A CRISPR/Cas9-BASED APPROACH FOR EDITING IMMORTALISED HUMAN MYOBLASTS TO MODEL DUCHENNE MUSCULAR DYSTROPHY IN VITRO

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Summary

We report two novel immortalised myoblast culture models for studying Duchenne muscular dystrophy (DMD), generated through CRISPR/Cas9 gene editing: one recapitulates a common *DYSTROPHIN* (*DMD*) deletion and the other a regulatory mutation leading to *UTROPHIN (UTRN)* ectopic upregulation.

Abstract

CRISPR/Cas9-mediated gene editing may allow treating and studying rare genetic disorders by respectively, correcting disease mutations in patients, or introducing them in cell cultures. Both applications are highly dependent on Cas9 and sgRNA delivery efficiency. While gene editing methods are usually efficiently applied to cell lines such as HEK293 or hiPSCs, CRISPR/Cas9 editing *in vivo* or in cultured myoblasts prove to be much less efficient, limiting its use. After a careful optimisation of different steps of the editing protocol, we established a consistent approach to generate human immortalised myoblasts disease models through CRISPR/Cas9 editing. Using this protocol we successfully created a coding deletion of exon 52 of the *DYSTROPHIN (DMD)* gene in wild type immortalised myoblasts modelling Duchenne muscular dystrophy (DMD), and a microRNA binding sites deletion in the regulatory region of the *UTROPHIN (UTRN)* gene leading to utrophin upregulation in in Duchenne muscular dystrophy patient immortalised cultures. Sanger sequencing confirmed the presence of the corresponding genomic alterations and protein expression was characterised using myoblots. To show the utility of these cultures as platforms for assessing the efficiency of DMD treatments, we used them to evaluate the impact of exon skipping therapy and ezutromid treatment. Our editing protocol may be useful to others interested in genetically manipulating myoblasts and the resulting edited cultures for studying DMD disease mechanisms and assessing therapeutic approaches.

KEYWORDS: Duchenne muscular dystrophy, Dystrophin, Utrophin, Gene editing

55 ***INTRODUCTION***

56 **Duchenne muscular dystrophy** (DMD) is a fatal X-linked recessive disease affecting
57 one out of 3.500-5.000 newborn males. It is commonly caused by deletions disrupting
58 the open reading frame of the *DYSTROPHIN (DMD)* gene causing a lack of dystrophin
59 protein (Hoffman et al., 1987). Patients carrying out of frame mutations present a
60 severe phenotype, while those carrying in-frame mutations may result in hypomorphic
61 alleles, a partially functional dystrophin and milder phenotypes, such as in Becker
62 muscular dystrophy (BMD)(Anthony et al., 2011). Dystrophin plays a major role in
63 membrane stabilization during muscle contraction, linking the actin cytoskeleton to
64 the sarcolemma (Muntoni et al., 2003) and also contributes to extracellular signalling
65 (Lai et al., 2009). Lack of dystrophin in DMD patients' muscles, leads to progressive
66 muscle wasting and degeneration. DMD children suffer from loss of ambulation in the
67 first or second decade of life and premature death by cardiac and respiratory
68 complications (Eagle et al., 2002).

69 Although no definitive cure for DMD is available, three drugs have been recently
70 approved by different regulatory agencies. Ataluren, approved by the European
71 Medicines Agency (EMA), induces readthrough of premature stop codons during
72 mRNA translation, generating a full length dystrophin protein (Finkel, 2010). Eteplirsen
73 and golodirsen, approved by the US Food and Drug Administration (FDA), are antisense
74 oligonucleotides. Eteplirsen targets *DMD* exon 51 and golodirsen, exon 53. Both
75 antisense oligonucleotides modulate splicing by exon skipping, restoring *DMD* reading
76 frame and leading to a shorter but functional protein (Kinali et al., 2009; Muntoni et
77 al., 2018). Exon skipping therapies aim to attenuate the phenotype and phenocopy
78 milder BMD-like genotypes, to potentially improve disease outcome. Ataluren,
79 eteplirsen and golodirsen are designed for rescuing specific patient mutations only
80 present respectively in 13%, 13% and 8% of patients (Aartsma-Rus et al., 2006). It is
81 therefore important to test and assess alternative exon-skipping strategies targeting
82 other *DMD* exons in different phases of clinical assays (Arechavala-Gomeza et al.,
83 2012). Alternatively, a number of compounds applicable to all DMD patients are also
84 under evaluation, targeting secondary DMD pathologies or trying to compensate for
85 the lack of dystrophin.

86 ***UTROPHIN*** *(UTRN)* is an autosomal paralog of dystrophin, expressed in skeletal muscle
87 cells during embryonic development, but restricted to neuromuscular and
88 myotendinous junctions in the mature muscle fibre (Tinsley et al., 1992).
89 Overexpression of utrophin in skeletal muscle in DMD animal models can partially
90 compensate the lack of dystrophin and improve DMD phenotype (Cerletti et al., 2003;
91 Tinsley et al., 1998; Tinsley et al., 1996). Importantly, ectopic and high levels of
92 utrophin in myoblasts are not associated with toxicity, making utrophin upregulation
93 an interesting therapeutic strategy applicable to all patients, no matter their particular
94 mutation (Fairclough et al., 2013; Fisher et al., 2001). Ezutromid/SMT-C1100 was the
95 first utrophin modulator evaluated in clinical assays, but was recently abandoned due
96 to lack of evidence of utrophin restoration, nor clinical improvement for patients
97 (Ricotti et al., 2016; Tinsley et al., 2015). Alternatively, other studies proposed new
98 strategies to upregulate *UTRN* by removing the binding sites of microRNAs repressing
99 *UTRN* expression through gene editing (Amenta et al., 2011; Goyenvalle et al., 2010;
100 Morgoulis et al., 2019; Pisani et al., 2018). Finally, utrophin upregulation has been
101 efficiently achieved in multiple animal models with non-immunogenic response using
102 gene therapy (Song et al., 2019).

103 **CRISPR/Cas9** currently represents the most efficient and versatile genome-engineering
104 tool, introducing small and large DNA modifications, including large genomic deletions
105 in different cell types and organisms (Wright et al., 2016). Hence, in the presence of
106 two single guide (sg) RNAs targeting two different loci on the same chromosome, Cas9
107 can induce two DNA double strand breaks (DDSBs), leading in some cases to deletion
108 of the excised DNA segment through repair by the non-homologous end joining (NHEJ)
109 pathway (He et al., 2015). Similar to antisense oligo-mediated exon skipping therapies
110 at RNA level, CRISPR/Cas9 can therefore be used to remove mutations by deleting
111 mutated exons and restore the open reading frame of the *DMD* gene (Amoasii et al.,
112 2018; Ousterout et al., 2015; Young et al., 2016). The advantage of this approach is
113 that the genetic modification, once introduced, is stable over cell cycles. However, it´s
114 efficiency is currently too low to provide a real therapeutic alternative *in vivo*, not even
115 mentioning immunogenicity and off-target problems linked with the use of Cas9
116 (Charlesworth et al., 2019) . These difficulties have been recently reported in the

117 golden retriever muscular dystrophy dog (GRMD), were no dystrophin restoration at
118 protein level was obtained after gene editing using this technology (Mata Lopez et al.,
119 2020).

120 In order to easily and rapidly assess the efficiency of current and novel therapies to
121 treat DMD, *in vitro* cellular models are particularly useful. However, only a few human
122 immortalized muscle cell lines derived from DMD patients are currently available
123 (Mamchaoui et al., 2011). Due to the wide spectrum of DMD mutations and the
124 difficulties to obtain DMD patient muscle biopsies, an efficient approach to edit DMD
125 mutations in immortalised myoblasts would be extremely valuable to generate a more
126 exhaustive panel of DMD-myoblast models. Such lines would provide a powerful
127 resource for *in vitro* drug screening and study disease rescue mechanisms.

128 Here, we report an optimised CRISPR/Cas9 approach to edit myoblasts to create DMD
129 disease models. We successfully used this protocol to created two new cell lines: in
130 objective 1, control myoblasts were edited to remove *DMD* exon 52 (a common
131 mutation in DMD patients); in objective 2, DMD patient's myoblasts were edited to
132 generate a utrophin ectopic expression rescue model by deleting miRNA binding sites
133 in *UTRN* regulatory region.

134 ***RESULTS***

135 **Optimisation of gene editing in myoblasts and generation of two new cell culture**
136 **models.**

137 • <u>Guide selection for CRISPR/Cas9 system.</u>

138 We had two different gene editing objectives: **objective 1** aimed to delete exon 52 of
139 the *DMD* gene to generate a disease model in control immortalised human myoblasts;
140 **objective 2** was to delete in the *UTRN* gene a binding site for *UTRN*-repressing
141 microRNAs in DMD immortalized human myoblasts.

142 Our strategy to perform CRISPR/Cas9 editing in myoblasts was to design two sgRNAs
143 flanking the region to be deleted in order to generate two DSBs leading to removal of
144 that region (Figure 1).

145

146

147 • Testing sgRNAs in HEK293 cultures.

148 As transfection of myoblast is very inefficient, all the different combinations of the
149 sgRNAs cutting before and after the target region (5x5), were tested in HEK293
150 cultures first (Figure 2). The combination of sgRNAs that was most efficient in HEK293
151 cells for each objective was selected to be used in the transfection of human
152 immortalized myoblasts.

153 • Myoblast transfection and single cell sorting workflow

154 Myoblasts were transfected with the two GFP-plasmids encoding each of the selected
155 sgRNA selected for the edition of the target region. After comparing four different
156 transfection reagents and an electroporation method, transfection of myoblasts with
157 ViaFect™ (Promega) was selected (See Supplementary figure 1. After transfection,
158 GFP-positive cells were sorted by FACS (See Supplementary figure 1), seeded as single
159 cells in 96 well plates (five plates per condition) and expanded in culture (see
160 schematic workflow in Figure 3).

161 • Selection of edited clones:

162 A limited number of clones derived from GFP-positive single cells grew enough for
163 further analysis: 20/314 for objective 1 and 35/480 for objective 2. To confirm the
164 presence of the desired deletions, a genomic PCR was performed with specific primers
165 for each targeted gene (Figure 4 and Supplementary Table 1). Amplicons
166 corresponding in size with the expected deletions were analysed by Sanger sequencing
167 and the expected deletions were confirmed in all the positive clones, which
168 corresponds to editing efficiency between 5-6%. To evaluate any potential off-target
169 effects, each selected sgRNA was analysed *in silico* using the bioinformatics web-tool
170 CRISPOR (Haeussler et al., 2016) . We selected the six more likely off-target sites for
171 each sgRNA and analysed each one of them through PCR, followed by Sanger
172 sequencing in edited clones (Table 1). We found no off-target effects in any of the 12
173 sites studied for each clone sites (Supplementary figure 2).

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

175 We compared dystrophin expression in myotubes of the DMDΔ52-Model to controls
176 and DMD cultures and confirmed that it was abolished by immunocytochemistry

177 (Figure 5A), western blot analysis (Figure 5B) and myoblots (Figure 5C). Dystrophin
178 levels in this model, where exon 52 had been removed by CRISPR/Cas9 editing, were
179 statistically no different than those seen in a culture from a DMD patient.
180
181 It was difficult to corroborate by immunocytochemistry the increase of utrophin
182 expression between unedited DMD and DMD-UTRN-Model myotubes (Figure 5D) but
183 this increase was evident by western blot (a 175% increase, Figure 5E) and myoblot
184 analysis (close to 50% increase, Figure 5F). We also evaluated myoblast differentiation
185 in all the cultures by myoblot, and we could observe a decrease in the MF20
186 differentiation marker in all the edited clones, no matter the deletion, compared with
187 their corresponding controls (Supplementary Figure 3).
188
189 **Evaluation of therapies in newly generated model cell lines.**
190 To assess if the DMDΔ52-Model cell culture could be useful to test potential mutation
191 specific therapies for DMD, we evaluated the exon skipping efficiency of an antisense
192 oligonucleotide in this culture. We treated the DMDΔ52-Model cultures with an
193 antisense oligonucleotide drug that can skip exon 51 (van Deutekom et al., 2007) and
194 restore *DMD* open reading frame. After treatment with this drug, we confirmed that
195 exon skipping had taken place at RNA level (Figure 6A), and the restoration of
196 dystrophin expression by myoblot analysis (Figure 6B).
197 To test our DMD-UTRN-Model as a positive utrophin overexpression control, we
198 cultured it alongside the original unedited DMD cultures, which we treated with
199 several concentrations of ezutromid and we evaluated the expression of utrophin in all
200 cultures. We observed that utrophin was hardly modified in DMD cultures treated with
201 ezutromid while a robust overexpression was confirmed in the DMD-UTRN-Model
202 compared to the unedited DMD cultures. (Figure 6C)

203 ***DISCUSSION***

204 CRISPR/Cas9 as a possible therapeutic approach for DMD has been explored in the
205 past in *in vitro* models of the disease. In most of these cases, a "permanent" exon
206 skipping approach was selected where a shorter protein would be produced
207 (Ousterout et al., 2015; Young et al., 2016), while in some others full length dystrophin

208 was the result of the edition (Lattanzi et al., 2017; Li et al., 2014; Wojtal et al., 2015).
209 Several studies have shown efficacy in mice models (Long et al., 2016; Nelson et al.,
210 2016; Tabebordbar et al., 2016), an most recently in dogs, which is currently the most
211 advanced example of its application to DMD (Amoasii et al., 2018) but there are also
212 examples of the challenges associated to gene editing (Mata Lopez et al., 2020). Before
213 this can be a credible therapeutic option, several delivery and manufacturing problems
214 will need to be overcome. In the meantime, this methodology is very useful for
215 researchers looking for disease models: muscle biopsies are not routinely collected
216 during diagnosis of this disorder and seldom cultured. This means there are few good
217 culture models of the disease. Another tool created to facilitate research, was the
218 immortalisation of some of the available cultures (Mamchaoui et al., 2011), which
219 increases the possibility of performing more experiments with a given culture. We
220 have used immortalised cultures to further increase this possibility.

221 Our preliminary experiments to perform gene editing in the *DMD* gene of HEK293 cells
222 were successful. However, a big roadblock was encountered in the form of very poor
223 transfection efficiency of our chosen gene editing reagents in myoblasts (Ousterout et
224 al., 2015; Wojtal et al., 2015). This is a problem shared by many research laboratories,
225 and we consider that our protocol, although still not very efficient, may help those
226 facing the same difficulties. We have successfully applied this protocol to edit 2
227 different regions in two different cell backgrounds (Control and DMD), and we
228 consider that those described and fully characterised in this manuscript could be
229 relevant research models that we would be happy to share. The first of these models is
230 an immortalised DMD disease cell culture model, (DMDΔ52-Model) that lacks exon 52
231 of the *DMD* gene, which disrupts the ORF and dystrophin expression. This model could
232 be useful to evaluate mutation-independent drug treatments, and also exon skipping
233 drugs that aim to skip exons 51 or 53 (Arechavala-Gomeza et al., 2007; Popplewell et
234 al., 2010) as skipping either exon in this case would restore the ORF and dystrophin
235 expression. We have demonstrated that DMDΔ52-Model lacks dystrophin expression
236 and that this can be reverted through treatment with an exon 51 skipping drug.

237 An immortalised cell culture model constitutively expressing utrophin, DMD-UTRN-
238 Model, is both a proof of principle of a possible therapeutic option to overexpress

239 utrophin as a substitute for dystrophin, and a valuable research tool. The search of
240 drugs that could overexpress utrophin is ongoing (Guiraud and Davies, 2017) after the
241 recent failure of ezutromid to show relevant results in clinical trials. Many research
242 projects, including drug re-purposing screening, are ongoing to find new candidates to
243 test in clinical trials. However, there are no reliable positive controls that could be used
244 to compare such treatments. We propose that our cell model could serve that
245 purpose, offering researchers useful custom controls for their studies. We have tested
246 this hypothesis and compared the stable utrophin overexpression quantified in the
247 DMD-UTRN-Model with the one that is obtained after treatment with ezutromid, the
248 lead market candidate in this field until very recently, with positive results. Previous
249 studies in muscle sections show that DMD patients already overexpress utrophin, in
250 many cases 4 to 5-fold the levels seen in control muscle sections (Arechavala-Gomeza
251 et al., 2010). Our choice to target this particular *UTR* region, increases basal
252 overexpression in DMD cultures, and the amount of overexpression varies significantly
253 when evaluated by western blot (more than 2.5 times) or our preferred method,
254 myoblots (close to 1.5 times). We like to consider that myoblot evaluation reflects
255 more closely the actual protein expression, as it is not subjected to many of the
256 inherent problems of western blotting when evaluating very large proteins (Ruiz-Del-
257 Yerro et al., 2018). This is why we cannot comment yet on the differences in
258 expression between our study and other published studies that also aimed to
259 overexpress utrophin by gene editing, but which 1) targeted different promoter
260 regions (*UTRN* A or *UTRN* B) of utrophin and 2) evaluated their results by western blot
261 analysis (Wojtal et al., 2015). We would be interested on studying this matter further
262 to analyse the differences in utrophin expression when targeting different regions.

263 As a conclusion, we have optimised a gene editing method to be applied to myoblasts,
264 a rather difficult target, and expect our experience to be useful to other muscle
265 researchers. The two new cell culture models described are already being used as
266 efficient tools in our search for new therapies for DMD and we are looking forward
267 expanding those experiments.

268 ***MATERIALS AND METHODS***

269 **CRISPR/Cas9 tools**

270 Each edition requires 2 sgRNAs. Specific sgRNA guides were designed using the online
271 bioinformatics tool http://crispr.mit.edu (Ran et al., 2013). Ten different guides (five
272 before and five after the target region) were designed targeting exon 52 flanking
273 regions in *DMD* gene and another ten targeting a repressor binding site in the UTR 3'
274 region of *UTRN* gene and selected according to their score number (Figure 1). They
275 were cloned into a plasmid containing *Cas9* from *S. pyogenes* with 2A-*EGFP*
276 pSpCas9(BB)-2A-GFP (PX458) (Addgene plasmid # 48138, deposited by Feng Zhang. All
277 sgRNAs were cloned using BbsI sites.

278 **Cell cultures**

279 Immortalized myoblasts derived from muscle biopsies from healthy controls and DMD
280 patients were provided by the CNMD Biobank, London, UK and the Institut de
281 Myologie Paris, France, and cultured using skeletal muscle medium (SMM) (Promocell,
282 Germany) and differentiation media as previously described (Ruiz-Del-Yerro et al.,
283 2018).

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

288 **Cell culture transfection**

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

294

295 **FACS Sorting of GFP positive myoblasts:**

296 48 hours after transfection myoblasts were trypsinised and collected for FACS sorting
297 (fluorescence activated cell sorting), at the Cell Analytics Facility (BD FACS Jazz)

298 Achucarro Basque Center for Neuroscience - (Leioa, Spain). GFP-positive cells were
299 seeded individually in 96 well plates for clonal selection. The first colonies were visible
300 around 7 days post-sorting. Clones were expanded from single cell to near-confluence
301 and expanded into larger well plates to be harvested 15-30 days post-sorting.
302 Myoblasts often developed elongated and stressed shapes during this clonal expansion
303 after single cell sorting. Harvested cultures were aliquoted: some aliquots were frozen
304 for archival; others were pelleted for DNA analysis, while replicates were cultured
305 further for characterization by immunocytochemistry, western blot and myoblots.
306

307 **Analysis of gene editing**

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

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

318 Potential off-target region loci of each sgRNA used were predicted using CRISPOR
319 bioinformatics tool http://crispor.tefor.net/. The six most probable off-target
320 sequences per guide were analysed in the edited clones using genomic PCR and Sanger
321 sequencing. Primer sets flanking off-target sites and the corresponding internal
322 primers used for Sanger sequencing are listed in table 2.

323 **Primary Antibodies**

324 Anti-dystrophin: Dys1 (Leica Biosystems), Mandys1, Mandys106 (The MDA Monoclonal
325 Antibody Resource)
326 Anti-utrophin: Mancho7 (The MDA Monoclonal Antibody Resource)
327
328

329

330 **Immunostaining assays**

331 Original and edited clones for objectives 1 (DMDΔ52-Model) and 2 (DMD-UTRN-
332 Model) were cultured and immunostained for dystrophin or utrophin expression.
333 Edited clones were seeded into chamber slides and treated with a MyoD virus,
334 (Applied Biological Materials Inc, Canada) to facilitate differentiation into myotubes
335 (Roest et al., 1996). After seven days differentiating, samples were fixed with 4% PFA.
336 Cultures were permeabilised with Triton 0.5% and then blocked for half an hour with
337 BSA 2%. Afterwards, immunostaining was performed overnight at 4ºC with the
338 required antibodies. Primary antibodies used for dystrophin staining were a mix of
339 Dys1, Mandys1 and Mandys106 at 1:100 dilution and for utrophin staining was
340 Mancho 7 diluted at 1:50. The following day, after being washed with PBS Tween 0.1%,
341 cells were stained with Alexa Fluor 488 goat anti-mouse (Invitrogen) for 1 hour at
342 room temperature. Hoechst 1/2000 was used for nuclei staining and chamber slides
343 were mounted with PermaFluor™ Aqueous Mounting Medium (Thermoscientific).
344 Images were captured with a LEICA DMI 6000B microscope at the Microscopy Platform
345 at Biocruces Bizkaia Health Research Institute.

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

347 Myoblots were performed as described before (Ruiz-Del-Yerro et al., 2018). In short,
348 clones were seeded in 96-well plates and incubated for 24 h in SMMC, after which they
349 were treated with MyoD virus and incubated in differentiation media for 7 days. Then,
350 plates were fixed with ice-cold methanol, permeabilised and blocked before incubation
351 with the required primary antibodies overnight: anti-dystrophin mix (Dys1, Mandys1
352 and Mandys106 at 1:100), anti-utrophin (Mancho 7 antibody at 1:50), and anti-myosin
353 heavy chain antibody (MF20 at 1:100) that was used to evaluate differentiation. Next
354 day, plates were incubated with the secondary antibodies. Biotin-mediated
355 amplification (Abcam 6788 goat antimouse IgG biotin 1:2000) was used to increase
356 dystrophin signal. Secondary antibodies, IRDye 800cw streptavidin 1:2000 and IRDye
357 800CW goat anti-mouse 1:500, were prepared together with CellTag 700 Stain (LI-
358 COR® Biosciences) at 1:1000 dilution and incubated for 1 hour at RT and protected

359 from light. After incubation, plates were analysed using the Odyssey® CLx Imager (LI-
360 COR® Biosciences).

361 **Treatment with antisense exon skipping drugs**

362 Cultures in 96 wells and P6 wells were treated with a 2'MOE-phosphorotioate
363 antisense oligonucleotide (AO) aiming to skip *DMD* exon 51 (5-[
364 T*C*A*A*G*G*A*A*G*A*T*G*G*C*A*T*T*T*C*T]-3, Eurogentec, Belgium) by
365 transfection with Lipofectamine as described in (Arechavala-Gomeza et al., 2007;
366 Popplewell et al., 2010) and analysed by either myoblot (96 well plates) or RT-PCR
367 (pellets extracted from 6 well plates).

368 **RT-PCR**

369 RNA was extracted from cell pellets (RNeasy mini kit, Qiagen) according to the
370 manufacturer's protocol. Reverse transcription of the samples was performed using
371 (SuperScrip™ IV Reverse Transcriptase, Invitrogen) according to the manufacturer's
372 protocol. cDNA samples were amplified by nested PCR using specific primers sets
373 (supplementary table 1) and Taq DNA Polymerase (Recombinant), Invitrogen, as
374 described in (Arechavala-Gomeza et al., 2007). Samples were resolved in TAE-agarose
375 and PCR amplicons of interest were first analysed with Gel Doc TM EZ Imager, BIORAD
376 and then purified with (QIAquick® Gel extraction Kit, QIAGEN) for sequencing analysis.
377 Before DNA extractions bands were semi-quantified using Image J.

378 **Treatment with utrophin overexpression drugs**

379 Ezutromid was diluted first in DMSO and finally in differentiation medium to different
380 concentrations and added to myoblasts in 96 well plates 7 days after differentiation.
381 Twenty four hours after treatment, medium was removed and plates were fixed with
382 ice-cold methanol for myoblot analysis.

383

384 **Western blot**

385 Cell cultures were seeded into P6 plates and trypsinized after 7 days of differentiation.
386 Then, cell pellets were solubilized in lysis/loading buffer and denatured at 95°C for 5
387 min. The samples were loaded onto a NuPAGE® Novex® 3–8% Tris-Acetate Gel3–8%
388 (Thermo Fisher Scientific) and run in Novex Tris-Acetate SDS Running Buffer (Thermo

389 Fisher Scientific) for 60 min at 70 V + 120 min at 150 V at 4ºC. Protein wet transfer was
390 performed overnight at 4°C using an Immobilon®-FL PVDF membrane (Merck™). Next
391 day, membranes were stained with Revert ™ 700 Total Protein Stain (Li-Cor) for total
392 protein measurement, blocked with Intercept (PBS) Blocking Buffer (Li-Cor) for 2 hours
393 and incubated overnight at 4°C with the primary antibodies (1/200 anti-dystrophin
394 antibody Abcam15277, 1/50 anti-utrophin antibody Mancho 7 or 1/500 anti a-actinin
395 antibody, Sigma-Aldrich A7732). After washing steps with PBS-Tween 0.1%,
396 membranes were incubated with secondary antibodies for 1 hour (1/5000 IRDye
397 800CW goat anti-rabbit 926–32211 or IRDye 680RD goat anti-mouse 926-68070, Li-
398 Cor) at room temperature, washed again and scanned using an Odyssey Clx imaging
399 system. Bands quantification was performed using Image Studio ™ software.
400

401 **Statistical analysis**

402 Mann-Whitney U test was used throughout this study to calculate P values for
403 determination of statistical significance (**P values<0.05, ****p-value<0.0001).
404 Statistical analysis was performed using GraphPad Prism software.

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411 Resource, which distributes free antibodies for research in neuromuscular diseases
412 worldwide from Oswestry, United Kingdom.

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414 obtained from the Developmental Studies Hybridoma Bank, created by the NICHD of
415 the NIH and maintained at The University of Iowa, Department of Biology, Iowa City, IA
416 52242.

Cell sorting experiments were performed at the Cell Analytics Facility at Achucarro-Basque Centre of Neuroscience (Leioa, Spain).

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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. 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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609

610

611 ***Figure legends***

612 **Table 1. Off-target sites analyses.**

613 Top 6 off-target sequences of Ob1sgRNA2, Ob1sgRNA6, Ob2sgRNA22 and
614 Ob2_sgRNA26 identified with CRISPOR webtool, including the mismatches between
615 sgRNAs, the off-target sequence, the chromosomes and loci targeted. All of them were
616 analysed by PCR and Sanger sequencing, and no off-targets were detected.

617 **Figure 1. Editing approach and sgRNA design**.

618 (A and B). Schematic representation of our strategy for editing the *DMD* (A) and the
619 *UTRN* loci (B). A pair of flanking sgRNAs are co-transfected in order to delete *DMD*
620 exon 52 (A) or the microRNA cluster contained in the 5'UTR of *UTRN* (B). (C and D). List
621 of sgRNAs for editing the *DMD* (C) and the *UTRN* loci (D), showing sgRNAs sequences,
622 PAM sequences and scores of all sgRNA tested. (E and F) Genomic location of the
623 sgRNAs targeting the *DMD* (E) and *UTRN* loci (F).

624 **Figure 2. sgRNAs pairs test in HEK293 cells.**

625 (A and B). Representation of all the different sgRNAs combinations tested for editing
626 the *DMD* (A) and the *UTRN* loci (B). (C and D) Representative PCR analysis of HEK293
627 cells transfected with some of the sgRNAs combinations tested. Upper bands
628 correspond to wild type or non-edited cells, while the lower bands correspond to the
629 edited ones. Selected combinations are highlighted: Obj1sgRNA2 (C); Obj2
630 sgRNA22+sgRNA26 (D)

631 **Figure 3. Cloning and editing workflow, and efficiency diagram.**

632 (A) Scheme of the workflow followed to obtain the edited myoblast cell lines. 48 hours
633 after plasmid transfection GFP positive myoblasts were single cell sorted using FACS.
634 Clones were expanded until confluence for DNA extraction. (B) Efficiency of the
635 different steps during the workflow process.

636 **Figure 4. Genotyping *DMD* and *UTRN* deletion breakpoints in edited myoblast clones.**

637 (A and B) PCR genotyping (A) and Sanger sequencing (B) of *DMD* edited clones (A). (C
638 and D) PCR genotyping (C) and Sanger sequencing (D) of *UTRN* edited clones (C).
639 Larger products in agarose gels (A and C) indicate non-edited clones, and shorter ones
640 correspond with the expected deletion. (B and D) Sequences of the smaller bands
641 confirmed the expected gene editing for objective 1: DMDΔ52-Model and objective 2:
642 DMD-UTRN-Model (B).

643 **Figure 5. Functional consequences of gene editing: dystrophin and utrophin**
644 **expression.**

645 Dystrophin expression in control myotubes compared to DMDΔ52-Model cultures and
646 DMD myotubes studied by immunocytochemistry (A), western blotting (B) and
647 myoblots (C) where n=24 wells per cell type were compared (****p-value<0.0001).
648 Utrophin expression in DMD myotubes compared to DMD-UTRN-Model studied by
649 immunocytochemistry (D), western blotting (E) and myoblots (F), where n=48 replicate
650 wells were included per cell type. (****p-value<0.0001) (P values were determined
651 with Mann-Whitney U test).

652 **Figure 6. Evaluation of potential therapies in the generated cell culture models.**

653 A) DMDΔ52-Model cell line was treated with an antisense to skip exon 51 and the
654 effect was evaluated by RT-PCR and nested PCR analysis. Gel picture shows a pattern
655 corresponding with the correct skipping, which was confirmed by Sanger sequencing.
656 The same experiment evaluated by myoblot using n=20 wells per condition (B) showed
657 the restoration of dystrophin expression in the treated cultures. (**P values<0.05. P
658 values were determined with Mann-Whitney U test).
659 C) The DMD-UTRN-Model was used as a positive control in an experiment in which
660 unedited DMD cultures were treated with different ezutromid concentrations to up-
661 regulate utrophin expression. Myoblot analysis using n=8 wells per condition of the
662 treated cultures shows that ezutromid had no significant effect in DMD cultures while
663 utrophin expression is significantly increased in DMD-UTRN-Model compared to
664 unedited DMD cultures. (**P values<0.05. P values were determined with Mann-
665 Whitney U test).
666

667

668 **Suplementary figure 1. Myoblasts transfection optimization and selection of positive**
669 **cells by FACS.**

670 A) GFP expression 48h after transfection in control myoblasts using four different
671 transfection reagents and following manufacturers conditions. B) Control myoblasts
672 expressing GFP 48h post transfection with ViaFect™ reagent and dot plot showing GFP
673 positive cells (3,22%) selected using FACS.

674

675 **Supplementary figure 2. PCR Analysis of Off-target Effects. Representative gel and**
676 **sequence of PCR analysis performed for all targets.**

677 A) Agarose gel showing the amplification of the six predicted off-targets regions for
678 sgRNA2 and the six for sgRNA6 (the combination used for our DMD edition model)
679 amplified in control myoblasts and DMDΔ52-Model. B) All the amplicons were
680 sequenced and no differences between control myoblasts and DMDΔ52-Model were
681 found.

682 **Supplementary figure 3. MF20 differentiation marker depletion in edited cells.**

683 MF20 expression determined by myoblot in DMDΔ52-Model compared to control
684 myoblasts (A) and in DMD-UTRN-Model compared to DMD myoblasts (B). Myoblot
685 analysis was performed using n=12 (A) or n=18 (B) wells for MF20 staining. (**P
686 values<0.05; **** P values<0.0001 P values were determined with Mann-Whitney U
687 test).

688 **Supplementary table 1. Gene edition and antisense experiments primer sets.**

689 **Supplementary table 2. Off-target primer sets.**

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
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

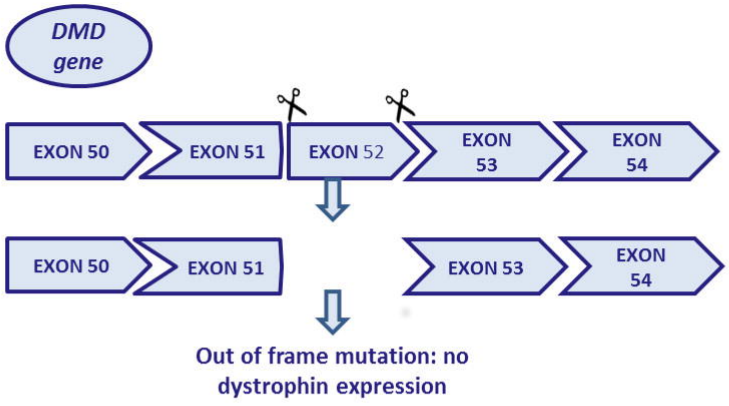
Ob2_22_Off4		GAATCCTTTTAGCTGGGATCAGG	4	chr19	intron:ZNF536	No off-target editing	Confirmed
Ob2_22_Off5		GGTTCATCTTAGCTGGGATATGG	4	chr13	intron:FLT1	No off-target editing	Confirmed
Ob2_22_Off6		TGTTCTCTCTAACTGGGGTCTGG	4	chr21	intergenic:PPP6R2P1-AP001347.6	No off-target editing	Confirmed
Ob2_26_Off1	GTGCTTTCTTGGGTATGACATGG (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

690

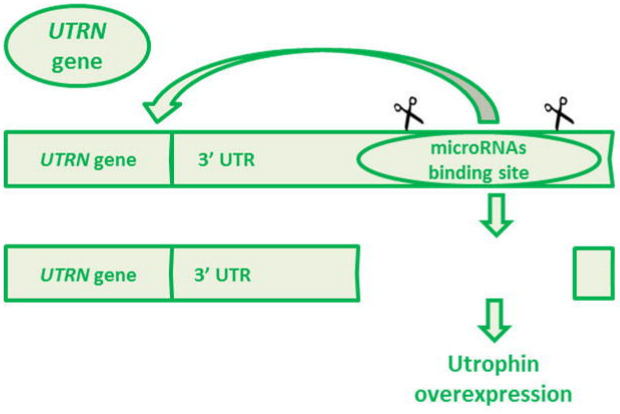
691 **Table 1. Potential off-target sites.**

692

A)



B)



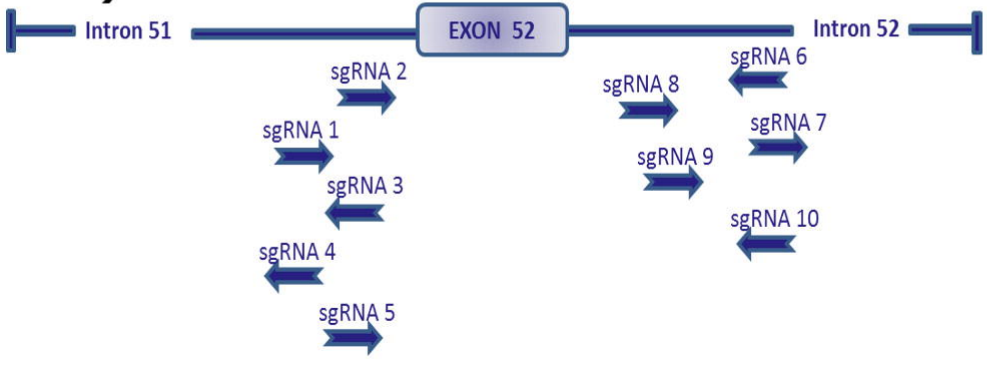
C)

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

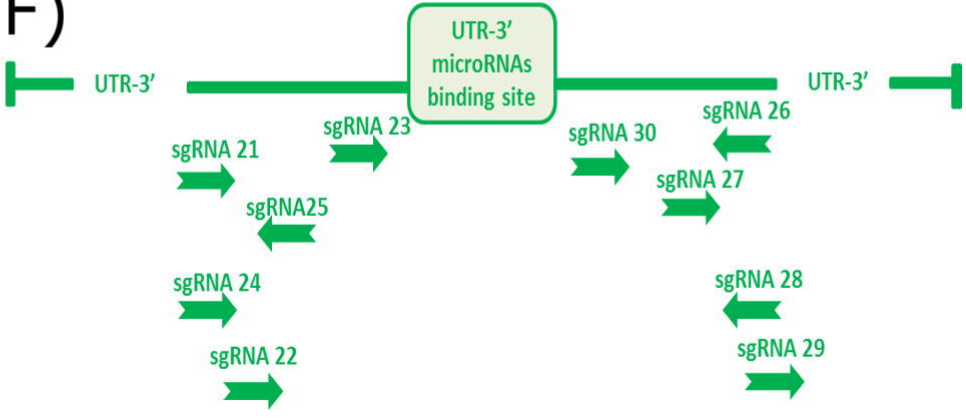
D)

CODE	SCORE	SEQUENCE	PAM
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

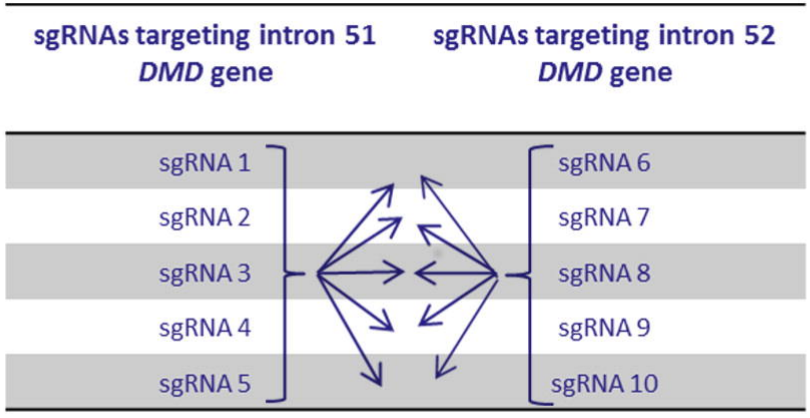
E)



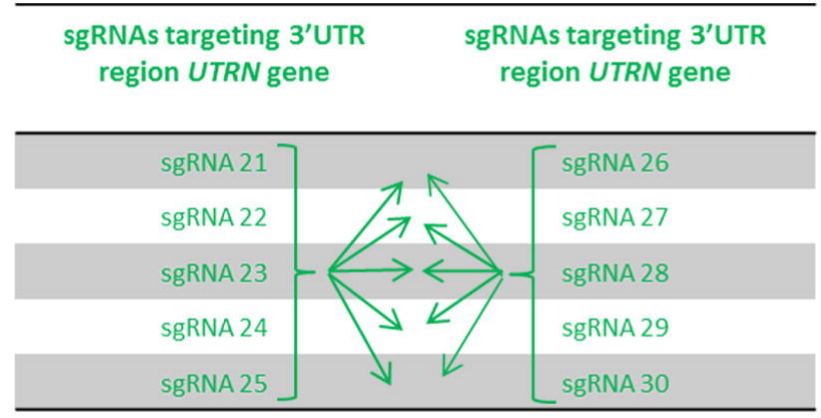
F)



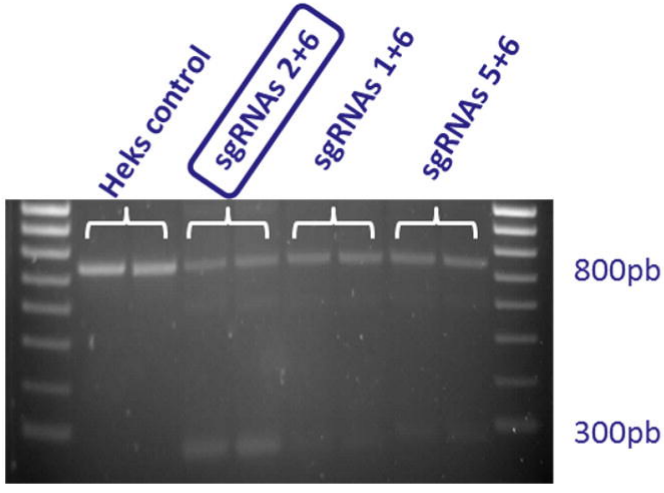
A)


sgRNAs targeting intron 51
DMD gene
sgRNAs targeting intron 52
DMD gene
sgRNA 1
sgRNA 2
sgRNA 3
sgRNA 4
sgRNA 5
sgRNA 6
sgRNA 7
sgRNA 8
sgRNA 9
sgRNA 10

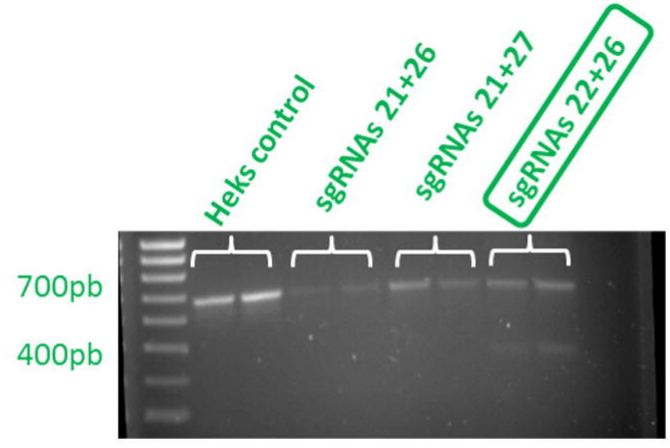
B)


sgRNAs targeting 3'UTR
region *UTRN* gene
sgRNAs targeting 3'UTR
region *UTRN* gene
sgRNA 21
sgRNA 22
sgRNA 23
sgRNA 24
sgRNA 25
sgRNA 26
sgRNA 27
sgRNA 28
sgRNA 29
sgRNA 30

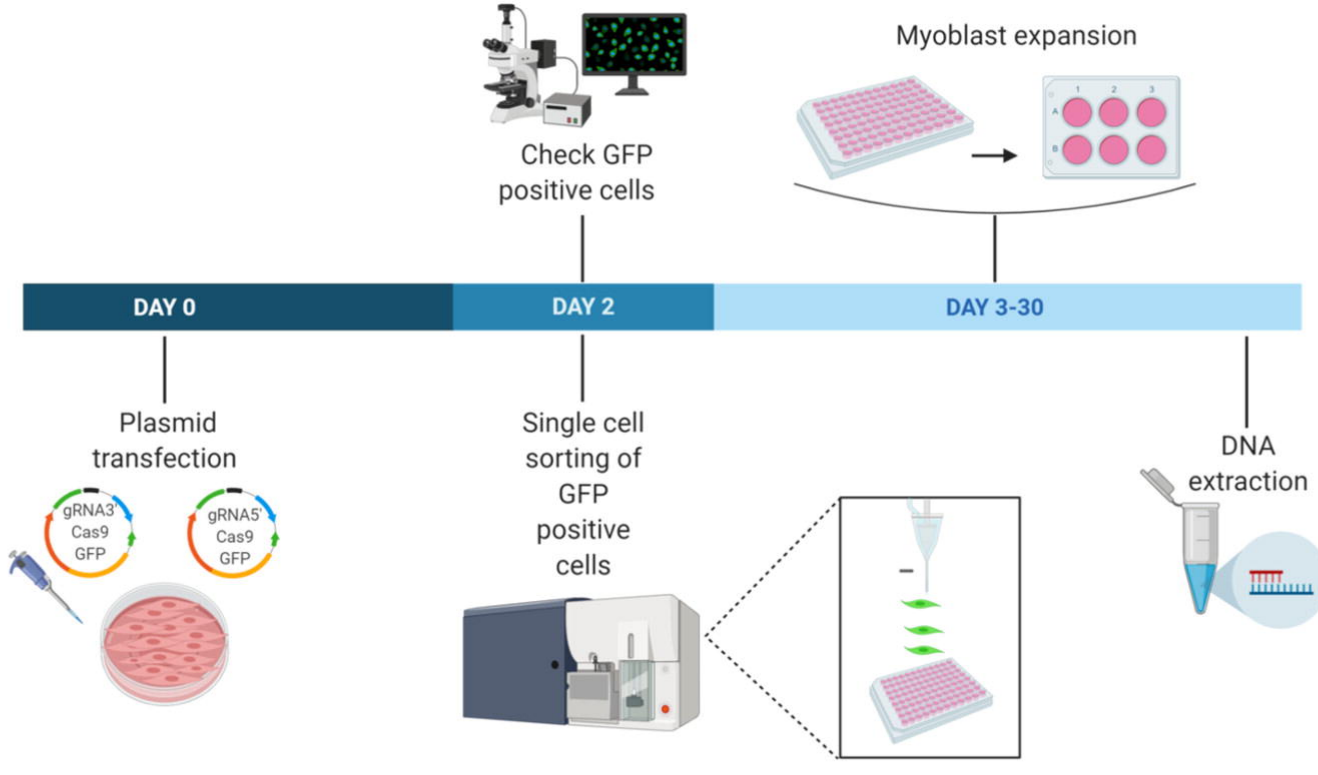
C)


Heks control
sgRNAs 2+6
sgRNAs 1+6
sgRNAs 5+6
800pb
300pb

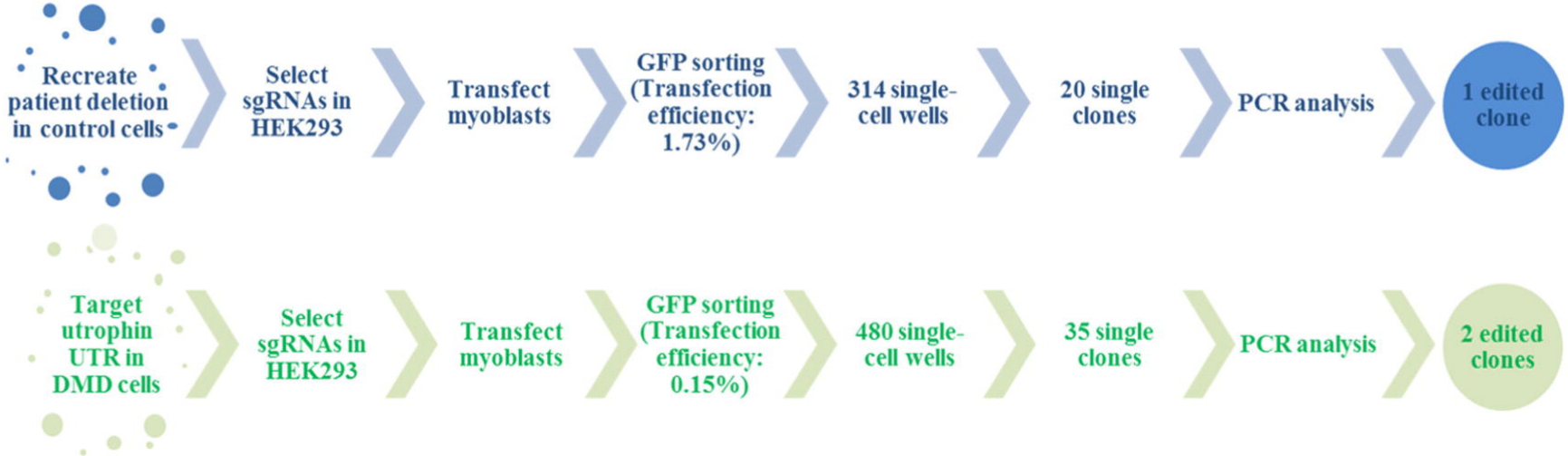
D)

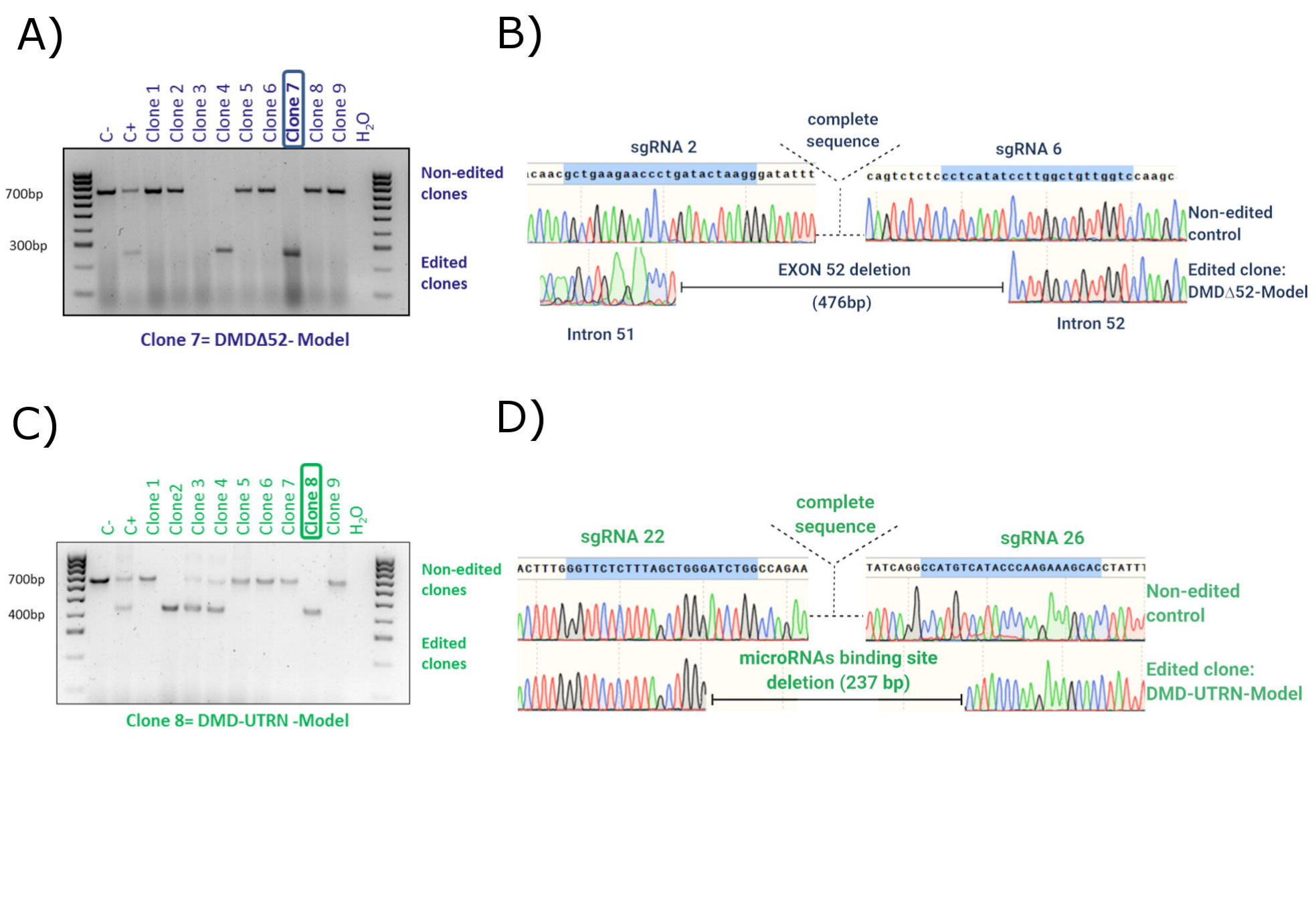

Heks control
sgRNAs 21+26
sgRNAs 21+27
sgRNAs 22+26
700pb
400pb

A)

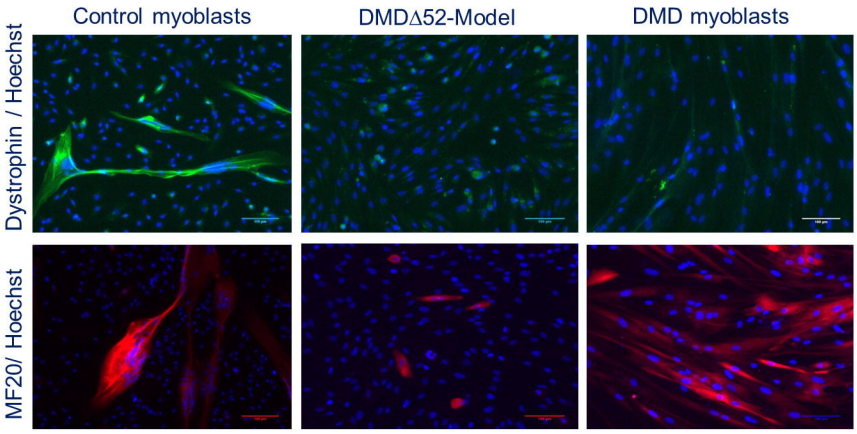

Check GFP positive cells
Myoblast expansion
DAY 0
DAY 2
DAY 3-30
Plasmid transfection
gRNA3 Cas9 GFP
gRNA5' Cas9 GFP
Single cell sorting of GFP positive cells
DNA extraction

B)

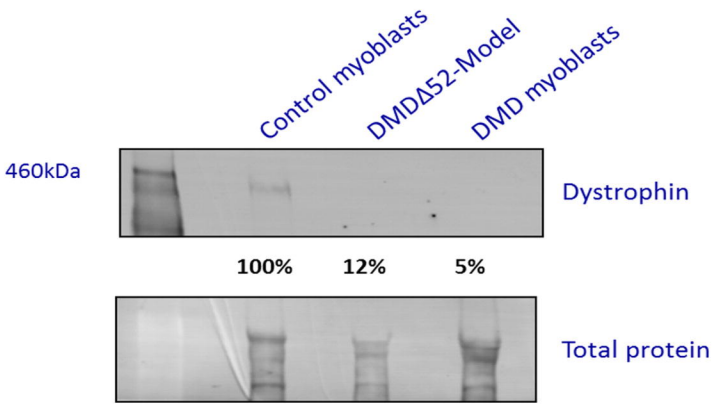

Recreate patient deletion in control cells
Select sgRNAs in HEK293
Transfect myoblasts
GFP sorting (Transfection efficiency: 1.73%)
314 single-cell wells
20 single clones
PCR analysis
1 edited clone
Target utrophin UTR in DMD cells
Select sgRNAs in HEK293
Transfect myoblasts
GFP sorting (Transfection efficiency: 0.15%)
480 single-cell wells
35 single clones
PCR analysis
2 edited clones


A)
C-
C+
Clone 1
Clone 2
Clone 3
Clone 4
Clone 5
Clone 6
Clone 7
Clone 8
Clone 9
H2O
700bp
300bp
Non-edited clones
Edited clones
Clone 7= DMDΔ52- Model
B)
complete sequence
sgRNA 2
sgRNA 6
caacgctgaagaaccctgatactaagggatattt
cagtctctccctcatatccttggctgttggtccaagc
Non-edited control
EXON 52 deletion
(476bp)
Edited clone: DMDΔ52-Model
Intron 51
Intron 52
C)
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
D)
complete sequence
sgRNA 22
sgRNA 26
ACTTTGGGTTCTCTTTAGCTGGGATCTGGCCAGAA
TATCAGGCCATGTCATACCCAAGAAAGCACCTATTT
Non-edited control
microRNAs binding site deletion (237 bp)
Edited clone: DMD-UTRN-Model

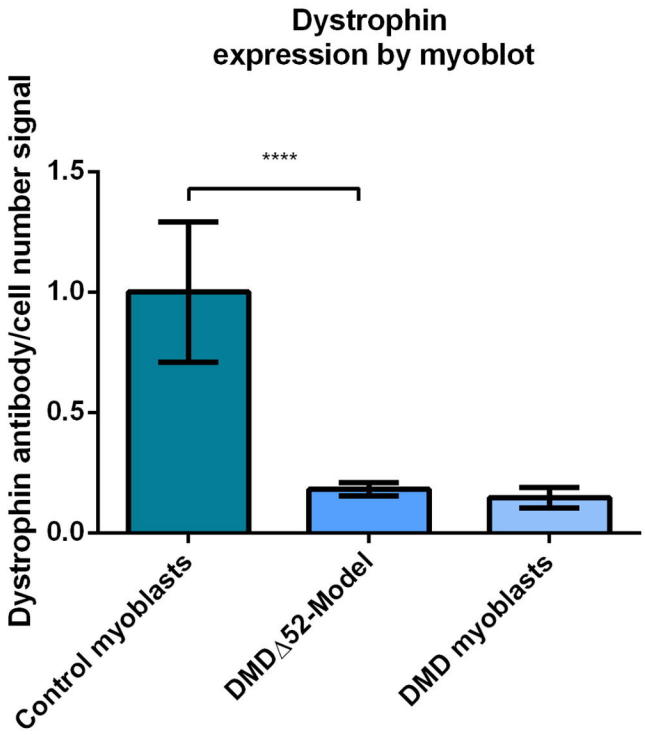
A)


Control myoblasts
DMDΔ52-Model
DMD myoblasts
Dystrophin / Hoechst
MF20/ Hoechst

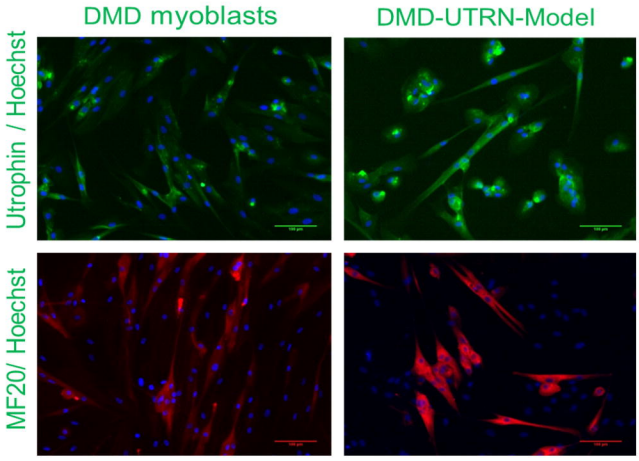
B)


Control myoblasts
DMDΔ52-Model
DMD myoblasts
460kDa
Dystrophin
100%
12%
5%
Total protein

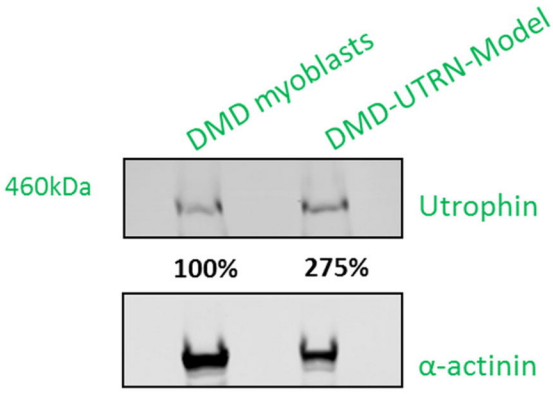
C)


Dystrophin expression by myoblot
Dystrophin antibody/cell number signal
****
1.5
1.0
0.5
0.0
Control myoblasts
DMDΔ52-Model
DMD myoblasts

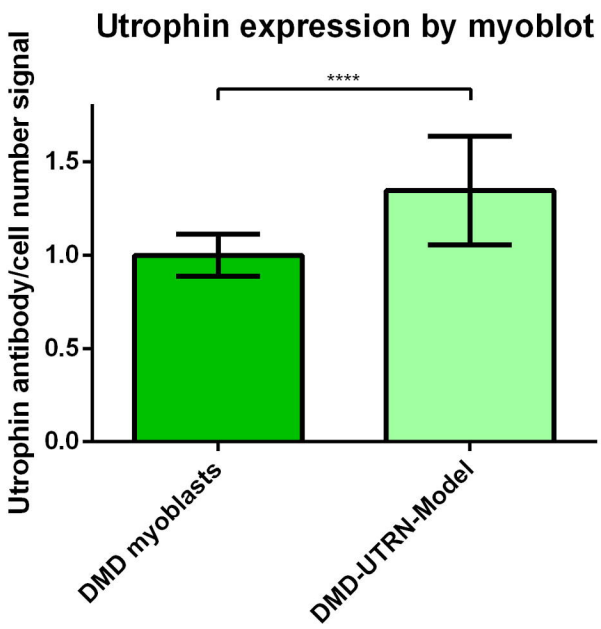
D)


DMD myoblasts
DMD-UTRN-Model
Utrophin / Hoechst
MF20/ Hoechst

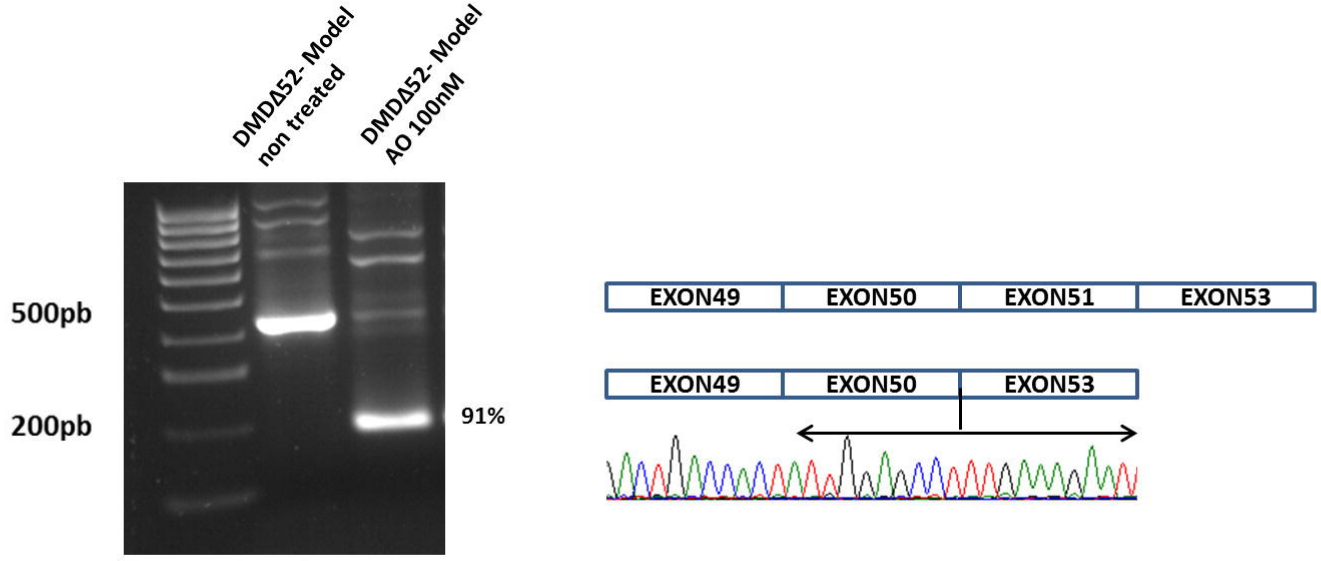
E)


DMD myoblasts
DMD-UTRN-Model
460kDa
Utrophin
100%
275%
α-actinin

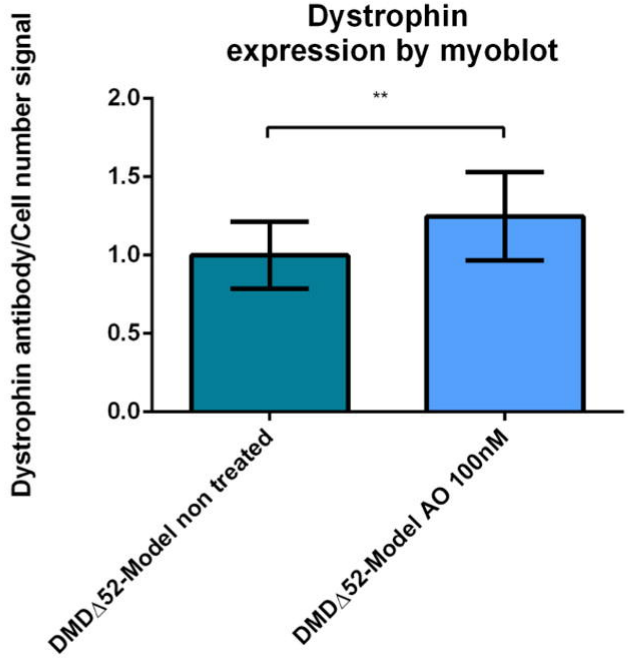
F)


Utrophin expression by myoblot
Utrophin antibody/cell number signal
****
1.5
1.0
0.5
0.0
DMD myoblasts
DMD-UTRN-Model

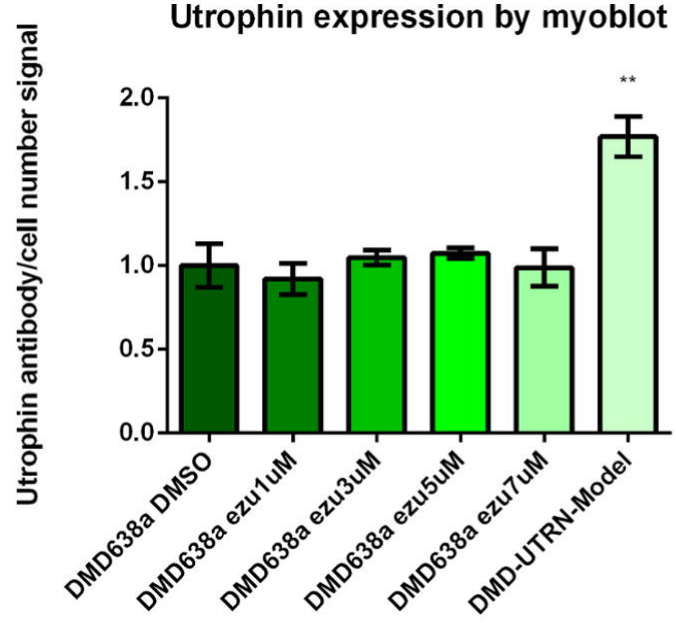
A)


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

B)


Dystrophin expression by myoblot
Dystrophin antibody/Cell number signal
2.0
1.5
1.0
0.5
0.0
**
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