Supplementary Materials for

**Optogenetic modeling of human neuromuscular circuits in Duchenne muscular dystrophy with CRISPR and pharmacological corrections**

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Published 10 September 2021, *Sci. Adv.* 7, eabi8787 (2021)
DOI: 10.1126/sciadv.abi8787

The PDF file includes:

- Figs. S1 to S6
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Other Supplementary Material for this manuscript includes the following:

- Movies S1 to S6
- Data files S1 and S2
Fig. S1. Characterization of a pair of DMD patient-derived and isogenic control ePSCs.

(A) *DMD* c.10141C>T (p.R3381X) mutation confirmed by sequencing analysis in DMD patient’s derived fibroblasts.

(B) Relative expression of *OCT4* and *NANOG* pluripotency markers in two independent DMD-R3381X ePSC clones. DMD fibroblasts did not express pluripotency genes. N=3, technical replicates, values are mean ± SD,

(C) Positive immunocytochemistry of *NANOG*, *OCT4*, *SOX2*, *SSEA4* and *TRA-1-60* in CORR-R3381X ePSCs. Scale bars are 100 µm.

(D) Microsatellite analysis confirmed common parental origin of the two independent CORR-R3381X ePSCs generated clones.
Fig. S2. Expression of myogenic markers in DMD- and CORR-R3381X MPCs and myotubes.

(A) Representative images of immunocytochemistry of PAX7 in DMD and CORR-R3381X MPCs and at 24, 48 and 120h in secondary differentiation medium. Scale bars are 50 μm.

(B) Representative images of immunocytochemistry of MYOD1 in DMD and CORR-R3381X MPCs and at 24, 48 and 120h in secondary differentiation medium. Scale bars are 50 μm.

(C) Representative images of immunocytochemistry of MYH stained with MF20 antibody in DMD and CORR-R3381X MPCs and at 24, 48 and 120h in secondary differentiation medium. Scale bars are 50 μm.

(D) Log2RPKM values of PAX7 gene do not significantly differ in DMD and CORR-R3381X muscle cells at MPCs (0h) stage, after 24 and 120h in secondary differentiation medium. N=3, values are mean ± SD. Two-way ANOVA followed by Sidak’s multiple comparisons test.

(E) Log2RPKM values of MYOD1 gene follow a similar trend in DMD and CORR-R3381X muscle cells at MPCs (0h) stage, after 24 and 120h in secondary differentiation medium. N=3, values are mean ± SD. Two-way ANOVA followed by Sidak’s multiple comparisons test.
(F) Log2RPKM values are significantly lower in DMD-R3381X muscle cells at the three stages of secondary differentiation when compared with CORR-R3381X cells. N=3, values are mean ± SD. Two-way ANOVA followed by Sidak’s multiple comparisons test, ****p < 0.0001.
**Fig. S3.** Heatmaps of core enrichment genes in GO MUSCLE CELL CELLULAR HOMEOSTASIS at 0, 24 and 120 hours. Both *DMD* and *CHRNA1* are down-regulated in DMD-R3381X compared with CORR-R3381X.
Fig. S4. SB-431542 treatment of DMD-R3381X muscle cells during secondary differentiation

(A) Log2RPKM values of \(TGFB1\) are significantly higher in DMD-R3381X MPCs during secondary differentiation. N=3, values are mean \(\pm\) SD. Two-way ANOVA followed by Sidak’s multiple comparisons test, \(*p < 0.05\).

(B) Heatmaps of core enrichment genes in GO NEGATIVE REGULATION OF MYOBLAST DIFFERENTIATION include \(TGFB1\) and genes involved in TGFβ signaling, which are up-regulated in DMD-R3381X at 24 and 120 hours of secondary differentiation.

(C) Representative images of immunocytochemistry for titin in 2D myogenic cultures of DMD-R3381X, DMD-R3381X treated with 10 \(\mu\)M SB-431542 and CORR-R3381X after 120h in secondary differentiation medium. Scale bars are 50 \(\mu\)m.

(D) Quantification of mean velocity of DMD-R3381X, DMD-R3381X + SB-431542 and CORR-R3381X myofibers upon optogenetic stimulation at day 5. The blue shading indicates the time during optogenetic stimulation. N=12. Values are mean \(\pm\) SEM, Two-way ANOVA followed by Sidak’s multiple comparisons test between DMD-R3381X + SB-431542 and CORR-R3381X samples. \(*p < 0.05, **p < 0.01, ****p < 0.0001\). The data is the same as in Figure 4D and 4F.
Fig. S5. Gentamicin or PTC124 treatment of DMD-R3381X muscle cells during secondary differentiation

(A) Representative immunocytochemistry images of dystrophin staining in DMD-R3381X, DMD-R3381X treated with a range of Gentamicin concentrations (10 µM, 100 µM, 200 µM and 600 µM) and CORR-R3381X cells after 120h in secondary differentiation medium. Scale bars are 50 µm.

(B) Quantification of percentage of dystrophin-positive area, mean fluorescence intensity multiplied by pixel number and percentage of normalized dystrophin levels in gentamicin treated
conditions. NT, Not treated. N=3. Values are mean ± SD. One-way ANOVA followed by Tukey’s multiple comparisons test, ***p < 0.001, ****p < 0.0001.

(C) Representative immunocytochemistry images of dystrophin staining in DMD-R3381X, DMD-R3381X treated with a range of PTC124 concentrations (1 µM, 5 µM, 10 µM, 17 µM, 25 µM and 35 µM) and CORR-R3381X cells after 120h in secondary differentiation medium. Scale bars are 100 µm.
**Fig. S6.** Heatmaps illustrating log$_2$ RPKM gene expression (row z-scores) of core enrichment genes for KEGG AXON GUIDANCE at each individual time point, columns represent samples and rows represent genes.
Table S1. Summary of DMD-R3381X patient’s mutation, symptoms and the reprogrammed ePSC line

| P1    |          |                |                |                |                |                |
|-------|----------|----------------|----------------|----------------|----------------|----------------|
|       | Fibroblasts | FB763 (P4)    | Mutations      | c.10141C>T (p.R3381X) |                |                |
|       | Exon     | 70             |                |                |                |                |
|       | Ensembl variant ID | rs104894790 |                |                |                |                |
|       | Sex      | Male           |                |                |                |                |
|       | Age at biopsy | 6 years old    |                |                |                |                |
|       | Muscular symptoms | Frequent falls  |                |                |                |                |
|       | Microscopic description | Abnormal round fibre size, necrosis, increased internal nuclei, increase in fat/connective tissue, |                |                |                |
|       | Dystrophin immunocytochemistry | Absent |                |                |                |                |
|       | CK       | 10,000 IU/L    | Normal range<200 IU/L |                |                |                |
|       | Brain symptoms | Severe learning difficulties |                |                |                |                |
|       | Cardiac symptoms | N/A            |                |                |                |                |
|       | PSC type  | ePSCs          |                |                |                |                |
|       | PSC growth medium | EPSCM         |                |                |                |                |
|       | PSC line  | DMD-R3381X     |                |                |                |                |
Table S2. Primary antibodies

| Antibodies                      | Species | Type       | Isotype | Supplier     | Cat Number | Working Dilution |
|---------------------------------|---------|------------|---------|--------------|------------|------------------|
| OCT4                            | Mouse   | Monoclonal | IgG2b   | Santa Cruz   | sc-5279    | 1:100            |
| NANOG                           | Rabbit  | Polyclonal | IgG     | Abcam        | AB80892    | 1:100            |
| SOX2                            | Mouse   | Monoclonal | IgG2a   | R&D          | MAB2018    | 1:100            |
| TRA-1-60                        | Mouse   | Monoclonal | IgM     | Santa Cruz   | sc-21705   | 1:100            |
| SSEA4                           | Mouse   | Monoclonal | IgG3    | BD Bioscience| 560796     | 1:50             |
| α-Smooth Muscle Actin           | Mouse   | Monoclonal | IgG2a   | R&D          | MAB1420    | 1:75             |
| β-III tubulin (TUBB3)           | Mouse   | Monoclonal | IgG2a   | R&D          | MAB1195    | 1:100            |
| α-Fetoprotein                   | Mouse   | Monoclonal | IgG1    | R&D          | MAB1368    | 1:100            |
| PAX7                            | Mouse   | Monoclonal | IgG1    | DSBH         | N/A        | 1:100            |
| MYOD1                           | Mouse   | Monoclonal | IgG1    | Dako         | M3512      | 1:100            |
| MYH (MF20)                      | Mouse   | Monoclonal | IgG2b   | DSBH         | N/A        | 1:100            |
| Titin                           | Mouse   | Monoclonal | IgM     | DSBH         | N/A        | 1:100            |
| Dystrophin (Immunocytochemistry) | Mouse   | Monoclonal | IgG2a   | Millipore    | MABT827    | 1:50             |
| Dystrophin (Immunoblotting)     | Rabbit  | Polyclonal | IgG     | Fisher Scientific | PA5-32388 | 1:750           |
| β-Actin                         | Mouse   | Monoclonal | IgG2a   | Sigma        | A5316      | 1:5,000          |
| Vinculin                        | Mouse   | Monoclonal | IgG1    | Sigma        | MAB3574    | 1:1000          |
| Acetylcholine receptor, nicotinic, muscle | Rat   | Monoclonal | IgG1    | DSBH         | mAb 35     | 1:200            |
| Synaptic vesicle glycoprotein 2A | Mouse   | Monoclonal | IgG1    | DSBH         | SV2        | 1:500            |
| Antibodies                  | Species | Supplier     | Cat Number | Working Dilution |
|----------------------------|---------|--------------|------------|------------------|
| Anti-mouse IgG1 488        | Goat    | Invitrogen   | A-21121    | 1:1,000          |
| Anti-mouse IgG 488         | Goat    | Invitrogen   | A-28175    | 1:1,000          |
| Anti-rabbit IgG 488        | Goat    | Invitrogen   | A-11034    | 1:1,000          |
| Anti-mouse IgG2a 488       | Goat    | Invitrogen   | A-21131    | 1:1,000          |
| Anti-mouse IgG2b 546       | Goat    | Invitrogen   | A-21143    | 1:1,000          |
| Anti-rat IgG 555           | Goat    | Invitrogen   | A-21434    | 1:1,000          |
| Anti-mouse IgM 594         | Goat    | Invitrogen   | A-21044    | 1:1,000          |
| Anti-mouse IgG1 647        | Goat    | Invitrogen   | A-21240    | 1:1,000          |
| IRDye 680RD Anti-mouse IgG | Goat    | LI-COR Bioscience | 926-68070 | 1:10,000         |
| IRDye 800CW Anti-rabbit IgG| Donkey  | LI-COR Bioscience | 926-32213 | 1:10,000         |
| Target Gene | Sequence (5’-3’) |
|-------------|------------------|
| **NANOG**   | F-AGAAAAACAACTGGCCGAAGAAT  
               R-GTTGAATTTGCCAGGTCTGGTT |
| **OCT4**    | F-CACTGTACTCTGGCTCCCTTTTC  
               R-CAACCAGTTGCCCCAAACTC |
| **TGFB1**   | F-TCGCCAGAGTGGTTATCTT  
               R-TAGTGAACCGTTGATGTC |
| **MUSK**    | F-GCCTTCAGCGGAACGTGAGAAA  
               R-GGCTGGGGTGGATGATTCA |
| **SLIT2**   | F-GACGACTGCCAGACACAAA  
               R-TGATAGCCAGGCAAACACTG |
| **SLIT3**   | F-AGCGCCTTGACCTGGACA  
               R-TCGCGGTGCTCTGGAAAA |
| **ROBO2**   | F-GGGTTACTACATCTGCCAGGCTT  
               R-AGGTGGAGGTCTATCTGTCAAAACAT |
| **EFNB2**   | F-GCAAGTTCTGCTGGATCAAC  
               R-AGGATGTTGGTCCCGAATG |
| **EPHB4**   | F-GTCTGACTTTGGCCTTTCCC  
               R-TGACATCACCCTCCACATCA |
| **SEMA3D**  | F-TGGGACATCGAAGACAGCAT  
               R-AAAGTGTGCCTCTGGGCTTT |
| **SEMA5A**  | F-GTCTATACTTGCCAGCG  
               R-GTAAATGCGCTTGTGGCCT |
| **ACTB**    | F-GCGAGAAAGATGACCCAGATC  
               R-CCAGTGTACGGCCAGGAGG |
Table S5. Oligonucleotide primer sequences used to amplify the fragments for Gibson Assembly

| Fragment          | Sequence (5’-3’)                                                                 | Product Length (bp) |
|-------------------|----------------------------------------------------------------------------------|---------------------|
| Left Arm          | F-CGC GCC GTACCTTAATTAAAACCTAAATGCTAGGCATTTC                                           | 1,040               |
|                   | R-GACTATCTTTCTAGGGTTAAGGAGGAGTGGGTGGTGTTGGA                                       |                     |
| Right Arm (1)     | F-TGATCTCACCATGATCTCCCTTTTAGACTACATCAGGAGAG AGTTCGAGACTTTTGCCAAAGGTACTAAAACCAAATTTTGGAA CCAAAAGGTATTTTGC | 950                 |
|                   | R-GGGGATCCACAGTTCTAGAGCGACCCCTTCAGCAAAAA                                           |                     |
| Right Arm (2)     | F-GATTATCTTTCTAGGGTTAATTACAAAACAAATGTGCATGGG GCAGAAGACTCGGAGGTACATTAGTTTTGAAATCATCCTGT CCTAAATCTGATCTCACC | 1,040               |
|                   | R-GGGGATCCACAGTTCTAGAGCGACCCCTTCAGCAAAAA                                           |                     |
| Backbone Vector   | F- TTTTTGCTGAAGGGTGGTCGTCTAGA ACTAGTGATGCCCTCC                                          | 3,013               |
|                   | R-GTAATGTGCCTAGCATTTATTAAATTAAAGGTACGGGCGG                                          |                     |
| Selection Cassette| F-TCACAACCACAACACTCTCCTAAACCTAGAAAAGATAGTC R-CCATGACACTTTGGTTATTAACCTAGAAAGATAATC | 3,277               |

Right arm (1) primer was used to introduce the corrected base (green underlined).
Other Supplementary Materials for this manuscript include the following:

**Movie S1.** DMD-R3381X contraction video_S1 for Fig 3

**Movie S2.** CORR-R3381X contraction video_S2 for Fig 3

**Movie S3.** DMD-R3381X contraction video_S3 for Fig 4

**Movie S4.** DMD-R3381X + SB-431542 contraction video_S4 for Fig 4

**Movie S5.** CORR-R3381X contraction video_S5 for Fig 4

**Movie S6.** CORR-R3381X + SB-431542 contraction video_S6 for Fig 4

**Data S1.** gsea_report_Mut_vs_Ctrl_cp

**Data S2.** gsea_report_Mut_vs_Ctrl_gobp