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 edition

INTRODUCTION

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

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

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

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

In order to easily and rapidly assess the efficiency of current and novel therapies to treat DMD, *in vitro* cellular models are particularly useful. However, only a few human

117 immortalized muscle cell lines derived from DMD patients are currently available
118 (Mamchaoui et al., 2011). Due to the wide spectrum of DMD mutations and the
119 difficulties to obtain DMD patient muscle biopsies, an efficient approach to edit DMD
120 mutations in immortalised myoblasts would be extremely valuable to generate a more
121 exhaustive panel of DMD-myoblast models. Such lines would provide a powerful
122 resource for *in vitro* drug screening and study disease rescue mechanisms.

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

129 ***RESULTS***

130 **Optimisation of gene edition in myoblasts and generation of two new cell culture**
131 **models.**

132 • Guide selection for CRISPR/Cas9 system.

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

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

140 • Testing sgRNAs in HEK293 cultures.

141 As transfection of myoblast is very inefficient, all the different combinations of the
142 sgRNAs cutting before and after the target region (5x5), were tested in HEK293
143 cultures first (Figure 2). The combination of sgRNAs that was most efficient in HEK293
144 cells for each objective was selected to be used in the transfection of human
145 immortalized myoblasts.
146

- Myoblast transfection and single cell sorting workflow

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

- Selection of edited clones:

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

Analysis of dystrophin and utrophin expression in edited clones.

We compared dystrophin expression in myotubes of the DMD Δ52-Model to controls and DMD cultures, and confirmed that it was abolished by immunohistochemistry (Figure 5A), western blot analysis (Figure 5B) and myoblots (Figure 5C). Dystrophin levels in this model, where exon 52 had been removed by CRISPR/Cas9 edition, were statistically no different than those seen in a culture from a DMD patient.

It was difficult to corroborate by immunocytochemistry the increase of utrophin expression between unedited DMD and DMD-UTRN-Model myotubes (Figure 5D) but

this increase was evident by western blot (a 175% increase, Figure 5E) and myoblot analysis (close to 50% increase, Figure 5F).

We also evaluated myoblast differentiation in all the cultures by myoblot, and we could observe a decrease in the MF20 differentiation marker in all the edited clones, no matter the deletion, compared with their corresponding controls (Supplementary Figure 2).

Evaluation of therapies in newly generated model cell lines.

To assess if the DMDΔ52-Model cell culture could be useful to test potential mutation specific therapies for DMD, we evaluated the exon skipping efficiency of an antisense oligonucleotide in this culture. We treated the DMDΔ52-Model cultures with an antisense oligonucleotide drug that can skip exon 51 (van Deutekom et al., 2007) and restore *DMD* open reading frame. After treatment with this drug, we confirmed that exon skipping had taken place at RNA level (Figure 6A), and the restoration of dystrophin expression by myoblot analysis (Figure 6B).

To test our DMD-UTRN-Model as a positive utrophin overexpression control, we cultured it alongside the original unedited DMD cultures, which we treated with several concentrations of ezutromid and we evaluated the expression of utrophin in all cultures. We observed that utrophin was hardly modified in DMD cultures treated with ezutromid while a robust overexpression was confirmed in the DMD-UTRN-Model compared to the unedited DMD cultures. (Figure 6C)

DISCUSSION

CRISPR/Cas9 as a possible therapeutic approach for DMD has been explored in the past in *in vitro* models of the disease. In most of these cases, a "permanent" exon skipping approach was selected where a shorter protein would be produced (Ousterout et al., 2015; Young et al., 2016), while in some others full length dystrophin was the result of the edition (Lattanzi et al., 2017; Li et al., 2014; Wojtal et al., 2015). After several studies showed efficacy also in mice models (Long et al., 2016; Nelson et al., 2016; Tabebordbar et al., 2016), a recent study in dogs is currently the most

advanced example of its application to DMD (Amoasii et al., 2018). Before this can be a credible therapeutic option, several delivery and manufacturing problems will need to be overcome. In the meantime, this methodology is very useful for researchers looking for disease models: muscle biopsies are not routinely collected during diagnosis of this disorder and seldom cultured. This means there are few good culture models of the disease. Another tool created to facilitate research, was the immortalisation of some of the available cultures (Mamchaoui et al., 2011), which increases the possibility of performing more experiments with a given culture. We have used immortalised cultures to further increase this possibility.

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

An immortalised cell culture model constitutively expressing utrophin, DMD-UTRN-Model, is both a proof of principle of a possible therapeutic option to overexpress utrophin as a substitute for dystrophin, and a valuable research tool. The search of drugs that could overexpress utrophin is ongoing (Guiraud and Davies, 2017) after the recent failure of ezutromid to show relevant results in clinical trials. Many research projects, including drug re-purposing screening, are ongoing to find new candidates to

test in clinical trials. However, there are no reliable positive controls that could be used to compare such treatments. We propose that our cell model could serve that purpose, offering researchers useful custom controls for their studies. We have tested this hypothesis and compared the stable utrophin overexpression quantified in the DMD-UTRN-Model with the one that is obtained after treatment with ezutromid, the lead market candidate in this field until very recently, with positive results. Previous studies in muscle sections show that DMD patients already overexpress utrophin, in many cases 4 to 5-fold the levels seen in control muscle sections (Arechavala-Gomeza et al., 2010). Our choice to target this particular *UTR* region, increases basal overexpression in DMD cultures, and the amount of overexpression varies significantly when evaluated by western blot (more than 2.5 times) or our preferred method, myoblots (close to 1.5 times). We like to consider that myoblot evaluation reflects more closely the actual protein expression, as it is not subjected to many of the inherent problems of western blotting when evaluating very large proteins (Ruiz-Del-Yerro et al., 2018). This is why we cannot comment yet on the differences in expression between our study and other published studies that also aimed to overexpress utrophin by gene edition, but which 1) targeted different promoters regions (*UTRN* A or *UTRN* B) of utrophin and 2) evaluated their results by western blot analysis (Wojtal et al., 2015). We would be interested on studying this matter further to analyse the differences in utrophin expression when targeting different regions.

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

MATERIALS AND METHODS

CRISPR/Cas9 tools

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

Cell cultures

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

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

Cell culture transfection

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

FACS Sorting of GFP positive myoblasts:

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

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

Analysis of gene edition

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

Off-target analysis of mutations in clonal lines

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

Primary Antobodies

Anti-dystrophin: Dys1 (Leica Biosystems), Mandys1, Mandys106 (The MDA Monoclonal Antibody Resource)

Anti-utrophin: Mancho7 (The MDA Monoclonal Antibody Resource)

Inmunostaining assays

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

In-cell western assay (myoblots)

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

Treatment with antisense exon skipping drugs

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

RT-PCR

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

Treatment with utrophin overexpression drugs

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

Western blot

Cell cultures were seeded into P6 plates and trypsinized after 7 days of differentiation. Then, cell pellets were solubilized in lysis/loading buffer and denatured at 95°C for 5 min. The samples were loaded onto a NuPAGE® Novex® 3–8% Tris-Acetate Gel3–8% (Thermo Fisher Scientific) and run in Novex Tris-Acetate SDS Running Buffer (Thermo Fisher Scientific) for 60 min at 70 V + 120 min at 150 V at 4ºC. Protein wet transfer was performed overnight at 4ªC using an Immobilon®-FL PVDF membrane (Merck™). Next day, membranes were stained with Revert ™ 700 Total Protein Stain (Li-Cor) for total

protein measurement, blocked with Intercept (PBS) Blocking Buffer (Li-Cor) for 2 hours and incubated overnight at 4°C with the primary antibodies (1/200 anti-dystrophin antibody Abcam15277, 1/50 anti-utrophin antibody Mancho 7 or 1/500 anti a-actinin antibody, Sigma-Aldrich A7732). After washing steps with PBS-Tween 0.1%, membranes were incubated with secondary antibodies for 1 hour (1/5000 IRDye 800CW goat anti-rabbit 926–32211 or IRDye 680RD goat anti-mouse 926-68070, Li-Cor) at room temperature, washed again and scanned using an Odyssey Clx imaging system. Bands quantification was performed using Image Studio ™ software.

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

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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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598 ***Figure legends***

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

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

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

605 (A and B). Schematic representation of our strategy for editing the *DMD* (A) and the
606 *UTRN* loci (B). A pair of flanking sgRNAs are co-tranfected in order to delete *DMD* exon
607 52 (A) or the microRNA cluster contained in the 5'UTR of *UTRN* (B). (C and D). List of
608 sgRNAs for editing the *DMD* (A) and the *UTRN* loci, showing sgRNAs sequences, PAM
609 sequences and scores of all sgRNA tested. (E and F) Genomic location of the sgRNAs
610 targeting the *DMD* (E) and *UTRN* loci (F).

611 **Figure 2. sgRNAs pairs test in 293 cells.**

612 (A and B). Representation of the different sgRNAs combinations tested for editing the
613 *DMD* (A) and the *UTRN* loci (B). (C and D) Representative PCR analysis of HEK293 cells
614 transfected with some of the sgRNAs combinations tested. Upper bands correspond to
615 wild type or non-edited cells, while the lower bands correspond to the edited ones.
616 Selected combinations are highlighted: Ob1sgRNA2+sgRNA6 (C);
617 Ob2sgRNA22+sgRNA26 (D).

618 **Figure 3. Cloning and edition workflow, and efficiency diagram.**

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

623

624

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

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

Figure 5. Functional consequences of gene edition: dystrophin and utrophin expression.

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

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

A) DMDΔ52-Model cell line was treated with an antisense to skip exon 51 and the effect was evaluated by RT-PCR and nested PCR analysis. Gel picture shows a pattern corresponding with the correct skipping, which was confirmed by Sanger sequencing. The same experiment evaluated by myoblot using n=20 wells per condition (B) showed the restoration of dystrophin expression in the treated cultures. (**P values<0.05. P values were determined with Mann-Whitney U test).

C) The DMD-UTRN-Model was used as a positive control in an experiment in which unedited DMD cultures were treated with different ezutromid concentrations to up-regulate utrophin expression. Myoblot analysis using n=8 wells per condition of the treated cultures shows that ezutromid had no significant effect in DMD cultures while utrophin expression is significantly increased in DMD-UTRN-Model compared to

654 unedited DMD cultures. (**P values<0.05. P values were determined with Mann-
655 Whitney U test).
656

657 **Supplementary figure 1. PCR Analysis of Off Target Effects. Representative gel and**
658 **sequence of PCR analysis performed for all targets.**

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

664 **Supplementary figure 2. MF20 differentiation marker depletion in edited cells.**

665 MF20 expression determined by myoblot in DMDΔ52-Model compared to control
666 myoblasts (A) and in DMD-UTRN-Model compared to DMD myoblasts (B). Myoblot
667 analysis was performed using n=12 (A) or n=18 (B) wells for MF20 staining. (**P
668 values<0.05; **** P values<0.0001 P values were determined with Mann-Whitney U
669 test).
670

671 **Suplementary figure 3. Myoblasts transfection optimization and selection of positive**
672 **cells by FACS.**
673 A) GFP expression 48h after transfection in control myoblasts using four different
674 transfection reagents and following manufacturers conditions. B) Control myoblasts
675 expressing GFP 48h post transfection with ViaFect® reagent and dot plot showing GFP
676 positive cells (3,22%) selected using FACS.

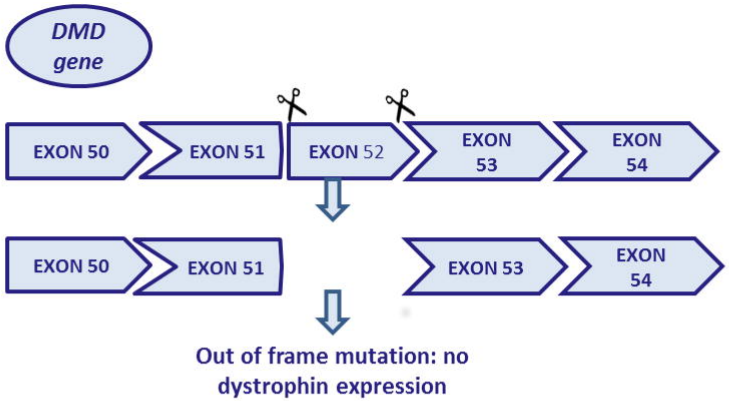
677

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 edition	Confirmed
Ob1_2_Off2		TCTGGAGAACCCTAATACTAAGG	3	chr8	intergenic:RP11-24P4.1-AC009695.1	No off target edition	Confirmed
Ob1_2_Off3		ACTGAAGAATCCAGAAACTAGGG	4	chr7	intergenic:NOBOX-RP4-545C24.5	No off target edition	Confirmed
Ob1_2_Off4		GCTAGAGAAACCTGAAACTAAGG	4	chr8	intergenic:RP11-536K17.1-EIF3H	No off target edition	Confirmed
Ob1_2_Off5		TCTGGAGAACCCTAATACTGTGG	4	chr3	intron:TMEM45A	No off target edition	Confirmed
Ob1_2_Off6		TCTGAAGAATCCTGATATTTTGG	4	chr2	intron:AC019100.3	No off target edition	Confirmed
Ob1_6_Off1	GACCAACAGCCAAGGATATGAGG (Obj1 sgRNA6)	CACCATCAGCCAAGAATATGCGG	3	chr11	intergenic:RP11-430H10.3-RP11-958J22.1	No off target edition	Confirmed
Ob1_6_Off2		TAACAACAGCCAAAGACATGAGG	4	chr14	exon:RP11-1012A1.4/RDH11	No off target edition	Confirmed
Ob1_6_Off3		GTAAAAGAGCCAAGGATATGAGG	4	chr10	intron:RP11-556E13.1	No off target edition	Confirmed
Ob1_6_Off4		TACTAGCAGCCAAGGATATCTGG	4	chr2	intergenic:AC007377.1-SLC8A1	No off target edition	Confirmed
Ob1_6_Off5		GAGCGACAGCCAAGAATATTCGG	4	chr3	intron:CD96	No off target edition	Confirmed
Ob1_6_Off6		AATCAACAGCCAAGAATGTGGGG	4	chr5	intergenic:CTD-2201E9.4-SEMA5A	No off target edition	Confirmed
Ob2_22_Off1	GGTTCTCTTTAGCTGGGATCTGG (Obj 2 sgRNA22)	TGTTCTCTCTAACTGGGATCTGG	3	chr18	intergenic:RP11-411B10.6-RP11-411B10.5	No off target edition	Confirmed
Ob2_22_Off2		TGTTCTCTAGAGCTGGGATCTGG	3	chr21	intron:LCA5L	No off target edition	Confirmed
Ob2_22_Off3		TGTTCTCTCCAACTGGGATCTGG	4	chr22	intron:PPP6R2	No off target edition	Confirmed

Ob2_22_Off4	GTGCTTTCTTGGGTATGACATGG (Obj2 sgRNA26)	GAATCCTTTTAGCTGGGATCAGG	4	chr19	intron:ZNF536	No off target edition	Confirmed
Ob2_22_Off5		GGTTCATCTTAGCTGGGATATGG	4	chr13	intron:FLT1	No off target edition	Confirmed
Ob2_22_Off6		TGTTCTCTCTAACTGGGGTCTGG	4	chr21	intergenic:PPP6R2P1-AP001347.6	No off target edition	Confirmed
Ob2_26_Off1		AAGCTTTCCTGGATATGACAAGG	4	chr4	intron:RNF150	No off target edition	Confirmed
Ob2_26_Off2		GTGCTTACTTGGGTAAGACGTGG	3	chr17	intergenic:RP11-212E8.1-RP11-642M2.1	No off target edition	Confirmed
Ob2_26_Off3		GAGTTAACTTGGGTATGACAGGG	4	chr4	intron:RGS12	No off target edition	Confirmed
Ob2_26_Off4		GTGCTCTCATGAGAATGACAGGG	4	chr4	intergenic:GABRG1-RP11-320H14.1	No off target edition	Confirmed
Ob2_26_Off5		GAGCTTTCCTGGGAATGACAGGG	3	chr1	intergenic:FOXO6-RNA5SP45	No off target edition	Confirmed
Ob2_26_Off6		GTGCTTTATAGGATATAACATGG	4	chr6	intron:GSTA3	No off target edition	Confirmed

Table 1. Potential off-target sites.

A)



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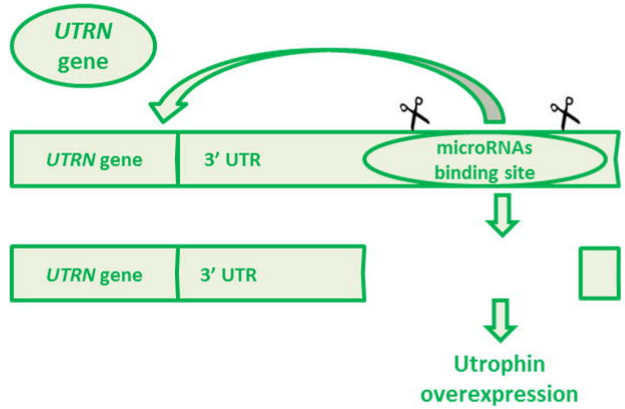


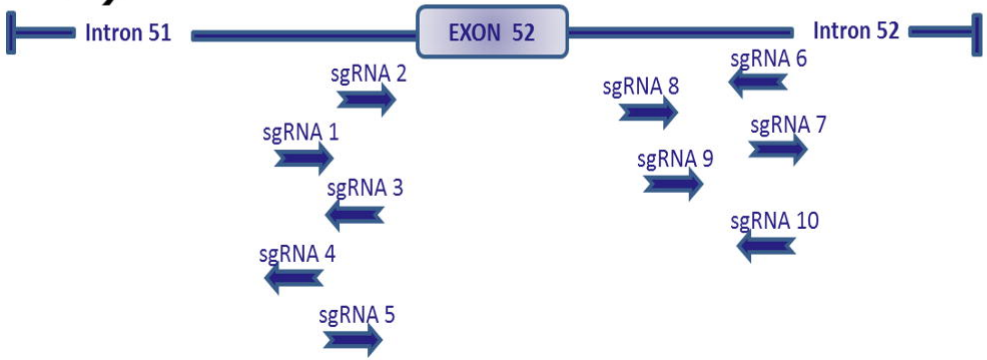
C)

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)



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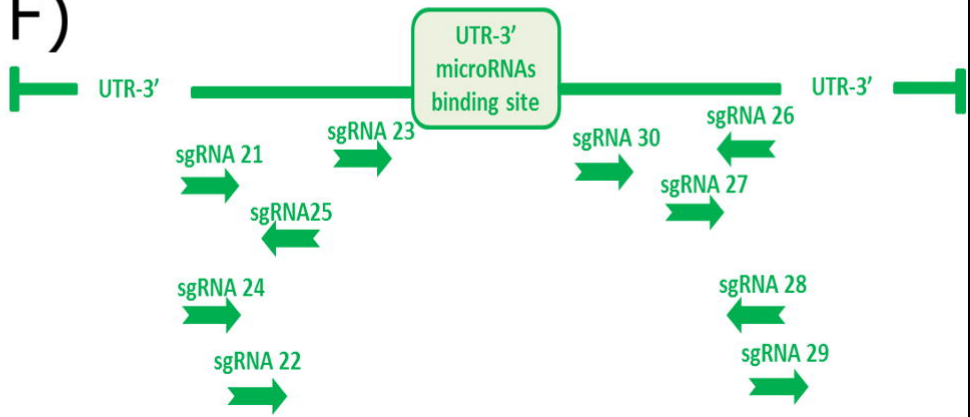

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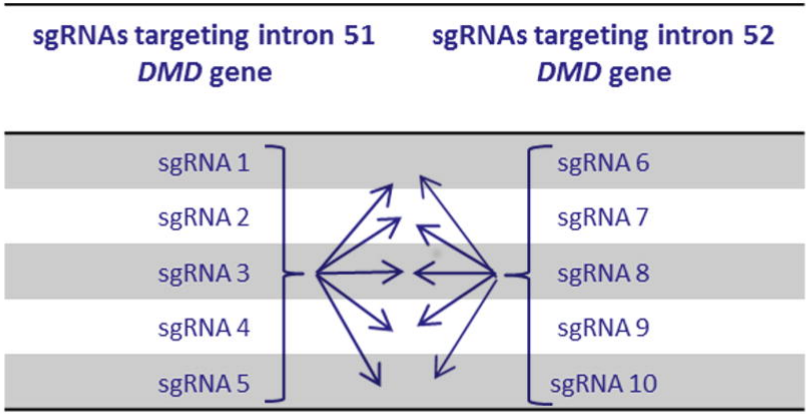
sgRNAs targeting intron 51
DMD gene
sgRNAs targeting intron 52
DMD gene
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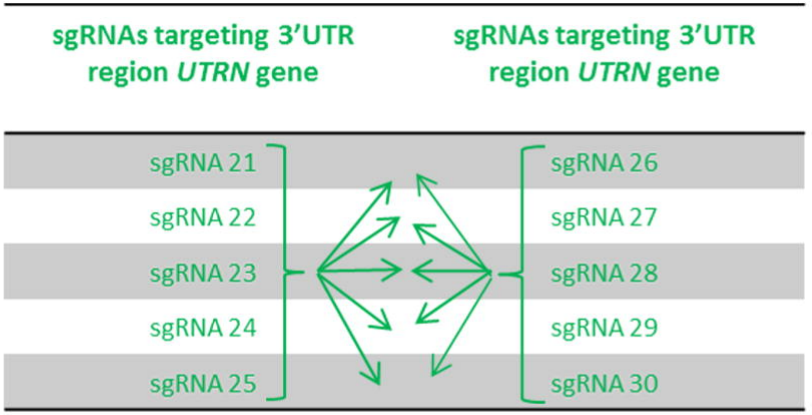
sgRNA 4
sgRNA 5
sgRNA 6
sgRNA 7
sgRNA 8
sgRNA 9
sgRNA 10

B)


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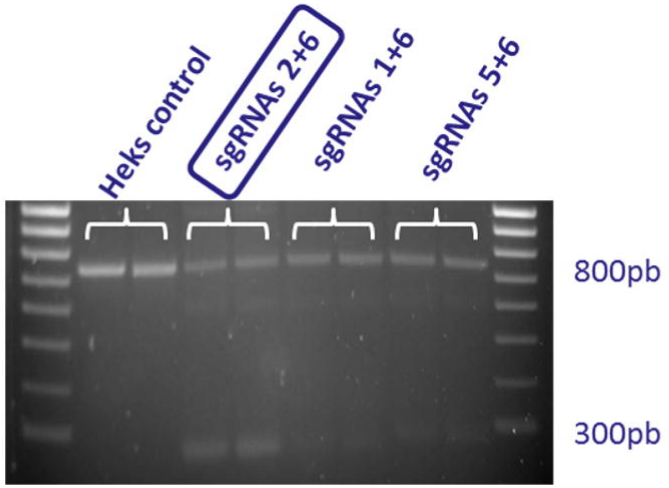
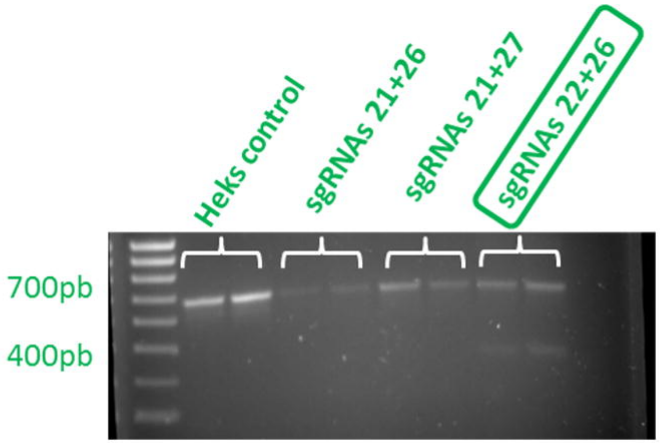
sgRNA 22
sgRNA 23
sgRNA 24
sgRNA 25
sgRNA 26
sgRNA 27
sgRNA 28
sgRNA 29
sgRNA 30

C)


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

D)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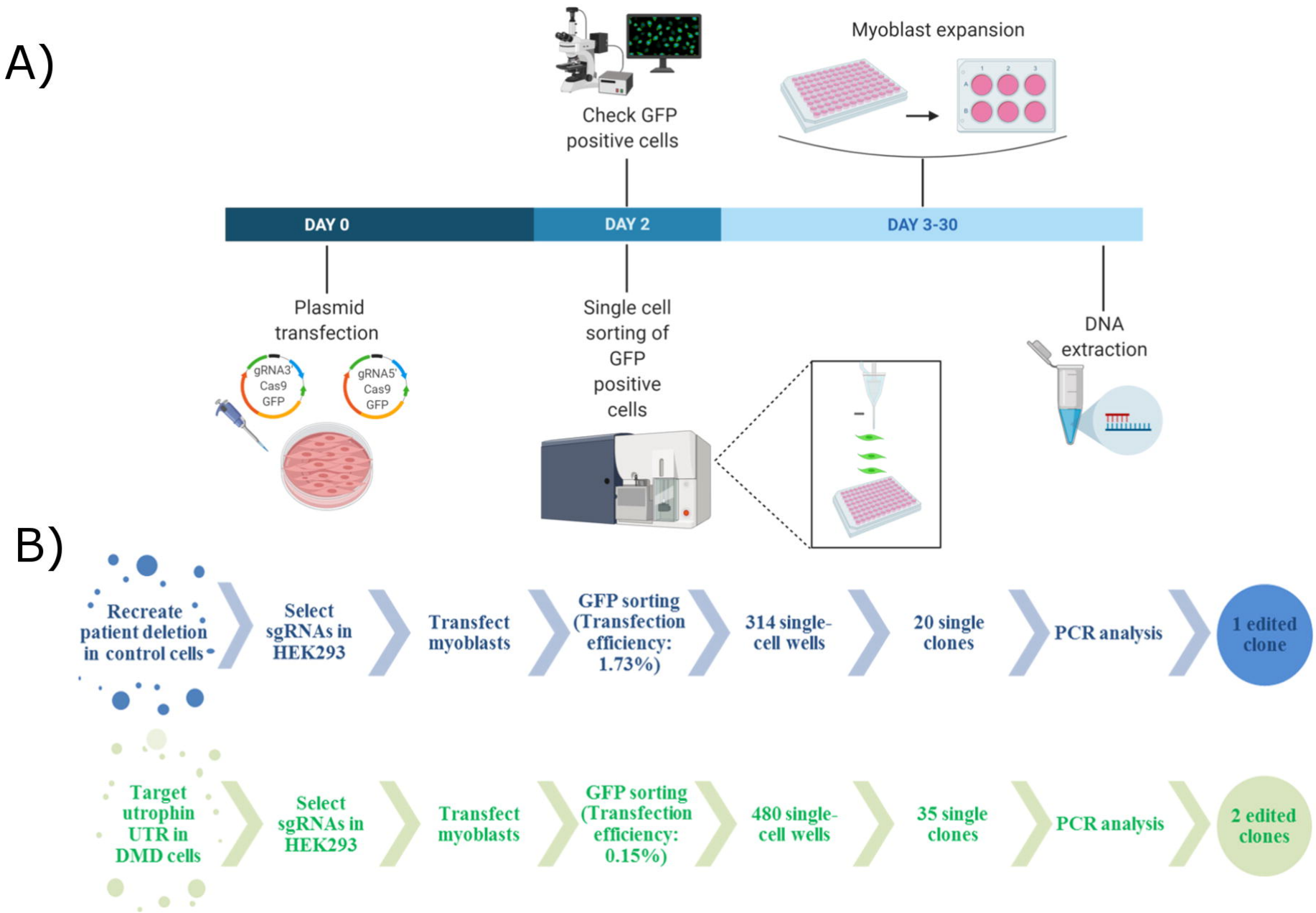
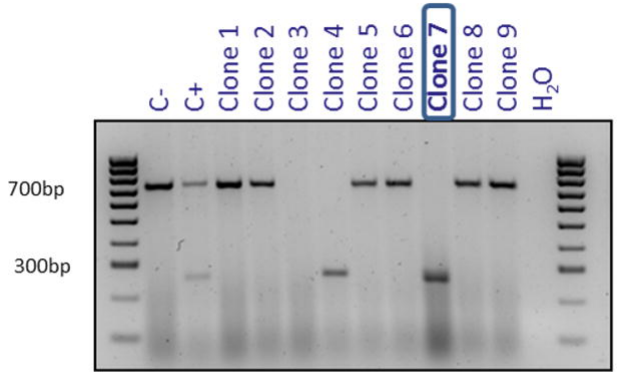
sgRNAs 21+26
sgRNAs 21+27
sgRNAs 22+26
700pb
400pb


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

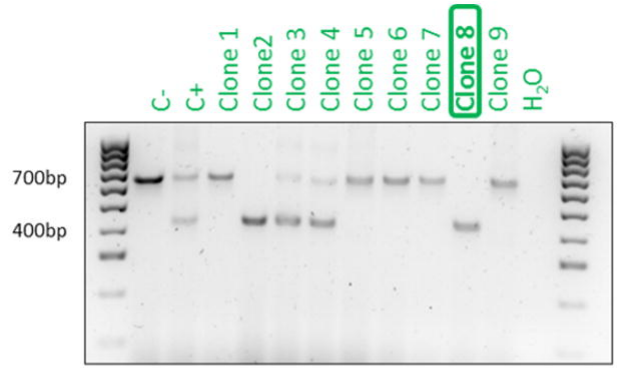
A)



Clone 7= DMDΔ52- Model

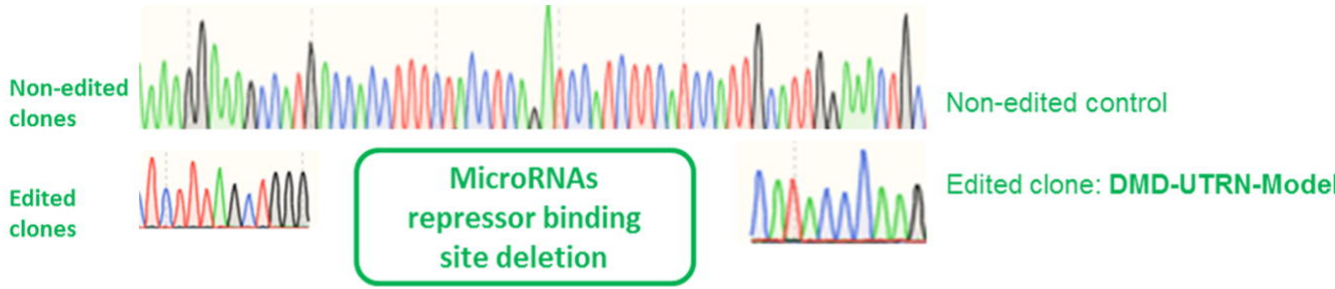
B)

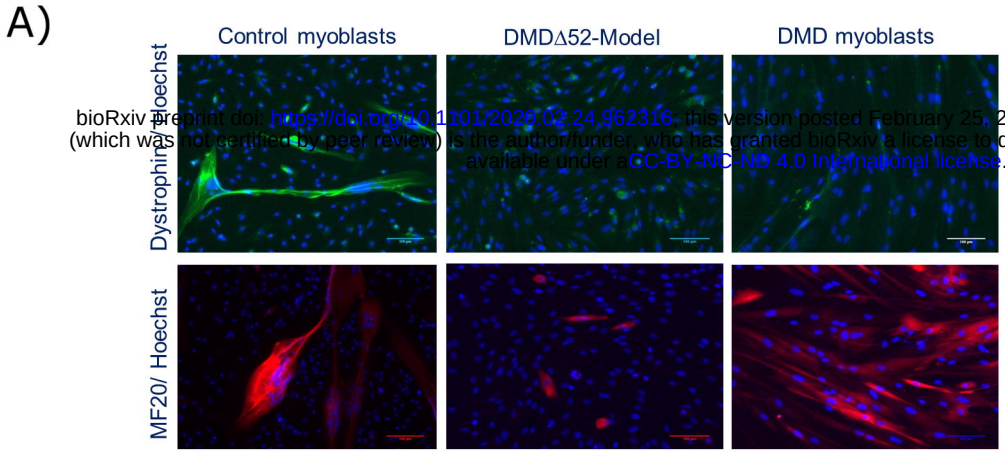
C)

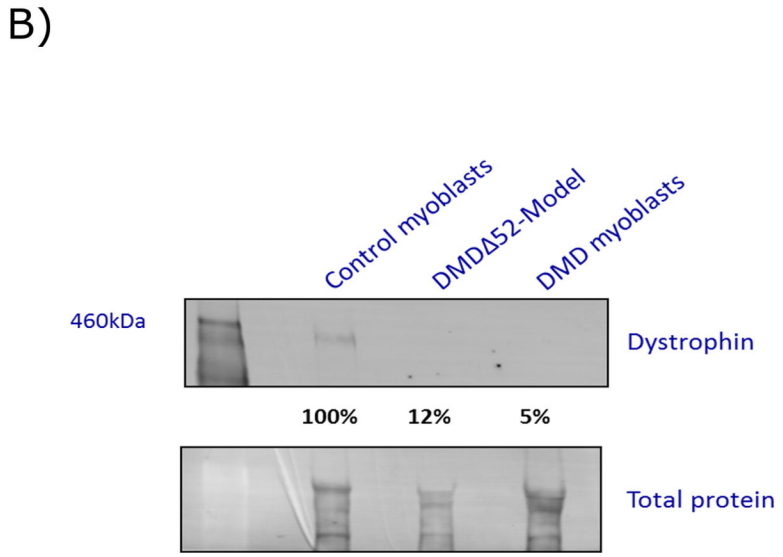


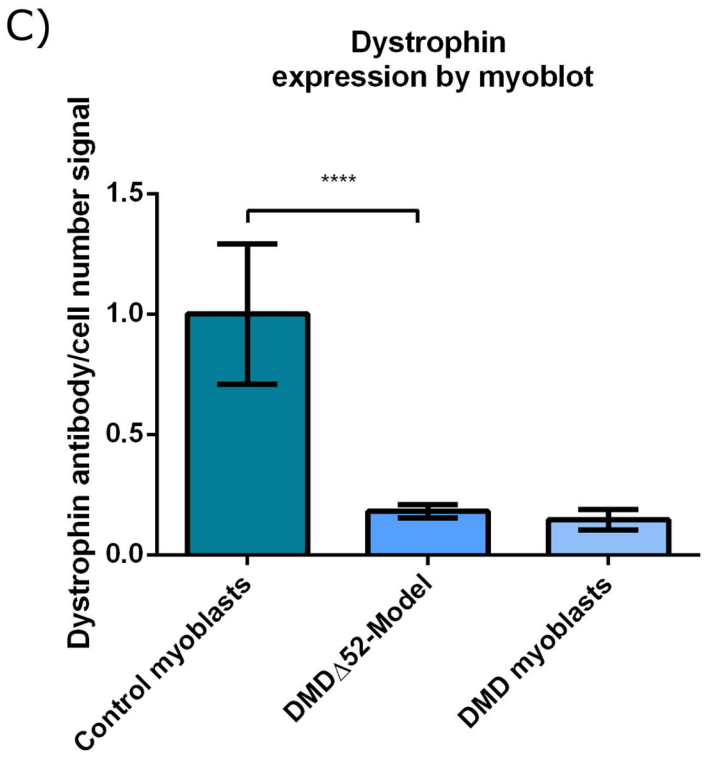
Clone 8= DMD-UTRN -Model

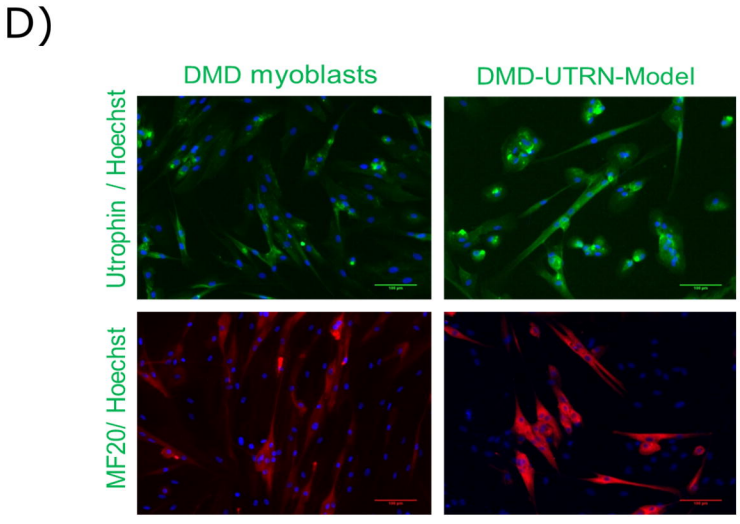
D)

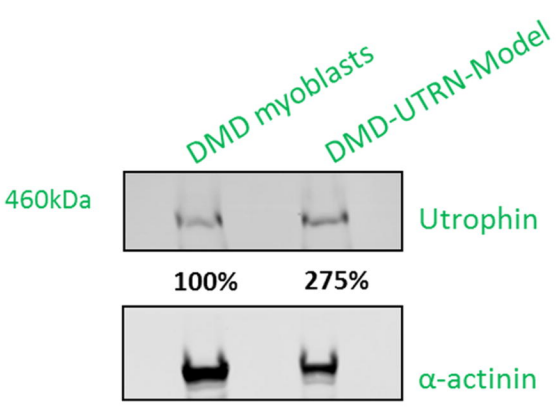


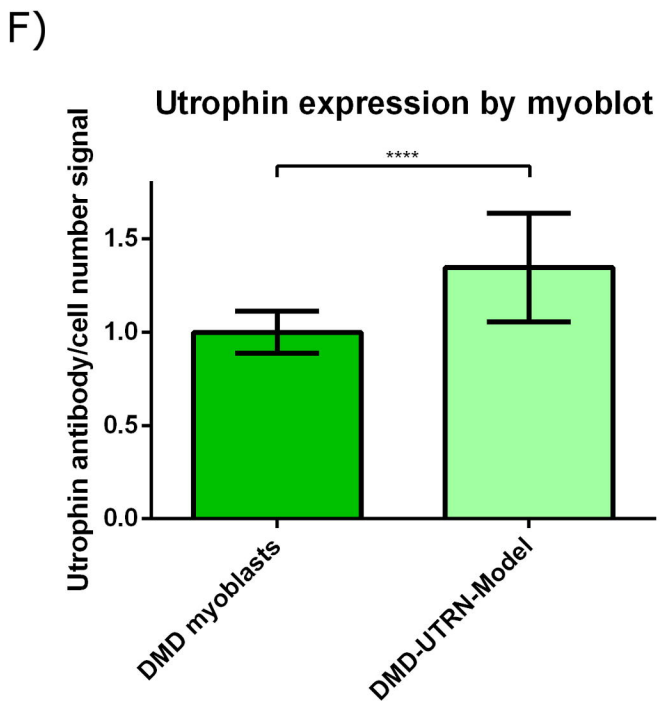




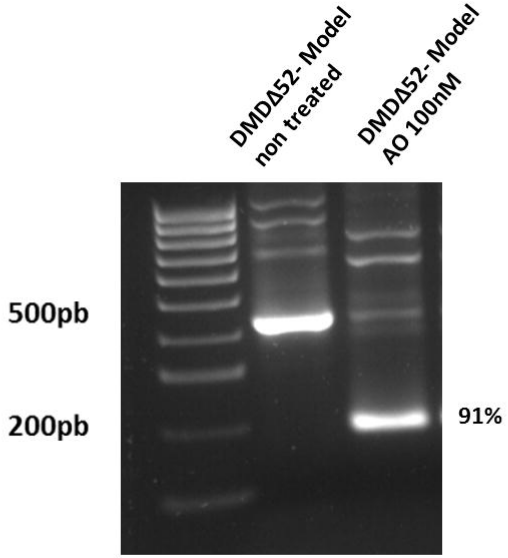


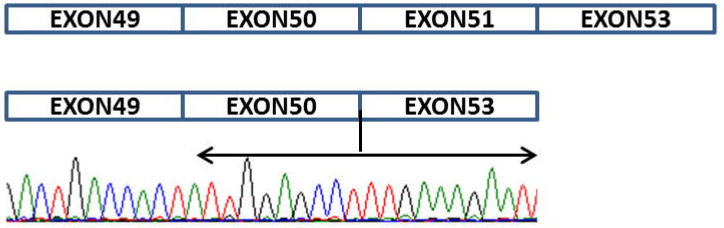




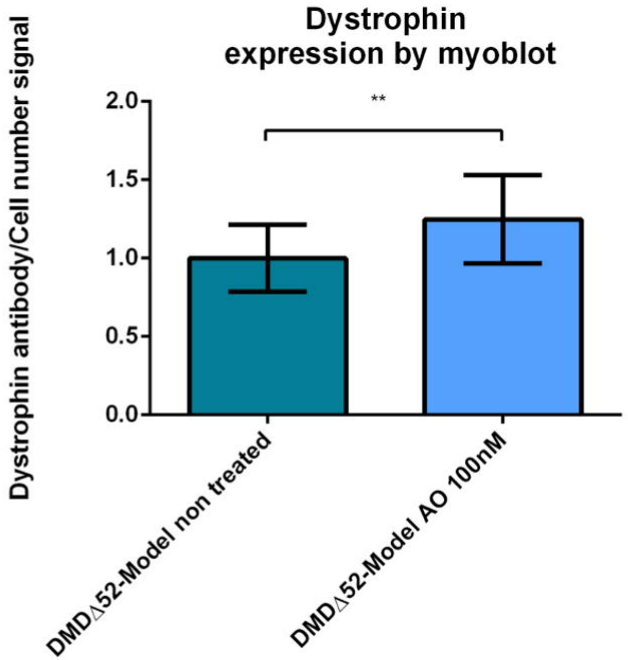


A)

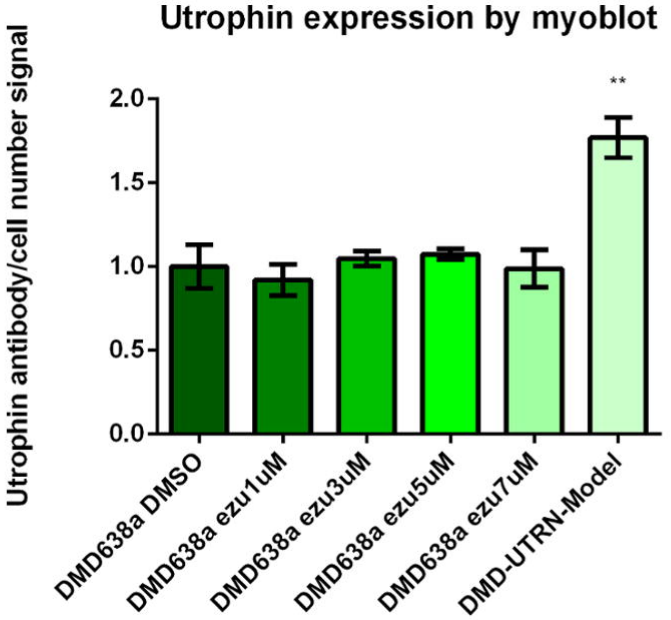

DMDΔ52- Model non treated
DMDΔ52- Model AO 100nM
500pb
200pb
91%


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