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Supplemental Information

CRISPR/Cas9-Induced $(CTG \cdot CAG)_n$ Repeat Instability in the Myotonic Dystrophy Type 1 Locus:

Implications for Therapeutic Genome Editing

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SUPPLEMENTARY MATERIAL

Supplementary Figures and Legends:

Figure S1. Low off-target cleavage by CRISPR-2 and -3 in LHCN cells. Cleavage of CRISPR-2 and -3 at predicted off-target sites in *CARMIL2*, *EBF3*, *DVL1* and *ALK* was assessed using T7E1 assays. DNA fragments containing these putative sites were PCR-amplified on DNA isolated from transfection-positive pools of CRISPR-treated LHCN cells. *DMPK* amplicons of non-CRISPR treated LHCN (two *DMPK* (CTG)5 alleles) and KM155C25 myoblasts (with (CTG)5 and (CTG)14 alleles) were included as controls. Note that essentially no cleavage products were observed.

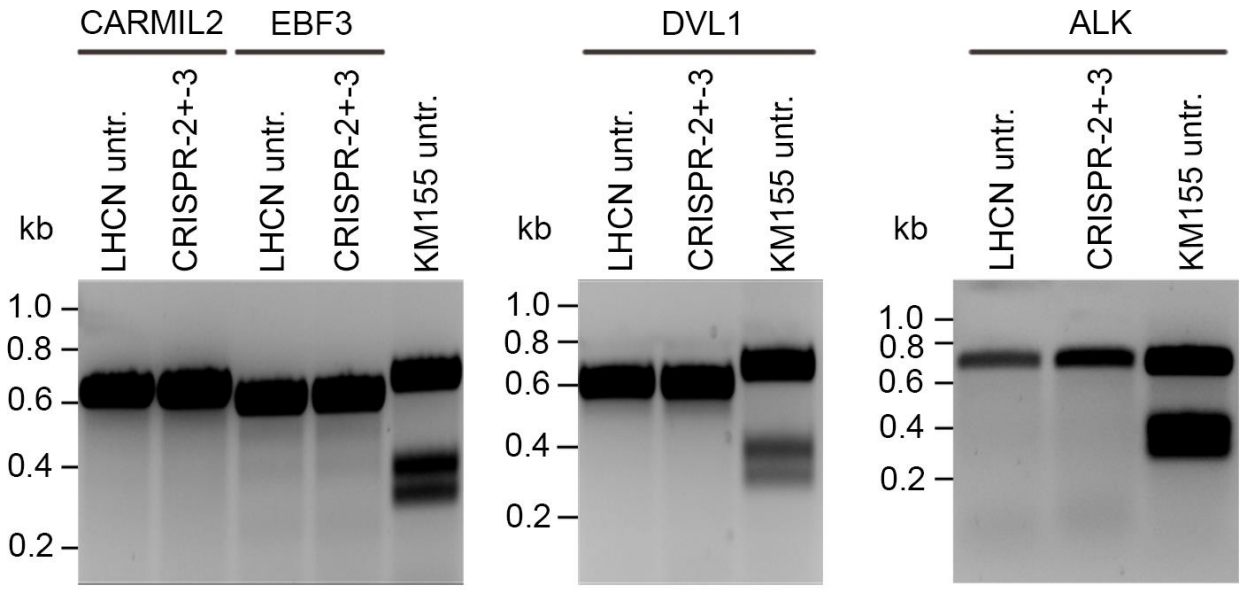
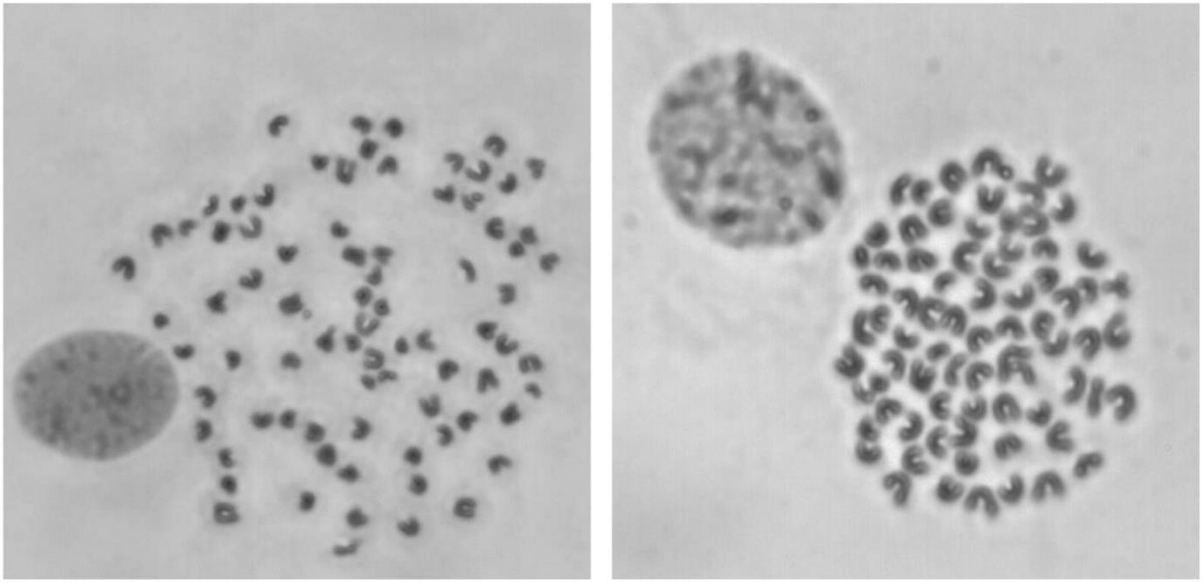


Figure S2. Karyotype analysis of DM500 cell clones. Top: Representative Giemsa-stained chromosome spreads for clone DM500-A2.4, containing two unmodified (CTG•CAG)540/610 repeats, and genome-edited clone DM500-3.6-Δ^2, which lacks the entire ~1.8 kbp area containing the (CTG•CAG)540/610 repeat and flanking segments from both chromosomes. Bottom: Listing of chromosome counts for these and other genome-edited DM500 cell clones described in the text (normal tetraploidy, 4n =80).

DM500-A2.4 DM500-A3.6-Δ^2

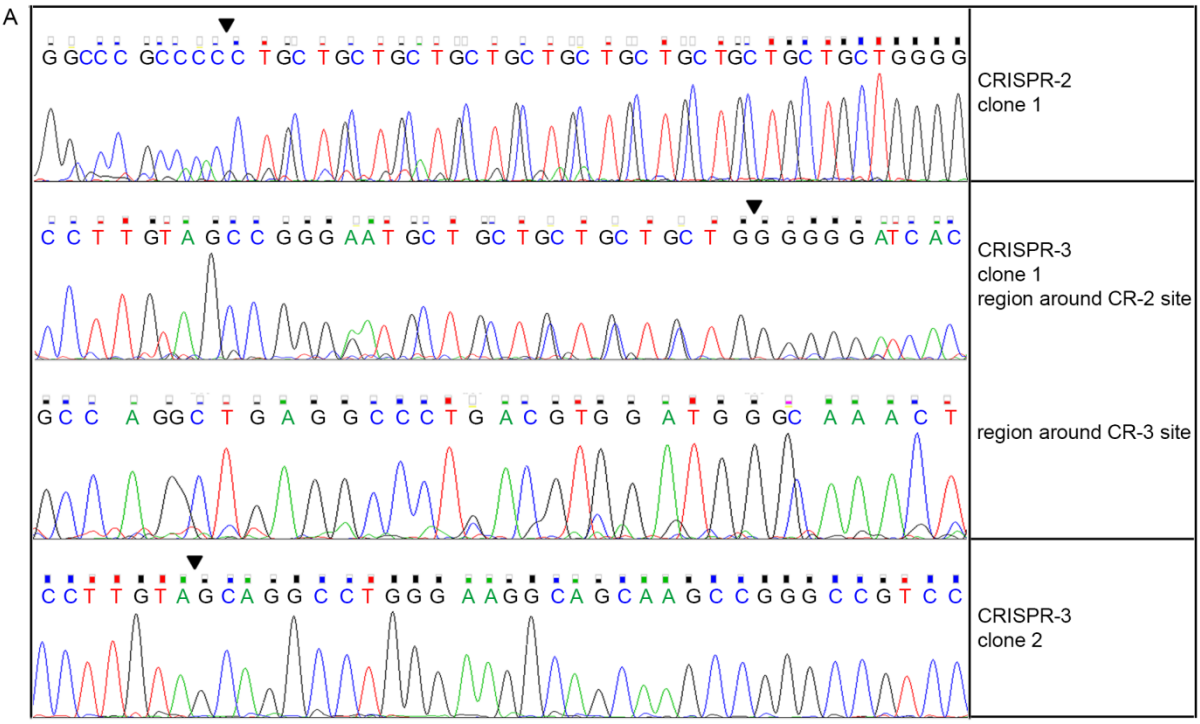


Chromosome count of DM500 myoblasts with and without (CTG•CAG)500

DM500-A1.4	DM500-A2.4	DM500-A2.6	DM500-A1.3-Δ^2	DM500-A2.3-Δ^2	DM500-A3.6-Δ^2
80	77	80	80	80	78
78	77	77	82	77	74
77	79	78	82	74	76
80	79	78	79	79	77
76	78	80	80	78	80
80	79	75	76	77	76
79	80	79	77	78	76
77	77	80	80	78	75
77	79	78	78	80	78
78	80	78	80	80	78

Figure S3. Deletion of the DM500 repeat region after cleavage with a single CRISPR. Sequence verification of excision of the repeat-containing segment in three different DM500 clones treated with CRISPR-2 or CRISPR-3 only (related to Fig 4). DNA sequencing profile (A) and sequences (B) of the *DMPK* exon 15 region in three DM500 clones. Fusion sites are indicated by arrow heads. The first two bases of the sequences shown in A are underlined in B.

A



B

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                    45,770,368
                    *
WT          CTCCAGTCCTGTGATCCGGGCCCGCCCCCTAGCGGCCGGGGAGGGAGGGGCCGGGTCCGCGGCCGGCGAACG
CR-2 cl1    CTCCAGTCCTGTGATCCGGGCCCGCCCC----------------------------------------------------------------------------------------
CR-3 cl1    CTCCAGTCCTGTGATCCGGGCCCGCCCCCTAGCGGCCGGGGAGGGAGGGGCCGGGTCCGCGGCCGGCGAACG
CR-3 cl2    CTCCAGTCCTGTGATCCGGGCCCGCCCCCTAGCGGCCGGGGAGGGAGGGGCCGGGTCCGCGGCCGGCGAACG

                                CR-2
                                 |                                (CTG)500
WT          GGGCTCGAAGGGTCCTTGTAGCCGGGAATG{CTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG}GGGGG
CR-2 cl1    ---------------------------------------------------- CTGCTGCTGCTGCTGCTGCTGCTGCTG GGGGG
CR-3 cl1    GGGCTCGAAGGGTCCTTGTAGCCGGGAATG CTGCTGCTGCTGCTG ---------------------------------- GGGGG
CR-3 cl2    GGGCTCGAAGGGTCCTTGTA ----------------------------------------------------------------------------------------

                                                                CR-3                    45,770,126
                                                                 |                      *
WT          ATCACAGACCATTTCTTTCTTTCGGCCAGGCTGAGGCCCTGACGTGGATGGGCAAACTGCAGGCCTGGGAAGGC
CR-2 cl1    ATCACAGACCATTTCTTTCTTTCGGCCAGGCTGAGGCCCTGACGTGGATGGGCAAACTGCAGGCCTGGGAAGGC
CR-3 cl1    ATCACAGACCATTTCTTTCTTTCGGCCAGGCTGAGGCCCTGACGTGGATGGGCAAACTGCAGGCCTGGGAAGGC
CR-3 cl2    ---------------------------------------------------------------------------------------- GCAGGCCTGGGAAGGC
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Figure S4. Inversion-deletion of the CRISPR-excised (CTG)13-containing segment in DM11 clone. (A) DNA sequencing profile, indicating inversion of the (CTG)13 repeat-containing segment in *DMPK* exon 15 of the normal-sized allele in clone DM11-1E8. Newly created fusion sites in the *DMPK* DNA sequence are indicated by arrow heads. Note that the double peaks are caused by the copurified PCR product from the edited expanded allele in this clone, from which the repeat sequence was removed and a TG was inserted. (B) *DMPK* sequence that contains the inverted (CTG)13 fragment, aligned with the normal *DMPK* sequence. Alignment shows that the sequence in red, containing a 10 nt deletion (dotted line), is reverse complementary to the WT sequence.

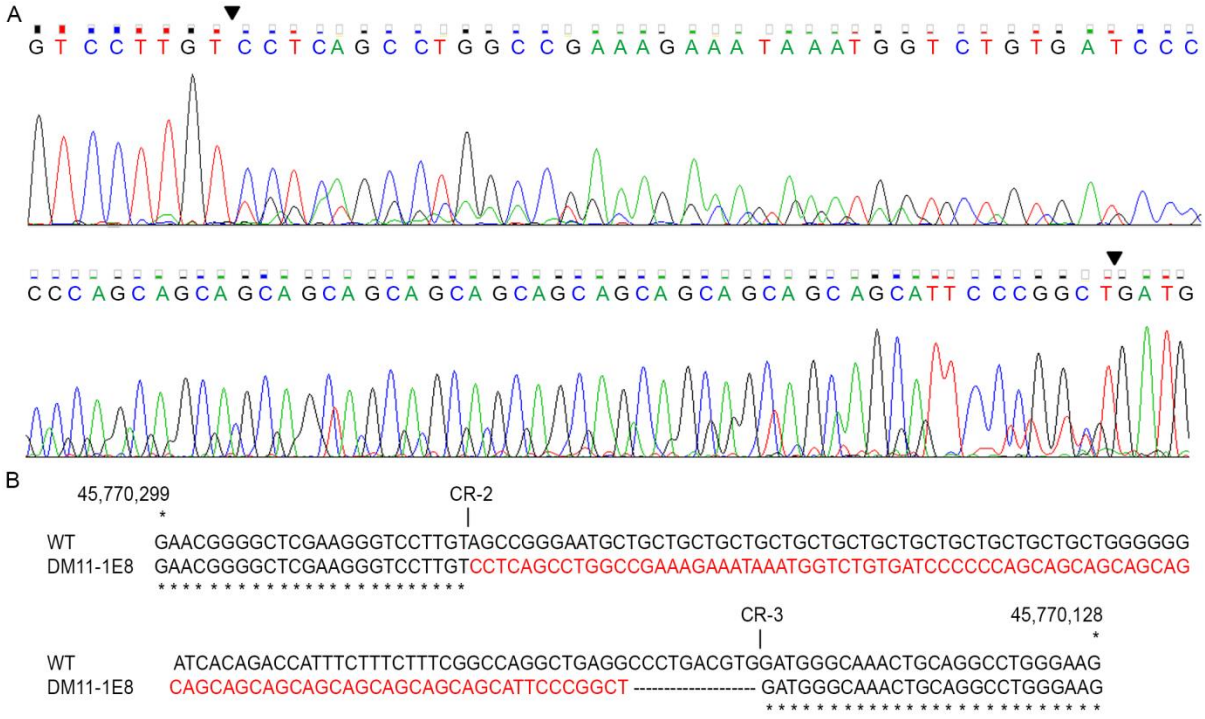


Figure S5. (CTG·CAG)n repeat excision normalizes RNA foci formation in DM11 myoblasts.

(A) Confocal microscope images of RNA-FISH on untreated DM11 cells, clone DM11-4F9 (CTG13/CTG2600) and two DM11-Δ/Δ clones (see Supplementary Table S4). Foci containing DMPK (CUG)2600 RNA were labeled using a (CAG)6-TYE563 LNA probe (red). Nuclei were stained with DAPI (blue). Scale bar: 10 µm. (B) Quantification of nuclear foci in DM11-derivative clones shown in (A). Each symbol represents the number of foci in one nucleus. Mean + SEM. *** $P < 0.005$.

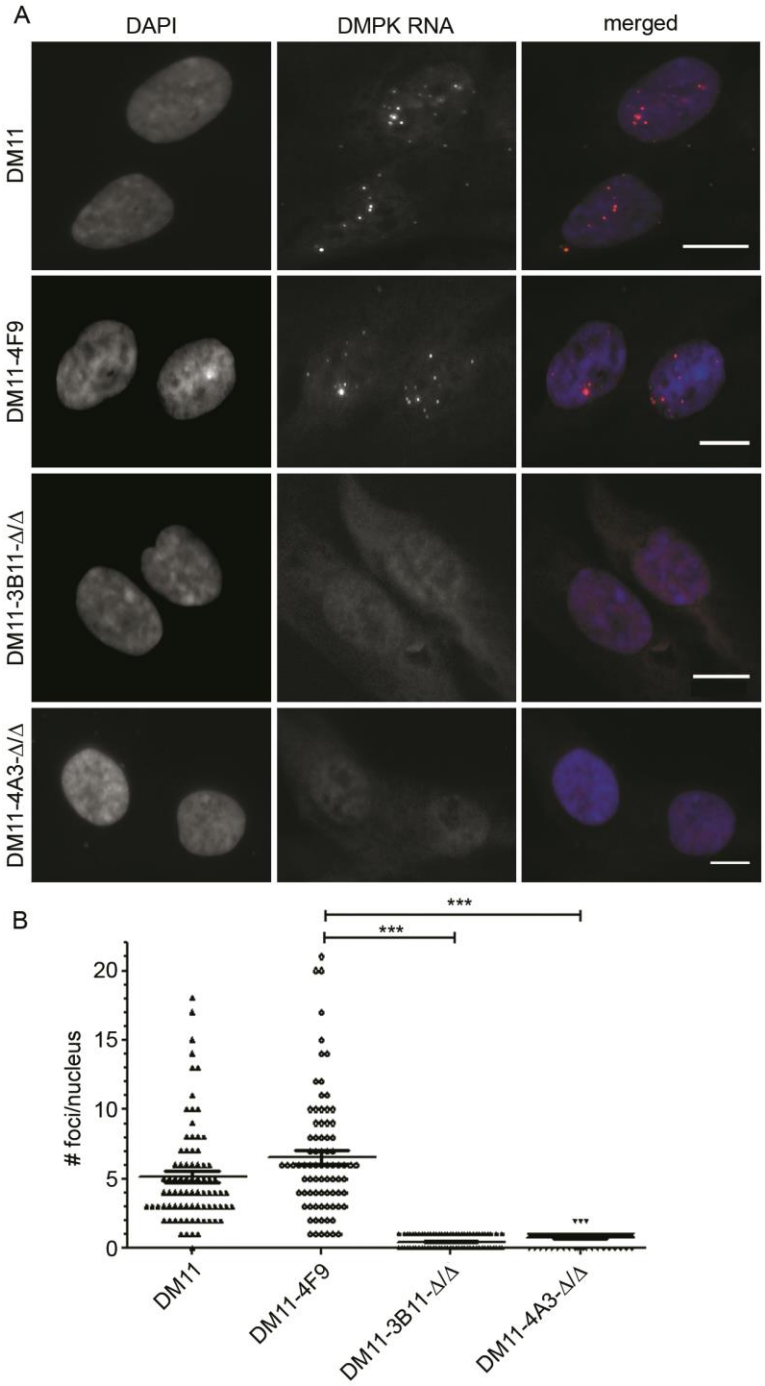


Figure S6. (CTG•CAG)n repeat excision prevents MBNL1 sequestration to RNA foci. (A) Immunofluorescence staining of MBNL1 (green) in untreated DM11 cells, in clone DM11-4F9 (CTG13/CTG2600) and in two DM11-Δ/Δ clones (see Supplementary Table S4). Nuclei were stained with DAPI (blue). Scale bar: 10 µm. (B) Quantification of nuclear foci stained for MBNL1 in DM11 clones by wide-field microscopy analysis shows a significant reduction in foci count in DM11 clones without the expanded repeat.

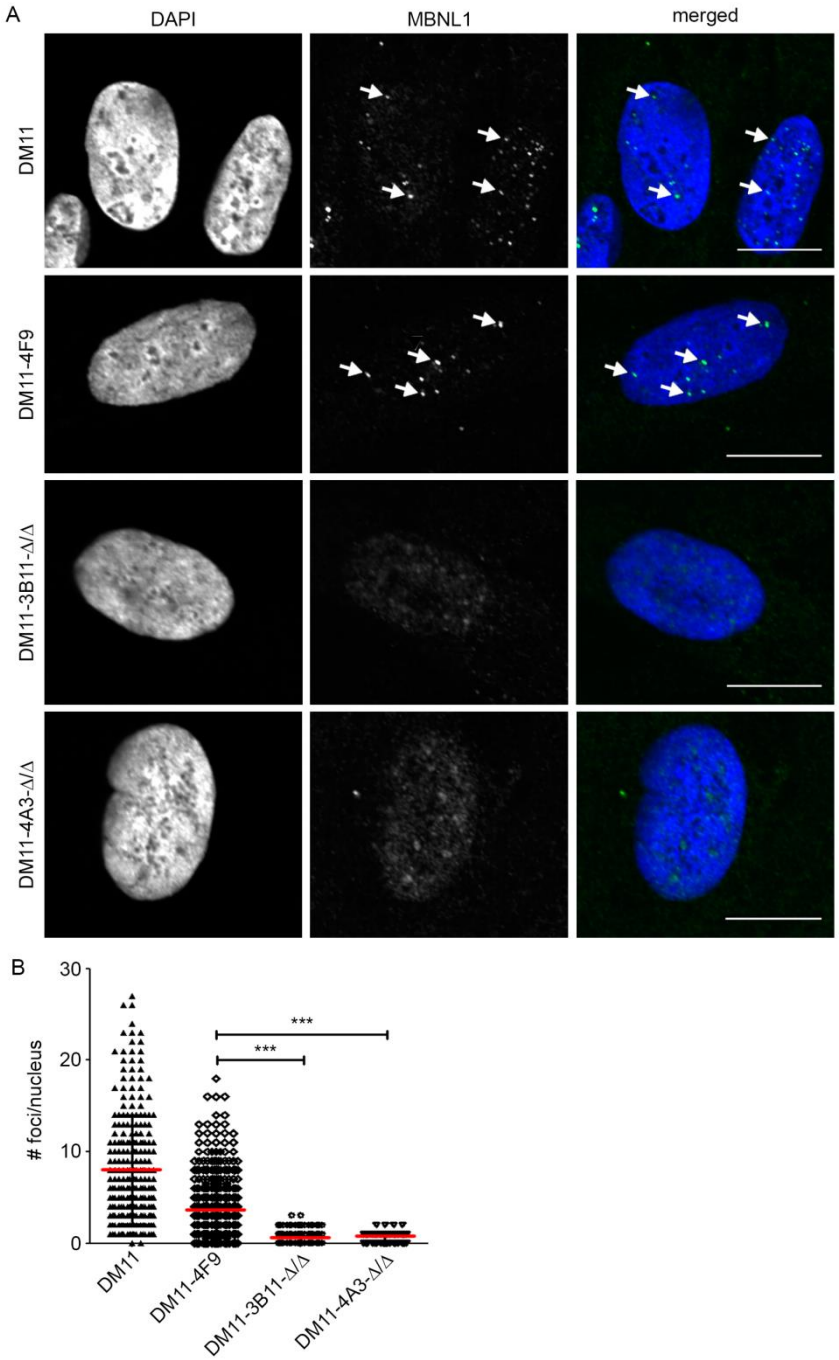
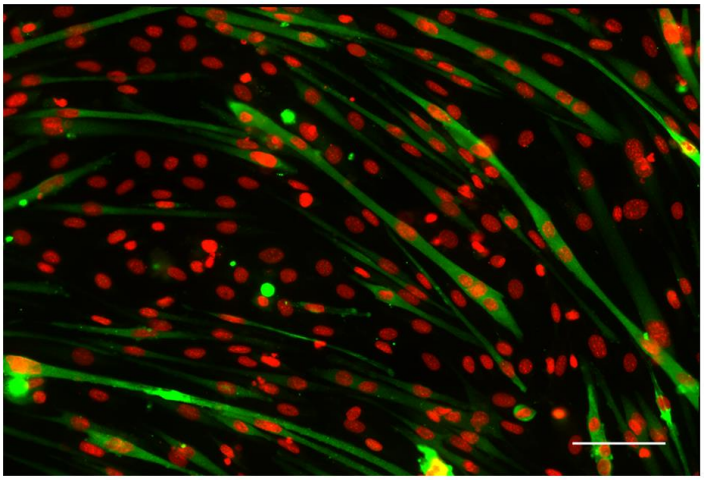
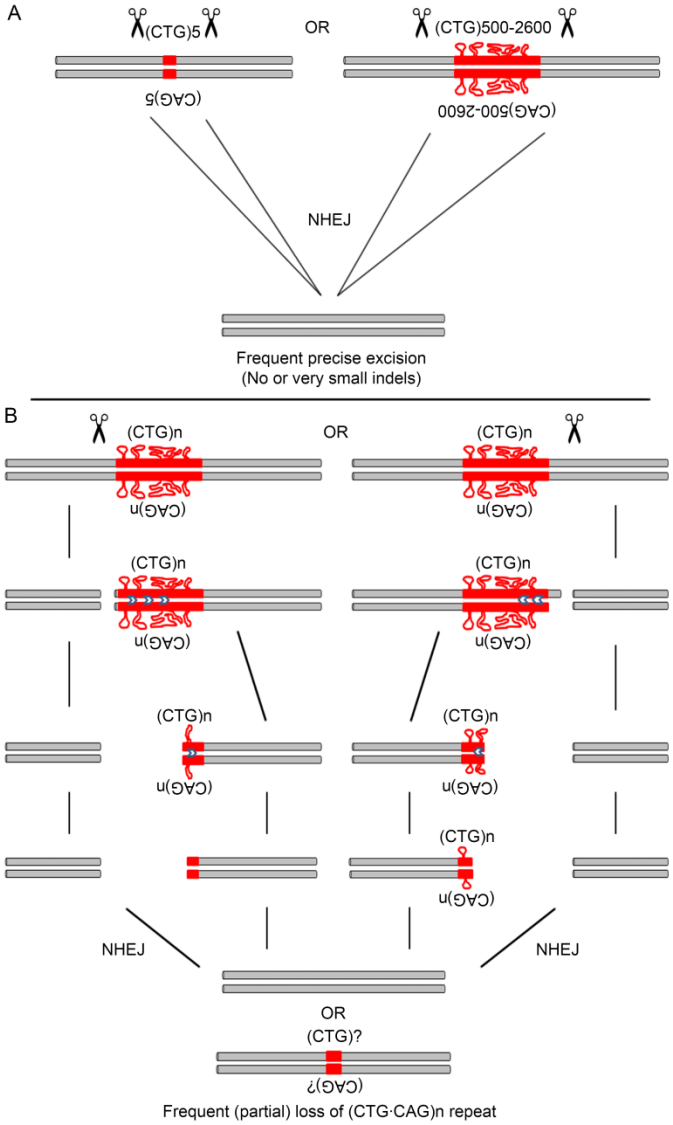


Figure S7. Myogenic capacity of representative gene-edited mouse myoblast clone without (CTG•CAG)n repeat. Immunostaining of MHC expression (green fluorescence) in DM500-A3.5-Δ^2 cells after 5 days in differentiation culture. Nuclei were stained with DAPI (red). Formation of myotubes and preservation of differentiation capacity in edited myoblasts is apparent from the expression of MHC in multinucleated cells. Scale bar: 100 µm.



DM500-A3.5-Δ^2

Figure S8. Model for behavior of the (CTG·CAG)n repeat during CRISPR-mediated genome-editing in the DM1 locus. (A) Dual cleavage with one CRISPR at either side of, and close to, a normal or an expanded (CTG•CAG)n repeat frequently results in clean loss of the entire repeat-containing segment. When the segment carrying the expanded repeat DNA with non-B DNA topology (red curved structure) is simultaneously cut at both ends, it probably gets lost in the nuclear environment and is degraded. Hence it exerts no perturbing effect on the efficiency of NHEJ-mediated resealing of the gap. (B) Single cleavage close to the expanded (CTG·CAG)n repeat promotes formation of one-sided large deletions. We hypothesize that these deletions occur because the DSB exposes abnormal DNA configuration at the side of the repeat segment, while at the same time keeping both ends of the DNA in close proximity. The non-B or slipped-strand structure of the DNA (red structure) is probably a difficult substrate for NHEJ and will first be trimmed by nucleases of the recombination-repair machinery (arrow heads in dsDNA). Note that expanded repeat DNA is uniformly represented as having abnormal topology, even though transitions between normal and non-B slipped strand configuration may be dynamic and cell-type and -state dependent, or be induced after CRISPR cleavage.



Supplementary Tables:

Table S1. Cleavage efficiency of gRNA-expressing vectors determined by T7E1 assay in LHCN cells.

	Target region	Target sequence 5' -> 3'	On-target cleavage efficiency
CRISPR-1	5’ flank	CCGCCCCCTAGCGGCCGGGGAGG	<1%
CRISPR-2	5’ flank	GCTCGAAGGGTCCTTGTAGCCGG	8-21%
CRISPR-3	3’ flank	GCTGAGGCCCTGACGTGGATGGG	~14%
CRISPR-4	3’ flank	GCCTGGCCGAAAGAAAGAAATGG	~18%
CRISPR-5	5’ flank	AGCAGCAGCAGCAGCATTCCCGG	~3%
CRISPR-6	5’ flank	CGAGCCCCGTTCGCCGGCCGCGG	~5%
CRISPR-7	(CTG•CAG)n repeat	TGCTGCTGCTGCTGCTGCTGGGG	<1%

Table S2. Potential off-target sites for CRISPR-2 and CRISPR-3 in the human genome. Mismatches are indicated in red, PAM sequences in blue.

CRISPR-2 target sequence	**GCTCG AAGGG TCCTT GTAGC CGG**
CARMIL2	**AGGGG AAGGG TCCTT GTAGC AGG**
EBF3	**TAGGG AAGGG TCCTT GTGGC TGG**
CRISPR-3 target sequence	**GCTGA GGCCC TGACG TGGAT GGG**
DVL1	**CCAAA ATGCC TGACG TGGAT GGG**
ALK	**AAACG GGCCC TGACG TGGTT TGG**

Table S3. **Summary of CRISPR-induced events across repeats in LHCN, DM500 and DM11 cells (healthy and mutant allele separately) after dual treatment with CRISPR-2 and -3.**

In the column '**Other**' all clones are listed which carry large insertions of unknown origin (>30 nucleotides), inversions, deletions that extend to over the CRISPR-2 or -3 sites and also removed primer sites used for PCR analysis, or combinations of these mutational events. The high percentage (25%) of anomalous editing events in the (CTG•CAG)2600 allele of DM11 cells is explained by a high frequency (21%) of partial deletions across the (CTG•CAG)2600 repeat segment between CRISPR-2 and -3 sites.

Cell line	**Clean deletion between CRISPR sites**	**Clones with deletion of the (CTG•CAG)n repeat tract but imperfect fusion of CRISPR-2 and -3 cleavage sites**	**Small indel at either one of both CRISPR sites**	**Other**	**Unable to call**
LHCN (CTG•CAG)5/5	83 %	0 %	17 %	0 %	0 %
DM500 (CTG•CAG)530/580	30 %	4 %	37 %	7 %	22 %
DM 11 (CTG•CAG)13	62 %	7 %	11 %	8 %	12 %
DM 11 (CTG•CAG)2600	46 %	5%	ND	25 %	24 %

Table S4. **Characteristics of CRISPR-edited clonal myoblasts employed in this study.**

Tetraploid DM500 mouse myoblasts contain two identical transgenic chromosomes, each with one repeat in the (CTG•CAG)540-610 length range; diploid human LHCN or DM11 myoblasts contain two parental chromosomes 19 with allelic (CTG•CAG)5/(CTG•CAG)5 or (CTG•CAG)13/(CTG•CAG)2600 repeats, respectively (see text). The number of nucleotides (nt) in small insertions or deletions (indels) found at CRISPR-2 or -3 cleavage sites is listed for each clone; N.D. is not determined.

Name clone	Species	Repeat length/Repeat fate	Indel types
LHCN-E2.3-Δ/Δ	human	deletion in both alleles	none
LHCN-B2.2-Δ/Δ	human	deletion in both alleles	none
DM500-A1.4	mouse	two (CTG•CAG)540-610 copies	CRISPR-3 site in one copy: +1nt
DM500-A2.4	mouse	two (CTG•CAG)540-610 copies	CRISPR-3 sites: 1x -5nt, 1x -10nt
DM500-A2.6	mouse	two (CTG•CAG)540-610 copies	CRISPR-3 sites: 1x -9nt, 1x -4nt
DM500-A1.3-Δ^2	mouse	deletion in both copies	none
DM500-A2.3-Δ^2	mouse	deletion in both copies	none
DM500-A3.5-Δ^2	mouse	deletion in both copies	none
DM500-A3.6-Δ^2	mouse	deletion in both copies	none
DM11-4F9	human	(CTG•CAG)13 and (CTG•CAG)2600	none
DM11-EA7	human	(CTG•CAG)13 and (CTG•CAG)2600	(CTG•CAG)13, CRISPR-3 site: 1x -1nt (CTG•CAG)2600, CRISPR-3 site: N.D.
DM11-EA11	human	(CTG•CAG)13 and (CTG•CAG)2600	(CTG•CAG)13, CRISPR-3 site: 1x -1nt (CTG•CAG)2600, CRISPR-3 site: 1x -10nt
DM11-3B11-Δ/Δ	human	deletion in both alleles	none
DM11-4A3-Δ/Δ	human	deletion in both alleles	none
DM11-3E3-Δ/Δ	human	deletion in both alleles	CRISPR-2 site in one allele: +1nt CRISPR-3 site in one allele: +1nt
DM11-1E6-13/Δ	human	(CTG•CAG)13 and deletion of (CTG•CAG)2600	(CTG•CAG)13, CRISPR-2 site: -11nt (CTG•CAG)2600, CRISPR-2 site: -15 nt and CRISPR-3 site: -16 nt