Assessing the Therapeutic Potential of CRISPR/Cas9-Mediated Gene Modulation in Merosin-Deficient Congenital Muscular Dystrophy Type 1A

By

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A thesis submitted in conformity with the requirements for the degree of Master of Science
Department of Molecular Genetics
University of Toronto

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Assessing the Therapeutic Potential of CRISPR/Cas9-Mediated Gene Modulation in Lama2-Deficient Congenital Muscular Dystrophy Type 1A

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ABSTRACT:
Merosin-deficient congenital muscular dystrophy type 1A is an autosomal recessive disease caused by mutations in the LAMA2 gene, which codes for the laminin α2 chain of the laminin-211 cell adhesion molecule. MDC1A is the most common type of congenital muscular dystrophy. MDC1A patients exhibit severe hypotonia, progressive muscle weakness and wasting, delayed developmental milestones and often die prematurely. Lama1 is a potential disease modifier gene for MDC1A that could be modulated to treat or prevent the disease phenotype. The Lama1 gene encodes the laminin α1 chain protein, which is structurally similar to laminin α2 chain. We hypothesize that using the S.aureus CRISPR/Cas9 system, we will be able to upregulate expression of Lama1 and compensate for the deficiency of laminin α2 chain in mice to re-establish the integrity of muscle fibers. We have evaluated the effectiveness of local intramuscular and systemic delivery of CRISPR/Cas9 components to modulate Lama1 expression in vivo to decrease disease severity in laminin alpha 2 deficient dy^2/J/dy^2/J mice. Successful results from this project will provide evidence to develop CRISPR/Cas9-mediated gene modulation as a therapeutic option for patients with MDC1A.
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LIST OF ABBREVIATIONS:

AAV9 – Adeno-Associated Virus, Serotype 9
BSA – Bovine Serum Albumin
Cas9 – CRISPR-Associated Protein 9
CMV – Cytomegalovirus
CRISPR – Clustered Regularly Interspaced Short Palindromic Repeat
CRISPRa – CRISPR Activation
CRISPRi – CRISPR Inactivation
DG - Dystroglycan
DMC/DMA – Dynamic Muscle Control/Analysis
DMD – Duchenne Muscular Dystrophy
DMEM – Dulbecco's Modified Eagle Medium
EDL – Extensor Digitorum Longus
FBS – Fetal Bovine Serum
GWA – Genome-wide association
HDR – Homology-Directed Repair
IGF-1 – Insulin-like Growth Factor 1
ITR – Inverted Terminal Repeat
Lama1 – Laminin-α1 chain
Lama2 – Laminin-α2 chain
MDC1A – Merosin-Deficient Congenital Muscular Dystrophy
NFH – Neurofilament H
NGS – Normal Goat Serum
NHEJ – Non-Homologous End-Joining (NHEJ)
OCT – Optimal Cutting Temperature
PAM – Protospacer-Adjacent Motif (PAM)
PBS – Phosphate Buffer Saline
SaCas9 – Staphylococcus aureus Cas9
SG - Sarcoglycan
sgRNA – Single Guide RNA (sgRNA)
SNP – Single Nucleotide Polymorphism
SpCas9 – Streptococcus pyogenes Cas9
SV40 – Simian Virus 40
TAR/TAL – Tibialis Anterior Right/Tibialis Anterior Left
TBST - Tris-Buffered Saline with Tween
TGF-β – Transforming Growth Factor Beta
TSS – Transcriptional Start Site
VGC – Viral Genome Copies
1 INTRODUCTION:

1.1 Introduction to Disease Modifier Genes:

In humans, a single mutation can cause devastating diseases that significantly impact an individual’s quality of life and can even be fatal. Genetic diseases are often classified as monogenic Mendelian diseases when a single mutation is thought to be the sole cause of the phenotype. However, both genetic and environmental factors can influence the outcome of a particular mutation. These genetic factors are known as modifier genes. A single gene mutation may be the determinant of whether an individual is affected or unaffected, yet the disease presentations, such as onset, severity and progression rate can be widely variable due to the effect of disease modifiers.

1.2 Identifying Modifier Genes:

The identification of modifier genes for specific diseases is commonly done through either candidate gene or genome-wide association (GWA) studies. Candidate gene studies begin with the formulation of a hypothesis about the influence of a gene on the disease phenotype. This is often based on prior knowledge of the gene’s encoded protein structure or physiological role. For example, Pinto et al. conducted a candidate-based study to identify genetic modifiers in Huntington’s disease, a fatal neurodegenerative disease, which is caused by expansion of CAG repeats within the HTT gene. Mutations in mismatch repair genes, Mlh1 and Mlh3, were hypothesized to contribute to increased CAG repeat size. When Huntington’s disease mice were crossed with knockouts of these mismatch repair genes, the progeny were found to have increases in CAG repeat instability, confirming the hypothesis that Mlh1 and Mlh3 are indeed genetic modifiers for Huntington’s disease. Similar studies can provide an in-depth understanding of how significantly, if at all, the candidate gene influences a disease phenotype.
However, these studies are often limited to genes that are well understood and can overlook genes that have subtler or unexpected phenotypic impacts.

Conversely, GWA analysis can be used to find every genetic variation between genomes of patients with a specific disease. By comparing sequencing data, difference in coding and non-coding regions can be used to identify genetic factors contributing to disease phenotype severity. For example, GWA analysis has helped to determine genetic factors causing phenotypic variability in Duchenne muscular dystrophy (DMD) patients with pathogenic DMD gene mutations. Pegoraro et al. compared the presence of single nucleotide polymorphisms (SNP) at loci of interest between severe and mild DMD patients. This study found that a polymorphism in the promoter of the SPP1 gene was associated with a more severe phenotype, thus providing evidence for its role as a genetic modifier in DMD. GWA analysis can help identify novel modifier gene candidates, however, it is important that each candidate modifier gene is further characterized, e.g. in animal models, to show its relevance to the pathophysiology of the disease. Once identified, disease modifiers can be potential therapeutic targets for treatment of various diseases, such as Huntington’s disease or many different types of muscular dystrophies.

1.3 Understanding Muscular Dystrophies:

Muscular dystrophies are a family of inherited genetic disorders characterized by progressive muscle weakness and wasting. Single gene defects can result in the inability of patients’ muscle fibers to sustain mechanical forces when conducting any kind of movement. This can result in rupturing of the cell membrane, which contributes to myofiber death. Muscle tissue attempts to regenerate the damaged tissue by mobilizing satellite cells, which are muscle precursor cells. They begin to differentiate and fuse together to form new fibers that replace the damaged fibers, which are subsequently phagocytized. This repair mechanism is typically unable to adequately compensate for damaged tissue. In the absence of muscle repair, adipose
tissue and fibrotic tissue accumulate in the damaged muscle while immune cells scavenge necrotic tissue.

1.4 Merosin-Deficient Congenital Muscular Dystrophy Type 1A:

One specific muscular dystrophy is Merosin-Deficient Congenital Muscular Dystrophy Type 1A (MDC1A). This is a severe congenital muscular dystrophy caused by deficiency of laminin-α2 chain protein due to a mutation in the LAMA2 gene. With a prevalence of 1:150000, MDC1A is one of the most common types of congenital muscular dystrophy. MDC1A patients exhibit severe hypotonia, progressive muscle weakness and wasting, delayed developmental milestones, respiratory distress, and often die prematurely. Deficiency of laminin-α2 chain (Lama2) also affects tissues other than skeletal muscle, such as the brain and peripheral nervous system. This leads to abnormal white matter organization and myelination defects in the peripheral nervous system, which may contribute to disease presentation through reduced signaling to skeletal muscle.

1.5 Laminin Alpha 2 Chain Deficiency Causes MDC1A:

To date there are over 350 described missense, nonsense, splice site, and deletion mutations in the LAMA2 gene that have caused MDC1A. These mutations often alter the amino acid sequence of laminin-α2 chain protein, which is crucial for connection of muscle and Schwann cell membranes to the basement membrane and extracellular matrix (Figure 1A). Normally, laminin-α2 polymerizes with a heterodimer containing laminin-β1 and laminin-γ1 within the endoplasmic reticulum to form Laminin-211, also known as merosin, which is then transported to the cell membrane and secreted. Once outside the sarcolemmal membrane of skeletal muscle, Laminin-211 heterotrimers associate with each other at the N-terminal LN
domains and form laminin networks \(^{16}\). These networks adhere to the cell surface through the binding of the C-terminal LG domains of Laminin-211 to sulfated glycolipids, integrins, and dystroglycan \(^{17}\).

**Figure 1** – Laminin-211 is a heterotrimeric complex necessary for adhesion of the sarcolemmal membrane and the extracellular matrix. A. Laminin-211 is incorporated into the dystrophin-associated complex. (DG: dystroglycan, SG: sarcoglycan) (http://www.stem-cell-
B. Domains and structure of Laminin-211. C. Laminin-211 polymers and interacting proteins at the sarcolemmal surface. Adapted from 18

Additional linkages are made to the cell membrane through agrin, which binds the coil-coiled domain of laminin-211 and connects to cell surface proteins (Figure 1B). The coil-coiled domain of Laminin-211 also binds nidogen proteins that then bind collagen VI, thus establishing a connection to the basement membrane (Figure 1C) 17. This intricate network of structural proteins stabilize the sarcolemma and mediate cell signaling between myofibers and the extracellular matrix.

One of major cell surface receptors in muscle cells is dystroglycan, which plays a role in preserving the stability of the sarcolemma in response to mechanical stress. Dystroglycan is composed of two subunits, α-dystroglycan, which binds Laminin-211 at the LG domain and β-dystroglycan, which binds an intracellular structural protein, dystrophin 19,20. Dystrophin connects the dystroglycan complex to the cytoskeleton within cells, making the dystroglycan complex a transmembrane linker that connects extracellular matrix to the intracellular components of myofibers 21. Additionally, the dystroglycan complex has a sarcoglycan-sarcospan complex that stabilizes α-dystroglycan within the sarcolemma 22. These dystroglycan mediated linkages serve to both protect the integrity of the sarcolemma and contribute to force production by muscle 23.

Like dystroglycan, integrin α7β1 is another cell surface receptor that links Laminin-211 to the cytoskeleton and contributes to force production by muscles 23,24. The LG domain of Laminin-211 binds integrin α7β1 and facilitates another type of connection between the basement membrane and the myofiber 25. These integrin α7β1 receptors are thought to help facilitate the regeneration pathway in muscles 26. Integrin α7β1 is downregulated in MDC1A
patients and *Lama2* deficient animal models, which likely contributes to the severity of the disease\textsuperscript{27}. Integrin $\alpha7\beta1$ may play a role in the molecular pathway that directs satellite cells to transition into myofibers\textsuperscript{26,28}, therefore, absence of this molecular complex could contribute to the disease severity as the pathophysiology of MDC1A involves impaired muscle regeneration.

### 1.6 Failed regeneration in MDC1A:

Typically muscle regenerates in response to injury by mobilizing satellite cells, which reside between the sarcolemma and basement membrane of muscles. In MDC1A, the dystrophic muscle is made up of myofibers that are susceptible to mechanical stress. As these myofibers rapidly undergo cell death over time, satellite cells are constantly being mobilized to regenerate the lost muscle tissue. However, this regeneration requirement could potentially go unfulfilled if the pool of available satellite cells is depleted from continuous cycles of degeneration and regeneration\textsuperscript{7}. Another possible reason for inadequate regeneration could be caused by impairment of the regeneration pathway. There has been evidence to suggest that laminin-$\alpha2$ chain deficiency results in delayed and impaired muscle regeneration\textsuperscript{29}. With decreased expression of integrin $\alpha7\beta1$ complexes, as well as the loss of its Laminin-211 connection to the extracellular matrix, satellite cells may not be effectively activated\textsuperscript{26}. The reduced regenerative capacity results in muscle having a net degeneration in MDC1A, which leads to a gradual decline in muscle mass and replacement of muscle tissue by fibrosis and fat observed in the disease phenotype.

### 1.7 Increased muscle degeneration in MDC1A:

The degeneration of muscle tissue in MDC1A is facilitated by abnormally high levels of apoptosis, proteasome activity, and autophagy. Relative to wildtype samples, *Lama2* deficiency
both in patients and mouse models results in elevated numbers of myofibers positive for apoptotic markers. Several proapoptotic pathways have been observed to be dysregulated in MDC1A. One such example is that apoptotic protein Bax has been determined to contribute to abnormal cell death in MDC1A. Another example is that the proapoptotic GAPDH-Siah1-CBP/p300-p53 signaling pathway is overactive in MDC1A. Additionally, proteasome and autophagy pathways are normally inhibited by phosphorylated AKT. In both Lama2 deficient humans and mice, proteasome and autophagy pathways have increased activity due to reduced AKT phosphorylation.

1.8 Accumulation of Fibrotic Tissue in Dystrophic Muscle:

In response to damage sustained by dystrophic muscle, inflammatory cells infiltrate the injured tissue and elicit an inflammatory response. Inflammatory macrophages then express profibrotic cytokines that facilitate the deposition of fibrotic tissue. Based on analysis of gene expression levels in Lama2 deficient human and mouse samples, it has been shown that there is an increase in expression of many extracellular matrix proteins, including biglycan, connective tissue growth factor, fibrin, fibronectin, galectin-1, galectin-3, lumican, matrix metalloproteinases-2 and -9, periostin, tenascin-C, thrombospondin-4, tissue inhibitor of metalloproteinases-1, and collagens I, III, and VI. Many of these proteins are associated with the connective tissue that accumulates within dystrophic muscles. It is currently believed that fibrosis is a non-reversible outcome of muscular dystrophy.

1.9 Dysregulation of Laminin Chains in MDC1A:

Due to mutations in the LAMA2 gene, MDC1A patients have a deficiency or absence of functional laminin-α2 chain protein. In the absence of functional laminin-α2 chain, as with
integrin α7β1 downregulation, the expression levels of various other structural proteins are
dysregulated. Other laminin-α chains, including laminin-α4 and -α5, are upregulated in attempt
to compensate for the missing cytoskeletal-extracellular matrix linkage \(^{14,43}\). This compensation
fails to adequately make up for the absence of laminin-α2 chain because other laminin-α chains
are unable to efficiently polymerize and bind α-dystroglycan \(^{44}\).

**1.10 MDC1A Animal Models to Study the Disease:**

Animal models of MDC1A with various *Lama2* mutations are available and readily used
to study MDC1A and potential therapies. There are several mouse models for laminin-a2 chain
deficiency: *dy/dy*, *dy\(^{2j}\)/*dy\(^{2j}\)*, *dy\(^{6j}/dy\(^{6j}\)*, *dy\(^{7j}/dy\(^{7j}\)*, *dy\(^{W}/dy\(^{W}\)*, and *dy\(^{3K}/dy\(^{3K}\)* mice (www.jax.org)
These animal models are summarized in table 1. Specifically, the *dy\(^{2j}/dy\(^{2j}\)* mouse model of
laminin-α2 chain deficiency is the model that our group has chosen to work with, as these mice
survive till late in life while still showing signs of paralysis and muscle weakness \(^{45}\). This is
essential to testing the longitudinal efficacy of our treatments.

The *dy\(^{2j}/dy\(^{2j}\)* mutants of C57BL/6J mouse strain have a point mutation at a splice donor
site (c.417+1g>a) that results in abnormal splicing and exclusion of exon 2 from mRNA. This
results in a partial loss of the LN domain of laminin-α2 chain protein \(^{45}\). This MDC1A mouse
model has a milder phenotype and longer lifespan relative to other *Lama2* mutant mice \(^{45}\). These
mice still begin to show signs of muscle weakness and hind limb paralysis at three weeks of age
and get progressively worse. Additionally, these mice have variations in muscle fiber size,
centrally located nuclei, muscle fiber necrosis, infiltration of connective tissue in the muscle,
and demyelination \(^{45}\).

**Table 1** – Summary of Lama2-deficient animal models. Adapted from \(^{46}\)
### 1.11 Application of Lama2 Mutant Animal Models to MDC1A

These Lama2 mutant mouse models have played a central role in determining potential therapeutic strategies to treat MDC1A. Many strategies have attempted to manipulate signaling pathways to increase regeneration or inhibit apoptosis, proteasome activity, autophagy, or inflammation and fibrosis.

Additionally, zebrafish models of Lama2 deficiency have provided insight into the cellular pathology of MDC1A and are powerful systems for identification of novel therapies. By genetically inactivating both dystroglycan and integrin adhesion systems in candyfloss zebrafish, Sztal et al. showed the importance of compensation by other laminin chains in preventing catastrophic failure of muscle attachment. Furthermore, Smith et al. discovered candyfloss zebrafish had abnormalities in spontaneous coiling, which are some of the earliest

<table>
<thead>
<tr>
<th>Animal model</th>
<th>Mutation</th>
<th>LM</th>
<th>Muscular dystrophy</th>
<th>Survival</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>$dy/dy$ mouse</td>
<td>Unknown</td>
<td>Reduced expression of normal laminin-α2</td>
<td>Moderate</td>
<td>Less than 6 months</td>
<td>Xu, Christmas, et al. (1994), Sunada et al. (1994)</td>
</tr>
<tr>
<td>$dy^{60}/dy^{60}$ mouse</td>
<td>?</td>
<td>Moderate</td>
<td>?</td>
<td>Less than 6 months</td>
<td>—</td>
</tr>
<tr>
<td>$dy^{7J}/dy^{7J}$ mouse</td>
<td>Missense mutation in LN domain</td>
<td>Normal levels</td>
<td>Mild</td>
<td>Normal</td>
<td>Patton et al. (2008)</td>
</tr>
<tr>
<td>$dy^{W}/dy^{W}$ mouse</td>
<td>Knock-out</td>
<td>Severely reduced expression of truncated laminin-α2 missing LN domain</td>
<td>Severe</td>
<td>10–15 weeks</td>
<td>Kuang et al. (1998)</td>
</tr>
<tr>
<td>$dy^{3K}/dy^{3K}$ mutant</td>
<td>Knock-out</td>
<td>Complete deficiency</td>
<td>Very severe</td>
<td>3 weeks</td>
<td>Miyagoe et al. (1997)</td>
</tr>
<tr>
<td>candyfloss zebrafish</td>
<td>Nonsense mutation in LG4</td>
<td>Severe reduction of $lama2$ mRNA</td>
<td>Severe</td>
<td>14–16 days postfertilization</td>
<td>Hall et al. (2007)</td>
</tr>
<tr>
<td>$lama^{2G01/G01}$ zebrafish</td>
<td>Splice site mutation in LN domain</td>
<td>Severely reduced</td>
<td>Severe</td>
<td>8–15 days postfertilization</td>
<td>Gupta et al. (2012)</td>
</tr>
</tbody>
</table>

LN, laminin N-terminal domain; ?, indicates that no information is available.

*The phenotype of $dy^{60}/dy^{60}$ mice is described at: [jaxmice.jax.org/strain/005889.html](http://jaxmice.jax.org/strain/005889.html).*
motor movements in the zebrafish. This abnormal coiling is fully penetrant and can be used in large-scale drug testing 48.

1.12 Increasing Muscle Regeneration in *Lama2* Mutant Mice:

In a study by Kumar et al., Insulin-like growth factor 1 (IGF-1) was transgenically expressed in *dy<sup>W</sup>/dy<sup>W</sup>* under a muscle specific promoter to stimulate muscle regeneration 49. IGF-1 triggers muscle regeneration by inducing proliferation and differentiation of satellite cells growth of muscle 50. This overexpression of IGF-1 increased the expression of the regeneration markers MyoD, myogenin and embryonic myosin and somewhat improved the pathophysiology of the *dy<sup>W</sup>/dy<sup>W</sup>* mice. There was an increase in body weight, ability to stand on hind limbs, and lifespan, yet there was no visible decrease in fibrosis 49. This treatment shows some potential as a therapy for MDC1A, however, the rate of muscle degeneration outweighs the protective effects of increased regeneration.

1.13 Preventing Muscle Degeneration in *Lama2* Mutant Mice:

To address muscle degeneration, many different groups have worked towards manipulating apoptosis, proteasome activity, and autophagy signaling pathways. Girgenrath et al. published a study where they inactivated expression of a proapoptotic protein, Bax, and increased expression of an antiapoptotic protein, Bcl-2. In this study, both treatments resulted in an increase in lifespan, decrease in contractures, improvement in muscle histology 32. Additionally, it was later found in a study by Jeudy et al. that Bcl-2 overexpression also results in decreased infiltration of the muscle by inflammatory cells 40. Although these antiapoptotic treatments showed some promising therapeutic potential for diminishing muscle pathology, the reduced regenerative capacity of muscle was still present 32. With reduced regenerative activity, these mice would likely continue worsen, albeit at a slower pace relative to untreated *Lama2*
mutant. The therapeutic potential of these antiapoptotic strategies have been successful enough, however, to merit the clinical trials of a pharmaceutical known as omigapil, which inhibits the GAPDH-Siah1-CBP/p300 signaling pathway.

As mentioned above, proteasome degradation and autophagy contribute to muscle degradation and are upregulated in MDC1A. Both the ubiquitin–proteasome system and autophagy-lysosome pathway are involved in protein degradation in skeletal muscle. Carmignac et al. found that components of the ubiquitin–proteasome system were upregulated in $dy^{3k}/dy^{3k}$ and subsequently inhibited the ubiquitin–proteasome system by treating mice with proteasome inhibitor MG-132. In a subsequent study, Carmignac et al. treated $dy^{3k}/dy^{3k}$ mice with 3-methyladenine, an autophagy inhibitor. Both studies showed that the treated mice had a phenotypic improvement and reduced muscle fibrosis, atrophy, apoptosis and increased muscle regeneration, muscle mass, and lifespan. A pharmaceutical, Bortezomib, is an FDA approved proteasome inhibitor currently used for treatment of multiple myeloma, has also been shown to partially improve laminin α2 chain deficient muscular dystrophy. These treatments had somewhat ameliorated the disease phenotype, however, proteasome activity and autophagy are used throughout the body and there may be complications associated with dysregulation of those systems.

### 1.14 Inhibition of Fibrosis:

Inflammation in dystrophic muscle results in the deposition of fibrotic tissue in skeletal muscle of muscular dystrophy patients and is thought of as an end stage for muscular dystrophies. Several pharmaceuticals have been tested for their effectiveness in preventing fibrosis. One drug, Halofuginone, blocks TGF-β-mediated collagen synthesis, one of the major components of fibrotic tissue, and was shown to decrease fibrosis in the dystrophic muscle of $dy^{2j}/dy^{2j}$ mice. Additionally, Losartan, an angiotensin II type I receptor blocker has been
shown inhibit the TGF-β pathway to have a similar effect on $dy^{2j}/dy^{2j}$ mice. Relative to other treatments, these strategies have only yielded a mild improvement in the Lama2 deficiency disease phenotype. They attempt to prevent an endpoint state of MDC1A, which is necessary, yet their need can be circumvented through early intervention with therapies that re-establish muscle fiber integrity.

1.15 Reintroducing Laminin α2 Chain Expression:

Many potential treatment strategies have been focused on managing the result of laminin α2 chain deficiency, however, there have also been other strategies focused on reintroducing normal Lama2 expression. Kuang et al. transgenically expressed human LAMA2 in $dy^W/dy^W$ and $dy^{2j}/dy^{2j}$ mice under a muscle specific promoter. The results showed a substantial improvement in muscle morphology, muscle integrity, lifespan and overall health of the mice. However, there was visible dysfunction in the hind limbs indicating the impact of having an untreated peripheral nervous system. Despite this peripheral neuropathy, this LAMA2 transgene treatment provided an excellent amelioration of the disease phenotype. However, this approach is not currently very promising as a clinical therapy for patients as the LAMA2 gene spans over 260 kb and the cDNA alone is approximately 9.5 kb in size. This large gene size makes it difficult to find safe and effective delivery methods, such as adeno-associated viral (AAV) vectors, that can transport the transgene to muscle fibers and Schwann cells of MDC1A patients.

1.16 Expression of Compensatory Molecules for Lama2 Deficiency:

In addition to expressing a LAMA2 transgene in Lama2 deficient mice, several other transgenes have been used to treat Lama2 deficient mice. Two of the most successful experiments involved the expression of a miniagrin transgene and expression of a Lama1 transgene. A miniagrin transgene, a shortened version of the agrin gene that can be packaged
into AAV vectors, was expressed in $dy^W/dy^W$ mice. The miniagrin protein works as a linker between the $\alpha$-dystroglycan molecule and laminin-411 molecules $^{59}$. Laminin $\alpha4$ chain is upregulated in Lama2 deficient mice, yet is unable to facilitate polymerization of laminin complex and binding to the sarcolemma via $\alpha$-dystroglycan, which is thought to be accomplished with the presence of miniagrin $^{44,59}$. This strategy significantly reduced muscle pathology and increased lifespan in $dy^W/dy^W$ mice $^{59}$.

One of the most successful experiments in ameliorating the Lama2 disease phenotype was conducted by Gawlik et al., as they showed that transgenic expression of laminin $\alpha1$ chain protein (Lama1) in $dy^{3k}/dy^{3k}$ mice had a highly beneficial effect on disease severity $^{60}$. Laminin $\alpha1$ chain is structurally the most similar laminin chain to laminin $\alpha2$, however, it is not expressed in adult skeletal muscle and, unlike other laminin $\alpha$ chains, is not upregulated in Lama2 deficiency. Instead, it is expressed in various tissues during embryogenesis and is limited to the brain and kidney after birth $^{61}$. The transgenic expression of laminin $\alpha1$ chain fully prevented the development of muscular dystrophy in several muscles, and partially in others. It reversed the appearance of histopathological features of the disease and greatly improved the overall health and lifespan of the mice $^{60}$. Additionally, transgenic expression of laminin $\alpha1$ was successful in mediating proper myelination of the peripheral nervous system and almost entirely preventing peripheral neuropathy associated with Lama2 deficiency $^{62}$. As laminin $\alpha1$ is structurally similar to laminin $\alpha2$, laminin-111 is capable of replacing laminin-211 and fulfilling its function by re-establishing the sarcolemma to extracellular matrix linkage. Further evidence of this was provided by Rooney et al. as their group systemically delivered Laminin-111 protein to $dy^W/dy^W$ mice and found that the treatment could prevent muscle pathology, improve muscle strength, and increase lifespan $^{63}$.

1.17 Adeno-associated Viral Vectors as Genetic Therapy Delivery Vehicles:
Expression of transgenic myomatrix components such as Lama2 and Lama1 have all yielded very promising results, both providing a near perfect amelioration of the disease phenotype. However, these treatments can only be applied to a clinical setting if they can be delivered safely and effectively to target tissues in MDC1A patients. Virus vectors, specifically AAV vectors, can fulfill this role, yet the size of these transgenes prevents them from being successfully packaged. Instead, Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)/Cas9 genome-editing constructs can be packaged into AAV vectors and facilitate a restoration of Lama2 deficiency.

Although the carrying capacity is approximately 35 kb and 9 kb for adenoviral and retroviral/lentiviral vectors, respectively, which far exceed the 4.9 kb carrying capacity of AAV, AAVs are a safer delivery vector. Adenoviral and retroviral/lentiviral vectors are constructed from attenuated forms of disease-causing agents that have the potential to cause illness. Conversely, AAV vectors do not contain viral genes and are relatively safe for use in gene therapy work. Additionally, retroviral/lentiviral vectors integrate their viral genome into the host genome, which can result in unnecessary long-term expression and potential disruption of endogenous genes. AAV vectors, however, do not integrate into the host genome and instead remain in an episomal state, which can remain in post-mitotic cells for nearly a decade.

In addition to being safe tools for gene therapy, AAV vectors can be altered to give rise to different serotypes with each having tropism for different tissues. The glycoprotein or lipoprotein antigens on the surface of AAV capsids can be manipulated to alter the tropism of a specific serotype. Specifically, our lab has chosen to work with AAV9, as it is a safe and effective viral vector that can be used to target skeletal muscle and the nervous system. Although the large Lama2 gene or cDNA cannot be packaged into the limited capacity of AAV vectors, the discovery of genome editing technology has provided the possibility of mutation correction therapies that can instead be delivered with these viral vehicles.
1.18 CRISPR-Cas9 Mediated Correction of Lama2:

The CRISPR/Cas9 genome editing technology consists of a CRISPR-associated protein 9 (Cas9) endonuclease that generates double stranded breaks in a specific genomic region, which is located adjacent to a protospacer-adjacent motif (PAM) and targeted by a complementary single guide RNA (sgRNA) (Figure 2). In the presence of a DNA repair template, a precise genomic modification can take place through homology-directed repair (HDR), whereas non-homologous end-joining (NHEJ) repairs Cas9-induced breaks in the absence of the exogenous template. Moreover, as mentioned above, two double stranded breaks can be simultaneously introduced to generate genomic deletions. The specific CRISPR/Cas9 species that we chose to conduct our experiments was the Staphylococcus aureus (SaCas9), which has a smaller size (3.2 kb) than the commonly used Cas9 from Streptococcus pyogenes (SpCas9). Although it has a larger PAM region (NNGRRT), the SaCas9 can be packaged into AAV vectors while maintaining similar genome-editing efficiencies.

One method to treat a genetic disease is to repair the causative mutation. Our lab has recently shown that the mutated Lama2 gene can be repaired in dy2/dy2 mice using the CRISPR/Cas9 system. This system was programmed to target the mutated splice donor site following exon 2 and create a double stranded break. Another double stranded break was introduced immediately before cryptic splice donor site downstream from exon 2. Through this method, the mutation was excised and the functional splice donor site was restored. Expression of laminin α2 chain was observed in treated dy2/dy2 mice. These mice also had significant improvement in muscle histopathology, strength and function without any signs of paralysis.
**Figure 2** – Dead Cas9 (dCas9) coupled with VP64 transactivator domains can be used to target and activate genes. A single guide RNA (sgRNA) contains a region complementary to region directly upstream of a protospacer-adjacent motif (PAM) and dictates the locus where the dCas9 binds. Adapted from (https://www.abmgood.com/marketing/knowledge_base/CRISPR-Cas9-dCas9-Gene-Regulation.php)

1.19 CRISPR/Cas9 Mediated Gene Modulation:

The type II CRISPR system can be harnessed to specifically target regulatory regions and modulate the expression of disease modifier genes. Two catalytic domains of Cas9, RuvC and HNH can be mutated to eliminate the DNA cleavage capability of Cas9 without affecting its ability to specifically bind a target. This dead Cas9 (dCas9) can be coupled with transcriptional activators or repressors to accomplish specific and targeted gene modulation. Engineered dCas9 can be coupled with transactivators such as VP64, four tandemly repeated VP16 domains, which helps recruit transcription initiation factors when it binds to the proximal promoter of a target gene. This is known as a CRISPR activation (CRISPRa) system. By helping to accelerate assembly of the transcription initiation complex, the target gene can be transcribed more efficiently, leading to increased overall expression.
1.20 CRISPR/Cas9 Mediated Modulation of Lama1 in Lama2 Deficient Mice:

In my thesis, I will explore the therapeutic potential of modulating the expression of disease modifier genes in MDC1A. Specifically, I aim to increase the expression of the disease modifier gene, *Lama1*, in the *dy*²/*dy*² mouse model of MDC1A using the CRISPRa system. Other potential therapeutic strategies can have significant drawbacks that can prevent them from being translated to a clinical therapy for MDC1A. For example, the CRISPR/Cas9 system can be implemented to correct a Lama2 in mice and potentially patients, however, MDC1A has high allelic heterogeneity. This would mean that each patient with a *LAMA2* mutation would need an independently tested CRISPR/Cas9 guide treatment. Our strategy offers a universal therapy for all MDC1A patients that can treat patients without altering the genome while simultaneously being able to be packaged in AAV vectors. Thus, I hypothesize that using the CRISPR/Cas9 system, we will be able to upregulate expression of Lama1 to re-establish the integrity of muscle fibers and improve pathophysiology of laminin α2 chain deficiency in *dy*²/*dy*² mice. My overall objectives are as follows:

1. Establish and optimize CRISPR/Cas9 systems from *S.aureus* to modify gene expression of the MDC1A disease modifier gene, *Lama1*, in *dy*²/*dy*² mouse myoblasts.

2. Evaluate whether local intramuscular and systemic delivery of CRISPR/Cas9 components can facilitate Lama1 upregulation *in vivo* to ameliorate the disease severity in *dy*²/*dy*² mice.
2 MATERIALS AND METHODS:

2.1 CRISPR Construct Cloning and Vector Production:

2.1.1 Engineering of Activation S. aureus dCas9:

Our lab engineered a catalytically inactive version of SaCas9 and coupled it to two flanking VP64 transactivator domains. The SaCas9 sequence was altered in silico to inactivate the RuvC and HNH catalytic sites and abolish their ability to create breaks in DNA. The nucleotide sequence was altered so that amino acid residues within each catalytic domain sequence were changed to nonpolar alanine residues. Furthermore, VP64 transactivator domain sequences were added in-frame immediately upstream and immediately downstream of the SaCas9 sequence. Additional components of the SaCas9 reading frame included an SV40 nuclear localization signal and a triple FLAG tag. The SaCas9 open reading frame was coupled to a CMV enhancer and CMV promoter for high and universal expression. An SV40 Poly A signal was placed downstream of the reading frame. This construct was synthesized by Bio Basic Canada Incorporated. We cloned this synthesized DNA cassette into a plasmid containing an ampicillin resistance gene and two inverted repeat regions (ITR). These ITRs flanked the inserted cassette. The final construct was cloned into stbl3 competent Escherichia coli cells (Cat# C737303, Thermo Fisher Scientific)

2.1.2 Designing Lama1 Upregulation Single Guide RNAs:

The Ensembl Genome Browser was used to determine the location and sequence of the Lama1 gene in the Mus musculus genome 9 database. The transcriptional start site (TSS) of the Lama1 gene was identified and used as a point of reference for designation of CRISPRa sgRNAs. Based on previous work by Gilbert et al., we designed our sgRNAs within 400 bp upstream from the Lama1 TSS for maximum gene activation. We identified all the S. aureus PAM regions within our target region and found nine hits. Based on the work of Ran et al. with
SaCas9, we designed five of the nine sgRNAs to be between 20 bp and 23 bp and began each with a guanine for optimal SaCas9 activity\(^{70}\). These guides, summarized in table 2, were cloned into a plasmid containing an ampicillin resistance gene and two inverted repeat regions (ITR). These ITRs flanked the inserted cassette. The final construct was cloned into stable 3 competent cells.

Table 2 – sgRNA sequences for targeting of SaCas9 to the Lama1 gene.

<table>
<thead>
<tr>
<th>sgRNA Number</th>
<th>Number of Base Pairs Upstream of the Lama1 TSS</th>
<th>Forward Sequence (5’ to 3’)</th>
<th>Reverse Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>43</td>
<td>GAAGGGCCTGCACCAGGC</td>
<td>GCCTGGTGCAGGCCCTTC</td>
</tr>
<tr>
<td>2</td>
<td>76</td>
<td>GCGGGGCGCAGCCAGGCAGGC</td>
<td>GCCTGGTCCTGAGCCCGCAGGC</td>
</tr>
<tr>
<td>3</td>
<td>131</td>
<td>TCCAGGGGAGCCCCGCGCTG</td>
<td>CACGGGCGGCTCCCCCTTGGAA</td>
</tr>
<tr>
<td>4</td>
<td>203</td>
<td>GGTGTGGCGGGGTTGCCTCTC</td>
<td>GAGAGGCACCCGCCACCAACC</td>
</tr>
<tr>
<td>5</td>
<td>260</td>
<td>ACCCAACTGGAGTGAGGGGT</td>
<td>ACCCTCCACTCCAGTTGGGT</td>
</tr>
</tbody>
</table>

2.1.3 AAV9 Viral Vector Production:

Our Lama1 CRISPRa constructs were packaged into AAV9 vectors by Vigene Biosciences Incorporated. The construct containing plasmids were prepared with the Qiagen Endofree Maxiprep kit. Once 1 mg of each plasmid was purified, it was sent to Vigene Biosciences Inc. They grew \(10^9\) HEK293 cells that produced the AAV9 packaged constructs and purified by Iodixanol gradient ultracentrifugation. The final products were shipped to us as 500 ul aliquots of AAV9 packaged constructs, each with viral titers of approximately \(10^{13}\) viral particles/ml. These AAV9 packaged constructs were stored in -80°C.

2.2 Cell Culture:

2.2.1 dy\(^{2i}\)/dy\(^{2i}\) Myoblast Isolation and Immortalization:
Immortalized $dy^{2j}/dy^{2j}$ mouse myoblasts were used for the in vitro CRISPR/Cas9-mediated upregulation of *Lama1*. The EDL (extensor digitorum longus) muscles from the hind limbs of $dy^{2j}/dy^{2j}$ mice were dissected and stored in sterile PBS. The wells of a 6-well plate were filled with dissociation media, DMEM (Cat# 31053028, Thermo Fisher Scientific) containing 0.2% collagenase type IV (Cat# 07909, StemCell Technologies). The EDL muscles were added to each of the wells in the 6-well plate and incubated for 90 minutes at 37°C. A 15 ml Falcon tube was coated with 5% BSA (Cat# 15260037, Thermo Fisher Scientific) in PBS by incubating for 30 minutes at 37°C. The muscles were then broken up by repeatedly pipetting and the mixture of muscle was added to the BSA coated 15 ml Falcon tube. The Falcon tube was centrifuged at 200 rpm for five minutes. The supernatant was aspirated and the pellet was resuspended in DMEM with 10% FBS (Cat# 16050-114, Thermo Fisher Scientific), 1% L-Glutamine, 1% streptomycin/penicillin.

The wells of a new 6-well plate were coated with 5% BSA in PBS by incubating for 30 minutes at 37°C. The wells were further coated with Matrigel Basement Membrane Matrix (Cat# 356234, BD Biosciences) by incubating for 30 minutes at 37°C. Myoblast media, DMEM with 10% FBS (Cat# 16050-114, Invitrogen), 1% L-Glutamine, 1% streptomycin/penicillin, 0.5% Chick Embryo Extract (Cat#100-163P, Gemini BioProduct-Cedarlane), was added to each well of the coated 6-well plate and warmed in a 37°C incubator. The Falcon tube containing the EDL muscle tissue remnants was centrifuged at 200 rpm for five minutes. The supernatant was aspirated and the pellet was resuspended in myoblast media. The EDL tissue mixture was added to a well of the Matrigel coated 6-well plate. The plate was incubated in in a 37°C incubator for four days, at which point myoblast colonies began to grow.

The media was removed and the cells were washed with PBS. These cells were then trypsinized by adding 0.25% trypsin and incubating in a 37°C incubator for five minutes.
Myoblast media was added to trypsinized plate to neutralize trypsin activity. The cells were centrifuged at 250xg for five minutes and the supernatant was aspirated. The cells were resuspended in myoblast media and plated into an uncoated 10 cm dish. After 30 minutes, the fibroblast cells would have bound the plastic plate while myoblasts remained in the supernatant. A 10 cm dish was coated with Matrigel and filled with myoblast media. This plate was pre-warmed and the myoblast supernatant was moved into this plate. These cells were incubated in a 37°C incubator for four days.

These cells were trypsinized and added to three 50 ml flasks containing myoblast media, which were sent to ABM Incorporated for immortalization. Once received by ABM Inc., the cells were plated into 6-well plates. The immortalization of the myoblasts was completed through viral transduction with Simian Virus 40 large T antigen (SV40). This SV40 T antigen can induce telomerase activity in the infected cells, which can prevent replicative senescence triggered by a DNA damage signal from short telomeres (www.abmgood.com). The growth rate of these transduced cells was compared to untransduced cells to confirm an increase in rate of proliferation. The cells were cryopreserved and shipped to our lab on dry ice and were stored in -80°C upon arrival.

2.2.2 HEK293 Cell Transfection:

HEK293 cells were grown for five days in DMEM+++ media (10% FBS, 1% L-glutamine, 1% streptomycin/penicillin) at 37°C. Cells were trysinized and 2.0 x10^5 cells were plated into a 12-well dish containing pre-warmed DMEM+++ media. These cells were grown overnight at 37°C to allow them to reach 70% confluency. Exactly 1 ug of DNA for transfection was mixed with DMEM to a volume of 50 ul. Exactly 4 ul of Lipofectamine 2000 reagent (Cat# 11668027, Thermo Fisher Scientific) was mixed with 46 ul of DMEM and mixed with the DNA
and media solution. The mixture was incubated for five minutes and added to a treatment well. The plate of transfected cells was incubated at 37°C and cells fluorescence expression was observed with the Zeiss Epifluorescence microscope.

2.2.3 Myoblast Transfection:

Immortalized dy²/dy² mouse myoblasts were transfected using the Invitrogen Neon Transfection System (Cat# MPK5000, Thermo Fisher Scientific). Exactly 1500 ng of each plasmid was used per treatment. Immortalized dy²/dy² mouse myoblasts were grown for five days in a 15 cm petri dish in myoblast media. These cells were then trypsinized by adding 0.25% trypsin and incubating in a 37°C incubator for five minutes. Myoblast media was added to trypsinized plate to neutralize trypsin activity. The cells were centrifuged at 250xg for five minutes. The supernatant was aspirated with a vacuum and the cells were resuspended in 3 ml of PBS. From the resuspended cells, 10 ul were taken and used to count the number of cells per milliliter using the Thermo Fisher Scientific Countess II Cell Counter. Once counted, 4 x 10⁵ cells/treatment were transferred to an Eppendorf microfuge tube and centrifuged at 250xg for five minutes. The PBS was aspirated using a vacuum and the cell pellet was resuspended using 15 ul of Resuspension Buffer R per treatment. Once the cells were resuspended, 15 ul of the cell mixture were added to Eppendorf microfuge tubes containing plasmids for each different treatment. Each mix of cells and DNA were drawn into a Neon transfection tip and placed into the Neon electrolyte buffer chamber. An electrical current (pulse voltage: 1400 V, pulse width: 20 ms, pulse number: 2) was run through the cell mixtures and the transfected cells were placed into a pre-warmed myoblast media containing well of a 12-well plate. These cells were incubated in a 37°C incubator for three days post-transfection.
2.3 Tissue Harvesting and Processing:

2.3.1 Tissue Isolation:

Mice used for these experiments were housed at the Toronto Center for Phenogenomics. Four weeks after receiving treatments, mice were sacrificed in a carbon dioxide chamber and dissected. Tissues were removed from the mice and covered with Tissue-Tek Optimal Cutting Temperature (OCT) compound (Cat# 4583, Sakura Finetechical Company). The OCT covered tissue was placed on a cork and frozen by immersing in isopentane pre-cooled in liquid nitrogen for 20 seconds. The frozen tissues were transported in liquid nitrogen and stored in -80°C for long-term storage.

2.3.2 Cryosectioning:

Tissues were sectioned in the Microm HM525 Cryostat (Cat# 956640, Thermo Fisher Scientific). The cryostat was set to have a cutting temperature between -25°C and -28°C, which is optimal for cutting muscles and nerves. Metal tissue holder discs were placed onto a fast cooling block in the cryostat. A large drop of OCT was added to the metal disc and allowed to partly freeze. The tissue was positioned and embedded into the semisolid OCT on the metal disc. This metal disc was further cooled until the OCT was completely solid and the tissue could not move. Once the tissue was fastened into the cryostat, the microtome was set to trim 40 nm sections. This function was used to remove unwanted OCT encasing the tissue. The microtome section size was changed to 8 nm and used to take sections for histological and immunofluorescence staining. Sections were placed onto superfrost plus slides (Cat# 48311-703, VWR) and stored in -80°C for long-term storage. Sections were also placed into tubes with zirconium beads (Cat# PFAW 1400-100-19, OPS Diagnostics) for protein extraction.
2.4 Protein Isolation and Western Blot:

2.4.1 Protein Extraction:

Protein was extracted from treated cells and from tissue sections of mice. Treated myoblast protein was extracted by aspirating media from plate wells containing transfected cells. Ripa buffer with detergent mixed with Roche cOmplete protease inhibiter cocktail (11697498001, Sigma Aldrich) to make lysis buffer and was kept cold. The well was filled with 200 ul of lysis buffer and cell scrapers were used to thoroughly scrape the bottom of each well. Each cell mixture was transferred into Eppendorf microfuge tubes. These tubes were incubated on ice for 30 minutes and then centrifuged at 12000xg for 15 minutes at 4°C. These samples were stored at -80°C.

Tissue sections of mouse muscles and nerves were placed into tubes with ceramic beads. Ripa buffer without detergent was mixed with Roche cOmplete protease inhibiter cocktail and 250 ul of this detergent-free lysis buffer was added to each tube. The tubes were placed in the Roche MagNA lyser and tissue was crushed twice at 7000 oscillations per minute for 20 seconds, cooling on ice between runs. Afterwards, 250 ul of Ripa buffer with 2x detergent was added to each tube and they were placed on rotator for an hour in the cold room. These tubes were then centrifuged at 12000xg for 15 minutes at 4°C. These samples were stored at -80°C.

2.4.2 Protein Quantification:

The whole protein concentrations of each of the cell and tissue samples were determined using the Pierce BCA protein assay kit (Cat# 23225, Thermo Fisher Scientific). Solution A and solution B were mixed with a volume ratio of 50:1 to make solution C. A 96-well plate had wells filled with 200 ul of solution C each. For each protein lysate sample, 10 ul were added to a
well in duplicate. The plate was incubated at 37°C for 30 minutes and then placed into a plate reader. The absorbance values at a wavelength of 562 nm were determined and protein concentrations were determined based on absorbance readings.

2.4.3 Western Blot Analysis:

Western blot analysis was used to assess the expression of laminin α1 chain and other proteins of interest from each sample. Per sample, 10 ug of whole cell or tissue protein lysates were added to individual tubes. Each of these samples were raised to a volume of 20 ul with Ripa buffer with detergent. Additionally, 20 ul of 2X Laemmli sample buffer with β-mercaptoethanol was added to each sample as a reducing agent. These samples were heated at 70°C for 10 minutes to allow proteins to denature. An NuPAGE 10-well 3-8% Tris-Acetate precast gel (Cat# EA0375BOX, Thermo Fisher Scientific) was placed into a XCell SureLock™ Mini-Cell Electrophoresis System (Cat# EI0002, Thermo Fisher Scientific). The gel apparatus was filled with NuPAGE Tris-Acetate SDS Running Buffer (Cat# LA0041, Thermo Fisher Scientific). A well was filled with 15 ul of HiMarK Pre-stained Protein Standard (Cat# LC5699, Thermo Fisher Scientific). The remaining wells were filled with each protein sample. The gel apparatus was plugged into a Bio-Rad PowerPac Basic Power Supply and run at 120 V for 90 minutes. The gel was then removed from its cast and placed onto nitrocellulose membrane (Cat# 88018, Thermo Fisher Scientific) in an XCell II Blot Module wet transfer apparatus with transfer buffer. The transfer apparatus was plugged into the Bio-Rad PowerPac and run at 150 mA for 20 hours in a cold room.

Following the transfer, the nitrocellulose membrane was blocked into 5% milk in TBST for 60 minutes on a rocker. The milk was removed and 5 ml of fresh 5% milk in TBST was added. A primary antibody for the proteins of interest were added to the milk. The antibodies
and dilutions used for western blot analysis are summarized in table 3. The membrane was incubated on a rocker in this antibody overnight in a cold room. The membrane was then washed three times with TBST for 10 minutes each. Afterwards, 5 ml of fresh 5% milk in TBST with a horseradish peroxidase secondary antibody was added to the membrane, which was then again incubated on a rocker in this antibody 60 minutes at room temperature. The membrane was then washed again three times with TBST for 10 minutes each. SuperSignal West Femto Maximum Sensitivity Substrate (Cat# 34095, Thermo Fisher Scientific) was added to the membrane and incubated for five minutes. The chemiluminescence signals on the membrane were detected using a Bio-Rad ChemiDoc.

**Table 3 – A summary of the antibodies used for the western blot protocol**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Antigen</th>
<th>Dilution</th>
<th>Species</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti LNA1 E3</td>
<td>Laminin alpha 1 E3 domain</td>
<td>0.6 ug/ml</td>
<td>Rabbit</td>
<td>The lab of Dr. Peter Yurchenco, Rutgers University</td>
</tr>
<tr>
<td>M2 anti FLAG</td>
<td>FLAG tag of dCas9</td>
<td>1:1000</td>
<td>Mouse</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Clone AA2, anti beta tubulin</td>
<td>Beta Tubulin</td>
<td>1:5000</td>
<td>Rabbit</td>
<td>Millipore</td>
</tr>
<tr>
<td>C4, anti beta actin</td>
<td>Beta Actin</td>
<td>1:5000</td>
<td>Mouse</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>HRP conjugate secondary</td>
<td>Rabbit Antibody</td>
<td>1:2500</td>
<td>Goat</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>HRP conjugate secondary</td>
<td>Mouse Antibody</td>
<td>1:2500</td>
<td>Goat</td>
<td>Santa Cruz</td>
</tr>
</tbody>
</table>

**2.5 Immunohistochemical and Morphometric Analysis:**

**2.5.1 Immunofluorescence Staining:**

Expression of Lama1 in treated and untreated muscle tissue and sciatic nerve was detected using immunofluorescence. Slides containing mouse muscle or nerve tissue sections were removed from -80°C storage and allowed to warm at room temperature for 30 minutes. Residual OCT was removed by washing the slides in PBS three times for five minutes each.
tissues were fixed in ice cold methanol for 10 minutes. The slides were washed once again with PBS three times for five minutes each. Sections were circled with elite PAP pen marks (Cat# 99990-104, VWR) and 20 ul of blocking buffer, 3% BSA, 1% NGS, 0.1% Triton 100-X in PBS, was added to each section. The slides were incubated at room temperature for 60 minutes in a humidifying chamber. Afterwards, primary antibodies, summarized in table 4, were diluted in blocking buffer and 20 ul were added to all but one section per tissue, which was covered again with blocking buffer as a secondary only negative control. The slides were incubated at room temperature for 60 minutes in a humidifying chamber. The slides were washed with PBS three times for five minutes each. Secondary antibodies, summarized in table 4, were diluted in blocking buffer and 20 ul were added to all the tissue sections. The slides were incubated at room temperature for 60 minutes in a dark humidifying chamber. The slides were washed in the dark with PBS three times for five minutes each. Each section was covered with 20 ul of 2ng/ul DAPI stain and incubated at room temperature for five minutes in a dark humidifying chamber. The slides were washed in the dark with PBS three times for five minutes each. Droplets of prolong gold were added to each slide and a coverslip was placed over the sections. These slides were stored in 4°C for 24 hours and then scanned with the 3dhistech Pannoramic 250 Flash digital scanner.

**Table 4** – A summary of the antibodies used for the immunofluorescence protocol

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Antigen</th>
<th>Dilution</th>
<th>Species</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat 200 LNα1</td>
<td>Laminin alpha 1</td>
<td>1:20</td>
<td>Rat</td>
<td>The lab of Dr. Madeleine Durbeej-Hjalt, Lund University</td>
</tr>
<tr>
<td>Anti-NFH SMI 31</td>
<td>Neurofilament H</td>
<td>1:1000</td>
<td>Mouse</td>
<td>Biolegend Inc.</td>
</tr>
<tr>
<td>Alexa 555 Anti-Rat</td>
<td>Rat</td>
<td>1:250</td>
<td>Goat</td>
<td>Thermo Scientific Fisher</td>
</tr>
<tr>
<td>Alexa 488 Anti-Mouse</td>
<td>Mouse</td>
<td>1:250</td>
<td>Goat</td>
<td>Thermo Scientific Fisher</td>
</tr>
</tbody>
</table>
2.5.2 Histological Analysis:

Mouse muscle tissue sections were stained with hematoxylin and eosin to observe histopathological features associated with Lama2 deficiency. Slides containing mouse muscle or nerve tissue sections were removed from -80°C storage and allowed to warm at room temperature for 30 minutes. The tissues were fixed in ice cold acetone for five minutes. The slides were then washed in MilliQ water and placed into hematoxyl for six minutes. The slides were, again, washed in MilliQ water and destained by dipping 10 times in acid ethanol. The slides were washed in tap water twice for one minute each, followed by a two-minute wash in MilliQ water. The slides were then dipped in eosin for 45 seconds. The slides were then washed for one minute in 80% ethanol, then 90% ethanol, and finally 100% ethanol. They were dipped 30 times in xylene and several droplets of permount were added to each slide. A coverslip was placed over the stained tissue sections and the slides were kept at room temperature for 24 hours and then scanned with the 3dhistech Pannoramic 250 Flash digital scanner.

2.6 Functional Analysis:

2.6.1 Open Field Activity Test:

Open field activity test and assessment of in vivo muscle force were performed on seven weeks-old mice (for systemically injected cohorts) at the Lunenfeld-Tanenbaum Research Institute’s Centre for Modeling Human Disease Mouse Phenotyping Facility. For the open field test, mice were placed in the frontal center of a transparent Plexiglas open field (41.25 cm x 41.25 cm x 31.25 cm) illuminated by 200 lx. Trained operator, who was unaware of the nature of the projects and treatments, performed the experiments. The VersaMax Animal Activity Monitoring System recorded vertical activities and total distance travelled for 20 minutes per animal.
2.6.2 Twitch Force Assay:

*In vivo* muscle contraction test was performed using 1300A: 3-in-1 Whole Animal System and analyzed using Dynamic muscle control/analysis (DMC/DMA) High throughput software suite (Aurora Scientific). The mice were anaesthetized with intraperitoneal injection of ketamine/xylazine cocktail at 100 mg/kg and 10 mg/kg of body weight, respectively. Contractile output was measured via percutaneous electrodes that stimulate specific nerves innervating the plantar flexors. Specific tetanic force (200 ms of 0.5-ms pulses at 125 Hz) was recorded and corrected to body weight.
3 RESULTS:

3.1 CRISPR/Cas9-mediated Expression Modulation \textit{in vitro}:

3.1.1 Generation and Characterization of CRISPR-mediated Modulation Construct:

Mutation-specific CRISPR/Cas9 genome-editing technology is a promising potentially curative therapy for many genetic diseases, including MDC1A. A modified CRISPR/Cas9 system can also be utilized to target and modulate expression of disease modifier genes. This mutation independent strategy can circumvent the challenge associated with developing and testing individualized genome editing treatments. Therefore, we set out to develop a CRISPRa system that could be used to increase expression of a disease modifier gene, \textit{Lama1}, and ameliorate the disease phenotype of Lama2 deficiency.

We began by engineering a catalytically inactive \textit{Sa}Cas9. This dCas9 has the capacity to target a gene specified by a sgRNA sequence immediately upstream of a PAM sequence (5’-NNGRRT-3’). The catalytic sites were mutated (D10A and H840A) to ablate the \textit{Sa}Cas9’s ability to induce breaks in the DNA phosphodiester backbone \textsuperscript{73}. Instead, it offers the opportunity to modulate expression of genes targeted by sgRNAs. Specifically, the N- and C-termini of this dCas9 were fused to two VP64 transcriptional activator domains that increase the expression of genes targeted by the dCas9 protein.

To determine if our CRISPR system could increase expression of target genes in practice, we attempted to increase the expression of a transfected dTomato gene in HEK293 cells (Figure 3). HEK293 cells were transfected with a plasmid containing a gene for a red fluorescent protein, dTomato, under a minimal CMV promoter (Figure 3A), which resulted in a weak fluorescent signal at basal level expression. Conversely, when co-transfected with a plasmid containing our engineered VP64-dCas9-VP64 (simply denoted as VP64-dCas9 from here onwards) and a sgRNA that targeted the minimal CMV promoter of the dTomato plasmid (Figure 3B), we observed fluorescent signal substantially stronger than the basal level. This
result provided evidence for the effectiveness of our CRISPR-Cas9-mediated upregulation system in increasing the expression of a target gene.

![Figure 3](image)

**Figure 3** – HEK293 cells were transfected with **A.** a plasmid containing dTomato under the control of a minimal CMV promoter or **B.** a plasmid containing dTomato under the control of a minimal CMV promoter and a plasmid containing VP64-dCas9 and a sgRNA for the minimal CMV promoter of the dTomato plasmid. Scale bar: 160 um.

### 3.1.2 The CRISPRa System Increases Expression of Lama1 *in vitro:*

Once the effectiveness of our CRISPRa system was validated, we hypothesized that it could be used to increase expression of our specific gene of interest, *Lama1*. Immortalized *dy^2j/*dy^2j* myoblasts were transfected with a VP64-dCas9 construct with and without sgRNA for *Lama1*, and different combinations of three different sgRNAs targeting *Lama1*, namely sgRNA 1, 2, and 5 and sgRNA 1, 3, and 4. The expression levels of Lama1 were assessed on western blot (Figure 4A). In the dCas9 only treatment, we observed a very low basal level expression of Lama1 (Figure 4B). Remarkably, with the expression of a sgRNA directing the VP64-dCas9
protein to the *Lama1* gene, we observed a substantial increase in the expression of *Lama1*. Similarly, cells transfected with combinations of sgRNAs 1, 2, and 5 and sgRNAs 1, 3, and 4 also resulted in increased *Lama1* expression. To correct for the differences in transfection efficiency, the expression of *Lama1* (normalized to Beta-Tubulin loading control) in each of the treatments was normalized to expression of FLAG-tagged VP64-dCas9 (also normalized to Beta-Tubulin loading control). We determined that the combination of sgRNAs 1, 2, and 5 treated samples consistently had higher levels of upregulation relative to other samples, thus we decided to proceed with this treatment as our multiple sgRNAs approach. The results of these experiments confirmed that our CRISPRa construct can be used to upregulate the *Lama1* gene in myoblasts from *dy<sup>2j</sup>/dy<sup>2j</sup>* mice.

<table>
<thead>
<tr>
<th>VP64-dCas9</th>
<th>i) dCas9 only</th>
<th>ii) sgRNA 1</th>
<th>iii) sgRNAs 1,2,5</th>
<th>iv) sgRNAs 1,3,4</th>
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<tr>
<td>Laminin alpha 1</td>
<td>450 kDa</td>
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<tr>
<td>FLAG-dCas9</td>
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<tr>
<td>Beta Tubulin</td>
<td>55 kDa</td>
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Figure 4 – Western blot analysis showing the efficacy of CRISPRa constructs for upregulation of the *Lama1* gene in transfected immortalized *dy^2j/dy^2j* myoblasts. A. From left to right, myoblasts were transfected with a construct without sgRNA for *Lama1* (negative control), with a single sgRNA for *Lama1*, with a combination of sgRNAs 1, 2, and 5, and with a combination of sgRNAs 1, 3, and 4. The blot was probed for expression of *Lama1*, FLAG tagged VP64-dCas9, and Beta-Tubulin as a loading control. B. Expression of *Lama1* (normalized to Beta-Tubulin loading control) in each treatment was normalized to the expression of FLAG tagged VP64-dCas9 (normalized to Beta-Tubulin loading control).

3.2 CRISPR/Cas9-mediated Upregulation of Lama1 in Lama2 Deficient Mice:

3.2.1 The CRISPRa System Upregulates Lama1 Expression Intramuscularly:

Following our successful *in vitro* upregulation of *Lama1* using the CRISPR/Cas9 system, we asked whether similar increase can be observed upon delivery of CRISPRa constructs into the skeletal muscles of *dy^2j/dy^2j* mice. To deliver our construct to muscle tissues of mice, we packaged them into AAV9 vectors. We injected 7.5 x 10^{11} viral genome copies
(vgc) per mouse into the right tibialis anterior muscle of three-week-old $dy^{2j}/dy^{2j}$ mice (Figure 5A). These mice received either a treatment of VP64-dCas9 only, VP64-dCas9 with sgRNA 1 for $Lama1$ (denoted as single guide in this section), and VP64-dCas9 with sgRNAs 1, 2, and 5 (denoted as combination guides).

Four weeks post injection, we analyzed Lama1 protein expression levels from treated and contralateral untreated tibialis anterior from these mice using western blot (Figure 5B). In all the injected mice, we determined that VP64-dCas9 was expressed in the right, injected tibialis anterior muscle. We also observed minor VP64-dCas9 expression in the left tibialis anterior, indicating that some virus leaked into circulation. Importantly, Lama1 was highly upregulated in only the right, injected tibialis anterior muscles of both single and combination sgRNA treated mice. These results provided evidence for feasibility of using the CRISPR/Cas9 system to modulate gene expression in vivo.
Figure 5 – Western blot analysis showing the efficacy of AAV9 packaged CRISPRa constructs for upregulation of the *Lama1* gene in the tibialis anterior muscles of *dy^2j/dy^2j* mice. A. A schematic depicting an overview of the local injection treatment plan. Mice were injected into the right tibialis anterior muscle at three-weeks-old and sacrificed at four weeks post injection. B. Samples from the tissues of mice differentially treated with local injections for upregulation of *Lama1* were used for western blot analysis. From left to right, both left (TAL) and right (TAR) tibialis anterior muscles of mice injected with an AAV9 packaged CRISPRa construct i) and ii) only (negative control), iii) and iv) with a single sgRNA for *Lama1*, v) and vi) with a combination of sgRNAs for *Lama1*. The blot was probed for expression of *Lama1*, FLAG tagged VP64-dCas9, and Beta-Actin as a loading control. Note that in some contralateral untreated muscles, low levels of VP64-dCas9 were detected.

### 3.2.2 Laminin Alpha 1 Chain Expression Improves Muscle Pathology in *dy^2j/dy^2j* mice:

Upon confirmation of *Lama1* upregulation in treated muscles of *dy^2j/dy^2j* mice with western blot, we sought to confirm whether *Lama1* expression was improving the muscle...
histopathology in Lama2 deficient mice. Immunofluorescence staining was used to determine the localization of Lama1 in the sgRNA treated muscles of dy2j/dy2j mice. The presence of Lama1 was not detected in the muscles VP64-dCas9 only treated mice (Figure 6A). In both single and combination of sgRNA treatments, a substantial increase in Lama1 expression was observed (Figure 6B,C).

Following this data, we asked whether Lama1 had a protective effect on the muscles of dy2j/dy2j mice. To determine the potential benefit of Lama1 expression in dy2j/dy2j mouse muscles, we assessed the histology of untreated and treated muscles. Tibialis anterior muscles from mice treated without sgRNA for Lama1 had large numbers of variably sized muscle fibers and substantial infiltration of fibrotic tissue, which are both common signs of damaged muscle tissue (Figure 6D). Conversely, muscles from tissues treated with either a single sgRNA or multiple sgRNAs for Lama1 upregulation appeared to have less variability in fiber size and less fibrotic area (Figure 6E,F). The qualitative improvements in muscle pathology supported our hypothesis that Lama1 overexpression has a therapeutic benefit for Lama2 deficiency.
Figure 6 – Tissue sections of treated $dy^2/dy^2$ mice tibialis anterior muscles express Lama1 and have improved muscle pathology. Immunofluorescence was used to assess Lama1 expression from muscle sections from mice locally treated with A. VP64-dCas9 only, B. VP64-dCas9 with a single sgRNA for Lama1, and C. VP64-dCas9 with a combination of sgRNAs for Lama1.
Muscle sections from mice treated with D. VP64-dCas9 only, E. VP64-dCas9 with a single sgRNA for Lama1, and F. VP64-dCas9 with a combination of sgRNAs for Lama1 were stained with Hematoxylin and Eosin to observe muscle tissue pathology of treated and untreated mice. Scale bar 190 um.

3.3 Systemic Upregulation of Lama1 in Lama2 Deficient Mice:

3.3.1 Dose Optimization for Systemic Injections:

Following the local treatment experiments, we attempted to deliver the CRISPRa constructs for Lama1 systemically via the blood stream to target all the skeletal muscles in the body, as well as target the Schwann cells in peripheral nervous system to prevent demyelination. First, we assessed whether the same treatment regime, including dose and treatment window, from local injections experiments could lead to efficient restoration when given systemically. We injected three-week-old dy2j/dy2j mice into the tail vein with CRISPRa constructs packaged into AAV9 vectors at a titre of 7.5 x 10^{11} viral genome copies per mouse (Figure 7A). Four weeks post injection, we harvested the tibialis anterior muscles of injected mice and assessed Lama1 expression with immunofluorescence staining. We found that Lama1 could be upregulated in vivo through systemic treatment with a CRISPRa system (Figure 7B, C, D). However, it was limited to a low percentage of myofibers. It is important to note that expression of Lama1 in combination sgRNA treated tissues consistently outperformed the single sgRNA treatment.

Subsequently, we tested whether increasing viral titer of the combination sgRNA treatment could increase Lama1 expression in muscle tissues. Two dosages were tested: 1.5 x 10^{12} viral genome copies per mouse (double), and 3.0 x 10^{12} viral genome copies per mouse (quadruple). We found an increase in the level of Lama1 expression in the both the double and quadruple dosage cohorts, as shown in immunofluorescence analysis, compared to the original
dose of $7.5 \times 10^{11}$ viral genome copies/mouse (Figure 8). The quadruple dosage treatment induced Lama1 expression nearly throughout the entire muscle. The double dose treatment, while having higher Lama1 expression than the starting dosage treatment, was notably less effective than the quadruple dose treatment.

**Figure 7** – Tissue sections of tibialis anterior muscles from systemically treated $dy^2j/dy^2j$ mice express Lama1. A. A schematic depicting an overview of the systemic injection treatment plan.
Mice were injected into the tail vein at three-weeks-old and sacrificed four weeks post injection. Immunofluorescence was used to assess Lama1 expression from muscle sections from mice treated systemically with A. VP64-dCas9 only, B. VP64-dCas9 with a single sgRNA for Lama1, and C. VP64-dCas9 with a combination of sgRNAs for Lama1. Scale bar 190 um.

**Figure 8** – Tissue sections of tibialis anterior muscles from systemically treated $dy^{2j}/dy^{2j}$ mice with increased viral titres express higher levels of Lama1. Mice were injected with AAV9 packaged CRISPRa constructs into the tail vein at three-weeks-old and sacrificed four weeks post injection. Mice were injected with a combination sgRNA treatment for Lama1 at a viral

\[ \text{Viral Titer: } 1.5 \times 10^{12} \] \[ \text{vgc/mouse} \] \[ \text{Viral Titer: } 3.0 \times 10^{12} \] \[ \text{vgc/mouse} \]
titer of A. $1.5 \times 10^{12}$ viral genome copies per mouse and B. $3.0 \times 10^{12}$ viral genome copies per mouse. The expression of Lama1 was determined using immunofluorescence staining. Scale 100 um.

3.3.2 Intravenous Injections of CRISPRa Constructs Increase Lama1 Expression in Skeletal Muscle and the Peripheral Nervous System:

We determined an optimal treatment dose for upregulation of Lama1. We sought to determine whether Lama1 could be upregulated in other tissues, in addition to the tibialis anterior muscles. Mice were injected with the highest viral dosage ($3.0 \times 10^{12}$ viral genome copies per mouse). These experiments were to test the CRISPRa system’s efficiency in multiple tissues, such as the gastrocnemius muscle and the sciatic nerve. Lama1 expression in the gastrocnemius muscles of systemically treated mice were assessed with immunofluorescence staining. Similar to the tibialis anterior, Lama1 expression could be observed throughout most of the muscle (Figure 9A). Interestingly, regions of the gastrocnemius muscle with low levels of Lama1 expression were observed to have higher levels of fibrosis (Figure 9B, C). This observation supports the hypothesis that Lama1 is protecting muscle fibers from damage in the areas that it is expressed while other regions accumulate damage.

We also tested peripheral nervous system tissue of treated mice to determine if Lama1 expression could be observed. Lama1 expression levels in the sciatic nerve were assessed using immunofluorescence. An increase in Lama1 expression was observed in sciatic nerves of mice treated with VP64-dCas9 and a combination of sgRNAs for Lama1 relative to the mice treated with VP64-dCas9 only (Figure 10). Neurofilament H is a component of the cytoskeleton of neural cells and staining for these denotes the presence of axons. The expression of Lama1 was found to surround points of neurofilament H staining, which may mean that Lama1 is expressed in Schwann cells and could potentially prevent demyelination. Taken together, these results
indicated that the AAV9 vectors effectively target and transduce the skeletal muscle and peripheral nervous system of dy^{2j}/dy^{2j} mice, and that our CRISPRa system successfully upregulates Lamal expression in both skeletal muscle and the peripheral nervous system.

Figure 9 – Tissue sections of gastrocnemius muscles from systemically treated dy^{2j}/dy^{2j} mice with Lamal expression and reduced muscle pathology. Mice were injected with a combination
sgRNA treatment for Lama1 into the tail vein at three-weeks-old and sacrificed four weeks post injection. **A.** Immunofluorescence stain for Lama1 expression in gastrocnemius muscle. Scale bar 100 um. **B.** Immunofluorescence stain for Lama1 expression in a complete cross-section of a gastrocnemius muscle. Scale bar 1000 um. **C.** Hematoxylin and Eosin staining of a complete cross-section of the same gastrocnemius muscle used for panel B. Scale bar 1000 um.

**Figure 10** – Tissue sections of a sciatic nerve from systemically treated \(dy^2/dy^2\) mice with Lama1 expression. Mice were injected with a combination sgRNA treatment for Lama1 into the tail vein at three-weeks-old and sacrificed four weeks post injection. Immunofluorescence staining for Lama1 in red and Neurofilament H in green. **A.** Sciatic nerve of mice treated with VP64-dCas9 only. **B.** Sciatic nerve of mice treated with a combination of sgRNAs for Lama1. 200x Magnification.
3.4 Assessment of Muscle Histopathology Changes upon Upregulation of Lama1

3.4.1 Treated Muscles Have Reduced Histopathology

The previous experiments confirmed the CRISPRa system used to treat these $dy^{2j}/dy^{2j}$ mice had induced a substantial amount of Lama1 expression in muscle tissues. Next, we assessed whether this upregulation could manifest in reduced dystrophic state of muscles. The percentage of centralized nuclei, as a marker of regeneration, and percentage of fibrotic area in a muscle, as a marker of muscle damage, were quantified to determine relative muscle pathophysiology. These parameters were quantified for the tibialis anterior muscles of untreated and treated mice (Figure 11A,B). The average percentage of myofibers with centralized nuclei was 10.9% (± 6.395%) for the untreated cohort and 3.3% (± 1.185%) for the treated cohort. The average percentage of fibrotic area in muscles was 32.5% (± 5.979%) and 25.9% (± 6.548%) for the treated cohort. While tibialis anterior muscle of treated mice show both decreased centralized nuclei and fibrotic area, relative to the muscles of untreated mice, a statistically significant difference could not be found in either of the quantifications. A statistically significant difference may be observed after increasing each of the sample sizes.

Percentage of centralized nuclei and percentage of fibrotic area were also quantified in the gastrocnemius muscles of untreated and treated mice (Figure 12A,B). We have found that the gastrocnemius muscles of $dy^{2j}/dy^{2j}$ mice typically have higher levels of fibrosis than the tibialis anterior muscles. The average percentage of myofibers with centralized nuclei was 9.6% (± 2.755%) for the untreated cohort and 8.0% (± 5.73%) for the treated cohort. The average percentage of fibrotic area in muscles was 36.0% (± 1.679%) and 24.4% (± 1.779%) for the treated cohort. A significant decrease in fibrotic area was observed in the treated cohort, relative to untreated cohort (p-value = 0.0003). This provides strong evidence for the effectiveness of Lama1 upregulation as a therapeutic option for reducing the dystrophic state of muscles in
Lama2 deficiency. We next tested if the reduced pathophysiology correlated to changes in overall severity of the disease phenotype.

Figure 11 – Quantification of pathophysiology in tibialis anterior muscles. Tibialis anterior muscles from $d\gamma^{2j}/d\gamma^{2j}$ mice treated with A. VP64-dCas9 only and B. a combination of sgRNAs for Lama1 were stained with hematoxylin and eosin. Scale bar 50 um. C. Percentage of...
myofibers with centrally located nuclei from (A) and (B). D. Quantification of fibrosis from (A) and (B). Data in (C) and (D) are presented as mean ± standard deviation on n = 4-5 mice/group. Statistical analyses were performed using Student’s t-test.
Figure 12 – Quantification of pathophysiology in gastrocnemius muscles. Gastrocnemius muscles from dy^{2j}/dy^{2j} mice treated with A. VP64-dCas9 only and B. a combination of sgRNAs for Lama1 were stained with hematoxylin and eosin. Scale bar 50 um. C. Percentage of myofibers with centrally located nuclei from (A) and (B). D. Quantification of fibrosis from (A) and (B). Data in (C) and (D) are presented as mean ± standard deviation on n = 2-4 mice/group. Statistical analyses were performed using Student’s t-test. ***p<0.0005.

3.5 Impact of CRISPR/Cas9-Mediated Upregulation of Lama1 on the Lama2 Deficiency Disease Phenotype:

3.5.1 Treated dy^{2j}/dy^{2j} Mice Have No Apparent Change in Motility and Hind Limb Functionality:

Based on the improved muscle pathophysiology observed from hematoxylin and eosin staining, we wanted to see if systemic upregulation of Lama1 in dy^{2j}/dy^{2j} mice could contribute to a global phenotypic improvement. The mobility and level of hind limb strength of treated, untreated, and wild-type mice was tested using the open field activity test. The total distance travelled during the testing was a marker or mobility, whereas the number of standing actions was an indicator of strength and paralysis of the hind limbs. The average total distance travelled for untreated mice was 5736.63 cm (± 787.96) and 5736.51 (± 1062 cm) for treated mice (Figure 13A). The average number of standing actions for untreated mice were 71.25 (± 11.75) and 65.67 (± 5.33) for treated mice (Figure 13B). Based on these results, the treated mice did not seem to acquire a significant phenotypic benefit from expressing Lama1. However, the open field activity test is a largely influenced by mouse behaviour, which may have masked the phenotypic benefit of our treatment. Additionally, our sample size was limited to n=3. We may be able to observe a significant difference with a larger sample size.
Open field activity tests were conducted to observe mouse mobility and hind limb function. Mice were allowed to move freely within empty chambers for 20 minutes. Four weeks post-injection, the mice were tested in open field chambers where A. total distance travelled and B. standing activity were assessed. Data is presented as mean ± standard deviation on n=2-4 mice per group. Statistical analyses were performed using a Student’s t-test.

**3.5.2 Lama1 Upregulation in dy²/dy² Mice Increased Specific Tetanic Force:**

Since open field activity test assesses more behavioural aspects of mouse physiology than specific muscle function, we tested mice with the twitch force assay, which is an assessment of specific limb muscles, and thus is a more objective measure of muscle strength of mice. This assay measures the potential of a muscle to produce a force when electrically stimulated. We measured the specific tetanic force, which is the force produced by a stimulated muscle before fatiguing that is then normalized to the body weight of the mouse. We tested the right tibialis anterior muscle of treated, untreated, and wild-type mice and found a statistically significant increase in the specific tetanic force produced by Lama1 upregulated mice, relative to untreated mice (Figure 14). This result reinforces the theory that upregulation of Lama1 expression functionally improves Lama2 deficiency disease phenotypes.
Figure 14 – Specific tetanic force was measured using in vivo muscle function analyzer. The specific tetanic force produced by the right tibialis anterior muscles of mice were tested. Electrodes were inserted into the tibialis anterior muscles of mice and 125 mV pulses were used to stimulate the muscle. The maximum force produced by the muscle was normalized to the mass of the mouse tested to determine the specific tetanic force. Data are presented as mean ± standard deviation on n=3-5 mice/group. Statistical analysis was performed using Student’s t-test.
4 DISCUSSION:

Muscular dystrophies are a group of genetic diseases causing progressive muscle weakness that often results in loss of ambulation, breathing and eating difficulties and premature death. Identification of the causative genetic mutations leads to understanding disease mechanisms as the basis for treatment targets, which can be either curative or disease-modifying. In diseases where curative genetic therapy is challenging due to heterogeneity of the mutations, such as in MDC1A, identification of disease modifier genes has proven to be key for the development of therapeutic strategies.

This project aims to increase expression of *Lama1*, a gene shown to be capable of compensating for Lama2 deficiency and significantly reducing disease severity \(^{60}\), by using a CRISPRa system in *dy^2j/dy^2j* mice. If successful, this strategy offers an approach that can help MDC1A patients independent of their mutation.

First, we engineered a CRISPRa system consisting of a catalytically inactive *S. aureus* Cas9 coupled to VP64 transactivation domains and tested its efficacy *in vitro*. In HEK293 cells, we observed upregulation of the expression of dTomato fluorescent protein (Figure 3). We then adapted our CRISPRa system to target the *Lama1* gene using a single sgRNA or a combination of three different sgRNAs. A combination of sgRNAs targeting the same gene can have a synergistic effect, which may contribute to an increased protective effect of the therapy \(^{77-79}\). In both kinds of treatments, single sgRNA and multiple sgRNAs, an increase in Lama1 protein expression levels was observed. A 24.7 fold increase was observed in the sgRNA 1 treatment and a 31.5 fold increase was observed in the sgRNA 1, 2, and 5 combination treatment (Figure 4B). This result is in accordance with previously reported studies that combination of guides have synergistic effects.

Once the efficacy of the CRISPRa system was validated *in vitro*, AAV9 particles containing our constructs were injected intramuscularly into right tibialis anterior of *dy^2j/dy^2j* mice.
mice and Lama1 expression was observed in only the sgRNA treated muscles (Figure 5). These vectors were chosen for their translational potential, as AAV vectors are currently one of the safest viral delivery options, and the AAV9 serotype has tropism for skeletal muscle and the nerves. This provides necessary evidence for the efficacy of our CRISPRa system \textit{in vivo}.

Furthermore, the expression of Lama1 in the treated muscles of intramuscularly injected $dy^2/dy^2$ mice was localized to the periphery of myofibers (Figure 6A-C). This localized expression is likely an indicator of the upregulated laminin alpha 1 chains forming Laminin-111 heterotrimers with laminin beta 1 and laminin gamma 1 chains and mediating a connection of the sarcolemma to the extracellular matrix. This observation supports the notion that Laminin-111 can compensate for the absence of Laminin-211 in Lama2 deficient muscle tissue. This compensation seems to have a protective effect on Lama2 deficient muscle as sgRNA treated tissues appeared to have less myofibers with centralized nuclei and reduced fibrotic tissue, indicating increased muscle regeneration and accumulation of muscle damage, respectively (Figure 6D-F).

Following the local administration experiments, we injected the AAV9 packaged CRISPRa system into the blood stream via the tail vein of $dy^2/dy^2$ mice (Figure 7A). The expression of Lama1 was successfully increased in the skeletal muscle of single and multiple sgRNA treated mice (Figure 7B-D). However, the expression was low and sparse throughout the muscle in both single and multiple sgRNA treatments, which was likely due to the packaged vectors being diluted throughout the bodies of mice. Importantly, we did observe a greater level of expression in multiple sgRNA treatment regimen. This led us to pursue higher dosage treatments using the three sgRNA approach. A substantial increase in Lama1 expression was observed in each of these systemic treatments, with the quadruple dose ($3.0 \times 10^{12}$ vgc/mouse) inducing Lama1 expression nearly throughout the entire tibialis anterior muscle (Figure 8A-B). This expression level was comparable to the Lama1 expression in \textit{Lama2} knockout mice with a
Lama1 transgene, which was shown to have a profound effect on reducing the disease severity (Figure S1) \(^{37}\).

In systemically treated mice, the expression of Lama1 in the gastrocnemius muscle was similar to the expression in the tibialis anterior muscle (Figure 9A). This result indicates that the CRISPRa system is likely able to reach different muscle groups throughout the bodies of treated mice via the blood stream. Additionally, by juxtaposing the Lama1 expression with the histology of the treated gastrocnemius muscles, we found a consistent pattern of reduced fibrosis in regions of high Lama1 expression, which supports hypothesis that Lama1 upregulation has a protective effect (Figure 9B-C). Expression of Lama1 was also found in the sciatic nerve of sgRNA treated dy\(^{2}/dy^{2}\) mice (Figure 10). Compensation of Lama1 for Lama2 deficiency in the peripheral nervous system can potentially prevent demyelination and reduce the peripheral neuropathy associated with the disease.

The previous experiments confirmed the CRISPRa system used to treat these dy\(^{2}/dy^{2}\) mice had induced a substantial amount of Lama1 expression in both skeletal muscle and the peripheral nervous system. We next wanted to see if a quantitative difference could be detected in muscle histology. Theoretically, the presence of Laminin-111 should have a protective effect on myofibers by re-establishing a connection between the sarcolemma and extracellular membrane and allowing them to better withstand mechanical forces. Additionally, Laminin-111 in Schwann cells should prevent demyelination of motor neurons and facilitate strong and rapid signal transduction from the central nervous system to skeletal muscles. Taken together, these compensatory methods should likely result in reduced muscle damage and increased muscle function.

To assess whether Lama1 upregulation could reduce muscle histopathology, we tested whether the treatment could reduce the percentage of myofibers with centralized nuclei and percentage of fibrotic area in a muscle. As muscle progenitor cells fuse to regenerate myofibers
in response to muscle damage, newly forming myofibers can be observed with centralized nuclei. Thus, a larger percentage of myofibers with centralized nuclei is indicative of high levels of regeneration occurring in the muscle. There was no significant difference between the percentage of centralized nuclei and fibrotic area in the tibialis anterior muscles of treated and untreated mice (Figure 11). However, tibialis anterior muscles often had fewer pathophysiological features relative to the gastrocnemius muscles. In quantifying the fibrotic area between treated and untreated gastrocnemius muscles, a significant reduction in fibrosis was observed in the treated cohort (Figure 12D). This result supports the hypothesis that Lama1 upregulation has a protective effect on myofibers and reduces degeneration of muscle, which further contributes to increased functional activity.

Finally, we analyzed the functional test data performed on treated, untreated, and wild-type mice. We found no significant difference between treated and untreated cohorts when testing the motility and hind limb strength with the open field activity test (Figure 13). Yet, qualitative differences between these cohorts of mice can be observed when they receive an external stimulus to become active (Figure S2). We believe this discrepancy may be a result of behavioural traits specific to dy²j/dy²j mice. From early stages in life, dy²j/dy²j mice seem resistant to movement. Therefore, it is feasible that during the long periods of testing without external stimulus these mice may choose not to move to the same degree as wild-type mice. We obtained a more objective measure of muscle strength through the twitch force assay, which determines the maximum force produced by stimulating the tibialis anterior muscle of mice with an electrical pulse. This force was normalized to mouse body weight and used as a measure of the contractile capacity of the tested muscle. We found a significant increase in the force produced by treated mice relative to untreated mice (Figure 14).

Taken together, this data suggests that the CRISPR/Cas9-mediated upregulation of Lama1 can offer amelioration of the Lama2 deficiency disease phenotype in mice. The
therapeutic benefit of this system can potentially be further increased by adjusting the treatment window to treat neonatal mice. Therefore, we will begin a new cohort of mice treated at P2 and observe their disease progression overtime. We will also continue treating mice at three weeks of age. However functional tests will be conducted at and later than four weeks post-injection. We may be able to better observe the protective effects of Lama1 expression as untreated mice may worsen while treated may remain stable or improve.

This project describes the potential of utilizing the CRISPR/Cas9 system to conduct gene modulation in vivo, which may offer an effective, mutation-independent therapeutic option for MDC1A patients. Furthermore, this CRISPRa system can be very easily adjusted to target a different gene that may improve the phenotype of a different genetic disease by simply designing a new sgRNA sequence. Our project shows the efficacy and therapeutic potential of this highly versatile gene modulation system in a mammalian model and provides support for its implementation in a clinical setting.

Going forward, we can identify novel disease modifiers of MDC1A and target them with either the CRISPRa and CRISPRi (CRISPR inactivation) systems. A CRISPRi system can be developed by replacing the VP64 transactivation domain with a KRAB transcriptional repression domain that can instead decrease the expression of a target gene. One such option is to downregulate a gene that contributes to accumulation of fibrotic tissue in dystrophic muscle. We plan to pursue this downregulation strategy in the future. We also plan to eventually implement both the CRISPRa and CRISPRi systems to increase Lama1 expression and decrease fibrosis, respectively, by utilizing two different species of dCas9.
APPENDICES:

Supplementary Figure 1 – Immunofluorescence staining for Lama1 expression in the gastrocnemius muscle of a dy3k mouse with a Lama1 transgene. This muscle sample was generously gifted to us by Dr. Durbeej from Lund University, Sweden.
Supplementary Figure 2 – **A.** Video of seven-week old untreated \(dy^2/dy^2\) mouse. **B.** Video of seven-week old \(dy^2/dy^2\) mice treated with three different sgRNA for Lama1 at a dosage of 3.0 x \(10^{12}\) vgc/mouse.
REFERENCES:


