperscript{10} probe, and immunofluorescence for nucleolin (top) and control omitting the primary antibody for nucleolin (bottom). Scale bars represent 20 \( \mu \text{m} \).
Figure S2. RCas9 Localizes to DMPK mRNA and Does Not Alter Polymerase II Occupancy at the DMPK Locus, Alter Expression of a Transcriptionally Downstream Gene, or Cause Gross Destabilization of the Repeat Expansion DNA in Patient Cells, Related to Figures 2, 3, and 5–7

(A) Quantification of RNA foci in the presence of doxycycline-inducible expression of the hexanucleotide repeat, and a non-targeting (NT) or targeting (GGGCC) sgRNA, and dCas9, as indicated.

(B) RNA polymerase II ChIP-qPCR with primers flanking sequences upstream and downstream of the CTG repeat expansion in control or DM1 patient myoblasts transduced with either non-targeting (NT) or CUG targeting (CUG) sgRNA and PIN-dCas9 compared to an intergenic region and the muscle marker MYOG. Error bars represent SD.

(C) DMPK gene structure with placement of primers used in the ChIP-PCR.

(D) qPCR of SIX5 transcript in DM1 patient and healthy cells treated with the RCas9 system.

(E) Repeat-primed PCR of the CTG repeat expansion in patient cells after treatment with the RCas9 system.
Figure S3. Elimination of RNA Foci with Various Cas9 Modifications, Related to Figure 2

(A) RNA-FISH in COS-M6 cells transfected with either (CCTG)300, (CAG)80 and (GGGGCC)120 with either non-targeting sgRNA or respective cognate targeting sgRNA and the indicated Cas9 constructs. Scale bars represent 20 μm.

(B) Quantification of cells containing RNA foci in same conditions as (A). 100 cells were counted in 3 different fields and results are shown as bar graph normalized by the repeat only condition. Error bars represent standard deviation.
Figure S4. Splicing Validation and Reversal in DM1 Cells after RCas9 Treatment, Related to Figure 7
(A) Electropherograms for splicing validation results shown in Figure 7G.
(B) RT-PCR based validation of splicing changes in AS events altered in DM1 and reversed by CTG-targeting sgRNA and RCas9-PIN in two different DM1 patient myoblast lines DM03 and DM05.
(C) Electropherograms for splicing validation results shown in (B).
Figure S5. Off-Target Effects of RCas9 on CTG and CAG-Repeat-Containing Genes, Related to Figure 7

(A) qPCR showing changes in *DMPK* (CTG), *TCF4* (CTG), and *AR* (CAG) expression after treatment with various combinations of sgRNAs and RCas9-PIN in COS-M6 cells expressing (CTG)_{105} repeats and the non-targeting (NT) or targeting (CUG) sgRNAs, as indicated. Error bars represent standard deviation.

(B) Heatmap of log_{2}RPKM values from RNA-seq analysis showing expression of CUG or CAG containing transcripts in human Control (Ctrl) or DM1 myoblasts treated with either non-targeting (NT) or CUG-targeting (CTG) sgRNA and PIN-dCas9.
Figure S6. Uncropped Electropherograms for Splicing Measurements, Related to Figure 6
(A) Uncropped electropherogram of RT-PCRs for TNNT2 and NAV3 DM1-linked alternative splicing events.
(B) Uncropped electropherogram of RT-PCRs for TNNT2 and NAV3 DM1-linked alternative splicing events with color balance adjusted to highlight TNNT2 splicing changes.
(C) Uncropped electropherogram of RT-PCRs for DST, SPTAN1, and TEAD1 DM1-linked alternative splicing events.