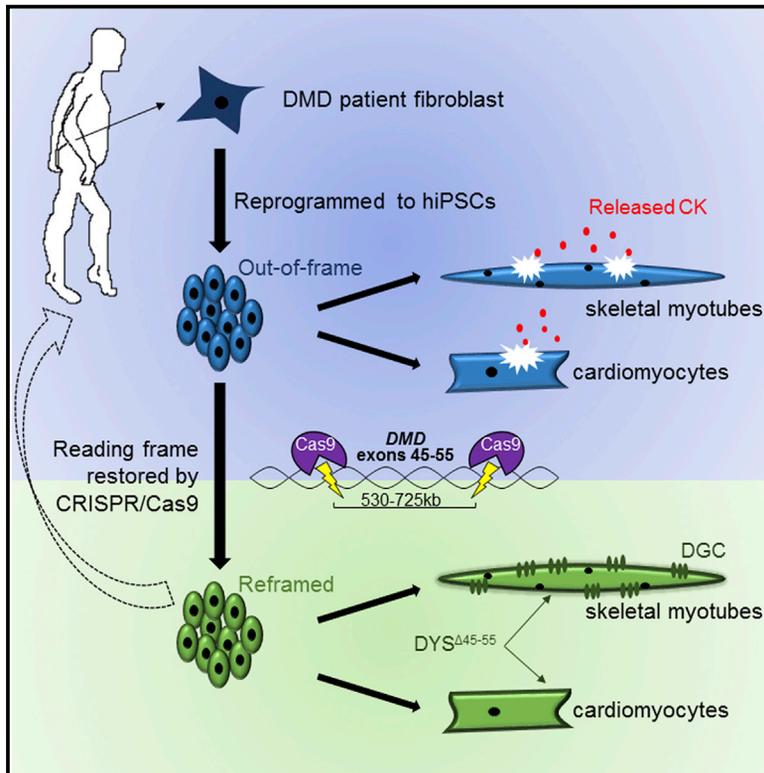# Cell Stem Cell

A Single CRISPR-Cas9 Deletion Strategy that Targets the Majority of DMD Patients Restores Dystrophin Function in hiPSC-Derived Muscle Cells

Graphical Abstract

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In Brief

Young et al. demonstrate restoration of the *DMD* reading frame by CRISPR/Cas9-mediated deletion of up to 725 kb in hiPSCs as a therapeutic strategy for 60% of Duchenne muscular dystrophy patients. The resulting internally deleted protein is shown to be functional in vitro and in vivo.

Highlights

- Largest CRISPR/Cas9-mediated deletion of 725 kb of *DMD*

- Reframed DMD hiPSCs differentiated to cardiac and skeletal muscle express dystrophin

- Internally deleted dystrophin demonstrates functionality in vitro and in vivo

- This single gRNA pair is therapeutically relevant to 60% of DMD mutations

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A Single CRISPR-Cas9 Deletion Strategy that Targets the Majority of DMD Patients Restores Dystrophin Function in hiPSC-Derived Muscle Cells

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SUMMARY

Mutations in *DMD* disrupt the reading frame, prevent dystrophin translation, and cause Duchenne muscular dystrophy (DMD). Here we describe a CRISPR/Cas9 platform applicable to 60% of DMD patient mutations. We applied the platform to DMD-derived hiPSCs where successful deletion and non-homologous end joining of up to 725 kb reframed the *DMD* gene. This is the largest CRISPR/Cas9-mediated deletion shown to date in *DMD*. Use of hiPSCs allowed evaluation of dystrophin in disease-relevant cell types. Cardiomyocytes and skeletal muscle myotubes derived from reframed hiPSC clonal lines had restored dystrophin protein. The internally deleted dystrophin was functional as demonstrated by improved membrane integrity and restoration of the dystrophin glycoprotein complex in vitro and in vivo. Furthermore, miR31 was reduced upon reframing, similar to observations in Becker muscular dystrophy. This work demonstrates the feasibility of using a single CRISPR pair to correct the reading frame for the majority of DMD patients.

INTRODUCTION

Duchenne muscular dystrophy (DMD) is the most common fatal genetic disease of childhood, affecting ~1 in 3,500–5,000 boys. In DMD, progressive muscle degeneration generally leads to death in the twenties, and there are currently no highly effective therapies. DMD is often caused by frameshifting exonic deletions in *DMD*, which encodes dystrophin. Dystrophin stabilizes the dystrophin glycoprotein complex (DGC) at the sarcolemma; loss of functional dystrophin leads to the degradation of DGC components, which results in muscle membrane fragility and leakage of creatine kinase (CK) (Pearce et al., 1964). Approximately 60% of mutations causing DMD occur between *DMD* exons 45–55 (Béroud et al., 2007). Multiple independent clinical reports in patients and dystrophic mice have revealed that in-frame deletions of exons 45–55 produce an internally deleted dystrophin protein and are associated with a very mild Becker muscular dystrophy (BMD) disease course, with some patients still asymptomatic in their sixties (Béroud et al., 2007; Echigoya et al., 2015; Nakamura et al., 2008; Taglia et al., 2015). Thus, genetic manipulation to create a large deletion of exons 45–55 is a therapeutic strategy to restore the reading frame for 60% of DMD patients with mutations in this region.

One promising approach to induce genetic correction of *DMD* is through the use of the bacterially acquired immune surveillance system known as clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated nuclease (Cas) 9. In this system a short guide RNA (gRNA), which is complimentary to a specific site in the genome, is used to target the Cas9 nuclease and induce double-stranded breaks (DSBs). The DSBs can be repaired through non-homologous end joining (NHEJ) or homology-directed repair.

Previous work has shown that CRISPR/Cas9 components can modify the *DMD* gene (Li et al., 2015; Long et al., 2014, 2016; Nelson et al., 2016; Ousterout et al., 2015; Tabebordbar et al., 2016; Wojtal et al., 2016; Xu et al., 2015). In this investigation, we describe a therapeutically relevant CRISPR/Cas9 platform that we designed to modify *DMD*. Our platform involves excision of exons 45–55 and NHEJ to reframe dystrophin through creation of an internally deleted protein that is stable and functional. The internally deleted protein mimics the naturally occurring exon 45–55 deletion observed in mild BMD patients and encompasses 60% of DMD patient mutations.

For the first time, we demonstrate CRISPR/Cas9-mediated deletion and NHEJ of up to 725 kb of the *DMD* gene in human induced pluripotent stem cell (hiPSC) lines. We show that CRISPR/Cas9 reframed, hiPSC-derived skeletal and cardiac

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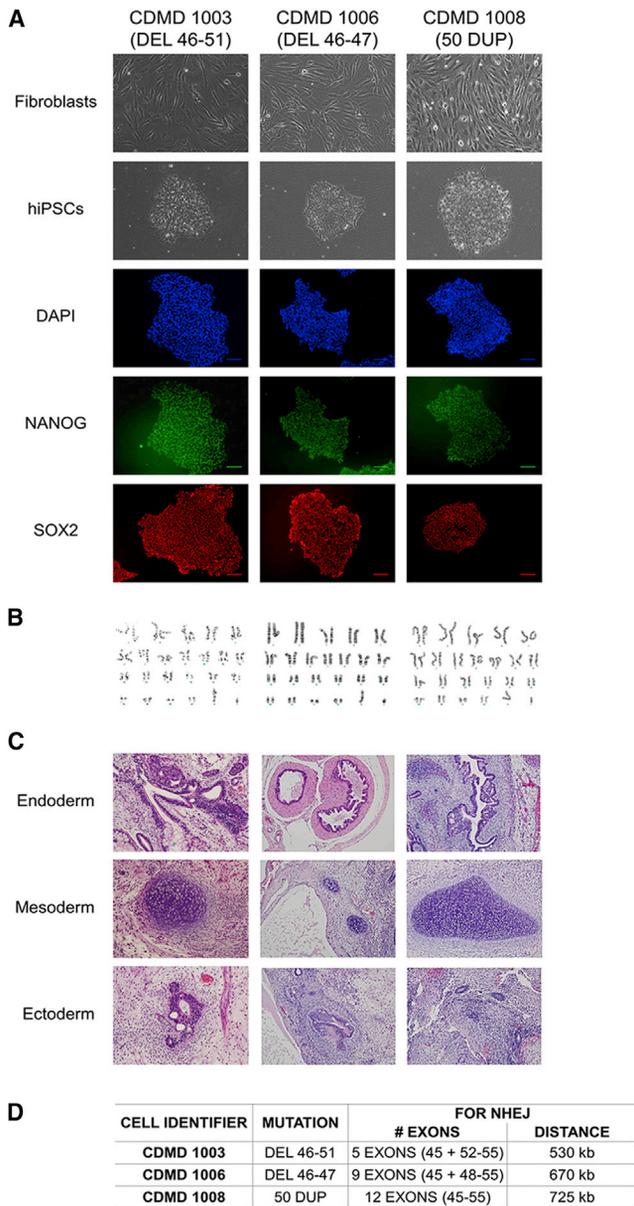
Figure 1. CDMD hiPSCs Are Pluripotent and Genetically Stable

(A) CDMD hiPSCs were generated from DMD fibroblasts. Brightfield images depict fibroblasts before and after reprogramming to hiPSCs. Immunocytochemical staining reveals that cells express pluripotency markers NANOG (green) and SOX2 (red). Scale bar, 100 μm.

(B) Karyotyping of all lines is shown.

(C) CDMD hiPSCs were injected into mice to test teratoma formation in vivo. Representative H&E stainings of the three germ layers (endoderm, mesoderm, and ectoderm) are shown.

(D) Patient mutations for each CDMD hiPSC line are shown. In addition, the number of exons and the approximate distance necessary for successful NHEJ is indicated, based on comparative genomic hybridization data for the patient's underlying mutation size.

muscle cells express stable dystrophin that improves membrane stability and restores a DGC member, β-dystroglycan. We also demonstrate reduced microRNA 31 (miR31) levels after the reading frame is restored, consistent with the observations

made in BMD patients (Cacchiarelli et al., 2011). Furthermore, we show restoration of dystrophin and β-dystroglycan in vivo after engraftment of reframed hiPSC-derived skeletal muscle cells into a mouse model of DMD. This work sets the stage for use of reframed DMD hiPSC-derived cells or in vivo correction strategies using CRISPR/Cas9 for direct translation to patients with DMD.

RESULTS

DMD hiPSC Lines Are Pluripotent and Genetically Stable

We have developed several xenobiotic-free hiPSC lines derived from wild-type and DMD patient fibroblasts using current good manufacturing practice protocols. Each DMD hiPSC line harbors a unique frameshifting *DMD* mutation within the exon 45–55 hotspot region. All hiPSC lines (Center for Duchenne Muscular Dystrophy [CDMD] 1003, 1006, and 1008) express pluripotency markers (NANOG and SOX2) and are karyotypically normal (Figures 1A and 1B). CDMD hiPSCs maintain pluripotency, as they form teratomas in vivo that represent all three germ layers (Figure 1C), and each harbor unique mutations (Figure 1D).

CRISPR/Cas9-Mediated Deletion and NHEJ of up to 725 kb in the *DMD* Gene

In order to delete exons 45–55 of *DMD*, gRNAs were designed to target introns 44 and 55. gRNA sites were chosen to only retain ~500 bp of the intron next to each of the flanking exons (44 and 56). The rationale for this design is to develop gRNAs applicable to as many patient mutations as possible and to ensure that a small functional chimeric intron is generated. During NHEJ, the 3′ end of intron 44 and the 5′ end of intron 55 join to create a ~1 kb chimeric intron (Figure 2A). We expect that introns generated in this manner are functional and splice correctly to create an in-frame transcript, with exon 44 joined with exon 56.

Since hiPSCs are challenging to genetically manipulate, human embryonic kidney (HEK) 293FT cells were used to screen five gRNAs at each intronic region. All gRNAs demonstrated individual cutting activity on Surveyor assay up to 34% (Figures S1A and S1B). Using multiplex PCR, gRNAs transfected in pairs were shown to effectively delete the entire 708 kb region encompassing exons 45–55 (Figures S1C and S1D).

In order to assess the feasibility of an exon 45–55 deletion across different patient mutations, we applied our gRNAs to three DMD hiPSC lines. The lines (CDMD 1003, 1006, and 1008) require ~530 kb, 670 kb, or 725 kb, respectively, for successful deletion and NHEJ of *DMD*. The gRNAs used were shown to be active in all three lines and effectively deleted exons 45–55 (Figures S2 and S3). Transient puromycin selection of cells nucleofected with the CRISPR plasmids improved the efficiency of deletion in CDMD 1003 and 1006 hiPSCs (Figure S3D).

Clonal Reframed DMD hiPSC Lines Contain No Off-Target Activity at Candidate Sites

Stably deleted DMD hiPSC lines were generated from CDMD 1003 and 1006 by clonal selection after nucleofection with the gRNA pair 44C4 and 55C3 (Figures 2B and 2C) and are pluripotent (Figures 2C and S4B). All reframed lines were karyotypically normal except for one clone (CDMD 1003-81), which was found to contain a 1q32 amplification confirmed via FISH analysis
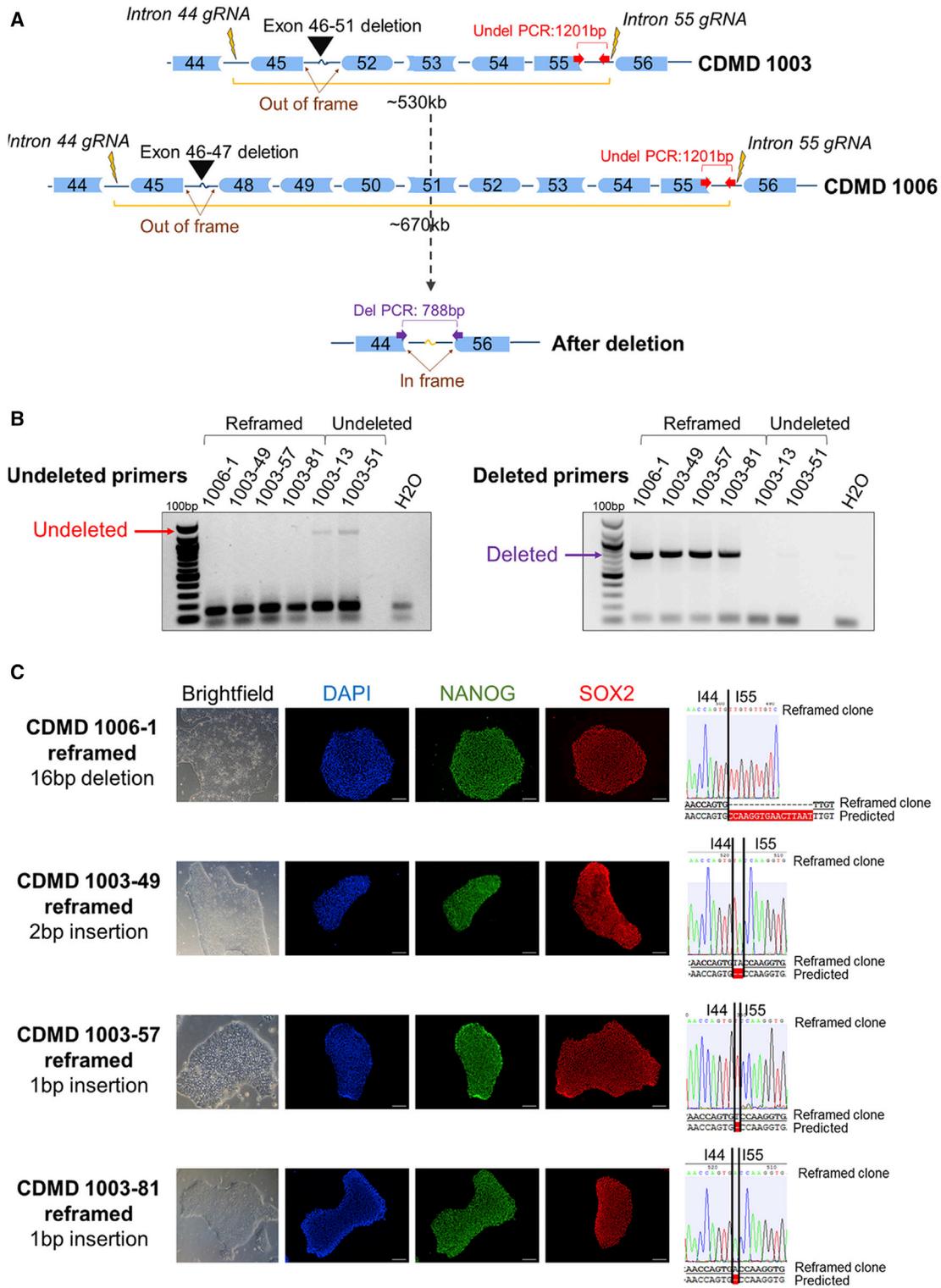
Figure 2. Generation of Stable, Pluripotent CDMD hiPSC Lines with an Exon 45–55 Deletion

(A) Shown is a cartoon (not to scale) of the region of *DMD* targeted for CRISPR/Cas9-mediated deletion using gRNAs specific to introns 44 and 55 (lightning bolts). Successful NHEJ deletes exons 45–55 and restores the reading frame for mutations within this region. Different deletion sizes are required depending on the patient's underlying mutation (black arrow heads).

(B) PCR genotyping of 117 and 109 single-cell clones from parental lines CDMD 1006 and 1003, respectively, was carried out on cells nucleofected with gRNAs 44C4 and 55C3. One clone from CDMD 1006 (CDMD 1006-1) and three from CDMD 1003 (CDMD 1003-49, 1003-57, and 1003-81) were identified as stably

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(Figure S4A), also observed in the original parental line and in all daughter clones after post hoc analysis. The 1q32 amplification is common in hPSCs after extended propagation in culture (Dekel-Naftali et al., 2012), and thus was not a result of CRISPR-mediated off-target activity. To determine off-target activity of our gRNAs, the top ten homologous sites per guide were determined by COSMID (Cradick et al., 2014) and sequenced in all clonal and parental lines. No off-target mutations were observed at any site (Table S2). All variants, besides a heterozygous SNP in chromosome 11, were detected in less than 1% of reads, which is consistent with error in the sequencing method.

Dystrophin (DYS$^{\Delta 45-55}$) Expression Is Restored in Reframed DMD hiPSC-Derived Cardiomyocytes and Skeletal Myotubes

CRISPR/Cas9-mediated deletion of *DMD* should result in an internally deleted dystrophin protein lacking exons 45−55 (hereafter referred to as DYS$^{\Delta 45-55}$). As hiPSCs do not express dystrophin, we differentiated the reframed DMD hiPSC clonal lines to two disease-relevant cell types, cardiomyocytes and skeletal muscle myotubes, using directed differentiation or overexpression (OE) of MyoD to evaluate rescue of DYS$^{\Delta 45-55}$. PCR and sequencing of the exon 44/56 boundary in cDNA from the reframed cardiomyocyte clones demonstrated correct splicing of the dystrophin transcript (Figures S4C and S4D). Additionally, both the reframed cardiac and skeletal muscle cell lines restored dystrophin expression as assayed by immunocytochemistry and western blot (Figures 3A–3C). Compared to wild-type CDMD 1002 or human skeletal muscle myotubes (HSMM), the band was truncated by ∼66 kDa as expected.

DYS$^{\Delta 45-55}$ Protein Restores Membrane Functionality to Cardiomyocytes and Skeletal Myotubes In Vitro

Cardiomyocytes or skeletal myotubes lacking dystrophin demonstrate membrane fragility in vitro and respond to osmotic stress by releasing elevated levels of CK (Guan et al., 2014; Menke and Jockusch, 1995), as is seen in human patients (Pearce et al., 1964). To determine whether DYS$^{\Delta 45-55}$ could restore stability to dystrophic plasma membranes, we subjected differentiated cardiomyocytes and skeletal muscle myotubes derived from reframed and out-of-frame hiPSCs to hypo-osmotic conditions. Cells were stressed by incubation in hypoosmolar solutions (66–240 mosmol) and CK release into the supernatant was measured to show functional improvement after dystrophin restoration. Both the reframed CDMD 1003-49 cardiomyocytes and skeletal muscle cells demonstrated reduced CK release, similar to wild-type (CDMD 1002), versus the out-of-frame CDMD 1003 cells, indicating that DYS$^{\Delta 45-55}$ was capable of reducing membrane fragility (Figure 4A). The same trend was also observed with CDMD 1006/1006-1 cardiomyocytes (Figure S4E). After normalizing and pooling all experiments, we observed that significantly less CK was released at 93, 135, and 240 mosmol in the reframed and wild-type cells compared to out-of-frame (Figure S4F).

CRISPR/Cas9 Reframing Correlates with miR31 Levels in Skeletal Myotubes In Vitro

Elevated levels of miR31 have been observed in DMD patient biopsies compared to wild-type or BMD (Cacchiarelli et al., 2011). We measured levels of miR31 using droplet digital PCR (ddPCR) after differentiation of out-of-frame and reframed CDMD hiPSCs to skeletal myotubes. Reframing *DMD* reduced levels of miR31 (similar to wild-type cells) compared to out-of-frame *DMD*, as is observed in human dystrophinopathies (Figure 4B). Thus, reframing the *DMD* gene normalizes miR31 levels similar to BMD, demonstrating functional rescue of the dystrophic phenotype to a BMD phenotype.

DYS$^{\Delta 45-55}$ Protein Restores the DGC In Vitro and In Vivo

As a third assay of DYS$^{\Delta 45-55}$ functionality, we evaluated its ability to restore the DGC in vitro and in vivo. The DGC member β-dystroglycan was restored and detected at the membrane of reframed hiPSCs, but not out-of-frame hiPSCs, after directed differentiation to skeletal muscle in vitro by immunostaining and western blot (Figures 4C and 4D). Additionally, skeletal muscle cells derived from a wild-type (CDMD 1002), out-of-frame (CDMD 1003), or reframed (CDMD 1003-49) hiPSC line were injected into the tibialis anterior (TA) of NOD *scid* IL2Rgamma (NSG)-mdx mice. Correctly localized dystrophin and β-dystroglycan was only observed in engrafted human cells (demarked by human lamin A/C and spectrin) from the reframed or wild-type lines (Figures 4E and 4F). These studies taken together with the hypo-osmotic stress assays demonstrate the ability of DYS$^{\Delta 45-55}$ to functionally reassemble the DGC and restore membrane stability in vitro and in vivo.

DISCUSSION

Using CRISPR/Cas9 gene editing, we have induced the largest deletion accomplished to date in DMD hiPSCs and restored a functional dystrophin protein. Deletion of *DMD* exons 45–55 has the potential to be therapeutically relevant to 60% of DMD patients. Since this internal deletion has been associated with a very mild disease course in multiple independent patients, a therapy utilizing this approach should create a highly functional dystrophin. We showed successful deletion of exons 45–55 using a single gRNA pair and did not identify any off-target activity at the top ten homologous sites; however, a more comprehensive and unbiased approach should be undertaken such as whole-genome sequencing. Importantly, removal of exons 45–55 resulted in stable dystrophin protein (DYS$^{\Delta 45-55}$) in both cardiomyocytes and skeletal myotubes in vitro. Functionality of

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deleted. Deletion PCR genotyping results for six hiPSC clonal lines is shown. One pair of primers (red arrows in A) was located internal to the deletion and only produced a 1,201 bp band in the undeleted clones CDMD 1003-13 and 1003-51. Another primer set (purple arrows in A) flanked the deletion region and produced a 788 bp band only when the deletion and NHEJ occurred successfully, as in the reframed clones CDMD 1006-1, 1003-49, 1003-57, and 1003-81.

(C) Each clonal line maintained normal morphology (brightfield) and expressed NANOG (green) and SOX2 (red) by immunocytochemistry. Scale bar, 100 μm. Shown to the right is the sequence of the gDNA at the rejoining site between introns 44 (I44) and 55 (I55). Sequencing revealed a 16 bp deletion in CDMD 1006-1, a 2 bp insertion in CDMD 1003-49, and 1 bp insertions in CDMD 1003-57 and CDMD 1003-81.

See also Figures S1, S2, S3, S4A, and S4B.
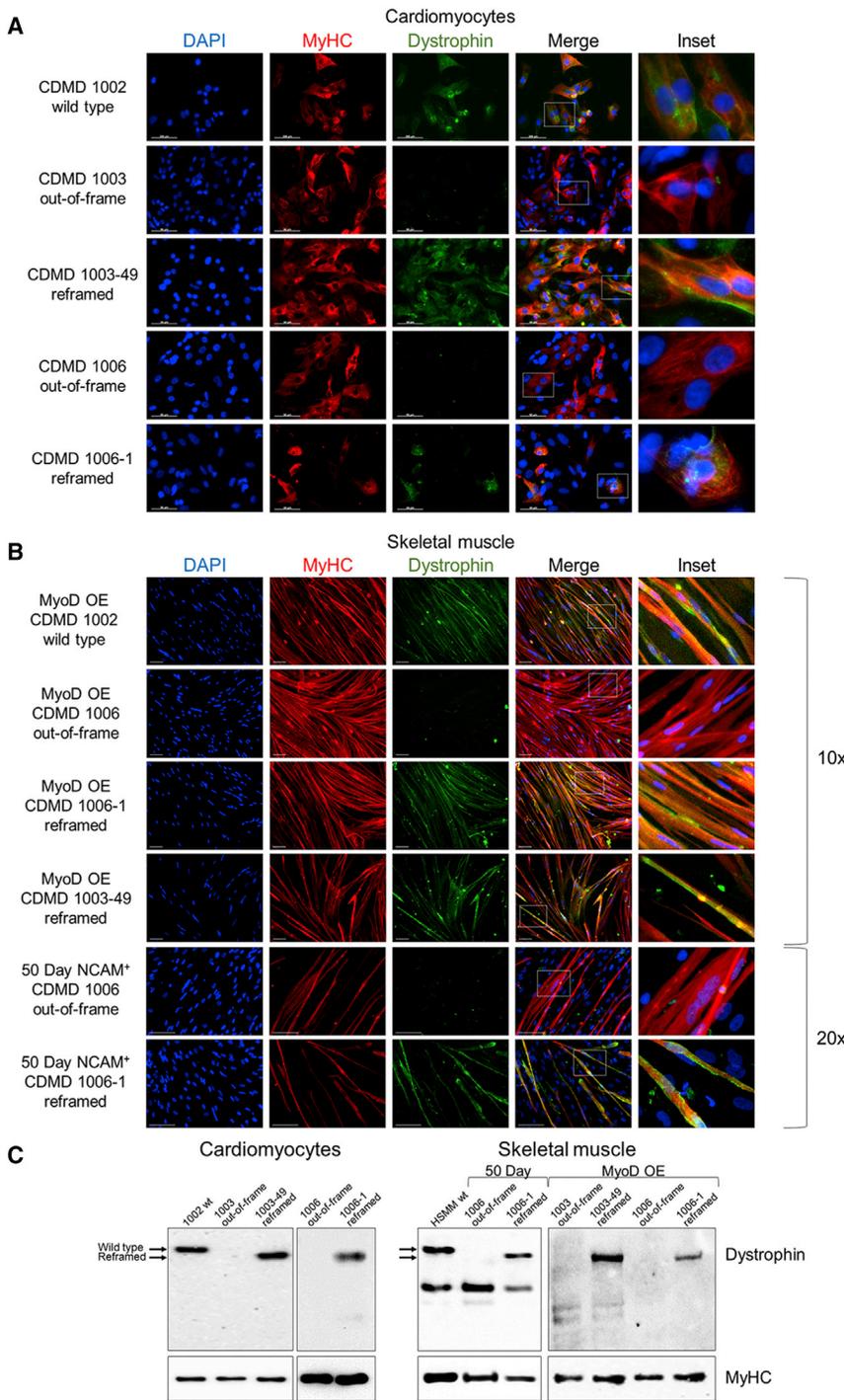
Figure 3. Reframed CDMD hiPSC-Derived Skeletal Muscle and Cardiomyocytes Restore Dystrophin Expression

(A) Immunocytochemical staining of human myosin heavy chain (MyHC, red) and dystrophin (green) of wild-type (CDMD 1002), out-of-frame (CDMD 1003 or 1006) or reframed (CDMD 1003-49 or 1006-1) cardiomyocytes derived from hiPSCs by directed differentiation. Inset depicts zoomed in region defined by the white box. Scale bar, 50 μm.

(B) Immunocytochemical staining of MyHC (red) and dystrophin (green) of wild-type (CDMD 1002), out-of-frame (CDMD 1006) or reframed (CDMD 1006-1 or 1003-49) skeletal muscle myotubes derived from hiPSCs. Myotubes were fused after MyoD OE or from sorted NCAM^{+} cells after an adapted directed differentiation 50-day protocol was used. Inset depicts zoomed-in region defined by the white box. Scale bar, 100 μm.

(C) Western blots of cell extracts probed with anti-dystrophin. Extracts were from out-of-frame and reframed cardiomyocytes (left) and skeletal muscle myotubes (right), derived from CDMD hiPSCs. Wild-type (wt) hiPSCs (CDMD 1002) or human skeletal muscle myotubes (HSMM) were used as a control for dystrophin. The molecular weight shift caused by the exon 45–55 deletion (1779 bp, ~66 kDa) is evident in reframed versus wild-type dystrophin (arrows). A non-specific band around 220 kDa was seen in some samples. Samples were also probed with anti-MyHC as a loading control (bottom panels).
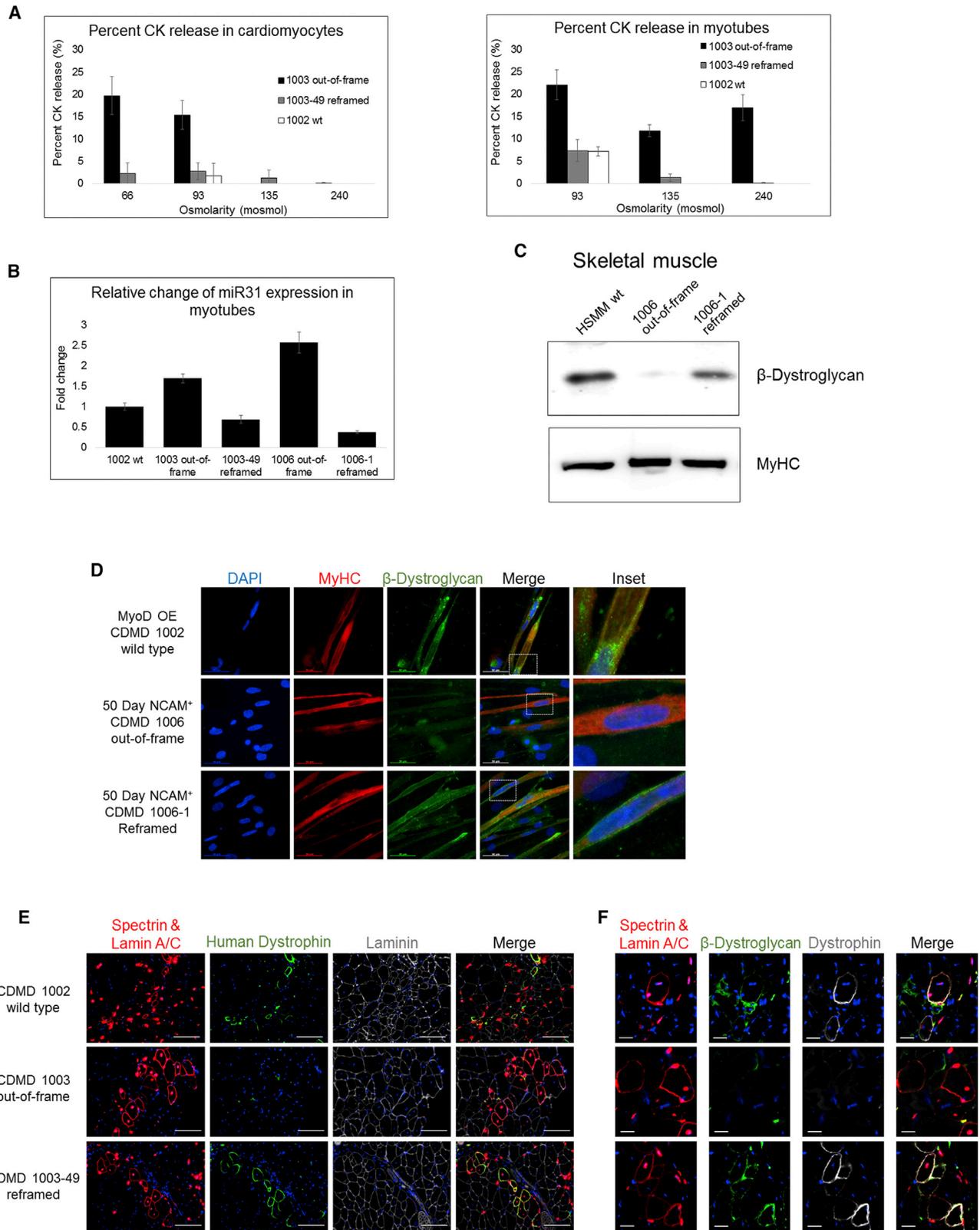
See also Figures S4C and S4D.

phin, after reading frame restoration, similar to what is observed in human BMD patients (Cacchiarelli et al., 2011). Finally, we show restored DGC localization in vitro and in vivo, which further validates the functionality of DYS$^{\Delta45–55}$.

Previous work by Ousterout et al. (2015) demonstrated that multiplexed gRNAs can restore the *DMD* reading frame in primary myoblasts. However, myoblasts do not provide a renewable source of stem cells, which is a requirement for long-term therapeutic efficacy (Partridge, 2002). In contrast, we used hiPSCs, which offer the opportunity to evaluate the internally deleted dystrophin protein in multiple cell types that are affected in DMD, and in future studies,

DYS$^{\Delta45–55}$ was tested in cardiomyocytes and skeletal muscle derived from reframed DMD hiPSCs and demonstrated improved membrane stability by a physiologically relevant measure of CK release, similar to wild-type. The ability to evaluate cardiomyocyte functionality is an advantage of using hiPSCs, as some current preclinical and clinical studies for DMD therapies do not efficiently target the heart (e.g., exon skipping; Arecha-vala-Gomeza et al., 2012). Additionally, we demonstrated a normalization in miR31 levels, a microRNA that inhibits dystro-

they may provide a renewal source of corrected progenitor cells. Our work is further distinguished from previous studies as we are the only group to show restoration of dystrophin function on membrane integrity, miR31 expression, and the DGC in cardiac and skeletal muscle cells following CRISPR-mediated gene editing.

An advantage of our CRISPR platform is the therapeutic potential of a single pair of gRNAs to treat the majority of DMD patients. By designing gRNAs that accomplish a

A

Percent CK release in cardiomyocytes

Percent CK release in myotubes

B

Relative change of miR31 expression in myotubes

C

Skeletal muscle

β-Dystroglycan

MyHC

D

DAPI MyHC β-Dystroglycan Merge Inset

MyoD OE CDMD 1002 wild type

50 Day NCAM+ CDMD 1006 out-of-frame

50 Day NCAM+ CDMD 1006-1 Reframed

E

Spectrin & Lamin A/C Human Dystrophin Laminin Merge

CDMD 1002 wild type

CDMD 1003 out-of-frame

CDMD 1003-49 reframed

F

Spectrin & Lamin A/C β-Dystroglycan Dystrophin Merge

Figure 4. Reframed hiPSC-Derived Cardiomyocytes and Skeletal Muscle Cells Demonstrate Restored Function In Vitro and In Vivo

(A) Representative graphs of CK release assays from cells exposed to hypo-osmotic conditions. Cardiomyocytes and skeletal muscle myotubes derived from hiPSCs were subjected to a range of osmolarities below 240 mosmol, and CK release to the supernatant was measured as an indication of membrane fragility. Data are presented as average ± SE.

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deletion that encompasses the majority of DMD mutations, this approach is optimized for future clinical studies. It would be unreasonable to design, validate, and evaluate off targets for every new CRISPR pair tailored for each individual patient. Additionally, CRISPR/Cas9 is advantageous over exon skipping, as it results in permanent restoration of the reading frame as opposed to transient effects on RNA splicing. Previously, Li et al. (2015) used CRISPR/Cas9 to induce exon skipping, frameshifting, or exon knockin to restore dystrophin in a DMD hiPSC line with an exon 44 deletion; however, their platform is only applicable to 3%–9% of DMD patients (Bladen et al., 2015), and two of their strategies relied on the creation of indels, which would be difficult to apply consistently to each patient. While Ousterout et al. deleted exons 45–55, they removed significantly less of the intervening region (336 kb) and thus their approach would cover fewer patient mutations within the hotspot region. This is because many mutations extend into the intronic region; thus, by designing gRNAs that encompass more of the intron, our platform is applicable to more patients.

Another benefit of using this platform to delete a large portion of *DMD*, as opposed to single exons, is the known correlation of DYS$^{\Delta 45-55}$ with a mild BMD phenotype. Large deletions in the rod domain of dystrophin often produce a more functional (more like wild-type) protein, than even very small deletions (Harper et al., 2002). Larger deletions, which remove hinge III (exons 50–51), are believed to lead to a milder BMD phenotype than smaller deletions, or those that retain hinge III (Carsana et al., 2005). Thus, in many cases larger deletions are more therapeutically beneficial than smaller ones, due to the way they affect the secondary structure of the protein.

In summary, we have developed a potentially therapeutic gene editing platform for DMD to permanently restore the dystrophin reading frame in multiple patient-derived hiPSCs. Our approach using CRISPR/Cas9 and NHEJ deletes up to 725 kb of *DMD* encompassing exons 45–55 and restores dystrophin protein function in both cardiomyocytes and skeletal muscle cells derived from reframed hiPSCs. A current limitation of this platform is that clinical protocols still need to be developed that allow rapid clonal line derivation and the utilization of hiPSC-derived cardiac and skeletal muscle progenitors combined with gene correction. Alternatively, CRISPR/Cas9 to restore the reading frame in DMD mouse models has been delivered directly in vivo (Long et al., 2016; Nelson et al., 2016; Tabebordbar et al., 2016). Thus, applications of this platform in the future will allow for the development of an in situ gene strategy or ex vivo gene correction followed by autologous cell transplantation, either of which offers tremendous potential for DMD.

EXPERIMENTAL PROCEDURES

Differentiation of hiPSCs to Skeletal Muscle Cells and Cardiomyocytes

Skeletal muscle differentiation from hiPSCs was induced using OE of a tamoxifen inducible MyoD-ERT lentivirus or an adapted 50 day directed differentiation protocol where NCAM$^+$ HNK1$^-$ cells underwent fluorescence-activated cell sorting at day 50. Cardiomyocytes were derived through aggregates over 30 days. See Supplemental Experimental Procedures.

Engraftment into Immunodeficient Mice

NSG immunodeficient mice (Jackson Laboratory) were crossed to mdx *scid* mice (Jackson Laboratory) to generate NSG-mdx mice (see Supplemental Experimental Procedures). Five- to seven-week-old NSG-mdx mice were pretreated with 50 μl of 10 μM cardiotoxin (Sigma-Aldrich) injected into the right TA 24 hr prior to engraftment. For MyoD OE cells, 100 μl of 5 mg/ml tamoxifen (Sigma-Aldrich) was i.p. injected for 5 days beginning on the day prior to engraftment. 1 × 10^6 cells in HBSS were injected intramuscularly and the TA was harvested after 30 days. See Supplemental Experimental Procedures.

Hypo-osmotic Stress CK Release Assay

Terminally differentiated skeletal muscle cells and cardiomyocytes plated in duplicate were stressed by incubation in hypo-osomolar solutions ranging from 66 to 240 mosmol (see Supplemental Experimental Procedures) for 20 min at 37°C. CK was measured in triplicate from the supernatant and cell lysate with the Creatine Kinase-SL kit (Sekisui Diagnostics) according to the manufacturer's instructions.

SUPPLEMENTAL INFORMATION

Supplemental Information for this article includes four figures, two tables and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.stem.2016.01.021.

AUTHOR CONTRIBUTIONS

(B) Fold change in expression of miR31 measured by ddPCR in myotubes derived from out-of-frame or reframed hiPSCs by MyoD OE, normalized to wild-type (CDMD 1002). Data are presented as average ± SD.

(C) Western blots of cell extracts probed with anti-β-dystroglycan. Extracts were from out-of-frame and reframed skeletal muscle myotubes derived by MyoD OE. HSMM was used as a positive control. Samples were also probed with anti-MyHC as a loading control (bottom panel).

(D) Immunocytochemical staining of MyHC (red) and β-dystroglycan (green), a component of the DGC, in wild-type (CDMD 1002), out-of-frame (CDMD 1006), or reframed (CDMD 1006-1) skeletal muscle myotubes. Inset depicts zoomed-in region defined by the white box. Scale bar, 50 μm.

(E) Assessment of human dystrophin restoration in wild-type (CDMD 1002), out-of-frame (CDMD 1003), and reframed (CDMD 1003-49) MyoD OE cells engrafted into the TA of NSG-mdx mice. Engrafted human cells were identified by co-immunostaining for human spectrin and lamin A/C (shown in red). Positive staining for human dystrophin is shown in green and all fibers are shown using laminin (gray). All sections were stained with DAPI (blue) to identify nuclei. Scale bar, 100 μm.

(F) Assessment of β-dystroglycan restoration in human fibers from wild-type (CDMD 1002), out-of-frame (CDMD 1003), and reframed (CDMD 1003-49) MyoD OE cells engrafted into the TA of NSG-mdx mice. Engrafted human cells were identified by co-immunostaining for human spectrin and lamin A/C (shown in red). Positive staining for dystrophin is shown in gray and β-dystroglycan is shown in green. All sections were stained with DAPI (blue) to identify nuclei. Cell order is the same as noted in (E). Scale bar, 20 μm.

See also Figures S4E and S4F.

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