Duchenne Muscular Dystrophy Cell Culture Models Created By CRISPR/Cas 9 Gene Editing And Their Application To Drug Screening

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Abstract

Gene edition methods are an attractive putative therapeutic option for Duchenne muscular dystrophy and they have an immediate application in the generation of research models. To generate two new edited myoblast cultures that could be useful *in vitro* drug screening, we have optimised a CRISPR/Cas9 gene edition protocol. We have successfully used it in wild type immortalised myoblasts to delete exon 52 of the dystrophin gene: DMDΔ52-Model, modelling a common Duchenne muscular dystrophy mutation; and in patient's immortalised cultures we have deleted an inhibitory microRNA target region of the utrophin UTR, leading to utrophin upregulation. We have characterised these cultures and, to show their use in the assessment of DMD treatments, we have performed exon skipping in the DMDΔ52-Model and have used the unedited cultures/ DMD-UTRN-Model combo to assess utrophin overexpression after drug treatment. While the practical use of DMDΔ52-Model is limited to the validation to our gene edition protocol, DMD-UTRN-Model offers a possible therapeutic gene edition target as well as a useful positive control in the screening of utrophin overexpression drugs.

KEYWORDS: Duchenne muscular dystrophy, CRISPR/Cas9 gene editing, inhibitory microRNA target region, dystrophin, utrophin.

48 ***INTRODUCTION***

49 **Duchenne muscular dystrophy** (DMD) is a fatal X-linked recessive disease affecting
50 one out of 5.000 newborn males. It is commonly caused by deletions disrupting the
51 open reading frame of the *DMD* gene causing a lack of dystrophin protein[1]. Patients
52 carrying out of frame mutations present a severe phenotype, while those carrying in-
53 frame mutations may result in hypomorphic alleles, a partially functional dystrophin
54 and milder phenotypes, such as in Becker muscular dystrophy (BMD)[2]. Dystrophin
55 plays a major role in membrane stabilization during muscle contraction, linking the
56 actin cytoskeleton to the sarcolemma[3] and also contributes to extracellular signalling[4].
57 Lack of dystrophin in DMD patients' muscles leads to progressive muscle wasting and
58 degeneration. DMD children suffer from loss of ambulation in the first or second
59 decade of life and premature death by cardiac and respiratory complications[5].

60 Although no definitive cure for DMD is available, four drugs have been recently
61 approved by different regulatory agencies: ataluren, approved by the European
62 Medicines Agency (EMA), induces readthrough of premature stop codons during
63 mRNA translation, generating a full length dystrophin protein[6]; and the US Food and
64 Drug Administration (FDA), has recently approved three antisense oligonucleotide
65 drugs, which modulate splicing by exon skipping, restoring *DMD* reading frame and
66 leading to a shorter but functional protein[7,8]: eteplirsen, golodirsen and viltolarsen.
67 Exon skipping therapies aim to attenuate the phenotype and phenocopy milder BMD-
68 like genotypes, to potentially improve disease outcomes. Eteplirsen targets *DMD* exon
69 51 and golodirsen and viltolarsen, exon 53. All approved drugs are mutation specific
70 and designed to rescue specific patient mutations only present, respectively, in 13%
71 (ataluren), 13% (eteplirsen) and 8% (golodirsen and viltolarsen) of patients[9]. It is
72 therefore important to assess exon-skipping strategies targeting other *DMD* exons, and
73 many such drugs are in different phases of clinical assays[10].

74 Other than these approved therapies, gene transfer has emerged as a promising
75 approach applicable to all DMD patients. SRP-9001, a human micro-dystrophin gene
76 transfer therapy, driven by the muscle-specific promoter MHCK7 and delivered by the
77 recombinant adeno-associated virus rAAVrh74 has recently got the Fast Track

78 designation by the FDA after showing a robust posttreatment increase of micro-
79 dystrophin expression in patients muscles and positive safety and tolerability data in
80 clinical trials[11] suggesting the potential of this therapy to provide clinically meaningful
81 functional improvement in DMD patients.

82 Alternatively to these therapies aiming to restore dystrophin expression, many
83 compounds targeting secondary effects of dystrophin deficiency or looking for
84 alternatives to dystrophin are also under evaluation.

85 *In vitro* cellular models are particularly useful to assess the efficiency of novel
86 therapies for DMD. However, only a few human immortalized muscle cell lines derived
87 from DMD patients are currently available[12]. Due to the wide spectrum of DMD
88 mutations and the difficulties to obtain DMD patient muscle biopsies, DMD-myoblasts
89 models would provide a powerful resource for *in vitro* drug screening and study
90 disease rescue mechanisms.

91 ***UTROPHIN*** *(UTRN)* is an autosomal paralog of dystrophin, expressed in skeletal muscle
92 cells during embryonic development, but restricted to neuromuscular and
93 myotendinous junctions in the mature muscle fibre[13]. Overexpression of utrophin in
94 skeletal muscle in DMD animal models can partially compensate the lack of dystrophin
95 and improve DMD phenotype[14-16]. Importantly, ectopic and high levels of utrophin in
96 myoblasts are not associated with toxicity, making utrophin upregulation an
97 interesting therapeutic strategy applicable to all patients, regardless of their specific
98 mutation[17,18]. Ezutromid/SMT-C1100 was the first utrophin modulator evaluated in
99 clinical assays, but was recently abandoned due to lack of evidence of utrophin
100 restoration, nor clinical improvement demonstrated in patients [19,20]. Alternatively,
101 other studies proposed new strategies to upregulate *UTRN* by blocking the inhibitory
102 target region of microRNAs repressing *UTRN* expression[21]. Recently, utrophin
103 upregulation has been efficiently achieved using gene therapy in multiple animal
104 models with non-immunogenic side effects[22]. Several new compounds that aim to
105 overexpress utrophin are currently being developed[23,24], and this preclinical
106 development could benefit from a gold standard or an adequate positive control to
107 use in these assays.

108 **CRISPR/Cas9** currently represents a very efficient and versatile genome-engineering
109 tool, introducing small and large DNA modifications, including large genomic deletions
110 in different cell types and organisms[25]. In the presence of two single guide (sg) RNAs
111 targeting two different loci on the same chromosome, Cas9 can induce two DNA
112 double strand breaks (DDSBs), leading in some cases to deletion of the excised DNA
113 segment through repair by the non-homologous end joining (NHEJ) pathway[26]. Similar
114 to antisense oligo-mediated exon skipping therapies at RNA level, CRISPR/Cas9 can
115 therefore be used to remove mutations by deleting mutated exons and restore the
116 open reading frame of the *DMD* gene[27-29]. The advantage of this approach is that the
117 genetic modification, once introduced, is stable over cell cycles. CRISPR/Cas9 has been
118 successfully employed to correct mutations and/or restore the open reading frame
119 recovering dystrophin expression both *in vitro* and *in vivo*[30,31]. However, some hurdles
120 have been reported, such as difficulties transfecting myoblast or a recent study in the
121 golden retriever muscular dystrophy dog (GRMD), where no dystrophin restoration at
122 protein level was evident after gene editing using this technology[32]. More importantly,
123 as well as these preclinical problems others such as possible off-target problems or
124 immunogenicity linked with the use of Cas9[33] may delay their clinical application.
125 However, while gene editing as therapeutic option still needs further development,
126 CRISPR/Cas9 methodology has been applied to provide a large number of new animal
127 models to further understand DMD pathology and perform preclinical studies[34]. In our
128 quest to optimise the preclinical development of new therapies for DMD, we have
129 developed a genome editing strategy applicable in control and patient myoblasts.
130 Here, we report two new cell cultured models that can be successfully used for
131 preclinical assessment of new DMD therapies: a culture that replicates a patient's
132 deletion **(DMDΔ52-Model)** and another that overexpresses utrophin **(DMD-UTRN-**
133 **Model)**.

134 ***RESULTS***

135 **Generation of cell culture models by gene editing**

136 We completed two different gene editing projects: **objective 1** aimed to delete exon
137 52 of the *DMD* gene (a common mutation in DMD patients) in control immortalised

138 human myoblasts to generate a disease model **(DMDΔ52-Model)**; **objective 2 (DMD-**
139 **UTRN-Model)** was to delete in the *UTRN* gene of DMD immortalized human myoblasts
140 an inhibitory microRNA target region to generate a utrophin ectopic expression rescue
141 model.

142 Each edition required to design two sgRNAs flanking the region to be deleted to
143 generate two DDSBs leading to the removal of that region (Figure 1A and D). All the 25
144 different combinations of the sgRNAs designed, cutting before (x5) and after (x5) the
145 target region, were tested in HEK293 cultures first (Supplementary figure 1A and B).
146 The most efficient combination of sgRNAs in HEK293 cells for each objective was
147 selected to be used in the transfection of human immortalized myoblasts
148 (Supplementary figure 1C and D).
149 To accomplish objective 1, two GFP-plasmids, each encoding Cas9 nuclease and either
150 sgRNA 2 or sgRNA 6, were transfected into human immortalized control myoblasts. For
151 objective 2, the selected GFP-plasmids, encoded Cas9 nuclease and either sgRNA 22 or
152 sgRNA 26 and were transfected into human immortalized DMD myoblasts. After FACS
153 sorting of individual GFP-positive cells, clones were expanded for DNA extraction
154 (Supplementary figure 2). Clones were analysed to confirm the presence of the desired
155 deletions by genomic PCR performed with specific primers for each targeted gene
156 (Figure 1B and 1E), amplicons corresponding in size with the expected deletions were
157 analysed by Sanger sequencing, and the expected deletions were confirmed in all the
158 positive clones (Figure 1C and 1F).
159 For **objective 1** two positive clones were obtained, clone number 2 and 7 but only
160 clone 7 was used for further analysis and called **DMDΔ52-Model** (Figure 1B and 1C).
161 For **objective 2,** two clones were edited in only one allele, corresponding to numbers 3
162 and 4 and other two were completely edited, numbers 2 and 8. In this case, clone
163 number 8 was selected to be used for further analysis and was called **DMD-UTRN-**
164 **Model** (Figure 1E and 1F).
165 To evaluate any potential off-target effects, each selected sgRNA was analysed *in silico*
166 using the bioinformatics web-tool CRISPOR[35]. We selected the six more likely off-target
167 sites for each sgRNA and analysed each one of them through PCR, followed by Sanger

168 sequencing in edited clones (Table 2 and 3). We found no off-target effects in any of
169 the 12 sites studied for each clone sites (Supplementary figure 3).

170 **Analysis of dystrophin and utrophin expression in edited clones.**

171 We compared dystrophin expression in myotubes of the DMDΔ52-Model to controls
172 and DMD cultures and confirmed that it was abolished by immunocytochemistry
173 (Figure 2A), western blot analysis (Figure 2B), myoblots (Figure 2C) and droplet digital
174 PCR (ddPCR) (Figure 2D). Dystrophin levels in this model, where exon 52 had been
175 removed by CRISPR/Cas9 editing, were statistically no different than those seen in a
176 culture from a DMD patient.

177 Immunocytochemistry showed the increase of utrophin expression between unedited
178 DMD and DMD-UTRN-Model myotubes (Figure 3A) and this increase was corroborated
179 by western blot (a 175% increase, Figure 3B), myoblot analysis (close to 50% increase,
180 Figure 3C) and ddPCR (Figure 3D).

181 We also quantified dystrophin and utrophin expression in the edited cultures by
182 myoblot during their differentiation process and compared these with control and
183 DMD myotubes (Supplementary figure 4, bar chart).

184 **Analysis of differentiation markers expression in edited clones.**

185 As we suspected that the editing and cloning process could have affected the
186 differentiation of the edited models, the fusion index (%) of edited and non-edited
187 myotubes after MF20 and Hoechst inmunocytochemistry was calculated (Figure 2E and
188 3E) and the expression of different myogenic regulatory factors like the myogenic
189 factor 5 (Myf5) and the myosin heavy chain isoform 3 (MyH3) were analysed at
190 different time points during myotube formation in DMDΔ52-Model and DMD-UTRN-
191 Model as well as in their corresponding unedited cultures by ddPCR (Figure 2F and 3F).
192 Fusion index was clearly lower in DMDΔ52-Model compared to control myoblasts
193 (Figure 2E) while no significant differences were found between DMD-UTRN-Model
194 and DMD cultures (Figure 3E). As expected, Myf5 expression decreased while MyH3
195 increased during the differentiation process in both models and followed the same
196 pattern in their origin cells, however we see that MyH3 marker is significantly lower in
197 the edited models at day 5 and day 7 after starting fusion (Figure 2F and 3F).

198 The MF20 differentiation marker was also analysed by myoblot in edited cultures and
199 we could observe a decrease in both edited clones, no matter the deletion, compared
200 with their corresponding controls (Supplementary figure 4, red lines).
201
202 **Evaluation of therapies in newly generated model cell lines.**
203 To assess if the DMDΔ52-Model cell culture could be useful to test potential mutation
204 specific therapies for DMD, we evaluated the exon skipping efficiency of an antisense
205 oligonucleotide in this culture. We treated the DMDΔ52-Model cultures with an
206 antisense oligonucleotide drug that can skip exon 51[36] and restore *DMD* open reading
207 frame. After treatment with this drug, we confirmed that exon skipping had taken
208 place at RNA level (Figure 4A), and a limited restoration of dystrophin expression by
209 myoblot analysis (Figure 4B).
210 To test our DMD-UTRN-Model as a positive utrophin overexpression control, we
211 cultured it alongside the original unedited DMD cultures, which we treated with
212 several concentrations of ezutromid and we evaluated the expression of utrophin in all
213 cultures. We observed that utrophin was hardly modified in DMD cultures treated with
214 ezutromid while a robust overexpression was confirmed in the DMD-UTRN-Model
215 compared to the unedited DMD cultures (Figure 4C). We also confirmed that this
216 overexpression was stable during a time course experiment (Supplementary figure 4D).

217 ***DISCUSSION***

218 CRISPR/Cas9 has been explored in the past as a possible therapeutic approach for
219 DMD in *in vitro* models of the disease. In most of these cases, a "permanent" exon
220 skipping approach was selected where a shorter protein would be produced[27,28], while
221 in some others full length dystrophin was the result of the edition[37-39]. Several studies
222 have shown efficacy in mice models[40-42], an most recently in dogs, which is currently
223 the most advanced example of its application to DMD[29] but there are also examples of
224 the challenges associated to gene editing[32]. Before this can be a credible therapeutic
225 option, several delivery and manufacturing problems will need to be overcome. In the
226 meantime, this methodology is very useful for researchers looking for cell culture
227 models: muscle biopsies are not routinely collected during diagnosis of this disorder
228 and seldom cultured. This means there are few good culture models of the disease.

229 Another tool created to facilitate research was the immortalisation of some of the
230 available cultures[12], which increases the possibility of performing more experiments
231 with a given culture. We have used immortalised cultures to further increase this
232 possibility.

233 Although we are concerned about the efficiency limitations of our gene editing
234 protocol, specially due to transfection difficulties in myoblasts reported also by other
235 laboratories[27,38]; we have successfully applied it to edit 2 different regions in two
236 different cell backgrounds (Control and DMD), and we consider that those described
237 and fully characterised in this manuscript could be relevant research models that we
238 would be happy to share. The first of these models is an immortalised DMD disease
239 cell culture model, (DMDΔ52-Model) that lacks exon 52 of the *DMD* gene, which
240 disrupts the ORF and dystrophin expression. This model could be useful to evaluate
241 mutation-independent drug treatments, and also exon skipping drugs that aim to skip
242 exons 51 or 53[43,44] as skipping either exon in this case would restore the ORF and
243 dystrophin expression. We have demonstrated that DMDΔ52-Model lacks dystrophin
244 expression and that this can be reverted through treatment with an exon 51 skipping
245 drug. However, the main interest of this model was demonstrating the efficacy of the
246 technique, as immortalised cultures from patients with the same deletion are currently
247 available. We have replicated this protocol to generate other DMD-like cultures
248 (details will be included in a manuscript being currently drafted).

249 An immortalised cell culture model constitutively expressing utrophin, DMD-UTRN-
250 Model, is both a proof of principle of a possible therapeutic option to overexpress
251 utrophin as a substitute for dystrophin, and a valuable research tool. The search of
252 drugs that could overexpress utrophin is ongoing[45] after the recent failure of
253 ezutromid to show relevant results in clinical trials. Many research projects, including
254 drug re-purposing screening, are ongoing to find new candidates to test in clinical
255 trials[46]. However, there are no reliable positive controls that could be used to compare
256 such treatments. We propose that our cell model could serve that purpose, offering
257 researchers useful custom controls for their studies. We have tested this hypothesis
258 and compared the stable utrophin overexpression quantified in the DMD-UTRN-Model
259 with the utrophin that is expressed after treatment of the unedited DMD patient

260 cultures with ezutromid, the lead market candidate in this field until very recently,
261 with positive results. Previous studies in muscle sections show that DMD patients
262 already overexpress utrophin, in many cases 4 to 5-fold the levels seen in control
263 muscle sections[47]. Our choice to target this particular *UTR* region, increases basal
264 overexpression in DMD cultures, and the amount of overexpression varies significantly
265 when evaluated by western blot (more than 2.5 times) or our preferred method,
266 myoblots (close to 1.5 times). We like to consider that myoblot evaluation reflects
267 more closely the actual protein expression, as it is not subjected to many of the
268 inherent problems of western blotting when evaluating very large proteins[48]. This is
269 why we cannot comment yet on the differences in expression between our study and
270 other published studies that also aimed to overexpress utrophin by gene editing, but
271 which 1) targeted different promoter regions (*UTRN* A or *UTRN* B) of utrophin and 2)
272 evaluated their results by western blot analysis[38]. We would be interested on studying
273 this matter further to analyse the differences in utrophin expression when targeting
274 different regions.

275 As a conclusion, we have created two new cell culture models that we have already
276 used as efficient tools in our search for new therapies for DMD, after optimization of a
277 gene editing method to be applied to myoblasts, a rather difficult target. We expect
278 our experience to be useful to other muscle researchers and we are looking forward
279 expanding those experiments.

280 ***METHODS***

281 **CRISPR/Cas9 tools**

282 Specific sgRNA guides were designed using the online bioinformatics tool
283 http://crispr.mit.edu[49]. Ten different guides (five before and five after the target
284 region) were designed targeting exon 52 flanking regions in *DMD* gene and another ten
285 targeting a repressor binding site in the UTR 3' region of *UTRN* gene and selected
286 according to their score number (Table 1). They were cloned into a plasmid containing
287 *Cas9* from *S. pyogenes* with 2A-*EGFP* pSpCas9 (BB)-2A-GFP (PX458) (Addgene plasmid #
288 48138, deposited by Feng Zhang). All sgRNAs were cloned using BbsI sites.

289

290 **Cell cultures**

291 Human embryonic kidney 293 (HEK 293) cells, used in the preliminary selection of the
292 best sgRNA combinations for our experiments, were purchased from the European
293 Collection of Authenticated Cell cultures (ECCAC) via Sigma-Aldrich, Spain, and
294 maintained following the manufacturer's protocols.

295 Immortalized myoblasts derived from muscle biopsies from healthy controls and DMD
296 patients were provided by the CNMD Biobank, London, UK and the Institut de
297 Myologie Paris, France, and cultured using skeletal muscle medium (SMM) (Promocell,
298 Germany) and differentiation media as previously described[48].

299

300 **Cell culture transfection**

301 All different sgRNAs combinations were transfected into HEK 293 cells using
302 lipofectamine 2000® (Thermo Fisher Scientific), according to the manufacturer's
303 protocol. Myoblasts seeded in 6 well plates at 70-80% confluence were transfected
304 with 1.5ug of each plasmid with the most efficient guide RNA combination using
305 ViaFect™ (Promega) transfection reagent (1:5 ratio).

306

307 **FACS workflow of GFP positive myoblasts**

308 48 hours after transfection, myoblasts were trypsinised and collected for fluorescence
309 activated cell sorting (FACS) at the Cell Analytics Facility (BD FACS Jazz) Achucarro
310 Basque Center for Neuroscience - (Leioa, Spain). GFP-positive cells were seeded

311 individually in 96 well plates for clonal selection. The first colonies were visible around
312 7 days post-sorting. Clones were expanded from single cell to near-confluence and
313 expanded into larger well plates to be harvested 15-30 days post-sorting. Myoblasts
314 often developed elongated and stressed shapes during this clonal expansion after
315 single cell sorting. Harvested cultures were aliquoted: some aliquots were frozen for
316 archival; others were pelleted for DNA analysis, while replicates were cultured further
317 for characterization by immunocytochemistry, western blot and myoblots (see
318 schematic workflow in Supplementary figure 2).
319

320 **Analysis of gene editing**

321 DNA was extracted from cell pellets using a QIAamp® DNA Mini Kit, Qiagen. PCR
322 amplification targeting the edited regions was carried out using Taq DNA Polymerase
323 (Recombinant), Invitrogen, under the following conditions: preheating 3' 94ºC, 25
324 cycles of 94º for 3', 94º 20'', 63º 20'', 72º 1' and 72º 5' and DMD-Seq-D52-DOWN-F2
325 and DMD-Seq-D52-DOWN-R2 primers (see Suplementary Table 1). PCR products were
326 resolved in 2% TAE-agarose gels and purified with QIAquick® Gel extraction Kit,
327 Qiagen. PCR amplicons corresponding to the expected length were analysed by Sanger
328 sequencing at the sequencing platform of Biocruces Bizkaia Health Research Institute
329 using DMD-Seq-D52-DOWN-F2 and DMD-Seq-D52-DOWN-R2 primers (see
330 Supplementary Table 1).

331

332 **Off-target analysis of mutations in clonal lines**

333 Potential off-target region loci of each sgRNA used were predicted using CRISPOR
334 bioinformatics tool http://crispor.tefor.net/. The six most probable off-target
335 sequences per guide (Table 2 and 3) were analysed in the edited clones using genomic
336 PCR and Sanger sequencing. Primer sets flanking off-target sites and the corresponding
337 internal primers used for Sanger sequencing are listed in Supplementary Table 2.

338

339 **Primary Antibodies**

340 Anti-dystrophin: Dys1 (Leica Biosystems), Mandys1, Mandys106 (The MDA Monoclonal
341 Antibody Resource) and anti-dystrophin antibody (Abcam 15277).
342 Anti-utrophin: Mancho7 (The MDA Monoclonal Antibody Resource).
343 Anti-myosin heavy chain (MF20: Developmental Studies Hybridoma Bank).
344 Anti a-actinin (Sigma-Aldrich A7732).
345

346 **Immunostaining assays**

347 Original and edited clones for objectives 1 (DMDΔ52-Model) and 2 (DMD-UTRN-
348 Model) were cultured and immunostained for dystrophin or utrophin expression.
349 Edited clones were seeded into chamber slides and treated with a MyoD virus,
350 (Applied Biological Materials Inc, Canada) to facilitate differentiation into myotubes[50].
351 After seven days differentiating, samples were fixed with 4% PFA. Cultures were
352 permeabilised with Triton 0.5% and then blocked for half an hour with BSA 2%.
353 Afterwards, immunostaining was performed overnight at 4ºC with the required
354 antibodies. Primary antibodies used for dystrophin staining were a mix of Dys1,
355 Mandys1 and Mandys106 at 1:100 dilution and for utrophin staining was Mancho 7
356 diluted at 1:50. The following day, after being washed with PBS Tween 0.1%, cells were
357 stained with Alexa Fluor 488 goat anti-mouse (Invitrogen) for 1 hour at room
358 temperature. Hoechst 1/2000 was used for nuclei staining and chamber slides were
359 mounted with PermaFluor™ Aqueous Mounting Medium (Thermoscientific). Images
360 were captured with a LEICA DMI 6000B microscope at the Microscopy Platform at
361 Biocruces Bizkaia Health Research Institute.

362

363 **In-cell western assay (myoblots)**

364 Myoblots were performed as described before[48]. In short, clones were seeded in 96-
365 well plates and incubated for 24 h in SMMC, after which they were treated with MyoD
366 virus and incubated in differentiation media for 7 days. Then, plates were fixed with
367 ice-cold methanol, permeabilised and blocked before incubation with the required
368 primary antibodies overnight: anti-dystrophin mix (Dys1, Mandys1 and Mandys106 at
369 1:100), anti-utrophin (Mancho 7 antibody at 1:50), and anti-myosin heavy chain

370 antibody (MF20 at 1:100) that was used to evaluate differentiation. Next day, plates
371 were incubated with the secondary antibodies. Biotin-mediated amplification (Abcam
372 6788 goat antimouse IgG biotin 1:2000) was used to increase dystrophin signal.
373 Secondary antibodies, IRDye 800cw streptavidin 1:2000 and IRDye 800CW goat anti-
374 mouse 1:500, were prepared together with CellTag 700 Stain (LI-COR® Biosciences) at
375 1:1000 dilution and incubated for 1 hour at RT and protected from light. After
376 incubation, plates were analysed using the Odyssey® CLx Imager (LI-COR® Biosciences).

377

378 **Treatment with antisense exon skipping drugs**

379 Cultures in 96 wells and P6 wells were treated with a 2'MOE-phosphorotioate
380 antisense oligonucleotide (AO) aiming to skip *DMD* exon 51 (5-[
381 T*C*A*A*G*G*A*A*G*A*T*G*G*C*A*T*T*T*C*T]-3, Eurogentec, Belgium) by
382 transfection with Lipofectamine as described in[43,44] and analysed by either myoblot (96
383 well plates) or RT-PCR (pellets extracted from 6 well plates).

384

385 **RT-PCR**

386 RNA was extracted from cell pellets (RNeasy mini kit, Qiagen) according to the
387 manufacturer's protocol. Reverse transcription of the samples was performed using
388 (SuperScrip™ IV Reverse Transcriptase, Invitrogen) according to the manufacturer's
389 protocol. cDNA samples were either used for digital droplet PCR analysis or amplified
390 by nested PCR using specific primers sets (Supplementary Table 1) and Taq DNA
391 Polymerase (Recombinant), Invitrogen, as described in[44] for exon skipping analysis.
392 Amplified samples were resolved in TAE-agarose and PCR amplicons of interest were
393 first analysed with Gel Doc TM EZ Imager, BIORAD and then purified with (QIAquick®
394 Gel extraction Kit, QIAGEN) for sequencing analysis. Before DNA extractions bands
395 were semi-quantified using Image J.

396

397 **Treatment with utrophin overexpression drugs**

398 Ezutromid was diluted first in DMSO and finally in differentiation medium to different
399 concentrations and added to myoblasts in 96 well plates 7 days after differentiation.
400 Twenty-four hours after treatment, medium was removed, and plates were fixed with
401 ice-cold methanol for myoblot analysis.
402
403 **Western blot**
404 Cell cultures were seeded into P6 plates and trypsinized after 7 days of differentiation.
405 Then, cell pellets were solubilized in lysis/loading buffer and denatured at 95°C for 5
406 min. Samples were loaded onto a NuPAGE® Novex® 3–8% Tris-Acetate Gel3–8%
407 (Thermo Fisher Scientific) and run in Novex Tris-Acetate SDS Running Buffer (Thermo
408 Fisher Scientific) for 60 min at 70 V + 120 min at 150 V at 4ºC. Protein wet transfer was
409 performed overnight at 4°C using an Immobilon®-FL PVDF membrane (Merck™). Next
410 day, membranes were stained with Revert ™ 700 Total Protein Stain (Li-Cor) for total
411 protein measurement, blocked with Intercept (PBS) Blocking Buffer (Li-Cor) for 2 hours
412 and incubated overnight at 4°C with the primary antibodies (1/200 anti-dystrophin
413 antibody Abcam15277, 1/50 anti-utrophin antibody Mancho 7 or 1/500 anti a-actinin
414 antibody, Sigma-Aldrich A7732). After washing steps with PBS-Tween 0.1%,
415 membranes were incubated with secondary antibodies for 1 hour (1/5000 IRDye
416 800CW goat anti-rabbit 926–32211 or IRDye 680RD goat anti-mouse 926-68070, Li-
417 Cor) at room temperature, washed again and scanned using an Odyssey Clx imaging
418 system. Bands quantification was performed using Image Studio ™ software.
419
420 **Droplet digital PCR (ddPCR)**
421 Gene expression was detected and quantified using a QX200™ Droplet Digital™ PCR
422 system (Bio-Rad). The reaction was performed using 2 µl of cDNA in a 20 µl reaction
423 volume containing: 1 µl of Utrophin Taqman probe (ID: Hs01125975, FAM labelled),
424 1µl of MYF5 ddPCR™ GEX Assay probe (ID: dHsaCPE5026295, HEX labelled) or MYH2
425 ddPCR™ GEX Assay probe (ID: dHsaCPE5050991, HEX labelled), 10 µl of ddPCR™
426 Supermix for Probes (no dUTP) (Bio-Rad) and 6 µl of DNase/RNase-free H2O.
427 To generate the droplets, 20 µL of the previous ddPCR reaction and 70 µL of Droplet
428 Generation Oil for Probes (Bio-Rad) were added to the 8 channel droplet generation
429 cartridge according to manufacturer instructions and this cartridge was placed in the

430 QX200 droplet generator (Bio-Rad). Then, 40 µL of the resulting droplet emulsion was
431 transferred to a semi-skirted 96 well PCR plate (Eppendorf), sealed with foil and
432 amplified on a thermal cycler using the following amplification conditions: enzyme
433 activation 10', 40 cycles of 94°C for 30'' and 55°C for 1', and heat deactivation 10' 98°C.
434 Plates containing the amplified droplets were loaded into the QX200 droplet reader
435 and results were analysed using QuantaSoft software™ (Bio-Rad).
436
437 **Statistical analysis**
438 Mann-Whitney U test was used throughout this study to calculate P values for
439 determination of statistical significance (*p-value<0.05, ** p-value<0.01, ****p-
440 value<0.0001). Statistical analysis was performed using GraphPad Prism software.
441

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COMPETING INTERESTS

The authors declare no competing interests

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DATA AVAILABILITY

Under request

AUTHOR CONTRIBUTIONS STATEMENT

P S-M, formal analysis, investigation, data curation, writing or original draft, review and editing, visualization, supervision. E. A-A, investigation, data curation, writing -review and editing. A. A-M, investigation, writing - review and editing. L. d-l-P-O, investigation, writing - review and editing. I. G-J, methodology, investigation, review and editing, supervision. G. G-I, investigation, writing - review and editing. I. L-A, investigation, writing - review and editing. A. L-M, investigation, writing - review and editing. J. P-G, investigation, writing - review and editing. E. R-D-Y, methodology, investigation, writing-review and editing, supervision. F. G conceptualization, formal analysis, writing-review and editing. V. A-G: conceptualization, methodology, formal analysis, investigation, resources, data curation, writing or original draft, review and editing, visualization, supervision, project administration and funding acquisition.

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642 ***Figure legends***

643 **Table 1. List of sgRNAs**

644 List of sgRNAs for editing the *DMD* and the *UTRN* loci, showing sgRNAs sequences,
645 PAM sequences and scores of all sgRNA tested.

646 **Table 2. Off-target sites analyses.**

647 Top 6 off-target sequences of Ob1sgRNA2 and Ob1sgRNA6 identified with CRISPOR
648 webtool, including the mismatches between sgRNAs, the off-target sequence, the
649 chromosomes and loci targeted. All of them were analysed by PCR and Sanger
650 sequencing, and no off-targets were detected.

651 **Table 3. Off-target sites analyses.**

652 Top 6 off-target sequences of Ob2sgRNA22 and Ob2_sgRNA26 identified with CRISPOR
653 webtool, including the mismatches between sgRNAs, the off-target sequence, the
654 chromosomes and loci targeted. All of them were analysed by PCR and Sanger
655 sequencing, and no off-targets were detected.

656

657 **Figure 1. Editing approach and genotyping *DMD* and *UTRN* deletion breakpoints in**
658 **edited myoblast clones**.

659 (A and D). Schematic representation of our strategy for editing the *DMD* (A) and the
660 *UTRN* loci (D). A pair of flanking sgRNAs were co-transfected in order to delete *DMD*
661 exon 52 (A) or the inhibitory microRNA target region contained in the 5'UTR of *UTRN*
662 (D). (B and C) PCR genotyping of *DMD* edited clones (B) and Sanger sequencing of
663 clone 7 or DMDΔ52-Model (C) . (E and F) PCR genotyping of *UTRN* edited clones (E) and
664 Sanger sequencing of clone 8 or DMD-UTRN-Model (F). Larger products in agarose gels
665 (B and E) indicate non-edited clones, and shorter ones correspond with the expected
666 deletion. (C and F) Sequences of the smaller bands confirmed the expected gene
667 editing for objective 1: DMDΔ52-Model (C) and objective 2: DMD-UTRN-Model (F).

668

669 **Figure 2. Characterization of DMDΔ52-Model cultures.**

670 Dystrophin expression in DMDΔ52-Model cultures compared to control myotubes and
671 DMD myotubes were studied by immunocytochemistry (A), western blotting (B) and
672 myoblots (C). Myoblot experiments, where n=24 wells per cell type were compared,
673 were performed twice. (D) Dystrophin expression in DMDΔ52-Model cultures was
674 compared to control myotubes by ddPCR. For ddPCR experiments three technical
675 replicates per sample and condition were run in parallel and a no template control
676 (NTC) was included as negative control. (E) Differentiated myotubes of DMDΔ52-
677 Model and control cultures were inmunostained with MF20 and Hoechst antibodies.
678 Fusion index was calculated as the ratio between the number of nuclei in
679 differentiated myotubes (defined as >2 nuclei and MF20-positive cells) compared to
680 the total number of nuclei. For quantification, five fields per cell line were randomly
681 chosen and more than 200 nuclei were counted. Analysis was performed using ImageJ
682 software. (F) Differentiation markers, Myf5 and MyH3, were studied by ddPCR at
683 different fusion times in DMDΔ52-Model cultures compared to control myotubes. For
684 ddPCR experiments three technical replicates per sample and condition were run in
685 parallel and a no template control (NTC) was included as negative control. (*p-value
686 <0.05, ** p-value <0.01, ****p-value<0.0001). (P values were determined with Mann-
687 Whitney U test).

688 **Figure 3. Characterization of DMD-UTRN-Model cultures.**

689 Utrophin expression in DMD myotubes compared to DMD-UTRN-Model studied by
690 immunocytochemistry (A), western blotting (B) and myoblots (C). Myoblot
691 experiments, where n=48 wells per cell type were compared, were performed twice.
692 (D) Utrophin expression in DMD-UTRN-Model cultures was compared to DMD
693 myotubes by ddPCR. For ddPCR experiments three technical replicates per sample and
694 condition were run in parallel and a no template control (NTC) was included as
695 negative control. (E) Differentiated myotubes of DMD-UTRN-Model and DMD cultures
696 were inmunostained for MF20 and Hoechst. Fusion index was calculated as the ratio
697 between the number of nuclei in differentiated myotubes (defined as >2 nuclei and
698 MF20-positive cells) compared to the total number of nuclei. For quantification, five

699 fields per cell line were randomly chosen and more than 200 nuclei were counted.
700 Analysis was performed using ImageJ software. (F) Differentiation markers, Myf5 and
701 MyH3, were studied by ddPCR at different fusion times in DMD-UTRN-Model cultures
702 compared to DMD myotubes. For ddPCR experiments three technical replicates per
703 sample and condition were run in parallel and a no template control (NTC) was
704 included as negative control. (*p-value <0.05, ** p-value <0.01, ****p-value<0.0001).
705 (P values were determined with Mann-Whitney U test).

706 **Figure 4. Evaluation of potential therapies in the generated cell culture models.**

707 (A) DMDΔ52-Model cell line was treated with an antisense to skip exon 51 and the
708 effect was evaluated by RT-PCR and nested PCR analysis. Gel picture shows a pattern
709 corresponding with the correct skipping, which was confirmed by Sanger sequencing.
710 The same experiment evaluated by myoblot using n=20 replicate wells per condition
711 (B) showed the restoration of dystrophin expression in the treated cultures compared
712 to non-treated (*P values<0.05. P values were determined with Mann-Whitney U test).
713 (C) The DMD-UTRN-Model was used as a positive control in an experiment in which
714 unedited DMD cultures were treated with different ezutromid concentrations to up-
715 regulate utrophin expression. Myoblot analysis using n=8 replicate wells per condition
716 of the treated cultures shows that ezutromid had no significant effect in DMD cultures
717 while utrophin expression is significantly increased in DMD-UTRN-Model compared to
718 unedited DMD cultures. (**P values <0.01. P values were determined with Mann-
719 Whitney U test).
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721

CODE	SCORE	SEQUENCE	PAM
sgRNA1	79	CTGAAGAACCCTGATACTAA	GGG
sgRNA2	78	GCTGAAGAACCCTGATACTA	AGG
sgRNA3	69	AACAAATATCCCTTAGTATC	AGG
sgRNA4	65	ACAAATATCCCTTAGTATCA	GGG
sgRNA5	60	TAAGGGATATTTGTTCTTAC	AGG
sgRNA6	48	ATTTCTAAAAGTGTTTTGGC	TGG
sgRNA7	44	AAAAAAGATGTTACTGTATA	AGG
sgRNA8	44	AAAAAGATGTTACTGTATAA	GGG
gsRNA9	29	TTTACTTTGTATTATGTAAA	AGG
sgRNA10	24	TTTTATTTCTAAAAGTGTTT	TGG
sgRNA21	71	AACTTTGGGTTCTCTTTAGC	TGG
sgRNA22	66	GGTTCTCTTTAGCTGGGATC	TGG
sgRNA23	63	TATTTTAGAATAGGTTGGGT	GGG
sgRNA24	62	ACTTTGGGTTCTCTTTAGCT	GGG
sgRNA25	62	TCTAACTTTAAGCCTCCTTC	TGG
sgRNA26	76	GTGCTTTCTTGGGTATGACA	TGG
sgRNA27	68	CAAAGTCTAGAGCTTTTATC	AGG
sgRNA28	66	CAACTTGGAGTTGAGAGCTC	AGG
sgRNA29	64	TCAACTCCAAGTTGTAGATT	TGG
sgRNA30	63	TCCATCTTCATCCATTGCAT	TGG

722 **Table 1. List of sgRNAs**

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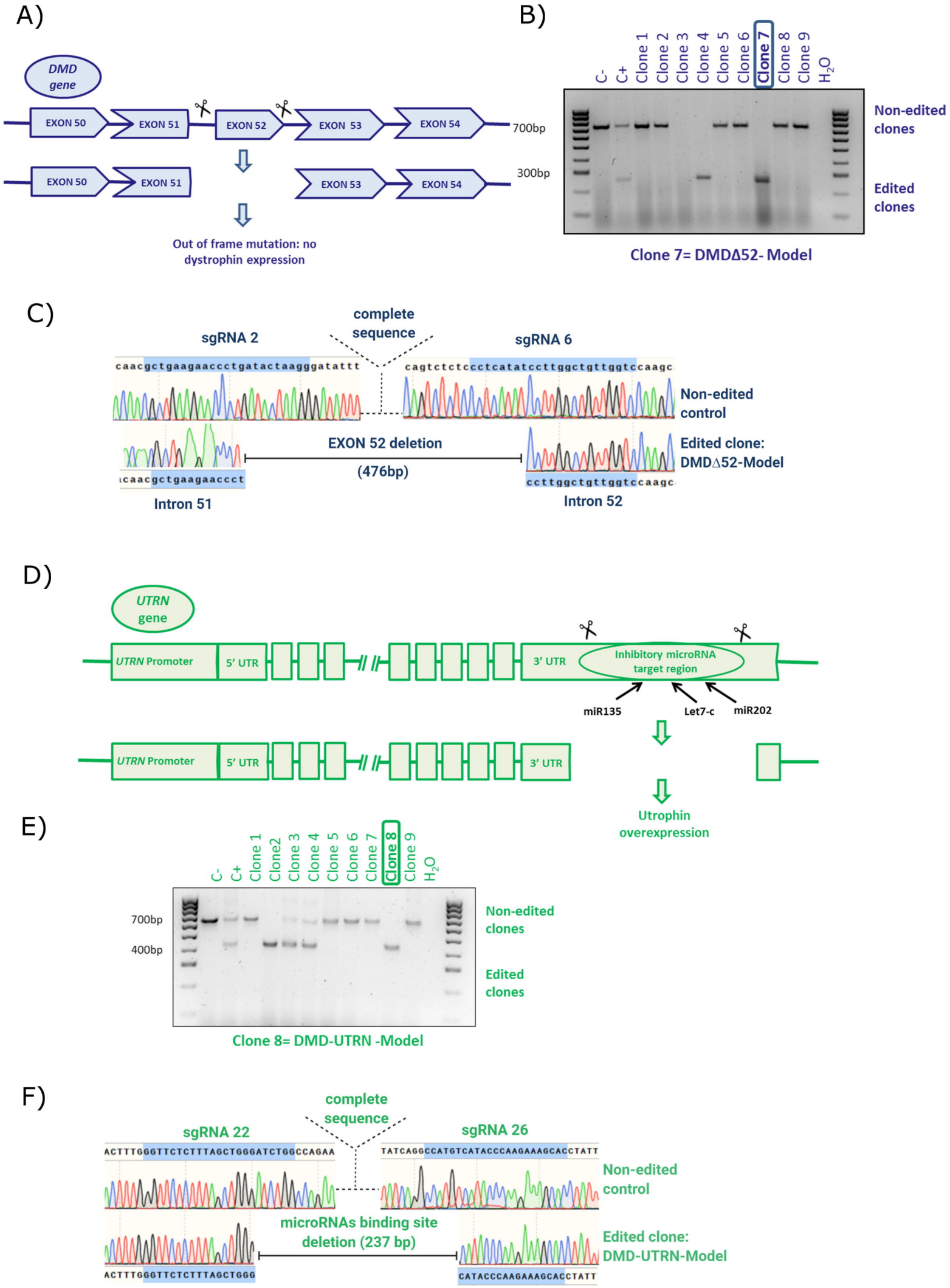
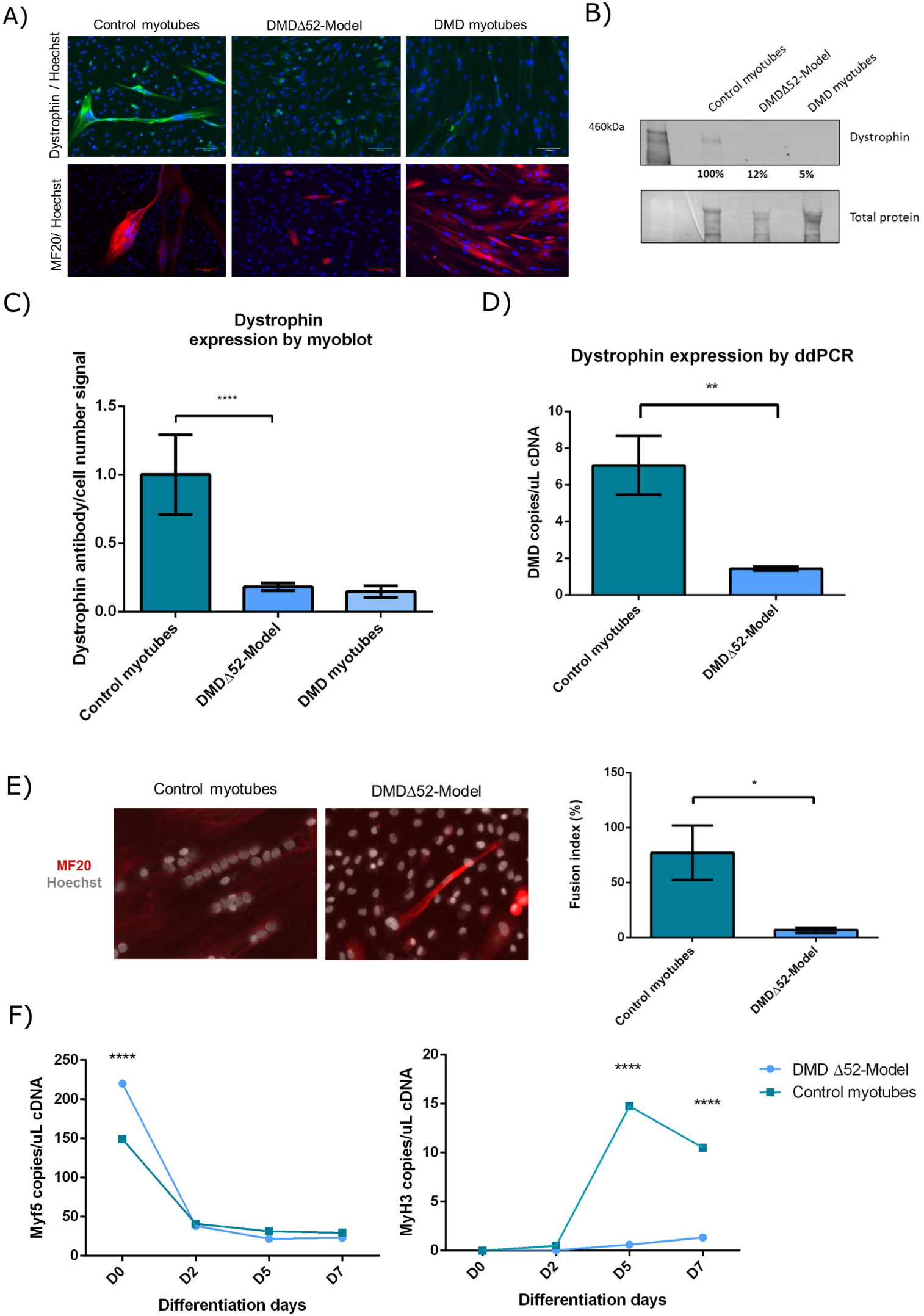
Off Target name	Guide sequence	Off target sequence	Mis	Chrom	Locus	PCR analysis	Sequencing
Ob1_2_Off1	GCTGAAGAACCCTGATACTAAGG (Ob1_sgRNA2)	GCTGGAGAACCCTGATACTGTGG	2	chr1	intergenic:RP4-781L3.1-RP4-706G24.1	No off-target editing	Confirmed
Ob1_2_Off2		TCTGGAGAACCCTAATACTAAGG	3	chr8	intergenic:RP11-24P4.1-AC009695.1	No off-target editing	Confirmed
Ob1_2_Off3		ACTGAAGAATCCAGAAACTAGGG	4	chr7	intergenic:NOBOX-RP4-545C24.5	No off-target editing	Confirmed
Ob1_2_Off4		GCTAGAGAAACCTGAAACTAAGG	4	chr8	intergenic:RP11-536K17.1-EIF3H	No off-target editing	Confirmed
Ob1_2_Off5		TCTGGAGAACCCTAATACTGTGG	4	chr3	intron:TMEM45A	No off-target editing	Confirmed
Ob1_2_Off6		TCTGAAGAATCCTGATATTTTGG	4	chr2	intron:AC019100.3	No off-target editing	Confirmed
Ob1_6_Off1	GACCAACAGCCAAGGATATGAGG (Obj1 sgRNA6)	CACCATCAGCCAAGAATATGCGG	3	chr11	intergenic:RP11-430H10.3-RP11-958J22.1	No off-target editing	Confirmed
Ob1_6_Off2		TAACAACAGCCAAAGACATGAGG	4	chr14	exon:RP11-1012A1.4/RDH11	No off-target editing	Confirmed
Ob1_6_Off3		GTAAAAGAGCCAAGGATATGAGG	4	chr10	intron:RP11-556E13.1	No off-target editing	Confirmed
Ob1_6_Off4		TACTAGCAGCCAAGGATATCTGG	4	chr2	intergenic:AC007377.1-SLC8A1	No off-target editing	Confirmed
Ob1_6_Off5		GAGCGACAGCCAAGAATATTCGG	4	chr3	intron:CD96	No off-target editing	Confirmed
Ob1_6_Off6		AATCAACAGCCAAGAATGTGGGG	4	chr5	intergenic:CTD-2201E9.4-SEMA5A	No off-target editing	Confirmed

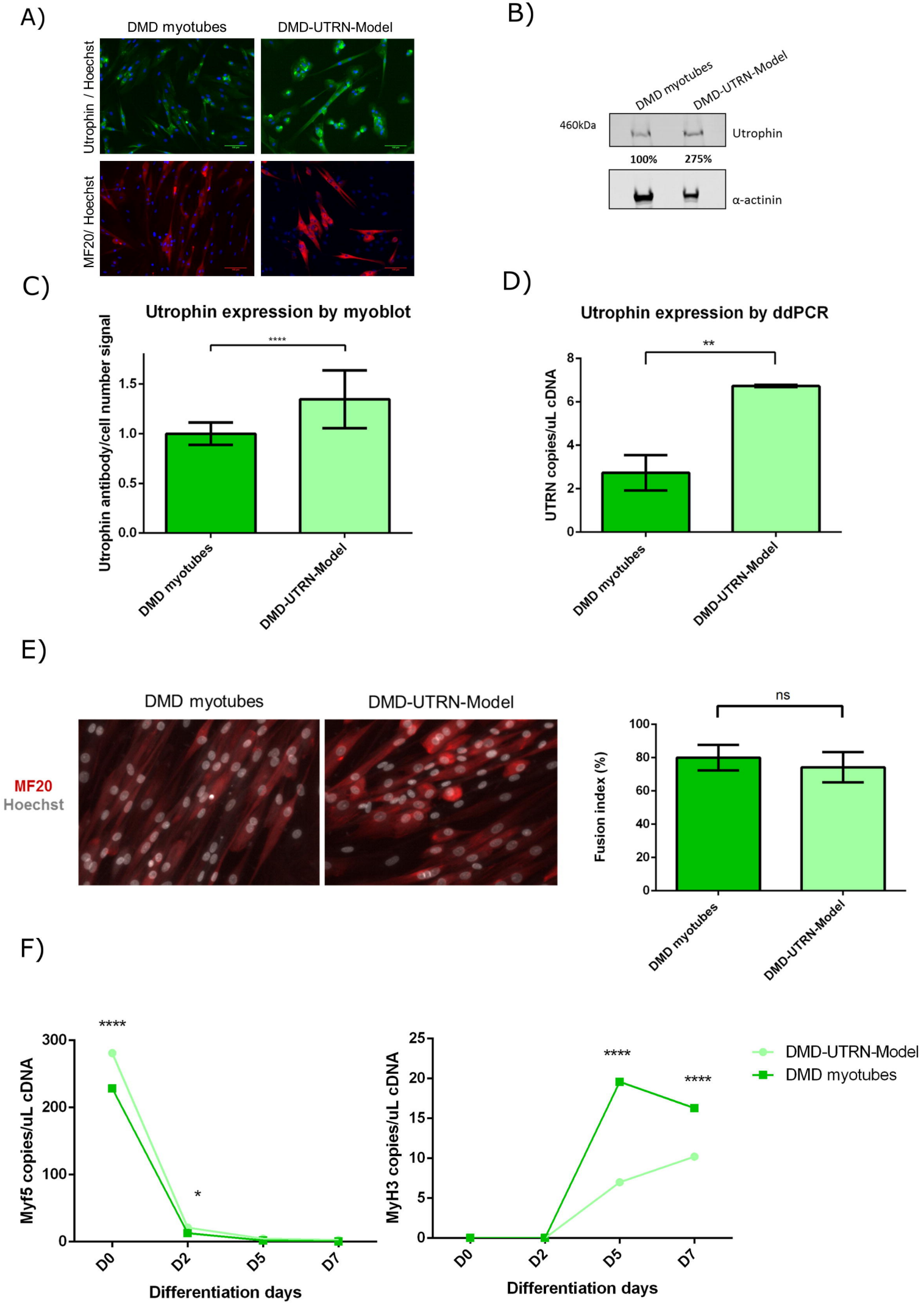
Table 2. Objective 1 potential off-target sites.

Off Target name	Guide sequence	Off target sequence	Mis	Chrom	Locus	PCR analysis	Sequencing
Ob2_22_Off1	GGTTCTCTTTAGCTGGGATCTGG (Obj2 sgRNA22)	TGTTCTCTCTAACTGGGATCTGG	3	chr18	intergenic:RP11-411B10.6-RP11-411B10.5	No off-target editing	Confirmed
Ob2_22_Off2		TGTTCTCTAGAGCTGGGATCTGG	3	chr21	intron:LCA5L	No off-target editing	Confirmed
Ob2_22_Off3		TGTTCTCTCCAACTGGGATCTGG	4	chr22	intron:PPP6R2	No off-target editing	Confirmed
Ob2_22_Off4		GAATCCTTTTAGCTGGGATCAGG	4	chr19	intron:ZNF536	No off-target editing	Confirmed
Ob2_22_Off5		GGTTCATCTTAGCTGGGATATGG	4	chr13	intron:FLT1	No off-target editing	Confirmed
Ob2_22_Off6		TGTTCTCTCTAACTGGGGTCTGG	4	chr21	intergenic:PPP6R2P1-AP001347.6	No off-target editing	Confirmed
Ob2_26_Off1	GTGCTTTCTTGGGTATGACATGG (Obj2 sgRNA26)	AAGCTTTCCTGGATATGACAAGG	4	chr4	intron:RNF150	No off-target editing	Confirmed
Ob2_26_Off2		GTGCTTACTTGGGTAAGACGTGG	3	chr17	intergenic:RP11-212E8.1-RP11-642M2.1	No off-target editing	Confirmed
Ob2_26_Off3		GAGTTAACTTGGGTATGACAGGG	4	chr4	intron:RGS12	No off-target editing	Confirmed
Ob2_26_Off4		GTGCTCTCATGAGAATGACAGGG	4	chr4	intergenic:GABRG1-RP11-320H14.1	No off-target editing	Confirmed
Ob2_26_Off5		GAGCTTTCCTGGGAATGACAGGG	3	chr1	intergenic:FOXO6-RNA5SP45	No off-target editing	Confirmed
Ob2_26_Off6		GTGCTTTATAGGATATAACATGG	4	chr6	intron:GSTA3	No off-target editing	Confirmed

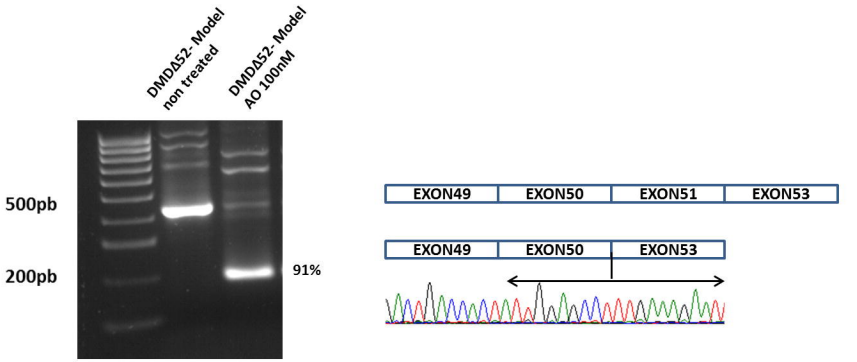
Table 3. Objective 2 potential off-target sites.


A)
DMD gene
EXON 50
EXON 51
EXON 52
EXON 53
EXON 54
700bp
300bp
Out of frame mutation: no dystrophin expression
B)
C-
C+
Clone 1
Clone 2
Clone 3
Clone 4
Clone 5
Clone 6
Clone 7
Clone 8
Clone 9
H2O
Non-edited clones
Edited clones
Clone 7= DMDΔ52- Model
C)
complete sequence
sgRNA 2
sgRNA 6
caacgctgaagaaccctgatactaagggatattt
cagtctctccctcatatccttggctgttggtccaagc
Non-edited control
EXON 52 deletion
(476bp)
Edited clone: DMDΔ52-Model
caacgctgaagaaccct
ccttggctgttggtccaagc
Intron 51
Intron 52
D)
UTRN gene
UTRN Promoter
5' UTR
3' UTR
Inhibitory microRNA target region
miR135
Let7-c
miR202
Utrophin overexpression
E)
C-
C+
Clone 1
Clone2
Clone 3
Clone 4
Clone 5
Clone 6
Clone 7
Clone 8
Clone 9
H2O
700bp
400bp
Non-edited clones
Edited clones
Clone 8= DMD-UTRN -Model
F)
complete sequence
sgRNA 22
sgRNA 26
ACTTTGGGTTCTCTTTAGCTGGGATCTGGCCAGAA
TATCAGGCCATGTCATACCCAAGAAAGCACCTATT
Non-edited control
microRNAs binding site deletion (237 bp)
Edited clone: DMD-UTRN-Model
ACTTTGGGTTCTCTTTAGCTGGG
CATACCCAAGAAAGCACCTATT

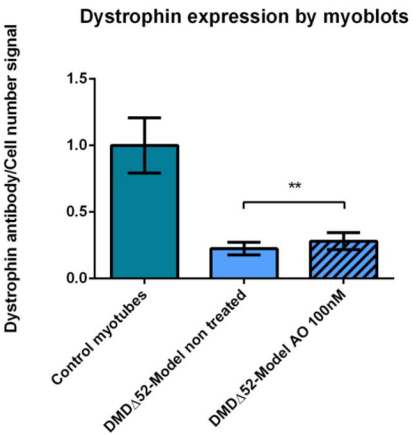

A)
Control myotubes
DMDΔ52-Model
DMD myotubes
Dystrophin / Hoechst
MF20/ Hoechst
B)
Control myotubes
DMDΔ52-Model
DMD myotubes
460kDa
Dystrophin
100%
12%
5%
Total protein
C)
Dystrophin expression by myoblot
Dystrophin antibody/cell number signal
****
1.5
1.0
0.5
0.0
Control myotubes
DMDΔ52-Model
DMD myotubes
D)
Dystrophin expression by ddPCR
DMD copies/uL cDNA
**
10
8
6
4
2
0
Control myotubes
DMDΔ52-Model
E)
Control myotubes
DMDΔ52-Model
MF20
Hoechst
Fusion index (%)
*
150
100
50
0
Control myotubes
DMDΔ52-Model
F)
Myf5 copies/uL cDNA
****
250
200
150
100
50
0
D0
D2
D5
D7
Differentiation days
MyH3 copies/uL cDNA
****
****
20
15
10
5
0
D0
D2
D5
D7
Differentiation days
DMD Δ52-Model
Control myotubes


A)
DMD myotubes
DMD-UTRN-Model
Utrophin / Hoechst
MF20/ Hoechst
B)
DMD myotubes
DMD-UTRN-Model
460kDa
Utrophin
100%
275%
α-actinin
C)
Utrophin expression by myoblot
Utrophin antibody/cell number signal
****
1.5
1.0
0.5
0.0
DMD myotubes
DMD-UTRN-Model
D)
Utrophin expression by ddPCR
UTRN copies/uL cDNA
**
8
6
4
2
0
DMD myotubes
DMD-UTRN-Model
E)
DMD myotubes
DMD-UTRN-Model
MF20
Hoechst
ns
Fusion index (%)
100
80
60
40
20
0
DMD myotubes
DMD-UTRN-Model
F)
Myf5 copies/uL cDNA
****
*
300
200
100
0
D0
D2
D5
D7
Differentiation days
MyH3 copies/uL cDNA
****
****
25
20
15
10
5
0
D0
D2
D5
D7
Differentiation days
DMD-UTRN-Model
DMD myotubes

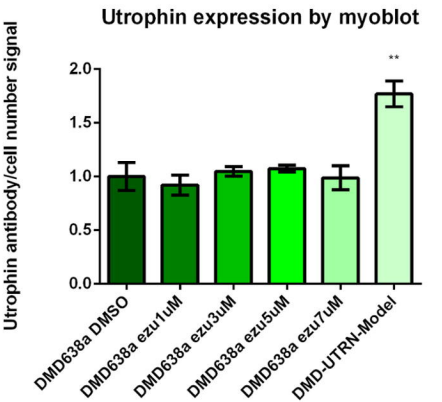
A)


DMDΔ52- Model non treated
DMDΔ52- Model AO 100nM
500pb
200pb
91%
EXON49
EXON50
EXON51
EXON53
EXON49
EXON50
EXON53

B)


Dystrophin expression by myoblots
Dystrophin antibody/Cell number signal
1.5
1.0
0.5
0.0
**
Control myotubes
DMDΔ52-Model non treated
DMDΔ52-Model AO 100nM

C)


Utrophin expression by myoblot
Utrophin antibody/cell number signal
2.0
1.5
1.0
0.5
0.0
**
DMD638a DMSO
DMD638a ezu1uM
DMD638a ezu3uM
DMD638a ezu5uM
DMD638a ezu7uM
DMD-UTRN-Model