Harnessing genome editing to restore full-length dystrophin and accelerate corrective DMD therapy development

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Abstract

Duchenne Muscular Dystrophy (DMD) is the most common pediatric neuromuscular disease, affecting 1 in 5000 boys. Mutations in the DMD gene which eliminate dystrophin expression cause DMD, with deletions and duplications being the most common. Without dystrophin, skeletal and cardiac muscles become vulnerable to contraction damage, resulting in systemic muscle wasting, loss of ambulation, and significantly reduced life expectancy. For my thesis, I will utilize the genome editing tool CRISPR/Cas9 to correct DMD mutations to restore full-length dystrophin. Homology-mediated end joining is promising for efficient knock-ins in post-mitotic cells and will be tested to correct a patient deletion of exons 52-54. The Cohn lab has also validated an elegant strategy to remove a DMD exon 18-30 duplication, although efficiency remains low. To overcome this, dystrophin overexpression via Cas9-mediated upregulation will be combined with our existing duplication removal strategy to reach clinically relevant levels of dystrophin.

In developing DMD gene therapies, current methods for validating their ability to restore dystrophin in vitro remains inefficient and time-consuming. This is because patient fibroblasts are used, but do not produce dystrophin, necessitating conversion into myotubes through a multifaceted and lengthy reprogramming process. I will utilize Cas9 activation to induce dystrophin expression in patient fibroblasts, negating the need for myotube reprogramming and greatly streamlining assessment of gene editing proof-of-concepts.

Collectively, this thesis project will demonstrate the efficacy of CRISPR/Cas9 gene editing for restoring full-length dystrophin for the two most prevalent mutations as well as accelerating the development of novel DMD gene therapies.

Background

Duchenne Muscular Dystrophy

Duchenne Muscular Dystrophy (DMD) is the most common pediatric neuromuscular disease, affecting 1 in 5000 boys due to its recessive X-linked inheritance[1]. Individuals experience progressive wasting of skeletal and cardiac muscles throughout the entire body starting from birth[1]. Diagnosis of DMD typically occurs around age five when symptoms begin manifesting, with patients demonstrating delayed motor milestones and muscle weakness[2]. DMD leads to escalating muscle weakness and a complete loss of ambulation around age ten[3]. Without aggressive medical intervention, fatal cardiorespiratory failure occurs in a patient's mid teens to early 20s[3]. There is no cure for DMD.

The role of dystrophin in healthy and dystrophic muscle

The molecular basis for DMD is the loss of functional dystrophin protein within myotubes[1]. Dystrophin is a large, 427 kDa protein composed of 79 exons encoded in 14kb of cDNA[1,4]. Dystrophin is localized to the sarcolemma at the myotube periphery, being a critical component of the dystrophin glycoprotein complex (DGC) (Fig. 1)[5]. The DGC provides myotubes with the necessary structural integrity to avoid damage from repeated muscle contractions[4]. Without dystrophin, the DGC is lost, leaving myotubes vulnerable to damage, culminating in cell death[4]. Hallmarks of DMD pathology are centralized nuclei, inflammation, fibrosis, smaller fibers, and elevated serum creatine kinase[6]. Mutations in the DMD gene, which encodes dystrophin, typically cause DMD[1]. DMD is the largest gene in the human genome at 2.4 Mb[1]. This gene experiences a high mutation rate due to its enormous introns which contain numerous repetitive sequences[7]. To date, 4000 unique mutations have been identified in DMD patients, and approximately a third of all cases arise from spontaneous mutations[8].

CRISPR/Cas9: a genome editing tool

The CRISPR/Cas9 system was discovered in archae and bacteria as an adaptive immune system for viruses[9]. Here, invading viral DNA is added to the host's CRISPR array, transcribed into CRISPR RNA (crRNA), and paired with a trans-activating crRNA (tracrRNA)[9]. The endonuclease, Cas9, targets

a DNA region homologous to the crRNA if a protospacer adjacent motif (PAM) sequence is present (Fig. 2A)[9]. Cas9 then introduces a double strand break (DSB)[9]. To simplify usage, the crRNA and tracrRNA can be combined into a single guide RNA (sgRNA)[9]. Genome editing is accomplished by taking advantage of the two predominant DSB repair systems – non-homologous end joining (NHEJ) and homologous recombination (HR). The primary mechanism is NHEJ, where the two free DNA ends are directly ligated[10]. However, when the ends are incompatible, their processing leads to small insertions or deletions (indels)[10]. HR on the other hand, is used to faithfully repair DNA using a template[10]. Due to the requirement of a template, HR is only active during S and G2 phases of cell division when the genome has been duplicated, limiting its utility in post-mitotic muscle[10]. The cumulative discoveries and advances in CRISPR/Cas9 technology have yielded a flexible tool which enables exceptional precision and efficiency in the manipulation of genomes.

Efficient Cas9-mediated DNA knock-in

Deletions affect the overwhelming majority of DMD patients, being ~70% of cases[1]. Knock-ins can correct these mutations at their genetic source, permanently restoring full-length dystrophin. Cas9 enables exogenous DNA integration when a template is present during repair of the DSB[11]. However, current methods suffer from poor efficiency, particularly in post-mitotic cells like skeletal and cardiac myotubes[12]. Homology-mediate end joining (HMEJ) is a promising system summarized in Fig. 3. Yao et al. used HMEJ to knock-in mCherry into the neurons of mice in vivo with an efficiency of ~52%[12]. Since neurons, like myotubes, are post-mitotic this efficiency may be possible in muscle using HMEJ.

Transcriptional upregulation with catalytically inactive and active Cas9

The utility of CRISPR/Cas9 has been expanded beyond simply cutting DNA and can be used to manipulate gene expression. Typically, this requires a dead Cas9 (dCas9), which is a variant whose nuclease domains have been inactivated by mutations, rendering it unable to cleave DNA but still bind it[13]. Fusing dCas9 to transcription factors allows for sgRNAs to target these factors to promoters and enhancers[13]. Recent studies have demonstrated a catalytically active Cas9 can be used instead of dCas9.

Cas9 DNA cleavage requires sgRNAs of 20 bases or longer, but when shortened to 12-16 bases, these dead sgRNAs (dsgRNAs) prohibit cutting yet retain DNA binding abilities (Fig. 2B)[14,15].

DMD duplication removal through Cas9 genome editing

Around 11% of those afflicted by DMD have duplication mutations[1]. Dystrophin can be restored by removing the duplicated region with two sgRNAs (Fig. 4A). But this necessitates an additional sgRNA and its applicability depends on the presence of a PAM at the duplication junction. We have overcome these limitations with a single sgRNA strategy which cuts at the same intronic point within the original and duplicated DNA (Fig. 4B)[16]. This removes the intervening region, which is equal to the duplication. The entire duplicated sequence is then made available for sgRNA design.

Adeno-associated viruses (AAVs) as a delivery system for CRISPR/Cas9 editing strategies

Adeno-associated viruses (AAVs) will be used to deliver all required Cas9 components to muscle in vitro and in vivo. They are the most promising vehicle for systemic in vivo gene therapies as they are non-pathogenic in humans, cannot replicate when modified, and can transduce dividing and non-dividing cells[17]. These viruses can package ~4.7 kb of single stranded DNA[17]. The AAV9 serotype is applicable to DMD gene therapy because of its ability to efficiently transduce skeletal and cardiac myotubes after local or systemic delivery[18,19]. The AAV-DJ serotype will be used for in vitro work due to its superior transduction of cultured myotubes[20].

Project Objectives and Relevant Experimental Progress

My thesis is divided into “Therapeutic” and “Pre-Therapeutic” sections. I will use two mouse models of DMD patient mutations: one of deletion of exons 52-54 (Δ52-54) by Tatianna Wong and the other a duplication of exons 18-30 (dup18-30) by Daria Wojtal. My objectives are as follows:

Therapeutic:

1) Restore full-length dystrophin through Cas9-mediated HMEJ knock-in of exons 52-54

2) Enhance our DMD exon 18-30 duplication removal strategy using simultaneous dystrophin upregulation in vivo

Screening:

3) Validate DMD duplication removal strategies directly in patient fibroblasts

1) Restore full-length dystrophin by Cas9 mediated HMEJ knock-in of exons 52-54

To explore the feasibility of using HMEJ for knock-ins in muscle, I aim to knock-in exons 52-54 of dystrophin into intron 54 of the DMD gene in our deletion mouse. Small flanking regions of introns 51 and 54 are included to enable correct splicing of the cDNA. First, I needed to identify an sgRNA which could efficiently cut within intron 54. Using the CRISPR design tool CHOPCHOP, I designed and screened six of the best predicted sgRNAs. One sgRNA possessed a cutting efficiency of ~97% as determined by sequencing analysis with the Synthego ICE online tool. Based on this sgRNA, I designed and assembled an HMEJ compatible knock-in construct, where exons 52-54 were flanked by 800 bp homology arms identical to the genomic region at the desired intron 54 insertion point. These homology arms were then flanked by cuts sites for my sgRNA to excise the construct from its vector.

To first validate knock-ins with my HMEJ construct were possible, I transfected murine neuro-2A (N2A) cells with two plasmids: one containing my knock-in construct, the other, a Cas9 with the sgRNA. These plasmids were delivered in various mass-to-mass ratios to assess if varying the proportion of one element of the system relative to the other would have an impact on knock-ins. I was successful in knocking-in exons 52-54 based on PCR analysis and sequencing of the knock-in band (Fig. 6). While this assay was not suitable to draw quantitative conclusions, it was notable there were fewer non-specific bands when the ratio of knock-in construct-to-Cas9 was increased (Fig. 6B). This finding will be considered when optimizing the ratio of the two AAVs for in vivo delivery.

My next step is to test my HMEJ approach in vitro in C_2C_{12} myotubes, allowing assessment of its knock-in efficiency in post-mitotic cells. The two plasmids have been sent for AAV-DJ packaging.

2) Enhance our DMD exon 18-30 duplication removal strategy by simultaneous dystrophin upregulation in vivo

We have previously validated the single sgRNA approach to remove duplications in DMD patient cells and our novel dup18-30 mouse[16,21]. A single AAV9, packaged with both the Cas9 and sgRNA, was delivered systemically via tail injection into neonatal pups. After 15 weeks, significant restoration of full-length dystrophin in skeletal muscles and the heart was observed with corresponding functional improvements (Fig. 5)[16]. However, optimization of this approach is necessary as the phenotype was only partially rescued and the overall dystrophin recovery sat well below ~20% in most muscle groups; which is the estimated minimum protective level[16,22]. Insufficient editing events and/or delivery of the AAV9 to muscle were likely major limitations. However, a sufficiently large increase in dystrophin production from the few edited nuclei may enable dystrophin to reach levels for complete DMD rescue. Our duplication removal approach could remain unaltered if dsgRNAs are used to target the dystrophin muscle promoter for upregulation. The only other addition would be the fusion of two VP64 activation domains to the Cas9 (Fig. 2B). This system would allow for both duplication removal and simultaneous upregulation of the corrected dystrophin using orthologous sgRNA variants.

I initially designed 12 sgRNAs targeting the mouse muscle DMD promoter and co-transfected various sgRNA triplets into N2A cells with dCas9-VPR; a more potent but larger activation system than VP64 (Fig. 7A)[23]. Western blot revealed dystrophin expression with sgRNAs that clustered around the CArG and TATA boxes (Fig. 7B). Next, I swapped the VPR system with dCas9-2xVP64, which was still capable of inducing dystrophin expression using the previous sgRNA combinations (Fig. 7C). Finally, I designed 15 nt dsgRNA variants of my sgRNAs and tested these with dCas9-2xVP64, yielding substantial dystrophin protein (Fig. 7D). I believe the discrepancy in dystrophin expression between my dsgRNA and sgRNA experiments was the result of superior enrichment of successfully transfected N2A cells.

Before moving the system in vivo, I needed to ensure these dsgRNAs do not allow Cas9 to generate DSBs. N2A cells were co-transfected with an active Cas9 and the dsgRNAs either individually or in their triplet combinations. DNA has been extracted and Cas9 cleavage analysis by Synthego ICE will be performed upon resumption of lab operations.

3) Validate DMD duplication removal and cDNA knock-in strategies directly in patient fibroblasts

The feasibility of genome editing approaches for DMD are initially evaluated by their restoration of dystrophin protein expression in DMD patient cells. This in vitro analysis is a critical step for moving into in vivo experimentation. Fibroblasts are commonly provided by DMD patients, as this circumvents the need to generate a cell model of the mutation. These cells are then corrected with the desired strategy. Fibroblasts however do not express dystrophin, which is problematic since dystrophin is the primary readout of these in vitro experiments. To overcome this, fibroblasts undergo a lengthy conversion into dystrophin expressing myotubes. First, they are transdifferentiated into myoblasts by MyoD overexpression for two weeks, then differentiated for an additional week into myotubes through serum reduction (Fig. 8)[24,25]. Thus, approximately three weeks are necessary before the myotubes are ready to be analyzed for dystrophin restoration.

This is an extremely time consuming and tedious process that has numerous points for costly errors to be made. If dystrophin can instead be produced by these patient fibroblasts, the entire reprogramming process can be circumvented. I aim to use dCas9-VPR to induce dystrophin expression in human fibroblasts, which will enable the rapid in vitro validation of DMD editing strategies.

After my success with upregulating dystrophin in N2A cells, I redesigned my 12 mouse sgRNAs to target the same regions within the human dystrophin muscle promoter. Initially, various sgRNA triplets were transfected with dCas9-VPR into human embryonic kidney (HEK) 293T cells. Dystrophin transcripts were detectable but no protein products (Fig. 9A, B). Interestingly, it takes ~16 hours just to transcribe one copy of dystrophin, while HEK293T double every ~22 hours[26,27,28]. It was possible that the dystrophin protein was unable to accumulate prior to division which is why none was

detected. I repeated the experiment with two conditions: incubation at 37℃ and at 33℃ to slow HEK293T growth. This still failed to yield dystrophin (Fig. 9C, D).

My next approach was to attempt upregulation directly in human fibroblasts, as their ability to be reprogrammed into muscle suggested they may be more amenable to VPR induction of dystrophin. I first needed to optimize an electroporation protocol as we had been unable to efficiently electroporate the large, 8.6 kb, dCas9-VPR construct into these cells. Using an 8 kb EGFP plasmid, I screened six different fibroblast electroporation conditions. Two were particularly efficient and will be used for delivering the dCas9-VPR and sgRNA plasmids once lab work restarts.

Rationale

There is a great unmet need for effective DMD therapies as most medical interventions are restricted to symptom management or delaying disease progression[29]. No curative therapy exists, although a few antisense oligo nucleotide (AON) strategies for exon skipping have become available[30]. Unfortunately, AONs have significant limitations, particularly in their transient nature requiring consistent re-administration to maintain dystrophin expression and whose levels are still quite low, ~1% or less[30]. DMD cannot be effectively treated without correcting its genetic source, which CRISPR/Cas9 can do. The overwhelming majority of DMD cases are caused by deletions (70%) or duplications (11%), thus I will focus on correcting these[1]. Many genome editing approaches for DMD currently utilize exon deletion or exon skipping with Cas9. This restores the reading frame, yielding a truncated, partially functional dystrophin. The result is conversion of DMD to the less severe Becker Muscular Dystrophy (BMD), which has a milder course than DMD but is not ideal as it is still a life-limiting disease[31]. By restoring full-length dystrophin, complete functionality is retained and may allow for enhanced recovery while avoiding conversion to BMD. Thus, I propose utilizing CRISPR/Cas9 to restore clinically relevant levels of full-length dystrophin in deletion and duplication DMD mouse models.

Owing to the size of the DMD gene and its inherently elevated mutation rate, approximately 4000 unique mutations in DMD patients have been documented[8]. This means a significant proportion of genome editing strategies will need to be personalized and tailored to each patient. A large catalog of corrective therapies will be necessary, but this demand cannot be met in any realistic timeframe with the current development pipeline. Several crucial steps are particularly time consuming and difficult which limits the speed of therapy development. One bottleneck that can be addressed with CRISPR/Cas9 is the movement from in vitro to in vivo assessment. Subsequently, I aim to accelerate the rate at which genome editing approaches for DMD can be produced by streamlining the in vitro validation phase of the development pipeline.

Specific Aims

Aim 1: Restore full-length dystrophin by Cas9 mediated HMEJ knock-in of exons 52-54

I aim to develop an efficient HMEJ approach to knock-in dystrophin exons 52-54 through in vitro and in vivo analyses. In dividing N2A cells, I have demonstrated knock-in of exons 52-54 with an HMEJ construct is feasible. This approach must next be evaluated in non-dividing myotubes followed by in vivo delivery into our Δ52-54 DMD mice to assess therapeutic efficacy.

Methods:

For in vitro knock-in experiments, differentiated C_2C_{12} mouse myotubes will be utilized. I have sent the necessary plasmids off for viral packaging and am awaiting shipment of the AAV-DJ vectors. C_2C_{12} myotubes will be transduced with both AAV-DJs: one carrying the Cas9 with sgRNA and the other the HMEJ knock-in construct. The ratio of the two viruses will be varied to identify the best combination. Knock-ins will be first validated through PCR and sequencing of the knock-in amplicon, with knock-in frequency calculated through digital droplet PCR (ddPCR).

My HMEJ system will be repackaged into dual AAV9 vectors for in vivo experiments. To assess HMEJ feasibility in muscle I will first proceed with local intramuscular (IM) injection of the

tibialis anterior (TA) in 4 week old wild-type (WT) control and Δ52-54 DMD mice. A GFP encoding AAV9 will serve as the negative control. Three weeks post-injection, the mice will be sacrificed. DNA, RNA, and protein will be isolated from the treated TA. Knock-ins will be validated by PCR and RT-PCR while knock-in rate will be determined by ddPCR. Dystrophin restoration will be evaluated through western blot and immunofluorescence (IF). Muscle histology will be analyzed via H&E staining on cryostat prepared muscle sections.

Following this, systemic delivery of the dual AAV9s will be performed via tail vein injection in P1 mice. Seven weeks later, mice will be functionally assessed through grip strength, open field tests, and specific tetanic force prior to being sacrificed. Several critical muscles will be isolated (TA, triceps, diaphragm, and heart) as well as the liver. Identical analysis as done for IM injected TA, will be performed on the isolated muscles. IF of DGC components will also be conducted to assess DGC restoration and the viral load in muscles and liver will be evaluated by quantitative PCR.

Expected outcomes and mitigation strategies:

For my in vitro assays, I anticipate knock-in rates of approximately 5-8%. While low, this is consistent with neuronal in vitro data obtained by Yao et al.[12]. As IM injection provides the highest concentration of AAVs, I anticipate robust knock-in rates and dystrophin recovery with local delivery. If HMEJ behaves similarly in muscle as in neurons, knock-in rates approaching 50% should be seen; though the multi-nucleated nature of myotubes may cause editing rates to fall short of this.

Systemic treatment dilutes the AAVs throughout the body, thus, decreases in knock-in rates and dystrophin restoration are expected. Despite this, I hypothesize significant phenotypic improvements if dystrophin expression remains above the minimum protective level of ~20%. In the event of poor dystrophin expression and phenotype recovery, the knock-in construct will be modified to enhance efficiency. Revisions would include shortening or lengthening the homology arms, re-orienting the sgRNA sites in the construct, and targeting a different region within intron 54.

Aim 2: Enhance our DMD exon 18-30 duplication removal strategy by simultaneous dystrophin upregulation in vivo

Our lab has validated that duplication removal with one sgRNA can, to some degree, rescue the dystrophic phenotype of dup18-30 mice[16]. To improve this strategy, I aim to simultaneously correct the exon 18-30 duplication and upregulate the corrected dystrophin protein using a single Cas9 coupled with orthologous sgRNA/dsgRNAs. Thus far, I have been successful in upregulating dystrophin in vitro, but before I can assess the system in vivo I need to verify the dsgRNAs prevent Cas9 DSBs.

Methods:

To confirm my dsgRNAs do not induce DSB formation, I have co-transfected each dsgRNA individually and in triplet combinations with an active Cas9 into N2A cells. DNA was extracted, which will be sequenced, and analyzed by Synthego ICE to determine the targeting efficiency of each dsgRNA.

Moving this approach in vivo will require packaging the constructs into two AAV9s. One will encode the Cas9-2xVP64, and the other the sgRNA for duplication removal as well as dsgRNAs 9, 10, and 11. Recent studies have revealed that for a two vector system, there is specific depletion of the AAV carrying multiple sgRNAs[32-34]. The cause warrants investigation, but Zhang et al. demonstrated that a self-complementary AAV (scAAV), which has a double-stranded genome, does not suffer disproportionate loss[32]. Thus, a scAAV9 will be used for the sgRNA/dsgRNA vector. The AAV9 pair will be delivered systemically via tail vein injection into P1 WT and dup18-30 DMD mice. My combinatorial approach will be compared to the original single AAV9 duplication removal strategy, with a GFP AAV9 serving as a negative control. Seven week old mice will be evaluated identically to those undergoing systemic delivery in Aim 1, with the exception that DNA analysis will identify the rate of duplication deletion.

Expected outcomes and mitigation strategies:

Based on several studies, dsgRNAs of 15 nt completely prevent Cas9 from cleaving DNA, therefore I expect editing efficiencies of 0% for all dsgRNAs. In the event any editing is detected, I will shorten the offending dsgRNA(s) to 14 nt and re-evaluate both cutting potential and dystrophin upregulation in N2A cells. I anticipate superior dystrophin recovery in all muscle groups and phenotype rescue with the combined editing/upregulation compared to our original editing only strategy. Our lab has extensive experience performing the in vivo data collection and analysis necessary for this project, and I do not anticipate any problems here. If improvement is not observed with the combinatorial treatment at seven weeks, I will analyze the mice at four and ten weeks old to assess if the approach is transient or requires additional time to manifest.

Aim 3: Validate DMD duplication removal and cDNA knock-in strategies directly in patient fibroblasts

The ability to upregulate dystrophin in N2A cells is extremely promising and suggests it will be possible to induce expression in patient non-muscle cells. Although my current approaches in HEK293T cells have been unsuccessful, I will be attempting upregulation directly in fibroblasts next,

Methods:

Electroporation with the Neon transfection system will be used to deliver the dCas9-VPR and sgRNA plasmids into WT human fibroblasts. I have previously determined the optimal system parameters to electroporate the large dCas9-VPR plasmid into these cells. All sgRNA triplets evaluated in HEK293T cells will be tested. Protein and RNA will be extracted after five days of upregulation and puromycin selection. Dystrophin expression will be assessed by western blot and RT-PCR.

My next step will be assessing if this system can detect dystrophin recovery in DMD patient fibroblasts by CRISPR/Cas9 genome editing. We have three patient fibroblast lines with unique DMD duplications, but all share a common region of intron 6. I will design an sgRNA which efficiently cuts within this region and assess duplication removal in the all patient fibroblasts. However, the active Cas9 for duplication removal can utilize the upregulation sgRNAs to cut the dystrophin promoter.

Thus, I will make 15 nt dsgRNA variants of the promoter targeting sgRNAs and assess their ability to induce dystrophin expression and prevent DBS generation with Cas9-VPR. Patient cells will be electroporated with two plasmids: one carrying an active Cas9-VPR, and another encoding the three dsgRNAs for upregulation and intron 6 targeting sgRNA. Five days post-treatment, DNA and protein will be isolated, with editing validated through PCR and dystrophin restoration by western blot.

Expected outcomes and mitigation strategies

Currently, I have been unable to express dystrophin in HEK293T cells, but fibroblasts may allow dystrophin expression. Thus, I expect to observe dystrophin protein in these human fibroblasts with one of my sgRNA combinations. If dystrophin remains undetected, I will extend the duration of upregulation to eight days. If protein still cannot be observed there are a plethora of other Cas9 activation systems I can utilize such as the $P300^{core}$ acetylase or TET1 demethylase[35,36]. As this will remain an in vitro assay, with no need for AAV delivery, the size of the Cas9 fusion proteins are not a concern. In our DMD patient fibroblasts, I anticipate that efficient duplication removal and detectable levels of dystrophin protein will be observed after delivery of the Cas9 activation system.

Summary and Potential Impact on the Field

DMD is the most common pediatric neuromuscular disease which impacts 1 in 5000 boys[1]. It is fatal and no cure or effective treatment is available[1]. This thesis will establish valuable pre-clinical data for the first efficient knock-in DMD therapy and a novel combinatorial approach to treat those with DMD duplications. Together, these two strategies could treat approximately 80% of DMD patients. Furthermore, the streamlining of the DMD genome therapy development pipeline will help lay the groundwork to meet the demand of the vast array of DMD mutations. This, together with effective CRISPR/Cas9 approaches to restore full-length dystrophin in vivo, will help advance the clinical translation of therapeutic genome editing for those afflicted by DMD.

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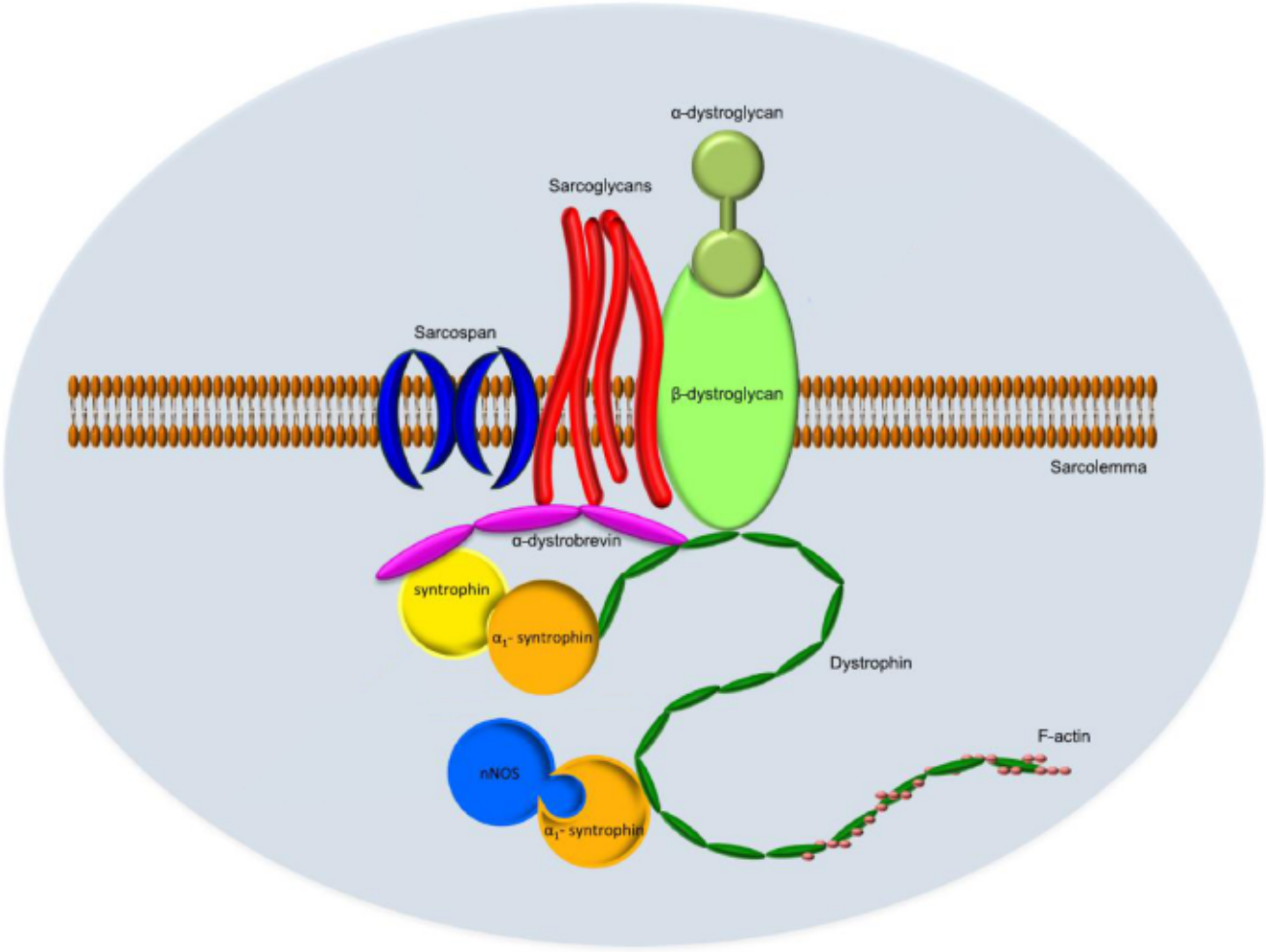

Figure 1. The dystrophin glycoprotein complex[5]. The dystrophin glycoprotein complex (DGC) is located at the sarcolemma of skeletal and cardiac muscle. Dystrophin pays a significant role in assembling and stabilizing the DGC.

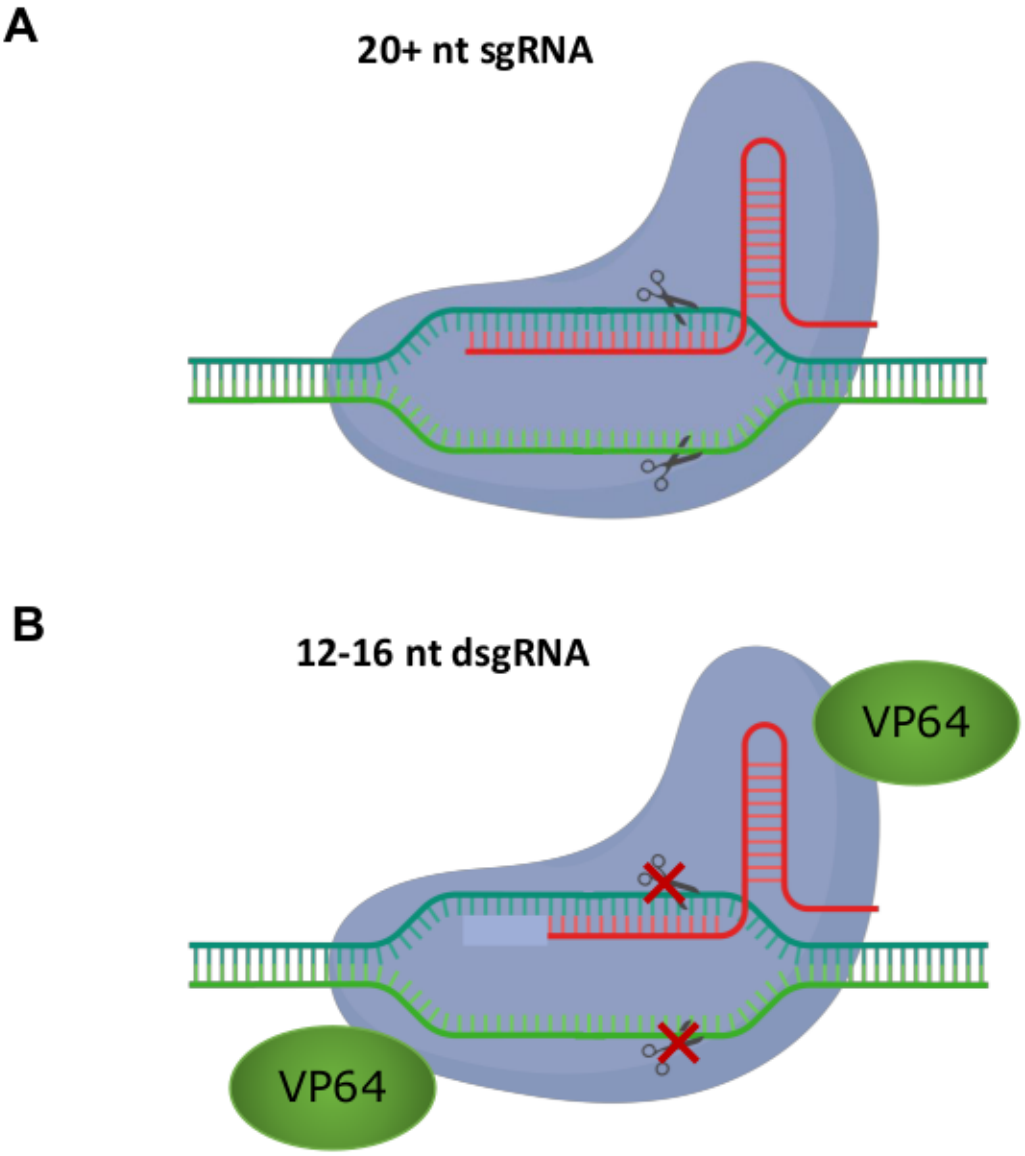

Figure 2. DNA cleavage and gene upregulation using the CRISPR/Cas9 system. Adapted from Lian et al.[9,13]. The CRISPR/Cas9 system enables the manipulation of genomic material. Hybridized crRNA and tracrRNA constitute the sgRNA in red. The sgRNA contains a sequence homologous to a region of the DNA immediately upstream of the PAM. Association of Cas9 with the sgRNA allows targeting of this DNA sequence and separation of the DNA strands (A) With a 20+ nucleotide (nt) homology region, Cas9 will cut both DNA strands using its two endonuclease domains, generating a DSB that can be manipulated to yield various desired editing events. (B) With a 12-16 nt sgRNA homology region, Cas9 will still bind the targeted DNA region but not cut. Therefore, using shortened sgRNAs, transcriptional activators (such as VPR or VP64) can be localized to DNA loci when fused to Cas9.

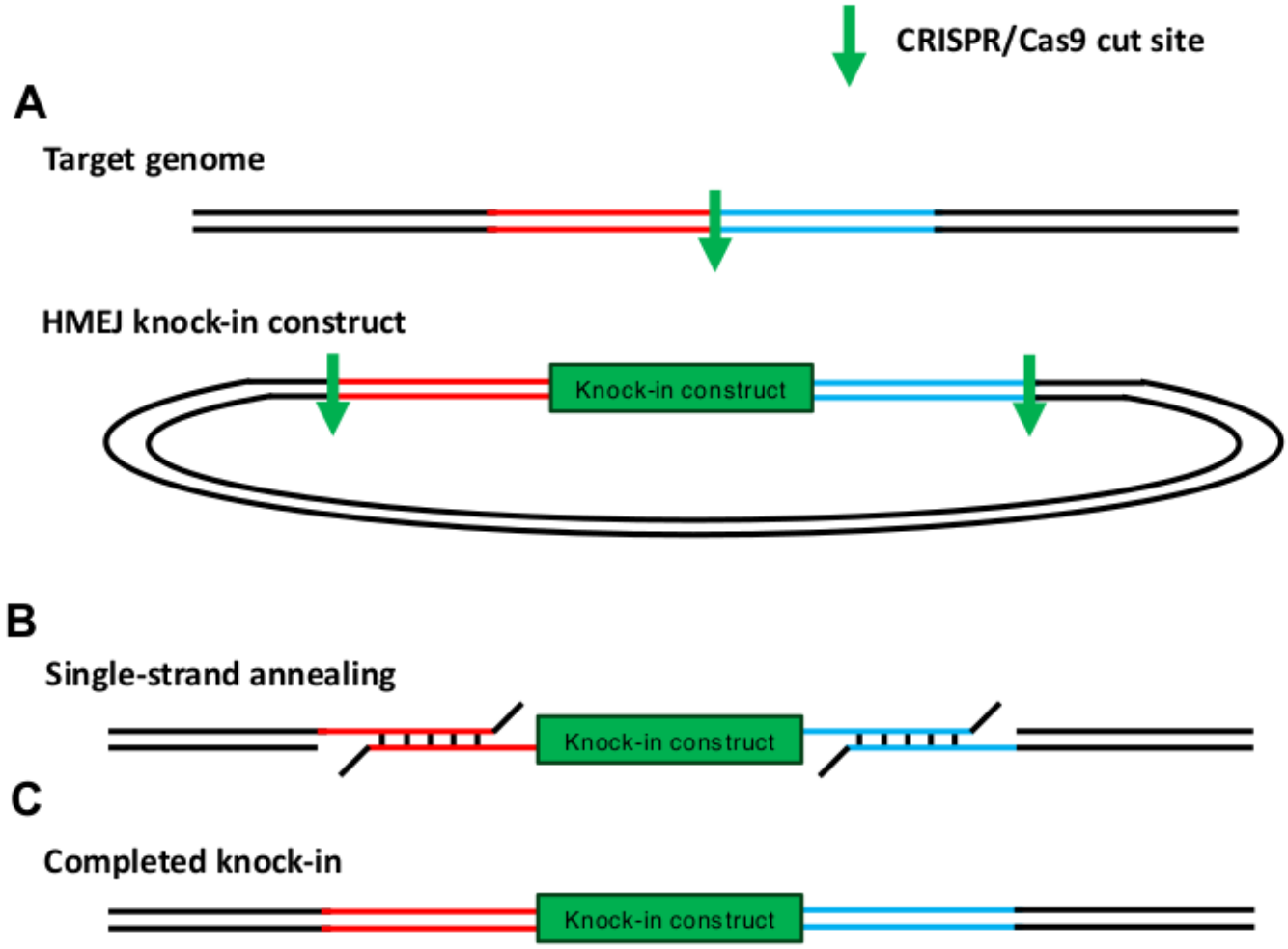

Figure 3. Current predicted mechanism of homology-mediated end joining (HMEJ) knock-in with CRISPR/Cas9[12]. Presented is a schematic of the current understanding of Cas9-mediated HMEJ knock-in events. The blue and red DNA are 800 base pairs long and homologous to regions of the same colour. The green arrows represent CRISPR/Cas9 cleavage sites using the same sgRNA. A) First, the desired insertion site in the genome is cut by CRISPR/Cas9. Simultaneously, the HMEJ knock-in construct is excised from its delivery vector by CRISPR/Cas9 cleavage at the ends of the flanking homology arms. B) Directional exonuclease activity degrades a single strand from the homology regions in the genome and knock-in construct. Annealing between complementary single-stranded homology regions of the genome and knock-in construct follows. C) Unannealed flaps are removed, gaps are filled by DNA polymerase, and ligase seals the DNA backbone to complete the HMEJ knock-in of the desired construct.

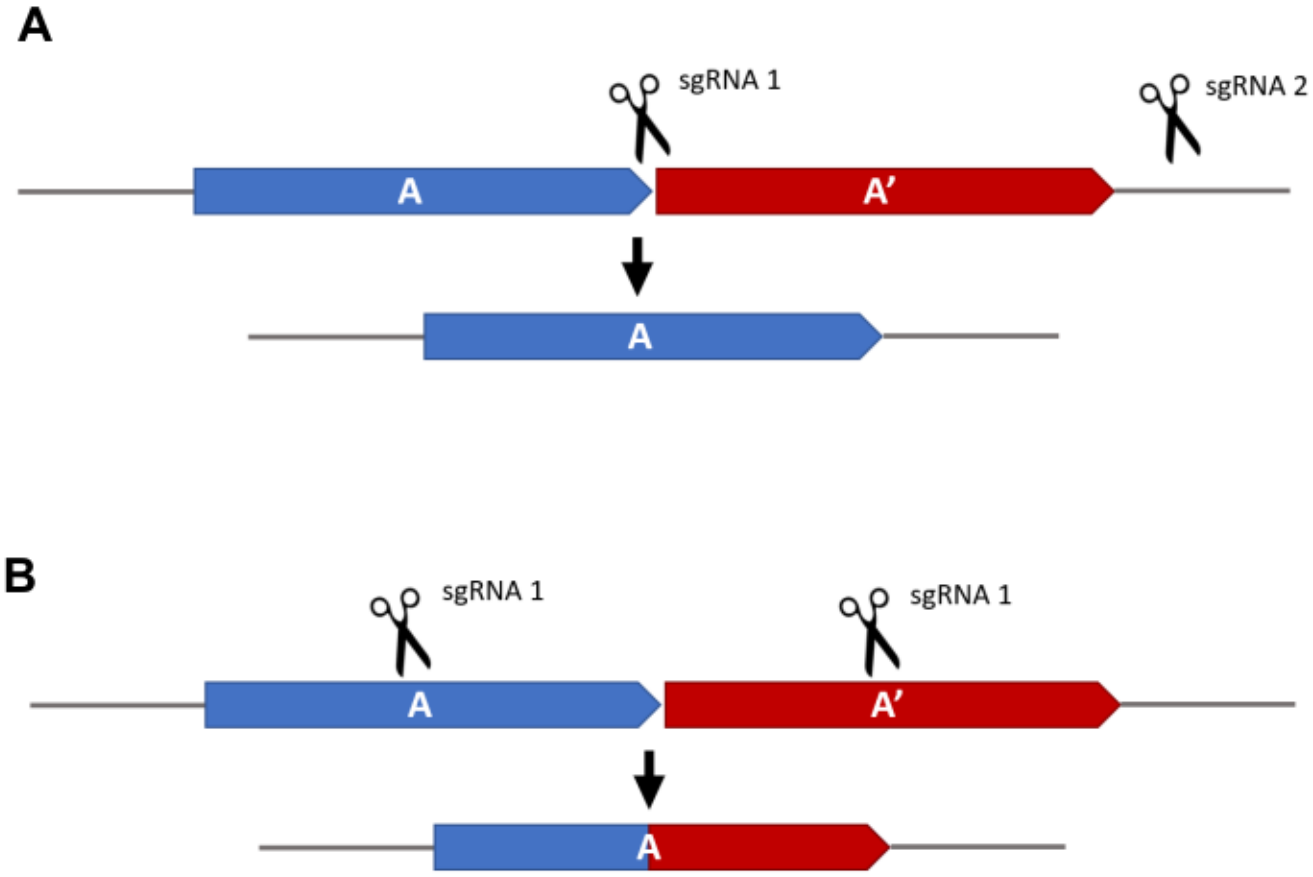

Figure 4. Double and single sgRNA strategies for correcting DNA duplications with CRISPR/Cas9[16]. In the above diagram, a wild-type DNA region labelled A in blue experiences a tandem duplication labelled *A'* in red. A) A schematic of a dual sgRNA duplication removal approach. Here sgRNA 1 cuts at the duplication junction whereas sgRNA 2 cuts after the duplicated region, excising the intervening duplication in its entirety. B) A schematic of a single sgRNA duplication removal approach. Here sgRNA 1 targets an intronic region within the wild-type sequence which is also targeted in the duplication. Upon cutting, the intervening region is removed with the corrected loci being a hybridization of the original and duplicated sequences.