Supplementary information

Generation of muscular dystrophy model rats with a CRISPR/Cas system

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Supplementary Fig. 1. Mutations in the rat *Dmd* gene generated by CRISPR/Cas.

Sequence analyses of detectable F0 male rats in target1 **(a)** and target2 **(b)** loci. Target sequences of gRNAs are labeled in magenta and protospacer adjacent motif (PAM) sequences are labeled in green. Insertions are represented as bold lowercase letters (blue) and mutations are labeled in cyan.

Supplementary Fig. 2. The same mutation patterns were observed in the tail tips and muscle.

(a) The mutations detected in the tail tip were the same as those detected in the tibialis anterior (TA) muscle. **(b)** F0 rat #9 exhibited overlapping of two types of waveform data from the tail tip near the target1 site, which indicates the occurrence of a mosaic pattern. This overlapping was also detected in the waveform data from the TA muscle. **(c)** The sequences detected by TA cloning from the tail tip and TA muscle.

Supplementary Fig. 3. Large-scale deletion pattern in the rat *Dmd* gene with the CRISPR/Cas system.

(a) PCR for the rat *Dmd* gene in *Dmd*-mutated F0 rats using the forward primer of the target1 site and the reverse primer of the target2 site. Amplification occurred only in one F0 rat (#3).

(b) Sequencing result and waveform data of the PCR products using the forward primer of the target1 site and the reverse primer of the target2 site in F0 rat #3. This mutation pattern was different from that shown in Supplementary Fig. 1, indicating the occurrence of a mosaic pattern in F0 rat #3.

Supplementary Fig. 4. All *Dmd*-mutated F0 rats exhibited dystrophic phenotypes in the skeletal muscle.

(a) Immunostaining for tibialis anterior (TA) muscles of 13-week-old wild type (WT) and *Dmd*-mutated F0 male rats. The signals of Dystrophin disappeared or were reduced in all of the F0 rats. Scale bar = 100 μm. **(b)** Masson's trichrome staining of TA muscles in *Dmd*-mutated F0 rats. Scale bar = 500 μm. **(c)** Immunostaining for laminin and embryonic myosin heavy chain (eMHC) in TA muscles of 13-week-old

WT and F0 male rats. The presence of eMHC-positive regenerating myofibers was observed in all of the F0 rats, but not in age-matched WT rats. Scale bar = 100 µm.

Supplementary Fig.5. Immunoblotting and immunostaining with Dys2 antibody in *Dmd*-mutated rats.

(a) Full-length image of immunoblotting for Dmd with Dys2 antibody. Coomassie Brilliant Blue (CBB) staining is shown as a sample processing control. **(b)** Immunostaining for Dmd.

Supplementary Fig. 6. Slight elongation of the right ventricle in *Dmd*-mutated rats.

Area plots of right ventricles in wild type (WT; n = 4) and *Dmd*-mutated F0 (n = 10) rats. Bars represent mean values.

Supplementary Fig. 7. All F1 male rats exhibited phenotypes similar to those of F0 male rats.

(a) Sequence results of F1 male rats born from *Dmd*-mutated F0 female rats in the target2 locus. The gRNA sequences (magenta) and protospacer adjacent motif (PAM; green) are labeled. Insertions are represented as bold lowercase letters (blue) and conversions are labeled in cyan. **(b)** Immunostaining for Dystrophin in tibialis anterior (TA) muscles of 4-week-old wild type (WT) and F1 male rats. Scale bar = 100 µm. **(c)** Immunostaining for laminin and embryonic myosin heavy chain (eMHC) in TA muscles of 4-week-old WT and F1 male rats. eMHC-positive regenerating myofibers were observed in all of the F1 rats, but not in age-matched WT rats. **(d)** Immunostaining for Dystrophin-glycoprotein complex (β-Dystroglycan, α-sarcoglycan and nNOS) in TA muscles of 4-week-old WT and F1 male rats. Scale bar = 100 µm.

Supplementary Fig. 8. Full-length images of gels and blots.

Full-length images of gels of RT-PCR for *Dmd* **(a)** and *Hprt* **(b)**. Water was used as a negative control (NC) instead of cDNA template. Full-length images of immunoblotting for Dmd **(c)** and α-tubulin **(d)**. Blots for α-tubulin are shown as a sample processing control. Full-length images of immunoblotting for Perilipin **(d)** and α-tubulin **(f)**. Blots for α-tubulin are shown as a sample processing control.

Supplementary Table 1. Generation efficiency of *Dmd*-mutated rats using CRISPR/Cas9. *Cas*9 mRNA and two types of gRNAs targeting *Dmd* were injected into fertile eggs. The resulting pups were used for sequence analyses of the target1 and target2 sites. *: One rat showed the large-scale deletion pattern.

Supplementary Table 2. Off-target effects in *Dmd*-mutated rats. Potential off-target loci, which have three mismatches to target1 and target2, were examined by sequence analyses in F0 male rats. Mismatches are labeled in magenta. At off-target site 1.3, rats #2, #3, #8, and #10 had the indels detected by the overlapping of waveform data.

Supplementary Table 3. List of primers used in this study.

Supplementary Fig.1

a Target 1

chrX: 51,878,371-51,878,393

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WT  5'CTTATTAATTGAAAGGGTGAAAT//CAGTGACCTGCAGGATGGGAAACGCCTCCTGGA 3'
#1    CTTATTAATTG------------//-----------------GGAAACGCCTCCTGGA      -162 bp

#2, #3: unsuccessful

WT      CATAGACAACCTCTTCAGTGACCTGCAGG//AATGAATATTTATATGTGGCTATATAA
#4      CATA-------------------------//--------------TGTGGCTATATAA    -178 bp
WT      AGTGACCTGCAGGATGGGAAACGCCTCC//ATAATTCATATTACAATTTTCATTTTTG
#5      AGTGAC----------------------//------------ACAATTTTCATTTTTG    -566 bp

WT      AGTGACCTGCAGGATGGGAAACGCCTCCTGGACCTCCTGGAAGGCCTGACAGGGCAAA
#6      AGTGACCTGCA---------ACGCCTCCTGGACCTCCTGGAAGGCCTGACAGGGCAAA      -9 bp
#7-1    AGTGACCTGC------------------TGGACCTCCTGGAAGGCCTGACAGGGCAAA     -18 bp
WT      AGTGACCTGCAGGATGGGAAACGCCTCCTGG//TATTTCCTTCTTTCCTTTCTTCCCA
#7-2    AGTGACCT-----------------------//--------TCTTTCCTTTCTTCCCA     -73 bp
WT      AGTGACCTGCAGGATGGGAAACGCCTCC//TTTTCATTTTTGCATTGTCTATCATGTG
#8      AGTGACCTGCA-----------------//---------TTGCATTGTCTATCATGTG    -577 bp
#9-1    AGTGACC----------------CCTCCTGGACCTCCTGGAAGGCCTGACAGGGCAAA     -16 bp
#9-2    AGTGACCTGC----tggaaAACGCCTCCTGGACCTCCTGGAAGGCCTGACAGGGCAAA   -9/+4 bp
#10     AGTGACCTGCAggGGATGGGAAACGCCTCCTGGACCTCCTGGAAGGCCTGACAGGGCA      +2 bp
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b Target 2

chrX: 52,203,342-52,203,364

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WT   5'GGAAAACTTTGCACAACGTTGGGATAATTTAACCCAAAAACTTGAAAAGAGTTCAGCA 3'
#1-1   GGAAAACTTTGCACAACGTTGGGATAATTTAACCCAAAAACTTGAAAAGAGTTCAGCA
#1-2   GGAAAACTTTGCACA--GTTGGGATAATTTAACCCAAAAACTTGAAAAGAGTTCAGCA      -2 bp
#1-3   GGAAAACTTTGCACAA-GTTGGGATAATTTAACCCAAAAACTTGAAAAGAGTTCAGCA      -1 bp

#2: unsuccessful

#3-1   GGAAAACTTTG---------GGGATAATTTAACCCAAAAACTTGAAAAGAGTTCAGCA      -9 bp
#3-2   GGAAAACTTT---------TGGGATAATTTAACCCAAAAACTTGAAAAGAGTTCAGCA      -9 bp
WT     GGAAAACTTTGCACAACGTTGGGATAATT//GTATTGGGAAATTGTGAAGAGGCAGAT
#4-1   GGAAAACTTTGCACAACG-----------//-----GGGAAATTGTGAAGAGGCAGAT    -186 bp
#4-2   GGAAAACTTTT----------GGATAATT//GTATTGGGAAATTGTGAAGAGGCAGAT     -10 bp
#5     GGAAAACTTTGCAC---------------------AAAAACTTGAAAAGAGTTCAGCA     -21 bp
#6     GGAAAACTTTGCACAACGTTGGGATAATTTAACCCAAAAACTTGAAAAGAGTTCAGCA
#7     GGAAAACTTTGCACAACGTTGGGATAATTTAACCCAAAAACTTGAAAAGAGTTCAGCA
#8     GGAAAACTTTG-------TTGGGATAATTTAACCCAAAAACTTGAAAAGAGTTCAGCA      -7 bp
#9-1   GGAAAACTTTGCACAACGTTGGGATAATTTAACCCAAAAACTTGAAAAGAGTTCAGCA
#9-2   GGAAAACTTTGCACAA--------------------------------GAGTTCAGCA     -32 bp
#9-3   GGAAAACTTTGCAGA-----TGGATAATTTAACCCAAAAACTTGAAAAGAGTTCAGCA      -5 bp
#10-1  GGAAAACTTTGCACA----------AATTTAACCCAAAAACTTGAAAAGAGTTCAGCA     -10 bp
#10-2  GGAAAACTTTGCACAAA--TGGGAT---TTAACCCAAAAACTTGAAAAGAGTTCAGCA      -5 bp
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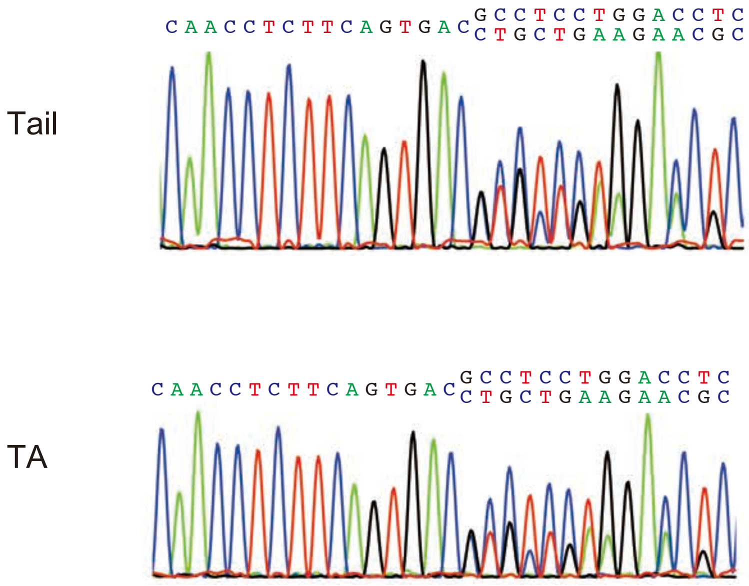
Supplementary Fig.2

a

	Target 1	
WT	CAACCTCTTCAGTGACCTGCAGGATGGGAAACGCCTCCTGGAC	
#7 tail	CAACCTCTTCAGTGACCTGC------------------TGGAC	-18 bp
#7 TA	CAACCTCTTCAGTGACCTGC------------------TGGAC	-18 bp

	Target 2	
WT	GGAAAACTTTGCACAACGTTGGGATAATTTAACCCAAAAACTT	
#8 tail	GGAAAACTTTG-------TTGGGATAATTTAACCCAAAAACTT	-7 bp
#8 TA	GGAAAACTTTG-------TTGGGATAATTTAACCCAAAAACTT	-7 bp

b Target 1

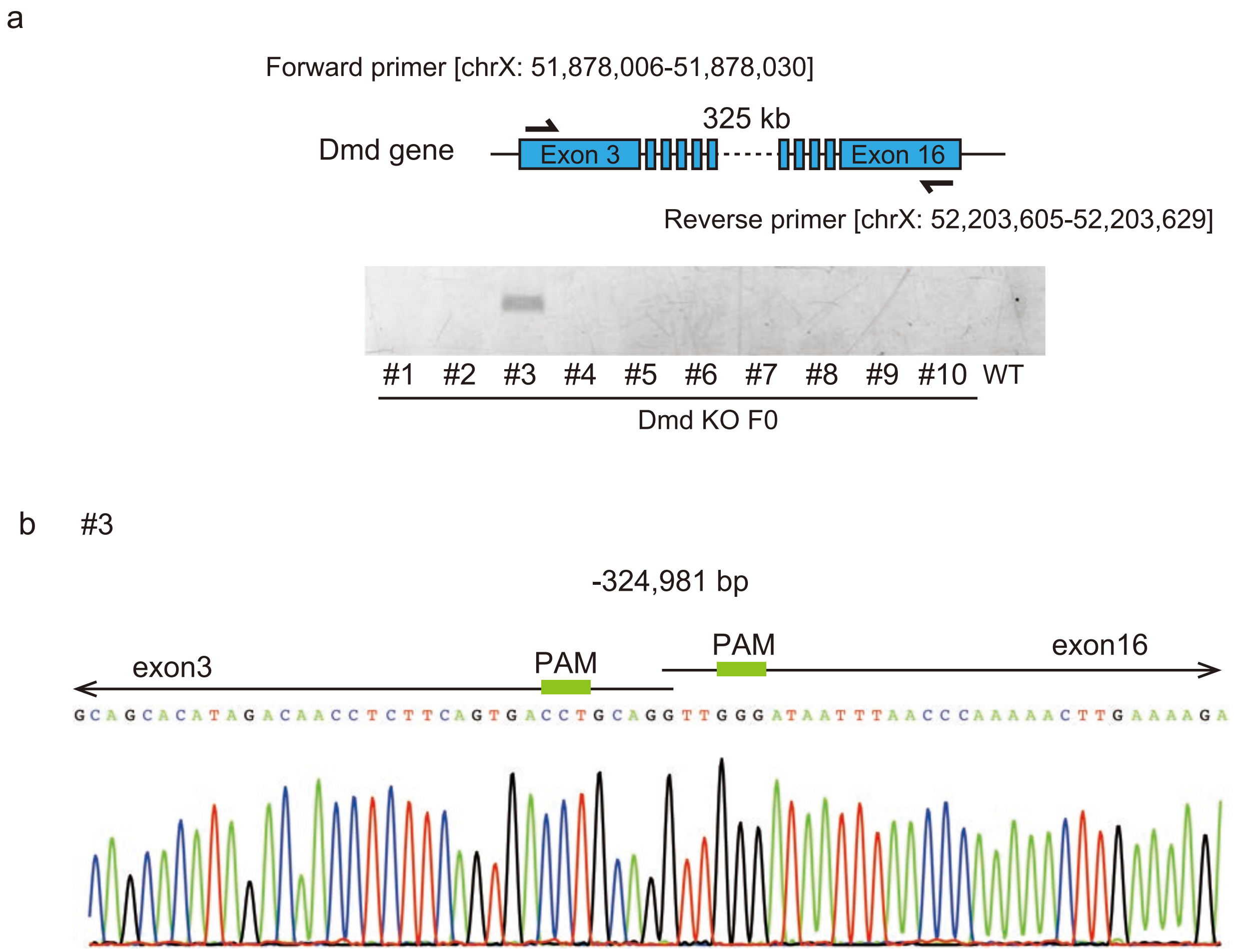


c

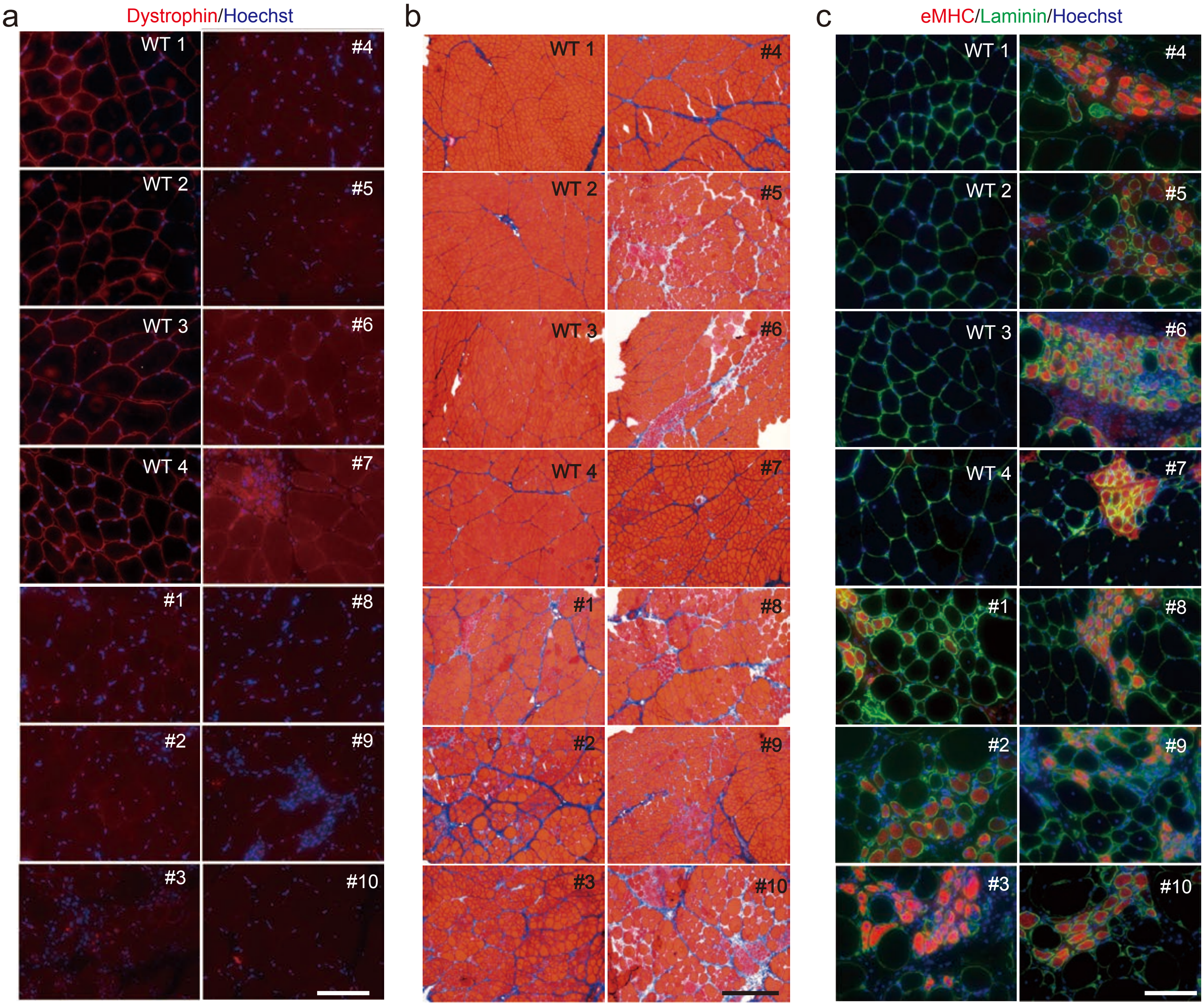
Tail		
WT	CAACCTCTTCAGTGACCTGCAGGATGGGAAACGCCTCCTGGACCTC	
#9-1	CAACCTCTTCAGTGACC----------------CCTCCTGGACCTC	-16 bp
#9-2	CAACCTCTTCAGTGACCTGC----***tggaa***AACGCCTCCTGGACCTC	-9/+5 bp

TA		
WT	CAACCTCTTCAGTGACCTGCAGGATGGGAAACGCCTCCTGGACCTC	
#9-1	CAACCTCTTCAGTGACC----------------CCTCCTGGACCTC	-16 bp
#9-2	CAACCTCTTCAGTGACCTGC----***tggaa***AACGCCTCCTGGACCTC	-9/+5 bp

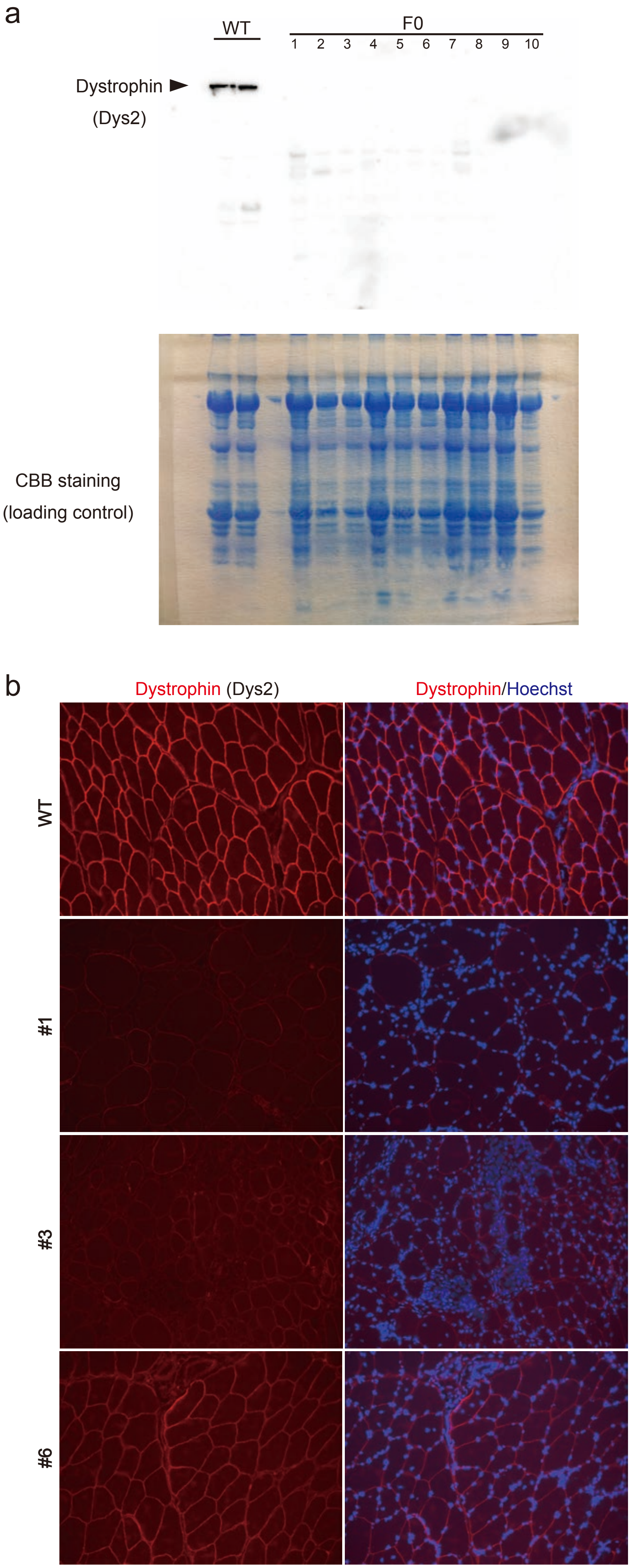
Supplementary Fig.3



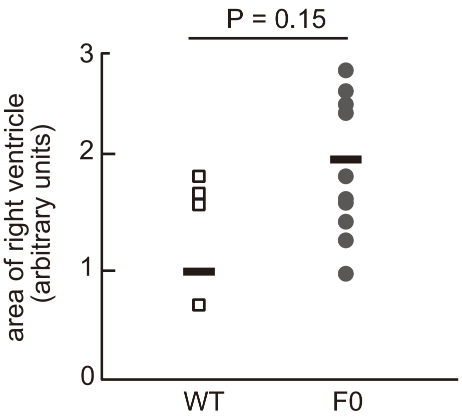
Supplementary Fig.4



Supplementary Fig.5



Supplementary Fig.6



Supplementary Fig.7

a

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      Target2             PAM
GTGGATGGAAAACTTTGCACAACGTTGGGATAA   WT
GTGGATGGAAAACTTTG----------GGATAA   F0 mother
GTGGATGGAAAACTTTG----------GGATAA   #1
GTGGATGGAAAACTTTG----------GGATAA   #2
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GTGGATGGAAAACTTTGCACA-ACGTTGGGATAA   WT
GTGGATGGAAAACTTTGCACAtAATTTGGGATAA   #3
GTGGATGGAAAACTTTGCACAtAATTTGGGATAA   #4
GTGGATGGAAAACTTTGCACAtAATTTGGGATAA   #5
********************* *  *********
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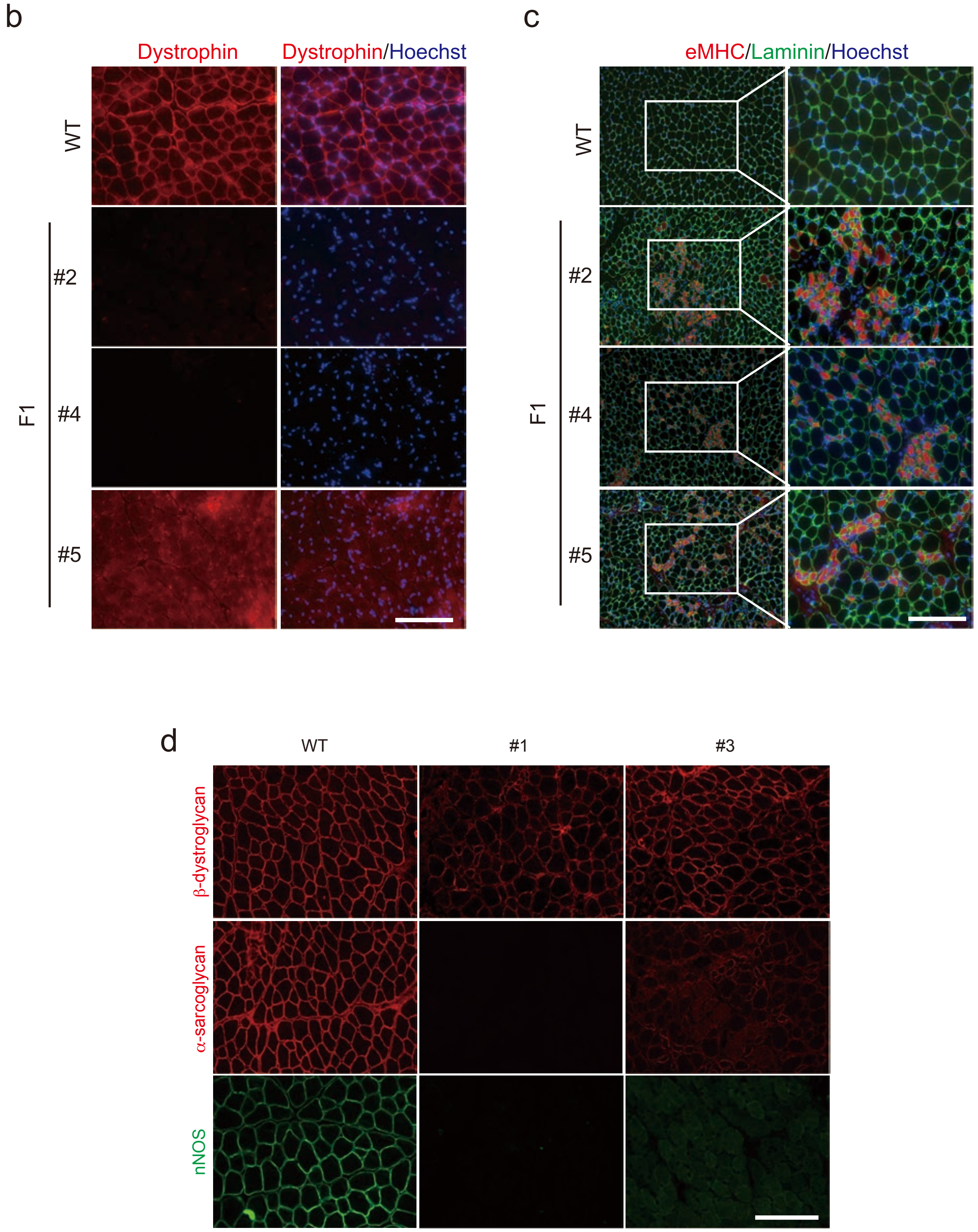
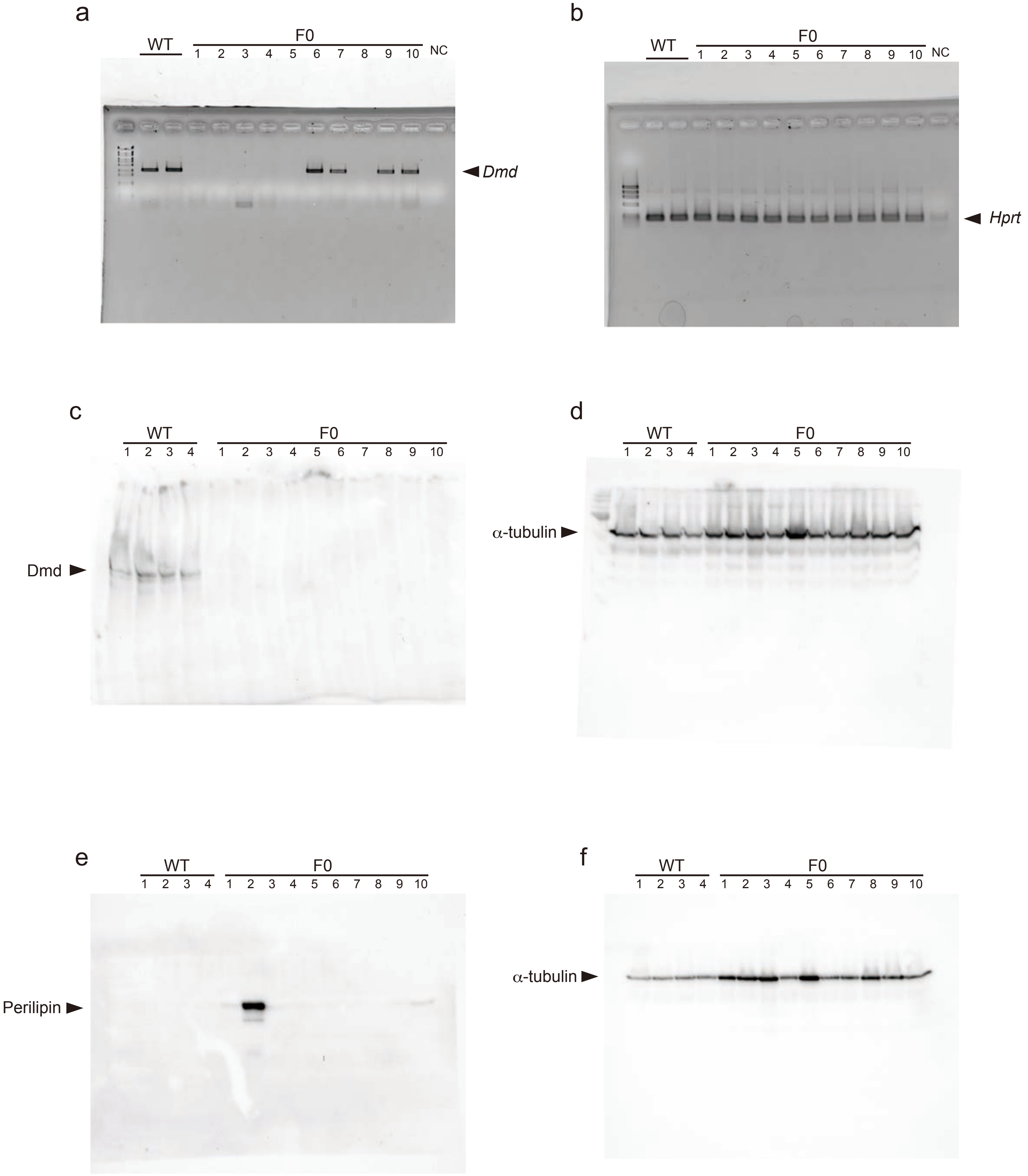


Supplementary Fig.8



Supplementary Table 1

Generation efficiency of *Dmd*- mutated rats using CRISPR/Cas9

males/newborns	detectable/newborn males		mutant allele/detectable rats		double mutated rats /newborn males
	target1	target2	target1	target2	
10/17	8/10	9/10	8/8	7/9	5/10*

*One rat had the large-scale deletion.

Supplementary Table 2

	sequence of off-target	locus	No. of mutated F0 male rats
1.1	CCTGCAGGATGGGAAAGGCATCT	chr14: 80,131,101-80,131,123	0/10
1.2	CCTGCAGGCTGGCCAACGCCTCC	chr20: 14,437,218-14,437,240	0/10
1.3	CCTGCAGGATGGGAAACACCAGC	chr8: 60,483,929-60,483,951	4/10
1.4	CCAGCAGGAAGGGAAACCCCTTC	chr8: 50,757,345-50,757,367	0/10
1.5	CCTGCAGGCTGGGCAACGCCTTC	chr2: 135,597,810-135,597,832	0/10
1.6	CCAGCAGGAGGGGAAATGGCTCC	chr6: 1,082,237-1,082,259	0/10
1.7	CCTGCAGGATGGGACACTCCACC	chr10: 57,594,822-57,594,844	0/10
1.8	CCTGCAGGACTGGAAATGCCTCC	chr13: 102,121,427-102,121,449	0/10
2.1	GGAAAACTCTGCACAACCCTTGG	chr18: 41,820,912-41,820,934	0/10
2.2	AGAAAAGTTTGCACAACTTTGGG	chr9: 117,853,303-117,853,325	0/10
2.3	GGCTAACTTTGCACAATGTTGGG	chr11: 31,967,242-31,967,264	0/10
2.4	GGTAACCTTTGCACAACGTTTGT	chr15: 2,132,713-2,132,735	0/10
2.5	GGCAAACTTTGCACAACATTGGC	chr1: 94,964,610-94,964,632	0/10
2.6	GGAAACCTTTGCACAAAGATGGG	chr2: 260,132,982-260,133,004	0/10

Supplementary Table 3

Target		**Forward**	**Reverse**
target 1	For #1, #4	5′-AGTTTCCATCAATAGCCATACCAAA	5′-TCTCAGTGTACAAGTGTGACGAACA
	For #5	5′-TATTGATGCACACTATCTCCCTTGA	5′-AGGAGGCAAGTGAGAGATAGGATTT
	For #8	5′-ATCGAAGTGCTGAAAAGAATCTCAT	5′-TATAACCATGTTTTCTCTCCCCAAA
	For others	5′-AAAAGGAGAACAGGAGTTTTTGAAT	5′-TACAGTAGCTGAGTCAATGAGGTTG
target 2		5′-GAATACCTTTGGGTGTGACTGTATC	5′-TACAGTTTTCCATTTCTGAAGAACC
target 1	off-target1.1	5′-CCCACTTCATAGATGAGAACACTGA	5′-CTGTAGCCAGGAAGTAAAGCTGTGT
	off-target1.2	5′-CCTCCTATACAACCCACATGTTTCT	5′-TACATATCTTCCCAGCCAGTGATCT
	off-target1.3	5′-AGGTCCCATCTCCTAGTCTCAAAGT	5′-TTCTTAGAAGTCTGATCCTGGCAGT
	off-target1.4	5′-TAACACGAGCACAGGCAATTTATTA	5′-GCTTCTCTCTTTACACCACCACATT
	off-target1.5	5′-TCACCGACCTAACATGTAAATCAAA	5′-GTGCAATGCTGATACATAATACACAA
	off-target1.6	5′-AAAAAGGAAGGGTAAATAGCACGAC	5′-ACCCTTGCACTGAGAACACATATT
	off-target1.7	5′-TGGCTAGTACATCAAGGTCTCTTGG	5′-CTTCCTCATTACTCAGACCCAAAAA
	off-target1.8	5′-AATACGACCTAGGTTAGGTGGGAAG	5′-ACTCCCATACAGGTGCTATCAAGAG
target 2	off-target2.1	5′-ACTCAATTTTGACTGAAGACCAACC	5′-CATGCTCTTCCGGTAGGAGATAGTA
	off-target2.2	5′-GCTACTTCACGTCAAATGAGTGGTA	5′-GCATAGGACTAGGAAGACATCCAGA
	off-target2.3	5′-TTCTTTCGACTGCCTTCATGATATT	5′-GGAGCGTTTCATATGTGAGATGAGAT
	off-target2.4	5′-AAAAAGGTTCTCATTCCCAAGTAGC	5′-TGGAAATGACACAGAAAGAAATGAA
	off-target2.5	5′-TAAGTGGTTTCAAAACCCTAAGCTC	5′-GGCTTAAGTGGACAATCATTCAACT
	off-target2.6	5′-AAACAGACGTAGCCTTCTCAGGTAA	5′-GCGTGACACATATTTCTATCCTGTG
Dmd mRNA		5′-AAAGCAACACATAGACAACCTCTTC	5′-GTTTTACCATGATTTGTTCCCTTGT
Hprt mRNA		5′-GCTGGTGAAAAGGACCTCT	5′-CACAGGACTAGAACRYCTGC