Development of Novel Splice Modulation Therapies for Muscular Dystrophy

by

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Abstract

The muscular dystrophies are a heterogeneous group of over 30 genetic diseases which are characterized by progressive weakening and deterioration of muscle tissue and which vary with respect to age of onset, pattern of muscle involvement, and severity. To date, no effective therapeutic options exist for either halting or reversing disease progression for any form of muscular dystrophy, although some emerging strategies are promising.

Antisense-mediated exon skipping therapy uses synthetic molecules called antisense oligonucleotides to modulate splicing, allowing exons harboring or near genetic mutations to be removed and the open reading frame corrected. Antisense-mediated exon skipping has made significant progress as a therapeutic platform in recent years, especially in the case of Duchenne muscular dystrophy (DMD). Despite FDA approval of eteplirsen – the first-ever antisense drug clinically marketed for DMD – exon skipping therapy still faces significant hurdles, including limited applicability and unknown function of truncated proteins.

Key to the success of an exon skipping strategy is the identification of appropriate exon targets – exons which are dispensable in terms of the stability and function of the resulting truncated proteins. In the case of DMD, in-frame exon skipping of *DMD* exons 45-55 represents a significant approach to treating DMD, as patients harboring *DMD* exons 45-55 deletion mutations are reported to have exceptionally mild to asymptomatic phenotypes. Additionally, a large proportion of patients harbor mutations within this "hotspot" region.

The dysferlinopathies are another form of muscular dystrophy, caused by mutations in the *dysferlin* (*DYSF*) gene which render dysferlin protein unable to facilitate plasma membrane repair. Unlike *DMD*, there is no reported mutation hotspot in *DYSF*, and it is largely unknown which exons are potentially amenable to exon skipping and which are not.

In this work, we sought to establish novel antisense-mediated exon skipping therapeutic approaches for two major forms of muscular dystrophy: Duchenne muscular dystrophy and dysferlinopathy. Here, we demonstrate that a cocktail of antisense oligonucleotides can

effectively skip *DMD* exons 45-55 *in vitro* in myotubes transdifferentiated from DMD patient fibroblast cells. We also report that removal of *DYSF* exons 26-27 or 28-29 does not impair plasma membrane resealing in dysferlinopathy patient fibroblasts, and a cocktail of antisense oligos is able to achieve multi-exon skipping of *DYSF* exons 28-29 and rescue plasma membrane resealing in patient cells. This is the first report of substantive *DMD* exons 45-55 skipping in DMD patient cells, as well as the first description of exons 26-27 and 28-29 as exon skipping targets in *DYSF*.

Taken together, these findings help validate the feasibility of *DMD* exons 45-55 skipping in DMD patients and *DYSF* exons 26-27 and 28-29 skipping in dysferlinopathy patients as potential therapeutic platforms for future translation into clinical practice.

This work also describes efforts undertaken by our lab to elucidate the underlying mechanisms behind the generation and expansion of dystrophin-positive revertant fibers (RFs) in *mdx* and *mdx52* mouse models of DMD. We assessed the number of RFs in these mice and determined that mutation types and aging differently affect RF expansion in *mdx* and *mdx52* mice. An understanding of how these rare dystrophin-positive fibers are generated and expand could be very beneficial to the development of future DMD therapies.

Preface

Topics covered in Chapter 1 are taken from published review articles written by Joshua Lee (Lee and Yokota 2013, Touznik, Lee, and Yokota 2014) and are reproduced here with permission.

Chapter 3 contains original work performed by Joshua Lee in collaboration with other members of the Yokota Lab: Yusuke Echigoya and Merryl Rodrigues. In this work, Yusuke Echigoya and Merryl Rodrigues performed cryosectioning of mouse tissues, immunohistochemical analysis of revertant fibers - with the associated statistical analysis - and co-wrote the manuscript. Joshua Lee performed HE staining of histological sections, imaging of HE histological sections, counting of centrally-nucleated fibers (CNFs), statistical analysis of CNFs, and co-wrote the corresponding manuscript. Mice were maintained by the Health Sciences Laboratory Animal Services (HSLAS) at the University of Alberta. The work is published in PLoS One (Echigoya *et al.* 2013) and is reproduced here with permission.

Chapter 4 contains original work conducted by Joshua Lee. Data in this chapter has been published in PLoS One (Lee, Echigoya, et al. 2018) and is reproduced here with permission.

Chapter 5 contains original work carried out by Joshua Lee and a corresponding manuscript is currently in preparation for submission to a scientific journal.

Original work carried out by Joshua Lee is also found in the appendix and is not currently being considered for publication.

All experimental work involving human patient samples or animals was approved by The Research Ethics Office of the University of Alberta. All animal work was conducted according to relevant national and international guidelines. Animal studies were approved by the Ethics Committee for the Treatment of Laboratory Animals of the National Center of Neurology and Psychiatry, and the Animal Care and Use Committee (ACUC) of the University of Alberta. All animals were euthanized by cervical dislocation by trained personnel.

This thesis is dedicated to Tenille Lee and Jeanne Lee for their sacrifice in my behalf and for their unfailing faith, love, and support.

In loving memory of my father, Kenneth Lee.

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Joshua Lee
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List of Abbreviations and Symbols

°C	Degrees Celsius
µM	Micrometer
µl	Microliter
AO	Antisense oligonucleotide
ALS	Amyotrophic lateral sclerosis
bp	Base pair
cDNA	Complementary deoxyribonucleic acid
CO2	Carbon dioxide
CNF	Centrally-nucleated fiber
CSF	Cerebrospinal fluid
DGC	Dystrophin-glycoprotein complex
DAPI	4′,6-diamidine-2-phenylindole
DM	Myotonic dystrophy
DMAT	Distal myopathy with anterior tibial onset
DMD	Duchenne muscular dystrophy
DMD	Human dystrophin gene
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
DYSF	Human dysferlin gene
eMHC	Embryonic myosin heavy chain
FBS	Fetal bovine serum
FCMD	Fukuyama congenital muscular dystrophy
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase

GC	Gastrocnemius
GFP	Green fluorescent protein
HD	Huntington's disease
HE	Hematoxylin and Eosin
LGMD2B	Limb-girdle muscular dystrophy type 2B
mL	Milliliter
MM	Miyoshi Myopathy
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
RF	Revertant fiber
RT-PCR	Reverse transcription polymerase chain reaction
RNA	Ribonucleic acid
RT	Room temperature
S.D.	Standard deviation
S.E.	Standard error
SMA	Spinal muscular atrophy
TA	Tibialis anterior
WT	Wildtype

CHAPTER 1: GENERAL INTRODUCTION

1 Chapter 1: General introduction

Antisense therapy is an approach to fighting diseases using short DNA-like molecules called antisense oligonucleotides (AOs). Recently, antisense therapy has emerged as an exciting and promising strategy for the treatment of various neurodegenerative and neuromuscular disorders (Lee and Yokota 2013, Rinaldi and Wood 2018). Previous and ongoing pre-clinical and clinical trials have provided encouraging results (Stein and Castanotto 2017). Spinal muscular atrophy (SMA), Huntington's disease (HD), amyotrophic lateral sclerosis (ALS), Duchenne muscular dystrophy (DMD), Fukuyama congenital muscular dystrophy (FCMD), dysferlinopathy (including limb-girdle muscular dystrophy 2B; LGMD2B, Miyoshi Myopathy; MM, and distal myopathy with anterior tibial onset; DMAT), and myotonic dystrophy (DM) are all reported to be promising targets for antisense therapy (Hua *et al.* 2007, Ottesen 2017, Kordasiewicz *et al.* 2012, Miller *et al.* 2013, Echigoya, Aoki, *et al.* 2015, Aartsma-Rus and Krieg 2017, Taniguchi-Ikeda *et al.* 2011, Dominov *et al.* 2014, Barthelemy *et al.* 2015, Jauvin *et al.* 2017). The overarching goal of this study was to identify novel therapeutic approaches for two major forms of muscular dystrophy: Duchenne muscular dystrophy and dysferlinopathy. To this end, we performed a number of *in silico* and *in vitro* analyses to determine whether AOs could facilitate exon skipping, rescue protein expression, and restore protein functionality. This thesis will demonstrate how cocktails of antisense drugs can facilitate multi-exon skipping in DMD and dysferlinopathy patient cells, as well as describe two novel therapeutic exon skipping targets for treating dysferlinopathy.

This thesis will also describe the effect of mutation types and aging on the expansion of dystrophin-positive revertant fibers (RFs) in two mouse models of DMD, in an effort to better understand the nature of these rare muscle fibers so that future therapies can be developed.

1.1 Overview of Duchenne muscular dystrophy, dystrophin, and the DGC

DMD is a lethal, recessive X-linked genetic disorder affecting about 1 in 3500 - 5000 males worldwide (Mah *et al.* 2014, Mendell *et al.* 2012). Characteristics of the disease include progressive deterioration of the skeletal muscles which results in delayed motor milestones (e.g. sitting, walking, talking), proximal weakness, hypertrophied calves, markedly elevated serum creatine kinase levels, and development of serious muscle weakness (Duchenne 1867). Affected children present with symptoms of muscle weakness as early as 2 years old, losing ambulation and becoming wheelchair-dependent at around puberty. Most patients eventually require assisted ventilation. In addition to the deterioration of the skeletal muscles, DMD is also hallmarked by progressive cardiomyopathy which presents clinically during early adolescence and is the main cause of death (Barber *et al.* 2013, Cheeran *et al.* 2017). The majority of patients affected by DMD pass away during their late teens to mid-thirties (Kieny *et al.* 2013). Mortality typically occurs due to cardiac problems or respiratory failure, although improvements in treating respiratory complications that are consequential to DMD, such as through the use of assisted ventilation, have significantly increased the survival of DMD patients (Yiu and Kornberg 2015, Bushby *et al.* 2010, Passamano *et al.* 2012).

DMD is mostly caused by deletions (~68% of cases), duplications (~11%) and small mutations, such as point mutations, splice site mutations and small intra-exon deletions (25-30%) in the dystrophin (*DMD*) gene, resulting in an absence of functional dystrophin protein (Hoffman, Brown, and Kunkel 1987, Juan-Mateu *et al.* 2015, Aartsma-Rus, Ginjaar, and Bushby 2016). The *DMD* gene is the largest known gene in humans, comprising 79 exons and spanning 2.2 megabases (Koenig *et al.* 1987). There exists phenotypic variation with respect to mutation pattern, with patients having the same mutation exhibiting a range of clinical severity, even within the same family (Ginjaar *et al.* 2000). Becker muscular dystrophy (BMD) is also caused by mutations in the DMD gene, but it is five times less frequent and is a milder form of the disease, characterized by mutations which generally maintain the reading frame. These in-frame

mutations can include deletions which result in truncated dystrophin protein that retains some functionality (Blake *et al.* 2002, Flanigan 2014). Studies involving BMD patients have speculated that as little as 10% of wild-type dystrophin levels are sufficient to ameliorate pathology (Anthony *et al.* 2011, van den Bergen *et al.* 2014), although it seems that mutation pattern and resulting protein function may be more important than the level of protein expression, since some patients harboring in-frame mutations developed more severe pathologies compared to patients having similar protein levels but with different in-frame mutations (Kaspar *et al.* 2009).

Dystrophin protein plays an essential role in maintaining muscle fiber integrity by linking the cytoskeleton of the muscle fiber and the extracellular matrix through the organization of a multiprotein complex known as the dystrophin-glycoprotein complex (DGC), located near the cell membrane (Rybakova, Patel, and Ervasti 2000, Watkins *et al.* 2000, Koenig, Monaco, and Kunkel 1988). There are four major functional domains in dysferlin: an actin-binding N-terminal domain, a central rod domain, a cysteine-rich domain, and a C-terminal domain (Gao and McNally 2015). Dystrophin connects to the subsarcolemmal actin network through binding of F-actin at its N-terminus (Way *et al.* 1992). The flexible central rod domain is composed of 24 spectrin repeats interspersed by four proline-rich spacers (called "hinges") and harbors an additional actin-binding motif (Broderick and Winder 2005, Koenig, Monaco, and Kunkel 1988, Amann, Renley, and Ervasti 1998, Koenig and Kunkel 1990). Spectrin repeats 20-23 mediate dystrophin's interaction with microtubules (Belanto *et al.* 2014, Prins *et al.* 2009). Dystrophin's central rod domain is also capable of binding phospholipids, facilitating targeting to the plasma membrane (Le Rumeur *et al.* 2003). The fourth hinge at the end of the rod domain contains a WW domain which, along with two adjacent EF-hands (located in the cysteine-rich domain), anchors dystrophin at the sarcolemma through direct binding with β-dystroglycan (Ilsley, Sudol, and Winder 2002, Rentschler *et al.* 1999). Within the cysteine-rich domain is a zinc finger (Znf) domain which binds calmodulin (Anderson, Rogers,

and Jarrett 1996). The cysteine-rich domain also binds ankyrin-B (Ayalon *et al.* 2008) and synemin (Bhosle *et al.* 2006). The C-terminus contains binding sites for dystrobrevin and syntrophin (Sadoulet-Puccio, Rajala, and Kunkel 1997).

There are three major components or groups which comprise the DGC, each with their own protein constituents: cytoplasmic (dystrophin, dystrobrevin, syntrophins, neuronal nitric oxide synthase); transmembrane (β-dystroglycan, sarcoglycans, sarcospan); and extracellular (α-dystroglycan) (Gao and McNally 2015).

α-dystroglycan and β-dystroglycan are translated from the same transcript and then processed into separate proteins (Ibraghimov-Beskrovnaya *et al.* 1992). α-dystroglycan acts as a receptor for extracellular ligands is closely associated with the transmembrane protein β-dystroglycan, which interacts with dystrophin (Gao and McNally 2015). α-dystroglycan is glycosylated on serine residues (O-glycosylation) and mutations which disturb the normal glycosylation of α-dystroglycan are associated with a spectrum of neuromuscular disorders referred to as dystroglycanopathies (Buysse *et al.* 2013, Carss *et al.* 2013, Di Costanzo *et al.* 2014, Longman *et al.* 2003, Ogawa *et al.* 2013, Willer *et al.* 2012). Dystroglycan also plays a major role in embryonic development and mice null for dystroglycan display embryonic lethality (Williamson *et al.* 1997).

Closely associated with β-dystroglycan is the sarcoglycan subcomplex, which is composed of four transmembrane proteins: α-sarcoglycan β-sarcoglycan γ-sarcoglycan and δ-sarcoglycan (Gao and McNally 2015). Loss of any particular sarcoglycan subunit causes instability of the complex and mutations in α-, β-, γ-, and δ-sarcoglycan result in limb-girdle muscular dystrophy type 2C-2F (LGMD2C-2F), respectively (Gao and McNally 2015).

Sarcospan (SSPN) is a small (25-kDa) transmembrane protein associated with the sarcoglycans and interacts with the α7β1 integrin complex (Crosbie *et al.* 1997, Crosbie *et al.*

1999, Marshall, Chou, *et al.* 2012, Marshall *et al.* 2015). Sarcospan also plays an important role in development of the muscular ventricular septum of the heart through interaction with the cardiac transcription factor NKX2-5 (Panzer *et al.* 2017). It has been demonstrated that overexpression of sarcospan increases the cell membrane localization of all three skeletal muscle adhesion complexes – the DGC, the utrophin-glycoprotein complex (UGC), and the α7β1 integrin complex (Peter, Marshall, and Crosbie 2008, Marshall, Holmberg, *et al.* 2012, Marshall *et al.* 2013). This makes sarcospan an important potential therapeutic target, as the upregulation of compensatory adhesion complexes can ameliorate dystrophic pathology that occurs in the absence of dystrophin protein (Rafael *et al.* 1998, Tinsley *et al.* 1998, Gilbert *et al.* 1999, Fisher *et al.* 2001, Burkin *et al.* 2005). In the *mdx* mouse model of DMD, overexpression of human sarcospan increases membrane expression of the UGC and α7β1 complex, increasing membrane stability and reducing markers of disease pathology (Peter, Marshall, and Crosbie 2008, Marshall, Holmberg, *et al.* 2012). Overexpression of mouse SSPN increased membrane stability and improved both muscle and pulmonary function in *mdx* mice (Gibbs *et al.* 2016).

Along with dystrophin, the other members of the cytoplasmic portion of the DGC are dystrobrevin, syntrophins, neuronal nitric oxide synthase. α-Dystrobrevin interacts with dystrophin at their respective C-terminals and its association with dystrophin anchors α-dystrobrevin to the sarcolemma (Blake *et al.* 1995). α-Dystrobrevin also interacts with the sarcoglycans and syntrophins (Newey *et al.* 2000, Yoshida *et al.* 2000). Although the DGC does not display significant abnormalities in α-dystrobrevin-deficient mice, mice develop skeletal and cardiac myopathies, with neuromuscular junction (NMJ) defects, irregular myotendinous junctions, and mislocalization of nNOS (Grady *et al.* 2003, Grady *et al.* 2000, Grady *et al.* 1999). To-date, there have been no reports of mutations in dystrobrevin causing neuromuscular disease.

Syntrophin associates with α-dystrobrevin and dystrophin and is involved in coordinating the signaling molecules nNOS, Grb2, calmodulin, and stress-activated protein kinase-3 (Brenman *et al.* 1996, Hasegawa *et al.* 1999, Madhavan and Jarrett 1999, Madhavan, Massom, and Jarrett 1992, Oak *et al.* 2001). Similar to α-dystrobrevin, mice with mutations in syntrophin show NMJ defects and mislocalization of nNOS, in addition to loss of utrophin. Unlike α-dystrobrevin mutant mice, syntrophin-null mice do not develop myopathy (Adams *et al.* 2000).

Neuronal nitric oxide synthase (nNOS) is the biosynthetic enzyme of nitric oxide (NO). NO is involved in the regulation of a wide range of physiological functions, including the cardiovascular, nervous, and immune systems (Zhou and Zhu 2009). The DGC anchors nNOS to the sarcolemma where it modulates vascular homeostasis (Thomas *et al.* 1998, Kurihara *et al.* 1998, Seddon *et al.* 2009, Hagioka *et al.* 2005). The loss of nNOS is a characteristic of many different forms of muscular dystrophy and aberrant nNOS can lead to misregulation of muscle development, blood flow, fatigue, fibrosis, and inflammation (Tidball and Wehling-Henricks 2014). The cause of nNOS deficiency in dystrophic muscle is unknown, although some evidence suggests that dystrophin deficiency in DMD leads to a reduction in nNOS transcription or mRNA instability (Chang *et al.* 1996, Kameya *et al.* 1999, Arning *et al.* 2004).

1.2 Overview of dysferlinopathy, dysferlin, and membrane repair

The dysferlinopathies are a category of muscular dystrophy arising due to mutations in the *dysferlin* (*DYSF*) gene (Liu *et al.* 1998, Aoki *et al.* 2001). Three clinically distinct autosomal recessive muscular dystrophies are attributed to *DYSF* mutations: limb-girdle muscular dystrophy type 2B (LGMD2B), Miyoshi Myopathy (MM), and distal myopathy with anterior tibial onset (DMAT) (Liu *et al.* 1998, Bashir *et al.* 1998, Illa *et al.* 2001). Dysferlinopathy is characterized by progressive muscle weakness and atrophy with onset usually beginning in adulthood and commencing in either the proximal or distal muscles, defining the clinical

phenotype. Although distinct initially, the clinical phenotypes of dysferlinopathy include a wide spectrum of pathology that becomes less divergent as the disease progresses, eventually including both proximal and distal muscle groups, becoming one indistinguishable disorder (Klinge *et al.* 2008, Nguyen *et al.* 2007). Dysferlin protein is a transmembrane protein that is ubiquitously expressed and is found abundantly in cardiac and skeletal muscle where it plays a pivotal role in plasma membrane re-sealing (Bansal and Campbell 2004, Bansal *et al.* 2003, Roche *et al.* 2010). Dysferlin is one member of the ferlin family of proteins, which also includes myoferlin and otoferlin. Myoferlin is known to facilitate myoblast fusion during muscle development and otoferlin regulates synaptic vesicle exocytosis in hair cells of the cochlea (Doherty *et al.* 2005, Wenzel *et al.* 2006, Johnson and Chapman 2010, Roux *et al.* 2006).

Although the precise mechanisms are still under investigation, several models describing the process of plasma membrane repair have been proposed (Blazek, Paleo, and Weisleder 2015). The activation of particular plasma membrane repair pathways is dependent upon the size and nature of the membrane lesion. For example, small perforations (<1 nm) may be repaired by either lateral recruitment of plasma membrane (thermodynamic resealing or lateral fusion model) or by the aggregation of proteins (protein clogging model) (McDade, Archambeau, and Michele 2014, Benninger and Piston 2013, Parsegian, Rand, and Gingell 1984). Larger membrane disruptions trigger an energetic, Ca^{2+}-dependent, repair response involving dysferlin-mediated vesicle fusion and the formation of a repair patch (repair patch model). In the repair patch model, disruption of the plasma membrane causes a rapid influx of extracellular Ca^{2+} which triggers the recruitment of several proteins (dysferlin, mitsugumin-53, annexins, and EDH proteins) that together form a "repair patch" complex at the site of membrane injury, as well as the fusion of intracellular vesicles or lysosomes (McNeil and Kirchhausen 2005, Demonbreun *et al.* 2016, McNeil and Khakee 1992, Rodriguez *et al.* 1997, Reddy, Caler, and Andrews 2001). Constriction of plasma membrane at the site of injury, budding and pinching off of injured membrane, endocytotic, and exocytotic mechanisms are

also proposed mechanisms of plasma membrane resealing (Bement, Forscher, and Mooseker 1993, Babiychuk *et al.* 2009, Jimenez *et al.* 2014, Keyel *et al.* 2011, Idone *et al.* 2008). These models of plasma membrane repair are not necessarily mutually exclusive and may work in concert to facilitate membrane repair, depending on cell-type and context. It is unlikely that there is a single, prevailing mechanism of plasma membrane repair at work in all instances of membrane injury (Blazek, Paleo, and Weisleder 2015).

Several proteins are known to interact with dysferlin as part of the plasma membrane repair machinery and will be briefly described as follows.

Mitsugumin 53 (MG53/TRIM72) belongs to the tripartite motif family of E3 ubiquitin ligases and is expressed predominantly in muscle tissue (as well as a few others) where it forms a lattice along with dysferlin to facilitate membrane resealing (Cai, Masumiya, *et al.* 2009, Cao *et al.* 2010, Duann *et al.* 2015, Kim *et al.* 2014, Jia *et al.* 2014, Lek *et al.* 2013). Following plasma membrane injury, MG53/TRIM72 along with recruited vesicles translocates to the site of injury where it interacts with a dysferlin/caveolin-3-containing complex to facilitate vesicle fusion and membrane repair (Cai, Masumiya, *et al.* 2009, Cai, Weisleder, *et al.* 2009, Waddell *et al.* 2011). Notably, MG53/TRIM72-null mice show progressive myopathy and defective plasma membrane repair (Cai, Masumiya, *et al.* 2009) and overexpression of recombinant MG53/TRIM72 in both muscle and non-muscle cells provides protection against plasma membrane damage and decreases muscle pathology in a mouse model of muscular dystrophy *in vivo* (Weisleder *et al.* 2012). Treatment with recombinant MG53/TRIM72 also reduces pathology in models of other diseases, including kidney injury (Duann *et al.* 2015), lung injury (Jia *et al.* 2014, Kim *et al.* 2014), ischemia-reperfusion injury (Corona *et al.* 2014), and myocardial infarct (Liu *et al.* 2015).

As noted above, caveolin-3 (CAV-3) interacts with dysferlin and MG53/TRIM72 (Cai, Weisleder, *et al.* 2009). CAV-3 is a muscle-specific member of the caveolin family of proteins,

which are involved in membrane transport and are found within invaginations of the plasma membrane (caveolae) (Kovtun *et al.* 2015, Blazek, Paleo, and Weisleder 2015). Mutations in CAV-3 are associated with autosomal-dominant limb-girdle muscular dystrophy type 1C (LGMD1C) and have been shown to cause retention of both dysferlin and MG53/TRIM72 in the Golgi and impaired plasma membrane resealing (Hernandez-Deviez *et al.* 2006, Cai, Weisleder, *et al.* 2009, Hernandez-Deviez *et al.* 2008).

Polymerase-1 and Transcriptase Release Factor (PTRF) is known to regulate caveolae membrane structure and has also been shown to bind dysferlin, with mutations in PTRF resulting in reduced dysferlin at the plasma membrane and a dystrophic phenotype (Cacciottolo *et al.* 2011). In membrane repair, PTRF acts as a docking protein, anchoring MG53/TRIM72 at the site of injury through binding of exposed membrane cholesterol (Zhu *et al.* 2011). Knockdown of PTRF reduces membrane repair capacity, while overexpression rescues dystrophic muscle membrane repair (Zhu *et al.* 2011).

Dysferlin associates with annexins A1 and A2 in a Ca^{2+}-dependent manner in response to membrane injury (Lennon *et al.* 2003). Although the precise mechanisms by which annexins participate in the process of membrane resealing remains unclear, they are believed to play roles in repair patch formation and vesicle movement/fusion (Gerke and Moss 2002, Han and Campbell 2007, McNeil *et al.* 2006). Annexin A5 has been shown to bind at sites of plasma membrane injury, forming two-dimensional arrays which could contribute to resealing (Bouter *et al.* 2011), and annexin A6 has been shown to localize to the plasma membrane following injury where it forms repair patch “cap” (Swaggart *et al.* 2014).

Affixin (β-Parvin) is involved in the linkage between integrin and the cytoskeleton and interacts with dysferlin at the sarcolemma (Matsuda *et al.* 2005). Despite this interaction, it remains unclear what specific role affixin might play in the process of plasma membrane repair,

although it is speculated that affixin might assist through the organization of the cytoskeleton (Matsuda *et al.* 2008, Mishima *et al.* 2004, Yamaji *et al.* 2004).

Dysferlin interacts with AHNAK (desmoyokin) and is believed to stabilize AHNAK at the plasma membrane (Huang *et al.* 2008, Huang *et al.* 2007). While its exact role in membrane resealing is unknown, AHNAK supposedly interacts with annexin A2 and is associated with enlargeosomes, suggesting it may play roles in organizing the cytoskeleton and in vesicle exocytosis (Benaud *et al.* 2004, Borgonovo *et al.* 2002, Davis, Loos, and Engelbrecht 2014). AHNAK is recruited to the plasma membrane by S100A10 (annexin A2 light chain), which also binds dysferlin, and together these form a scaffold for membrane repair (Dempsey *et al.* 2012, Kobayashi *et al.* 2012, Ozorowski, Milton, and Luecke 2013, Rezvanpour, Santamaria-Kisiel, and Shaw 2011).

Vesicle fusion with the plasma membrane via the interaction of synaptotagmins and soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) is implicated in the process of plasma membrane resealing (Sudhof and Rothman 2009, Steinhardt, Bi, and Alderton 1994, Detrait *et al.* 2000, Yoo *et al.* 2003, Shen *et al.* 2005).(Chakrabarti *et al.* 2003). Dysferlin has been shown to interact directly with ubiquitously-expressed SNARE proteins syntaxin 4 and SNAP-23, facilitating syntaxin 4/SNAP-23 heterodimerization and acting as a calcium-sensing SNARE effector for plasma membrane fusion (Codding *et al.* 2016). These observations are consistent with the fact that dysferlin deficiency results in decreased exocytosis and an increase in unfused vesicles (Bansal *et al.* 2003).

In addition to its major role in plasma membrane repair, dysferlin also participates in vesicle trafficking, focal adhesion, membrane turnover, endocytosis, modulation of the immune system, and intercellular signaling (Covian-Nares *et al.* 2010, de Morree *et al.* 2010, Demonbreun *et al.* 2011, Evesson *et al.* 2010, Han *et al.* 2010, Nagaraju *et al.* 2008, Wenzel *et al.* 2006, Wenzel *et al.* 2005).

1.3 Overview of antisense therapy

Antisense oligonucleotides (AOs) are short, synthetic nucleic acid sequences that can selectively hybridize to target sequences in pre-messenger RNA (mRNA). AOs can cause inhibition or redirection of splicing and inhibition of protein synthesis through various mechanisms, including disruption of the cell's splicing machinery, interference with the ribosomal complex, and/or by activation of RNase H1-mediated degradation of the oligo-RNA heteroduplex (Kuzmiak and Maquat 2006, Kole, Krainer, and Altman 2012). Antisense therapy is an approach to fighting diseases using DNA-like molecules (AOs). After initially observing antisense-mediated RNA regulation in nature, investigations using model systems to test the feasibility of using synthetic AOs to reduce levels of specific mRNA transcripts quickly followed. Early experiments showed that AOs were effective in reducing target transcripts and protein synthesis (Bennett *et al.* 1994). However, despite promising early results, the use of AOs in disease therapy has been stymied by technical challenges and progress has been slow. Despite more than 30 years of research and clinical investigations, the United States Food and Drug Administration (FDA) has only approved six AO drugs to-date (Stein and Castanotto 2017).

1.4 Current clinical AO drugs

The first clinically-marketed AO drug was Vitravene (Fomivirsen), developed by Isis Pharmaceuticals (now Ionis Pharmaceuticals) and Novartis Ophthalmics for the treatment of cytomegalovirus retinitis in immunocompromized Acquired Immune Deficiency Syndrome (AIDS) patients with human immunodeficiency virus (HIV) infection (Jiang 2013). Vitravene was a 21-mer phosphorothioate oligodeoxynucleotide targeting CMV immediate-early (IE)-2, which is required for replication of the virus (Stein and Castanotto 2017). Vitravene was administered weekly via injection into the vitreous humor and proved effective at reducing symptoms of CMV retinitis (Vitravene Study 2002). Despite approval in 1998, Vitravene was eventually removed from the market in 2006 after the development of high-activity anti-

retroviral therapy (HAART), which drastically reduced the number of CMV cases (Stein and Castanotto 2017).

Macugen (formerly pegaptanib) is a 27-mer atapmer targeting vascular endothelial growth factor (VEGF165) (Ng *et al.* 2006). Macugen was approved in 2004 for the treatment of age-related macular degeneration of the retina. The drug binds the heparin binding site of VEGF165, inhibiting the binding of VEGF165 with VEGFR1 and VEGFR2, preventing the neovascularization of the choroid (Stein and Castanotto 2017). Macugen is currently marketed by Bausch and Lomb.

Kynamro (Mipomersen) is a 20-mer phosphorothioate 2′-methoxyethoxy (MOE) gapmer developed by Isis (Ionis) Pharmaceuticals and was approved by the FDA in 2013 for the treatment of homozygous familial hypercholesterolemia (HoFH) (Stein and Castanotto 2017). HoFH is caused by mutations in both LDL-receptor genes, causing reduced uptake of plasma LDL cholesterol and resulting in heart disease which kills untreated individuals by age 30 (Raal *et al.* 2010). Apolipoprotein B (apoB) is the primary protein of LDL particles and Kynamro functions by targeting apoB mRNA in an effort to reduce circulating LDL-C (Crooke *et al.* 2005). Despite FDA approval, the European Medicines Agency (EMA) refused marketing authorization for Kynamro, citing concerns regarding adverse effects (injection site irritation, liver toxicity, hepatic steatosis (fatty liver), and the development of serious cardiovascular events following treatment) and concluding that the risks did not outweigh the benefits of lowered cholesterol (Stein and Castanotto 2017).

Defibrotide (Defitelio) was approved by the FDA in 2016 for treating hepatic veno-occlusive disease (sVOD) (also known as sinusoidal obstruction syndrome (SOS)). Characterized by organ failure and associated with a mortality rate of >80% sVOD/SOS can develop after chemotherapy and myeloablative hematopoietic stem cell transplantation (Dalle and Giralt 2016). DFT is not a single molecule, it is a mixture of single-stranded phosphodiester

oligonucleotides (90%) and double stranded phosphodiester oligonucleotides (10%) derived from the depolymerization of porcine intestinal mucosal DNA (Pescador *et al.* 2013). While the mechanism of action of defibrotide (DFT) is complex and controversial, it is known to display antithrombotic, anti-inflammatory, and profibrinolytic properties (Pescador *et al.* 2013). The effects of intravenously-delivered DFT on sVOD/SOS were evaluated in a Phase III clinical trial involving 102 patients with organ failure (Richardson *et al.* 2016). Patient survival at 100 days post-transplantation were 38% in the DFT-treated group and 25% in the case-matched historical-control group. Complete resolution of sVOD/SOS symptoms was 26% in the DFT-treated group and 13% in the control group.

In 2016, eteplirsen (Exondys 51) became the first-ever clinically-approved antisense drug for treating Duchenne muscular dystrophy (DMD). Developed by Sarepta Therapeutics, eteplirsen is a 30-mer phosphorodiamidate morpholino oligomer (PMO) which facilitates the splicing (or skipping) of *DMD* exon 51 in an effort to correct out-of-frame mutations and restore expression of functional dystrophin protein (Stein 2016, Mendell *et al.* 2013). Just prior to approval, a Phase III study reported the therapeutic effectiveness of eteplirsen as measured by the 6-minute walk test (6MWT), the primary endpoint metric accepted by regulatory agencies (Mendell *et al.* 2016). In this double-blind study, patients were divided into three groups (n = 4 each) and received either placebo, eteplirsen 30 mg/kg, or 50 mg/kg each week for 24 weeks. All patients, including placebo patients, then received open-label drug (30 or 50 mg/kg) for over 3 years. Pooled historical controls were used for comparison, as all patients eventually received eteplirsen. The results of this study showed that the distance lost on the 6MWT test at 36 months in eteplirsen-treated patients was 151 m less than in the historical controls ($p = <0.01$). After 36 months, only 17% of eteplirsen-treated patients had lost ambulation, compared with 46% of historical controls. This study also showed that eteplirsen was well tolerated by patients, with no significant adverse events resulting in treatment interruption or dosage adjustment reported. Despite these promising results, several concerns were raised following this clinical

study. First, the study relied on data from a small pool of treated patients (n = 12) and historical control patients (n = 13). Second, eteplirsen had never demonstrated significant rescue of dystrophin protein in patient muscle biopsy (0.3% of normal). Lastly, it was suggested that eteplirsen altered the natural history of the disease in treated patients (Stein and Castanotto 2017). The FDA has since directed Sarepta to pursue a larger clinical trial with more patients and submit biopsy samples from eteplirsen-treated patients in order to maintain approval.

Nusinersen (Spinraza) is an 18-mer phosphorothioate 2′-O-methoxyethoxy AO which facilitates the inclusion of exon 7 in SMN1 and SMN2 mRNA by targeting an internal splice silencing motif (Touznik, Lee, and Yokota 2014). Approved in late 2016 and marketed by Biogen, nusinersen is indicated for children with Type I, II, and III spinal muscular atrophy (SMA). Data from recent clinical trials have demonstrated promising therapeutic results in both infantile and late-onset forms of SMA. A randomized, double-blind, sham-controlled phase III study demonstrated that infants treated with nusinersen had a higher motor-milestone response (as defined by the Hammersmith Infant Neurological Examination) than the control group ($p = <0.001$, 37 of 73 infants vs. 0 of 37) (Finkel *et al.* 2017). The likelihood of event-free survival and overall survival was also significantly higher in the nusinersen cohort (hazard ratio 0.53; $p = 0.005$ and hazard ratio 0.37; $p = 0.004$, respectively). A recent multicenter, double-blind, sham-controlled, phase III study was performed in 126 children with SMA symptom onset after 6 months of age (Mercuri *et al.* 2018). In this study, children received intrathecal injections of 12 mg nusinersen or sham on days 1, 29, 85, and 274. Motor function was assessed using the Hammersmith Functional Motor Scale–Expanded (HFMSE) score following 15 months of treatment. A significant proportion of children in the nusinersen group (57%) demonstrated a clinically meaningful increase from baseline to month 15 in the HFMSE in comparison to the control group (26%; $p = 0.001$).

1.5 Challenges associated with antisense therapy

Although promising, the headway of antisense therapy in the clinical realm has been quite slow. To better appreciate the current status of AO drug therapies, it is important to consider the hurdles that AOs have had to overcome. The first of these hurdles is drug delivery. First generation AOs do not easily cross the lipid bilayer of the cell, making intracellular potency via systemic delivery problematic since these AOs cannot readily penetrate to their intracellular targets at significant concentrations to be effective (Bendifallah *et al.* 2006, Miller, Braiterman, and Ts'o 1977, Shiraishi and Nielsen 2011, Torchilin 2006). Furthermore, the systemic administration of AOs (as is most commonly performed in AO clinical trials) often results in a large amount of AO uptake in the liver, kidneys, lymph nodes, and bone marrow (Martin-Armas *et al.* 2006, Geary *et al.* 2015) In the case of certain neurodegenerative diseases, such as Huntington's disease and Alzheimer's, the limited permeability of the blood-brain barrier further compounds the difficulty of effective drug administration to target cells of the central nervous system (CNS) (Kazantsev and Thompson 2008). Another problem associated with some AO chemistries is off-target toxic effects (Muntoni and Wood 2011). DNA and RNA can be immunostimulatory, binding to and activating toll-like receptors or other receptors involved in innate immunity in a sequence- and chemistry-dependent manner (Iwasaki and Medzhitov 2004). Sequence-specific toxicity has been observed in Locked nucleic acid (LNA) chemistries, which have displayed severe hepatotoxicity and liver damage in some cases (Swayze *et al.* 2007, Kakiuchi-Kiyota *et al.* 2014, Stanton *et al.* 2012). Chemistry-dependent toxicological differences are highlighted by phosphorothioate AOs, which are known to activate proinflammatory responses (Frazier 2015), and phosphorodiamidate morpholino AOs, which do not (Carver *et al.* 2016). Other biological barriers include uptake and sequestration of AOs in the reticuloendothelial system and intracellular sequestration in oligo-protein complexes and phagolysosomes (Hoffman *et al.* 2011). Furthermore, to achieve biochemical efficacy, a large proportion of RNA targets must be hybridized and silenced—this number can vary widely, but

can be as high as >90 percent (Juliano *et al.* 2009), resulting in potential toxic effects from AO accumulation following high dosages.

To overcome these challenges, AOs have been designed such that the ribose backbone (normally present in RNA and DNA) is replaced with other chemistries. Oligo delivery is enhanced through various chemical modifications and through the conjugation of other moieties, such as cell-penetrating peptides (Guidotti, Brambilla, and Rossi 2017). These constructs are so distinct from classical nucleic acid structures that they are not readily targeted by nucleases or DNA/RNA-binding proteins. These modifications result in increased stability and help prevent most off-target toxic effects. Various AO chemistries and their associated modifications will be discussed in-depth in the next section. Regarding issues of delivery to CNS tissues, studies have shown the feasibility of AO-mediated RNA silencing in CNS tissues by AO drug administration into cerebrospinal fluid (CSF) via cerebral ventricles and intrathecal injection (Broaddus *et al.* 1998, Wahlestedt *et al.* 2000). Drug administration into CSF via cerebral ventricles is a common medical practice in humans (Pardridge 1997). Studies involving administration of AOs into cerebral ventricles have shown significant oligonucleotide concentrations present not only in the brain and brainstem but also in many levels of the spinal cord after delivery in rats and nonhuman primates, providing evidence of delivery efficacy and sidestepping the hurdle of permeating the blood-brain barrier (Smith *et al.* 2006).

1.6 Comparative AO chemistries

To avoid nuclease degradation, facilitate stronger base-pairing with target mRNA sequences, increase stability, and enable easier delivery into the cell, a variety of AO chemistries have been developed (**Figure 1.1**). One of the most widely used oligo chemistries is the 2'O-methylphosphorothioate- modified (2'OMePS) antisense oligo. These oligos contain a 2'-modification of the ribose ring as well as phosphorothioate linkages throughout their length (**Figure 1.1C**). The 2'OMePS AOs exhibit improved stability and increased cellular uptake via

conventional delivery reagents. These AOs have also been shown to be very efficient *in vivo* (Lu *et al.* 2005, Heemskerk *et al.* 2009). The safety of this particular AO chemistry has been well characterized through a number of preclinical and clinical trials for several diseases (Yokota, Lu, *et al.* 2009, Goemans *et al.* 2011, van Deutekom *et al.* 2007). Of note, BioMarin's DMD AO drug drisapersen (Kyndrisa), designed to skip *DMD* exon 51, was a 2'OMePS oligo chemistry. Unlike Sarepta's eteplirsen, drisapersen failed to reach its primary endpoint (the 6MWT) in a phase III clinical trial and the FDA concluded that there was no clinical benefit to the drug, so BioMarin abandoned development in 2016 (Mendell, Sahenk, and Rodino-Klapac 2017).

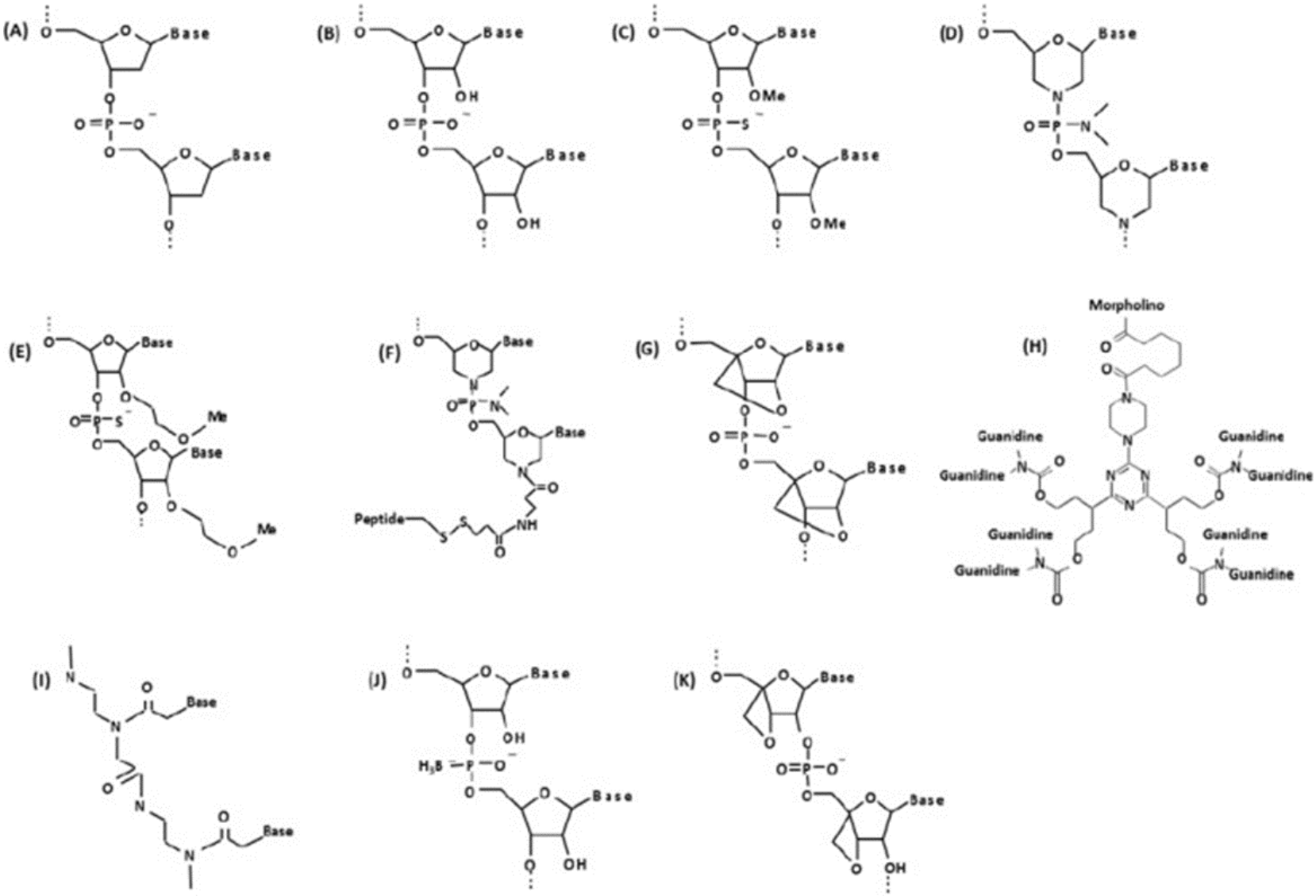

(A)
Base
(B)
OH
(C)
OMe
(D)
(E)
Me
(F)
Peptide
NH
(G)
(H)
Morpholino
Guanidine
(I)
(J)
(K)

Figure 1.1 Chemical structure of biological and synthetic oligonucleotides.

(A) DNA; (B) RNA; (C) 2'O-methylphosphorothioate (2'O-MePS); (D) Morpholino (PMO); (E) 2'-methoxyethoxy (2'-MOE); (F) PMO with peptide conjugate (PPMO); (G) Locked nucleic acid (LNA); (H) Vivo-morpholino (vPMO); (I) Peptide nucleic acid (PNA); (J) Boranophosphate-oligodeoxy-nucleoside (BH3-ODN); (K) Oxetane-modified AO. From (Lee and Yokota 2013).

Another oligo chemistry that has gained significant popularity, especially in recent years, is the phosphorodiamidate morpholino oligomer (PMO, morpholino). The PMO chemistry differs from traditional DNA/RNA chemistry in that the nucleic acid bases are bound to morpholine moieties as opposed to deoxyribose/ribose rings and the phosphodiester backbone is replaced by a phosphorodiamidate linkage (**Figure 1.1D**). Like other oligos, the chemical modifications to PMOs render them sufficiently different from conventional nucleic acid chemistries so that they are not recognized by nucleases, making them very stable. PMOs are also less susceptible to metabolic degradation. Moreover, PMOs do not activate toll-like receptors, the nuclear factor (NF)-κB-mediated inflammatory response, or the interferon system (Sazani *et al.* 2011, Summerton 1999, Ekker 2000). As mentioned previously, eteplirsen, currently the only clinically-approved AO drug for treating muscular dystrophy, is a PMO chemistry oligo.

There are several groups of next generation antisense compounds that have shown very promising results in animal models. For example, 2'-methoxyethoxy (2'-MOE)-modified oligonucleotides containing lipophilic 2'-O-alkyl-substituted nucleobase modifications demonstrate high RNA binding affinity and metabolic stability and can be used as gapmers to catalyze RNase H1-mediated degradation of target nucleic acids (Altmann *et al.* 1996, Monia *et al.* 1993, Prakash and Bhat 2007) (**Figure 1.1E**). 2'-MOE oligos have been used in vivo to target toxic mRNA triplet repeats in myotonic dystrophy (Wheeler *et al.* 2012).

Vivo-morpholinos (vPMOs) are octa guanidine (cell-penetrating moiety) conjugated PMOs (**Figure 1.1H**) and have shown very efficient splicing modulation in studies targeting the *FCMD* gene, *DMD* exons 6 and 8 multi skipping in dystrophic dogs, and exons 45–55 in *mdx52* mice (Aoki *et al.* 2012, Taniguchi-Ikeda *et al.* 2011, Yokota, Nakamura, *et al.* 2012).

PMOs with peptide conjugates (PPMOs) (**Figure 1.1F**) act similarly to vPMOs and efficiently rescued cardiac muscle as well as skeletal muscles in *mdx* mice (Yin *et al.* 2008, Yin,

Saleh, *et al.* 2011, Yin *et al.* 2009, Yin, Moulton, *et al.* 2011, Yin, Lu, and Wood 2008, Yin *et al.* 2010, Wu *et al.* 2008).

Peptide nucleic acids (PNAs) are another class of antisense oligo in which the phosphodiester-linked deoxyribose/ribose backbone is replaced by peptide-linked repeating N-(2-aminoethyl)-glycine units, to which the nucleobases are attached (Egholm *et al.* 1993) (**Figure 1.1I**). PNAs exhibit greater binding strength than many other AOs and are extremely stable, though their solubility in water is much lower (Karkare and Bhatnagar 2006, Ivanova *et al.* 2008).

Locked nucleic acid (LNA) AOs contain a 2'-C, 4'-C-oxymethylene-linkage which "locks" the deoxyribo/ribo sugar structure in an N-type conformation (Pfundheller and Lomholt 2002) (**Figure 1.1G**). LNAs are stable against exonucleolytic degradation, exhibit high thermostability and hybridize strongly with target nucleic acids (Singh, Kumar, and Wengel 1998, McTigue, Peterson, and Kahn 2004). Several LNA analogs have been developed (Singh, Kumar, and Wengel 1998, Kumar *et al.* 1998). The characteristics of LNA constructs have made them the oligo of choice for several molecular applications, including microarrays (Tolstrup *et al.* 2003), genotyping assays (Johnson, Haupt, and Griffiths 2004, Latorra *et al.* 2003, Simeonov and Nikiforov 2002), and for the stabilization of DNA triplex formation in gene silencing (Petersen and Wengel 2003).

In 1992, Sood *et al.* first reported an antisense oligo chemistry containing a boronated phosphate backbone (boranophosphate) (Sood *et al.* 1992). Known as boranophosphate-oligodeoxy-nucleosides (BH_3^--ODN), these AOs differ from classical DNA/RNA constructs in that they contain a borane group in place of a non-bridging oxygen species in the phosphodiester backbone (**Figure 1.1J**). Boranophosphates have been shown to activate RNase H1-mediated RNA cleavage (Rait and Shaw 1999). Furthermore, experiments have demonstrated the highly lipophilic nature of boranophosphates (Rait *et al.* 1999), thus

facilitating their transport across the bilipid membrane to target nucleic acids. This characteristic is likely due to the increased hydrophobicity of BH_3 compared with oxygen. Boron-modified dNTPs have also been successfully employed in DNA sequencing assays — by taking advantage of the nuclease-resistant nature of boranophosphates (Li and Shaw 2002, Shaw *et al.* 2003), researchers are able to sequence resultant nucleic acid fragments following exonuclease digestion (Shaw *et al.* 2000).

Oxetane-modified oligonucleotides (**Figure 1.1K**) are another form of AO which have proven their feasibly as antisense molecules by exhibiting resistance to nuclease digestion, the ability to activate RNase H1-mediated cleavage of the AO/RNA heteroduplex, tightly bind to their target nucleic acid sequences, and efficiently silence gene expression *in vitro* (Opalinska *et al.* 2004, Opalinska and Gewirtz 2005). Continued development of more effective and less toxic AOs will be a key to the future success of AO-based therapies.

1.7 Overview of antisense therapy in neurology

In this section, the use of antisense oligos for Duchenne muscular dystrophy (DMD), Fukuyama congenital muscular dystrophy (FCMD), myotonic dystrophy (DM), spinal muscular atrophy (SMA), dysferlinopathy, Amyotrophic lateral sclerosis (ALS), and Huntington's disease (HD) will be briefly reviewed. Although the aforementioned diseases are all targeted by antisense therapy, the therapeutic strategies for these disorders are quite different. For example, to treat DMD, antisense-mediated exon skipping can remove nonsense mutations or frame-shifting mutations from mRNA (Yokota *et al.* 2007, Yokota, Duddy, *et al.* 2012, Malerba, Boldrin, and Dickson 2011). To treat the mutation in the *FCMD* gene, a cocktail of vivo-morpholino AOs targeting splice enhancer sites and splice silencer sites led to correction of the aberrant splicing pattern in cell and mouse models (Taniguchi-Ikeda *et al.* 2011). RNase H1-mediated degradation of toxic RNA with 2'-MOE antisense for myotonic dystrophy type 1 showed very promising results in the mouse model (Wheeler 2008). A unique "knock up"

approach (exon inclusion) targeting the *SMN2* gene with 2'-MOE antisense or PMOs has been used to treat SMA cell and mouse models and has received FDA approval for clinical use (Hua *et al.* 2007, Porensky *et al.* 2012, Stein and Castanotto 2017).

1.8 Exon skipping therapy for DMD

Exon skipping has been heavily researched for the treatment of DMD (Lu *et al.* 2011, Aartsma-Rus 2010, Niks and Aartsma-Rus 2017). Exon skipping employs antisense oligos as "DNA Band-Aids" to splice out or "skip" mutation-carrying exons and/or flanking exons that would otherwise prevent the effective creation of functional proteins, maintaining the open reading frame (**Figure 1.2**). In fact, such exon skipping of disease-causing mutations occurs spontaneously in DMD patients and animal models to some extent (Yokota *et al.* 2006, Hoffman *et al.* 1990, Klein *et al.* 1992, Lu *et al.* 2000, Echigoya *et al.* 2013). The efficacy of exon skipping has been rigorously tested in several animal models, including dystrophic *mdx* mice and dystrophic dogs, as well as in human DMD cells (Yokota, Nakamura, *et al.* 2012, Yin, Lu, and Wood 2008, Aoki *et al.* 2010, Saito *et al.* 2010, Aartsma-Rus *et al.* 2005, Aartsma-Rus *et al.* 2003, Bertoni, Lau, and Rando 2003, Bremmer-Bout *et al.* 2004, Fletcher *et al.* 2007, Mann *et al.* 2001, McClorey, Fall, *et al.* 2006, McClorey, Moulton, *et al.* 2006, Mitrpant *et al.* 2009, van Deutekom *et al.* 2001, Wilton *et al.* 2007, Takeshima *et al.* 2005). Systemic rescue of animal models with exon skipping has been demonstrated in dystrophic dogs (exons 6 and 8 multi-skipping), *mdx* mice (exon 23), and *mdx52* mice (exon 51 and exons 45–55 multi-skipping) (Lu *et al.* 2005, Aoki *et al.* 2012, Aoki *et al.* 2010, Yokota, Hoffman, and Takeda 2011). To-date, the only clinically-available exon-skipping AO drug for treating DMD is eteplirsen, although other antisense-mediated exon skipping approaches are currently being investigated across various clinical trials, targeting *DMD* exons 44 (NCT02958202), 45 (NCT02667483), 51 (NCT03375255), and 53 (NCT03167255). Most recently, a phase 1, open-label, dose-escalation clinical trial investigating the safety, pharmacokinetics, and activity of Nippon Shinyaku's exon 53 skipping morpholino, NS-065/NCNP-01, was completed (Komaki *et al.* 2018). Ten patients

receiving 1.25, 5, or 20 mg/kg weekly for 12 weeks did not show any adverse reactions to drug administration. Furthermore, muscle biopsy revealed that treated patients showed a dose-dependent increase in *DMD* exon 53-skipped mRNA and increased dystrophin/spectrin ratio.

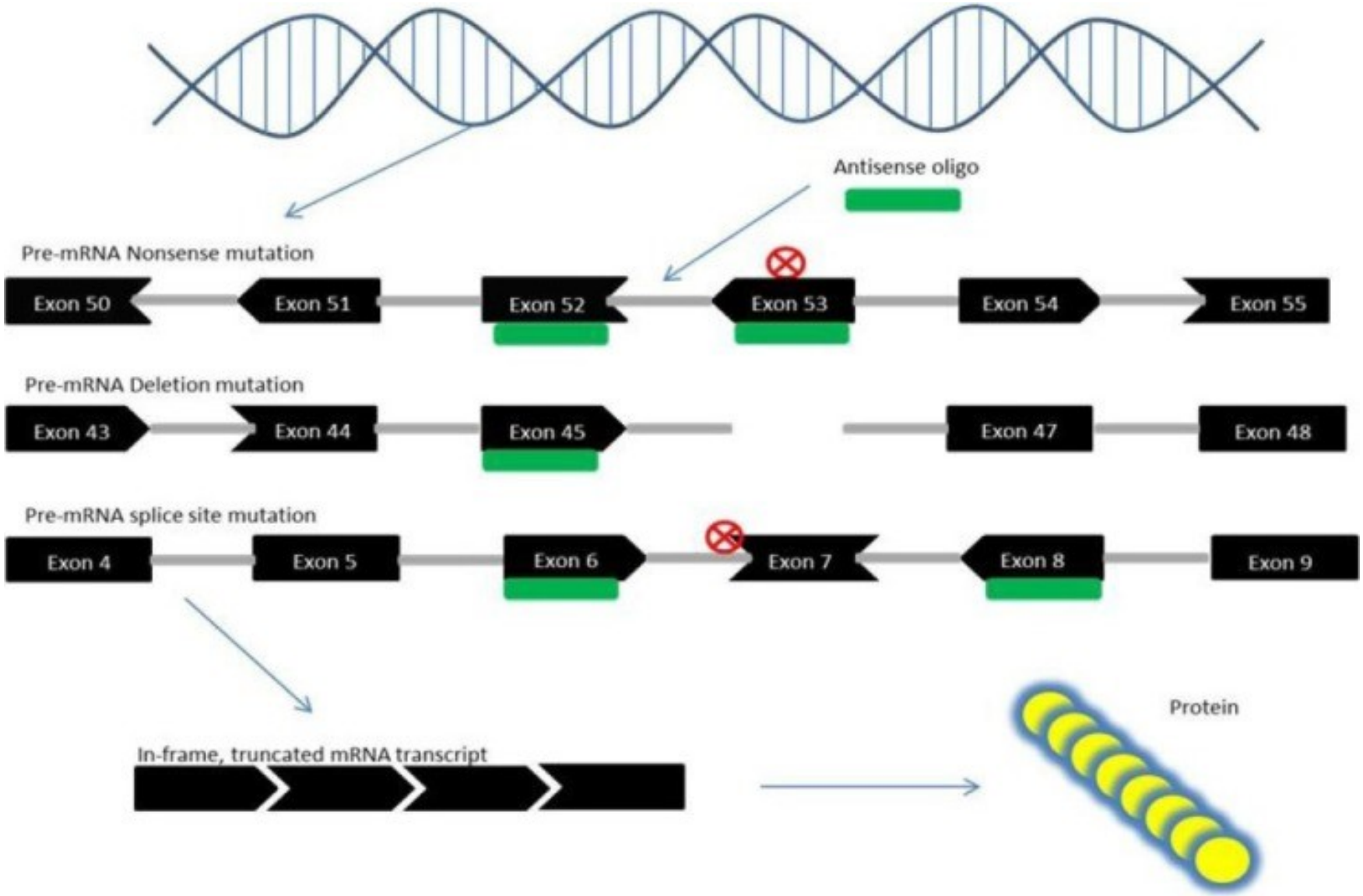

Antisense oligo
Pre-mRNA Nonsense mutation
Exon 50
Exon 51
Exon 52
Exon 53
Exon 54
Exon 55
Pre-mRNA Deletion mutation
Exon 43
Exon 44
Exon 45
Exon 47
Exon 48
Pre-mRNA splice site mutation
Exon 4
Exon 5
Exon 6
Exon 7
Exon 8
Exon 9
In-frame, truncated mRNA transcript
Protein

Figure 1.2: Mechanism of exon skipping therapy for Duchenne muscular dystrophy (DMD).

Nonsense mutations in the DMD gene can create a novel STOP codon which results in the loss of dystrophin protein. Exon skipping corrects this error when exons (black) that are bound to antisense oligos (green) are spliced out of the pre-mRNA, and the resulting exon sequences "fit together", i.e., are in-frame (denoted by the shape of each exon—ends that fit together are in-frame). Out-of-frame mutations caused by the loss of exonic sequences, through deletion or splice site mutations, can also be corrected through exon skipping, which removes exons adjacent to the mutation site so that the remaining exons are in-frame. The result is a truncated yet partly functional protein, as in the case of Becker muscular dystrophy (BMD). From (Lee and Yokota 2013).

1.9 Splice correction therapy in FCMD

Fukuyama congenital muscular dystrophy (FCMD) is an autosomal recessive form of muscular dystrophy mainly described in Japan (Kamoshita *et al.* 1976). The gene responsible for FCMD encodes the protein fukutin (Kobayashi *et al.* 1998). Fukutin is believed to add chains of sugar molecules (glycosylation) to α-dystroglycan, a member of the dystrophin glycoprotein complex (Michele *et al.* 2002, Hayashi *et al.* 2001). Interestingly, most patients (87%) with mutated *FCMD* gene bear chromosomes that have a 3-kb retrotransposon insertion into the 3'-untranslated region (UTR) of the gene derived from a single ancestral founder (Colombo *et al.* 2000, Kobayashi *et al.* 2001). The aberrant mRNA splicing induced by the SINE-VNTR-Alu (SVA) retrotransposon exon-trapping is responsible for the pathogenesis of FCMD (Taniguchi-Ikeda *et al.* 2011) (**Figure 1.3**). The insertion induces splicing errors and cryptic splice site activation with a new splice donor in exon 10 and a new splice acceptor in the SVA insertion site. This results in aberrant splicing and truncation of exon 10. A cocktail of vPMOs targeted against intronic and exonic splicing enhancer sites led to normal fukutin mRNA expression and protein production in human patient cells as well as in a mouse model *in vivo* (Taniguchi-Ikeda *et al.* 2011).

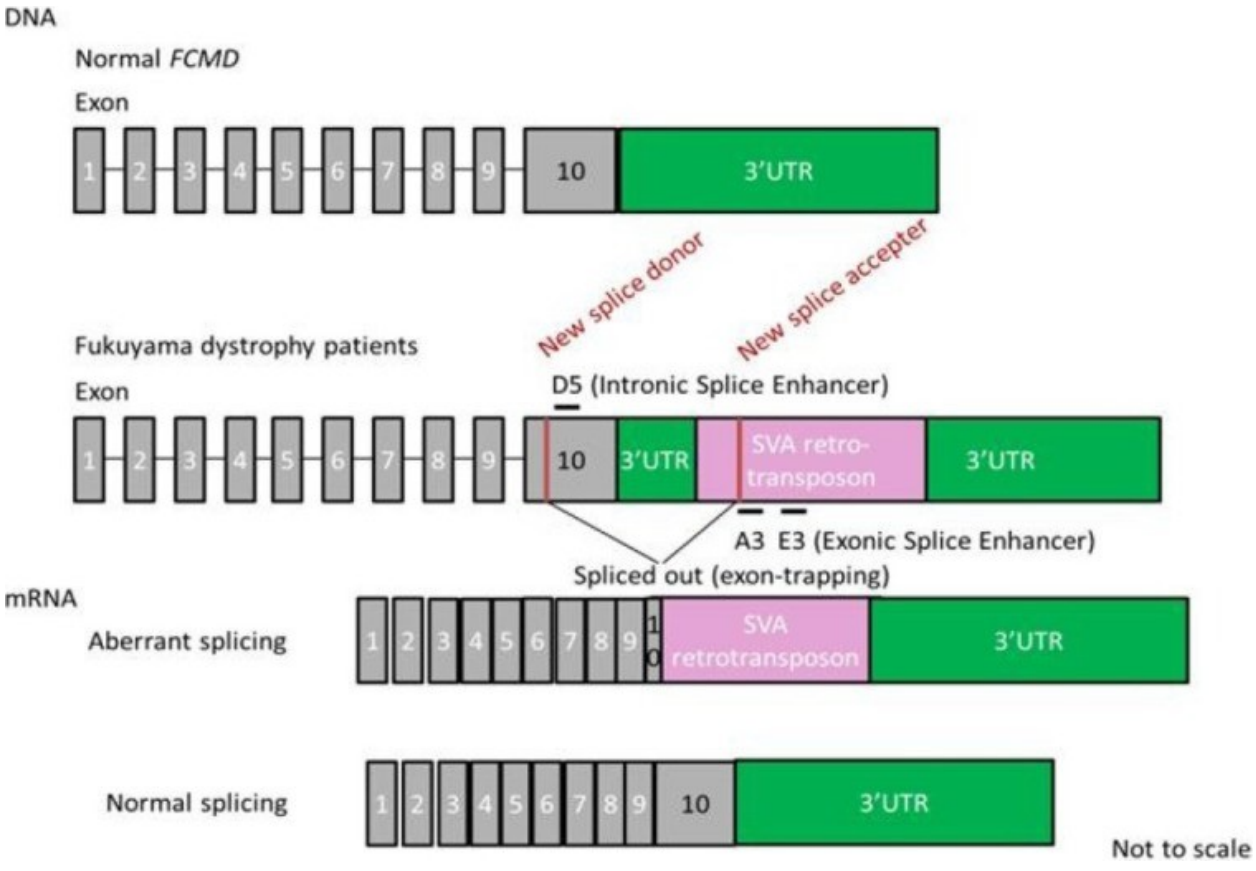


Figure 1.3: Strategy of antisense therapy for Fukuyama dystrophy

Retrotransposon insertion in the *FCMD* gene leads to aberrant splicing. An antisense vivo-morpholino cocktail (A3, E3 and D5) restores normal splicing. From (Lee and Yokota 2013).

1.10 Antisense therapy for myotonic dystrophy

Myotonic dystrophy (DM) is the most common adult form of muscular dystrophy and is characterized by myotonia (slow relaxation of the muscles), progressive muscle weakness, and atrophy (Bhagavati *et al.* 1997). DM can also cause dysfunction of heart, eye, and brain tissues, as well as the gastrointestinal and endocrine systems (Meola 2000a, b). Myotonic dystrophy type 1 (DM1) and myotonic dystrophy type 2 (DM2) are multisystemic microsatellite expansion disorders caused by an expanded CTG tract in the 3' UTR of the *dystrophia myotonica-protein kinase* gene (*DMPK*) and an expanded CCTG tract in the first intron of the *CCHC-type zinc finger, nucleic acid binding protein* gene (*CNBP*, also known as *ZNF9*), respectively (Schoser and Timchenko 2010, Cho and Tapscott 2007, Brook *et al.* 1992, Fu *et al.* 1992, Liquori *et al.* 2001, Mahadevan *et al.* 1992). Disease phenotype (including age of onset and severity) is highly correlated with repeat number. In the case of DM1, unaffected individuals tend to have CTG repeats between 5 and 35 while DM1 patients often present with expansions between 50 and >2,000 (Hamshere *et al.* 1999). DM follows an autosomal dominant pattern of inheritance and, although the precise molecular mechanisms are unknown, symptoms are thought to arise owing to the toxic gain-of-function of RNA transcripts containing expanded repeats, which causes the transcripts to be retained and accumulate in the nucleus (Taneja *et al.* 1995). Other evidence suggests a possible dominant-negative effect of expansion-containing mutant RNA transcripts (Wang *et al.* 1995). Protein-level gain-of-function is not likely, as the CTG expansion region lies outside of the *DMPK* coding region in the 3' UTR. Antisense-mediated suppression of *DMPK* RNA transcripts is, therefore, a promising therapeutic approach (Magana and Cisneros 2011, Gao and Cooper 2013) (**Figure 1.4**). Importantly, there is considerable evidence implicating diminished *DMPK* transcripts in DM1 pathology, with a consensus among several studies that production and processing of *DMPK* mRNA is inhibited by expansion-containing mutant transcripts (Carango *et al.* 1993, Hofmann-Radvanyi *et al.* 1993, Koga *et al.* 1994, Krahe *et al.*

1995, Maeda *et al*. 1995, Novelli *et al*. 1993). In their study utilizing homozygous *DMPK*-null mice, Reddy *et al*. showed that these mutants develop a progressive myopathy that is pathologically similar to DM, underscoring the importance of DMPK in maintaining proper skeletal muscle condition(Reddy *et al*. 1996).

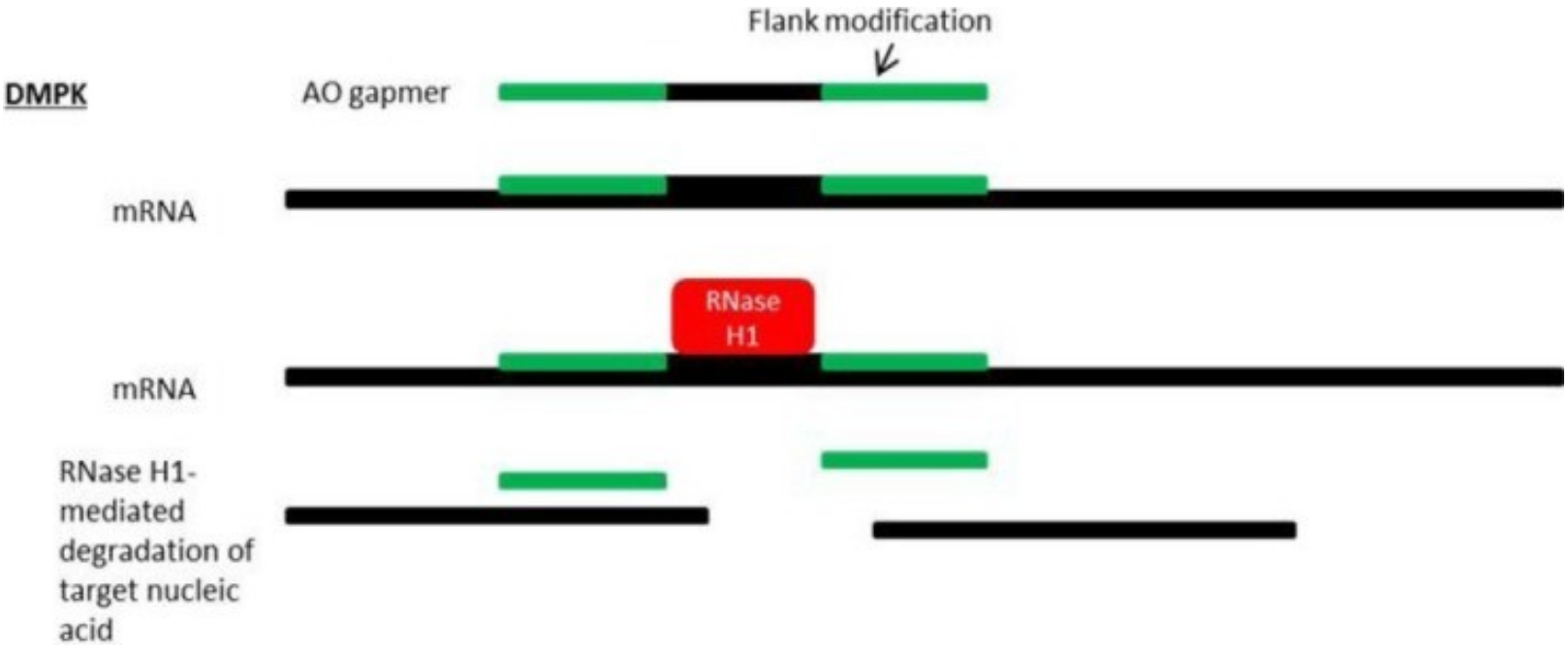

Flank modification
DMPK
AO gapmer
mRNA
RNase H1
mRNA
RNase H1-mediated degradation of target nucleic acid

Figure 1.4: Mechanism of antisense silencing via RNase H1 activity.

Myotonic dystrophy (DM1) is caused by RNA gain-of-function due to an expanded CUG repeat in the *dystrophia myotonica-protein kinase* (*DMPK*) gene transcript. RNase H1-mediated degradation of target nucleic acids is facilitated by AO "gapmers", composed of a central gap region which supports RNase H1 activity and flanking nucleotides at the 5' and 3'-ends which are resistant to RNase H1 degradation and display strong binding affinity for target RNA. From (Lee and Yokota 2013).

Several *in vitro* and *in vivo* studies have highlighted the therapeutic potential of various AO chemistries, such as 2'-MOE, LNA, and PPMO, targeted against the microsatellite expansion of DM1, which have demonstrated efficient, long-lasting antisense-mediated knockdown of mutant RNA transcripts, as well as amelioration of physiological and transcriptomic abnormalities (Gonzalez-Barriga *et al.* 2013, Wheeler *et al.* 2012, Lee, Bennett, and Cooper 2012, Leger *et al.* 2013, Pandey *et al.* 2015). Recently, a research group partnered with Ionis Pharmaceuticals demonstrated successful rescue of muscle strength in a mouse model of DM1 through targeted knockdown of mutant *DMPK* transcripts with 2′-4′-constrained ethyl (cEt) and 2′-O-methoxyethyl (MOE) gapmers, without overt toxicity (Jauvin *et al.* 2017). Ionis recently concluded a Phase I/II clinical trial (NCT02312011) and reported that their antisense drug, IONIS-DMPKRx, was well-tolerated at all dose levels; however, drug levels measured from muscle biopsies indicated that therapeutic benefit was unlikely, and therefore Ionis will no longer continue investigating IONIS-DMPKRx but will instead focus on a New Ligand-Conjugated Antisense (LICA) oligo chemistry they have developed.

1.11 Exon inclusion therapy for SMA

Spinal muscular atrophy (SMA) is a lethal autosomal recessive disease caused by a genetic defect in the *SMN1* (*survival motor neuron*) gene (Wirth 2000, Wirth *et al.* 1999). SMA is characterized by the deterioration of spinal motor neurons, followed by weakness and wasting of the voluntary muscles in the arms and legs of infants and children, resulting in death during childhood (Zellweger 1971). Interestingly, SMA patients retain at least one copy of a highly homologous gene called *SMN2* (Monani *et al.* 1999). *SMN2*, an inverted duplicate copy nearly identical to *SMN1*, is unable to compensate for the loss of *SMN1* due to a C-T transition in exon 7 which interferes with a splice modulator, causing exon 7 to be lost and rendering the resultant SMN protein nonfunctional; however, some full-length *SMN* transcripts (~10%) and functional SMN proteins are still produced. The *SMN2* gene differs from *SMN1* by only five base pair changes (Khoo and Krainer 2009). Consequently, upregulation of SMN by modification of

SMN2 exon 7 splicing is a promising therapeutic approach (**Figure 1.5**), an approach that has demonstrated favorable results in animal models (Hua *et al.* 2007, Edens *et al.* 2015); for example, antisense PMOs targeting splice silencing motifs that promote exon 7 retention successfully rescued the phenotype in a severe mouse model of SMA after intracerebroventricular delivery (Mitrpant *et al.* 2013). As noted previously, these promising preclinical investigations paved the way for clinical trials which in turn led to the recent FDA approval of the first antisense oligo drug for treating SMA, nusinersen.

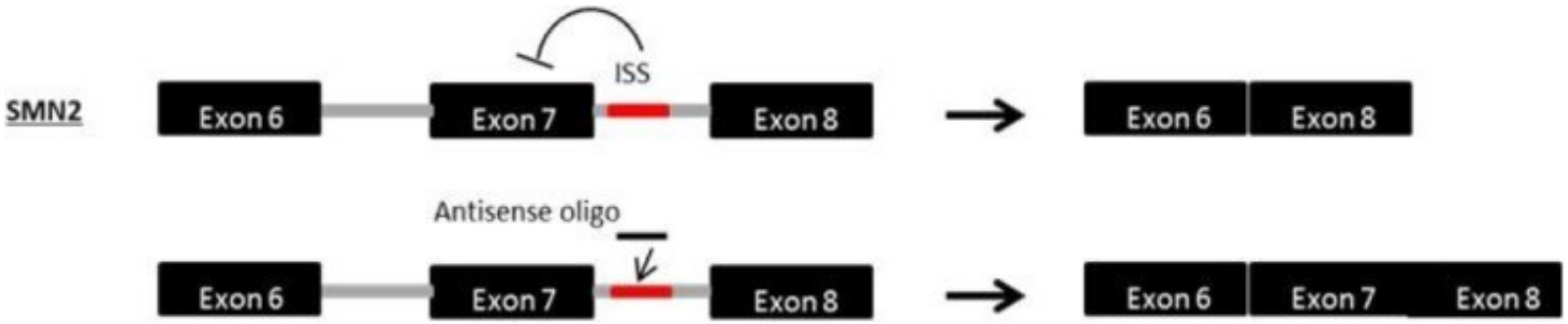


Figure 1.5: Mechanism of antisense exon 7 inclusion in *SMN2*.

Spinal muscular atrophy (SMA) is caused by a loss-of-function mutation in the *SMN1* gene. Within the *SMN2* gene, a paralogue of *SMN1*, a single nucleotide substitution in exon 7 interferes with an exonic splicing enhancer, impairing production of normal SMN protein. AOs targeted to the intronic splice silencer site (ISS) in intron 7 of *SMN2* facilitate the retention of exon 7 within the mature mRNA, increasing the production of functional SMN protein. From (Lee and Yokota 2013).

1.12 Exon Skipping Therapy for Dysferlinopathy

The dysferlinopathies are a category of muscular dystrophy arising due to mutations in the *dysferlin* (*DYSF*) gene which manifest clinically as three distinct autosomal recessive muscular disorders: limb-girdle muscular dystrophy type 2B (LGMD2B), Miyoshi Myopathy (MM), and distal myopathy with anterior tibial onset (DMAT) (Liu *et al.* 1998, Aoki *et al.* 2001, Anderson *et al.* 1999, Argov *et al.* 2000, Foxton, Laval, and Bushby 2004, Guglieri *et al.* 2008, Illa *et al.* 2001). The sarcolemmal protein dysferlin is a transmembrane protein that is ubiquitously expressed and is found abundantly in cardiac and skeletal muscle where it plays a pivotal role in plasma membrane re-sealing (Anderson *et al.* 1999, Bansal *et al.* 2003, Bansal and Campbell 2004, Cai, Weisleder, *et al.* 2009, Han *et al.* 2007, Lennon *et al.* 2003). Exon skipping is a promising therapeutic avenue to treating dysferlinopathies and has been successfully demonstrated *in vitro* using dysferlinopathy patient-derived cells (Aartsma-Rus *et al.* 2010, Wein *et al.* 2010). Encouragingly, Sinnreich *et al.* reported a case wherein a mildly affected mother with two severely affected daughters, both having LGMD2B with homozygous *DYSF* null mutations, was found to carry a lariat branch point mutation that resulted in the in-frame exon skipping of exon 32. The action of the resulting dysferlin protein is thought to account for her mild phenotype (Sinnreich, Therrien, and Karpati 2006). Therefore, at least *DYSF* exon 32 is thought to be a promising target of exon skipping therapy, although there are currently no ongoing or pending clinical trials involving AO-mediated therapy for dysferlinopathy.

1.13 Antisense Therapy for ALS

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease affecting upper and lower motor neurons in the brain and spinal cord (Rothstein 2007). Though associated with some clinical heterogeneity, ALS typically manifests during adulthood and is characterized by progressive neuronal death, spasticity, muscle atrophy, paralysis, and death

within ~5 years of diagnosis (Turner and Al-Chalabi 2002, Cleveland 1999, Cheah *et al.* 2011). Most cases of ALS are sporadic; however, ~10% of cases are familial and follow an autosomal-dominant pattern of inheritance (Morariu 1977, Armani *et al.* 1987) – of these, 20% are caused by mutations in the *Cu/Zn superoxide dismutase* (*SOD1*) gene, resulting in a toxic gain-of-function via a currently unknown mechanism (Penco *et al.* 1999, Wong *et al.* 1995). Although currently believed to be the result of a gain-of-function mechanism, initial investigations into the role of *SOD1* in ALS supported a loss-of-function mechanism (Deng *et al.* 1993, Rosen 1993). Belief in a loss-of-function model waned significantly following in vivo experiments involving transgenic mice expressing human SOD1 protein, which exhibited progressive neurodegeneration, mirroring human ALS clinical pathology (Wong *et al.* 1995, Bruijn *et al.* 1998, Gurney *et al.* 1994). Clinical observations which failed to support a connection between SOD1 activity and disease progression further eclipsed the idea of loss-of-function (Ratovitski *et al.* 1999). Saccon *et al.* compiled previous and more recent findings to provide a compelling argument for the existence of a possible modifying role of loss-of-function in ALS (Saccon *et al.* 2013). They note that SOD1 activity is significantly reduced in ALS patients and that *SOD1*-null mice exhibit neuropathology similar to human ALS. Although a loss of SOD1 activity does not appear directly responsible for ALS phenotype, these data support the idea of a possible synergistic relationship between gain-of-function and loss-of-function in ALS disease progression. The interplay between gain- and loss-of function has also been described in a host of other neurodegenerative disorders, including Huntington's disease and Parkinson's disease (Winklhofer, Tatzelt, and Haass 2008, Zuccato, Valenza, and Cattaneo 2010). As such, the implications to antisense therapy in neurology, and especially the antisense-mediated reduction of SOD1, are profound. The long-term effects of downregulating SOD1, therefore, should be an important focus of future clinical trials.

Previously, Ionis Pharmaceuticals (then Isis Pharmaceuticals) concluded a Phase 1 placebo-controlled, double-blind, dose-escalation, safety and tolerability clinical trial

(NCT01041222) for their antisense drug ISIS-SOD1Rx. The oligo employed in this study was a 2'-MOE modified antisense oligo targeted to the first exon (19th–38th bps) of *SOD1* (regardless of mutation) and catalyzed RNase H1-mediated degradation (Miller *et al.* 2013). The study involved patients from four US centers aged 18 years or older and carrying *SOD1* mutations. Participants were given 12-h intrathecal infusions of ISIS-SOD1Rx at varying concentrations, or placebo. No clinically significant adverse effects associated with oligo administration were reported. Following administration, AO was detected in the CSF of all AO-treated participants and increased with dosage concentration. SOD1 concentrations in the CSF did not change significantly, though achieving SOD1 reduction was never an aim of the study. Currently, Ionis, in collaboration with Biogen, has begun a larger Phase 1 clinical trial (NCT02623699) aimed at confirming the safety and tolerability of IONIS-SOD1Rx and measuring levels of SOD1 in the spinal fluid of treated patients. Following this study, patients will have the option to join a subsequent long-term safety and tolerability study (NCT03070119).

In addition to the *SOD1* gene, several other genes have also been implicated in ALS pathogenesis, including the *TAR DNA binding protein* (*TARDBP*), *fused in sarcoma* (*FUS*), *angiogenin* (*ANG*), *ubiquilin 2* (*UBQLN2*), and *valosin-containing protein* (*VCP*) genes (Deng *et al.* 2011, Johnson *et al.* 2010, Koppers *et al.* 2012, Kwiatkowski *et al.* 2009, Mosca *et al.* 2012, Neumann *et al.* 2006, van Es *et al.* 2009, Van Langenhove *et al.* 2010, Vance *et al.* 2009). Most notably, it was discovered that a GGGGCC hexanucleotide repeat expansion in the first intron of the *C9orf72* gene is the most common genetic cause of ALS pathogenesis, more common than all other known ALS gene mutations combined, accounting for between 37%–50% of familial ALS cases among studied cohorts (Byrne *et al.* 2012, DeJesus-Hernandez *et al.* 2011, Harms *et al.* 2013, Majounie *et al.* 2012, Mok *et al.* 2012, Renton *et al.* 2011, Smith *et al.* 2013). Although both loss-of-function and gain-of-function mechanisms have been postulated, the underlying etiology by which these *C9orf72* expanded repeats result in neurodegeneration is still under investigation; however, evidence suggests a pathogenic

threshold of hexanucleotide repeats may exist, though such a threshold has not yet been fully demarcated (DeJesus-Hernandez *et al.* 2011, Harms *et al.* 2013, Renton *et al.* 2011, Garcia-Redondo *et al.* 2013, Rutherford, DeJesus-Hernandez, *et al.* 2012, Rutherford, Heckman, *et al.* 2012). In a recent paper, Shi *et al.* demonstrated that repeat-expanded C9ORF72 was haploinsufficient in ALS, and that C9ORF72 interacted with endosomes and was required for normal vesicle trafficking and lysosomal biogenesis (Shi *et al.* 2018). C9ORF72 expression was reduced due to repeat expansion, which triggered neurodegeneration through the accumulation of glutamate receptors (leading to excitotoxicity) and impaired clearance of neurotoxic dipeptide repeat proteins, underscoring the postulated interplay between both gain- and loss-of-function mechanisms in C9ORF72-ALS.

Because of the high prevalence of *C9orf72* mutations in cases of ALS, and because mutations in *C9orf72* have also been associated with other neurodegenerative disorders, such as Parkinson's disease and frontotemporal dementia (FTD), *C9orf72* is a promising candidate for targeted antisense therapy (DeJesus-Hernandez *et al.* 2011, Renton *et al.* 2011, Cruts *et al.* 2013, Nuytemans *et al.* 2013, Rademakers 2012, Mis *et al.* 2017). Multiple research groups have partnered with Ionis Pharmaceuticals to develop an antisense strategy for *C9orf72*-based ALS, working under the hypothesis that reducing mutant *C9orf72* transcripts using AOs will ameliorate toxic aggregations of expanded repeat mRNA, which present as nuclear foci in brain and spinal cord in affected patients (DeJesus-Hernandez *et al.* 2011). Early investigations using AOs have yielded promising results, reducing the frequency of *C9orf72* expanded repeat aggregates and stabilizing gene expression *in vitro* (Sareen *et al.* 2013, Donnelly *et al.* 2013, Lagier-Tourenne *et al.* 2013).

1.14 Antisense Therapy for Huntington's Disease

Huntington's disease (HD) is an adult-onset, lethal, progressive neurodegenerative disease that follows an autosomal dominant pattern of inheritance. Clinical manifestations of

HD include cognitive decay, such as the diminished ability to perform executive functions, motor deficits, such as chorea (involuntary, spastic movements), the inability to manage prehensile controls, and psychiatric disturbances, such as dysphoria, anxiety, irritability, mania and psychosis (Craufurd, Thompson, and Snowden 2001, Frank *et al.* 2008, Arnulf *et al.* 2008, Carlock *et al.* 1995, Burns *et al.* 1990, Marder *et al.* 2000). Neuropathological features of HD include widespread neuronal atrophy and the formation of nuclear/intranuclear inclusions in neural tissues of the brain (Arnulf *et al.* 2008, Reiner *et al.* 1988, Rosas *et al.* 2005, Cha *et al.* 1998, Ross and Shoulson 2009, Liu and Zeitlin 2011, Urbaniak Hunter, Yarbrough, and Ciacci 2010). Although the precise etiology of HD is still unknown, the disease is caused by a trinucleotide CAG-expansion in the first exon of the *Huntingtin (HTT)* gene, which results in a toxic gain-of-function of the resultant mutant huntingtin protein (mHTT) (Rubinsztein *et al.* 1993, Aronin *et al.* 1995). The inclusion bodies are composed of aggregates of misfolded mHTT and their density is highly correlated with repeat length (Becher *et al.* 1998, Gutekunst *et al.* 1999, Hoogeveen *et al.* 1993). HTT is vital to proper embryonic development and neurogenesis, and also plays a role in protecting CNS cells from apoptosis, vesicular trafficking, axonal transport, and synaptic transmission (Ferrante *et al.* 1997, Nasir *et al.* 1995, White *et al.* 1997, Rigamonti *et al.* 2000, Zhang *et al.* 2006, Gauthier *et al.* 2004, Velier *et al.* 1998, Gunawardena *et al.* 2003, Trushina *et al.* 2004, Parker *et al.* 2007). Because the loss of HTT is associated with several deleterious consequences, the allele-specific silencing of mHTT is a promising therapeutic approach to treating HD (Carroll *et al.* 2011, Sah and Aronin 2011, Zuccato, Valenza, and Cattaneo 2010, Dragatsis, Levine, and Zeitlin 2000), although some studies have shown significant beneficial effects from the co-suppression of both mutant and wild-type alleles (Boudreau *et al.* 2009, Drouet *et al.* 2009, Kordasiewicz *et al.* 2012).

The two foremost therapeutic approaches to allele-specific silencing of mHTT are the targeting of single nucleotide polymorphisms (SNP) and direct targeting of the expanded CAG region (Lombardi *et al.* 2009, Pfister *et al.* 2009, van Bilsen *et al.* 2008, Hu, Matsui, and Corey

2009, Hu, Matsui, *et al.* 2009, Gagnon *et al.* 2010). *In vivo* studies have demonstrated successful selective reduction of mHTT and a concomitant amelioration of HD neuropathology and behavioral/motor dysfunctions in mouse models (DiFiglia *et al.* 2007, Harper *et al.* 2005, Yamamoto, Lucas, and Hen 2000). Since AOs were first used to downregulate the expression of HTT, much attention has been given to developing antisense strategies aimed at selectively reducing mHTT levels (Boado *et al.* 2000). A variety of AO chemistries, including PNA, LNA, 2'-MOE, and morpholino chemistries have been used *in vitro* and *in vivo* to selectively reduce levels of mHTT (Carroll *et al.* 2011, Kordasiewicz *et al.* 2012, Hu, Matsui, and Corey 2009, Gagnon *et al.* 2010, Hu, Dodd, *et al.* 2009, Nellemann *et al.* 2000, Fiszer *et al.* 2012). Notably, similar to DM1, 2'-MOE modified antisense oligo infusion into the cerebrospinal fluid of HD mouse models successfully reversed the disease progression with RNase H1-mediated degradation of huntingtin mRNA (Kordasiewicz *et al.* 2012). A recently-concluded phase I/II clinical trial conducted by Ionis Pharmaceuticals (NCT02519036) investigating their 2'-MOE antisense drug, IONIS-HTTRx, produced very encouraging results. The 13-week, randomized, placebo-controlled, dose escalation study evaluated the safety, tolerability, and bodily localization of IONIS-HTTRx in 46 patients with HD. Patients received various doses of the drug (between 10 mg and 120 mg), or placebo, as monthly injections into their cerebrospinal fluid (CSF). Patients receiving the two highest doses showed a significant decrease (~40-60%) in mHTT levels, without significant adverse reactions. Although clinical outcome was not the aim of this study, a post-hoc analysis showed a correlation between reduced mHTT and improved scores in several clinical measures, including the Composite Unified Huntington's Disease Rating Scale (cUHDRS) (rho=-0.41, p=0.004). Ionis has now partnered with Roche to pursue a subsequent phase II open-label extension trial (NCT03342053) for patients who completed the phase I/II study.

1.15 Alternative therapeutic strategies for neuromuscular disease

In addition to antisense therapy, there are other potential therapeutic approaches being investigated for treating neuromuscular diseases; of these, one of the most promising is viral-mediated gene delivery using adeno-associated viruses (AAVs). Non-replicating, non-integrating AAV vectors are the mechanism of choice for many gene therapy studies, owing to their lack of pathogenicity, low immunogenicity, and maintenance of prolonged gene expression (Hollinger and Chamberlain 2015). To-date, several AAV serotypes have been identified or developed, each with unique transfection efficiencies and tissue tropism (Hollinger and Chamberlain 2015). Despite a plethora of AAV options, one limitation to this form of gene therapy approach is the limited packaging capacity of AAV vectors, which is only ~5 kb. While AAV vectors can be less immunogenic than other viruses (e.g. adenovirus), they can nevertheless trigger cellular and humoral immune responses against the viral capsid and transgene product (Zaiss and Muruve 2005, Mingozzi and High 2013). Further compounding this hurdle is the fact that individuals can have a preexisting adaptive response to AAV administration, having already been exposed to a particular AAV variant, thereby reducing clinical applicability and efficacy (Louis Jeune *et al.* 2013, Naso *et al.* 2017).

AAV-mediated gene therapy has been explored for DMD. One major obstacle to this approach has been the very large size of the full-length muscle isoform of *dystrophin* (427 kDa encoded on 14 kb mRNA). To overcome this challenge, several mini- and micro-dystrophins have been developed and investigated in preclinical and clinical trials (Harper *et al.* 2002, Mendell, Campbell, *et al.* 2010). AAV vectors are the vector of choice for neuromuscular disorders such as DMD because they are the only vector shown to be able to systemically-target adult muscles in mammals (Seto, Bengtsson, and Chamberlain 2014). In addition to the mini- and micro-dystrophin approach, efforts have been made to deliver the full-length dystrophin by splitting the coding sequence into smaller portions and reconstructing them into the full-length sequence *in vivo* using a multiple vector system (Odom *et al.* 2011, Lostal *et al.* 2014, Koo *et al.*

2014). Although feasible, the multiple vector approach can be less efficient, since each vector must transduce the same cell for successful reconstitution of the full-length sequence.

Despite its challenges, AAV-mediated gene therapy is a widely-investigated therapeutic approach and holds great promise for the muscular dystrophies. There have been previous and there are ongoing clinical trials involving AAV-mediated gene therapy for DMD (NCT00428935, NCT02354781) (Mendell, Campbell, *et al.* 2010), BMD (NCT01519349) (Mendell *et al.* 2015, Al-Zaidy *et al.* 2015), limb girdle muscular dystrophy type 2D (NCT00494195) (Mendell, Rodino-Klapac, *et al.* 2010), limb girdle muscular dystrophy type 2C (NCT01344798), and SMA (NCT02122952).

Recently, the FDA approved the first-ever AAV-based gene therapy: Spark Therapeutics' luxturna (voretigene neparvovec-rzyl). The drug is marketed for one-time treatment of confirmed biallelic *RPE65* mutation-associated retinal dystrophy and comes with a hefty price tag of $425,000 USD per eye. Despite the staggering cost, approval of luxturna represents a significant milestone for AAV-based therapies and will hopefully provide momentum for other gene therapies seeking clinical approval.

Another gene therapy technique which has gained significant popularity in recent years, and which is highly amenable to neuromuscular disease, is gene editing via targeted nucleases, including zinc finger nucleases (ZNFs) (Kim, Cha, and Chandrasegaran 1996, Kim, Lee, and Carroll 2010), meganucleases (Posfai *et al.* 1999), transcription activator–like effector nucleases (TALENs) (Christian *et al.* 2010), and the CRISPR/Cas9 system (Cong *et al.* 2013, Mali, Yang, *et al.* 2013, Long, Amoasii, Bassel-Duby, *et al.* 2016). In this approach, engineered nucleases facilitate single- or double-strand breaks which are then repaired by either non-homologous end joining (NHEJ) or homology-directed repair (HDR). In NHEJ, insertion/deletion mutations can occur, and when breaks are repaired using HDR an exogenous DNA template can be used to precisely modify a target locus (Long, Amoasii, Bassel-Duby, *et al.* 2016). While NHEJ can

facilitate repair throughout the cell cycle, HDR occurs during the cell's S and G2 phases, using sister chromatids as repair templates (Symington and Gautier 2011).

Of the aforementioned approaches, CRISPR-based gene editing is arguably the most popular and recent advances in this technology have allowed for specific genetic manipulations in a variety of *in vitro* and *in vivo* model systems, including muscle and heart (Schwank *et al.* 2013, Swiech *et al.* 2015, Yin *et al.* 2016, Yang *et al.* 2016, Long, Amoasii, Mireault, *et al.* 2016, Nelson *et al.* 2016, Tabebordbar *et al.* 2016). With respect to muscular dystrophy, CRISPR has been used to correct the causative nonsense mutation in the *mdx* mouse model of DMD (Long *et al.* 2014, Long, Amoasii, Mireault, *et al.* 2016, Nelson *et al.* 2016, Tabebordbar *et al.* 2016) and CRISPR has facilitated correction of mutations in muscle cells and induced pluripotent stem cells (iPSCs) derived from human DMD patients (Li *et al.* 2015, Ousterout *et al.* 2015, Wojtal *et al.* 2016, Maggio *et al.* 2016, Iyombe-Engembe *et al.* 2016, Young *et al.* 2016). CRISPR also holds great promise as a therapeutic strategy for other neuromuscular diseases, such as HD (Yang *et al.* 2017, Kolli *et al.* 2017), ALS (Mutihac *et al.* 2015, Wang *et al.* 2017), SMA (DiMatteo, Callahan, and Kmiec 2008), and DM1 (Provenzano *et al.* 2017, van Agtmaal *et al.* 2017).

Notwithstanding the significant progress of gene editing technologies, several key hurdles remain. The first such hurdle is delivery. As with previously-mentioned mini- and micro-dystrophins, the mechanism of choice for delivery of CRISPR gene editing components is typically viral vector, with AAV again being the vector of choice (Maggio, Chen, and Goncalves 2016). Again, issues regarding immune response can be limiting to this approach. Though less prevalent by comparison, a few effective non-viral delivery systems have been developed which utilize microcarriers to deliver gene editing components into cells (Timin *et al.* 2018, He *et al.* 2017, Wang *et al.* 2018).

In addition to immunogenic responses to viral vectors, the potential immunogenicity of the delivered gene editing components themselves creates another issue with gene editing therapy. Furthermore, the rescue of protein products otherwise absent or abnormal could potentially introduce novel epitopes which can also trigger an immune response (Dasgupta *et al.* 2008).

Another major hurdle of gene editing therapy is off-target effects and unintended alterations of the genome. ZFNs and TALENs tend to be less prone to off-target effects, owing to their long target sequences (>30 bp) and the fact that only heterodimers of their nuclease domains will be functional (Long, Amoasii, Bassel-Duby, *et al.* 2016). CRISPR/Cas9, however, utilizes a much shorter 20 bp guide RNA (sgRNA) sequence to bind its target and reports of off-target activity in RNA-guided nucleases have ranged from low to >50% (Hsu *et al.* 2013, Zhang *et al.* 2015). Several approaches have been developed in an effort to reduce off-target effects, including predictive software (Shen *et al.* 2014, Grissa, Vergnaud, and Pourcel 2007, Bland *et al.* 2007), modification of Cas9 nucleases (Mali, Aach, *et al.* 2013, Shen *et al.* 2014, Ran *et al.* 2013, Kleinstiver *et al.* 2016, Slaymaker *et al.* 2016, Cho *et al.* 2014, Frock *et al.* 2015, Guilinger, Thompson, and Liu 2014, Tsai *et al.* 2014, Wyvekens *et al.* 2015), truncation of sgRNAs (Fu *et al.* 2014, Cho *et al.* 2014), and titration of Cas9 and sgRNA concentrations (Hsu *et al.* 2013, Pattanayak *et al.* 2013, Fu *et al.* 2013).

1.16 Study rationale

The overarching objective of the present studies was to help develop novel strategies for treating neuromuscular diseases. In the first study (**Chapter 3**), we investigated the generation and expansion of rare dystrophin-positive revertant fibers in two mouse models of DMD. Very little is known about the origin and development of RFs. Through the characterization of RF expansion in these mouse models of DMD we hope to better understand the underlying mechanisms and factors which influence RF expansion, thereby benefiting potential future

therapies aimed at modulating the evolution of these fibers and rescuing dystrophin protein expression.

In the second study (**Chapter 4**), we examined the *in vitro* feasibility of antisense-mediated exon skipping of *dystrophin* exons 45-55 in DMD patient cells. DMD is a lethal disease without any known cure. Eteplirsen, the only clinically-marketed antisense drug for treating DMD, is regarded by some as ineffective (Lim, Maruyama, and Yokota 2017) and has limited therapeutic applicability (~15% of patients). Effective skipping of exons 45-55 holds great promise as a therapeutic approach for treating DMD as a majority of patients harbor mutations within this particular genetic region (Beroud *et al.* 2007, Nakamura *et al.* 2017). Furthermore, it is known that patients harboring natural exons 45-55 deletion mutations exhibit very mild symptoms, or are asymptomatic, suggesting that the removal of this genetic region does not have a significant detrimental effect on protein function (Taglia *et al.* 2015). To-date, no other group has demonstrated significant *dystrophin* exons 45-55 skipping in human DMD patient cells. Demonstration of *dystrophin* exons 45-55 in human cells helps establish the feasibility of *dystrophin* exons 45-55 skipping as a potential therapeutic approach for treating DMD.

In the third study (**Chapter 5**), we investigated novel *dysferlin* (*DYSF*) exon skipping approaches in dysferlinopathy patient cells. Dysferlinopathy is a very debilitating neuromuscular disease, with no curative therapy and only a few palliative treatment options. To-date, only one potential therapeutic exon skipping target in *DYSF* has been described (exon 32) (Sinnreich, Therrien, and Karpati 2006). Which other exons are dispensable to protein function, and therefore amenable to exon skipping therapy, is unknown. Here, we characterized potential exon skipping patterns through the functional assessment of mutant dysferlin proteins generated from *DYSF* exon deletion constructs. These insights will help identify novel exon skipping targets, providing a foundation for future *in vivo* work and, eventually, translation into clinical utility.

CHAPTER 2: MATERIALS AND METHODS

2 Chapter 2: Materials and Methods

2.1 Animals

Mdx mice with C57BL/6 background, *mdx52* mice, and C57BL/6 mice as controls were used in Chapter 3. The genetic background of *mdx* mice was changed into C57BL/6 as previously described (Wang *et al.* 2011). Male and female *mdx* and *mdx52* mice at 2, 6, 12, and 18months of age were used in this study. Homozygous mutation of the *DMD* gene was confirmed in female mice by genotyping. Male C57BL/6 mice at 2months of age were used as controls. Mice were euthanized by cervical dislocation; then, tibialis anterior (TA) and gastrocnemius (GC) muscles were excised and immediately frozen in liquid nitrogen-cooled isopentane. Samples were stored at −80°C until used for immunohistochemistry and histochemistry.

2.2 Cell lines

All protocols involving human cell lines were carried out in the Yokota laboratory with approval from The Research Ethics Office of the University of Alberta.

Human DMD patient fibroblast cells harboring out-of-frame deletion mutations of dystrophin exons 45-50 (ID: GM05017) and exons 46-50 (ID: GM05162), as well as healthy human fibroblasts (ID: GM23815) were originally obtained from the Coriell Institute for Medical Research (Camden, NJ, USA). Human embryonic kidney cells (HEK293T) were obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA).

Human dysferlinopathy patient fibroblast cells harboring trans-heterozygous c.1958delG/ c.2997G>T mutations (ID: MM-Pt2) and fibroblast cells harboring a homozygous c.5077C>T mutation (ID: MM-Pt1) were supplied by Dr. Hidetoshi Sakurai.

2.3 Cell culture

Fibroblasts (including patient and healthy) and human embryonic kidney cells were cultured in DMEM/F-12 growth media (Invitrogen) containing 10% fetal bovine serum (FBS) and 0.5% penicillin/streptomycin and stored in a CO2 incubator at 37°C. For myotube differentiation of DMD fibroblasts, FACS-sorted fibroblasts were cultured in DMEM/F-12 (Invitrogen) containing 2% horse serum, 1X ITS Liquid Media Supplement (Sigma-Aldrich), and 0.5% penicillin/streptomycin.

2.4 Assessment of RFs and eMHC-positive fibers

The muscle RF assessment was performed according to our previous study (Yokota *et al.* 2006). RFs and eMHC-positive fibers in entire TA or GC muscle sections were observed using a fluorescence microscope (Nikon Eclipse TE 2000-U; Nikon, Tokyo, Japan) with a 20X objective lens. Muscle fibers were regarded as dystrophin-positive only when more than half the membrane circumference was stained in cross-sections. RFs immediately adjacent to each other were characterized as a single cluster. For closer comparison of RFs and eMHC-positive fibers in mice of different groups, at least 8 serial sections at every 100μm from the muscle belly were stained with antibodies to assess the following: the number of RFs in a cross-section, the number of revertant clusters (which reflects the frequency of RF generation), the maximum number of RFs in a cluster (which reflects the clonal expansion of RFs), and the number of eMHC-positive fibers. For data per mouse, the highest number among serial sections was averaged between left and right muscles for each of the aforementioned criteria. Large clusters of eMHC-positive fibers (clusters composed of more than one hundred eMHC-positive fibers, which arose due to severe degeneration and were independent of age) were excluded from counting.

2.5 Hematoxylin and Eosin staining

Hematoxylin and eosin (HE) staining was performed with Mayer's hematoxylin and eosin solutions as previously described (Aoki *et al.* 2012). A DMR microscope (Leica Micro-systems) was used for bright field microscopy with a 20x objective lens. The percentage of CNFs was calculated in 300 to 1,000 fibers in each left and right muscle and was averaged between two muscles per mouse.

2.6 Antisense oligonucleotide design and transfection

The *in silico* design of 30-mer AOs targeting *DMD* exons 45-55 was performed using a predictive software algorithm developed by our group (Echigoya, Mouly, *et al.* 2015) and PMOs were synthesized by Gene Tools (Oregon, USA). PMO sequences are shown in **Table 1**. For our oligo design, we also considered the binding free energy values between AOs and selected oligos with >-9 ΔG (**Table 2**). PMOs at 1, 3 or 10 μM each were transfected as a cocktail into differentiated DMD myotubes using 6 μM Endo-Porter transfection reagent (Gene Tools). Cells were incubated with PMOs in DMEM (Invitrogen) for 48 h and then media was changed to fresh media and cells were incubated an additional 72 h before harvesting for analysis.

PMO sequences targeting *DYSF* exons were designed using the same predictive software tool. The top three PMOs per exon with the highest predicted exon skipping ability, and which met technical criteria for synthesis (e.g. GC content, Tm, self-complementarity), were supplied by Gene Tools (Philomath, Oregon). PMO sequences are shown in **Table 1**. For molecular analysis, PMO cocktails were transfected into 70-80% confluent patient fibroblast cells using 6 μM Endo-Porter (Gene Tools) transfection reagent, at a final concentration of 10 μM each PMO in DMEM (Invitrogen). Cells were incubated in PMOs for 48 h before harvesting. For the two-photon membrane wounding assay, 40-50% confluent patient fibroblast cells were incubated with PMO in GM for 48 hours before being subjected to the wounding assay.

Table 1. PMO sequences used for exon skipping.

Exon Target	Distance from acceptor splice site	Sequence (5' to 3')	Predicted Skipping (%)
DMD Ex45	Ac9	GACAACAGTTTGCCGCTGCCCAATGCCATC	76.2
DMD Ex51	Ac5	AGGTTGTGTCACCAGAGTAACAGTCTGAGT	73.0
DMD Ex52	Ac24	GGTAATGAGTTCTTCCAACTGGGGACGCCT	90.1
DMD Ex53	Ac9	GTTCTTGTACTTCATCCCACTGATTCTGAA	73.9
DMD Ex54	Ac42	GAGAAGTTTCAGGGCCAAGTCATTTGCCAC	62.0
DMD Ex55	Ac0	TCTTCCAAAGCAGCCTCTCGCTCACTCACC	120.4
DYSF Ex28	Ac7	ACTCAATGTCATCCTTGGGAAGCACCTTCT	70.5
DYSF Ex28	Ac11	GGGCACTCAATGTCATCCTTGGGAAGCACC	68.2
DYSF Ex28	Ac41	CATTCCTCATCTTCCCACTTCCAGCCCAGT	61.01
DYSF Ex29	Ac36	TCTTCTCAGCAGGGACCCAGTGCTTCGGCT	86.4
DYSF Ex29	Ac32	CTCAGCAGGGACCCAGTGCTTCGGCTTCCG	79.7

***DYSF* Ex29**	**Ac40**	**TACATCTTCTCAGCAGGGACCCAGTGCTTC**	**62.1**

Table 2. Binding free energies between PMOs used for *DMD* exons 45-55 skipping.

	Binding Free Energy (ΔG) between PMOs					
Exon Target	**45**	**51**	**52**	**53**	**54**	**55**
45	-4.5	-6.7	-6.7	-2.9	-3.3	-6.2
51		-8.7	-5.4	-6.5	-5.9	-7.9
52			-4.2	-3.2	-7.2	-5.3
53				-6.2	-7.4	-2.1
54					-8.4	-5.0
55						-2.7

2.7 RNA extraction and cDNA synthesis

Total RNA was collected from cells using Trizol (Invitrogen) and 200 ng of total RNA was used for analyzing exon skipping efficiency via SuperScript III One-Step RT-PCR System with Platinum Taq DNA Polymerase (Invitrogen) according to manufacturer's instructions. Primers are shown in **Table 3**. PCR products were separated by electrophoresis on a 1.5% agarose gel and bands were excised using either a Wizard® SV Gel and PCR Clean-Up kit (Promega) or PureLink Quick Gel Extraction Kit (Invitrogen).

Table 3. Primers used in site-directed mutagenesis and RT-PCR

Primer name	**Primer Sequence**
Site-Directed Mutagenesis	

hDYSF DSC-B GFP REV	CTACTACTTGTACAGCTCGTCCATGC
hDYSF DSC-B GFP FWD	GGCCGCTCGAGTCTAGAGGG
hDYSF DSC-B Ex49 FWD	GTTTTTCCTGCGTTGTATTATC
hDYSF DSC-B Ex45 REV	ACACAGTAGGTCTGTGGG
hDYSF DSC-B Ex22 FWD	GAAGCTGGCCTGGAGCAG
hDYSF DSC-B Ex18 REV	CTTGCCTGTGTTGAGCTCTG
hDYSF DSC-B Ex19 REV	CTCCACCCGGAGGATGTC
hDYSF DSC-B Ex28 FWD	AACGGGGAGAAGGTGCTT
hDYSF DSC-B Ex25 REV	GGTTTCAGCAAAGACAGACAG
hDYSF DSC-B Ex30 FWD	CACAGGCAGGCGGAGGCG
hDYSF DSC-B Ex27 REV	CACATCGGTGTAGTTGTCACTCATGTAGATCC
RT-PCR	
hDYSF Ex26 FWD	CGAGACTAAGTTGGCCCTTG
hDYSF Ex30 REV	GGCATCTGTCTTGCGGTACT
Ex43/44_167-12_hDMD_Fwd	GACAAGGGCGATTTGACAG
Ex56_135-154_hDMD_Rv	TCCGAAGTTCACTCCACTTG
hGAPDH_662-81_Fwd1	TCCCTGAGCTGAACGGGAAG
hGAPDH_860-79_Rv1	GGAGGAGTGGGTGTCGCTGT

2.8 Immunofluorescence

Dystrophin-positive RFs were detected by immunohistochemistry. Transverse frozen sections (7µm thick) from TA and GC muscles were cut using a Leica CM1900 cryostat (Leica Micro-systems, Wetzlar, Germany). Serial sections were picked up on poly-L-lysine-coated glass microscope slides and air-dried for 30min. Unfixed sections were then blocked in phosphate-buffered saline (PBS) with 20% goat serum, 0.1% TritonX-100 for one hour at room temperature. Dystrophin was detected with rabbit polyclonal primary antibody against human dystrophin C-terminal (1:400) in the blocking solution by overnight incubation at 4°C. After washing 3 times with PBS, the primary antibody was detected with AlexaFluor 488-conjugated goat anti-rabbit IgG secondary antibody (1:2,000) (Molecular Probes, OR, USA) with one-hour room temperature incubation. Nuclear counterstaining was performed with 4',6-diamidino-2-

phenylindole (DAPI) in a mounting agent (Vectashield; Vector Laboratories, CA, USA). Expression of eMHC and its co-localization with RFs were assessed by triple staining using anti-developmental MHC antibody (1:20), anti-dystrophin antibody (1:400), and DAPI after blocking with Mouse on Mouse (M.O.M.) blocking reagent (Vector Laboratories). The rabbit polyclonal antibody against C-terminal domain (position at 3,661–3,677 amino acids) in human dystrophin was used to detect revertant fibers (Abcam, Bristol, UK). eMHC expressed in newly regenerated muscle fibers was detected with mouse monoclonal anti-rabbit developmental MHC antibody (Leica Biosystems, Newcastle upon Tyne, UK).

DMD patient cells were fixed with 4% paraformaldehyde (PFA) for 5 min at room temperature, then permeabilized and blocked with 0.5% Triton X-100 and 10% goat serum for 20 minutes at room temperature. Cells were incubated in primary antibody for 1 h at room temperature at a 1:30 ratio for anti-desmin (ab8592, Abcam), anti-dystrophin (NCL-DYS1, Novocastra), and anti-myosin heavy chain (NCL-MHCf, Novocastra). Cells were incubated with 1:500 anti-rabbit or mouse IgG (H+L) highly cross-adsorbed secondary antibody, Alexa Fluor 594 (Invitrogen). SlowFade Gold Antifade Mountant with DAPI (Invitrogen) was added and cells were stored at 4°C.

2.9 Immunoblot

Protein (1.5 μg) from PMO-treated MM-Pt2 fibroblasts was loaded onto a NuPAGE Novex 3–8% Tris-Acetate Midi Gel (Invitrogen) and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis at 150 V for 70 minutes. For comparison, 1.5, 0.75, 0.38, and 0.19 μg of protein from healthy fibroblasts was also loaded. Proteins were transferred onto an Immobilon PVDF membrane (MilliporeSigma, Darmstadt, Germany) by semidry blotting using 20 V for 45 minutes. The membrane was blocked with phosphate-buffered saline containing 0.05% Tween 20 and 5% skimmed milk, then incubated with the NCL Hamlet monoclonal antibody (Leica Biosystems, Wetzlar, Germany; 1:4000 in blocking solution) or alpha-tubulin

ab7291 antibody (Abcam, Cambridge, United Kingdom; 1:8000 in blocking solution) at 4 °C overnight. Membranes were then incubated with 1:10000 Goat anti-Mouse IgG (H+L) Secondary Antibody, HRP (Thermo Fisher), followed by incubation with ECL Select (GE Healthcare, Little Chalfont, United Kingdom). Bands were detected using a ChemiDoc system (Bio-Rad, Hercules, California).

2.10 Plasmid design and transfection

A cDNA expression construct containing N′-terminally GFP-tagged *DYSF* (termed full-length *DYSF* plasmid) was generously provided by Dr. Katherine Bushby (Newcastle University). All *DYSF* constructs investigated in this study (Δ19-21, Δ20-21, Δ26-27, Δ28-29, Δ46-48, GFP-only) were generated using a Q5 Site-Directed Mutagenesis Kit (New England Biolabs, Ipswich, Massachusetts) and this original full-length plasmid, according to manufacturer's instructions (for primers, see **Table 3**). Expression constructs were transfected into cells using a Lipofectamine LTX with Plus Reagent kit (Thermo Fisher) according to manufacturer's instructions. In brief, fibroblast cells were seeded into 35 mm collagen-coated glass-bottom dishes (MatTek, Ashland, MA) and cultured in 2 mL of growth media for 24 h. After incubation, media was changed to 2.3 mL of Opti-MEM (Thermo Fisher) containing 3 µL Lipofectamine LTX Transfection Reagent, 3.5 µL of PLUS Reagent, and 3.5 µg of plasmid. Cells were then incubated for 24 h, after which, media was changed to fresh growth media and cells were incubated an additional 24 h before imaging and membrane wounding analysis.

2.11 Membrane wounding assay

Human fibroblast plasma membranes were subjected to laser-induced injury using two-photon laser microscopy as described previously (Lee, Maruyama, et al. 2018). In brief, cells in glass-bottom dishes were prepared for wounding by rinsing once with Tyrode's salts solution (MilliporeSigma) followed by addition of 1 mL of Tyrode's salts containing 2.5 mM FM 4-64 dye (Invitrogen). Using a Zeiss LSM 710 inverted confocal laser scanning microscope and Zeiss ZEN

software, a 0.2 μm x 2 μm target was placed at the edge of the cell membrane. A 5 min time series of sequential image scans was performed, with cells imaged every 5 s. Cells were ablated 25 s after the beginning of the time series using a two-photon laser set to 820-nm, using 15% laser power with 10 iterations. Fluorescence values at sites of injury were quantified using Zeiss ZEN software and for each time point relative fluorescence values were determined by subtraction of the background value (mean of t = 0-25 s) and division of the net increase in fluorescence by the background fluorescence value.

2.12 Statistical Analysis

In Chapter 3, all data were reported as mean values ± standard deviation (S.D.). The statistical differences between the age groups or the strains were assessed by one-way ANOVA with a Tukey–Kramer multiple comparison test. Pearson correlation coefficient was performed between the number of RFs in a section, the number of RF clusters, and the maximum number of RFs in a single cluster. $P<0.05$ was considered statistically significant.

In Chapter 5, all data were reported as mean values ± standard error (S.E.). The statistical differences between treatment groups were assessed by one-way ANOVA with a Tukey–Kramer multiple comparison test. $P<0.05$ was considered statistically significant.

CHAPTER 3: Mutation Types and Aging Differently Affect Revertant Fiber Expansion in Dystrophic *Mdx* and *Mdx52* Mice

3 Chapter 3: Mutation Types and Aging Differently Affect Revertant Fiber Expansion in Dystrophic *Mdx* and *Mdx52* Mice

3.1 Introduction

Duchenne muscular dystrophy (DMD) is the most common genetic muscular disease and is characterized by progressive muscle degeneration. It occurs with a frequency of about 1 out of 3,500 boys and is caused by mutations in the *dystrophin* (*DMD*) gene (Lovering, Porter, and Bloch 2005, Biggar *et al.* 2002, Duchenne 1867, Hoffman, Brown, and Kunkel 1987). The mutation leads to progressive myopathy and muscle weakness coupled with cycles of muscle degeneration. Death eventually occurs due to severe respiratory and/or cardiac failure at approximately 20–30 years of age (McNally and Pytel 2007). The *DMD* gene is located on the X-chromosome and is one of the largest and more complex genes in humans, containing 79 exons and spanning more than 2.4 million base pairs (Koenig *et al.* 1987, Hoffman, Brown, and Kunkel 1987). The *DMD* gene encodes at least 18 protein isoform products of dystrophin with tissue-specific alternative promoters (Holder, Maeda, and Bies 1996, Feener, Koenig, and Kunkel 1989, D'Souza *et al.* 1995, Nishio *et al.* 1994, Blake *et al.* 2002, Muntoni, Cau, Congiu, *et al.* 1993, Muntoni, Cau, Ganau, *et al.* 1993, Wheway and Roberts 2003, Nudel *et al.* 1989, Monaco *et al.* 1986, Gorecki *et al.* 1992, Lidov, Selig, and Kunkel 1995, Byers, Lidov, and Kunkel 1993, Lederfein *et al.* 1992, Austin *et al.* 1995, Tinsley, Blake, and Davies 1993). The main skeletal muscle isoform is the largest known, consisting of 3,685 amino acids (427 kDa). In skeletal muscles, dystrophin plays a central role in organizing a multi-protein complex at the

sarcolemma and linking cytoskeleton proteins to extracellular matrix proteins (Rybakova, Patel, and Ervasti 2000, Watkins *et al.* 2000). The full-length dystrophin protein can be divided into actin-binding NH_2- (N) terminal, rod, cysteine-rich, and COOH- (C) terminal domains (Nishio *et al.* 1994, Monaco *et al.* 1986). Interestingly, most of the known functions of the protein are assigned to the N- and C- terminals and the cysteine-rich domain (Bies, Caskey, and Fenwick 1992). In contrast, the central rod domain, consisting of 24 spectrin repeats with 4 hinges, and spanning about half the length of the protein, appears to be less essential to proper function (Bhasin *et al.* 2005).

Most *DMD* mutations which cause disruption of the open reading frame occur within the central rod domain, thus preventing translation of the crucial C-terminal domain (Yokota, Takeda, *et al.* 2009, Walmsley *et al.* 2010, Gualandi *et al.* 2006). In the case of Becker muscular dystrophy (BMD), a milder form of muscular dystrophy also caused by mutations in the *DMD* gene, most mutations occur in the same rod domain regions but the mutated mRNA transcripts preserve the open reading-frame and are thus translated into a truncated yet partially-functional protein (Covone, Lerone, and Romeo 1991). The *mdx* mouse, an animal model of human DMD, harbors a natural nonsense point mutation in exon 23, while the *mdx52* mouse has a deletion mutation of exon 52 owing to a gene-targeting method (Bulfield *et al.* 1984, Sicinski *et al.* 1989, Araki *et al.* 1997, Aoki *et al.* 2010). *Mdx* mice were originally discovered with C57BL/10 background but they were later backcrossed with a C57BL/6 background for comparison with other mouse models, including *mdx52* mice with a C57BL/6 background (Wang *et al.* 2011). Both mutants exhibit a lack of dystrophin expression and cycles of muscle degeneration/regeneration (Yokota *et al.* 2006, Aoki *et al.* 2012). Thus, the comparison between *mdx* and *mdx52* mice with the same genetic background should be quite useful in understanding pathogenic mechanisms dependent on different types of nonsense and exon-deletion mutations, which are found in approximately 10–15% and 50–60% of DMD patients, respectively (Yokota, Duddy, *et al.* 2012, Den Dunnen *et al.* 1989).

Interestingly, skeletal muscles in most DMD patients and animal models, including mouse and dog models, display sporadic dystrophin-positive muscle fibers called "revertant fibers" (RFs) in an otherwise dystrophin-negative background (Hoffman *et al.* 1990, Nicholson *et al.* 1993, Sherratt *et al.* 1993, Yokota, Hoffman, and Takeda 2011, Arechavala-Gomeza *et al.* 2010, Winnard *et al.* 1995, Danko, Chapman, and Wolff 1992, Yokota, Lu, *et al.* 2009). Danko *et al* reported distinct frequencies of RFs in different *mdx* mutants (*mdx2cv*, *3cv*, *4cv*, and *5cv* mutants) (Danko, Chapman, and Wolff 1992). These dystrophin-positive fibers arise from spontaneous exon skipping (alternative splicing) with a loss of up to 30 exons, leading to the production of in-frame truncated proteins (Lu *et al.* 2000). In *mdx* mice, these RFs expand with age through cycles of muscle degeneration/regeneration and the activation of muscle precursor cells (Yokota *et al.* 2006, Lu *et al.* 2000). In human DMD patients, the increase of RFs correlates significantly with age, up to the early teens (Fanin *et al.* 1995). These revertant events are thought to arise within a subset of muscle precursor cells which proliferate in response to muscle degeneration and participate in the regeneration of muscle fibers (Yokota *et al.* 2006). These fibers often produce clonal clusters that can expand to up to 100 fibers, measuring more than 1 mm in length by 18 months of age (Lu *et al.* 2000). To date, there is no report of either genomic deletion or secondary mutation as the mechanism facilitating the restoration of dystrophin in RFs. Our previous studies using various transgenic mouse models, including micro-dystrophin transgenic *mdx* mice and utrophin overexpressing mice, as well as irradiated *mdx* models, clearly demonstrated that expansion of RF clusters is dependent on muscle regeneration (Yokota *et al.* 2006, Wakeford, Watt, and Partridge 1991, Tinsley *et al.* 1996, Sakamoto *et al.* 2002, Pagel and Partridge 1999). The expansion of RF clusters reflects the cumulative history of skeletal muscle regeneration and is thought to provide a useful index for functional evaluation of therapies that diminish muscle degeneration (Yokota *et al.* 2006).

In this study, we employed two mouse models: *mdx* mice with C57BL/6 background, and *mdx52* mice (Araki *et al.* 1997, Wang *et al.* 2011). We compared their RF generation and

long-term expansion up to 18 months of age. Interestingly, *mdx* mice exhibited a higher number of RFs compared to *mdx52* mice, indicating the occurrence of revertant events largely depends on the type of mutation present in the *DMD* gene. To our surprise, although both mouse models showed an increase in RFs through 18 months, the number of centrally nucleated fibers (CNFs) and embryonic myosin heavy chain (eMHC)-positive fibers (as cumulative and current indicators of muscle regeneration, respectively) decreased with age in both the strains, except for an increase in CNFs between 2 to 6months of age in *mdx* mice. Overall, the dynamics of muscle regeneration associated with age was more markedly altered in *mdx* than *mdx52* mice. These data reported here show that mutation types and aging differently affect RF expansion and muscle regeneration in *mdx* and *mdx52* mice.

3.2 Results

3.2.1 Distinct patterns of revertant fiber expression and clustering in mdx and mdx52 mice

To investigate the effect of different mutation types of the *DMD* gene on the generation and expansion of RF clusters, we examined RFs in tibialis anterior (TA) and gastrocnemius (GC) muscles of *mdx* mice with C57BL/6 background and *mdx52* mice (also with C57BL/6 background). *Mdx* mice harbor a nonsense mutation in exon 23 and *mdx52* mice contain a deletion mutation in exon 52 (Sicinski *et al.* 1989, Araki *et al.* 1997). RFs were observed in all age groups in both *mdx* and *mdx52* mice and centrally-located nuclei were found in most RFs (**Figure 3.1**). A rabbit polyclonal antibody against the C-terminal domain of dystrophin was used to detect revertant dystrophin expression because this amino acid-region is reported to be retained in most of the truncated dystrophin or RF proteins in *mdx* and DMD patients (Winnard *et al.* 1995, Lu *et al.* 2000, Klein *et al.* 1992). *Mdx* mice clearly showed a higher number of RFs and RF clusters in TA and GC muscles across all age groups in comparison to *mdx52* mice (**Figure 3.1, Figure 3.2A, 3.2B**). There were significantly fewer RFs and RF clusters in *mdx52* mice when compared to *mdx* mice. *Mdx* mice also showed a significantly higher

number of RFs per cluster compared with *mdx52* mice, except at 2 months of age in GC muscle (**Figure 3.2C**).

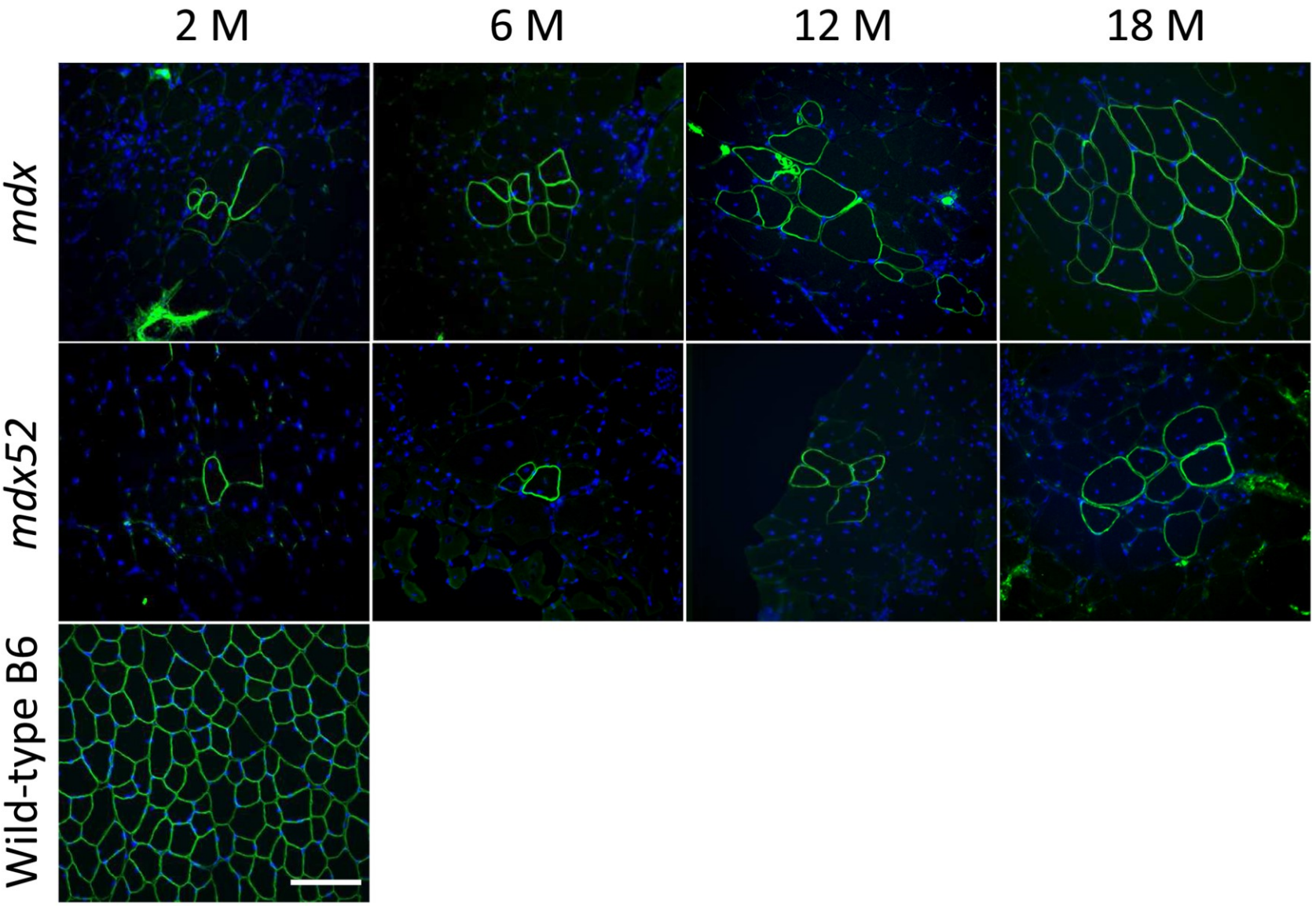

2 M
6 M
12 M
18 M
mdx
mdx52
Wild-type B6

Figure 3.1: Dystrophin-positive revertant fibers with central nuclei at ages of 2, 6, 12, and 18 months in *mdx* and *mdx52* mice.

Representative immunohistochemical images of maximum clusters of RFs in TA muscles are shown in each group. *Mdx* shows a higher maximum number of RFs than *mdx52* in all age groups. Wild-type C57BL/6 muscle at 2 months old is displayed as a control. An anti-dystrophin C-terminal antibody (green) and DAPI staining (blue) were used. M: months. 20x objective lens, scale bar = 100μm. From (Echigoya *et al*. 2013).

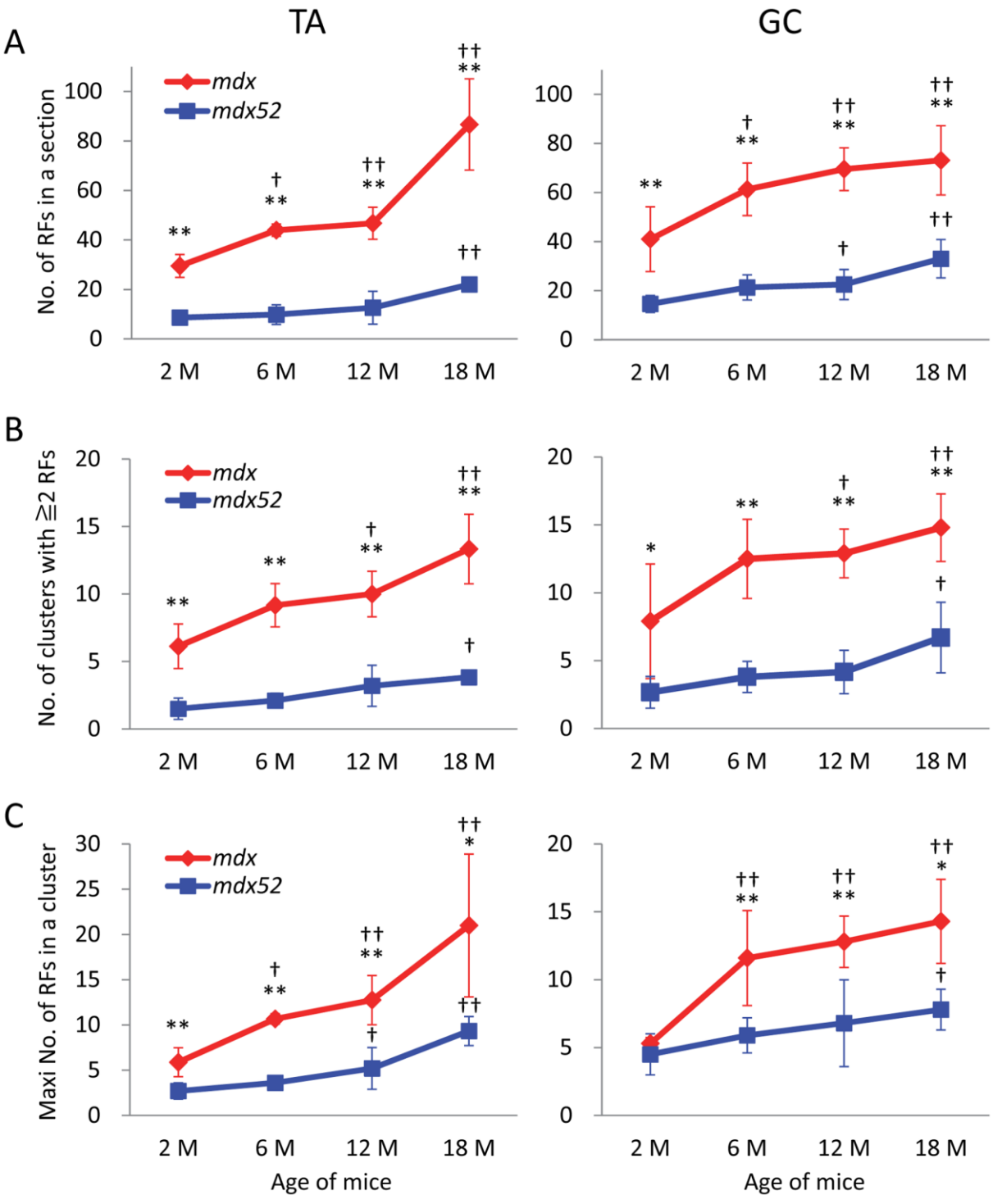

A
TA
GC
No. of RFs in a section
mdx
mdx52
2 M
6 M
12 M
18 M
B
No. of clusters with ≧2 RFs
C
Maxi No. of RFs in a cluster
Age of mice

Figure 3.2: Mutation- and age-related expression of dystrophin-positive revertant fibers in TA and GC muscles from *mdx* and *mdx52* mice.

(A) The number of RFs in one TA or GC section. (B) The number of RF clusters containing 2 or more positive fibers. (C) The maximum number of RFs in a single cluster. *Mdx* mice have a significantly higher number of RFs in all criteria than *mdx52* mice except for 2 months of age in maximum number of RFs per cluster. The number of RFs in all criteria increases with age. Values are mean ± S.D. (n=3–6 mice per each group). *P<0.05, **P<0.01 between *mdx* and *mdx52* mice; †P<0.05, ††P<0.05 compared to 2 months old. M: months. From (Echigoya *et al.* 2013).

We then compared the expression and clustering levels of RFs at 2, 6, 12, and 18 months of age for *mdx* and *mdx52* strains individually. In TA and GC muscles from *mdx* and *mdx52* mice, the number of RF, RF clusters, and the maximum number of RFs per cluster significantly increased compared to 2 months of age (**Figure 3.2**). The Pearson correlation coefficient showed a strong correlation among these three criteria regarding expression and clustering levels of RFs in each strain (squared correlation coefficient; $R^2 = 0.27–0.84$, $P<0.05$). A significant increase in the number of RFs and the maximum number of RFs per cluster at 6 months of age was found only in *mdx* mice. *Mdx* mice at 12 months also showed a significant increase in the number of RF clusters compared to 2 months old. In contrast to the dramatic RF expansion with age in *mdx* mice, *mdx52* mice showed only a moderate increase in RFs with age across all measured criteria. A significant increase in RF expression and clustering was found at 18 months in *mdx* mice compared with *mdx52* mice. Levels of RF expression/clustering in *mdx52* mice never reached levels seen in *mdx* mice. This result is not attributable to genetic background, since both mouse strains have a C57BL/6 background. The *mdx52* mouse, which has fewer RFs and shows little change in RF expansion with age, may be a good model for the testing of new therapies aimed at restoring dystrophin expression because pre-existing RFs can disturb accurate assessments of restored dystrophin expression owing to therapeutic treatment.

3.2.2 Dynamics of muscle regeneration with age is different between mdx and mdx52 mice

To examine whether muscle regeneration is physiologically correlated with RF expansion, we assessed centrally-nucleated fibers (CNFs) and eMHC-positive fibers, which are cumulative and current indicators of muscle regeneration, respectively, in *mdx* and *mdx52* mice. During muscle regeneration, regenerated skeletal muscle fibers are centrally-nucleated as opposed to mature muscle fibers which are peripherally-nucleated (Narita and Yorifuji 1999, Wroblewski, Edstrom, and Mair 1982). Also, centrally-located nuclei in *mdx* mice are reported to persist long-term, indicating that the percentage of CNFs represents the accumulated history of

regeneration up to the present (McGeachie *et al.* 1993, Shavlakadze *et al.* 2004). Following HE staining, *mdx* mice showed a significantly lower percentage of CNFs than *mdx52* mice at 2 months, followed by an increase in percent CNFs, resembling observations in *mdx52* mice at 6 months, and a subsequent decrease in CNFs at 12 and 18 months (**Figure 3.3**). At 18 months in *mdx* mice, a significant decrease in CNFs was found in both TA and GC muscles when compared to 6 months (the age of peak CNF percentage). In contrast to the dynamics of CNFs in *mdx* mice, the percentage of CNFs in *mdx52* mice consistently and significantly decreased over time from the peak at 2 months old. This finding indicates that there is a different peak in muscle regeneration between *mdx* and *mdx52* mice which is before 6 months of age and before 2 months of age, respectively.

To examine the possible correlation between ongoing muscle regeneration and RF expansion, we analyzed the co-localization of eMHC with revertant dystrophin and the number of eMHC-positive fibers, which are found short-term during muscle regeneration. Interestingly, eMHC was not expressed in RFs at any age in *mdx* and *mdx52* mice (**Figure 3.4A**). The number of eMHC-positive fibers was similar between *mdx* and *mdx52* mice at each age (**Figure 3.4**). However, expression dynamics of eMHC with age was different between the strains. In *mdx* mice, the number of eMHC-positive fibers was the highest at 2 months of age and significantly decreased at 18 months of age in TA and GC muscles, while in *mdx52* mice the number was slightly but not significantly decreased through 18 months. These data from CNFs and eMHC-positive fibers indicate that in *mdx* and *mdx52* mice the dystrophic skeletal muscles actively regenerate throughout their life span, but the muscle regeneration activity with age is different between the strains.

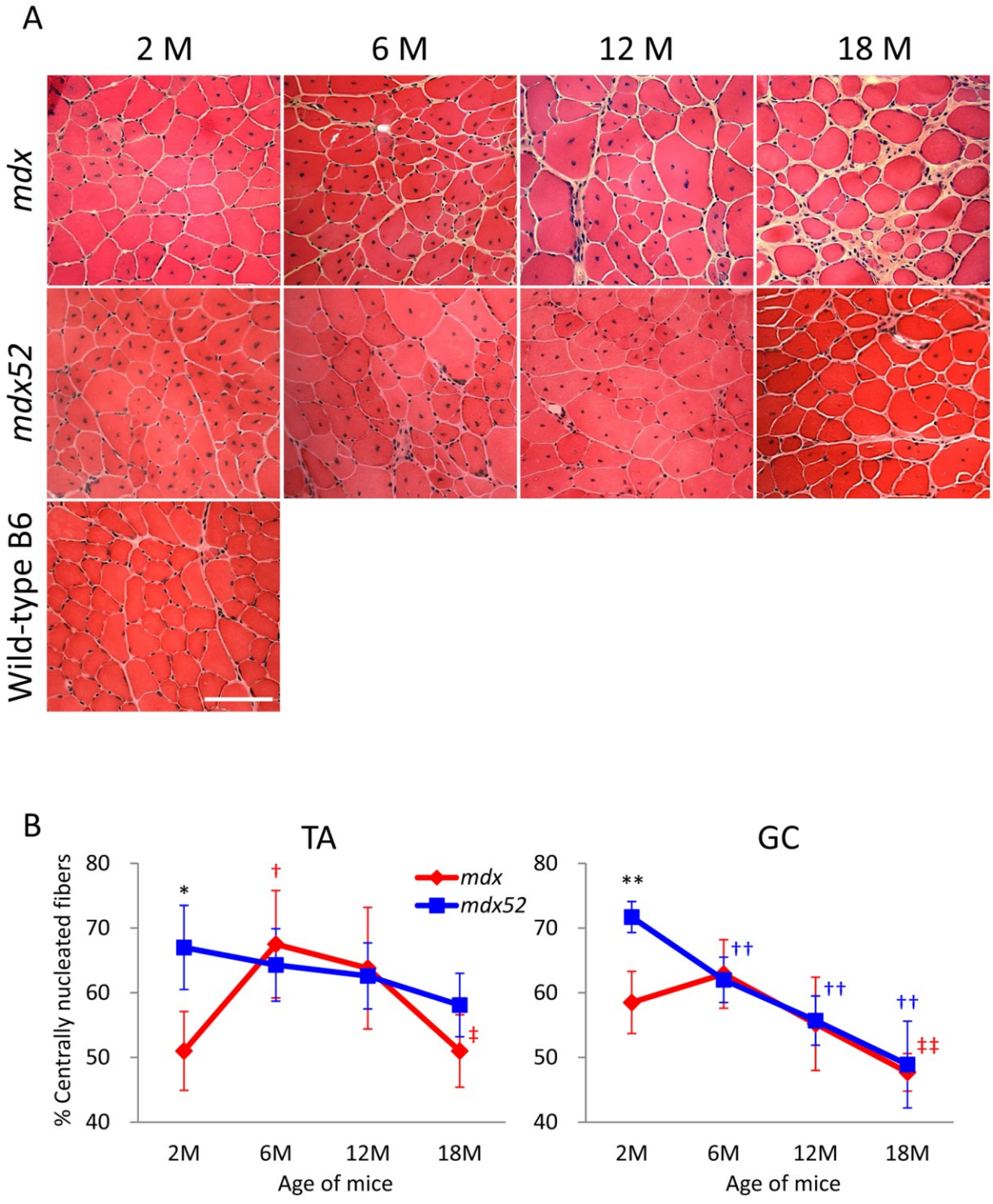
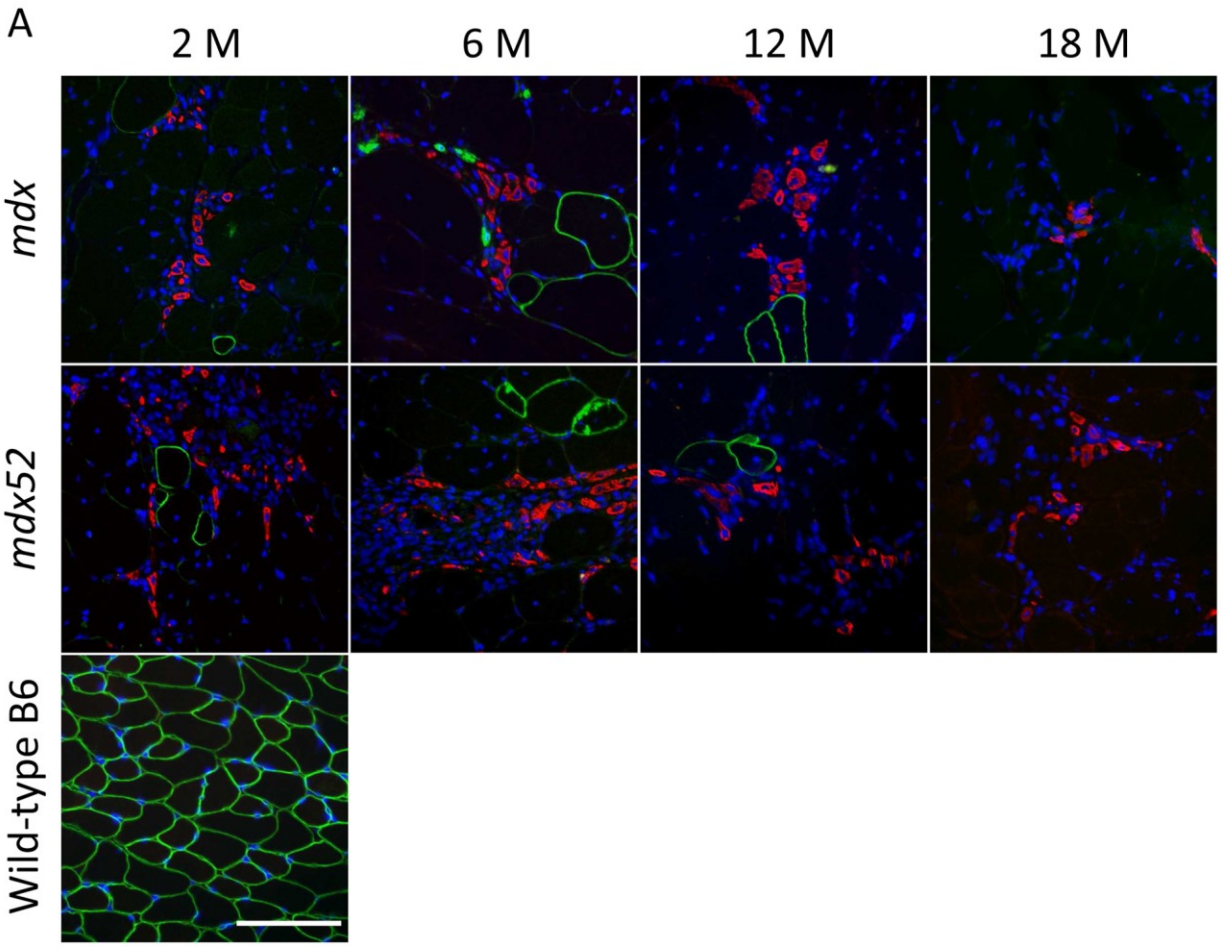

A
2 M
6 M
12 M
18 M
mdx
mdx52
Wild-type B6
B
TA
GC
% Centrally nucleated fibers
80
70
60
50
40
mdx
mdx52
*
†
‡
**
††
‡‡
2M
6M
12M
18M
Age of mice

Figure 3.3: Distinct changes in the percentage of centrally-nucleated fibers by mutations and age in *mdx* and *mdx52* mice.

(A) Representative images of TA muscles from *mdx* and *mdx52* mice at ages 2, 6, 12 and 18 months with hematoxylin and eosin staining. Wild-type C57BL/6 muscle at 2 months of age is displayed as a control. M: months. Scale bar = 100μm. (B) The percentage of centrally-nucleated fibers in TA and GC muscles from *mdx* and *mdx52* mice. Three hundred to one thousand myofibers were counted in left and right muscles and the percentage of CNFs was averaged between the two muscles per mouse. Values are mean ± S.D. ($n = 3–6$ mice per group). *$P<0.05$, **$P<0.01$ between *mdx* and *mdx52* mice; †$P<0.05$, ††$P<0.01$ compared to 2 months old; ‡$P<0.05$, ‡‡$P<0.01$ compared to 6 months old. Symbol colors are accordant with the color of mice (red; *mdx*, blue; *mdx52*). From (Echigoya *et al.* 2013).


A
2 M
6 M
12 M
18 M
mdx
mdx52
Wild-type B6

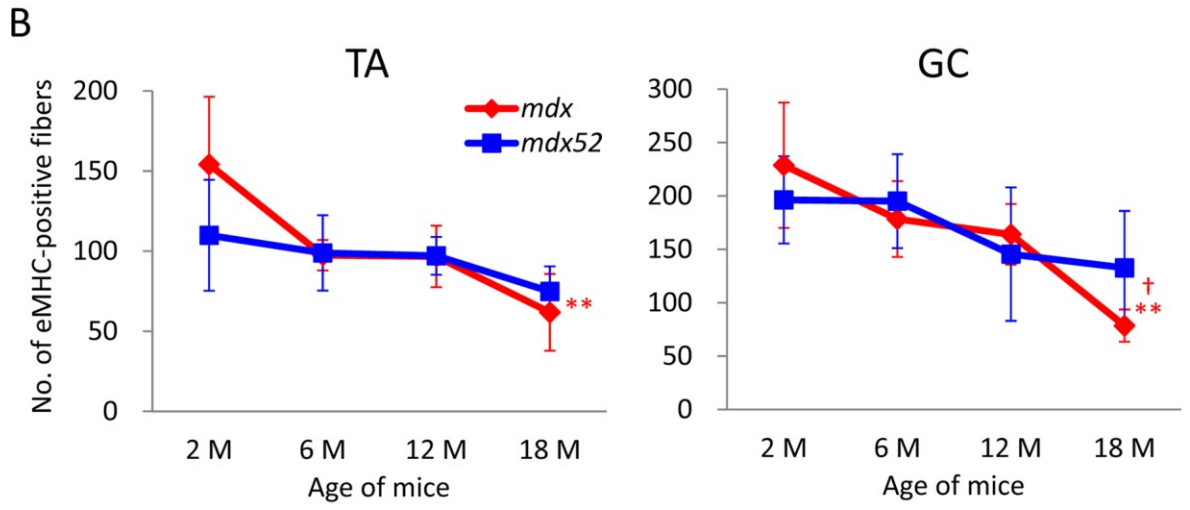

B
TA
GC
mdx
mdx52
No. of eMHC-positive fibers
200
150
100
50
0
300
250
200
150
100
50
0
2 M
6 M
12 M
18 M
Age of mice
**
†
**

Figure 3.4: No expression of eMHC in RFs and attenuation of ongoing muscle regeneration in aged *mdx* and *mdx52* mice.

(A) Triple staining of *mdx* and *mdx52* mice for RF (green), eMHC (red), and nucleus (blue). Revertant dystrophin is not co-localized with newly regenerated eMHC-positive fibers in TA and GC muscles from *mdx* and *mdx52* mice at any age. The pictures are representative GC muscles from *mdx* and *mdx52* mice at each age. 20x objective lens, scale bar = 100μm. (B) The number of eMHC-positive fibers. Values are mean ± S.D. (n = 3–6 mice per group). A significant decrease in the number of eMHC-positive fibers is found only at 18 months old in *mdx* mice (**P<0.01 compared to 2 months old, †P<0.05 compared to 6 and 12 months old). Symbol colors are accordant with the color of mice (red; *mdx*, blue; *mdx52*). From (Echigoya *et al.* 2013).

3.3 Discussion

Dystrophin RFs were first described more than 20 years ago by Hoffman *et al* (Hoffman *et al.* 1990), however, the mechanisms behind both the generation and expansion of RFs remain poorly understood. Lu *et al* previously reported that revertant events arise in individual muscle satellite cells at around birth (Lu *et al.* 2000). RFs appear at around birth as short segments, approximately 10 μm in length, of sporadic single muscle fibers. One of the most interesting characteristics of the RFs is their substantial expansion with age. In *mdx* mice, RFs continuously expand at least up to 18 months, resulting in clusters containing more than 100 fibers and traversing 1 mm or more of muscle fiber length. However, RFs never accumulate to a sufficient number to significantly ameliorate dystrophic muscle pathology (Yokota *et al.* 2006).

Our previous study with irradiated *mdx* mice aged up to 2 months supported the hypothesis that muscle regeneration is essential for RF expansion in *mdx* mice (Yokota *et al.* 2006). The expansion of RFs has been attributed to the combined effects of cycles of muscle degeneration/regeneration and increased survival of the fibers containing truncated dystrophin (Yokota *et al.* 2006, Garcia *et al.* 1999). However, we demonstrated that mutation types and aging differently affect the generation and expansion of dystrophin-positive RFs in *mdx* and *mdx52* mice.

Our paradoxical result reported here strongly challenges our previous hypothesis, since rapid expansion of RFs and a decrease in regenerated CNFs and eMHC-positive fibers occur concurrently at older ages (**Figures 3.1 – 3.4**). This is in sharp contrast to previous experiments with transgenic *mdx* muscles (Yokota *et al.* 2006, Crawford *et al.* 2001). No expansion of RFs was observed in any transgenic *mdx* mice expressing mini- or micro-dystrophin in spite of the significant muscle growth before the age of 5 weeks. Instead, the number of RFs decreased with age in these transgenic *mdx* mice. Our observations here lead us to propose two possibilities to explain RF expansion with age: first, mechanisms other than

muscle regeneration, such as secondary DNA mutation, up-regulation of short dystrophin isoforms, or age-related changes affecting splicing machinery may be involved in RF expansion in older muscle fibers. The expansion of RFs independent of muscle regeneration supports the existence of a mechanism in aged muscles that Lu *et al* described previously; i.e. RF expansion may represent the progressive increase in a territory of factors, each of which determines a specific pattern of exon skipping (alternative splicing), and which spread by diffusion within each fiber and between neighboring fibers (Lu *et al.* 2000). This hypothesis predicts that RF clusters grow within the existing stable (mature) muscle fibers. A second possibility is that the increase of RFs with age may be related to RF stability combined with the expression of partially functional dystrophin proteins; thus, more stable RFs would accumulate with age and prevent degeneration/regeneration cycles in themselves and in closely surrounding fibers. This could lead to a decrease in muscle regeneration with age in both mouse strains. Nevertheless, muscle regeneration appears to be fundamentally required for RF expansion because central nucleation was found in most RFs through 18 months in this study, and our previous study demonstrated that RF expansion does not occur in the absence of regeneration, even when degeneration continues after irradiation (Yokota *et al.* 2006). Another hypothesis to possibly explain these conflicting observations is that regenerating muscle fibers with central nucleation and/or eMHC in older dystrophic mice might change to mature fibers without them more rapidly than in younger dystrophic mice; thus, a reduced number of CNFs and eMHC-positive fibers might not necessarily reflect the frequency of regeneration in older muscles, although there is currently no evidence to support this hypothesis.

As no such phenomenon like RFs has been described in other genetic disorders, the mechanism of their genesis and expansion is still open to speculation, although it seems that modification of splicing is involved. Thus, RFs could be an interesting model with which to investigate the mechanisms of spontaneous exon skipping (alternative splicing). Future molecular analysis of RFs will also provide invaluable information toward the development of

molecular therapies, such as antisense-mediated exon skipping, which are aimed at inducing the production of revertant-like dystrophin-positive fibers for the treatment of DMD (Hoffman *et al.* 2011, Fall *et al.* 2006, Yokota, Duddy, and Partridge 2007).

CHAPTER 4: Antisense PMO cocktails effectively skip *dystrophin* exons 45-55 in myotubes transdifferentiated from DMD patient fibroblasts

4 Chapter 4: Antisense PMO cocktails effectively skip dystrophin exons 45-55 in myotubes transdifferentiated from DMD patient fibroblasts

4.1 Introduction

Duchenne muscular dystrophy (DMD) is a lethal, progressive myopathy affecting approximately 1 in every 3600-5000 male births and is caused by deleterious mutations in the *dystrophin* (*DMD*) gene (Duchenne 1867, Hoffman, Brown, and Kunkel 1987, Mah *et al.* 2014, Mendell *et al.* 2012). Mutations in *DMD* can also cause another milder form of muscular dystrophy known as Becker muscular dystrophy (BMD) (Koenig *et al.* 1989). Typically, DMD arises from out-of-frame mutations (~65% of patients) while BMD generally arises from in-frame mutations (~82% of patients) (Magri *et al.* 2011, Nakamura *et al.* 2013, Flanigan 2014).

The observation that truncated dystrophin protein arising from in-frame mutant *DMD* transcripts could still maintain partial functionality – as in the case of BMD – helped provide the rationale for a therapeutic approach involving splice modulation via synthetic polymers. Antisense oligonucleotides (AOs) are chemically modified nucleic acids which can hybridize to pre-mRNA and can affect splicing and protein synthesis (Lee and Yokota 2013, Miller and Harris 2016). By utilizing AOs, mutation-carrying exons and flanking exons can be selectively removed or "skipped" from the final messenger transcript, restoring the reading frame and producing a truncated protein which may retain some functionality – in essence, exon skipping could convert a DMD phenotype to a BMD phenotype (Yokota, Takeda, *et al.* 2009, Lee and Yokota 2013, Niks and Aartsma-Rus 2017). Several *in vitro* and *in vivo* studies have now demonstrated the feasibility of antisense-mediated exon skipping in *DMD* (Saito *et al.* 2010, Echigoya, Aoki, *et al.* 2015, Aoki *et al.* 2012, Yokota, Nakamura, *et al.* 2012, Aartsma-Rus *et al.* 2003, van Deutekom *et al.* 2001) and the first-ever clinical AO drug for treating DMD, eteplirsen (Exondys 51), was approved by the FDA in 2016 (Aartsma-Rus and Krieg 2017).

Exon skipping as a therapy for treating DMD is not without its challenges. The first major drawback is the requirement for specifically-targeted AO sequences for any given exon. This means a great deal of time and money needs to be spent in evaluating individual AO sequences to address a wide range of patient mutation patterns. Another big challenge is determining the stability and functionality of the truncated proteins (Hoffman *et al.* 2011, Yokota, Duddy, and Partridge 2007). A solution to the specific issue of validating oligo sequences for accommodating multiple mutation patterns is using analytical software algorithms to predict AO exon skipping efficiency, as has been described (Echigoya, Mouly, *et al.* 2015). In conjunction with *in silico* predictive tools, another possible avenue to circumventing these issues is multi-exon skipping of *DMD* exons 45-55. First, in terms of applicability, a large proportion of all DMD patients (~47%) harbor mutations within this "hot-spot" region of exons 45-55, and up to ~63% of DMD patients with deletion mutations could benefit from skipping exons 45-55 (Beroud *et al.* 2007, Nakamura *et al.* 2017). Multi-exon skipping of exons 45-55 would also address the issue of unknown truncated protein stability/function, as patients exhibiting this particular pattern of deletion mutation present with exceptionally mild symptoms or are asymptomatic (Nakamura *et al.* 2017, Beroud *et al.* 2007, Nakamura *et al.* 2008).

As more AOs are required to bind to the same transcript to ensure maintenance of the open reading frame, dosage levels must be sufficiently high enough to facilitate skipping multiple exons while avoiding overt toxicity. While several AO chemistries have been designed and tested (Lee and Yokota 2013, Shen and Corey 2017), one of the most promising antisense chemistries developed is the phosphorodiamidate morpholino oligomer (morpholino or PMO). The stability, safety, and tolerability of PMOs have been well-documented (Sazani *et al.* 2011, Heald *et al.* 2014, Moulton and Moulton 2010), even at high doses (Wu *et al.* 2010, Wu *et al.* 2011), and it is worth noting that the clinically-utilized eteplirsen is a PMO chemistry (Mendell *et al.* 2016). PMOs could therefore address issues regarding the potential toxicity of AO cocktails

as they could be delivered at concentrations high enough to facilitate exons 45-55 skipping while avoiding toxic effects. Our group has demonstrated this previously in a mouse model of DMD, using a cocktail of PMOs to effectively skip multiple exons *in vivo* without overt toxicity (Echigoya, Aoki, *et al.* 2015).

The first step toward clinical utility of any AO drug is the demonstration of its efficacy *in vitro*; thus, the establishment of a suitable *in vitro* exon skipping assay for determining *dystrophin* exon skipping is essential. To this end, muscle cell types (myoblasts and myotubes) expressing dystrophin are typically used. However, the collection of patient muscle tissue via biopsy is highly invasive, requires specialized equipment and preservation techniques (Joyce, Oskarsson, and Jin 2012), and yields only a small amount of what are often poorly proliferative cells (Paasuke *et al.* 2016). Myotubes converted from fibroblasts via *MYOD1* transduction express dystrophin at levels sufficient to determine the effectiveness of AOs at facilitating *DMD* exon skipping and protein rescue (Saito *et al.* 2010) and represent an alternative approach to biopsied patient muscle tissue at evaluating exon skipping efficiency *in vitro*.

In this chapter, we tested the exons 45-55 skipping efficacy of PMO sequences designed using a predictive software tool by transfecting PMO cocktails into transdifferentiated DMD patient myotubes harboring deletion mutations amenable to reading frame correction via exons 45-55 skipping. We observed a dose-dependent production of exons 45-55 skipped transcripts as well as the rescue of dystrophin protein in DMD patient cells. This is the first-ever demonstration of robust dystrophin exons 45-55 skipping in transdifferentiated patient cells.

4.2 Results

4.2.1 Patient mutation analysis and exon skipping approach

DMD fibroblast cell lines GM05162 and GM05017 supplied by the Coriell Institute were originally sampled from two clinically affected males aged 13 and 12, respectively. Both individuals exhibited progressive muscle weakness and were wheelchair bound before or at age

10. GM05162 harbors a deletion of dystrophin exons 46-50, resulting in an out-of-frame product which requires a cocktail of 6 PMOs and skipping of exons 45, 51-55 to restore the reading frame (**Figure 4.1A**). GM05017 contains an out-of-frame deletion of exons 45-50, requiring a cocktail of 5 PMOs and skipping of exons 51-55 (**Figure 4.1B**). Based on these mutation patterns, we utilized our exon skipping predictive tool (Echigoya, Mouly, *et al*. 2015) to calculate the expected exon skipping efficiencies for PMO sequences of 30-mer length covering all possible target sites for the corresponding exons. PMO sequences used are described in **Table 1**. To further optimize our oligo design, we also considered the binding free energy values between AOs and selected only oligos with >-9 ΔG (**Table 2**). The in-frame skipping of dystrophin exons 45-55 produces a truncated product containing a hybrid rod repeat that is known to retain similar function to the full-length protein (**Figure 1.1C**).

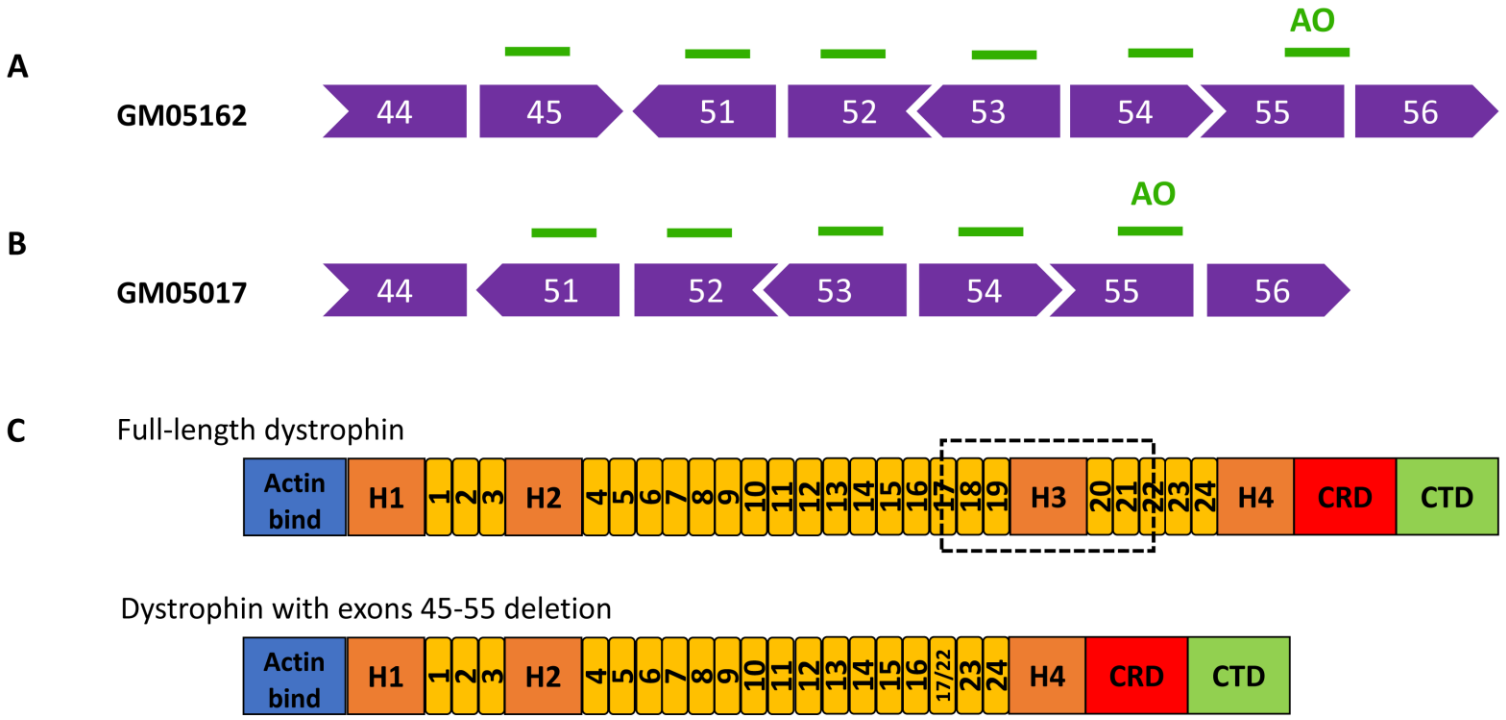

A
AO
GM05162
44
45
51
52
53
54
55
56
B
AO
GM05017
44
51
52
53
54
55
56
C
Full-length dystrophin
Actin bind
H1
1
2
3
H2
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
H3
20
21
22
23
24
H4
CRD
CTD
Dystrophin with exons 45-55 deletion
Actin bind
H1
1
2
3
H2
4
5
6
7
8
9
10
11
12
13
14
15
16
17/22
23
24
H4
CRD
CTD

Figure 4.1: Mutation analysis and exon skipping approach.

(A) Cell line GM05162 harbors an out-of-frame deletion mutation of dystrophin exons 46-50, which requires skipping 6 exons via PMOs to correct the reading frame. Sides of schematic boxes represent the codon phase. (B) Cell line GM05017 harbors an out-of-frame deletion mutation of dystrophin exons 45-50, which requires skipping 5 exons via PMOs to correct the reading frame. (C) Structure of full-length dystrophin and exons 45-55-deleted dystrophin. The truncated dystrophin generated by exons 45-55 skipping contains a hybrid rod repeat (yellow bars) of rods 17 and 22. Actin bind, actin-binding domain; H1-4, hinge domain 1-4; CRD, cysteine-rich domain; CTD, C-terminal domain. From (Lee, Echigoya, et al. 2018).

4.2.2 MYOD1 transduction of DMD fibroblasts and conversion to myotubes

An expression vector containing GFP and the human *MYOD1* coding sequence was delivered via retrovirus into human DMD patient fibroblast cells and healthy human fibroblasts (**Figure 4.2A**) (Miller and Buttimore 1986, Saito *et al.* 2010). Following transduction, GFP-positive cells were sorted via flow cytometry (**Figure 4.2B**) and seeded into collagen-coated 12-well plates. After adherence, cells were cultured in reduced-serum media to induce myogenic differentiation. Morphological examination showed that GFP-positive *MYOD1*-transduced cells had become elongated and contained multiple nuclei (**Figure 4.2C**) – hallmarks of myotube morphology. To confirm successful transdifferentiation of fibroblasts to muscle cell type, we performed immunostaining for several markers of muscle identity. These cells expressed muscle-specific proteins myosin heavy chain and desmin, and healthy cells expressed dystrophin (**Figure 4.2D**). We then utilized a time-course expression assay to compare dystrophin mRNA expression between transdifferentiated healthy and patient cells. In both healthy and DMD patient cells, dystrophin mRNA expression was detectable by RT-PCR as early as 3 d after addition of differentiation media (**Figure 4.2E**).

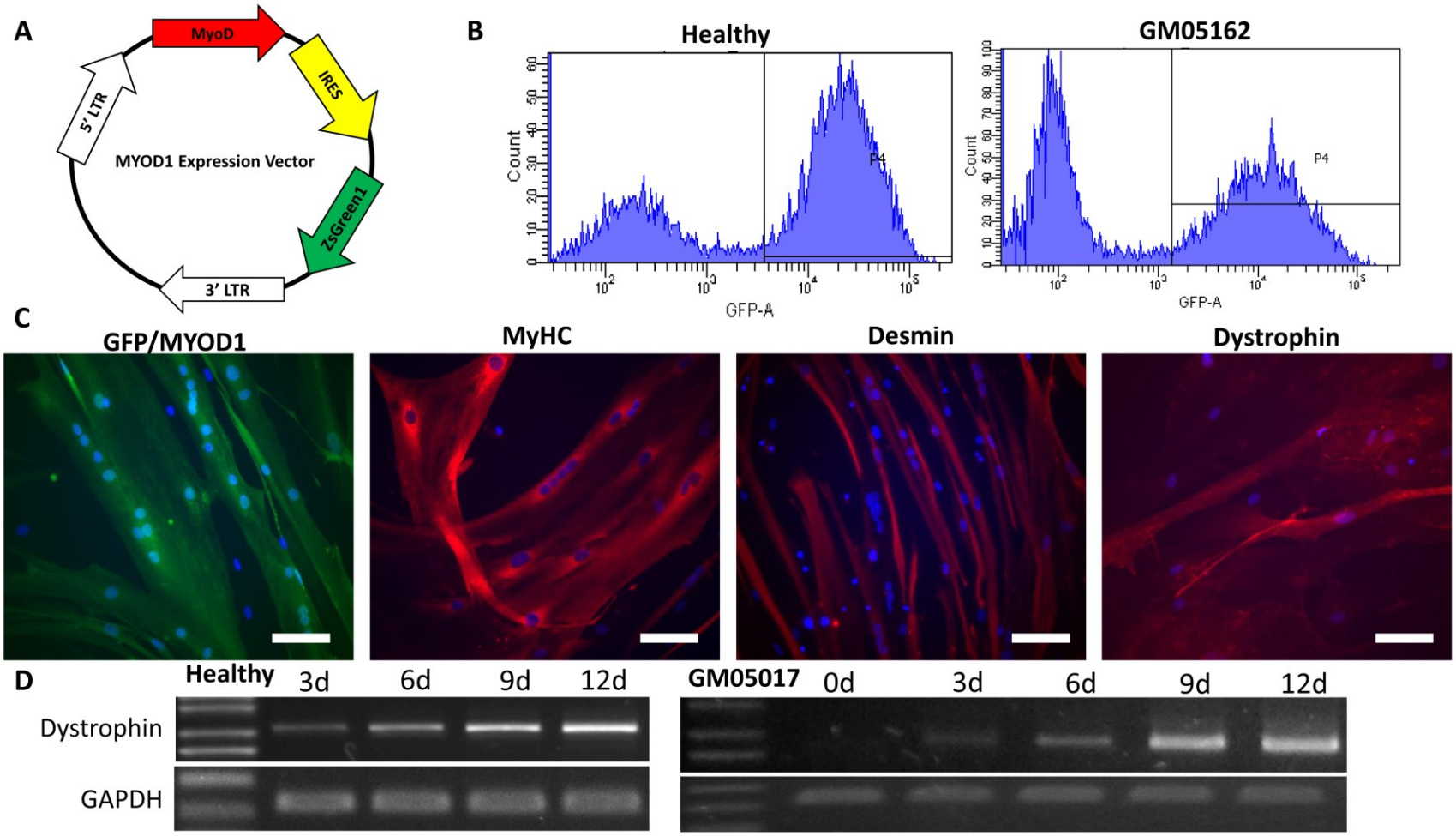

A
MyoD
IRES
ZsGreen1
3' LTR
5' LTR
MYOD1 Expression Vector
B
Healthy
GM05162
Count
GFP-A
P4
C
GFP/MYOD1
MyHC
Desmin
Dystrophin
D
Healthy
3d
6d
9d
12d
GM05017
0d
3d
6d
9d
12d
Dystrophin
GAPDH

Figure 4.2 Transdifferentiation of DMD fibroblasts to myotubes.

(A) Schematic diagram of *MYOD1* expression vector. (B) Histogram comparison of GFP fluorescence signal vs cell number between healthy and DMD patient fibroblast cells transduced with *MYOD1* expression vector. (C) Immunofluorescence of transduced fibroblasts following 18 d (ZsGreen, indicating MyoD), 15 d (MyHC, myosin heavy chain), 18 d (desmin), and 24 d (dystrophin) differentiation, respectively. Nuclei counterstained with DAPI. Scale bars: 100 μm. N = 3 (D) RT-PCR time-course analysis of dystrophin expression in healthy and patient DMD transdifferentiated fibroblasts. From (Lee, Echigoya, et al. 2018).

4.2.3 Antisense-mediated multi-exon skipping of dystrophin exons 45-55 in DMD patient cell lines using PMO cocktails

Based on respective mutation patterns, skipping of dystrophin exons 45-55 in cell line GM05162 requires a cocktail of 6 PMOs, while skipping of exons 45-55 in cell line GM05017 requires a cocktail of 5 PMOs (**Figure 4.1A, 4.1B**). Following transfection of PMO cocktails, RT-PCR analysis showed exon skipped products of the expected molecular weight in both cell lines in a dose-dependent manner (**Figure 4.3A**). Sanger sequencing of exon-skipped products revealed that the skipped products contained in-frame concatenations of *DMD* exons 44 and 56 (**Figure 4.3A**). Immunostaining showed some rescue of dystrophin protein in both PMO-treated cell lines (**Figure 4.3B**).

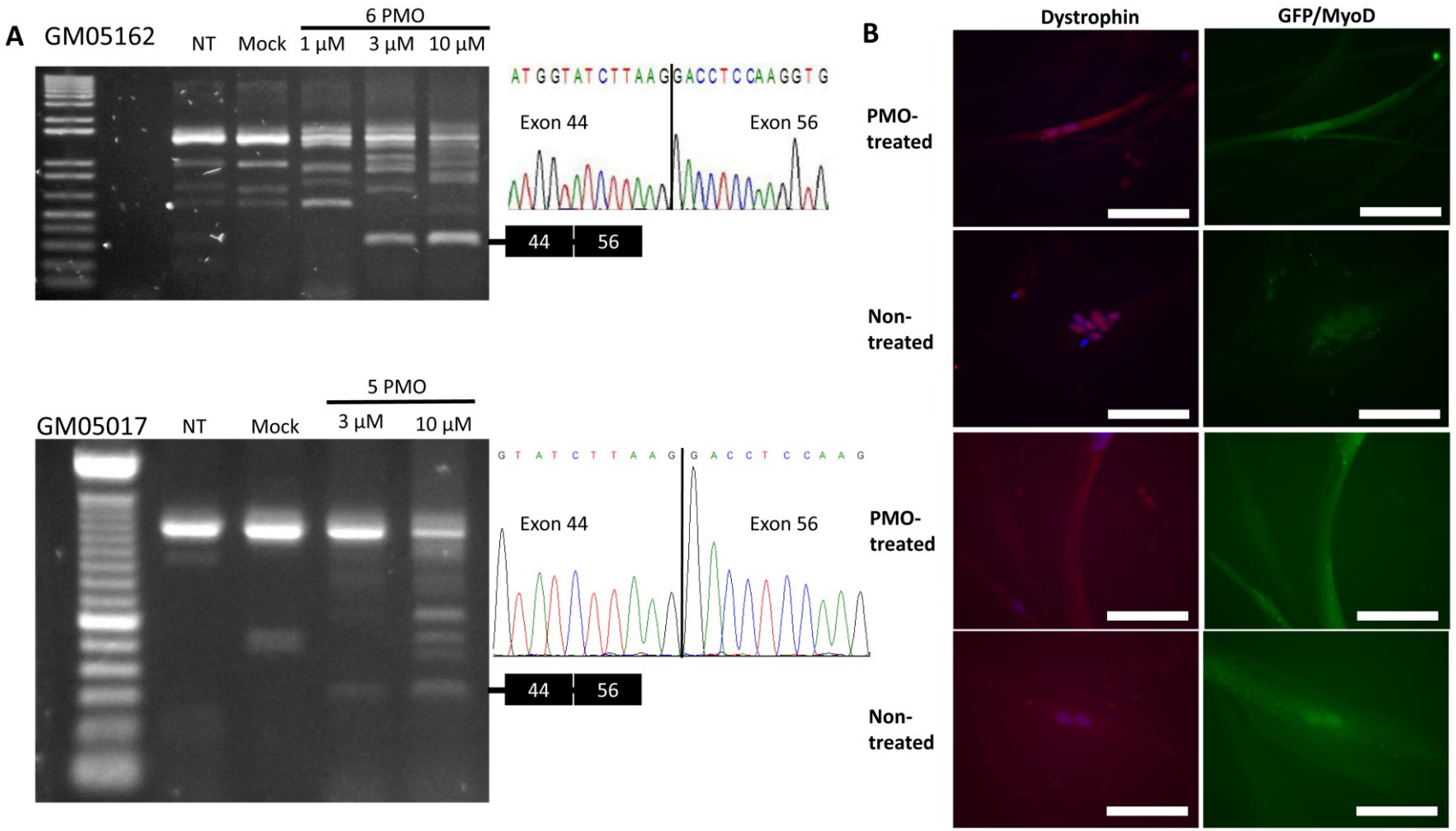

A
GM05162
NT
Mock
6 PMO
1 µM
3 µM
10 µM
ATGGTATCTTAAGGACCTCCAAGGTG
Exon 44
Exon 56
44
56
GM05017
NT
Mock
5 PMO
3 µM
10 µM
GTATCTTAAGGACCTCCAAG
Exon 44
Exon 56
44
56
B
Dystrophin
GFP/MyoD
PMO-treated
Non-treated
PMO-treated
Non-treated

Figure 4.3 Multi-exon skipping of dystrophin exons 45-55 in transdifferentiated DMD patient cells.

(A) RT-PCR for *dystrophin* following cocktail PMO transfection in transdifferentiated DMD patient cells. Cells were treated with 1, 3, or 10 μM each PMO. Expected molecular weight of *dystrophin* exons 45-55 skipped mRNA is 308 bp. NT = Non-treated; Mock = random oligo. N = 3. (B) Representative immunocytochemistry of transduced DMD fibroblasts following PMO cocktail transfection. Nuclei counterstained with DAPI. Scale bars: 100 μm. N = 3. From (Lee, Echigoya, et al. 2018).

4.3 Discussion

In this chapter, we demonstrated the feasibility of skipping dystrophin exons 45-55 *in vitro* using human DMD patients' myotubes converted from fibroblasts. This is the first successful demonstration of robust, dose-dependent dystrophin exons 45-55 skipping in DMD patient cells. While earlier attempts at exons 45-55 skipping in patient cells were unsuccessful (van Vliet *et al.* 2008), our results emphasize the importance of AO sequence optimization and highlight the utility of in silico predictive screening for potential AO sequences.

This is also the first demonstration of exons 45-55 skipping in transdifferentiated cells, which underscores the suitability of using patient fibroblast cells as an alternative to cells obtained via muscle biopsy for evaluating exon skipping. This method of measuring exon skipping efficiency in transdifferentiated myotubes offers advantages to other assays, such as easy monitoring of *MYOD1* transduction efficiency via GFP signal and effective induction of dystrophin expression, which can be difficult to induce (Cooper *et al.* 2007).

Our group previously reported the efficacy of antisense-mediated dystrophin exons 45-55 skipping and rescue of dystrophin protein *in vivo* using a cocktail of vivo-morpholinos (vPMOs) in a mouse model of DMD (Echigoya, Aoki, *et al.* 2015, Aoki *et al.* 2012). Before such new-generation antisense oligos can be effective and safe *in vivo* they require rigorous sequence optimization *in vitro*. Here, we emphasize an effective method for *in vitro* assessment of exon skipping efficacy in transdifferentiated human DMD patient cells which can pave the way for subsequent *in vivo* and clinical studies. By utilizing muscle cells obtained through fibroblast transdifferentiation, researchers can access an effective cell model for assessing the exon skipping ability of novel antisense chemistries and gene sequence targets while avoiding challenges associated with utilizing muscle harvested from patient biopsies, such as the limited availability and poor proliferative ability of patient muscle samples (Muntoni 2001, Paasuke *et al.* 2016).

Currently, the only clinically available exon skipping therapy is Sarepta's exon 51 skipping drug, eteplirsen (Exondys 51). Notwithstanding FDA approval of the drug in 2016 (Aartsma-Rus and Krieg 2017), eteplirsen has remained surrounded by controversy, with concerns being raised as to its clinical efficacy (Lim, Maruyama, and Yokota 2017). Furthermore, exon 51 skipping is limited in its therapeutic applicability, with an estimated ~13% of all DMD patients being able to benefit from such an approach (Aartsma-Rus *et al.* 2009). Several antisense-mediated exon skipping approaches are currently being evaluated across various clinical trials, targeting *DMD* exons 44 (NCT02958202), 45 (NCT02667483), 51 (NCT03375255), and 53 (NCT03167255); the respective therapeutic applicability of these targets is ~6%, ~8%, ~13%, and ~8% (Aartsma-Rus *et al.* 2009). Notably, while the combined applicability of the aforementioned exon skipping approaches is ~35%, a single exon skipping approach targeting exons 45-55 would theoretically be amenable to ~47% of all DMD patients (Nakamura *et al.* 2017). In addition to increased patient applicability, another advantage of skipping *DMD* exons 45-55 is that the resulting truncated protein is remarkably stable, as evidenced by patients harboring exons 45-55 deletion mutations who are either asymptomatic or display exceptionally mild symptoms (Nakamura *et al.* 2017).

In conclusion, our findings support the feasibility of future translation of the dystrophin exons 45-55 skipping approach into clinical practice for treating DMD. Potential hurdles such as possible side-effects of intermediate transcripts, toxicity assessment, and optimized PMO cocktail delivery will need to be addressed in future investigations. The development of a drug cocktail approach may also necessitate modification of existing regulatory body guidelines which currently consider individual AO sequences to be separate drugs.

CHAPTER 5: *DYSF* multi-exon skipping rescues membrane resealing in dysferlinopathy patient fibroblasts

5 Chapter 5. *DYSF* multi-exon skipping rescues membrane resealing in dysferlinopathy patient fibroblasts

5.1 Introduction

The dysferlinopathies are a heterogeneous group of recessive myopathies caused by mutations in the *dysferlin* (*DYSF*) gene (Liu *et al.* 1998, Bashir *et al.* 1998). Characterized by progressive muscle weakness which typically begins during the second decade of life, the dysferlinopathies can be clinically divided into at least three types: Miyoshi Myopathy (MM), Limb-girdle muscular dystrophy type 2B (LGMD2B), and distal myopathy with anterior tibial onset (DMAT) (Bashir *et al.* 1998, Liu *et al.* 1998, Illa *et al.* 2001). The dysferlinopathies are clinically distinguished based on the initial pattern of muscle weakness, originating in either the proximal (shoulder and pelvic girdle; LGMD2B) or distal musculature (gastrocnemius and soleus; MM). The disease can also present initially, and often advances to include, both the proximal and distal muscles (Klinge *et al.* 2008, Aoki 1993, Nguyen *et al.* 2007). While most patients experience a gradual decline over decades, several atypical phenotypes have been reported, including rapid loss of ambulation in less than five years (Nguyen *et al.* 2007). Variable age of onset has also been reported, ranging from 10 to 73 years (Jethwa *et al.* 2013, Klinge *et al.* 2008, Nguyen *et al.* 2007).

The *DYSF* gene codes for dysferlin protein, which is a large (~240 kDa) type II transmembrane protein containing seven lipid/protein-binding C2 domains, multiple Dysf and Fer domains, and a C-terminal transmembrane domain (**Figure 5.1**) (Therrien *et al.* 2009, Davis *et al.* 2002, Lennon *et al.* 2003, Azakir *et al.* 2010, Cai, Weisleder, *et al.* 2009, Matsuda *et al.* 2001, Huang *et al.* 2007, Bansal and Campbell 2004, Therrien *et al.* 2006). Dysferlin is ubiquitously expressed but is most abundant in skeletal and cardiac muscle (Anderson *et al.* 1999, Bansal and Campbell 2004). Dysferlin is predominantly localized to the plasma membrane but is also observed in cytoplasmic vesicles and is associated with the t-tubule

network (Anderson *et al.* 1999, Piccolo *et al.* 2000, Bansal and Campbell 2004, Kerr *et al.* 2013, Klinge *et al.* 2007). Dysferlin protein plays an essential role in plasma membrane repair and loss of dysferlin results in compromised membrane resealing and deterioration of muscle fibers (Bansal *et al.* 2003, Bansal and Campbell 2004, Roche *et al.* 2010). There is currently no cure for dysferlinopathy. Existing disease management consists mainly of physical therapy, orthopaedic surgery, and use of mechanical and respiratory aids (Aoki 1993, Kobayashi *et al.* 2012).

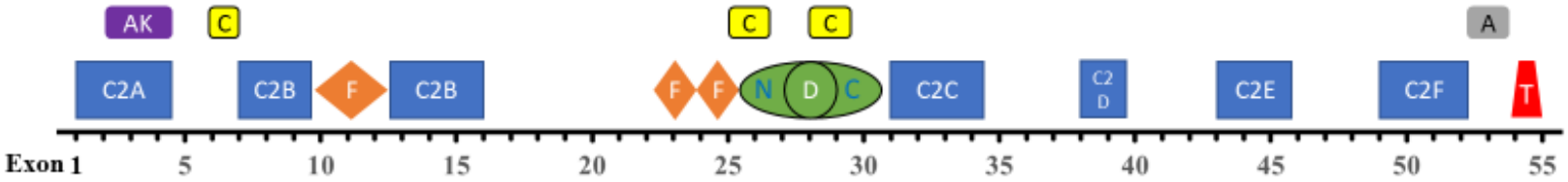


Figure 5.1: Human dysferlin domains relative to exons. There are 55 exons in the DYSF gene; dysferlin protein contains seven C2 domains (C2A-C2F), Ferlin family domains (F), an inner Dysf domain (D) and a second outer Dysf domain (N- and C-terminal), and a transmembrane domain (T). Also depicted are putative binding sites for various dysferlin interacting proteins, such as AHNAK (AK), Caveolin-3 (C), and Affixin (Beta-parvin; A). Predicted dysferlin domain positions and protein-protein interacting domains are based on coordinates available from the Universal Mutation Database (http://www.umd.be/DYSF/W_DYSF/Protein.html) and from Wein *et al.* 2010.

A promising therapeutic strategy which has gained traction in recent years, especially with respect to neuromuscular disorders, is antisense-mediated exon skipping. Exon skipping utilizes synthetic nucleic acids called antisense oligonucleotides (AOs) to modulate pre-mRNA splicing and can be used to remove mutation-carrying exons and flanking exons to maintain an open reading frame (Lee and Yokota 2013, Niks and Aartsma-Rus 2017). In 2016, the United States Food and Drug Administration (FDA) approved eteplirsen (Exondys 51), the first-ever antisense drug for the treatment of Duchenne muscular dystrophy (DMD) (Aartsma-Rus and Krieg 2017). Eteplirsen facilitates in-frame skipping of *dystrophin* exon 51 and utilizes the phosphorodiamidate morpholino oligomer (PMO) antisense chemistry. The rationale for exon skipping in dysferlinopathy is supported by reports of mildly-affected patients harboring in-frame *DYSF* deletion mutations, such as exon 32 (Sinnreich, Therrien, and Karpati 2006). Even large deletions of *DYSF* have been associated with a milder disease course (Krahn *et al.*). Exon skipping progress in dysferlinopathy has been limited to the *in vitro* skipping of *DYSF* exon 32 in human dysferlinopathy patient cells (Barthelemy *et al.* 2015). To date, no other therapeutic exon skipping targets have been identified for dysferlinopathy.

In this chapter, we undertook to identify novel exon skipping targets in *DYSF* by characterizing the relationship between exon deletion pattern and plasma membrane resealing ability. We created GFP-conjugated *DYSF* cDNA expression constructs lacking certain exons and transfected these into dysferlinopathy patient cells, then subjected the cells to a membrane wounding assay. We demonstrate that *DYSF* exon combinations 19-21, 20-21, and 46-48 are required for proper plasma membrane resealing, while exons 26-27 and 28-29 are dispensable for membrane resealing. After identifying which exons were not required for proper membrane resealing, we designed PMOs using a predictive software algorithm and tested the ability of PMO cocktails to facilitate multi-exon skipping and restore membrane resealing ability in patient cells. We show that a PMO cocktail targeting *DYSF* exons 28-29 restores membrane resealing in patient cells. Our results provide a foundation for future *in vivo* investigations and

possible clinical translation of *DYSF* exons 26-27 and 28-29 skipping approaches for treating dysferlinopathy.

5.2 Results

5.2.1 Mutation analysis and exon skipping approach for dysferlinopathy cell lines

We first assessed the mutation patterns present in two dysferlinopathy patient cell lines. The first cell line, MM-Pt1, was originally collected from a patient with Miyoshi Myopathy. Sanger sequencing confirmed a homozygous missense mutation in *DYSF* exon 46 (**Figure 5.2A**). To remove the mutated exon while maintaining the reading frame would involve multi-exon skipping of *DYSF* exons 46-48 (**Figure 5.2A**). While exons 46 and 47 do not code for any known protein domain, exon 48 codes for a portion of the C2F domain (**Figure 5.2A**). The second cell line, MM-Pt2, was also collected from a patient with Miyoshi Myopathy. Sanger sequencing confirmed that this cell line is transheterozygotic, carrying a frameshift-causing point mutation in exon 21 on one allele and a missense mutation in exon 28 on the other allele (**Figure 5.2B**). To remove the mutant exons while maintaining the reading frame would involve double exon skipping of exons 20 and 21, or 28 and 29 (**Figure 5.2B**). Exons 20 and 21 do not code for any known protein domain of dysferlin, while exons 28 and 29 code for portions of the inner Dysf domain (**Figure 5.2B**).

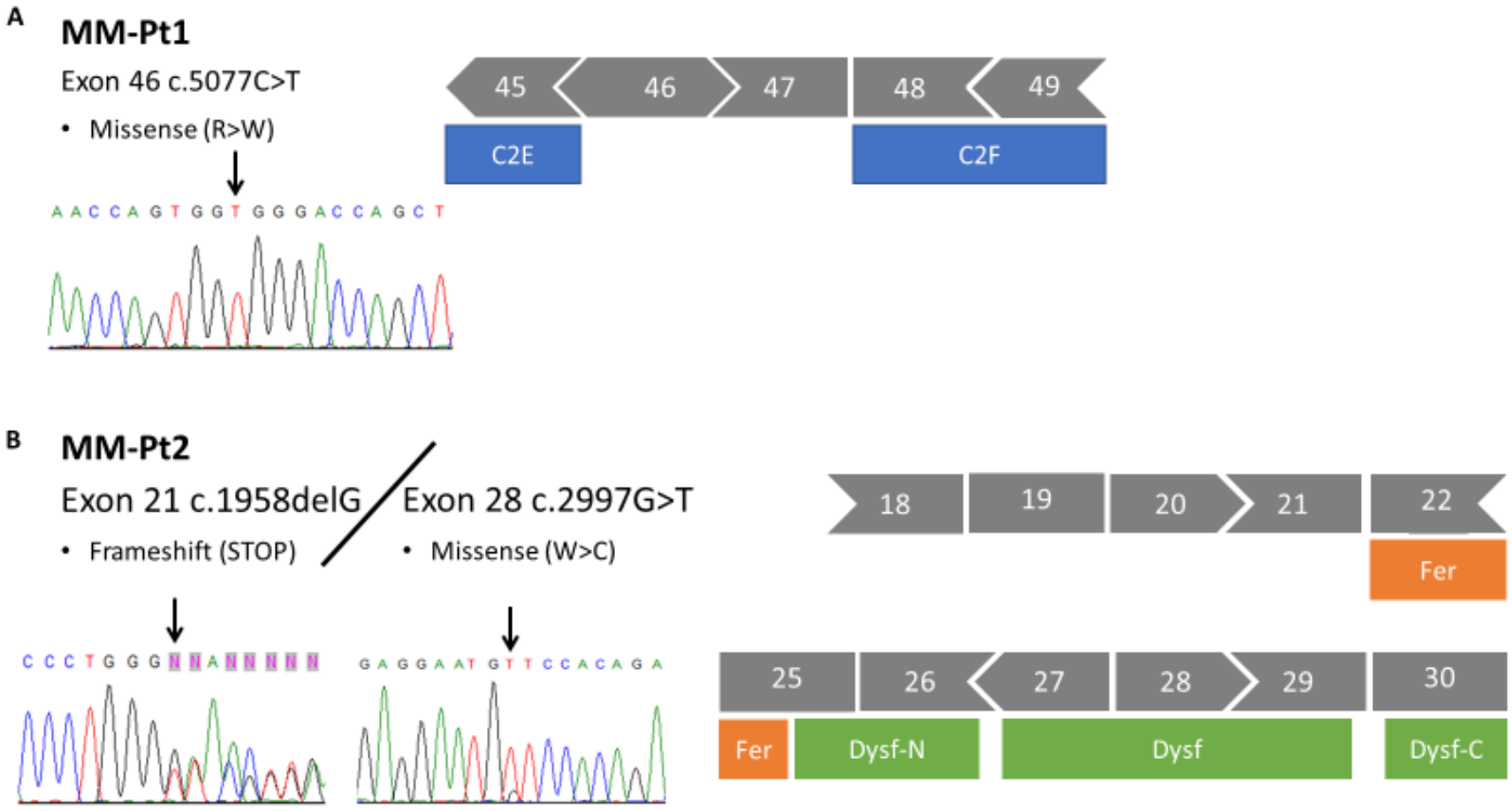

A
MM-Pt1
Exon 46 c.5077C>T
• Missense (R>W)
45
46
47
48
49
C2E
C2F
B
MM-Pt2
Exon 21 c.1958delG
• Frameshift (STOP)
Exon 28 c.2997G>T
• Missense (W>C)
18
19
20
21
22
Fer
25
26
27
28
29
30
Fer
Dysf-N
Dysf
Dysf-C

Figure 5.2 Dysferlinopathy patient cell line mutation analysis.

(A) Cell line MM-Pt1 was generated from a patient with Miyoshi Myopathy and harbors a homozygous missense mutation in *DYSF* exon 46. (B) Cell line MM-Pt2 was generated from a patient with Miyoshi Myopathy and contains two mutations: a frameshift mutation in exon 21 on one allele, and a missense mutation in exon 28 on the second allele. Sanger sequencing was used to confirm the mutation patterns in both cell lines. Grey boxes represent exons and ends of boxes denote corresponding phasing; colored boxes below represent predicted protein domains.

5.2.2 Determining the feasibility of exon skipping approaches in DYSF through transfection of exon-deleted constructs

Before proceeding with the application of an exon skipping approach for our patient cell lines, we undertook to identify which *DYSF* exons could be removed without negatively impacting protein function. To do this, we mimicked the effect of exon skipping by using site-directed mutagenesis to generate GFP-fused *DYSF* cDNA expression constructs lacking exons corresponding to an exon skipping approach amenable to each cell line. We therefore generated constructs lacking *DYSF* exons 20-21, 28-29, and 46-48. We also generated an exon 19-21 deletion construct, as this pattern of exon skipping would also restore the reading frame in MM-Pt2 (**Figure 5.2B**). Additionally, we generated an exon 26-27 deletion construct, based on our later procurement of another dysferlinopathy patient cell line with a mutation amenable to this exon skipping approach. The deletion of *DYSF* exons 26-27 is in-frame (**Figure 5.2B**). As a control, we generated a GFP-only plasmid. To identify whether the exons deleted in the above constructs would have an impact on protein function we transfected the constructs into cell line MM-Pt1, which exhibited impaired plasma membrane resealing ability compared to healthy cells (**Figure 5.3**) and observed whether any of these plasmids could rescue membrane resealing ability. To assess membrane resealing ability, we incubated cells in FM 4-64 dye and generated lesions in the plasma membranes of GFP-positive cells using a two-photon laser microscope, then quantified the relative fluorescence values of FM dye over time (Lee, Maruyama, et al. 2018). Our results show that Δ26-27, and Δ28-29 were able to rescue membrane resealing ability to a degree similar to that of healthy cells and cells transfected with the full-length *DYSF* plasmid, as measured by changes in relative fluorescence intensity over time (**Figure 5.3A, 5.3B**). In contrast, Δ19-21, Δ20-21, and Δ46-48 were not able to rescue membrane resealing ability (**Figure 5.3A, 5.3B**).

We next considered that while great care is taken to ensure that all plasma membrane wounding parameters are consistent between experiments (e.g. laser power, wavelength,

number of iterations, etc.) there can still be some disparity with respect to the degree of membrane damage following laser ablation. This might generate some small variation regarding quantification, in terms of the amount of fluorescent dye which infiltrates the cell. We therefore utilized an additional measure of membrane resealing ability which we termed "time to steady-state", defining steady-state as the point at which raw fluorescence values peak following laser wounding without any significant increase over time (**Figure 5.3C**). Time to steady-state is calculated by subtracting the time prior to laser wounding from the timepoint when fluorescence values peak. We observed that Δ26-27, and Δ28-29 constructs were able to rescue membrane resealing, while the Δ19-21, Δ20-21, and Δ46-48 constructs were not, as measured by time to steady-state (**Figure 5.3D**). Taken together, these results show that *DYSF* exons 26-27 and 28-29 are dispensable for plasma membrane resealing, suggesting that these exons are promising therapeutic targets for exon skipping, while exons 19-21, 20-21, and 46-48 are required for proper membrane resealing, suggesting that these are not promising exon skipping targets.

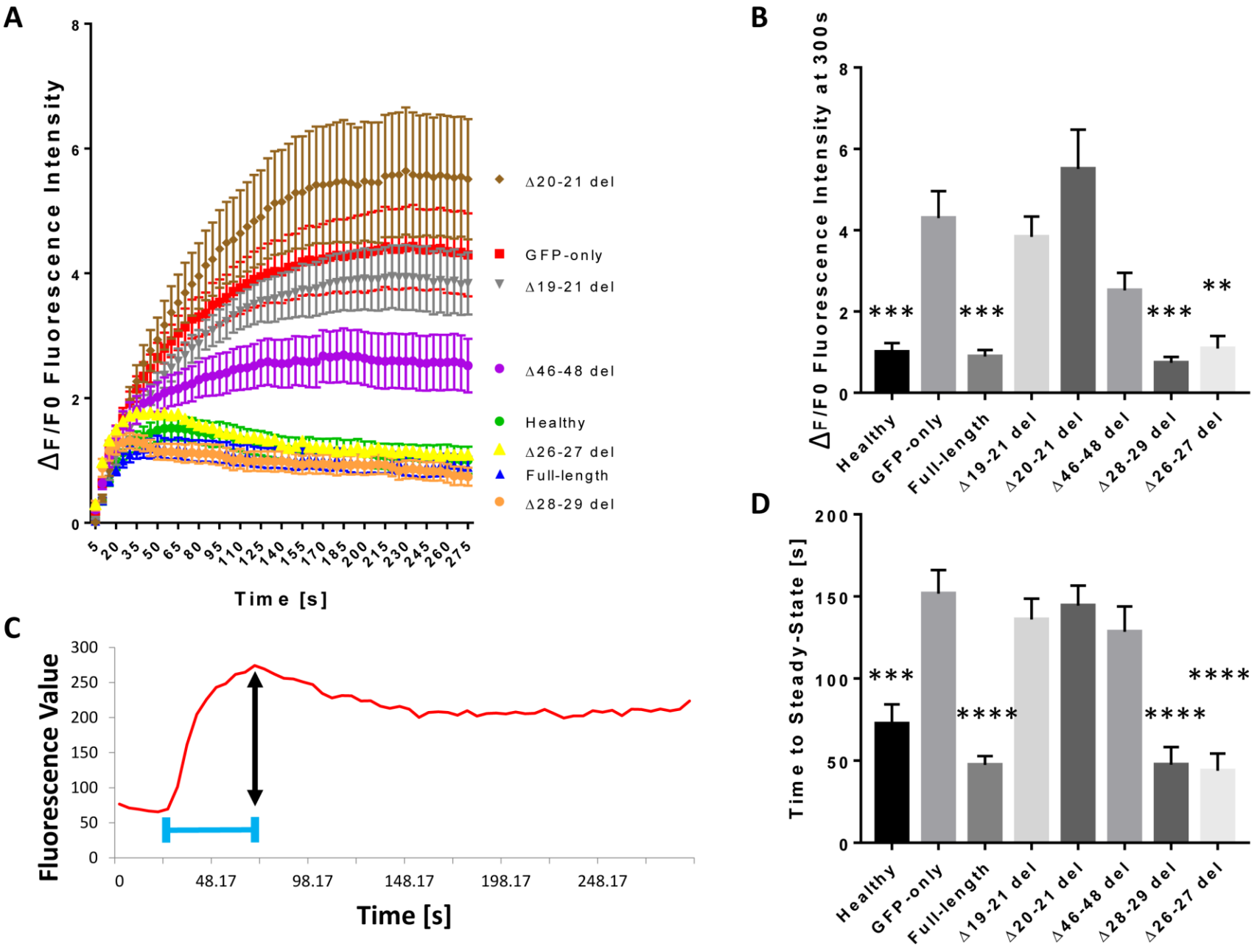

A
ΔF/F0 Fluorescence Intensity
Time [s]
Δ20-21 del
GFP-only
Δ19-21 del
Δ46-48 del
Healthy
Δ26-27 del
Full-length
Δ28-29 del
B
ΔF/F0 Fluorescence Intensity at 300s
***
***
***
**
Healthy
GFP-only
Full-length
Δ19-21 del
Δ20-21 del
Δ46-48 del
Δ28-29 del
Δ26-27 del
C
Fluorescence Value
Time [s]
D
Time to Steady-State [s]
***
****
****
****

Figure 5.3 Transfection with Δ26-27 or Δ28-29 *DYSF* plasmid restores membrane resealing ability in dysferlinopathy patient cells.

(A) A membrane wounding assay was performed on dysferlinopathy patient cells (MM-Pt1) transfected with various plasmids. Relative fluorescence intensity (ΔF/F0) over time is shown as means ± S.E. (B) Graphical representation of *A* showing relative fluorescence intensity at final timepoint (t = 300 s). (C) Representative image of raw fluorescence values over time generated by membrane wounding assay. Line with double arrows depicts the final timepoint from which fluorescence values no longer significantly increase over time. Blue bar represents the time from laser wounding until the timepoint when fluorescence values peak, termed "steady-state". (D) Graphical representation of steady-state means ± S.E. Statistics: One-Way ANOVA & Tukey-Kramer Multiple Comparisons Test. ** $p<0.005$, *** $p<0.0005$, **** $p<0.0001$ compared to GFP-only.

5.2.3 In silico and in vitro screening of PMOs for DYSF exons 28-29 skipping

Our exon skipping predictive algorithm (Echigoya, Mouly, *et al.* 2015) projected the expected exon skipping efficiencies for 191 PMO sequences of 30-mer length, covering all possible target sites for exons 28 and 29. From these, the top three PMO sequences with the highest predicted exon skipping efficiency which also met synthesis criteria (e.g. CG content, Tm, self-complementarity) were selected by us and produced by GeneTools (Philomath, Oregon) (**Table 1**). As measured by RT-PCR, all nine possible combinations of PMO cocktails were able to efficiently skip *DYSF* exons 28 and 29 using 10 μM each oligo in MM-PT2 cells, suggesting that substantive multiple exon skipping can be achieved in *DYSF* through the use of PMO cocktails (**Figure 5.4A**). To examine rescue of dysferlin protein we performed Western blot analysis and found that there was no detectable change in protein levels between control and 10 μM each oligo treatment groups, while mutant cells also expressed detectable levels of dysferlin (**Figure 5.4B**).

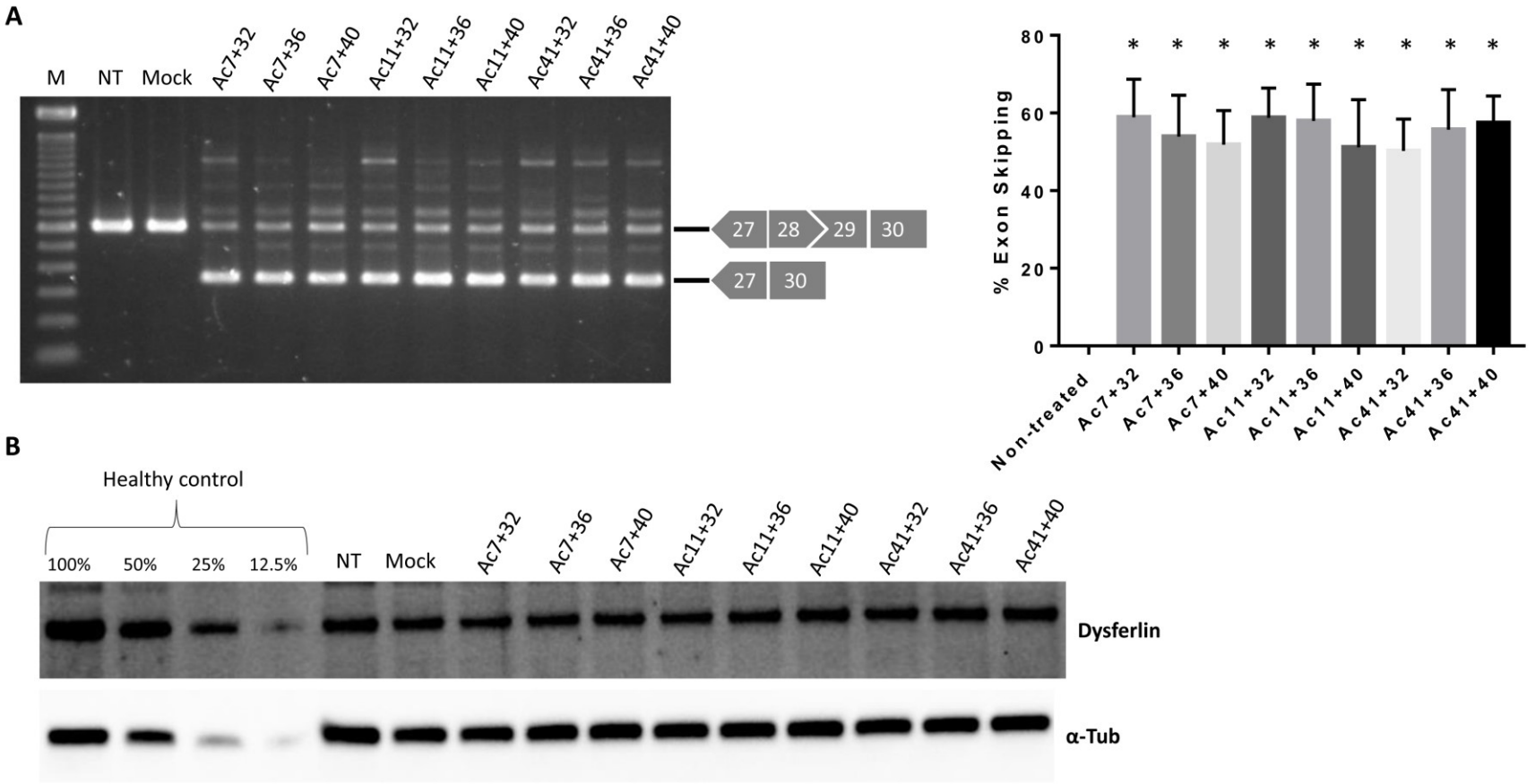

A
M
NT
Mock
Ac7+32
Ac7+36
Ac7+40
Ac11+32
Ac11+36
Ac11+40
Ac41+32
Ac41+36
Ac41+40
27
28
29
30
27
30
% Exon Skipping
80
60
40
20
0
Non-treated
B
Healthy control
100%
50%
25%
12.5%
Dysferlin
α-Tub

Figure 5.4 PMO cocktails facilitate multi-exon skipping of *DYSF* exons 28-29 in patient cells.

All transfections were performed using 10 μM each PMO in MM-Pt2 cells. (A) Efficiency of exons 28-29 skipping as measured by one-step RT-PCR. Representative image shown. M, 100 bp marker. Values are scored as mean ± S.E. (n = 3). Statistics: One-Way ANOVA & Tukey-Kramer Multiple Comparisons Test. * $p<0.0001$ compared to non-treated. (B) Western blot assessment of dysferlin protein expression following PMO cocktail transfection.

5.2.4 Rescue of plasma membrane resealing in PMO cocktail-treated dysferlinopathy patient cells

We selected PMO cocktail Ac11+32, which showed a high degree of *DYSF* exon 28-29 skipping, as measured by RT-PCR (**Figure 5.4A**), for transfection into patient cells. We observed that Ac11+32 was able to rescue plasma membrane resealing in dysferlinopathy patient fibroblasts (MM-Pt2), as measured by changes in relative fluorescence intensity over time (**Figure 5.5A, 5.5B**). Ac11+32 was also able to significantly reduce the time to membrane resealing, as measured by time to steady-state (**Figure 5.5C**). These results show that functional recovery of membrane wounding *in vitro* is possible through antisense-mediated exon skipping of *DYSF* exons 28-29 via PMO cocktail, suggesting that these PMOs might be promising therapeutic agents for treating patients with mutations amenable to *DYSF* exons 28-29 skipping.

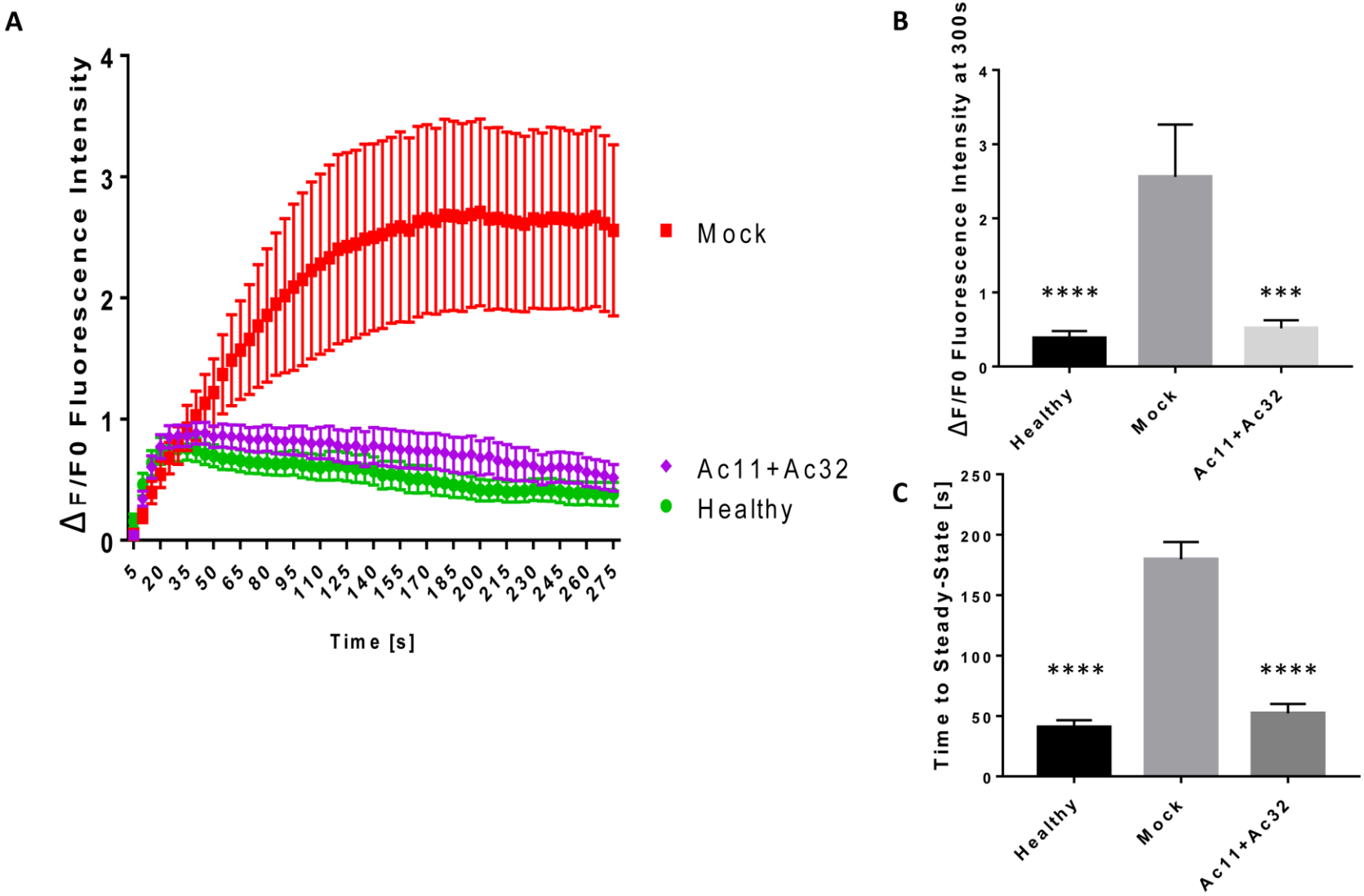

A
ΔF/F0 Fluorescence Intensity
Time [s]
Mock
Ac11+Ac32
Healthy
B
ΔF/F0 Fluorescence Intensity at 300s
****
***
Healthy
Mock
Ac11+Ac32
C
Time to Steady-State [s]
****
****
Healthy
Mock
Ac11+Ac32

Figure 5.5 Antisense-mediated skipping of *DYSF* exons 28-29 via PMO cocktail rescues membrane resealing ability in dysferlinopathy patient cells.

(A) Dysferlinopathy patient cells (MM-Pt2) transfected with PMO cocktail. Relative fluorescence intensity (ΔF/F0) over time is shown as means ± S.E. (B) Graphical representation of A showing relative fluorescence intensity at final timepoint (t = 300 s). (C) Graphical representation of steady-state means ± S.E. Statistics: One-Way ANOVA & Tukey-Kramer Multiple Comparisons Test. *** $p<0.0005$, **** $p<0.0001$ compared to mock-treated.

5.3 Discussion

The first identification of a potential therapeutic exon skipping target in dysferlinopathy was *DYSF* exon 32, described by Sinnreich *et al* (Sinnreich, Therrien, and Karpati 2006) in 2006. Since then, no new therapeutic exon skipping targets have been described for *DYSF*. While some groups have attempted to identify redundant protein domains for the purpose of mini or nano-dysferlin delivery via AAV vector (Llanga *et al.* 2017), the relationship between exon deletion pattern and protein functionality has gone largely uncharacterized. In this report, we described not only the first-ever success of multiple exon skipping in *DYSF*, but also the identification of two novel potential therapeutic exon skipping targets for treating dysferlinopathy – *DYSF* exons 26-27 and 28-29. Successful translation of these findings into the development of clinical AO drugs would establish new therapeutic approaches that would be applicable to approximately 5-7% (exons 26-27 skipping) and 8% (exons 28-29 skipping) of dysferlinopathy patients worldwide, according to reported variant data in the Leiden Open Variation Database (LOVD) (http://www.dmd.nl/) and Universal Mutation Database (UMD) (http://www.umd.be/DYSF/) (**Figure 5.6**).

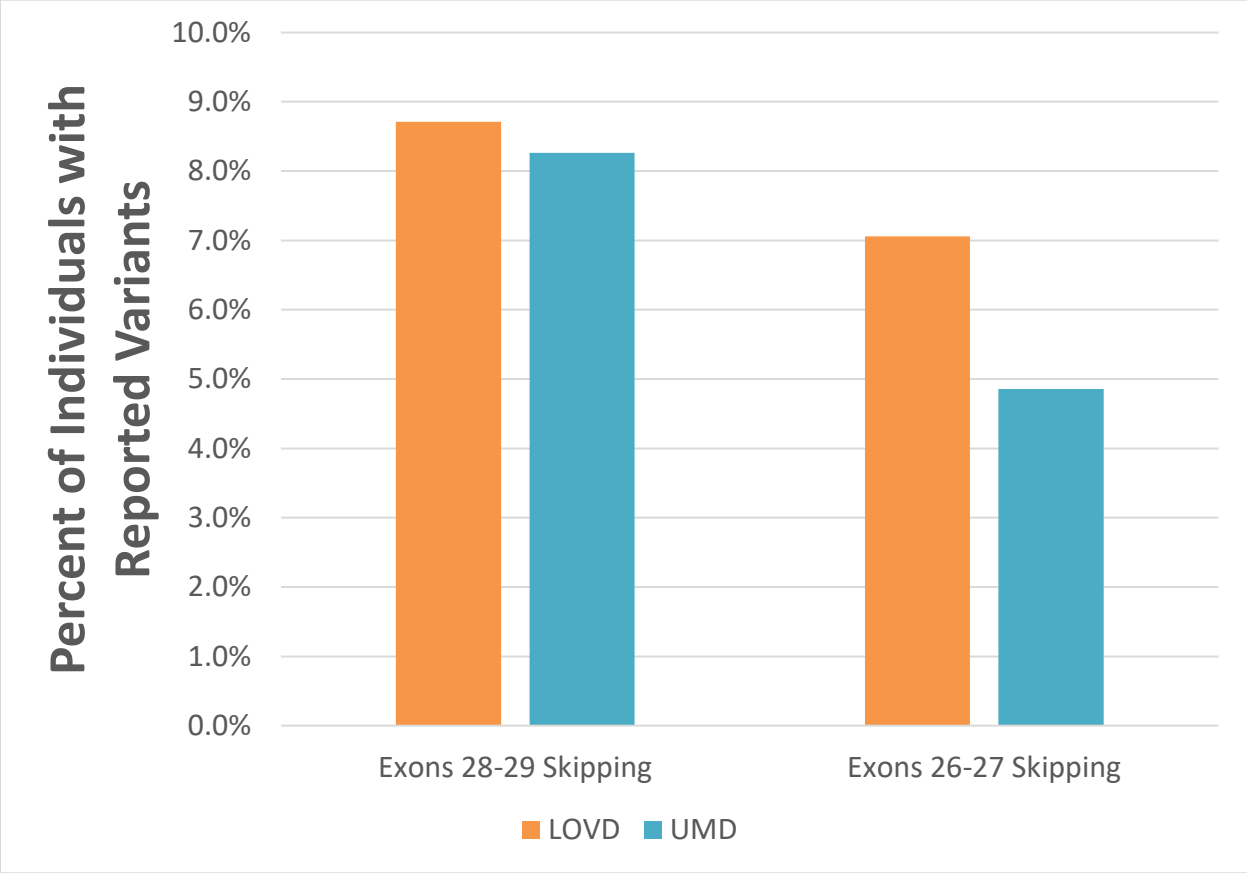


Figure 5.6 Projected therapeutic applicability of *DYSF* exons 28-29 and 26-27 skipping.

Potential therapeutic applicability of *DYSF* exons 28-29 and 26-27 skipping is based on mutation variants reported in the Leiden Open Variation Database (LOVD) and Universal Mutation Database (UMD). Applicability was calculated by taking the number of reported variants within *DYSF* exons 28-29 (116 in LOVD and 97 in UMD) or 26-27 (94 in LOVD and 57 in UMD) and dividing by the total number of reported individuals with variants (1332 total reported in LOVD and 1174 total reported in UMD). LOVD data was obtained in May 2018 and UMD data available in May 2018 was taken from UMD-DYSF v1.4 data from June 16, 2015.

This work further supports the use of dysferlinopathy patient fibroblasts in screening novel AO sequences for the identification of therapeutic exon skipping drugs, as fibroblasts expressed readily-detectable amounts of *DYSF* transcripts at levels sufficient for *in vitro* assessment of exon skipping efficiencies. While our AO sequences were able to facilitate robust exon skipping in fibroblasts, it remains to be seen whether the same sequences will be comparably effective when transfected into muscle cells and *in vivo*.

Our study also further validates the use of dysferlinopathy fibroblasts as an effective alternative to myoblasts or myotubes for the purpose of assessing plasma membrane repair (Matsuda et al. 2015, Lee, Maruyama, et al. 2018). Here, healthy and dysferlinopathy patient fibroblasts displayed significant differences in their ability to reseal plasma membranes following two-photon laser wounding. Furthermore, this study highlights how the membrane wounding assay can be used to validate the *in vitro* effectiveness of newly-designed AOs at rescuing dysferlin protein function.

Since as little as 10% of wild-type protein has been associated with very mild pathology in dysferlinopathy (Sinnreich, Therrien, and Karpati 2006), and our cell line MM-Pt2 expresses dysferlin protein somewhere between 25-50% of wild-type (**Figure 5.4B**), it is reasonable to assume that while the endogenous missense mutation may not affect protein stability, there is an appreciable effect on protein functionality, as evidenced here by the significant difference in membrane resealing ability between healthy and patient cells. The observation that our PMO cocktail was able to rescue plasma membrane resealing in patient cells despite no difference in protein expression between treated and non-treated cells is consistent with the idea that the proteins produced here via exon skipping could be more functional than non-treated proteins. Future studies aimed at characterizing the intracellular differences between native and exon-

skipped proteins, such as their respective subcellular localization and interaction with other proteins, will help shed light on this issue. It would also be beneficial to test our PMO cocktail in a *DYSF*-null cell line with a mutation pattern amenable to exons 28-29 skipping in order to determine the degree of protein rescue following transfection.

Dysferlin is a type II transmembrane protein containing several C2 domains, Fer and Dysf domains, and a C-terminal anchor to the sarcolemma (**Figure 5.1**). Dysferlin reportedly exists in its oligomeric state as a dimer, mediated by its C2 domains (Xu *et al.* 2011). While its C2 domains are implicated in calcium and lipid binding, the two Dysf domains have no known function. In humans, gene duplication has resulted in one Dysf domain residing within the other, creating a nested Dysf domain surrounded by an outer Dysf domain (Ponting *et al.* 2001). The dysferlin Dysf domains comprise the region from amino acids 874 to 1101, corresponding to *DYSF* exons 25-30 (Wein *et al.* 2010). Dysf domains have been reported in yeast peroxisomal protein, fer-1, and myoferlin (Nagase *et al.* 2000, Wood *et al.* 2002). Myoferlin is associated with the plasma membrane, is expressed at higher levels in skeletal and cardiac muscle, and is involved in myoblast fusion (Davis *et al.* 2000, Doherty *et al.* 2005). The inner Dysf domain of human dysferlin shares 61% sequence homology with myoferlin (Sula *et al.* 2014). To-date, mutations in myoferlin have not been directly implicated in human disease. Fer-1 is a homologue of dysferlin and is required for sperm vesicle fusion in *Caenorhabditis elegans*; mutations in this gene negatively affect spermatozoa motility (Achanzar and Ward 1997).

Notably, the Dysf domain is not found in otoferlin, one of the three human ferlins (along with dysferlin and myoferlin), suggesting that whatever role this domain plays it is not universal within the ferlin family. Like dysferlin, otoferlin is characterized by the presence of C2 domains, which bind calcium and mediate fusion of vesicles with the plasma membrane (Johnson and Chapman 2010). Mutations in otoferlin are associated with hearing loss, which results from mutated otoferlin's inability to facilitate synaptic vesicle exocytosis at the auditory inner hair cell ribbon synapse (Roux *et al.* 2006, Avraham 2016).

The human dysferlin Dysf domains contain multiple positively-charged and aromatic residues which exhibit a high degree of conservation in comparison to ferlin homologues myoferlin and fer-1 (Therrien *et al.* 2006). The secondary structure of the human inner Dysf domain consists of two antiparallel β-strands, one at each terminus (Sula *et al.* 2014). This secondary structure is conserved in the inner Dysf domain of myoferlin (Patel *et al.* 2008). The solution structure of the myoferlin Dysf domain indicates the presence of stacked W/R motifs, and mutations in this region are predicted to result in unfolding and protein degradation (Patel *et al.* 2008). Like myoferlin, the dysferlin inner Dysf domain is also held together by stacking of arginine/aromatic sidechains and disruption of this region is also predicted to result in instability and unfolding (Sula *et al.* 2014). Notably, the majority of residues of this flat domain region contribute to the surface, suggesting that perhaps the Dysf domain is involved in protein-protein interactions (Sula *et al.* 2014). While this region is evidently susceptible to deleterious mutations, our demonstration that removal of exons 26-27 and 28-29 does not impact dysferlin protein function suggests that removal of portions of the Dysf domains is a possible therapeutic strategy. The hypothesis that this region of dysferlin may be superfluous is supported by the use of a mini-dysferlin which does not contain the Dysf domain but is able to rescue membrane resealing and dysferlin expression in a mouse model of dysferlinopathy (Krahn *et al.* 2010).

While dysferlin is known to have several binding partners (Cacciottolo *et al.* 2011, Han and Campbell 2007, Ono *et al.* , Matsuda *et al.* 2005), the most likely interacting partner at the Dysf domains is caveolin-3 (CAV-3) (Couet *et al.* 1997, Matsuda *et al.* 2001). Mutations in *CAV-3* are implicated in several forms of muscular dystrophy (Galbiati *et al.* 2000, Galbiati *et al.* 1999, Minetti *et al.* 1998). Caveolin-3 is the only caveolin family member expressed in striated muscle and belongs to the dystrophin-glycoprotein complex, forming scaffolding along with t-tubules and caveolar membranes (Rothberg *et al.* 1992, Sargiacomo *et al.* 1995, Parton *et al.* 1997). CAV-3 is expressed primarily in muscle where it plays a role in regulating sarcolemma stability, vesicular trafficking, signal transduction, and regulation of nitric oxide-dependent

functions (Song *et al.* 1996, Scherer and Lisanti 1997, Aravamudan *et al.* 2003, Williams and Lisanti 2004) Importantly, there exist two putative CAV-3 binding sites within the Dysf domains of dysferlin, with one found in the region of exons 25-26 and the other in the region of exons 28-29 (**Figure 5.1**) (Wein *et al.* 2010). An additional CAV-3 binding site is located in a region which corresponds to exon 6. Our analyses were performed in fibroblasts, ruling out interactions with CAV-3 as influencing membrane resealing in this setting; however, it may be worth investigating possible Dysf domain/CAV-3 interactions in muscle cells.

It is also worth noting that patients with the exon 28 c.2997G>T missense mutation are reported to have significantly later disease onset (32.2 ± 4.8 years) and a patient homozygous for the c.2997G>T mutation did not use a cane at age 46 (Takahashi *et al.* 2003, Takahashi *et al.* 2013). In this study, we observed that the cell line harboring a c.2997G>T mutation (MM-Pt2) exhibited milder membrane resealing defects, as measured by change in relative fluorescence intensity over time (**Figure 5.5A**), when compared with the cell line containing a homozygous c.5077C>T mutation (**Figure 5.3A**). Our results further underscore the therapeutic potential of a *DYSF* exons 28-29 skipping approach and suggest that whatever unknown function(s) the dysferlin Dysf domains serve, they are either not directly related to the process of plasma membrane resealing, or their function is redundant.

In conclusion, these observations represent a significant achievement in the development of novel therapeutic strategies for treating dysferlinopathy. There are currently no ongoing or planned clinical trials involving exon skipping for dysferlinopathy, despite the successful translation of exon skipping therapy into several clinical trials for other forms of muscular dystrophy, such as DMD (see clinical trial ID: NCT02958202, NCT02667483, NCT03375255, and NCT03167255). Our identification of two novel exon skipping targets *in vitro* paves the way for future *in vivo* work which will help establish a foundation for the future clinical implementation of antisense-mediated exon skipping in dysferlinopathy

CHAPTER 6: OVERALL DISCUSSION AND CONCLUSIONS

6 Chapter 6. Overall Discussion and Conclusions

Elucidating the mechanisms behind the generation and expansion of rare dystrophin-positive revertant fibers (RFs) could aid the development of novel therapeutic strategies for treating DMD. One hypothesis is that revertant satellite stem cells located adjacent to RFs and encoding alternatively-spliced dystrophin are activated in response to myofiber degeneration and facilitate the clonal expansion of RFs (Rodrigues *et al.* 2016, Yokota *et al.* 2006). Our group previously demonstrated that irradiated mouse muscle containing damaged satellite cells does not regenerate or produce new RFs (Yokota *et al.* 2006), suggesting a negative relationship between the depletion of muscle stem cells and the expansion of revertant fibers. In the work described here, we compared RF expansion in two mouse models having different genetic mutations but the same genetic background. In another study, our group investigated RF expansion in *mdx* mice with the same genetic mutation (an exon 23 nonsense mutation) but with different genetic backgrounds (C57BL/6 and DBA/2) and described a significantly diminished regenerative capacity and reduced RF expansion in *mdx* mice having a DBA/2 background (Rodrigues *et al.* 2016). Taken together, these observations describe how multiple factors such as age, mutation type, and genetic background, contribute to the rise and expansion of RFs, and highlight the considerations which should be made when determining suitable model systems for assessing the effect of therapeutic interventions, since RF expansion could potentially confound such studies.

One of the primary goals in our laboratory is to develop novel antisense-based therapeutic approaches for treating various forms of muscular dystrophy. Specifically, this thesis focused on the development of exon skipping strategies for two major forms of muscular dystrophy: Duchenne muscular dystrophy and dysferlinopathy. In our study, we demonstrated that a cocktail of multiple phosphorodiamidate morpholino oligomers (PMOs) were able to effectively skip *DMD* exons 45-55, producing in-frame transcripts as well as dystrophin protein. These

results were demonstrated in DMD patient cells which had been transdifferentiated from fibroblasts into myotubes. Together, these findings support the feasibility of *DMD* exons 45-55 skipping in human cells, underscoring previous success with exons 45-55 skipping in animal models (Aoki *et al.* 2012, Echigoya, Aoki, *et al.* 2015). In addition, these observations further establish transdifferentiated patient cells as a suitable *in vitro* system for testing AO-mediated exon skipping (Saito *et al.* 2010).

While there are currently no other groups pursuing antisense-mediated multi-exon skipping of *DMD* exons 45-55for therapeutic application, other *DMD* exon skipping approaches are currently under investigation. In May of 2018, Daiichi Sankyo announced the results of their Phase 1/2 clinical trial in Japan of DS-5141, an ethylene-bridged nucleic acid designed to skip *DMD* exon 45 (Takaishi *et al.* 2017) (NCT02667483). Subcutaneous weekly injections (over 12 weeks) of the drug in patients with DMD did not illicit clinically significant adverse events; however, rescue of dystrophin protein was not readily observed. Another clinical trial investigating *DMD* exon skipping was also completed this year by Nippon Shinyaku for their morpholino exon 53-skipping drug, NS-065/NCNP-01 (Komaki *et al.* 2018). Researchers reported no significant adverse events in DMD patients treated with NS-065/NCNP-01 over 12 weeks and muscle biopsy showed a dose-dependent increase in exon 53-skipped mRNA. The respective therapeutic applicability of these exon skipping approaches in patients is approximately 8%, respectively (Aartsma-Rus *et al.* 2009).

As mentioned previously, another promising therapeutic approach for DMD is gene delivery of mini/micro-dystrophin via AAV vector (Harper *et al.* 2002, Zhang and Duan 2012). In June of 2018, Sarepta Therapeutics announced preliminary results from three patients enrolled in a Phase 1/2a clinical trial of AAVrh74.MHCK7.micro-Dystrophin (NCT02376816). Treated patients showed robust expression of transduced micro-dystrophin and a rescue of dystrophin protein to between 38% and 53% of wild-type levels, as detected by Western blot. A marked reduction (87%) of serum creatine kinase (CK) was also observed and no serious adverse events

were reported. Notably, Pfizer and Solid Biosciences are developing similar gene therapy approaches for treating DMD. Pfizer recently announced the start of a Phase 1b clinical trial for its mini-dystrophin gene therapy drug, PF-06939926 (NCT03362502). In May 2018, Solid Biosciences announced promising preclinical data involving its lead micro-dystrophin gene transfer candidate, SGT-001 (Schneider *et al.* 2017), and a Phase 1/2 clinical trial is currently in progress (NCT03368742).

Potential limitations of this study which will need to be investigated in the future include variable stability between exon-skipped transcripts and possible epigenetic mechanisms affecting therapeutic responses. Although beyond the scope of the present study, the variable stability of intermediate exon-skipped mRNA transcripts is an important consideration when assessing overall exon-skipping efficiency and protein rescue. In our experiments we did not achieve 100% efficiency at producing *DMD* exons 45-55 skipped products and observed several intermediate products, and the potential effects of these intermediates should be considered when translating into therapeutic applications. Additionally, the DNA methylation status of oligo-targeting regions may interfere with AO effectiveness, generating inter-individual variation. To account for this, treatment with histone-deacetylase inhibitor, trichostatin A (TSA), or another methylation inhibitor may be beneficial.

While we were able to demonstrate rescue of dystrophin protein following PMO cocktail transfection, we were not able to quantify the amount of protein rescued. In the future, our laboratory will seek to further optimize the exons 45-55 skipping approach to achieve robust dystrophin protein expression by testing other antisense chemistries and determining whether fewer oligos can be used in an AO cocktail to achieve exons 45-55 skipping.

We also characterized the relationship between *DYSF* exon deletion pattern and dysferlin protein function via recovery of plasma membrane resealing in dysferlinopathy patient cells. We discovered that *DYSF* exons 26-27 and 28-29 are not required for proper membrane resealing, implicating these exons as suitable targets for exon skipping therapy. We

substantiated this hypothesis through the design and *in vitro* assessment of PMOs targeting *DYSF* exons 28 and 29 and found that a cocktail of PMOs was able to effectively skip exons 28-29 and restore membrane resealing in patient cells.

Previous work on exon skipping in *DYSF* has centered around exon 32 and successful rescue of plasma membrane resealing has been reported in dysferlinopathy patient cells using 2′-O-methyl antisense oligonucleotides (Barthelemy *et al.* 2015). To date, no group has reported *in vivo DYSF* exon 32 skipping nor multiple exon skipping in *DYSF*.

Like DMD, another promising approach for treating dysferlinopathy is gene delivery of mini-dysferlin via AAV vector (Krahn *et al.* 2010). Since it remains unknown which regions of *DYSF* can be removed without negatively impacting protein function, current approaches for gene delivery involve the use of dual AAV vectors which serve as substrate for reconstitution of the complete 6.5 kb cDNA sequence, and these have been shown to promote robust dysferlin expression and functional improvement in systemically-treated mice (Potter *et al.* 2017).

Another group has investigated non-viral delivery of full-length *DYSF* by utilizing a specialized delivery method called hydrodynamic limb vein injection, which allows for direct contact between plasmid DNA and muscle fibers where it can be taken up and transcribed (Hagstrom *et al.* 2004, Ma *et al.* 2017). Co-delivery of full-length *DYSF* and *follistatin* (*FST*), which plays a role in muscle anabolism and has been shown to improve therapeutic efficacy when co-delivered with mini-dystrophin (Lee and McPherron 2001, Rodino-Klapac *et al.* 2013), led to widespread dysferlin protein expression and improved muscle pathology in treated mice (Ma *et al.* 2017).

A potential limitation in our approach is that we were not able to quantify the amount of exon-skipped dysferlin protein in cells transfected with either GFP-expression constructs or PMOs, and some variation in protein expression is therefore possible between membrane-wounded cells. We suggest, however, that such variation is likely minimal, and the possible confounding effects with respect to membrane resealing are greatly mitigated by having a larger

sample of wounded cells, which was between 10 and 25 in our experiments. Additionally, potential differences with respect to the mechanism of plasma membrane resealing between muscle and non-muscle cells may need to be taken into account, and it remains to be confirmed whether the same pattern of multiple exon skipping in dysferlin-deficient muscle cells will also rescue plasma membrane resealing.

Collectively, these results support the establishment of *DYSF* exons 28-29 and 26-27 skipping as potential therapeutic approaches for treating dysferlinopathy patients with amenable mutation patterns, which, according to available patient database information, is suggested to be ~8% (exons 28-29 skipping) and ~5-7% (exons 26-27 skipping).

Taken together, the results of these investigations provide new insights into the development of novel therapeutic strategies for treating muscular dystrophy. Future *in vivo* work examining *DMD* exons 45-55 skipping in mice harboring humanized *DMD* will help translate these findings and others into clinical utility, and the development of a novel mouse model with a mutation pattern amenable to *DYSF* exons 26-27 and/or 28-29 skipping will help establish the feasibility of this new therapeutic approach.

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APPENDIX

8 Appendix

The appendix section contains additional experiments carried out by Joshua Lee that did not merit publication in scientific journals and were therefore not included in the thesis chapters. These experiments will be briefly described and summarized in the appendix.

8.1 Other non-therapeutic multi-exon skipping approaches in *DYSF*

Although our experiments showed that *DYSF* exons 19-21, 20-21, and 46-48 were not promising therapeutic exon skipping targets, based on the inability of patient cells to reseal following transfection with the corresponding exon-deleted plasmids (**Figure 5.3A, 5.3B**), we designed and tested PMO cocktails to see whether these exons could nevertheless be skipped *in vitro*.

8.1.1 Multi-exon skipping of DYSF exons 19-21 and 20-21 in dysferlinopathy patient cells

We first designed three PMOs targeting each of *DYSF* exons 20 and 21 to restore the reading frame in MM-Pt2 cells, and tested the exon skipping ability of each combination of two-PMO cocktails (for PMO sequences, see **Table 4**). We found that PMO cocktails were able to achieve double exon skipping of *DYSF* exons 20-21 in MM-Pt2 cells (**Figure 8.1A**). We also designed three additional PMOs to facilitate *DYSF* exon 19 skipping, as this exon is in-frame and either exons 19-21 or 20-21 skipping strategy would maintain the reading frame. Unfortunately, no combination of three-PMO cocktails were able to achieve exons 19-21 skipping (data not shown). We then considered that perhaps a cocktail involving multiple PMOs targeting the same exon would be able to facilitate skipping of exons 19-21, as utilizing multiple oligos to target the same exon has been necessary in other exon skipping approaches (Yokota, Lu, *et al*. 2009, Yokota, Hoffman, and Takeda 2011). We therefore transfected a nine-PMO cocktail, with three PMOs targeting each of *DYSF* exons 19, 20, and 21, and assessed exon skipping potential. We

observed that this nine-PMO cocktail (termed "Supercocktail") was able to facilitate robust exons 19-21 skipping in patient cells (**Figure 8.1B)**.

Table 4. Additional PMO sequences used for exon skipping

Exon Target	Distance from acceptor splice site	Sequence (5' to 3')	Predicted Skipping (%)
***DYSF* Ex19**	**Ac13**	**CCAGGGAGAGCAGAAGCCGGCCACGATAAG**	**88.0**
***DYSF* Ex19**	**Ac42**	**CTGTTCACTGTGCTCCACCAGCTTGGTCTC**	**80.8**
***DYSF* Ex19**	**Ac46**	**CCTTCTGTTCACTGTGCTCCACCAGCTTGG**	**78.7**
***DYSF* Ex20**	**Ac20**	**GCTGAGTAGAAGGCCGCAAACAGGGAGTAC**	**86.0**
***DYSF* Ex20**	**Ac49**	**TGGCATCATCCACATCCTGCAGCATGGTGG**	**72.5**
***DYSF* Ex20**	**Ac17**	**GAGTAGAAGGCCGCAAACAGGGAGTACTTG**	**65.6**
***DYSF* Ex21**	**Ac39**	**CCTCCCAGTAGGATGACAGCACCACCACAG**	**80.2**
***DYSF* Ex21**	**Ac52**	**CTATGGCTGATGTCCTCCCAGTAGGATGAC**	**77.9**
***DYSF* Ex21**	**Ac83**	**AATCCCAAGCAGCTGGTTCTGAGTCTCGAT**	**67.9**
***DYSF* Ex46**	**Ac80**	**ACATTACACGGTCTGTCCGGTACACAGGTG**	**35.2**
***DYSF* Ex46**	**Ac2**	**GGAGCTGGTCCCGCCACTGGTTCGGTCCAG**	**67.6**
***DYSF* Ex46**	**Ac59**	**ACACAGGTGCCTTGACTCTATGCTGCTGGC**	**65.7**
***DYSF* Ex47**	**Ac36**	**GAAGCACATGCAGAGCCAGACGCTCCTCCA**	**56.4**
***DYSF* Ex47**	**Ac39**	**GCTGAAGCACATGCAGAGCCAGACGCTCCT**	**51.0**

***DYSF* Ex47**	**Ac31**	**ACATGCAGAGCCAGACGCTCCTCCACTGGG**	**60.6**
***DYSF* Ex48**	**Ac19**	**GCCGCCCCAGGGCCTTCGGAAATAGGTCGA**	**70.7**
***DYSF* Ex48**	**Ac73**	**GCTGGGCAAGTCACCTTCTGGCTCTCCGTG**	**N/A**
***DYSF* Ex48**	**Ac40**	**TGATGTTGAAGGGAGGTCCAGGCCGCCCCA**	**69.9**

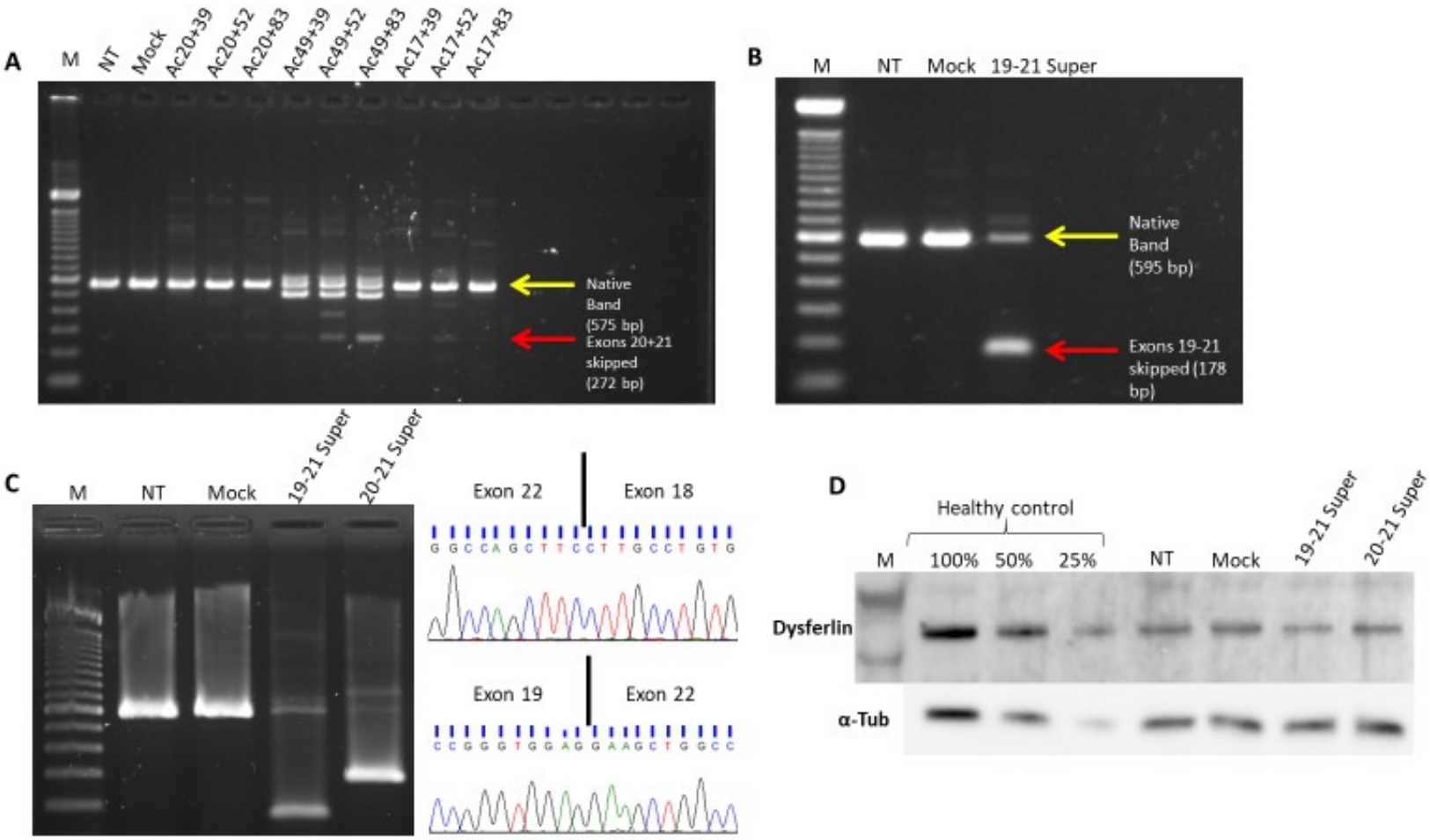
A
M
NT
Mock
Ac20+39
Ac20+52
Ac20+83
Ac49+39
Ac49+52
Ac49+83
Ac17+39
Ac17+52
Ac17+83
Native Band (575 bp)
Exons 20+21 skipped (272 bp)
B
M
NT
Mock
19-21 Super
Native Band (595 bp)
Exons 19-21 skipped (178 bp)
C
M
NT
Mock
19-21 Super
20-21 Super
Exon 22
Exon 18
Exon 19
Exon 22
D
Healthy control
M
100%
50%
25%
NT
Mock
19-21 Super
20-21 Super
Dysferlin
α-Tub

Figure 8.1: *DYSF* exons 20-21 and 19-21 skipping in MM-Pt2. All PMO concentrations tested were 10 μM each PMO. (A) RT-PCR of two-PMO cocktail-treated MM-Pt2 cells. (B) RT-PCR of "supercocktail"-treated (nine-PMO cocktail) MM-Pt2 cells, with three PMOs each targeting *DYSF* exons 19, 20, and 21. (C) A supercocktail of nine PMOs and six PMOs targeting *DYSF* exons 19-21 and 20-21 skipping, respectively. Exon-skipped products confirmed via Sanger sequencing. (D) Western blot of supercocktail-treated MM-Pt2 cells. NT = non-treated; Mock = random oligo.

We also decided to test a "supercocktail" for *DYSF* exons 20-21 skipping, using a six-PMO cocktail with three PMOs targeting each of exons 20 and 21 and again observed robust multi-exon skipping (**Figure 8.1C**). Sanger sequencing confirmed the presence of exons 19-21 and 20-21 skipped products. As our PMO supercocktails seemed more effective at facilitating exon skipping than cocktails employing only one PMO per exon, as measured by RT-PCR, we decided to test whether our supercocktails could affect dysferlin protein levels. Western blot showed that our supercocktails had no appreciable effect on protein levels when transfected into MM-Pt2 patient cells (**Figure 8.1D**). Taken together, our results show that multi-exon skipping of *DYSF* exons 19-21 and 20-21 skipping is possible *in vitro*. While this is the first demonstration of *DYSF* exons 19-21 and 20-21 skipping *in vitro*, this exon skipping approach is not likely to translate into clinical utility as our observations suggest that these exons are somehow required for proper plasma membrane resealing (**Figure 5.2**).

8.1.2 Multi-exon skipping of DYSF exons 46-48 in dysferlinopathy patient cells

We designed three PMOs targeting each of *DYSF* exons 46, 47, and 48 (nine PMOs in total) to restore the reading frame in MM-Pt1 cells, and tested the exon skipping ability of every combination of three-PMO cocktails (for PMO sequences, see **Table 4**). We found that three-PMO cocktails were able to produce *DYSF* exons 46-48 skipped products, as measured by RT-PCR, and confirmed these observations via Sanger sequencing (**Figure 8.2**). As mentioned previously, these observations are not likely to translate into clinical practice as we observed impaired membrane resealing following transfection with *DYSF* exons 46-48 deleted constructs (**Figure 8.2**).

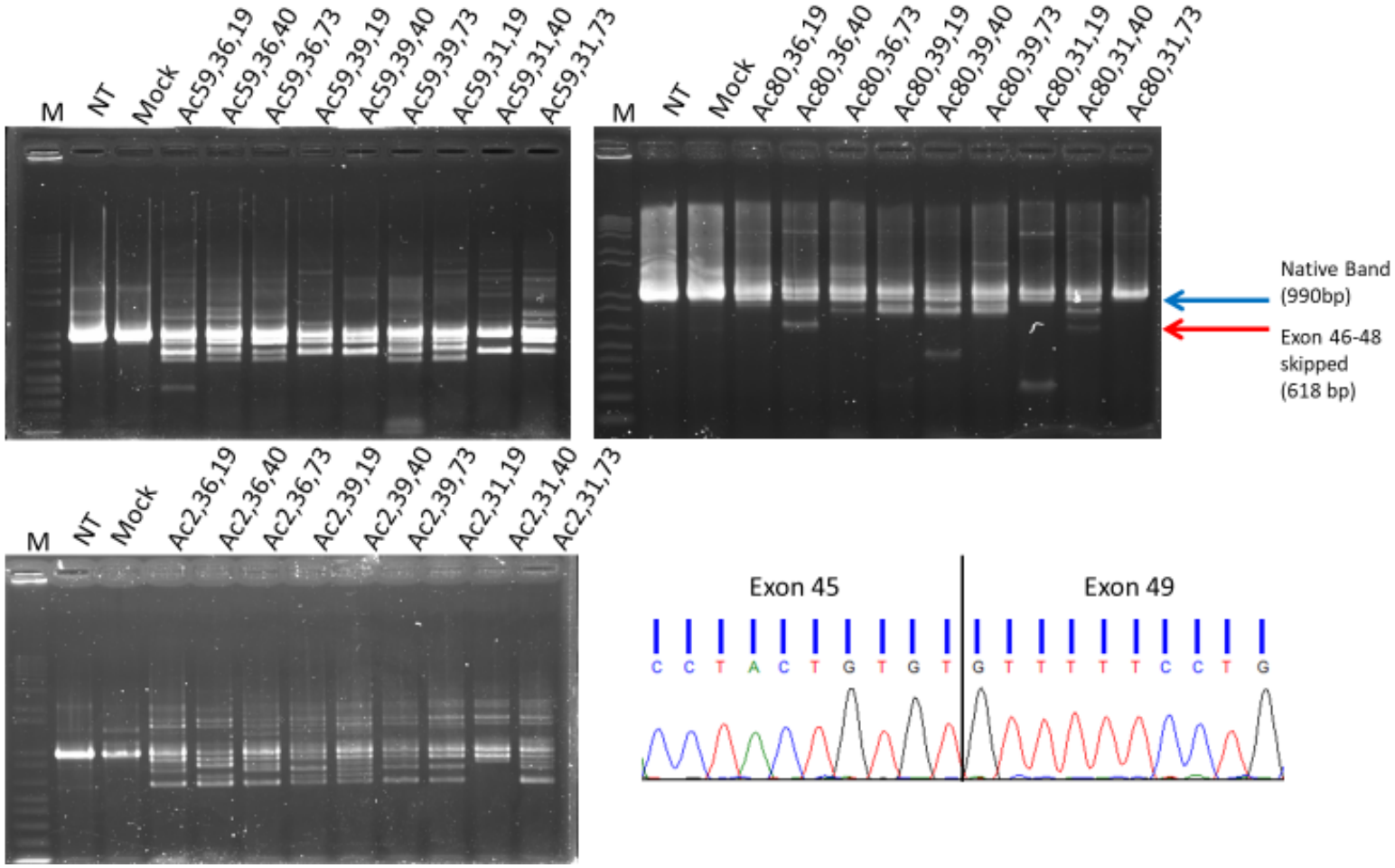
M
NT
Mock
Ac59,36,19
Ac59,36,40
Ac59,36,73
Ac59,39,19
Ac59,39,40
Ac59,39,73
Ac59,31,19
Ac59,31,40
Ac59,31,73
M
NT
Mock
Ac80,36,19
Ac80,36,40
Ac80,36,73
Ac80,39,19
Ac80,39,40
Ac80,39,73
Ac80,31,19
Ac80,31,40
Ac80,31,73
Native Band
(990bp)
Exon 46-48
skipped
(618 bp)
M
NT
Mock
Ac2,36,19
Ac2,36,40
Ac2,36,73
Ac2,39,19
Ac2,39,40
Ac2,39,73
Ac2,31,19
Ac2,31,40
Ac2,31,73
Exon 45
Exon 49
C C T A C T G T G T G T T T T T C C T G

Figure 8.2: *DYSF* Exons 46-48 skipping in MM-Pt1. RT-PCR showing every combination of three-PMO cocktails targeting *DYSF* exons 46-48 for exon skipping, using three different PMOs to target each individual exon. All PMOs were transfected at 10 μM each oligo. NT = non-treated; Mock = random oligo.