Development of AAV-Based CRISPR/Cas9 Therapies for Correcting Duchenne Muscular Dystrophy by Targeted Genomic Integration

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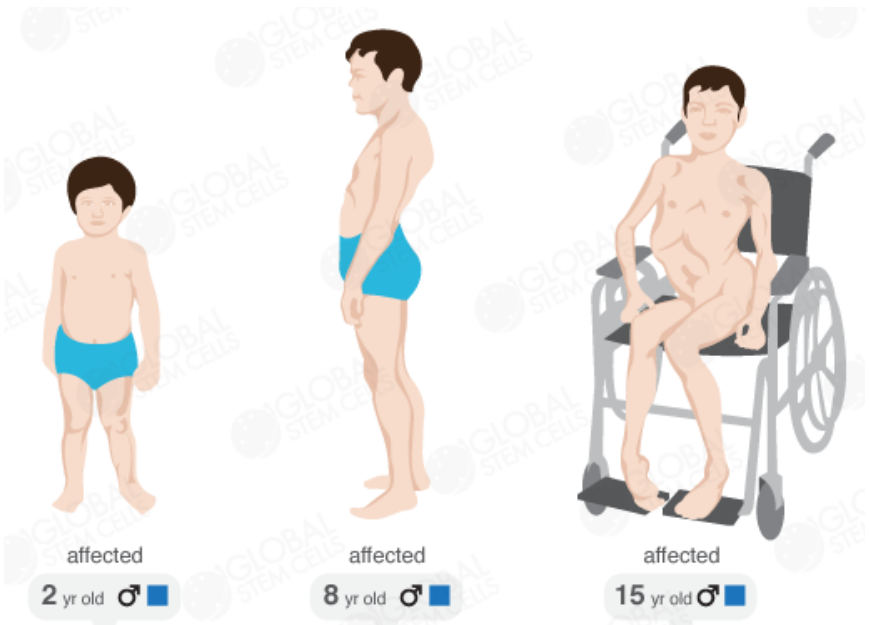
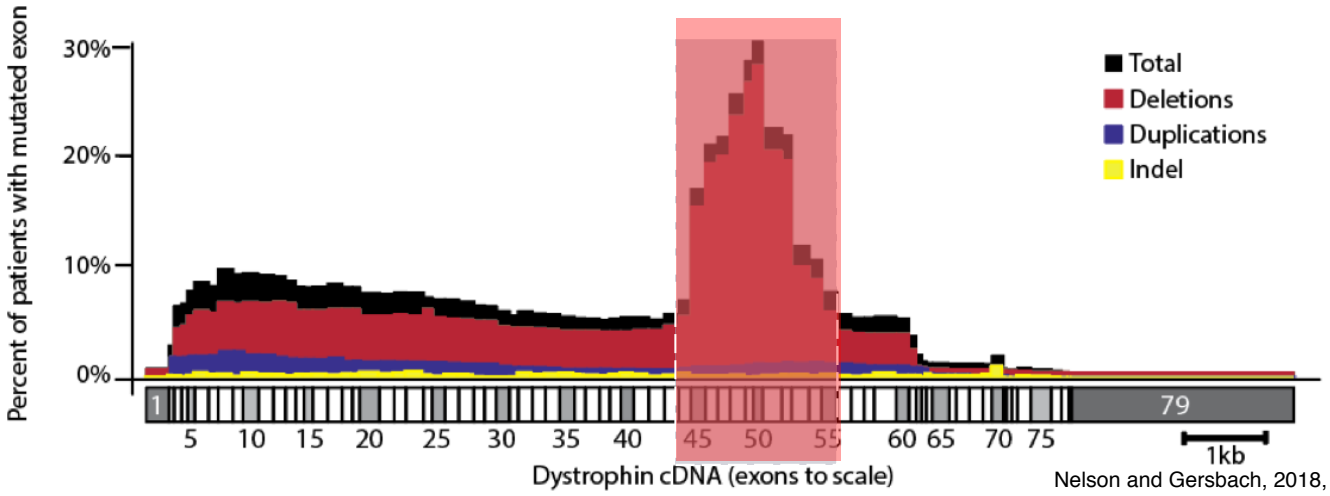
Duchenne muscular dystrophy (DMD)

- Most prevalent lethal heritable childhood disease
  - ~ 1:5000 newborn males
- Characterized with progressive muscle weakness leading to mortality in patients' mid-20s
  - Due to lack of functional dystrophin protein
- Mutations in the X-linked dystrophin gene
  - 79 exons cover 2.2 million bases
  - Most mutations are deletions that disrupt reading frame
  - Exons 45-55 mutational hotspot
- Need for corrective therapeutic options



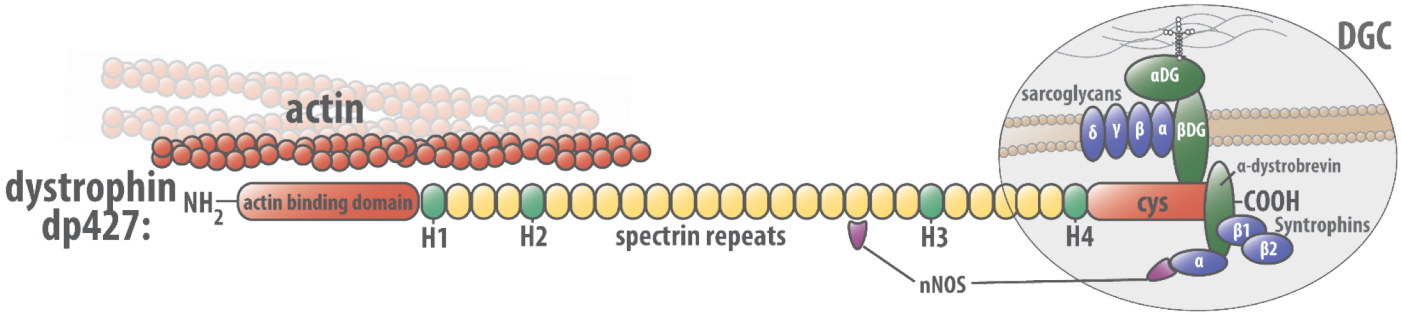
https://globalstemcells.com/treatment/muscular-dystrophy/

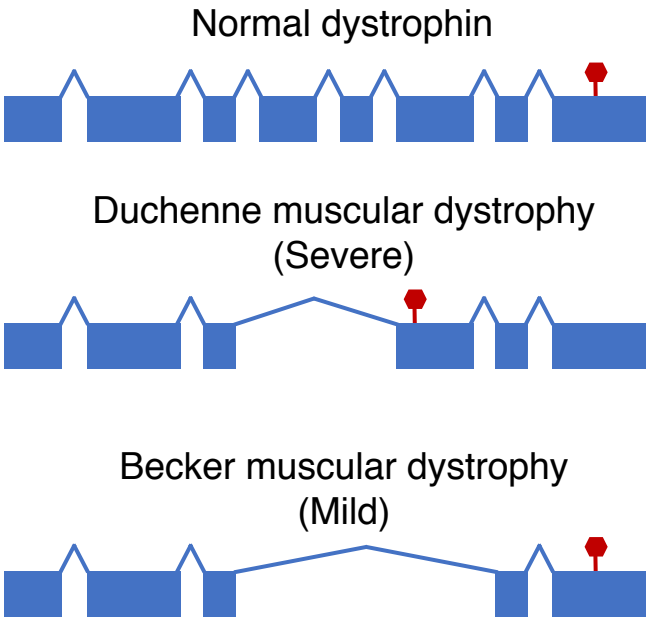


Nelson and Gersbach, 2018, *Muscle Gene Therapy*

DMD gene therapy strategies

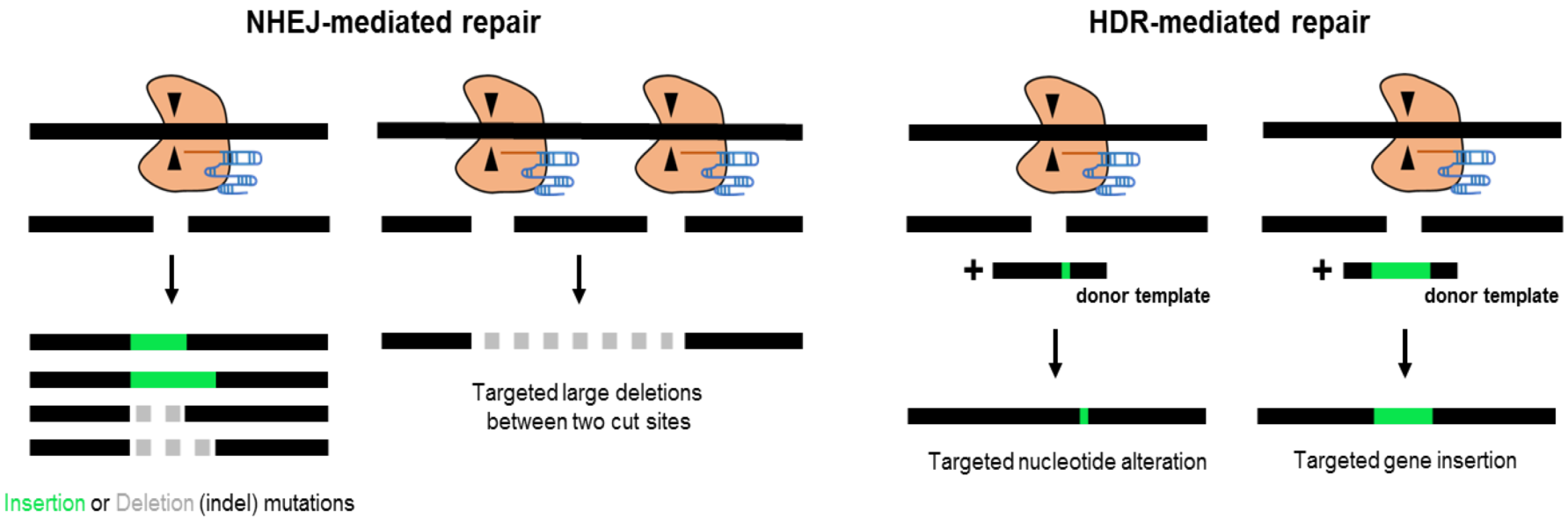
Nelson and Gersbach, 2018, *Muscle Gene Therapy*





- Delivery of mini/micro-dystrophin genes
- Restore reading frame
  - Enlarge DMD deletion for nearest in-frame BMD counterpart
    - Oligonucleotide-mediated exon-skipping strategies
    - Genome editing for gene deletions
- **Restore full-length dystrophin expression**
  - **Genome editing strategies for targetable gene insertion**

Genome editing: DNA double-strand break repair pathways



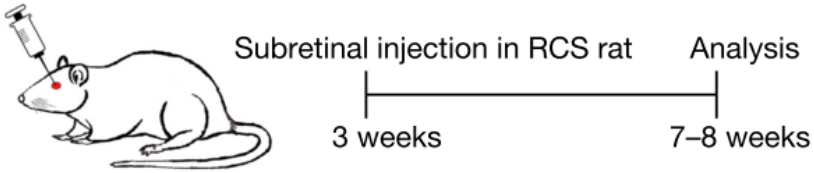
Site-specific transgene integration

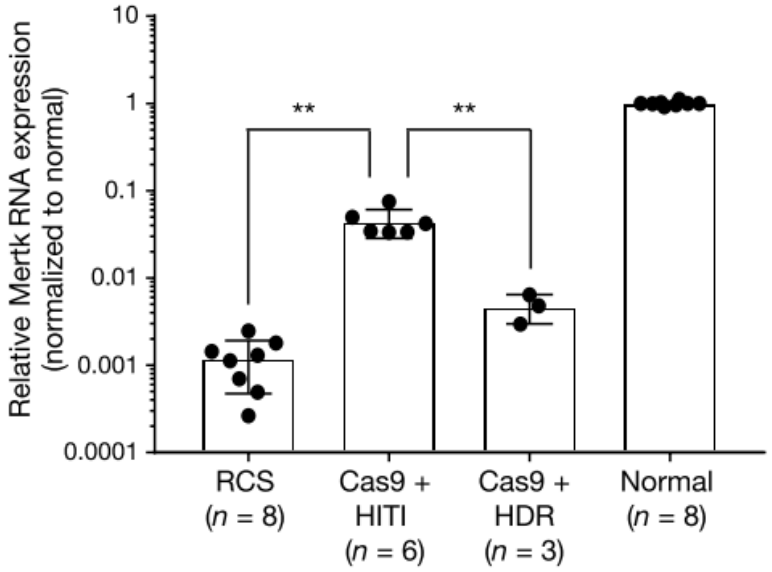
- Typically achieved by HDR pathway
  - Inefficient
  - Not readily accessible to non-dividing cells
- Utilize NHEJ pathway for gene knock-ins?
  - Generally more efficient than HDR in mammalian species
  - Active in proliferating and post-mitotic cells
  - NHEJ-based homology-independent strategy demonstrated in vivo

Genome editing: DNA double-strand break repair pathways

HITI

Homology-independent targeted integration





Suzuki et al, 2016, *Nature*

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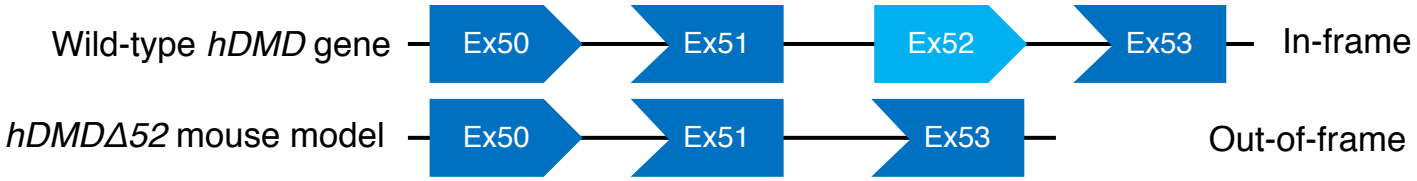
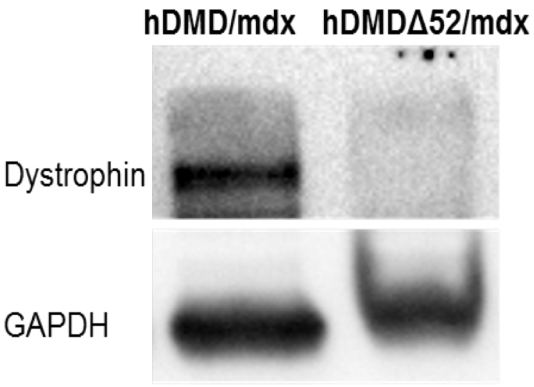
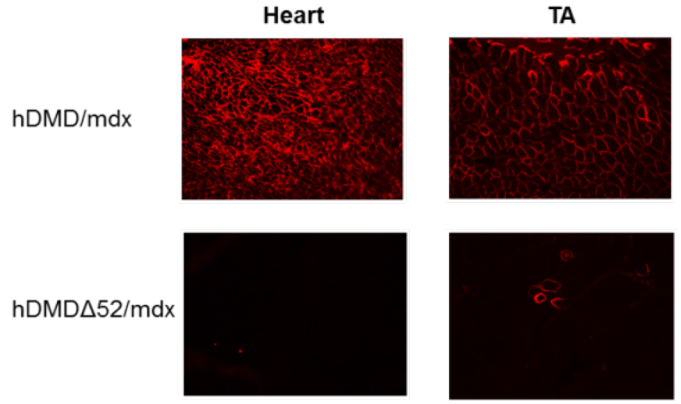
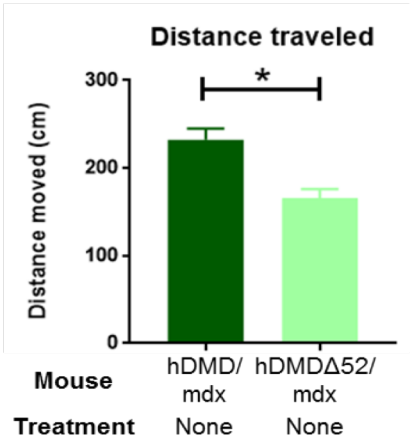
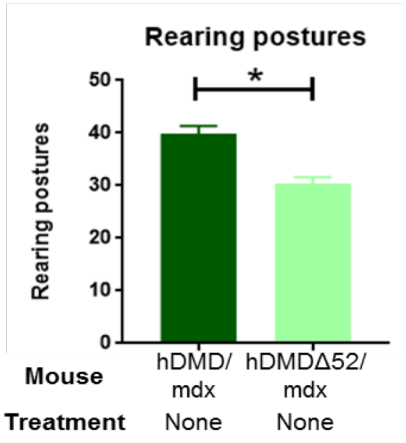
Humanized DMD mouse model: hDMDΔ52/mdx mice

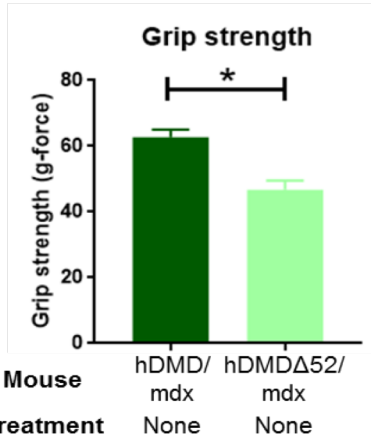






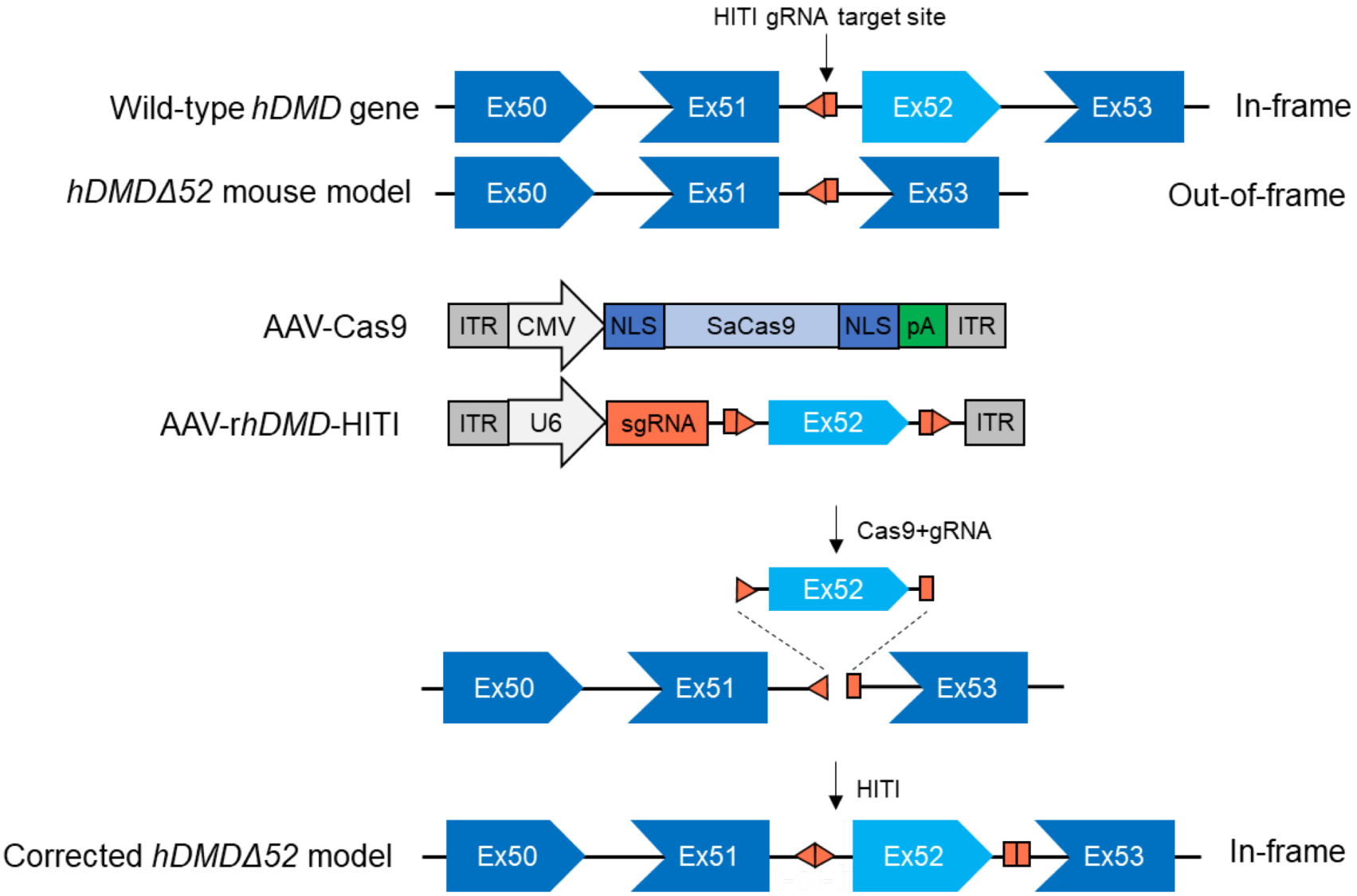




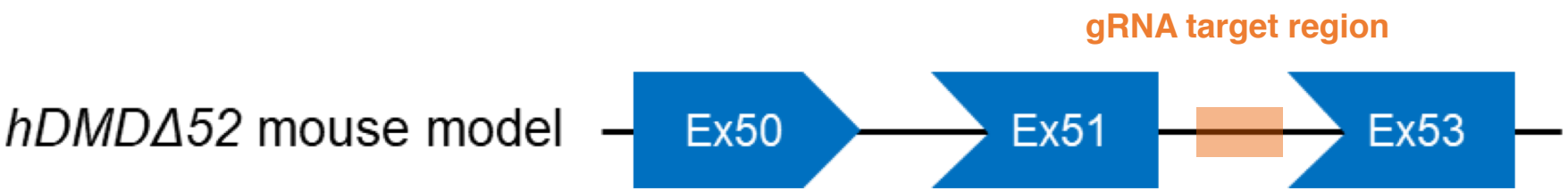


Robinson-Hamm et al., unpublished

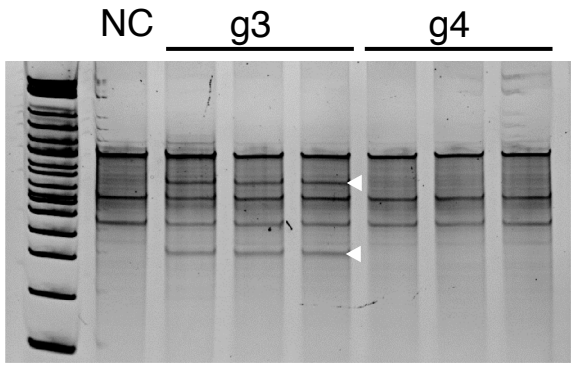
HITI-mediated strategy to correct humanized DMD mouse model

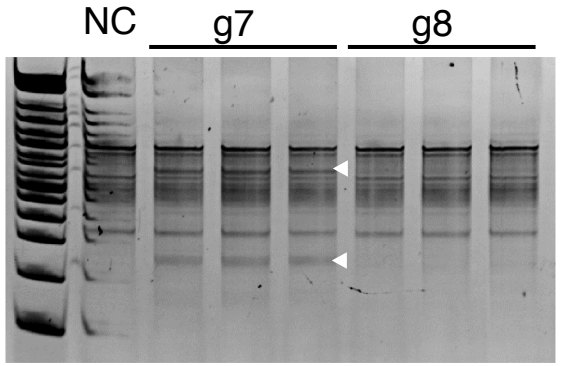


Indel editing efficiencies of SaCas9 gRNAs targeting hDMD Intron 51



1. Designed 10 gRNAs targeting Intron 51
2. Evaluated indel efficiency in transfected HEK293Ts



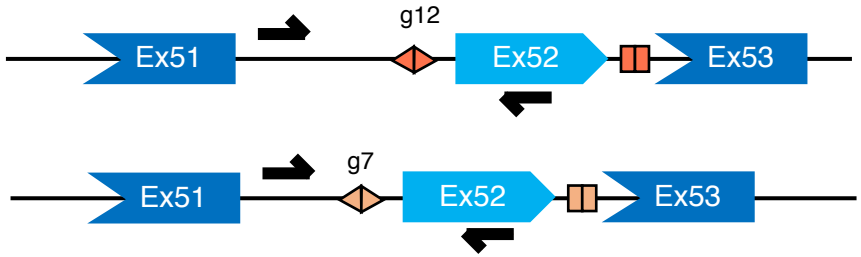


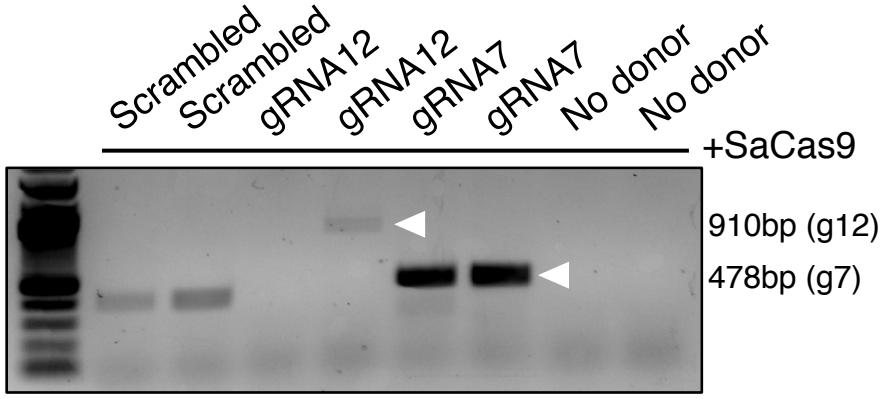
3. Additional testing of top gRNAs
   - Optimization of gRNA target spacer length (15-25 nucleotides)
   - Tested in patient myoblasts
   - Top hits:
     gRNA7
     gRNA12

HITI-based targeted integration in hDMDΔ52/mdx primary myoblasts

- Isolated primary myoblasts from hDMDΔ52/mdx mice
- Electroporated AAV plasmids (Cas9 + gRNA/donor)
- HITI-mediated targeted integration detected by PCR of genomic DNA

Sample	Amplicon size
g12-Ex52 donor integration	*910 bp*
g7-Ex52 donor integration	*478 bp*





HITI-based Ex52 insertion for correction of hDMDΔ52 mice

GOALS:

1. Confirm in vivo editing
2. Determine best gRNA/donor (g7 vs g12)
3. Determine best ratio of AAV-Cas9 to AAV-donor (1:1 vs 1:5)

hDMDΔ52/mdx mice
6-8 week old males
AAV9

Day 0
Local TA injection

PBS

AAV-Cas9 + AAV-gRNA7-Donor
1:1 and 1:5

AAV-Cas9 + AAV-gRNA12-Donor
1:1 and 1:5

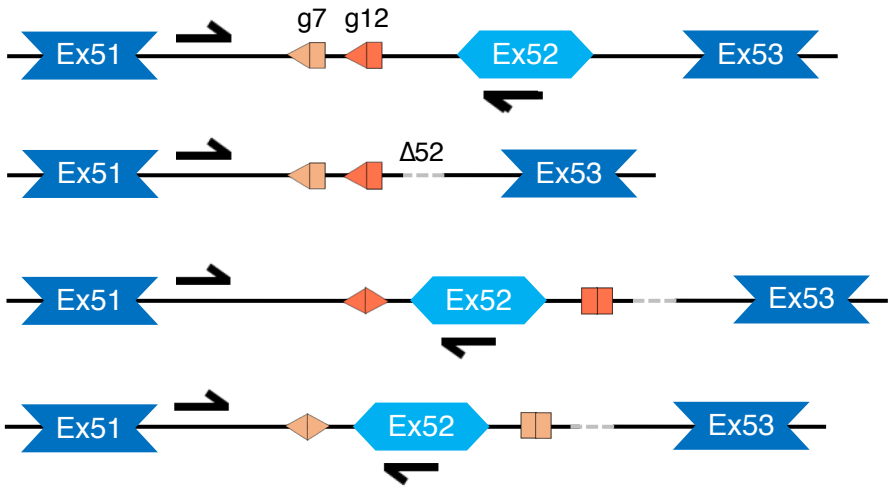
4 weeks post injection
Terminate

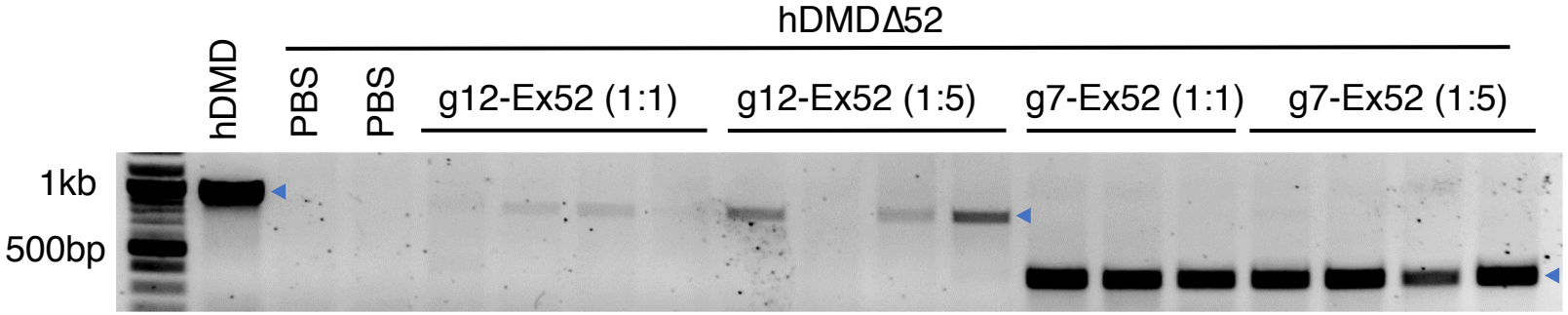
1:1 = 9.6e11 total vg/mouse
1:5 = 1.1e12 total vg/mouse

Targeted Ex52 insertion in hDMDΔ52/mdx mice

Genomic DNA

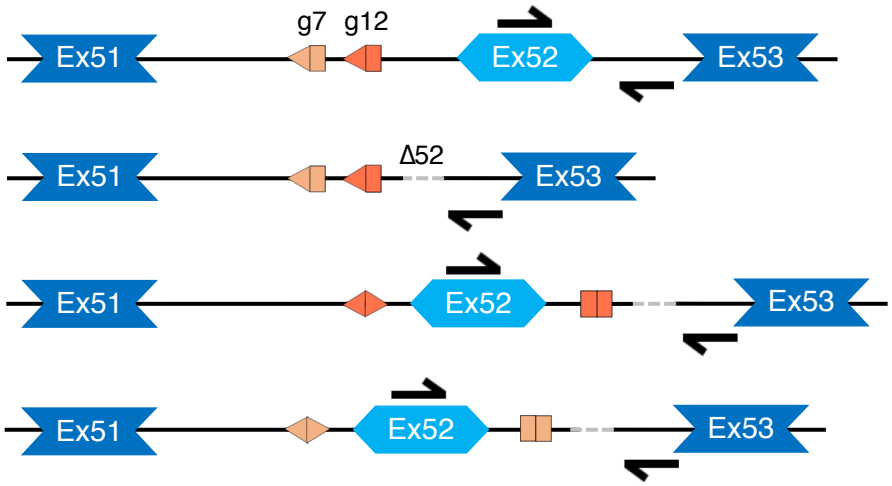
Sample	Amplicon size
Wild-type hDMD mouse model	*978 bp*
hDMDΔ52 mouse model	*No band*
g12-Ex52 donor integration	*896 bp*
g7-Ex52 donor integration	*465 bp*



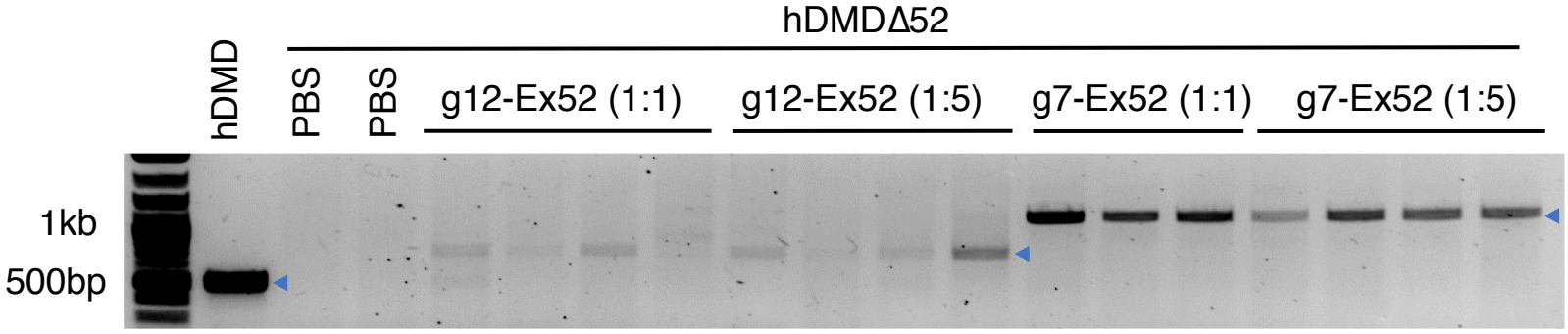


Targeted Ex52 insertion in hDMDΔ52/mdx mice

Genomic DNA



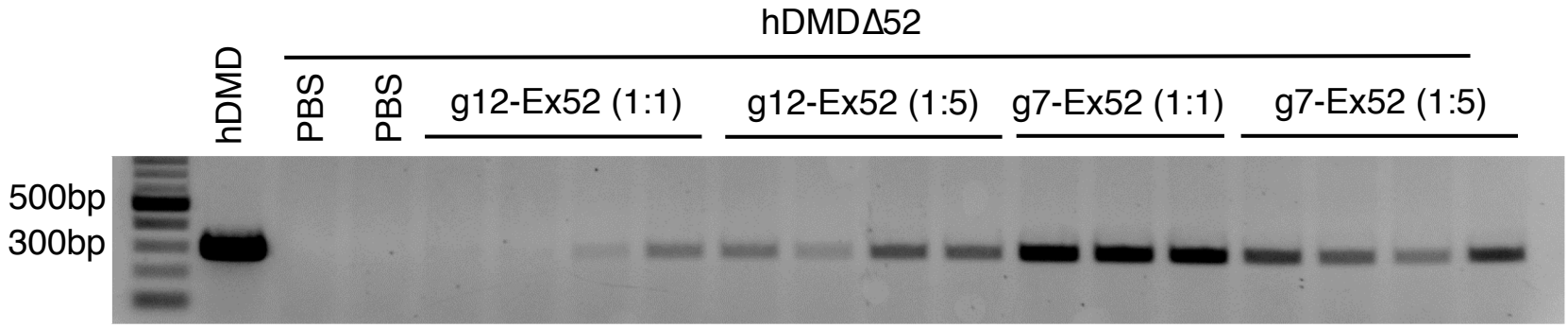
Amplicon size	Sample
521 bp	*Wild-type hDMD* mouse model
No band	*hDMDΔ52* mouse model
789 bp	*g12-Ex52 donor integration*
1222 bp	*g7-Ex52 donor integration*



Ex52 genomic integration spliced into dystrophin mRNA

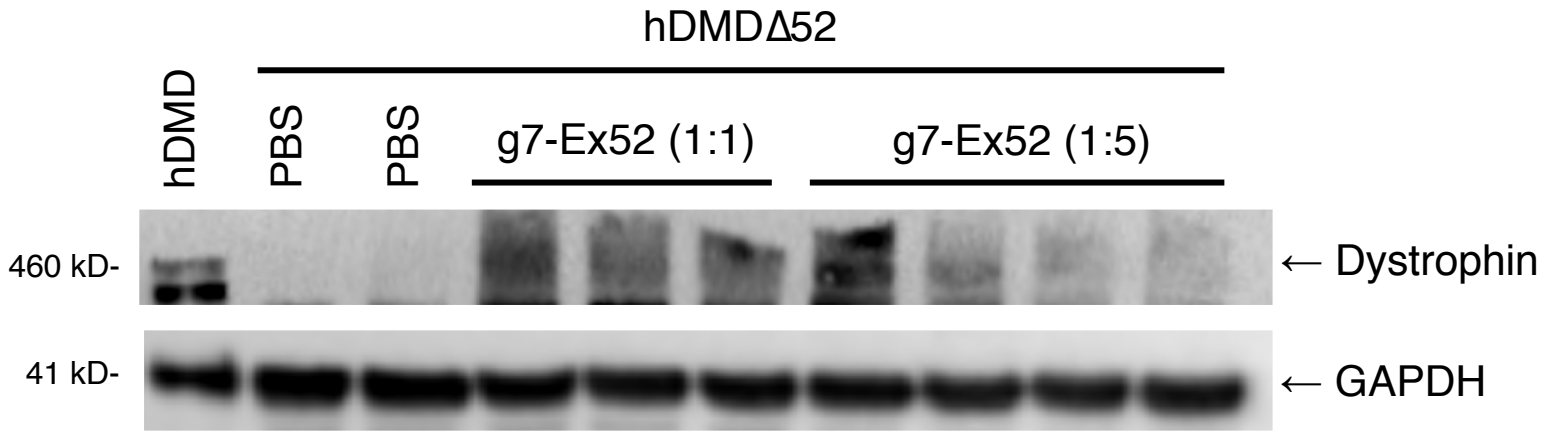
mRNA → cDNA

Expected size:
300bp



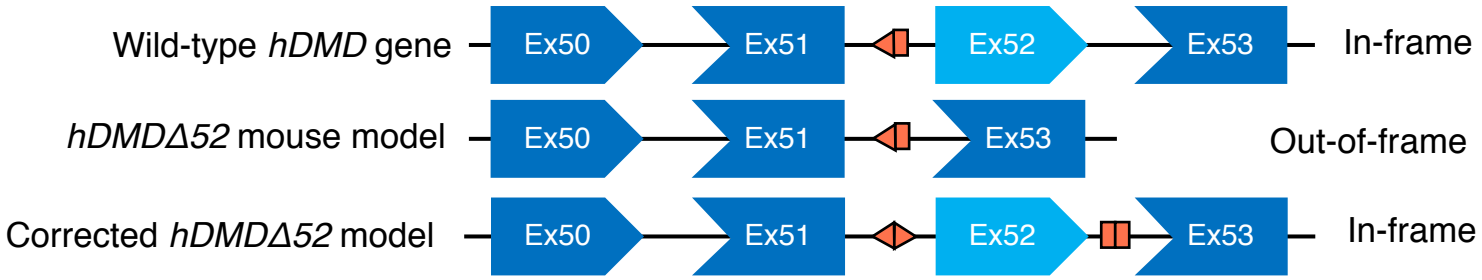
Dystrophin protein restoration in edited hDMDΔ52 mice

Protein



25ug total protein from TA muscles
**hDMD protein = 3.125 ug (12.5% of other samples)

Summary and future directions



1. AAV delivery of SaCas9 and gRNA-Ex52 donor can achieve HITI-mediated hDMD exon 52 targeted genomic integration in hDMDΔ52/mdx mice
   - Deep sequencing to quantify editing efficiencies between treatment groups
   - Quantify intended vs unintended edits

2. Targeted Ex52 insertion results in integration of exon 52 in hDMD mRNA
   - Continuing to evaluate isoform profiles between treatment groups

3. Targeted Ex52 insertion results in dystrophin protein restoration
   - Continuing to evaluate protein restoration via western blot and histological analysis of tissue samples

Future directions

- **Characterize functional improvement in edited *hDMDΔ52/mdx* mice following system injection**
  - **Short term and long term studies**

Acknowledgements

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