CRISPR-Cpf1 correction of muscular dystrophy mutations in human cardiomyocytes and mice

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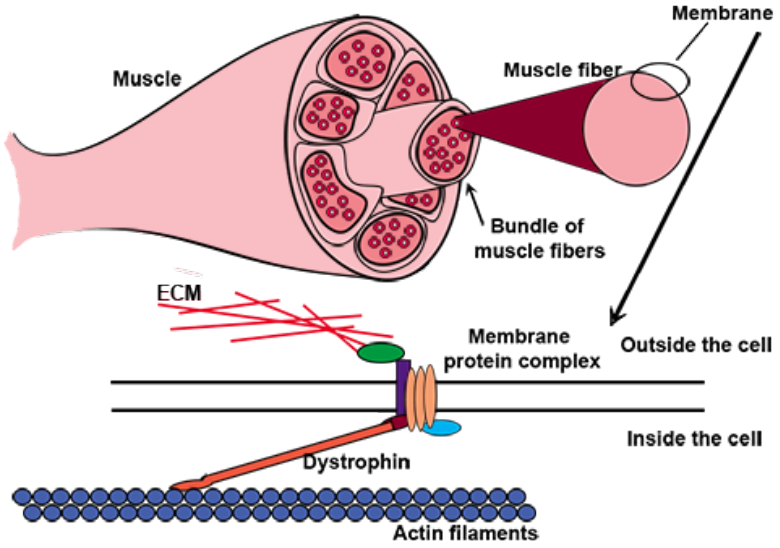
Introduction

- Duchenne muscular dystrophy (DMD) is an X-linked recessive disease caused by mutations in the gene coding for dystrophin
- dystrophin: large cytoskeletal protein essential for the integrity of muscle cell membranes
- DMD causes progressive muscle weakness, premature death by the age of 30 (cardiomyopathy)

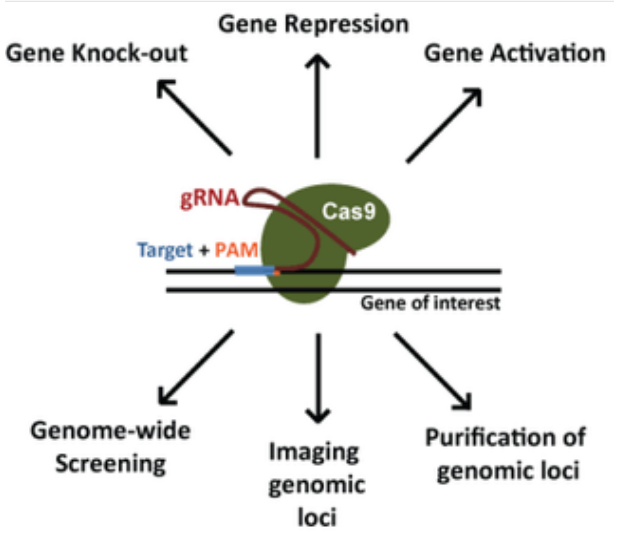


https://www.khanacademy.org/test-prep/mcat/biological-sciences-practice/biological-sciences-practice-tut/e/inherited-disorders--duchenne-muscular-dystrophy

- no effective treatment for this disease
- treatments: delivery of truncated dystrophin, utrophin by recombinant adeno-associated virus (AAV), skipping of mutant exons with antisense oligonucleotides and small molecules

Introduction

- these approaches cannot correct DMD mutations / permanently restore dystrophin expression
- new genome editing method as therapy?: **CRISPR-Cas9 system** (clustered regularly interspaced short palindromic repeats) for the correction of diverse genetic defects
  - adaptive immune system in bacteria that defends against phage infection
  - endonuclease (Cas9) guided to specific genomic sequences by guide RNA (gRNA) resulting in DNA cutting near a protospacer adjacent motif (PAM)
  - large size of SpCas9 reduces efficiency of packaging and delivery in AAV vectors
  - smaller CRISPR enzymes would facilitate precision gene editing
  - new RNA-guided endonuclease Cpf1 (CRISPR from *Prevotella* and *Francisella* 1)



https://www.addgene.org/crispr/guide/

Methods

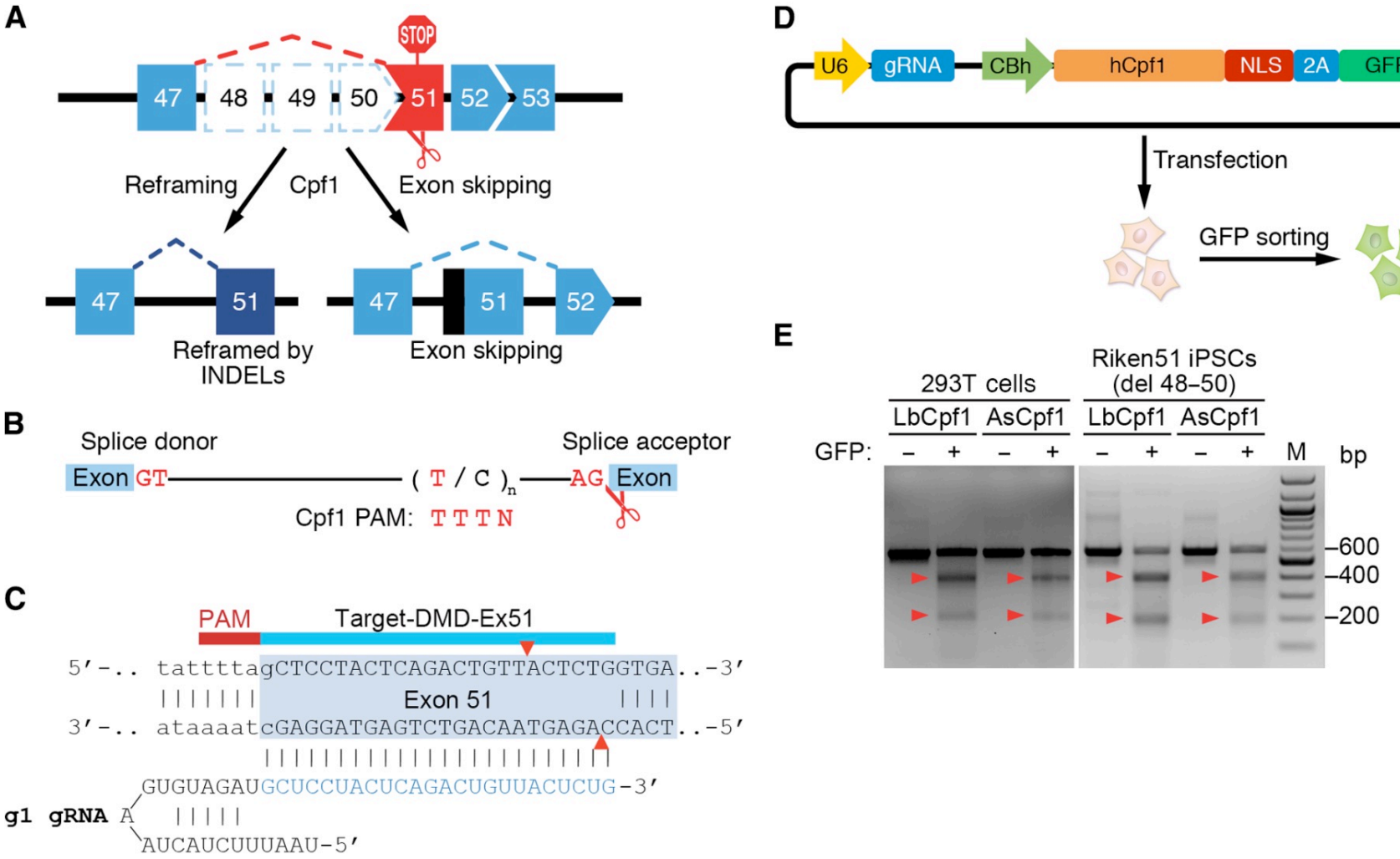
- generation of pLbCpf1-2A-GFP and pAsCpf1-2A-GFP plasmids: LbCpf1 (*Lachnospiraceae bacterium ND2006*) and AsCpf1 (*Acidaminococcus sp. BV3L6*)
- human iPSCs from DMD patient fibroblasts (Riken HPS0164, deletion of exons 48 to 50, introducing a STOP codon within exon 51): nucleofection with plasmids, GFP+ and GFP- cells were sorted, single clones from GFP+ cells → sequencing, iPSCs induced to differentiate into cardiomyocytes (chemical induction)
- genomic DNA isolation (mouse fibroblasts, human DMD iPSCs)
- reverse transcription PCR (primers flanking DMD exons 47 and 52; amplified from WT cardiomyocytes, uncorrected cardiomyocytes and exon 51-skipped cardiomyocytes)
- dystrophin Western blot analysis
- dystrophin immunocytochemistry and immunohistochemistry

Methods

- mitochondrial DNA (mtDNA) copy number quantification (qPCR) → functional analysis of DMD iPSC-derived cardiomyocytes
- cellular respiration rates: oxygen consumtion rates (OCR) determined in human iPSC-derived cardiomyocytes using XF24 Extracellular Flux Analyzer (Seahorse Bioscience)
- in vitro transcription of LbCpf1 mRNA and gRNA
- single-stranded oligodeoxynucleotide: ssODN used as a HDR template (template for correction of mutations)
- CRISPR-Cpf1-mediated genome editing by one-cell embryo injection in *mdx* mice
- PCR amplification of genomic DNA
- T7E1 assay (mismatch-specific T7 endonuclease I assay)
- Tse I RFLP analysis (restriction fragment length polymorphism analysis)
- mouse forelimb grip strength test, serum CK measurement

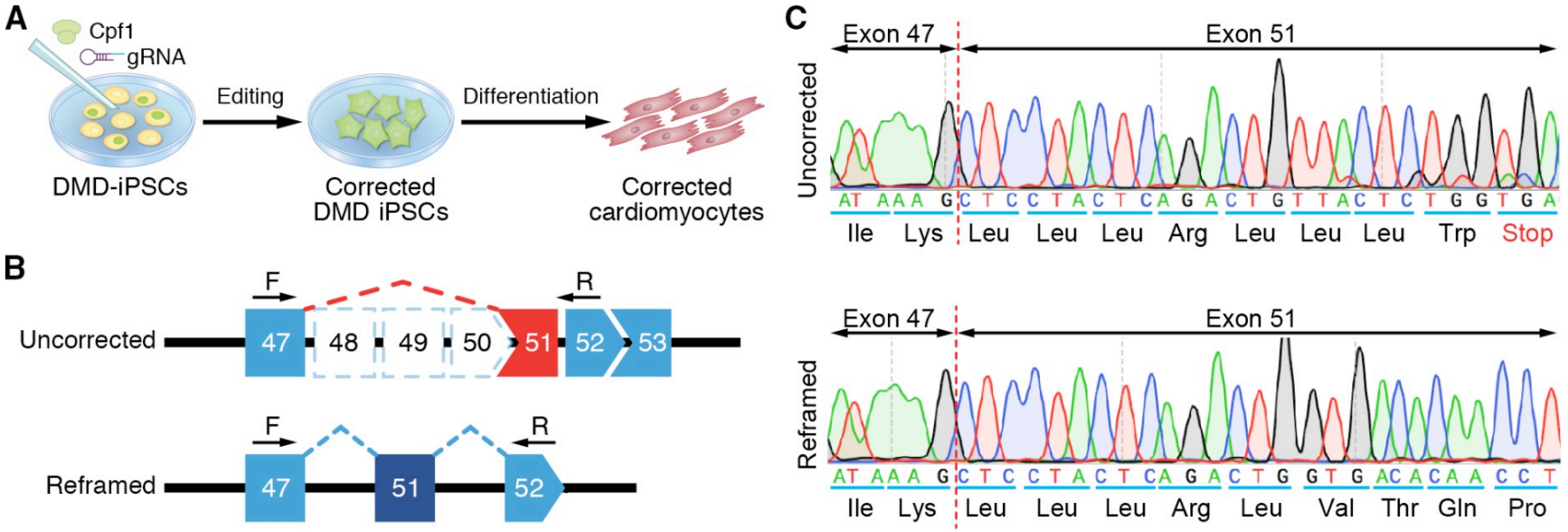
Results – Figure 1

Correction of DMD mutations by Cpf1-mediated genome editing



Results – Figure 2

DMD iPSC-derived cardiomyocytes express dystrophin after Cpf1-mediated genome editing by reframing



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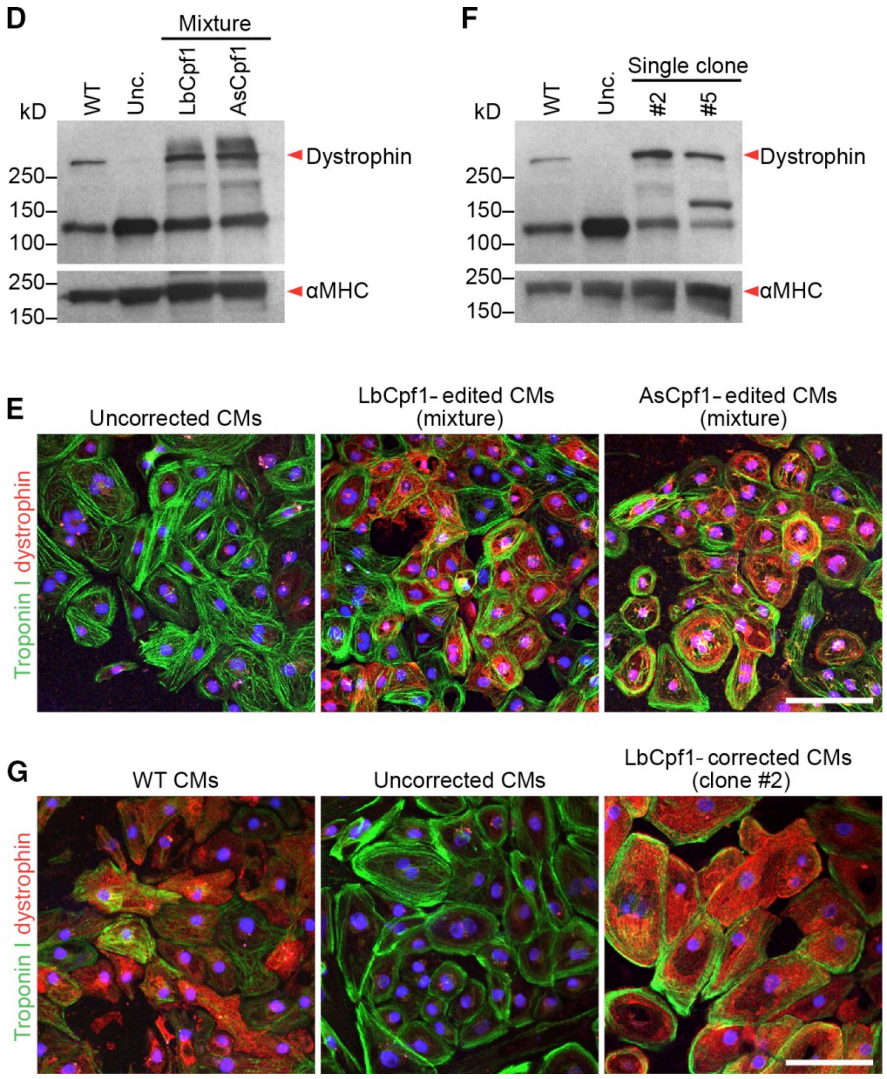
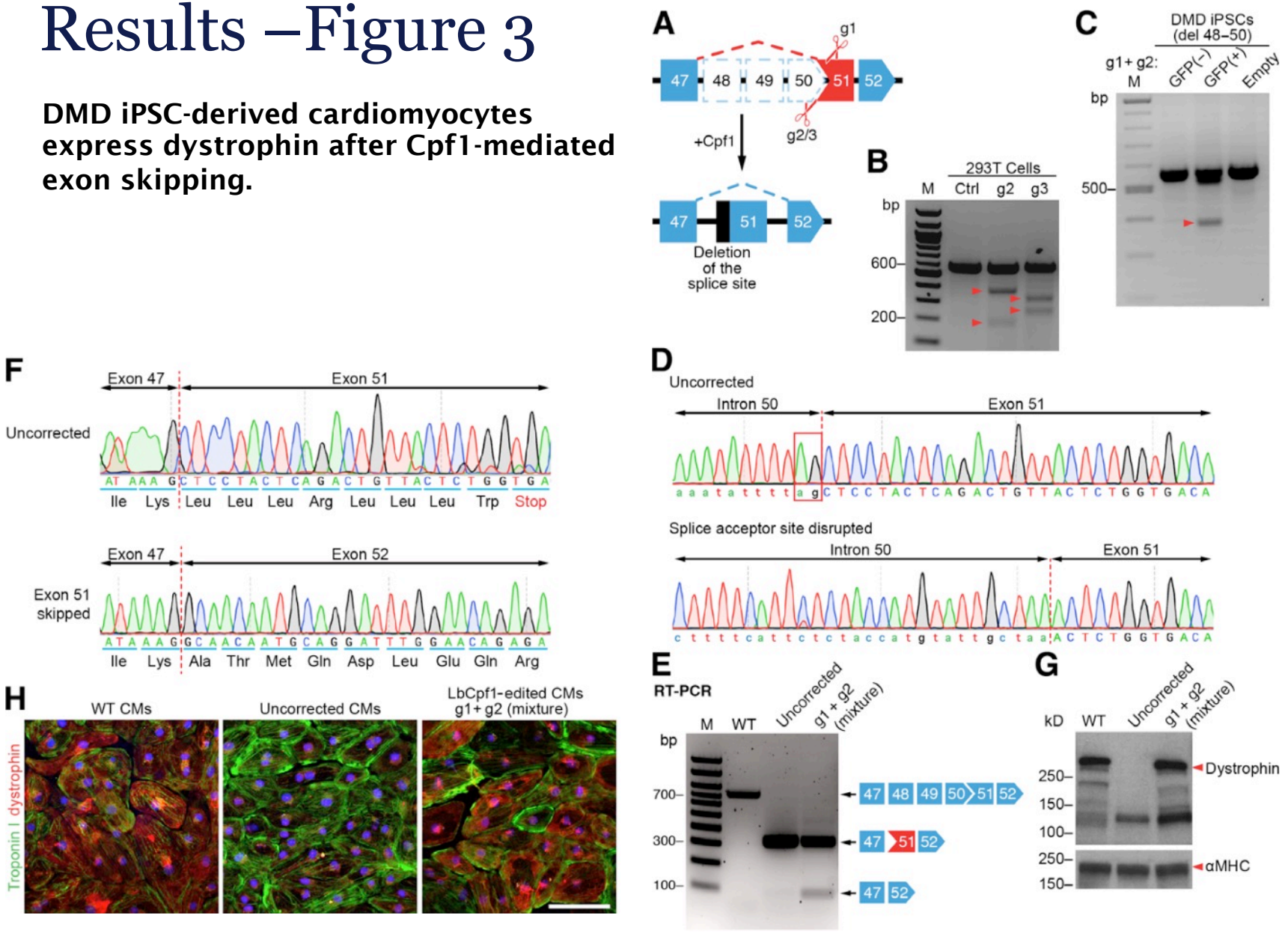
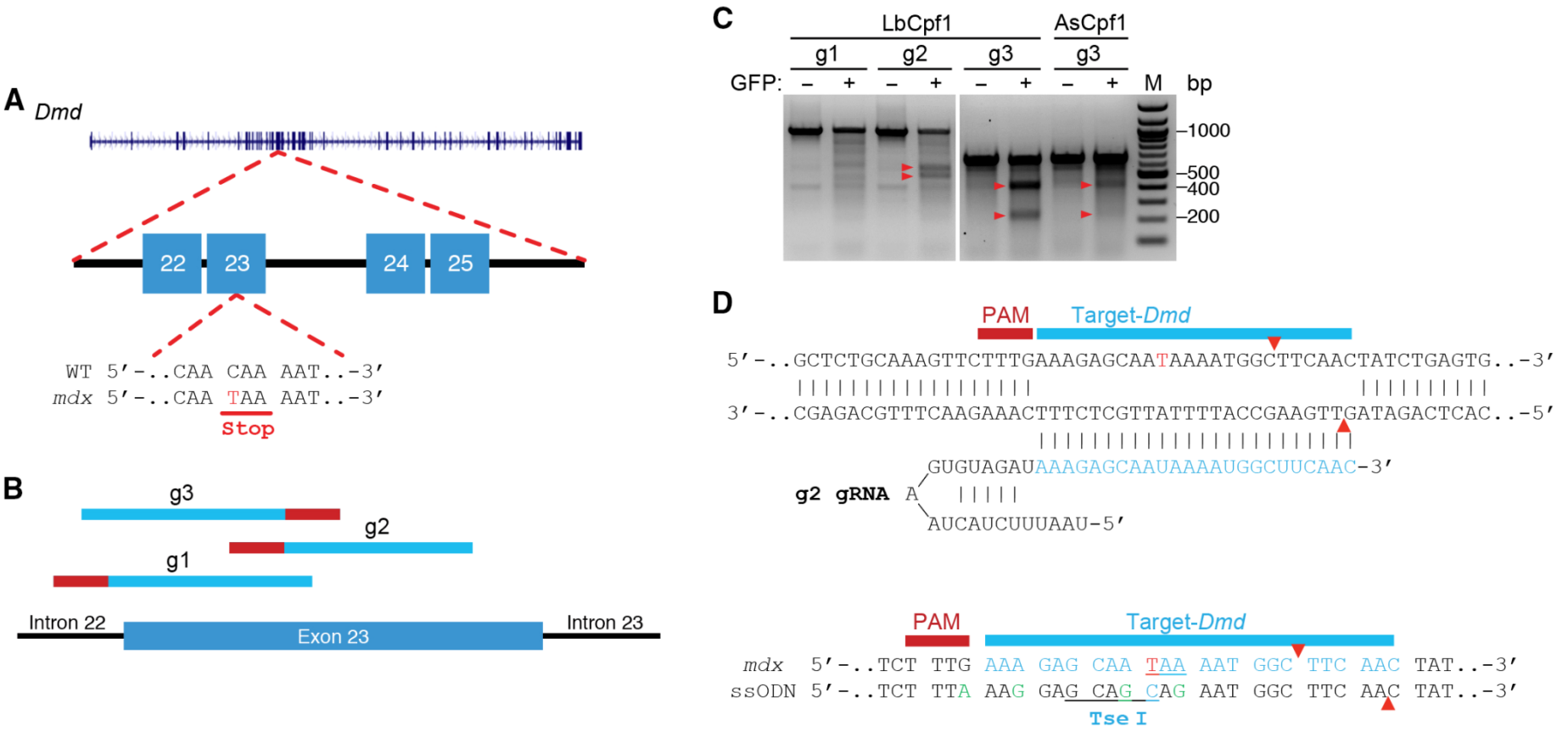


Results –Figure 3

DMD iPSC-derived cardiomyocytes express dystrophin after Cpf1-mediated exon skipping.

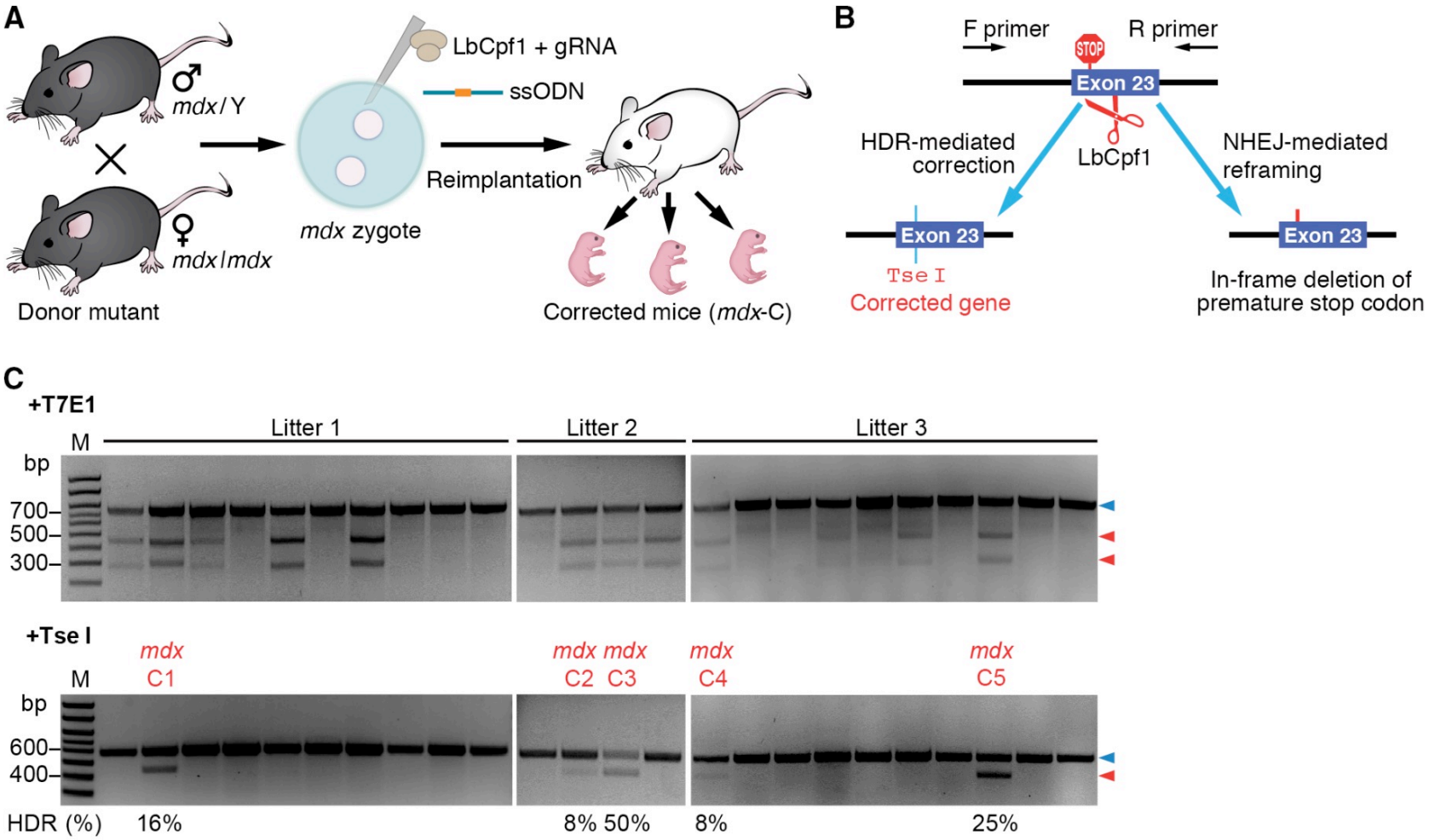


Results – Figure 4



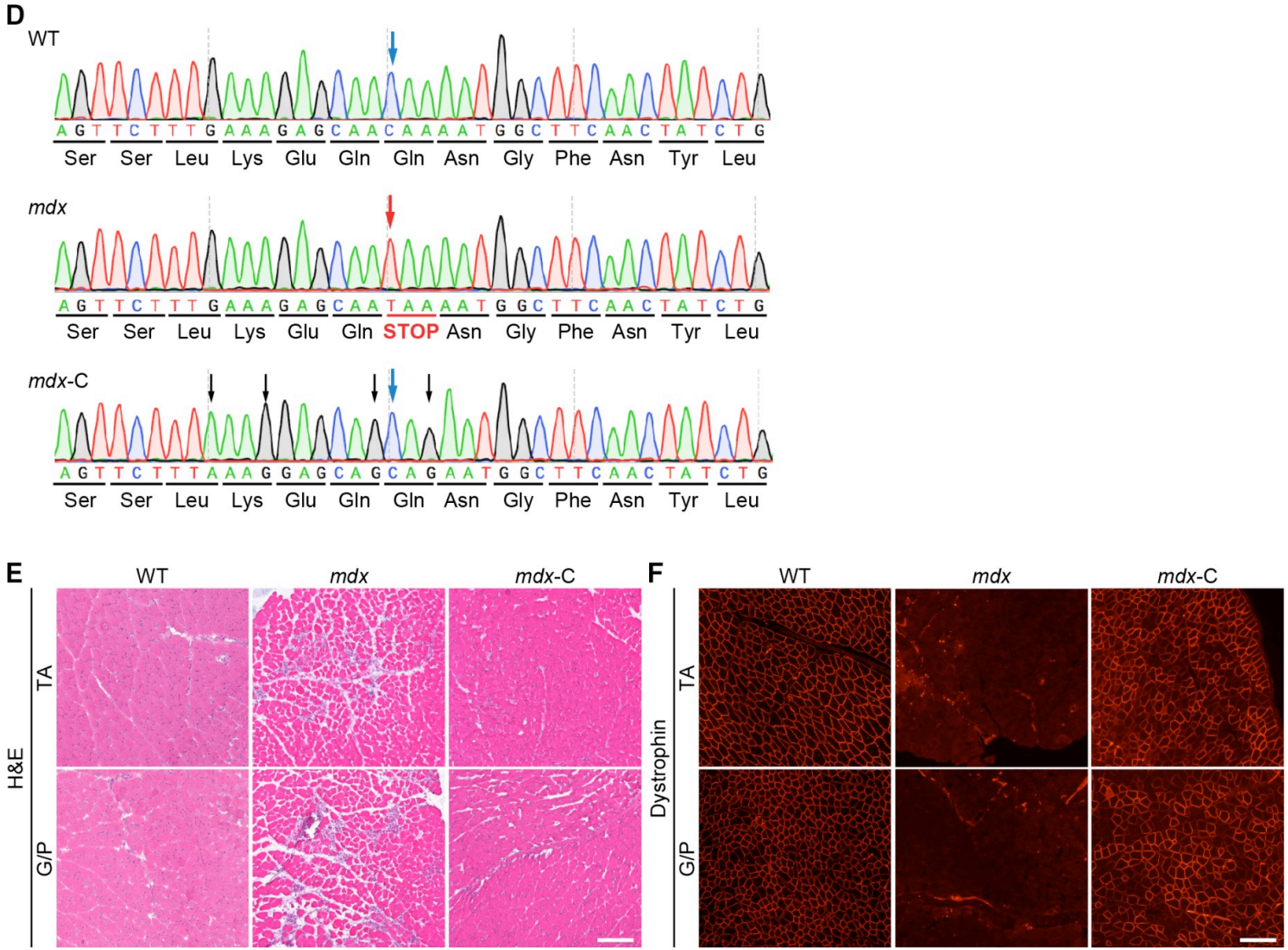
Results – Figure 5

CRISPR-LbCpf1–mediated *Dmd* correction in *mdx* mice



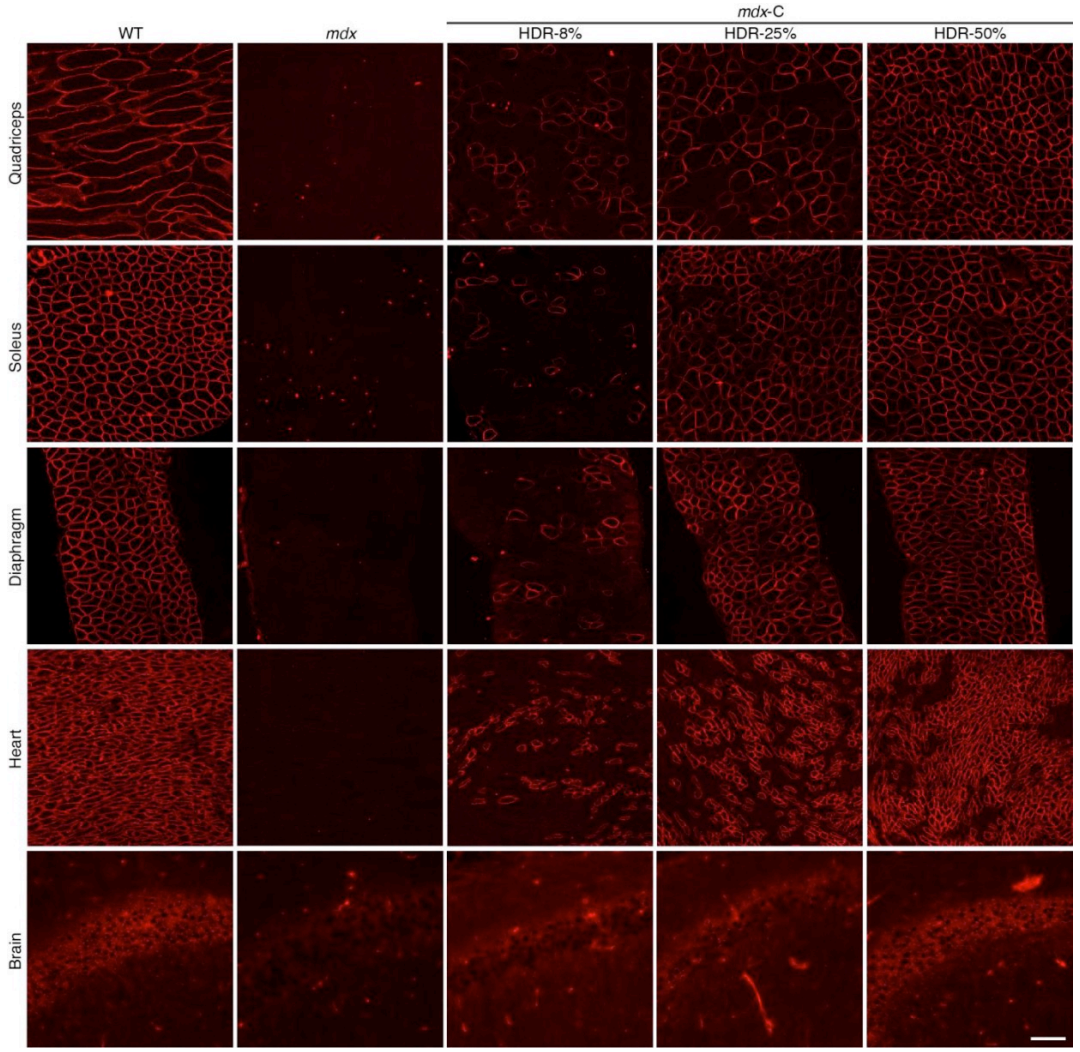
Results – Figure 5

CRISPR-LbCpf1–mediated Dmd correction in mdx mice



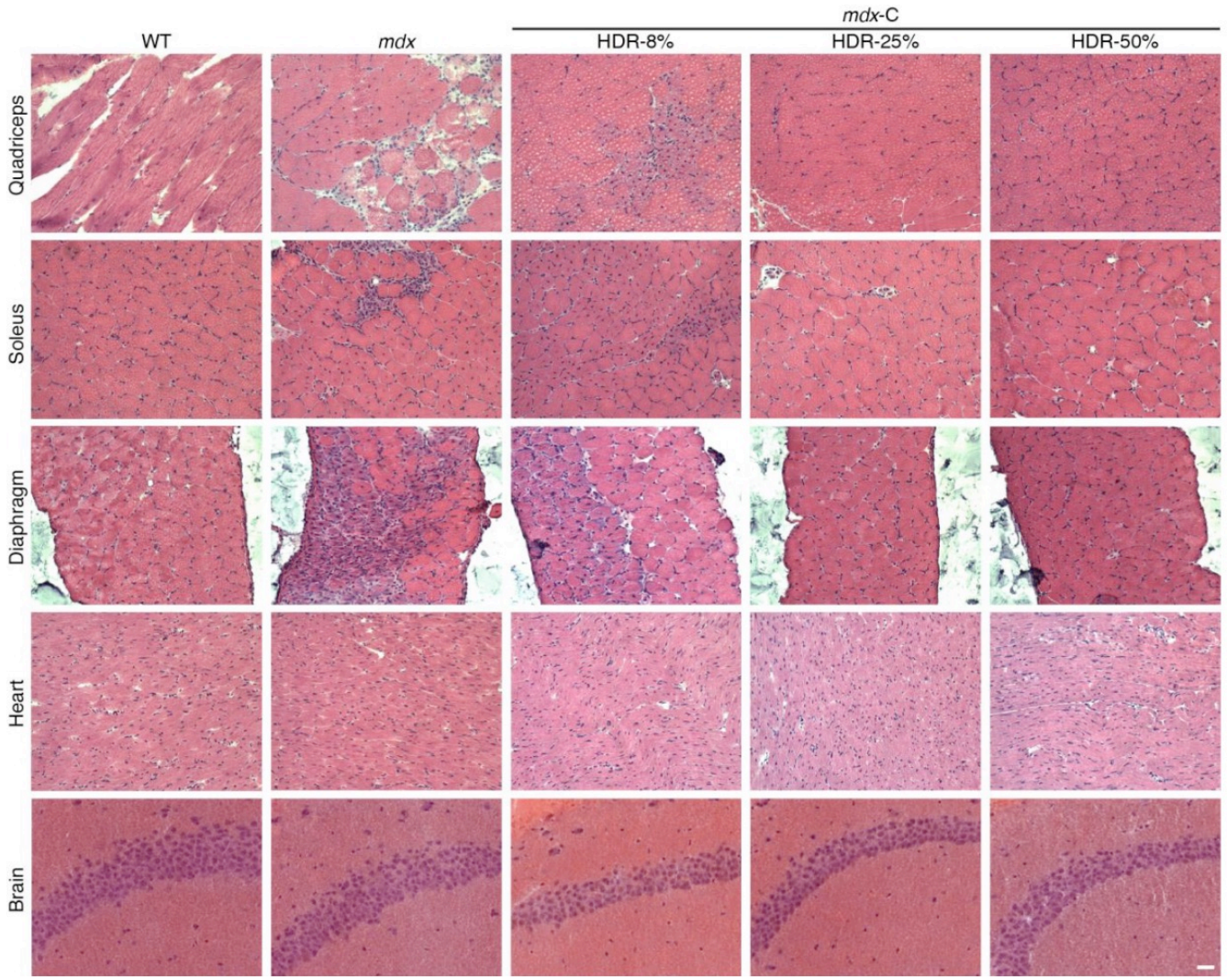
Results – Supplement 6

Immunohistochemistry of skeletal muscles, heart, and brain from WT, *mdx*, and LbCpf1-edited mice (*mdx*-C)



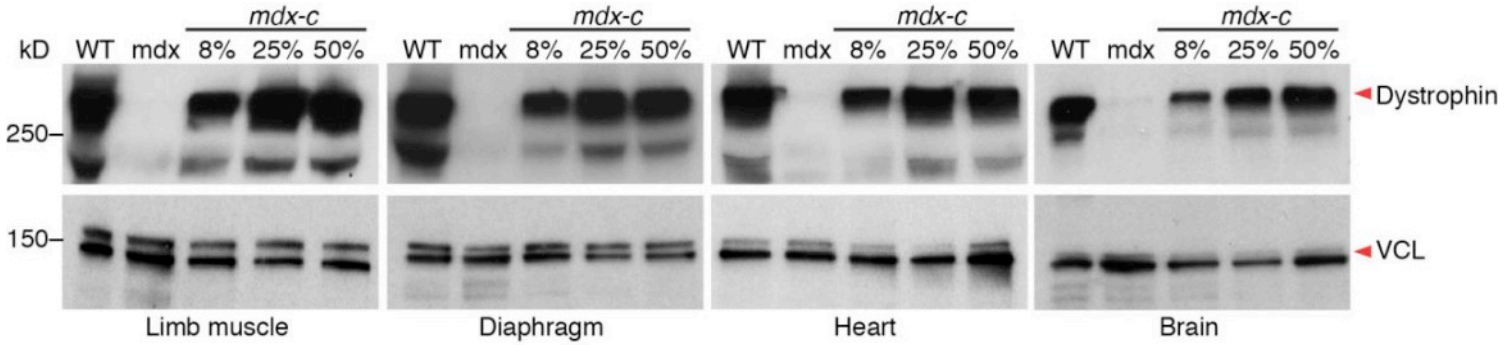
Results – Supplement 7

H&E staining of skeletal muscles, heart, and brain from WT, *mdx*, and LbCpf1-edited mice (*mdx*-C)



Results – Supplement 8

Western blot analysis of skeletal muscles, heart, and brain from WT, *mdx*, and LbCpf1-edited mice (*mdx*-C)



Results – Table 1

Table 1. Serum CK measurement and forelimb grip strength of WT, *mdx*, and LbCpf1-corrected *mdx*-C mice. M, male; F, female.

Mouse no.	Percent correction by HDR	Sex	Weight (g)	CK (U/liter)	Forelimb grip strength (grams of force)						
					Trial 1	Trial 2	Trial 3	Trial 4	Trial 5	Trial 6	Average ± SD
WT-1	—	M	16.6	455	103	106	97	71	88	82	91.2 ± 13.4
WT-2	—	M	19.5	220	87	95	73	73	74	78	80.0 ± 9.1
WT-3	—	M	18.7	306	79	97	74	78	84	84	82.7 ± 8.0
WT-4	—	F	15.5	184	86	97	85	83	85	88	87.3 ± 5.0
WT-5	—	F	15.4	175	88	85	100	96	88	86	90.5 ± 6.1
WT-6	—	F	12.6	157	76	75	73	78	64	61	71.2 ± 7.0
mdx-1	0	M	18.8	8579	76	92	86	33	32	29	58.0 ± 29.9
mdx-2	0	M	21.0	9440	62	58	54	45	45	47	51.8 ± 7.3
mdx-3	0	M	21.5	5936	77	54	69	57	56	61	62.3 ± 8.9
mdx-4	0	F	16.1	6306	69	63	69	61	67	60	64.8 ± 4.0
mdx-5	0	F	15.8	6349	83	88	85	59	55	54	70.7 ± 16.2
mdx-6	0	F	16.2	4168	69	71	53	59	59	57	61.3 ± 7.1
mdx-C1	16%	F	21.7	1233	112	111	112	115	101	109	110.0 ± 4.8
mdx-C2	8%	F	19.7	4920	119	109	108	95	86	85	100.3 ± 13.8
mdx-C3	50%	F	20.2	248	110	115	114	112	112	104	111.2 ± 3.9
mdx-C4	8%	M	22.7	6607	49	45	42	30	25	21	35.3 ± 11.5
mdx-C5	25%	F	17.7	3239	92	110	96	91	110	95	99.0 ± 8.7

Discussion

- newly discovered CRISPR-Cpf1 nuclease can efficiently correct DMD mutations in patient-derived iPSCs and *mdx* mice, allowing for restauration of dystrophin expression
- robustness and efficiency of Cpf1 in mouse genome editing → HDR-mediated correction, ORF of mouse *Dmd* gene was completely restored → fibrosis, inflammatory infiltration rescued
- two different strategies: reframing (only one gRNA needed) and exon skipping
- but differences in dystrophin expression level, mtDNA quantity, OCR in different edited clones → reframed dystrophin may not be structurally or functionally identical to WT dystrophin

Discussion

- use of one or two gRNA?
  - two are more effective for disruption of splice acceptor site
  - removes deleterious “AG” nucleotides → pseudosplice acceptor site generation eliminated
  - →both gRNAs should cleave simultaneously which may not occur
  - one gRNA → uncertainty of length of INDELs?
- unique T-rich PAM sequence (G-rich PAM sequence in SpCas9)
- LbCpf1 is 140 AA smaller → enhances packaging and delivery by AAV
- LbCpf1 and AsCpf1 had high genome-wide targeting efficiency and high specificity comparable to those of SpCas9
- new powerful approach to permanently eliminate genetic mutations and rescue abnormalities associated with DMD and other disorders

Pro-Cons

- interesting new approach to eliminate genetic mutations, safe therapy? possible risks?
- iPSCs from just one patient? why not generating more iPSC clones from different patients?
- out of 24 pups born only 5 carried corrected alleles with different correction rates → just 8 to 50%, no 100% correction? and 50% only in one mouse
- for me personally difficult to read because of genetic engineering science vocabulary

Thank you for your attention!