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(71) Applicant: UNIVERSITY OF MASSACHUSETTS [US/US]; One Beacon Street, 31st Floor, Boston, MA 02108 (US).

(72) Inventors: BROWN, Robert, H., Jr.; 16 Oakland Street, Needham, MA 02492 (US). DOMINOV, Janice, A.; 7 Westward Circle, North Reading, MA 01864 (US).

(74) Agent: YOUNG, Daniel, W. et al.; Wolf, Greenfield & Sacks, P.C., 600 Atlanitc Avenue, Boston, MA 02210-2206 (US).

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(54) **Title:** ANTISENSE OLIGONUCLEOTIDES TO RESTORE DYSFERLIN PROTEIN EXPRESSION IN DYSFERLINOPATHY PATIENT CELLS

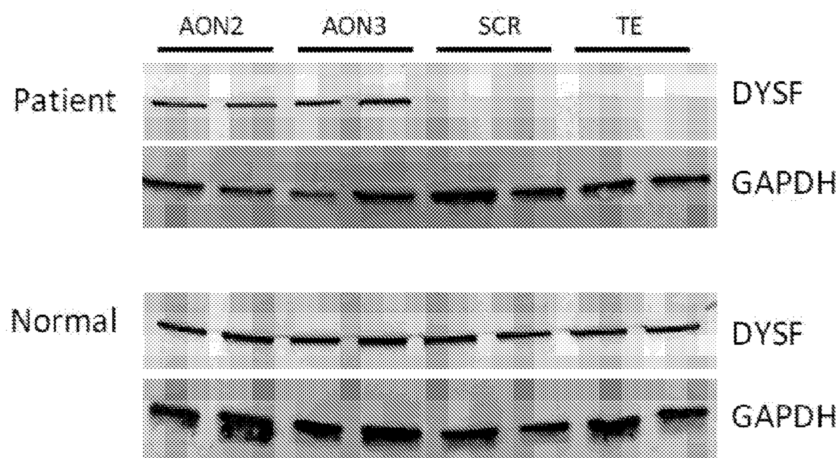

FIG. 4A

(57) **Abstract:** The relates, in some aspects, to antisense oligonucleotide compositions and methods for modifying pre-mRNA splicing in a *DYSF* gene using the same. In some embodiments, the *DYSF* gene comprises a novel mutation that results in a pseudoexon between exons 50 and 51.

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ANTISENSE OLIGONUCLEOTIDES TO RESTORE DYSFERLIN PROTEIN EXPRESSION IN DYSFERLINOPATHY PATIENT CELLS

RELATED APPLICATION

This application claims the benefit of the filing date of U.S. Provisional Application No. 62/682,681, entitled "ANTISENSE OLIGONUCLEOTIDES TO RESTORE DYSFERLIN PROTEIN EXPRESSION IN DYSFERLINOPATHY PATIENT CELLS" filed on June 8, 2018, the entire content of which is incorporated by reference herein in its entirety.

BACKGROUND OF INVENTION

Dysferlin is a large transmembrane protein that functions in critical processes of membrane repair and vesicle fusion. Dysferlin-deficiency due to mutations in the dysferlin gene leads to muscular dystrophy (Miyoshi myopathy (MM), limb girdle muscular dystrophy type 2B (LGMD2B), distal myopathy with anterior tibial onset (DMAT)), typically with early adult onset. At least 416 pathogenic dysferlin mutations are known, but for approximately 17% of patients, one or both of the pathogenic variants remain undefined following standard exon sequencing methods that interrogate exons and nearby flanking intronic regions but not the majority of intronic regions.

Antisense oligonucleotides (AONs) are short nucleic acids or modified nucleic acids that have the potential to modify the expression of specific genes based on complementarity to regions of those genes, either within the protein coding region or in regulatory regions necessary for expression. AONs have thus been recognized as potential therapeutic agents to treat genetic disorders, possibly providing a means to modify the expression of disease causing mutant genes. AON reagents are currently in clinical use to treat Duchenne muscular dystrophy, a lethal X-linked neuromuscular disease affecting young boys that is caused by dystrophin deficiency that currently has no cure.

SUMMARY

The disclosure relates, in some aspects, to compositions and methods for utilizing antisense nucleic acids to alter the splicing of pre-messenger RNA (pre-mRNA) transcribed from a *DYSF* gene in a cell, wherein the *DYSF* gene contains a pseudoexon inserted between exons 50 and 51. In some embodiments, methods described by the disclosure are useful to diagnose and/or treat muscular dystrophies, such as Miyoshi myopathy or limb girdle muscular dystrophy type 2B, which result from abnormal expression of the *DYSF* gene.

In some aspects the disclosure provides a method of modulating splicing in a cell which contains a *DYSF* gene comprising a c.5668-824(C>T) mutation. The method comprises delivering to the cell an antisense nucleic acid that targets a prem-RNA such that exons 50 and 51 of the pre-mRNA are spliced together without an intervening pseudoexon. In some embodiments, a cell is heterozygous for the c.5668-824(C>T) point mutation, and comprises a second *DYSF* gene encodes a wild-type DYSF protein. In some embodiments, a second *DYSF* gene comprises a mutation that causes a premature stop codon. In some embodiments, a premature stop codon is within a C2G domain of DYSF protein. In some embodiments a second *DYSF* gene is a human *DYSF* gene, wherein the mutation that causes the premature stop codon is a c.3444_3445delTGinsAA mutation.

In some embodiments, a cell is *in vitro*, while in other embodiments, a cell is *in vivo*. In some embodiments, a cell is a non-human cell engineered to contain the *DYSF* gene comprising the c.5668-824(C>T) mutation. In some embodiments, a cell is a human cell engineered to contain the *DYSF* gene comprising the c.5668-824(C>T) mutation. In some embodiments, a human cell is from a subject having a muscular dystrophy. In some embodiments, a muscular dystrophy is of the Miyoshi myopathy-type, limb girdle muscular dystrophy type 2B (LGMB2B), or other muscular dystrophy caused by abnormal expression of the *DYSF* gene.

In some embodiments, an antisense nucleic acid is an oligonucleotide of 10 to 25 nucleotides in length comprising a region of complementarity that is complementary with at least 8 contiguous nucleotides of a sequence within a region between exons 50 and 51 encoded by a *DYSF* gene. In some embodiments, an oligonucleotide comprises a sequence of AON1 to AON20 as in Tables 5 and 6. In some embodiments, an AON is chemically modified (*e.g.*, contains one or more 2'-O-methyl-modified nucleobases and/or one or more phosphorothioate linkages). In some embodiments, an antisense nucleic acid is expressed from a transgene, optionally wherein the transgene is delivered to the cell using a viral vector. In some embodiments, a viral vector is a recombinant AAV (rAAV) vector.

In some aspects, the disclosure provides a method of altering RNA splicing in a subject. In some embodiments, the method comprises administering to a subject an antisense nucleic acid that targets a pre-mRNA encoded by a *DYSF* gene and alters splicing of the pre-mRNA such that exons 50 and 51 of the RNA are spliced together without an intervening pseudoexon. In some embodiments, the human *DYSF* gene comprises a c.5668-824(C>T). In some aspects, methods described herein include a step of detecting that the subject has the human *DYSF* gene which comprises a c.5668-824(C>T) mutation prior to administering the antisense nucleic acid. In some embodiments, a human *DYSF* gene is detected using a hybridization assay that

discriminates between the presence of a cytosine and a thymidine at position c.5668-824(C>T) of the human *DYSF* gene. In further embodiments, the hybridization assay is a polymerase chain reaction (PCR) assay, wherein the PCR assay comprises using a primer that is at least partially complementary with a nucleic acid having a sequence as set forth in SEQ ID NO: 103 or a complementary sequence thereof.

In some embodiments, an antisense nucleic acid is an oligonucleotide (*e.g.*, a synthetic oligonucleotide) of 10 to 25 nucleotides in length comprising a region of complementarity that is complementary with at least 8 contiguous nucleotides of a sequence within a region between exons 50 and 51 encoded by a *DYSF* gene. In some embodiments, an oligonucleotide comprises a sequence of AON1 to AON20 as in Tables 5 and 6. In some embodiments, an antisense nucleic acid is expressed from a transgene, optionally wherein the transgene is delivered to the cell using a viral vector. In still further embodiments, a viral vector is a recombinant AAV vector.

In some aspects, the disclosure provides an oligonucleotide of 10 to 25 nucleotides in length comprising a region of complementarity that is complementary with at least 8 contiguous nucleotides of a sequence within a region between exons 50 and 51 encoded by a *DYSF* gene. In some embodiments, the oligonucleotides comprise at least one modified nucleotide or at least one modified internucleotide linkage. In some embodiments, the human *DYSF* gene comprises a c.5668-824(C>T). In further embodiments, the region of complementarity is complementary with at least 8 nucleotides of a sequence as set forth as SEQ ID NO: 103. In other embodiments, the region of complementarity is complementary with at least 8 nucleotides of a sequence as set forth as SEQ ID NO: 104 or 105.

In some embodiments, a region of complementarity is complementary with an exonic splice enhancer sequence. In some embodiments, a region of complementarity is complementary with a splice donor motif (*e.g.*, of a *DYSF* gene). In some embodiments, a region of complementarity is complementary with a splice acceptor motif (*e.g.*, of a *DYSF* gene). In some embodiments, a region of complementarity is complementary with a lariat branch point (*e.g.*, of a *DYSF* gene).

In some embodiments, a human *DYSF* gene comprises a mutation that results in an in-frame pseudoexon between exons 50 and 51. In other embodiments, an oligonucleotide, when present in a cell that contains the human *DYSF* gene, alters splicing of a pre-mRNA expressed from the human *DYSF* gene such that the pseudoexon is not incorporated between exons 50 and 51. In some embodiments, an oligonucleotide comprises at least one modified nucleotide, optionally wherein the at least one modified nucleotide is a 2'-modified nucleotide. In some

embodiments, the 2'-modified nucleotide is a 2'-deoxy, 2'-fluoro, 2'-O-methyl, 2'-O-methoxyethyl, 2'-amino and 2'-aminoalkoxy modified nucleotides. In other embodiments, the 2'-modified nucleotide comprises a 2'-O-4'C methylene bridge. In some embodiments, an oligonucleotide is fully 2'-O-methyl-modified (*e.g.*, each nucleotide base of the oligonucleotide is 2'-O-methyl-modified).

In some embodiments, an oligonucleotide comprises at least one modified internucleotide linkage. In some embodiments, a modified linkage is a phosphorothioate modified linkage. In some embodiments, an oligonucleotide is fully phosphorothioate modified (*e.g.*, all linkages of the oligonucleotide are phosphorothioate). In some embodiments, an oligonucleotide is fully 2-O-methyl and fully phosphorothioate modified.

In some embodiments, an oligonucleotide is a morpholino (or modified morpholino, *e.g.* peptide conjugated morpholino, phosphorodiamidate morpholino, *etc.*). In other embodiments, the oligonucleotide comprises alternating LNA and RNA nucleotides, LNA and DNA nucleotides, or RNA and DNA nucleotides.

In still other embodiments, an oligonucleotide, when present in a cell that contains the human *DYSF* gene, is capable of hybridizing with RNA expressed from the human *DYSF* gene without inducing cleavage of the RNA by an RNAse. In some embodiments, the disclosure provides a composition comprising an antisense oligonucleotides further comprising a carrier, for example a pharmaceutically-acceptable carrier.

In some aspects, the disclosure provides a preparation of oligonucleotides, wherein at least 95% of the oligonucleotides are 10 to 25 nucleotides in length and comprise a region of complementarity that is complementary with at least 8 nucleotides of a sequence within a region between exons 50 and 51 encoded by a human *DYSF* gene. In some embodiments, the region of complementarity is complementary with an exonic splice enhancer sequence. In some embodiments, the region of complementarity is complementary with a splice donor motif. In some embodiments, the region of complementarity is complementary with a splice acceptor motif. In some embodiments, the region of complementarity is complementary with a lariat branch point. In some embodiments of a preparation, a human *DYSF* gene comprises a mutation that results in an in-frame pseudoexon between exons 50 and 51. In some embodiments, the oligonucleotide, when present in a cell that contains the human *DYSF* gene, alters splicing of an RNA expressed from the human *DYSF* gene such that the pseudoexon is not incorporated between exons 50 and 51. In some embodiments of the preparation, the oligonucleotides are lyophilized. In some embodiments, at least 95% of the oligonucleotides are identical. In some

embodiments, the disclosure provides a container housing the preparation of oligonucleotides as described above.

In some aspects, the disclosure provides one or more (*e.g.*, pairs) of PCR primers having sequences selected from the primer sequences set forth in Table 3. In some embodiments, the disclosure provides a composition comprising the pair of PCR primers and a template comprising at least a portion of a human *DYSF* gene.

In some aspects, the disclosure provides an expression construct encoding an antisense nucleic acid having a region of complementarity that is complementary with a sequence between exons 50 and 51 encoded by a human *DYSF* gene. In some embodiments, the human *DYSF* gene comprises a c.5668-824(C>T) mutation. In some embodiments of the expression construct, the region of complementarity is complementary with at least 8 contiguous nucleotides of a sequence as set forth as SEQ ID NO: 103, 104, or 105. In some embodiments of the expression construct, the region of complementarity comprises a sequence complementary with an exonic splice enhancer sequence. In some embodiments, the region of complementarity comprises a sequence complementary with a splice donor motif. In some embodiments, the region of complementarity comprises a sequence complementary with a splice acceptor motif. In some embodiments of the expression construct, the region of complementarity comprises a sequence complementary with a lariat branch point. In some embodiments, the antisense nucleic acid expressed from the expression construct vector, when present in a cell that contains the human *DYSF* gene, alters splicing of a prem-RNA expressed from the human *DYSF* gene such that the pseudoexon is not incorporated between exons 50 and 51. In some embodiments, the disclosure provides a recombinant AAV comprising an expression construct as described herein (*e.g.*, an expression construct engineered to express an oligonucleotide, such as an antisense oligonucleotide, as described by the disclosure).

In some aspects, the disclosure provides an engineered cell comprising an exogenous human *DYSF* gene having a c.5668-824(C>T) mutation, wherein the cell expresses a pre-mRNA from the exogenous *DYSF* gene containing a pseudoexon between exons 50 and 51. In some embodiments, the engineered cell is not of a human origin. In other embodiments, the engineered cell is of a human origin. In other embodiments, the disclosure provides a composition comprising cells containing a human *DYSF* gene having a c.5668-824(C>T) mutation and an artificial cell culture medium. In other embodiments, the disclosure provides a tissue culture system comprising a composition further comprising oligonucleotides as described above.

BRIEF DESCRIPTION OF DRAWINGS

FIG. 1 shows the identification of the *DYSF* intron 50i mutation. RT-PCR of differentiated inducible fibroblast-derived myogenic (iFDM) cDNA from two patients (P1 (JF196), P2 (JF23)) shows novel amplicons (arrow) in patient samples that are not in a normal control sample (N). The amplicons were produced using two primer sets (SEQ ID NOs: 29 & 30, 31 & 32), whose amplicons overlap, but not with any other sets (e.g. SEQ ID NOs: 33 & 34), demonstrating additional sequence within patient cDNA in this region. Each PCR product was sequenced to identify the inserted sequence.

FIGs. 2A-2C show that dysferlin mRNA splicing is altered in patients P1 and P2, leading to inclusion of pathogenic pseudoexon PE50.1. **FIG. 2A** shows cDNA sequencing in which patients P1 and P2 have a 180 nucleotide insertion at the junction of exons 50 and 51, revealing the inclusion of a novel pseudoexon, PE50.1, splicing into the coding sequence. PE50.1 encodes 46 additional amino acids followed by a stop codon. **FIG. 2B** shows that *DYSF* intron 50i genomic DNA sequencing showed patients are heterozygous for a point mutation (c.57680824 C>T) (asterisk *) that creates a novel splice donor consensus site at the 3' end of the PE50.1 sequence. Both normal and mutant PE50.1-containint mRNA are expressed through alternative splicing of the *DYSF* transcripts. The mRNA structure within *DYSF* exon 50-intron 50i-exon 51 and the site of the 50i mutations is shown (insert, upper right), along with the normal and mutant splicing patterns. Numbers along the RNA indicate the size of each element in base pairs. **FIG. 2C** show the normal and mutant *DYSF* mRNA splice products and proteins in the region of exons 40-55. PE50.1 results in a 46 amino acid insertion and premature translation termination within the C2G domain and loss of the C-terminal transmembrane (TM) domain, both of which are required for dysferlin function. The predicted truncated protein would be 1935 amino acids rather than the normal 2081 amino acids.

FIGs. 3A-3B show antisense oligonucleotide-mediated skipping of PE50.1 in patient iFDM cells. **FIG. 3A** show pre-mRNA *DYSF* transcript and AON1, AON2, and AON3 (**Table 5**), which target potential exonic splicing enhancers in PE50.1 as shown. The primers in exon 50 (SEQ ID NO: 31) and 51 (SEQ ID NO: 30) amplify cDNAs to distinguish normal mRNA transcripts (88 base pair product containing exon 50 + 51) from mutant PE50.1 transcripts (268 base pair product containing exon 50 + PE50.1 + 51). **FIG. 3B** shows RT-PCR analysis of mRNA splicing. Patient iFDM cells treated with AON1, AON2, and AON3 (duplicate cultures for each) expressed reduced amounts of PE50.1 mutant mRNA and higher normal *DYSF* mRNA compared with a non-specific scrambled (SCR) AON-treated, TE-treated, or no treatment cells.

iFDM cells were allowed to differentiate in differential media (DM) for 6 days, then treated with AONs in DM for an additional 2 days, wherein (-RT) is no reverse transcriptase, H_2O is no RNA used in RT reactions.

FIGs. 4A-4B show that treatment of patient iFDM cells with AON2 and AON3 directed
5 to PE50.1 induces dysferlin protein expression. iFDM cells (duplicate cultures) were allowed to differentiate in DM for 6 days then treated with AONs in DM (or TE buffer as controls) for 3 days, and cells were collected for protein analysis 7 days after AON addition. **FIG. 4A** shows Western blots after 7 days of AON3 treatment (4 days after AON removal), where there was a dramatic increase in DYSF protein expression compared with control cells. GAPDH expression
10 served as a control for protein loading (5 μg protein/lane). Protein levels in normal control iFDM cultures are not affected by AON treatments. **FIG. 4B** shows the quantitation of DYSF protein expressed in **FIG. 4A**, normalized to GAPDH levels and shown relative to normal cells treated with TE. The mean relative DYSF expression in patient cells was significantly higher in both AON2- and AON3-treated cells compared with SCR- or TE-treated controls ($p < 0.05$, one-
15 way ANOVA, Tukey's Multiple Comparison Test).

FIG. 5 shows analysis of genomic DNA from patients P1 (JF196) and P2 (JF23), which revealed that both are heterozygous for the c.5768-824C>T mutation deep within *DYSF* intron 50i. Genomic DNAs from patients' fibroblasts were amplified and sequenced using primer sets that tiled through 50i (**Table 4**), revealing this mutation in the P1 and P2 cells, but not in
20 fibroblasts from an unrelated patients (8597: patient with other dysferlin mutations, RB19895: amyotrophic lateral sclerosis patient) or normal human dermal fibroblasts (NHDF-3). Samples were sequenced using sense (forward) and antisense (reverse) primers as indicated.

FIG. 6 shows that the intronic sequence upstream of pseudoexon PE50.1 contains additional consensus sites required for mRNA splicing. These include a splice acceptor
25 sequence (ag) at the 5' end of PE50.1, an adjacent pyrimidine-rich region and two potential branch point consensus sequences (underlined) that could be used to promote splicing. These sequences, in the presence of c.5768-824 C>T mutation, likely allow PE50.1 to be spliced between exons 50 and 51.

DETAILED DESCRIPTION

30 Aspects of the disclosure relate to methods for altering RNA splicing in a subject. In some embodiments, the disclosure provides compositions and methods for modulating splicing

in a cell that contains a *DYSF* gene with a mutation that results in a pseudoexon between exons 50 and 51 which results in defects in dysferlin protein expression. There are a number of specific features of the dysferlin protein that contribute to its function and interaction with other proteins. Dysferlin has seven Ca^{+2}-sensitive phospholipid binding C2 domains (C2A to C2G) which vary in phospholipid binding characteristics, relative importance for dysferlin dimerization and membrane interaction but collectively may place a role in altering the structure or curvature of lipid bilayers facilitating membrane fusion and interaction with other membrane associated proteins. Dysferlin interacts with numerous proteins which function in membrane trafficking and fusion including caveolin-3, annexins, affixin, calpain-3, and AHNAK. Thus, dysferlin plays an important role in sarcolemma repair following membrane damage, as well as vesicle trafficking, membrane turnover, and T-tubule formation and stability.

A novel mutation was previously identified in the dysferlin gene (*DYSF*) that causes either Miyoshi myopathy or limb girdle muscular dystrophy type 2B (LGMB2B) due to the lack of normal dysferlin protein levels in patients' muscles (US 15/316,027, the contents of which are incorporated herein by reference). This mutation is within intron 44 of the dysferlin gene and leads to incorporation of an extra segment of protein coding sequence derived from sequences within intron 44 (pseudoexon 44.1 (PE44.1)). Inclusion of PE44.1 results in disruption of the normal dysferlin protein sequence by the insertion of additional amino acids.

This disclosure describes a second pathogenic deep mutation in *DYSF* intron 50i that leads to inclusion of pseudoexon 50.1 (PE50.1) (**FIG. 1**). This mutation was found in patients from 17 families, making it one of the more prevalent known *DYSF* mutations. Other aspects of the disclosure provide methods of administering antisense oligonucleotides (AONs) to alter pre-mRNA splicing of the *DYSF* gene such that the pseudoexon (PE50.1) is not incorporated between exons 50 and 51 significantly restoring normal RNA expression and dysferlin protein levels.

Antisense oligonucleotides

As used herein, the term, "antisense nucleic acid," refers to a nucleic acid that has sequence complementarity to a target sequence and is specifically hybridizable, *e.g.,* under stringent conditions, with a nucleic acid having the target sequence. An antisense nucleic acid is specifically hybridizable when binding of the antisense nucleic acid to the target nucleic acid is sufficient to produce complementary based pairing between the antisense nucleic acid and the target nucleic acid, and there is a sufficient degree of complementarity to avoid non-specific binding of the antisense nucleic acid to non-target nucleic acid under conditions in which

specific binding is desired, *e.g.,* under physiological conditions in the case of *in vivo* assays or therapeutic treatment, and in the case of *in vitro* assays, under conditions in which the assays are performed.

In some embodiments, an antisense nucleic acid is used that has a region of
5 complementarity that is perfectly complementary to a portion of a target nucleic acid (e.g., target RNA). However, it should be appreciated that in some embodiments, an antisense nucleic acid may be used that has less than 100% sequence complementarity with a target nucleic acid. An antisense nucleic acid oligonucleotide may comprise a region of complementarity that is complementary with sequence as set forth in SEQ ID NO: 103, 104, or 105. The region of
10 complementarity of the antisense nucleic acid may be complementary with at least 6, *e.g.*, at least 7, at least 8, at least 9, at least 10, at least 15 or more consecutive nucleotides of a target nucleic acid. In addition, to minimize the likelihood of off-target effects, an antisense nucleic acid may be designed to ensure that it does not have a sequence (*e.g.*, of 5 or more consecutive nucleotides) that is complementary with an off-target nucleic acid.
15 Complementary refers to the capacity for precise pairing between two nucleotides. For example, if a nucleotide at a certain position of an antisense nucleic acid is capable of hydrogen bonding with a nucleotide at the corresponding position of a target nucleic acid (e.g., target RNA), then the antisense nucleic acid and target nucleic acid are considered to be complementary to each other at that position. The antisense nucleic acid and target nucleic acid
20 are complementary to each other when a sufficient number of corresponding positions in each molecule are occupied by nucleotides that can hydrogen bond with each other through their bases. Thus, "complementary" is a term that is used to indicate a sufficient degree of complementarity or precise pairing such that stable and specific binding occurs between the antisense nucleic acid and target nucleic acid. However, it should be appreciated that 100%
25 complementarity is not required. For example, in some embodiments, an antisense nucleic acid (e.g., an oligonucleotide) may be at least 80% complementary to (*e.g.*, at least 85%, 90%, 91%, 92%, 93%, 940%, 95%, 96%, 97%, 98%, 99% or 100% complementary to) the consecutive nucleotides of a target nucleic acid.

Thus, it is understood in the art that a complementary nucleotide sequence need not be
30 100% complementary to that of its target to be specifically hybridizable. In some embodiments, a complementary nucleic acid sequence for purposes of the present disclosure is specifically hybridizable when binding of the sequence to the target nucleic acid produces the desired alterations in splicing to occur and there is a sufficient degree of complementarity to avoid non-

specific binding to non-target nucleic acids under conditions in which avoidance of non-specific binding is desired, *e.g.*, under physiological conditions in the case of *in vivo* assays or therapeutic treatment, and in the case of *in vitro* assays, under conditions in which the assays are performed under suitable conditions of stringency.

Sequence identity, including determination of sequence complementarity for nucleic acid sequences, may be determined by sequence comparison and alignment algorithms known in the art. To determine the percent identity of two nucleic acid sequences, the sequences are aligned for optimal comparison purposes (*e.g.*, gaps can be introduced in the first sequence or second sequence for optimal alignment). The nucleotides at corresponding nucleotide positions are then compared. When a position in the first sequence is occupied by the same residue as the corresponding position in the second sequence, then the molecules are identical at that position. In some embodiments, the percent identity between the two sequences is a function of the number of identical positions shared by the sequences (*e.g.*, % homology=# of identical positions/total # of positions$\times$100), optionally penalizing the score for the number of gaps introduced and/or length of gaps introduced.

In some embodiments, an antisense nucleic acid is an antisense oligonucleotide (AON), which may be referred to simply as an oligonucleotide. For example, in some embodiments, oligonucleotides are provided that comprise a region of complementarity that is complementary with at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15 or more contiguous nucleotides of a sequence within a region between exons 50 and 51 encoded by a human *DYSF* gene. Such oligonucleotides are useful for modulating splicing of dysferlin and prevent incorporation of a pseudoexon between exons 50 and 51. In some embodiments, an antisense nucleic acid is an antisense oligonucleotide (AON) recited in Table 5 or Table 6.

In some embodiments, oligonucleotides of the disclosure have a length in a range of 5 to 40 nucleotides, 5 to 30 nucleotides, 10 to 30 nucleotides, 10 to 25 nucleotides, or 15 to 25 nucleotides. In some embodiments of the disclosure, oligonucleotides have a length of 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40 or more. In some embodiments, the oligonucleotide comprises a region of complementarity that is complementary with a region within 5, 10, 15, 25, 50, 100 or 200 nucleotides of a c.5668-824 (C>T) mutation in a human *DYSF* gene. In some embodiments, the oligonucleotide comprises a region of complementarity that is complementary with at least 8 contiguous nucleotides of a sequence as set forth as SEQ ID NO: 103. In some embodiments, the oligonucleotide comprises a region of complementarity that is complementary with at least 8 contiguous nucleotides of a sequence as set forth as SEQ ID NO: 104 or 105. The region of

complementarity may be complementary with an exonic splice enhancer or inhibitor sequence, a splice donor motif, a splice acceptor motif or a lariat branch point encoded by a human *DYSF* gene (*e.g.*, within a region spanning from exon 50 to exon 51).

In some embodiments, antisense nucleic acids (*e.g.*, oligonucleotides) are provided in a homogeneous preparation, *e.g.*, in which at least 85%, at least 90%, at least 95%, or at least 99% of the oligonucleotides are identical. For example, in some embodiments, homogeneous preparations of oligonucleotides are provided in which at least 85%, at least 90%, at least 95%, or at least 99% of the oligonucleotides in the preparation are 10 to 25 nucleotides in length and comprise a region of complementarity that is complementary with at least 8 contiguous nucleotides of a sequence within a region between exons 50 and 51 encoded by a human *DYSF* gene.

Antisense nucleic acids of the disclosure may be modified to achieve one or more desired properties, such as, for example, improved cellular uptake, improved stability, reduced immunogenicity, improved potency, improved target hybridization, susceptibility to RNAse cleavage, *etc*. In some embodiments, an antisense nucleic acid is modified such that when present in a cell that contains a human *DYSF* gene, it is capable of hybridizing with RNA expressed from the human *DYSF* gene without inducing cleavage of the RNA by an RNase. Antisense nucleic acids can be modified at a base moiety, sugar moiety and/or phosphate backbone. Accordingly, antisense nucleic acids may have one or more modified nucleotides (*e.g.*, a nucleotide analog) and/or one or more backbone modifications (*e.g.*, a modified internucleotide linkage). Antisense nucleic acids may have a combination of modified and unmodified nucleotides. Antisense nucleic acids may also have a combination of modified and unmodified internucleotide linkages.

Antisense nucleic acids may include ribonucleotides, deoxyribonucleotides, and combinations thereof. Examples of modified nucleotides which can be used in antisense nucleic acids include, for example, 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xanthine, 4-acetylcytosine, 5-(carboxyhydroxylmethyl)uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5′-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic

acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, and 2,6-diaminopurine.

In some embodiments, a modified nucleotide is a 2'-modified nucleotide. For example , the 2'-modified nucleotide may be a 2'-deoxy, 2'-fluoro, 2'-O-methyl, 2'-O-methoxyethyl, 2'-amino and 2'-aminoalkoxy modified nucleotides. In some embodiments, the 2'-modified nucleotide comprises a 2'-O-4'-C methylene bridge, such as a locked nucleic acid (LNA) nucleotide. In some embodiments of a 2' modified nucleotide the 2′-hydroxyl group is linked to the 3′ or 4′ carbon atom of the sugar ring thereby forming a bicyclic sugar moiety. In such embodiments, the linkage may be a methelyne $(\text{—CH2—})_n$ group bridging the 2′ oxygen atom and the 3′ or 4′ carbon atom wherein n is 1 or 2.

Antisense nucleic acids may include combinations of LNA nucleotides and unmodified nucleotides. Antisense nucleic acids may include combinations LNA and RNA nucleotides. Antisense nucleic acids may include combinations LNA and DNA nucleotides. A further preferred oligonucleotide modification includes Locked Nucleic Acids (LNAs) in which the 2′-hydroxyl group is linked to the 3′ or 4′ carbon atom of the sugar ring thereby forming a bicyclic sugar moiety.

Antisense nucleotide acids may also include nucleobase-modified nucleotides, *e.g.*, nucleotides containing a non-naturally occurring nucleobase instead of a naturally occurring nucleobase. Bases may be modified to block the activity of adenosine deaminase, for example. Examples of modified nucleobases include, but are not limited to, uridine and/or cytidine modified at the 5-position, *e.g.*, 5-(2-amino)propyl uridine, 5-bromo uridine; adenosine and/or guanosines modified at the 8 position, *e.g.*, 8-bromo guanosine; deaza nucleotides, *e.g.*, 7-deaza-adenosine; O- and N-alkylated nucleotides, *e.g.*, N6-methyl adenosine are suitable. It should be noted that the above modifications may be combined.

Within antisense nucleic acids (*e.g.*, oligonucleotides) of the disclosures, as few as one and as many as all nucleotides can be modified. For example, an oligonucleotide (*e.g.*, an oligonucleotide of 20 nucleotides in length) may contain 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 modified nucleotides. In some embodiments, a modified oligonucleotide will contain as few modified nucleotides as are necessary to achieve a desired level of *in vivo* stability and/or bioaccessibility or other desired property.

Certain antisense nucleic acids may include nonionic DNA analogs, such as alkyl- and aryl-phosphates (in which the charged phosphonate oxygen is replaced by an alkyl or aryl group), phosphodiester and alkylphosphotriesters, in which the charged oxygen moiety is alkylated. Nucleic acids which contain a diol, such as tetraethyleneglycol or

hexaethyleneglycol, at either or both termini have also been shown to be substantially resistant to nuclease degradation and may be used herein. In some embodiments, antisense nucleic acids may include at least one lipophilic substituted nucleotide analog and/or a pyrimidine-purine dinucleotide.

5 In some embodiments, antisense nucleic acids (*e.g.*, oligonucleotides) may have one or two accessible 5′ ends. It is possible to create modified oligonucleotides having two such 5′ ends, for instance, by attaching two oligonucleotides through a 3′-3′ linkage to generate an oligonucleotide having one or two accessible 5′ ends. The 3′3′-linkage may be a phosphodiester, phosphorothioate or any other modified internucleoside bridge. Additionally, 3′3′-linked
10 oligonucleotides where the linkage between the 3′ terminal nucleosides is not a phosphodiester, phosphorothioate or other modified bridge, can be prepared using an additional spacer, such as tri- or tetra-ethylenglycol phosphate moiety.

A phosphodiester internucleotide linkage of an antisense nucleic acid can be replaced with a modified linkage. The modified linkage may be selected from, for example,
15 phosphorothioate, phosphorodithioate, NR1R2-phosphoramidate, boranophosphate, α-hydroxybenzyl phosphonate, phosphate-(C1-C21)—O-alkyl ester, phosphate-[(C6-C12)aryl-(C1-C21)—O-alkyl]ester, (C1-C8)alkylphosphonate and/or (C6-C12)arylphosphonate bridges, and (C7-C12)-α-hydroxymethyl-aryl.

A phosphate backbone of the antisense nucleic acid can be modified to generate peptide
20 nucleic acid molecules. As used herein, the terms “peptide nucleic acids” or “PNAs” refer to nucleic acid mimics, *e.g.*, DNA mimics, in which the deoxyribose phosphate backbone is replaced by a pseudopeptide backbone and only the four natural nucleobases are retained. The neutral backbone of PNAs has been shown to allow for specific hybridization to DNA and RNA under conditions of low ionic strength. The synthesis of PNA oligomers can be performed using
25 standard solid phase peptide synthesis protocols, for example.

Antisense nucleic acids can also be formulated as morpholino oligonucleotides. In such embodiments, the riboside moiety of each subunit of an oligonucleotide of the oligonucleotide reagent is converted to a morpholine moiety. Morpholinos may also be modified, *e.g.* as a peptide conjugated morpholino, a phosphorodiamidate morpholino, *etc*.

30 In other embodiments, the antisense nucleic acid (*e.g.*, oligonucleotide) can be linked to functional groups such as peptides (*e.g.*, for targeting host cell receptors *in vivo*), or agents facilitating transport across the cell membrane or the blood-brain barrier. Oligonucleotide reagents of the disclosure also may be modified with chemical moieties (*e.g.*, cholesterol) that improve the *in vivo* pharmacological properties of the oligonucleotide reagents.

Dysferlinopathies

Dysferlinopathy is a degenerative muscle disease cause by insufficient expression of dysferlin protein in skeletal muscles. This is a recessively-inherited disorder with onset in late teens to early adulthood as muscle weakness due to muscle degeneration, which progressively worsens, typically leading to significant loss of mobility. There is currently no cure for dysferlinopathy, and treatment is currently palliative. Clinical trials have recently begun to evaluate a virally-mediated gene replacement strategy to restore dysferlin expression.

Dysferlin is a member of the ferlin family of Ca^{+2}-dependent phospholipid-binding proteins. Dysferlin is a large (237 kilodaltons, kDa) transmembrane protein critical in membrane repair, vesicle trafficking, and T-tubule structure. The dysferlin protein is derived from a ~6.2 kb transcript assembled from up to 55 exons. There are 14 known human isoforms of dysferlin, generated by altered exon splicing and the use of two alternate promoters, with isoform 8 being the predominant form in muscle. In addition to skeletal muscle, dysferlin is expressed in other tissues, and dysferlin-deficiency has been associated with immune cell migration changes such as increased motility and phagocytosis by blood monocytes. The dysferlin expression level in blood monocytes can be used as a diagnostic tool for dysferlinopathy.

At least 416 different pathogenic dysferlin variants are listed in the Universal Mutation Database (UMD-DYSF, http://www.umd.be/DYSF/), most lying within one of the 55 exons that are spliced together to form isoform 8 that is critical for muscle function. For approximately 17% of patients, a complete understanding of the genetic lesions underlying the disease is lacking; at least one of the pathogenic mutations has not been identified. It is likely that these mutations lie within dysferlin introns or areas other than exons that regulate dysferlin expression. These gene regions are not examined by standard exonic screening methods.

A pathogenic variant deep within dysferlin intron 44 (44i) (c.4866+1249G>T) in dysferlinopathy patients was previously identified (see WO 2015/187825A2, the contents of which are incorporated herein by reference). This intronic variant induces alternate splicing of the dysferlin transcript, allowing the inclusion of a 177 bp pseudoexon (PE44.1) within the mature transcript, resulting in an in-frame insertion of 59 amino acids within the C2F domain of the dysferlin protein.

The disclosure is based, in part, on the identification of a new deep intronic mutation located between dysferlin exons 50 and 51. This intronic variant induces alternative splicing of the dysferlin transcript, allowing the inclusion of a 180 bp pseudoexon 51 (also referred to as

PE50.1) within the mature transcript, resulting in an in-frame insertion of 46 amino acids within the C2G region of the dysferlin protein.

In some embodiments, at least 14 variants (*e.g.*, isoforms) of *DYSF* transcripts that arise from use of two separate promoters and/or alternate exon splicing events. In some embodiments, *DYSF* mRNA variant 8 (NM_003494.3) is used as a reference for RNA and cdRNA analyses and/or as a context for specifying exon and intron numeric assignments and nomenclature.

A dysferlinopathy is a muscular dystrophy caused mutations in the dysferlin gene. Examples of dysferlinopathies included limb-girdle muscular dystrophy type 2B, Miyoshi myopathy, and distal anterior compartment myopathy. In limb-girdle muscular dystrophies, the muscles in the shoulder and pelvic girdle are most affected and early symptoms include difficulty running, climbing stairs, standing, and walking. In Myoshi myopathy, early symptoms are most pronounced in distal portions of the legs and as the disease worsens, progress through the thighs, gluteal muscles, forearms, and shoulder girdle muscles.

Treatment Methods

Aspects of the disclosure relate to methods of altering RNA splicing in a subject involving administering to the subject an antisense nucleic acid that targets a pre-messenger RNA encoded by a human *DYSF* gene and alters splicing of the pre-messenger RNA such that exons 50 and 51 of the RNA are spliced together without an intervening pseudoexon. In some embodiments, the subject is heterozygous a c.5668-824 (C>T) mutation in the *DYSF* gene. However, in some embodiments, the subject is homozygous a c.5668-824 (C>T) mutation in the *DYSF* gene. Often the subject has or is suspected of having muscular dystrophy (*e.g.*, of the Miyoshi Myopathy-type) caused by abnormal expression of the dysferlin gene product and the methods are being implemented for purposes of treating the muscular dystrophy. Treating, in this case, includes improving dysferlin expression and/or ameliorating one or more symptoms of muscular dystrophy.

Any appropriate antisense nucleic acid disclosed herein may be administered. For example, the antisense nucleic acid may be an oligonucleotide (*e.g.*, of 10 to 25 nucleotides in length) comprising a region of complementarity that is complementary with a sequence within a region between exons 50 and 51 encoded by a human *DYSF* gene. An antisense oligonucleotide may for example comprise a sequence of AON1 to AON20 as set forth in Tables 5 and 6.

In some embodiments, an antisense nucleic acid is expressed from a transgene, *e.g.*, as an antisense RNA transcript. A transgene may be administered to a subject in a DNA expression

construct that is engineered to express an antisense RNA transcript in a subject. A DNA expression construct may be administered directly or using a viral vector (*e.g.*, a recombinant AAV (rAAV) vector) or other suitable vector. Viral vectors that have been used for gene therapy protocols include, but are not limited to, retroviruses, other RNA viruses such as poliovirus or Sindbis virus, adenovirus, adeno-associated virus (AAV), herpes viruses, SV 40, vaccinia, lentivirus and other DNA viruses.

Alternatively, a transgene may be express *ex vivo* and the resulting antisense RNA transcript may be administered directly to the subject.

As disclosed herein antisense nucleic acids (including DNA expression constructs that may be used to expressed them) may be administered by any suitable route. For use in therapy, an effective amount of the antisense nucleic acid (*e.g.* oligonucleotide) and/or other therapeutic agent can be administered to a subject by any mode that delivers the agent to the desired tissue, *e.g.*, muscle tissue. In some embodiments, agents (*e.g.*, antisense nucleic acids) are administered intramuscularly. Other suitable routes of administration include but are not limited to oral, parenteral, intravenous, intraperitoneal, intranasal, sublingual, intratracheal, inhalation, subcutaneous, ocular, vaginal, and rectal. Systemic routes include oral and parenteral. Several types of devices are regularly used for administration by inhalation. These types of devices include metered dose inhalers (MDI), breath-actuated MDI, dry powder inhaler (DPI), spacer/holding chambers in combination with MDI, and nebulizers.

For oral administration, the agents can be formulated readily by combining the active compound(s) with pharmaceutically acceptable carriers well known in the art. Such carriers enable the agents of the disclosure to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a subject to be treated. Pharmaceutical preparations for oral use can be obtained as solid excipient, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl cellulose, sodium carboxymethylcellulose, and/or polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate. Optionally oral formulations may also be formulated in saline or buffers for neutralizing internal acid conditions or may be administered without any carriers.

Pharmaceutical preparations that can be used orally include push fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push fit capsules can contain the active ingredients in admixture with filler such as lactose, binders such as starches, and/or lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active agents may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added. Microspheres formulated for oral administration may also be used. Such microspheres have been well defined in the art. Formulations for oral administration are typically in dosages suitable for such administration.

For buccal administration, the compositions may take the form of tablets or lozenges formulated in conventional manner.

For administration by inhalation, agents (*e.g.*, antisense nucleic acids) for use according to the present disclosure may be conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebulizer, with the use of a suitable propellant, *e.g.*, dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of *e.g.* gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

The agents (*e.g.*, antisense nucleic acids), when it is desirable to deliver them systemically, may be formulated for parenteral administration by injection, *e.g.*, by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, *e.g.*, in ampoules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents.

Pharmaceutical formulations for parenteral administration include aqueous solutions of agents (*e.g.*, antisense nucleic acids) in water-soluble form. Additionally, suspensions of agents may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions may contain substances that increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also contain suitable stabilizers or agents that increase the solubility of the agents to allow for the preparation of highly concentrated solutions.

Alternatively, agents (*e.g.*, antisense nucleic acids) may be in powder form for constitution with a suitable vehicle, *e.g.*, sterile pyrogen-free water, before use. Agents (*e.g.*, antisense nucleic acids) may also be formulated in rectal or vaginal compositions such as suppositories or retention enemas, *e.g.*, containing conventional suppository bases such as cocoa butter or other glycerides.

5 Other delivery systems can include time-release, delayed release or sustained release delivery systems. Such systems can avoid repeated administrations of the agents (*e.g.*, antisense nucleic acids), increasing convenience to the subject and the physician. Many types of release delivery systems are available. They include polymer base systems such as poly(lactide glycolide), copolyoxalates, polycaprolactones, polyesteramides, polyorthoesters,

10 polyhydroxybutyric acid, and polyanhydrides. Delivery systems also include non-polymer systems that are: lipids including sterols such as cholesterol, cholesterol esters and fatty acids or neutral fats such as mono , di , and tri glycerides; hydrogel release systems; silastic systems; peptide-based systems; wax coatings; compressed tablets using conventional binders and excipients; partially fused implants; and others disclosed herein.

15

Analytical Methods

In some aspects, the disclosure relates to the methods for detecting presence of a mutation causing an insertion in between exon 50 and exon 51 of the *DYSF* gene. In some embodiments, a clinical sample is obtained from a subject for the purpose of identifying a

20 mutation. As used herein, a clinical sample refers to a specimen of biological matter obtained from a subject for the purpose of analysis or diagnosis. Non-limiting examples of clinical samples include blood, saliva, urine, feces, tissue, semen, cerebral spinal fluid, nucleic acids, epithelial cells, sweat, tears, hair and mucous.

In some embodiments, a clinical sample may be obtained from the blood of the patient. In

25 some embodiments, a clinical sample may be obtained from the cells in the blood of a subject. In some embodiments, the cells may be blood cells. In some embodiments, a clinical sample may be obtained from the tissue of a subject. In some embodiments, the tissue of the subject is muscle tissue. In some embodiments, the muscle tissue comprises skeletal muscle. In some embodiments, the muscle tissue comprises smooth muscle. In some embodiments, the muscle

30 tissue comprises cardiac tissue. In some embodiments the clinical sample is obtained from a tissue that is not a muscle tissue. In some embodiments the non-muscle tissue comprises mesenchymal cells. In some embodiments, the tissue of the subject is skin tissue. In some embodiments, the non-muscle tissue comprises fibroblasts.

In some embodiments, a non-muscle tissue comprises stem cells, including, for example, embryonic stem cells, tissue stem cells, umbilical cord stem cells, mesenchymal stem cells, induced pluripotent stem cells, multipotent stem cells, totipotent stem cells, unipotent stem cells, progenitor cells, blastocysts, bone marrow stromal cells, hematopoietic stem cells, oligopotent
5 stem cells, neural stem cells, and trophoblast stem cells.

In some embodiments, methods may involve genotyping a subject with respect to the human *DYSF* gene for purposes of selecting an appropriate treatment for the subject. For example, a subject may be administered an antisense nucleic acid disclosed herein if it is determined that the subject has a *DYSF* gene having a mutation that results an in-frame
10 pseudoexon being coded for between exons 50 and 51 (*e.g.*, a c.5668-824 (C>T) mutation.) Often the subject has or is suspected of having muscular dystrophy (*e.g.*, of the Miyoshi Myopathy-type) caused by abnormal expression of the dysferlin gene product.

The genotype of the subject may be assessed using a hybridization assay that discriminates between the presence of a guanosine and a thymidine at position c.5668-824 of the
15 human *DYSF* gene. An example of a suitable hybridization is a polymerase chain reaction (PCR) based allelic discrimination assay. A PCR based assay may be performed, for example, by using a primers that are at least partially complementary with a nucleic acid having a sequence as set forth in SEQ ID NO: 103, 104, or 105 or a complementary sequence thereof together with a suitable probe for detecting presence or absence of a particular mutation. In some embodiments,
20 one or more PCR amplicons may be sequenced and the obtained sequence may be evaluated for purposes of detecting presence or absence of a particular mutation. In some embodiments, a pair of primers disclosed in Table 3 or 4 be used to amplify one or more regions of the *DYSF* gene for purposes of determining the sequence of the *DYSF* and/or detecting presence or absence of a particular mutation in the *DYSF* gene.

25

Cells

In some aspects, the present disclosure relates to the delivery of antisense nucleic acids (*e.g.* oligonucleotides) to a target cell. In some embodiments, the cell is of a subject having a *DYSF* gene containing a c.5668-824 (C>T) mutation. The cell may be heterozygous for the
30 mutated gene or may be homozygous. The cell may have a second *DYSF* gene (a second allele of the gene) encoding a wild-type *DYSF* protein. The cell may have a second *DYSF* gene (a second allele of the gene) having a different mutation than the c.5668-824 (C>T) mutation. For example, the cell may contain a *DYSF* gene comprising a mutation that causes a premature stop codon (*e.g.*, within a region encoding the C2D domain of *DYSF* protein). For example, the

premature stop codon may be a c.3444_3445delTGinsAA mutation. The cell to which the antisense nucleic acid is delivered may be *in vitro* or *in vivo*.

In some embodiments, the cell is a mammalian cell. In some embodiments the mammalian cell is a human cell. The cell may be from a subject having a muscular dystrophy that is associated at least in part with the *DYSF* gene comprising the c.5668-824 (C>T) mutation. For example, the muscular dystrophy is of the Miyoshi Myopathy-type, or other muscular dystrophy caused by abnormal expression of the dysferlin gene product. In some embodiments, the cell is a myoblast or other muscle progenitor cell. In some embodiments, the cells are muscle cells. In some embodiments of the disclosure the muscle cells are striated (*e.g.* skeletal) muscle cells (*e.g.*, myotubes). In some embodiments, the muscle cells are cardiac smooth muscle cells. In some embodiments, the muscle cells are smooth muscle cells. In some embodiments the cells are not muscle cells. In some embodiments, cells are of the brain, heart, kidneys, lungs, uterus, spleen, pancreas or muscle tissue of a subject. In some embodiments, the cell is a non-human cell (*e.g.*, a non-human mammalian cell, *e.g.*, a mouse cell). For example, the cell may be a non-human cell (*e.g.*, a mouse cell) engineered to contain one or more copies of the human *DYSF* gene comprising the c.5668-824 (C>T) mutation.

Pharmaceutical Compositions

According to some aspects of the disclosure, compositions are provided that comprise an agent (*e.g.*, an antisense nucleic acid (*e.g.*, an oligonucleotides) or vector comprising the same) and a carrier. As used herein, the term, "carrier" refers to an organic or inorganic ingredient, natural or synthetic, with which the active ingredient is combined to facilitate an intended use. For example, pharmaceutical compositions are provided that comprise an antisense nucleic acid and a pharmaceutically-acceptable carrier. As used herein the term "pharmaceutically acceptable carrier" refers to a carrier that is suitable for pharmaceutical administration. The term pharmaceutically-acceptable carrier includes compatible solid or liquid fillers, diluents or encapsulating substances that are suitable for administration to a human or other vertebrate animal.

Components of pharmaceutical compositions also are capable of being commingled with the agents of the present disclosure, and with each other, in a manner such that there is no interaction that would substantially impair the desired pharmaceutical efficiency. Pharmaceutical compositions may include solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and other suitable components compatible with pharmaceutical administration. Supplementary active agents can also be

incorporated into the compositions. Active ingredients (*e.g.*, oligonucleotides) may be admixed or compounded with any conventional, pharmaceutically acceptable carrier or excipient. Pharmaceutical compositions are generally sterile and prepared using aseptic technique. A sterile phosphate-buffered saline is one example of a pharmaceutically acceptable carrier. Other suitable carriers may be used. Pharmaceutical compositions also may comprise suitable solid or gel phase carriers or excipients. Examples of such carriers or excipients include but are not limited to calcium carbonate, calcium phosphate, various sugars, starches, cellulose derivatives, gelatin, and polymers such as polyethylene glycols.

Antisense nucleic acids may be administered per se (neat) or in the form of a pharmaceutically acceptable salt. When used in medicine the salts are generally pharmaceutically acceptable, but non-pharmaceutically acceptable salts may conveniently be used to prepare pharmaceutically acceptable salts thereof. Such salts include, but are not limited to, those prepared from the following acids: hydrochloric, hydrobromic, sulphuric, nitric, phosphoric, maleic, acetic, salicylic, p-toluene sulphonic, tartaric, citric, methane sulphonic, formic, malonic, succinic, naphthalene-2-sulphonic, and benzene sulphonic. Also, such salts can be prepared as alkaline metal or alkaline earth salts, such as sodium, potassium or calcium salts of the carboxylic acid group.

Suitable buffering agents include: acetic acid and a salt (1-2% w/v); citric acid and a salt (1-3% w/v); boric acid and a salt (0.5-2.5% w/v); and phosphoric acid and a salt (0.8-2% w/v). Suitable preservatives include benzalkonium chloride (0.003-0.03% w/v); chlorobutanol (0.3-0.9% w/v); parabens (0.01-0.25% w/v) and thimerosal (0.004-0.02% w/v).

The compositions may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art of pharmacy. All methods include the step of bringing the agents into association with a carrier that constitutes one or more accessory ingredients. In general, the compositions are prepared by uniformly and intimately bringing the agents into association with a liquid carrier, a finely divided solid carrier, or both, and then, if necessary, shaping the product. Liquid dose units are vials or ampoules. Solid dose units are tablets, capsules and suppositories.

An effective amount, also referred to as a therapeutically effective amount, of an antisense nucleic acid (*e.g.* oligonucleotide) capable of modulating splicing in a cell in which the *DYSF* gene is expressed is an amount sufficient to ameliorate at least one adverse effect associated with expression, or reduced expression, of the gene in a cell or in an individual in need of such modulation. The therapeutically effective amount to be included in pharmaceutical compositions may be selected based upon several factors, *e.g.*, the type, size and condition of the

patient to be treated, the intended mode of administration, the capacity of the patient to incorporate the intended dosage form, *etc.*

In some cases, antisense nucleic acids may be prepared in a colloidal dispersion system. Colloidal dispersion systems include lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes. An example of a colloidal system that may be used in methods provided herein is a liposome. Liposomes are artificial membrane vessels that are useful for delivering antisense nucleic acids *in vivo* or *in vitro*. It has been shown that large unilamellar vesicles can encapsulate large macromolecules. Nucleic acids and other components (*e.g.*, viral vectors) can be encapsulated within the aqueous interior and be delivered to cells in a biologically active form. Liposomes may be targeted to a particular tissue by coupling the liposome to a specific ligand such as a monoclonal antibody, sugar, glycolipid, or protein. Ligands which may be useful for targeting a liposome to, for example, a smooth muscle cell or skeletal muscle cell include, but are not limited to: intact or fragments of molecules that interact with muscle cell specific receptors and molecules, such as antibodies, which interact with the cell surface markers. Lipid formulations for transfection are commercially available from QIAGEN, for example, as EFFECTENE™ (a non-liposomal lipid with a special DNA condensing enhancer) and SUPERFECT™ (a dendrimeric technology). Liposomes are commercially available from Invitrogen, Life Technologies, for example, as LIPOFECTIN™, which is formed of cationic lipids such as N-[1-(2, 3 dioleyloxy)-propyl]-N, N, N-trimethylammonium chloride (DOTMA) and dioleoyl phophotidylethanolamine (DOPE), as well as other lipid-based reagents including Lipofectamine and Oligofectamine. Certain cationic lipids, including in particular N-[1-(2, 3 dioleoyloxy)-propyl]-N,N,N-trimethylammonium methyl-sulfate (DOTAP), may be advantageous when combined with the antisense nucleic acids (*e.g.* oligonucleotides) analogs of the disclosure.

In one embodiment, antisense nucleic acids may be formulated with a biocompatible microparticle or implant that is suitable for implantation or administration to a recipient. Bioerodible implants may include a biodegradable polymeric matrix, for example, for containing an exogenous expression construct engineered to express an antisense nucleic acid under the control of an appropriate promoter. The polymeric matrix can be used to achieve sustained release of the therapeutic agent in the subject. A polymeric matrix may be in the form of a microparticle such as a microsphere, in which an antisense nucleic acid and/or other therapeutic agent is dispersed throughout a solid polymeric matrix, or a microcapsule, in which antisense nucleic acid and/or other therapeutic agent is stored in the core of a polymeric shell. Other forms of the polymeric matrix for containing a therapeutic agent include films, coatings, gels, implants,

and stents. The matrix composition also can be selected not to degrade, but rather, to release by diffusion over an extended period of time. In some embodiments, antisense nucleic acids are administered to the subject via an implant while the other therapeutic agent is administered.

Both non-biodegradable and biodegradable polymeric matrices can be used to deliver antisense nucleic acids (*e.g.* oligonucleotides) and/or the other therapeutic agent to a subject. Biodegradable matrices are preferred. Such polymers may be natural or synthetic polymers. The polymer is selected based on the period of time over which release is desired, generally in the order of a few hours to a year or longer. Typically, release over a period ranging from between a few hours and three to twelve months may be used. A polymer may be in the form of a hydrogel, *e.g.*, a hydrogel that can absorb up to about 90% of its weight in water and which is optionally cross-linked with multi-valent ions or other components, *e.g.*, polymers.

Other exemplary compositions that can be used to facilitate uptake of a nucleic acid include calcium phosphate and other chemical mediators of intracellular transport, microinjection compositions, electroporation and homologous recombination compositions (*e.g.*, for integrating a nucleic acid into a preselected location within the target cell chromosome).

In addition to the formulations described previously, the agents may also be formulated as a depot preparation. Such long-acting formulations may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

Other suitable liquid or solid pharmaceutical preparation forms are, for example, aqueous or saline solutions for inhalation, microencapsulated, encochleated, coated onto microscopic gold particles, contained in liposomes, nebulized, aerosols, pellets for implantation into the skin, or dried onto a sharp object to be scratched into the skin. The pharmaceutical compositions also include granules, powders, tablets, coated tablets, (micro)capsules, suppositories, syrups, emulsions, suspensions, creams, drops or preparations with protracted release of active compounds, in whose preparation excipients and additives and/or auxiliaries such as disintegrants, binders, coating agents, swelling agents, lubricants, flavorings, sweeteners or solubilizers are customarily used as described above. The pharmaceutical compositions are suitable for use in a variety of drug delivery systems.

Kits

Agents (*e.g.*, antisense nucleic acids) described herein may, in some embodiments, be assembled into pharmaceutical or diagnostic or research kits to facilitate their use in therapeutic, diagnostic or research applications. A kit may include one or more containers housing the

components and instructions for use. Specifically, such kits may include one or more agents (*e.g.*, antisense nucleic acids) described herein, along with instructions describing the intended therapeutic application and the proper administration of these agents. In certain embodiments agents in a kit may be in a pharmaceutical formulation and dosage suitable for a particular application and for a method of administration of the agents.

The kit may be designed to facilitate use of the methods described herein by physicians and can take many forms. Each of the agents of the kit, where applicable, may be provided in liquid form (*e.g.*, in solution), or in solid form, (*e.g.*, a dry powder). In certain cases, some of the compositions may be constitutable or otherwise processable (*e.g.*, to an active form), for example, by the addition of a suitable solvent or other species (for example, water or a cell culture medium), which may or may not be provided with the kit. As used herein, "instructions" can define a component of instruction and/or promotion, and typically involve written instructions on or associated with packaging of the disclosure. Instructions also can include any oral or electronic instructions provided in any manner such that a user will clearly recognize that the instructions are to be associated with the kit, for example, audiovisual (*e.g.*, videotape, DVD, etc.), Internet, and/or web-based communications, etc. The written instructions may be in a form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which instructions can also reflects approval by the agency of manufacture, use or sale for human administration.

The kit may contain any one or more of the components described herein in one or more containers. As an example, in one embodiment, the kit may include instructions for mixing one or more components of the kit and/or isolating and mixing a sample and applying to a subject. The kit may include a container housing agents described herein. The agents may be in the form of a liquid, gel or solid (powder). The agents may be prepared sterilely, packaged in syringe and shipped refrigerated. Alternatively it may be housed in a vial or other container for storage. A second container may have other agents prepared sterilely. Alternatively the kit may include the active agents premixed and shipped in a syringe, vial, tube, or other container. The kit may have one or more or all of the components required to administer the agents to a patient, such as a syringe, topical application devices, or IV needle tubing and bag.

The kit may have a variety of forms, such as a blister pouch, a shrink wrapped pouch, a vacuum sealable pouch, a sealable thermoformed tray, or a similar pouch or tray form, with the accessories loosely packed within the pouch, one or more tubes, containers, a box or a bag. The kit may be sterilized after the accessories are added, thereby allowing the individual accessories

in the container to be otherwise unwrapped. The kits can be sterilized using any appropriate sterilization techniques, such as radiation sterilization, heat sterilization, or other sterilization methods known in the art. The kit may also include other components, depending on the specific application, for example, containers, cell media, salts, buffers, reagents, syringes, 5 needles, a fabric, such as gauze, for applying or removing a disinfecting agent, disposable gloves, a support for the agents prior to administration etc.

EXAMPLES

10 *Example 1, Identification of a deep intronic mutation in DYSF intron 50i*

Skin fibroblast cultures from sibling dysferlinopathy patients JF196 and JF23 were converted to myogenic iFDM cell lines and induced to differentiate into myotubes. RNA was then isolated and isolated by RT-PCR to determine the sequence of any dysferlin expressed in these cells. The dysferlin mRNA was analyzed using PCR primers that generated overlapping 15 amplicons tiling through the entire mRNA. Two of the overlapping amplicon products from these patients were larger than products from normal iFDM cells, indicating an insertion of extra sequence within the dysferlin transcript in these patients (**FIG. 1**).

As shown in **FIG. 2A**, sequence analysis of these RT-PCR products revealed the insertion of 180 base pairs (bp) of additional sequence between *DYSF* exons 50 and 51. This 20 sequence is present within intron 50i and is aberrantly spliced into the *DYSF* mRNA as a novel pseudoexon (PE) which is referred to as pseudoexon 50.1 (PE50.1). Sequence analysis of the remainder of the cDNA products confirmed the other known pathogenic variant in these patients (c.5698_5699delAG, exon 51) and revealed no additional novel variants in the coding sequence. Inclusion of PE50.1 in the mRNA transcript leads to insertion of 46 additional amino acids 25 followed by a stop codon by the pseudoexon sequence (**FIGs. 2B and 2C**). In this aberrantly spliced *DYSF* transcript that contains PE50.1, translation termination within the DYSF C2G domain, resulting in loss of the C-terminus, which contains the transmembrane domain which is critical for DYSF protein function.

Further sequence analysis of genomic DNA from these patient cells revealed that PE50.1 30 inclusion in the mRNA transcripts is caused by a novel point mutation (c.5668-824C>T) deep within intron 50i (**FIG. 5**). As shown in **FIG. 2B**, this point mutation creates a consensus splice donor site that promotes the splicing of the 180 bp PE50.1 sequence between exons 50 and 51. Additionally, a consensus “YAG” splice acceptor sequence exists immediately upstream of the

PE50.1 sequence, alone with a pyrimidine rich region and potential branch points as predicted by Human Splicing Finder, all of which are critical for splicing (**FIG. 6**).

Genomic DNA and cDNA from differentiated iFDM cells derived from an unrelated dysferlinopathy patient (TDM57) were analyzed. This unrelated patient also had only one known pathogenic dysferlin variant identified (c.2998T>C). Genomic DNA sequence analysis showed that this patient also carries that c.5668-824C>T point mutation in intron 50i, and RT-PCR analysis of iFDM cell mRNA showed the aberrant inclusion of PE50.1 in the mRNA of this patient. Additionally sequence variants present within intron 50i of patients JF196, JF23, and TDM57 are summarized in **Table 1** below. Each of these variants are reported in the dbSNP database and have no known pathogenicity.

Table 1- Genomic DNA sequence variants in *DYSF* intron 50i of dysferlinopathy patients.

SNP name	Ref.	Var.	JF196 (P1)	JF23 (P2)	TDM57	NHDF-2	nt Position	Sequence	Primers	Global MAF	Chromosomal location	SEQ ID NOs
Novel	C	T	C|T	C|T	C|T	C|C	c.5668-824C>T	TTGTTCCAGG[C/T]GAGCTGGTCT	50i 10 F+R (SEQ ID NO: 19, 20)		2:71673373	106/107
rs379182 9	A	G	G|G	G|G	A|G	G|G	c.5667-199A>G	CTGTCTCAGC[A/G]TTGTGTGATG	50i 1 F+R (SEQ ID NO: 1, 2)	A=0.1677/840 A=0.1425/4148	2:71669945	108/109
rs379183 0	G	A	A|A	A|A	G|A	A|A	c.5667-1075G>A	TATGCTGGTT[A/G]TTGTGTGATG	50i 3 F+R (SEQ ID NO: 5, 6)	G=0.2298/1151 G=0.2242/6527	2:71670821	110/111
rs379183 2	C	A	A|A	A|A	C|A	A|A	c.5668-2222C>A	GCAGGGGTGG[A/C]GAGGAGGAAG	50i 6 F+R (SEQ ID NO: 11, 12)	C=0.2151/1077 C=0.2033/5918	2:71671975	112/113
rs728297 66	C	T	C|C	C|C	C|C	C|T	c.5668-913C>T	CTGAGGGGTG[C/T]TCC	50i 9 F+R (SEQ ID NO: 17,	T=0.0505/253	2:71673284	114/115

								CCGGCCT	18)	T=0.0513/1494		
rs7604764	G	A	G|A	G|A	G|A	A|A	c.5668-642G>A	CCTTACAGCA[A/G]CGTGCTGGGA	50i 10 F+R (SEQ ID NO: 19, 20)	G=0.43.61/2184 G=0.4676/13616	2:71673555	116/117
rs13024390	T	G	T|G	T|G	T|G	G|G	c.5668-522T>G	AGAGCAGGAC[G/T]CTGGAACCCA	50i 10 F+R (SEQ ID NO: 19, 20)	T=0.4357/2182 T=0.4674/13610	2:71673675	118/119
rs882973	C	T	C|T	C|T	C|T	T|T	c.5668-366C>T	AGAGGGCCAA[C/T]GCATAGGAAG	50i 11 F+R (SEQ ID NO: 21, 22)	C=0.2718/1361 C=0.2665/7760	2:71673831	120/121
rs2559081	C	T	C|T	C|T	C|T	T|T	c.5668-41C>T	TCTCTCTAAC[C/T]TTGCTTCCTT	50i 12 F+R (SEQ ID NO: 23, 24)	C=0.2720/1362 C=0.2660/7743	2:71674156	122/123

Genomic variations within *DYSF* intron 50i in patients are listed. The novel mutation c.5668-824C>T results in PE50.1 inclusion in mature mRNAs. Other variations listed are reported in the dbSNP database and have not been shown to be pathogenic. Global MAF values: top, 1000 Genomes; bottom, TOPMED. The primer sets used to amplify and sequence these regions are shown. The *DYSF* reference sequence used: GRCh38.p7; mRNA isoform8, NM_003494.3.

Example 2, Additional dysferlinopathy patients with the intron 50i c.5668-824C>T mutation.

The genomic DNA was examined from additional dysferlinopathy patients that had only one or neither of their pathogenic variants identified to determine if they carried the 50i c.5668-824C>T intronic variant, including two additional siblings of JF196 and JF23. As summarized in **Table 2** below, a total of 22 patients from seventeen families were found to carry this mutation, wherein nine of these patients are homozygous.

Table 2- Summary of patients with the new c.5668-824C>T mutation in intron 50i

<table>
<tr><th>Patient ID</th><th>Patient Origin</th><th>Intron 50i Mutation</th><th>Second DYSF Mutation</th><th>Laboratory</th><th></th></tr>
<tr><td>JF23</td><td>UK</td><td>c.5668-824C>T</td><td>c.5698_5699delAG</td><td>1,5</td><td rowspan="4">Siblings</td></tr>
<tr><td>JF196</td><td>UK</td><td>c.5668-824C>T</td><td>c.5698_5699delAG</td><td>1,5</td></tr>
<tr><td>IGM7</td><td>UK</td><td>c.5668-824C>T</td><td>c.5698_5699delAG</td><td>5</td></tr>
<tr><td>IGM8</td><td>UK</td><td>c.5668-824C>T</td><td>c.5698_5699delAG</td><td>5</td></tr>
<tr><td>JF404</td><td>USA</td><td>c.5668-824C>T</td><td>c.2997G>T</td><td>2,4</td><td rowspan="2">Siblings</td></tr>
<tr><td>JF1729</td><td>USA</td><td>c.5668-824C>T</td><td>c.2997G>T</td><td>2</td></tr>
<tr><td>JF4228</td><td>USA</td><td>c.5668-824C>T</td><td>c.5668-824C>T</td><td>2</td><td></td></tr>
<tr><td>TDM48</td><td>India</td><td>c.5668-824C>T</td><td>c.5668-824C>T</td><td>3</td><td></td></tr>
<tr><td>TDM57</td><td>India</td><td>c.5668-824C>T</td><td>c.2998T>C</td><td>1,3</td><td rowspan="2">Siblings</td></tr>
<tr><td>TDM58</td><td>India</td><td>c.5668-824C>T</td><td>c.2998T>C</td><td>3</td></tr>
<tr><td>TDM63</td><td>India</td><td>c.5668-824C>T</td><td>c.5668-824C>T</td><td>3</td><td></td></tr>
<tr><td>TDM93</td><td>India</td><td>c.5668-824C>T</td><td>c.5668-824C>T</td><td>3</td><td></td></tr>
<tr><td>TDM180</td><td>India</td><td>c.5668-824C>T</td><td>c.5668-824C>T</td><td>3</td><td></td></tr>
<tr><td>TDM182</td><td>India</td><td>c.5668-824C>T</td><td>c.5668-824C>T</td><td>3</td><td></td></tr>
<tr><td>TDM196</td><td>India</td><td>c.5668-824C>T</td><td>c.5668-824C>T</td><td>2</td><td></td></tr>
<tr><td>TDM230</td><td>India</td><td>c.5668-824C>T</td><td>c.1911C>G</td><td>2</td><td></td></tr>
<tr><td>F1-170-1-2</td><td>France</td><td>c.5668-824C>T</td><td>c.855+1delG</td><td>4</td><td rowspan="2">Siblings</td></tr>
<tr><td>F1-170-2-2</td><td>France</td><td>c.5668-824C>T</td><td>c.855+1delG</td><td>4</td></tr>
</table>

F1-436-1-0	India	c.5668-824C>T	c.5668-824C>T	4	
IGM1	UK	c.5668-824C>T	c.1911C>G	5	
IGM2	UK	c.5668-824C>T	c.5668-824C>T	5	
IGM3	UK	c.5668-824C>T	c.2434dup	5	
IGM4	UK	c.5668-824C>T	c.937+1G>A	5	

The initial patients found to carry the mutation in bold type. Laboratory that identified the patient's mutation: 1-University of Massachusetts Medical School Worcester; 2-Emory University School of Medicine Atlanta; 3-Emory University School of Medicine Atlanta, described in Dastur et al. 2017; 4-Marseille Medical Genetics, Aix Marseille University Marseille; 5-Institue of Genetic Medicine, Newcastle.

Example 3, Antisense oligonucleotides targeting PE50.1 restore production of normal DYSF mRNA.

Given that aberrant splicing caused by the c.5668-824C>T intron 50i mutation leads to inclusion of the disruptive PE50.1 sequence, blocking this aberrant splicing should restore normal *DYSF* RNA and protein. Three antisense oligonucleotides (AONs) were designed targeting different possible exonic splice enhancer (ESE) regions within PE50.1 that could promote PE50.1 splicing into the mature mRNA. Therefore, targeting these ESEs with AONs would prevent PE50.1 inclusion. RT-PCR analysis of patient's iFDM myotubes treated for two days with AONs reveals that AONs indeed inhibit expression of the mutant PE50.1 *DYSF* mRNA spliced form and restore higher levels of the normal mRNA splice form that lacks PE50.1 (**FIG. 3**).

The presence and absence of PE50.1 in the mRNA transcripts was confirmed by sequencing the larger and smaller RT-PCR products, respectively. AON treatment for three days (followed by four days without AONs) also restored DYSF protein expression to levels similar to that in normal myotubes (**FIG. 4**), likely as a result of the higher amounts of normally-spliced transcripts induced by PE50.1-skipping. The premature translational stop codon within PE50.1 likely leads to nonsense-mediated mRNA decay, whereby mRNA carrying a premature stop codon is transcribed and spliced but degraded during the translation process, leading to reduced mRNA levels (typically detectable using sensitive RT-PCR methods) along with

aborted protein synthesis. The overall reduced levels of RT-PCR products observed in untreated or control-treated patient cells compared with normal cells (**FIG. 3**) is consistent with this. It is unlikely that the mutation leads to significant synthesis of a truncated protein be expression of a shorter DYSF protein is not detected on Western blots using an antibody specific to the DYSF
5 N-terminal region (data not shown).

Methods for Examples 1 – 3.

Patients. Samples used in this study (blood or skin biopsy samples) were from patients
10 clinically diagnosed as either distal dominant Miyoshi myopathy (MM) or proximal dominant limb-girdle muscular dystrophy (LGMD) 2B dysferlinopathies. These patients exhibited progressive limb muscle weakness beginning in adolescence or early adulthood, elevated blood creatine kinase levels indicating muscle damage, and demonstrated reduced or absent dysferlin protein expression in muscle biopsies and/or blood monocyte cells when screened. Patient
15 materials were collected in accordance with ethical guidelines and protocols approved by the University of Massachusetts Medical School Institutional Review Board, and similar review boards at Emory University School of Medicine, Aix-Marseille University, or Institute of Genetic Medicine, Newcastle. For all of the patients described, only one or neither of the pathogenic variants had been defined by exon sequencing. The dysferlinopathy patients initially
20 screened, JF196 and JF23 (designated P1 and P2, respectively), are siblings participating in an ongoing international "Clinical Outcome Study for Dysferlinopathy", a collaboration with the Jain Foundation and Newcastle upon Tyne Hospitals. Patient skin fibroblasts were obtained from the Newcastle-upon-Tyne Hospital BioBank. These patients are heterozygous for one pathogenic dysferlin variant identified by exon sequencing (c.5698_5699delAG), but the other
25 pathogenic allele had not been identified. Similarly, dysferlinopathy patient TDM57 had only one pathogenic allele defined by exon sequencing (c.2998T>C). TDM57 skin biopsy tissue was obtained via the Jain Foundation as part of a diagnostic program at the Centre for Advanced Molecular Diagnostics in Neuromuscular Disorders, Mumbai, Maharashtra, India. TDM57 and other TDM patients reported here (except TDM196 and TDM230) were described previously.
30 All TDM patient genomic DNA was sequenced at Emory University School of Medicine as described. Additional patient genomic DNA sequencing was performed at Aix-Marseille University or Newcastle-upon-Tyne Hospitals. Fibroblasts from unrelated individuals served as DNA sequence analysis controls (8597: dysferlinopathy patient with different dysferlin mutations; NHDF-3: normal; RB19895: amyotrophic lateral sclerosis patient).

Cell culture. Skin biopsy samples were placed in culture dishes to establish fibroblast cultures. We obtained normal adult human dermal fibroblasts (termed NHDF-2 and NHDF-3 cells) from the American Type Culture Collection (ATCC, Manassas, VA, USA). Fibroblasts were
5 transduced with lentivirus vectors carrying tamoxifen-inducible MyoD to generate inducible fibroblast-derived myogenic cells (iFDMs). These cells proliferate as fibroblasts and can be induced to differentiate into myotubes by treatment with 4-hydroxytamoxifen (TMX). TMX induces MyoD expression and myotube formation when cells are cultured in low serum differentiation medium (DM) ((DMEM Glutamax with pyruvate: Medium 199 (Gibco, Grand
10 Island, NY, USA) (3:1), 2% horse serum (HyClone, Logan UT, USA), 20 mM HEPES, and 20 μg/mL insulin, 11 μg/mL transferrin, 1.3 μg/mL selenium (2X ITS, Gibco)).

Nucleic acid purification. Genomic DNA was purified from cells using Gentra Puregene (Qiagen, Valenica, CA, USA) reagents following manufacturers' protocols. RNA was purified
15 from patient and normal iFDM cells using TRIzol reagent (Life Technologies, Grand Island, NY, USA), digested with DNAse (TURBO DNA-Free, Ambion, Austin, TX, USA) to remove contaminating DNA, then RNA was reverse transcribed (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems Foster City, CA, USA) using manufacturers' protocols to generate cDNA for PCR analyses. For all analyses, *DYSF* mRNA variant 8 (NM_003494.3), the
20 predominant skeletal muscle isoform was used as the reference sequence.

PCR amplification. PCR was used to amplify and sequence the complete dysferlin cDNA derived from myogenic cell (iFDM cell) RNA. For this, 17 primer sets generating 450-500 bp amplicons that tiled across the *DYSF* cDNA were used as described to sequence and screen the
25 cDNA for mutations. Additionally, 12 primer sets (**Table 4**) that tiled across *DYSF* intron 50i were used to sequence and screen the patient genomic DNA for variants. Additional primers (**Table 4**) that can be used to sequence patient DNA for the mutated sequence are also listed. M13 sequence tails were added to primers to facilitate sequencing (**Table 4**).

30 **Antisense oligonucleotide treatment of cell cultures.** Antisense oligonucleotides (AONs) were designed that target potential exonic splicing enhancer (ESE) sequences within PE50.1 using Human Splicing Finder v.3.0 and Rescue-ESE online tools. Blocking these ESEs within AONs could inhibit the splicing of PE50.1 into mature processed mRNAs, thereby leading to the synthesis of normally-spliced *DYSF* transcripts. Three AONs targeting PE50.1 (**Table 5**) were

used along with a non-specific scrambled AON control (SCR). These AONs were synthesized as 2'-O-methyl RNA with full-length phosphorothioate backbones (Integrated DNA Technologies, Coralville, IA, USA). iFDM cells derived from patients JF196 (P1), JF23 (P2), and normal NHDF-2 fibroblasts were allowed to differentiate for the indicated lengths of time to
5 form myotubes. Cells were transfected with AON (600 nM) (or TE buffer as control) using Oligofectamine (Life Technologies) and the manufacturers' protocol. After the indicated times, RNA was extracted and evaluated by RT-PCR using primers (SEQ ID NOs: 30 & 31) to amplify either the normal *DYSF* sequence (88 bp) or the mutant version containing pseudoexon50.1 (PE50.1) (268 bp). Proteins were extracted from similar cultures and evaluated on Western
10 blots using antibodies against the dysferlin C-terminal region (NCL-Hamlet, Leica, Buffalo Grove, IL, USA, 1/1000) and GADPH (G9545, Sigma, St. Louis, MO, USA, 1/1000) as described. Protein expression levels were calculated from Western blot images using a Li-COR Odyssey infrared imager and Image Studio Software (Li-COR).

15 **Table 3.** Primers (forward (F) and reverse (R)) used to generate overlapping amplicons that span entire dysferlin cDNA.

Primer Name	Sequence	SEQ ID NO:
1F	AGGTGCAAAATGCCGTGT	1
1R	TTCACCCCTGCAAACACC	2
2F	CACACCCGACACCGACAT	3
2R	CTCCGCCTCATCTCCAGTG	4
3F	CGACTCTGCCTGACCTGGA	5
3R	AATGGTGCCCACGTCCAT	6
4F	TCGTTCTCTCAGGACAGATGC	7
4R	CTGAGGGTTGGCCGTCTT	8
5F	GACCCCTTTGTGGAGGTCA	9
5R	GCTCCACCAGCTTGGTCTC	10
6F	GGGGGAAGGTGTGGCTTAT	11
6R	CAGCGAGTCCACGTCCTC	12
7F	CCAGCTGCTTGGGATTGC	13
7R	TCCCACAATTCTTGCCACA	14
8F	GCCCACCAAGTCCTCTTCTC	15
8R	AAGCCGGGTCTGGTTCTC	16
9F	TCACCTGAGCTTCGTGGAA	17

9R	TTCTCCAGTGGCTCCATG C	18
10F	CCACCTCGAGTACCGCAAG	19
10R	CGTACAGCTCCACCACAATG	20
11F	AACACCCTTAACCCCACCTG	21
11R	CGGAGGTTCCTGATGACACA	22
12F	CCCCAGCCTCGTGGTAGA	23
12R	ACCTTCAGGGTGTCAAAATCC	24
13F	TGCCTCCATAGGGGAGAGG	25
13R	TGCAGGTCAGCTCGAACA	26
14F	TGGAGCCCGTATTTGGAA	27
14R	TGCAGGGGGCTGTAGAGG	28
15F	CGTCTGGCTCTGCATGTG	29
15R	CCACTCGTGCTGGGATTTT	30
16F	CTGCCAGCTGAGCAAGTCTG	31
16R	GCCGCCACAGGATGAACT	32
17F	CCGACACCTCCTTCCTGTG	33
17R	TTGTGGTTCCAACTGTTTTATACTGA	34

Table 4. Primers (forward (F) and reverse (R)) used to generate overlapping amplicons that span dysferlin intron 50i, and an additional primer set (PE50.1-F, PE50.1-R) for genotyping.

Primer Name	Sequence	SEQ ID NO:
50i.1F	TGGGAGGTGAAGGCAACTT	35
50i.1R	TGGAAAAGGGGTGGATGC	36
50i.2F	TCATGGCACTACCGTGGTC	37
50i.2R	GGAGGGTGATGGCTGTGG	38
50i.3F	GCCTATGGTCACCGTCCA	39
50i.3R	AATCGGGCCAGCAGAATC	40
50i.4F	GTCCCACCCGGCATTAAA	41
50i.4R	TTGGGGCAGATGCAACCT	42
50i.5F	GAAAGCATGGGCCGTTTG	43
50i.5R	AATGGAGCCACCCCAAAT	44
50i.6F	AGAGAGGGTTACCCGGCAGT	45
50i.6R	GTTTCTGTCTCCGCCTTCG	46
50i.7F	GGGAGAAGGTGGCTGGAA	47

50i.7R	CTGACGTGCAGGGTGTGC	48
50i.8F	GCGGCTTTGAACCACCAC	49
50i.8R	AGCTCCCGAACCGAAGAC	50
50i.9F	CCAACCCAGGCAGCAGTC	51
50i.9R	CAGCTCGCCTGGAACAAG	52
50i.10F	CAGTCCCACACCGCTCAG	53
50i.10R	TATGCGTTGGCCCTCTACTG	54
50i.11F	CAGCTGCCAGGGTTTGAG	55
50i.11R	GGATGCAAGGAAGCAAGGT	56
50i.12F	CCCTTGGGGACATCCTACTC	57
50i.12R	ACTTGCGTCCCTCGCTTAC	58
PE50.1-F	CCCGGCACTCAGGACTTG	59
PE50.1-R	TGCTGGGAAGTTCCGTCTC	60
M13-F	GTAAAACGACGGCCAGT	61
M13-R	CAGGAAACAGCTATGAC	62

Table 5. AONs targeting human exonic splicing enhancer sequences (ESE) in DYSF PE50.1. AONs for *in vitro* studies are 2'-O-methyl RNA with full-length phosphorothioate backbones.

Primer Name	AON Sequence	SEQ ID NO:	Target Sequence	SEQ ID NO:
AON1	AGUCUUGUUCUCUGUUUUCUCA	63	TGAGAAAACAGAGAACAAGACT	64
AON2	ACUGAGGCUUCAUGGAGCAAC	65	GTTGCTCCATGAAGCCTCAGT	66
AON3	GGUCAUCUUGGGCUUCCUCCCAC	67	GTGGGAGGAAGCCCAAGATGACC	68

5 **Table 6.** AONs to effect pseudoexon PE50.1 expression and ability to enhance expression of normal dysferlin mRNA and protein.

Primer Name	AON Sequence	SEQ ID NO:	Target Sequence	SEQ ID NO:
AON4	CGGAGGCCGGGGAGCACCCCUCAG	69	CTGAGGGGTGCTCCCCGGCCTCCG	70
AON5	GAGCAAGGGCCUGGACCCAACUCU	71	AGAGTTGGGTCCAGGCCCTTGCTC	72
AON6	CCAAGUCCUGAGUGCCGGGUCAGA	73	TCTGACCCGGCACTCAGGACTTGG	74

AON7	GACCACCCUGCAGAUGGUGGGUGC	75	GCACCCACCATCTGCAGGGTGGTC	76
AON8	UGGCAAGGACAGGAAACAGUGUGA	77	TCACACTGTTTCCTGTCCTTGCCA	78
AON9	ACUAUAACUCUCCCCUGGGUGAGU	79	ACTCACCCAGGGGAGAGTTATAGT	80
AON10	GUCACUAGGGCUUUCCAGCGCAGU	81	ACTGCGCTGGAAAGCCCTAGTGA	82
AON11	CCACACUCAACCCACAGCACUGUC	83	GACAGTGCTGTGGGTTGAGTGTGG	84
AON12	ACCCCUCAGUCCAGCCACACUCAA	85	TTGAGTGTGGCTGGACTGAGGGGT	86
AON13	UGAGCGGUGUGGGACUGCACAUGC	87	GCATGTGCAGTCCCACACCGCTCA	88
AON14	GGGCUUCCUCCCACUGAGGCUUCA	89	TGAAGCCTCAGTGGGAGGAAGCCC	90
AON15	AGGUCAUCUUGGGCUUCCUCCCAC	91	GTGGGAGGAAGCCCAAGATGACCT	92
AON16	UCAGACCAGCUCGCCUGGAACAAG	93	CTTGTTCCAGGCGAGCTGGTCTGA	94
AON17	UUCUCUCCCCACCCCACCUCCCUC	95	GAGGGAGGTGGGGTGGGGAGAGAA	96
AON18	GCUGAAAGGAGAGGAGGGGCCCUU	97	AAGGGCCCCTCCTCTCCTTTCAGC	98
AON19	AGUGCUGGGAAGUUCCGUCUCCUG	99	CAGGAGACGGAACTTCCCAGCACT	100
AON20	GGGCAAAGCCAAAGCCUGAAGUGC	101	GCACTTCAGGCTTTGGCTTTGCCC	102

ADDITIONAL SEQUENCES

>Pseudoexon 50i.1: SEQ ID NO: 103

GGGAGAGTTATAGTGAGAAAACAGAGAACAAGACTGCGCTGGAAAGCCCTAGTGA
CAGTGCTGTGGGTTGAGTGTGGCTGGACTGAGGGGTGCTCCCCGGCCTCCGCATGT
GCAGTCCCACACCGCTCAGTTGCTCCATGAAGCCTCAGTGGGAGGAAGCCCAAGAT
GACCTTGTTCCAG

>Intron 50 in the context of a c.5668-824 (C>T): SEQ ID NO: 104

CACCATCTGCAGGGTGGTCACACTGTTTCCTGTCCTTGCCACTCACCCAGGGGAGAG
TTATAGTGAGAAAACAGAGAACAAGACTGCGCTGGAAAGCCCTAGTGACAGTGCTG
TGGGTTGAGTGTGGCTGGACTGAGGGGTGCTCCCCGGCCTCCGCATGTGCAGTCCC
ACACCGCTCAGTTGCTCCATGAAGCCTCAGTGGGAGGAAGCCCAAGATGACCTTGT
TCCAGG(**c/t**)AGCTGGTCTGAGGGAGGTGGGGTGGGGAGAGAAGGGCCCCTCCTCTC

>Intron 50: SEQ ID NO: 105

CACCATCTGCAGGGTGGTCACACTGTTTCCTGTCCTTGCCACTCACCCAGGGGAGAG
TTATAGTGAGAAAACAGAGAACAAGACTGCGCTGGAAAGCCCTAGTGACAGTGCTG
TGGGTTGAGTGTGGCTGGACTGAGGGGTGCTCCCCGGCCTCCGCATGTGCAGTCCC
ACACCGCTCAGTTGCTCCATGAAGCCTCAGTGGGAGGAAGCCCAAGATGACCTTGT
5 TCCAGGAGCTGGTCTGAGGGAGGTGGGGTGGGGAGAGAAGGGCCCCTCCTCTC

While several embodiments of the present invention have been described and illustrated herein, those of ordinary skill in the art will readily envision a variety of other means and/or structures for performing the functions and/or obtaining the results and/or one or more of the advantages described herein, and each of such variations and/or modifications is deemed to be
10 within the scope of the present invention. More generally, those skilled in the art will readily appreciate that all parameters, dimensions, materials, and configurations described herein are meant to be exemplary and that the actual parameters, dimensions, materials, and/or configurations will depend upon the specific application or applications for which the teachings of the present invention is/are used. Those skilled in the art will recognize, or be able to
15 ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. It is, therefore, to be understood that the foregoing embodiments are presented by way of example only and that, within the scope of the appended claims and equivalents thereto, the invention may be practiced otherwise than as specifically described and claimed. The present invention is directed to each individual feature,
20 system, article, material, and/or method described herein. In addition, any combination of two or more such features, systems, articles, materials, and/or methods, if such features, systems, articles, materials, and/or methods are not mutually inconsistent, is included within the scope of the present invention.

The indefinite articles "a" and "an," as used herein in the specification and in the claims,
25 unless clearly indicated to the contrary, should be understood to mean "at least one."

The phrase "and/or," as used herein in the specification and in the claims, should be understood to mean "either or both" of the elements so conjoined, i.e., elements that are conjunctively present in some cases and disjunctively present in other cases. Other elements may optionally be present other than the elements specifically identified by the "and/or" clause,
30 whether related or unrelated to those elements specifically identified unless clearly indicated to the contrary. Thus, as a non-limiting example, a reference to "A and/or B," when used in conjunction with open-ended language such as "comprising" can refer, in one embodiment, to A

without B (optionally including elements other than B); in another embodiment, to B without A (optionally including elements other than A); in yet another embodiment, to both A and B (optionally including other elements); etc.

As used herein in the specification and in the claims, “or” should be understood to have
5 the same meaning as “and/or” as defined above. For example, when separating items in a list, “or” or “and/or” shall be interpreted as being inclusive, i.e., the inclusion of at least one, but also including more than one, of a number or list of elements, and, optionally, additional unlisted items. Only terms clearly indicated to the contrary, such as “only one of” or “exactly one of,” or, when used in the claims, “consisting of,” will refer to the inclusion of exactly one element of
10 a number or list of elements. In general, the term “or” as used herein shall only be interpreted as indicating exclusive alternatives (i.e. “one or the other but not both”) when preceded by terms of exclusivity, such as “either,” “one of,” “only one of,” or “exactly one of.” “Consisting essentially of,” when used in the claims, shall have its ordinary meaning as used in the field of patent law.

15 As used herein in the specification and in the claims, the phrase “at least one,” in reference to a list of one or more elements, should be understood to mean at least one element selected from any one or more of the elements in the list of elements, but not necessarily including at least one of each and every element specifically listed within the list of elements and not excluding any combinations of elements in the list of elements. This definition also
20 allows that elements may optionally be present other than the elements specifically identified within the list of elements to which the phrase “at least one” refers, whether related or unrelated to those elements specifically identified. Thus, as a non-limiting example, “at least one of A and B” (or, equivalently, “at least one of A or B,” or, equivalently “at least one of A and/or B”) can refer, in one embodiment, to at least one, optionally including more than one, A, with no B
25 present (and optionally including elements other than B); in another embodiment, to at least one, optionally including more than one, B, with no A present (and optionally including elements other than A); in yet another embodiment, to at least one, optionally including more than one, A, and at least one, optionally including more than one, B (and optionally including other elements); etc.

30 In the claims, as well as in the specification above, all transitional phrases such as “comprising,” “including,” “carrying,” “having,” “containing,” “involving,” “holding,” and the like are to be understood to be open-ended, i.e., to mean including but not limited to. Only the

transitional phrases "consisting of" and "consisting essentially of" shall be closed or semi-closed transitional phrases, respectively, as set forth in the United States Patent Office Manual of Patent Examining Procedures, Section 2111.03.

Use of ordinal terms such as "first," "second," "third," etc., in the claims to modify a
5 claim element does not by itself connote any priority, precedence, or order of one claim element over another or the temporal order in which acts of a method are performed, but are used merely as labels to distinguish one claim element having a certain name from another element having a same name (but for use of the ordinal term) to distinguish the claim elements.

CLAIMS

What is claimed is:

1. A method of modulating splicing in a cell that contains a *DYSF* gene comprising a c.5668-824 (C>T) mutation, the method comprising:

delivering to the cell an antisense nucleic acid that targets a pre-messenger RNA expressed from the *DYSF* gene and alters splicing of the pre-messenger RNA such that exons 50 and 51 of the pre-messenger RNA are spliced together without an intervening pseudoexon.

2. The method of claim 1, wherein the cell is heterozygous for the c.5668-824 (C>T) point mutation.

3. The method of claim 2, wherein the cell contains a second *DYSF* gene encoding a wild-type DYSF protein.

4. The method of claim 2, wherein the cell contains a second *DYSF* gene comprising a mutation that causes a premature stop codon.

5. The method of claim 4, wherein the premature stop codon is within a region encoding the C2G domain of DYSF protein.

6. The method of claim 5, wherein the second *DYSF* gene is a human *DYSF* gene, and wherein the mutation that causes the premature stop codon is a c.3444_3445delTGinsAA mutation.

7. The method of any one of claims 1 to 6, wherein the cell is *in vitro*.

8. The method of any one of claims 1 to 6, wherein the cell is *in vivo*.

9. The method of any one of claims 1 to 8, wherein the cell is a non-human cell engineered to contain the *DYSF* gene comprising the c.5668-824 (C>T) mutation.

10. The method of any one of claims 1 to 8, wherein the cell is a human cell.

11. The method of claim 10, wherein the human cell is engineered to contain the *DYSF* gene comprising the c.5668-824 (C>T) mutation.

12. The method of claim 10, wherein the human cell is from a subject having a muscular dystrophy that is associated at least in part with the *DYSF* gene comprising the c.5668-824 (C>T) mutation.

13. The method of claim 12, wherein the muscular dystrophy is of the Miyoshi Myopathy-type, or other muscular dystrophy caused by abnormal expression of the dysferlin gene product.

14. A method of modulating splicing in a cell containing a *DYSF* gene that encodes a pre-messenger RNA having a pseudoexon incorporated between exons 50 and 51, the method comprising:

delivering to the cell an antisense nucleic acid that targets the pre-messenger RNA encoded by the human *DYSF* gene and alters splicing of the pre-messenger RNA such that the pseudoexon is not incorporated between exons 50 and 51 of the pre-messenger RNA.

15. The method of claim 14, wherein the *DYSF* gene is a human *DYSF* gene that comprises a c.5668-824 (C>T) mutation.

16. The method of any one of claims 1 to 15, wherein the antisense nucleic acid is an oligonucleotide of 10 to 25 nucleotides in length comprising a region of complementarity that is complementary with at least 8 contiguous nucleotides of a sequence within a region between exons 50 and 51 encoded by the *DYSF* gene.

17. The method of any one of claims 1 to 15, wherein the oligonucleotide is an oligonucleotide of any one of claims 33 to 55.

18. The method of any one of claims 1 to 16, wherein the oligonucleotide comprises a sequence of AON1 to AON20 as set forth in Tables 5 and 6.

19. The method of any one of claims 1 to 15, wherein the antisense nucleic acid is expressed from a transgene.

20. The method of claim 19, wherein the transgene is delivered to the cell using a viral vector.

21. The method of claim 20, wherein the viral vector is a recombinant AAV vector.

22. A method of altering RNA splicing in a subject, the method comprising:

administering to the subject an antisense nucleic acid that targets a pre-messenger RNA encoded by a human *DYSF* gene and alters splicing of the pre-messenger RNA such that exons 50 and 51 of the RNA are spliced together without an intervening pseudoexon, wherein the human *DYSF* gene comprises a c.5668-824 (C>T) mutation.

23. The method of claim 22 further comprising, prior to administering the antisense nucleic acid, detecting that the subject has the human *DYSF* gene that comprises the c.5668-824 (C>T) mutation.

24. The method of claim 23, wherein the human *DYSF* gene is detected using a hybridization assay that discriminates between the presence of a guanosine and a thymidine at position c.5668-824 (C>T) of the human *DYSF* gene.

25. The method of claim 24, wherein the hybridization assay is a polymerase chain reaction (PCR) assay.

26. The method of claim 25, wherein the PCR assay comprises using a primer or probe that is at least partially complementary with a nucleic acid having a sequence as set forth in SEQ ID NO: 103 or a complementary sequence thereof.

27. The method of any one of claims 22 to 26, wherein the antisense nucleic acid is an oligonucleotide of 10 to 25 nucleotides in length comprising a region of complementarity that is complementary with at least 8 contiguous nucleotides of a sequence within a region between exons 50 and 51 encoded by a human *DYSF* gene.

28. The method of any one of claims 22 to 27, wherein the antisense nucleic acid is an oligonucleotide of any one of claims 33 to 55.

29. The method of any one of claims 22 to 27, wherein the oligonucleotide comprises a
5 sequence of AON1 to AON20 as set forth in Tables 5 and 6.

30. The method of any one of claims 22 to 26, wherein the antisense nucleic acid is expressed from a transgene.

10 31. The method of claim 29, wherein the transgene is administered to the subject using a viral vector.

32. The method of claim 30, wherein the viral vector is a recombinant AAV vector.

15 33. An oligonucleotide of 10 to 25 nucleotides in length comprising a region of complementarity that is complementary with at least 8 contiguous nucleotides of a sequence within a region between exons 50 and 51 encoded by a human *DYSF* gene, wherein the oligonucleotide comprises at least one modified nucleotide or at least one modified internucleotide linkage.

20

34. The oligonucleotide of claim 33, wherein the human *DYSF* gene comprises a c.5668-824 (C>T) mutation.

35. The oligonucleotide of claim 33 or 34, wherein the region of complementarity is
25 complementary with at least 8 contiguous nucleotides of a sequence as set forth as SEQ ID NO: 103.

36. The oligonucleotide of claim 33 or 34, wherein the region of complementarity is complementary with at least 8 contiguous nucleotides of a sequence as set forth as SEQ ID NO:
30 104 or 105.

37. The oligonucleotide of claim 33, 34 or 35, wherein the region of complementarity is complementary with an exonic splice enhancer sequence.

38. The oligonucleotide of claim 33, 34 or 35, wherein the region of complementarity is complementary with a splice donor motif.

39. The oligonucleotide of claim 33, 34 or 35, wherein the region of complementarity is
5 complementary with a splice acceptor motif.

40. The oligonucleotide of claim 33, 34 or 36, wherein the region of complementarity is complementary with a lariat branch point.

10 41. The oligonucleotide of claim 33, wherein the human *DYSF* gene comprises a mutation that results in an in-frame pseudoexon between exons 50 and 51.

42. The oligonucleotide of any one of claims 33 to 41, wherein the oligonucleotide, when present in a cell that contains the human *DYSF* gene, alters splicing of a pre-messenger RNA
15 expressed from the human *DYSF* gene such that the pseudoexon is not incorporated between exons 50 and 51.

43. The oligonucleotide of any one of claims 33 to 42, wherein oligonucleotide comprises at least one modified nucleotide.

20

44. The oligonucleotide of claim 43, wherein the at least one modified nucleotide is a 2'-modified nucleotide.

45. The oligonucleotide of claim 44, wherein the 2'-modified nucleotide is a 2'-deoxy, 2'-
25 fluoro, 2'-O-methyl, 2'-O-methoxyethyl, 2'-amino and 2'-aminoalkoxy modified nucleotides.

46. The oligonucleotide of claim 44, wherein the 2'-modified nucleotide comprises a 2'-O-4'-C methylene bridge.

30 47. The oligonucleotide of any one of claims 33 to 46, wherein the oligonucleotide comprises at least one modified internucleotide linkage.

48. The oligonucleotide of claim 47, wherein the at least one modified internucleotide linkage is a phosphorothioate modified linkage.

49. The oligonucleotide of any one of claims 33 to 48, wherein the oligonucleotide is a morpholino or a modified morpholino (*e.g.* peptide conjugated morpholino, phosphorodiamidate morpholino, *etc.*).

5

50. The oligonucleotide of any one of claims 33 to 48, wherein the oligonucleotide comprises alternating LNA and RNA nucleotides.

51. The oligonucleotide of any one of claims 33 to 48, wherein the oligonucleotide comprises
10 alternating LNA and DNA nucleotides.

52. The oligonucleotide of any one of claims 33 to 48, wherein the oligonucleotide comprises alternating RNA and DNA nucleotides.

15 53. The oligonucleotide of any one of claims 33 to 52, wherein the oligonucleotide, when present in a cell that contains the human *DYSF* gene, is capable of hybridizing with RNA expressed from the human *DYSF* gene without inducing cleavage of the RNA by an RNase.

54. A composition comprising the oligonucleotide of any one of claims 33 to 53 further
20 comprising a carrier.

55. A pharmaceutical composition comprising the oligonucleotide of any one of claims 33 to 53 further comprising a pharmaceutically-acceptable carrier.

25 56. A preparation of oligonucleotides, wherein at least 95% of the oligonucleotides are 10 to 25 nucleotides in length and comprise a region of complementarity that is complementary with at least 8 contiguous nucleotides of a sequence within a region between exons 50 and 51 encoded by a human *DYSF* gene.

30 57. The preparation of claim 56, wherein the region of complementarity is complementary with an exonic splice enhancer sequence.

58. The preparation of claim 56, wherein the region of complementarity is complementary with a splice donor motif.

59. The preparation of claim 56, wherein the region of complementarity is complementary with a splice acceptor motif.

60. The preparation of claim 56, wherein the region of complementarity is complementary with a lariat branch point.

61. The preparation of claim 56, wherein the human *DYSF* gene comprises a mutation that results in an in-frame pseudoexon between exons 50 and 51.

62. The preparation of claim 61, wherein the oligonucleotide, when present in a cell that contains the human *DYSF* gene, alters splicing of an RNA expressed from the human *DYSF* gene such that the pseudoexon is not incorporated between exons 50 and 51.

63. The preparation of any one of claims 56 to 62, wherein the oligonucleotides are lyophilized.

64. The preparation of any one of claims 56 to 63, wherein at least 95% of the oligonucleotides are identical.

65. A container housing the preparation of any one of claims 56 to 64.

66. A pair of PCR primers having sequences selected from the primer sequences set forth in Table 3.

67. A composition comprising the pair of PCR primers of claim 66 and a template comprising at least a portion of a human *DYSF* gene.

68. An expression construct encoding an antisense nucleic acid having a region of complementarity that is complementary with a sequence between exons 50 and 51 encoded by a human *DYSF* gene.

69. The expression construct of claim 68, wherein the human *DYSF* gene comprises a c.5668-824 (C>T) mutation.

70. The expression construct of claim 68 or 69, wherein the region of complementarity is complementary with at least 8 contiguous nucleotides of a sequence as set forth as SEQ ID NO: 103.

5

71. The expression construct of claim 69 or 70, wherein the region of complementarity is complementary with at least 8 contiguous nucleotides of a sequence as set forth as SEQ ID NO: 104 or 105.

10 72. The expression construct of any one of claims 69 to 70, wherein the region of complementarity comprises a sequence complementary with an exonic splice enhancer sequence.

73. The expression construct of any one of claims 69 to 70, wherein the region of complementarity comprises a sequence complementary with a splice donor motif.

15

74. The expression construct of any one of claims 69 to 70, wherein the region of complementarity comprises a sequence complementary with a splice acceptor motif.

75. The expression construct of any one of claims 68, 69 or 71, wherein the region of
20 complementarity comprises a sequence complementary with a lariat branch point.

76. The expression construct of any one of claims 68 to 75, wherein the antisense nucleic acid expressed from the vector, when present in a cell that contains the human *DYSF* gene, alters splicing of a pre-messenger RNA expressed from the human *DYSF* gene such that the
25 pseudoexon is not incorporated between exons 50 and 51.

77. A recombinant AAV comprising the expression construct of any one of claims 68 to 76.

78. An engineered cell comprising an exogenous human *DYSF* gene having a c.5668-824
30 (C>T) mutation, wherein the cell expresses a pre-messenger RNA from the exogenous *DYSF* gene containing a pseudoexon between exons 50 and 51.

79. The engineered cell of claim 78, wherein the cell is not of a human origin.

80. The engineered cell of claim 78, wherein the cell is of a human origin.

81. A composition comprising cells containing a human *DYSF* gene having a c.5668-824 (C>T) mutation and an artificial cell culture medium.

5

82. A tissue culture system comprising the composition of claim 81.

83. The composition of claim 81 further comprising an oligonucleotide of any one of claims 33 to 55.

10

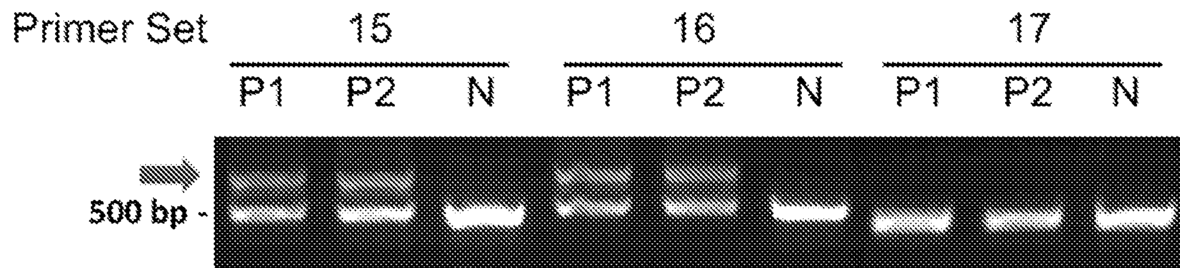
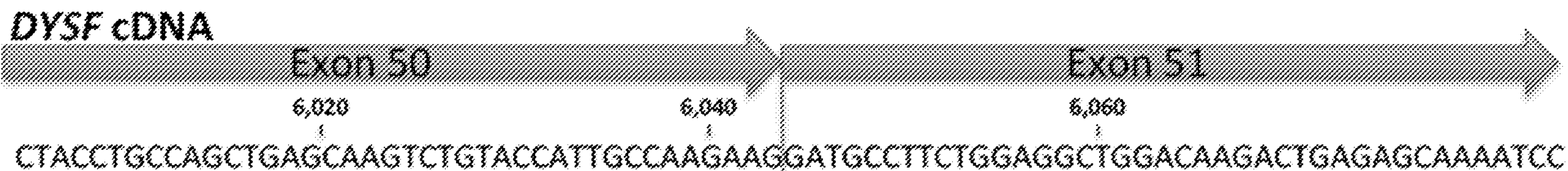


FIG. 1

DYSF **Pseudoexon PE50.1 (c.5768-824 C>T)**



cDNA
180 bp insert

GGGAGAGTTATAGTGAGAAAACAGAGAACAAGACTGCGCTGGAAA
GCCCTAGTGACAGTGCTGTGGGTTGAGTGTGGCTGGACTGAGGGGT
GCTCCCCGGCCTCCGCATGTGCAGTCCCACACCGCTCAGTTGCTCCA
TGAAGCCTCAGTGGGAGGAAGCCCAAGATGACCTTGTTCCAG

Protein
46 aa insert
then stop*

GRVIVRKQRTRLRWKALVTVLWVECGWTEGCSPASACAVPHRSVAP * SLSGRKPKMTLFQ

180 bps (46 aa + stop codon) terminates translation prematurely
= pseudoexon PE50.1

FIG. 2A

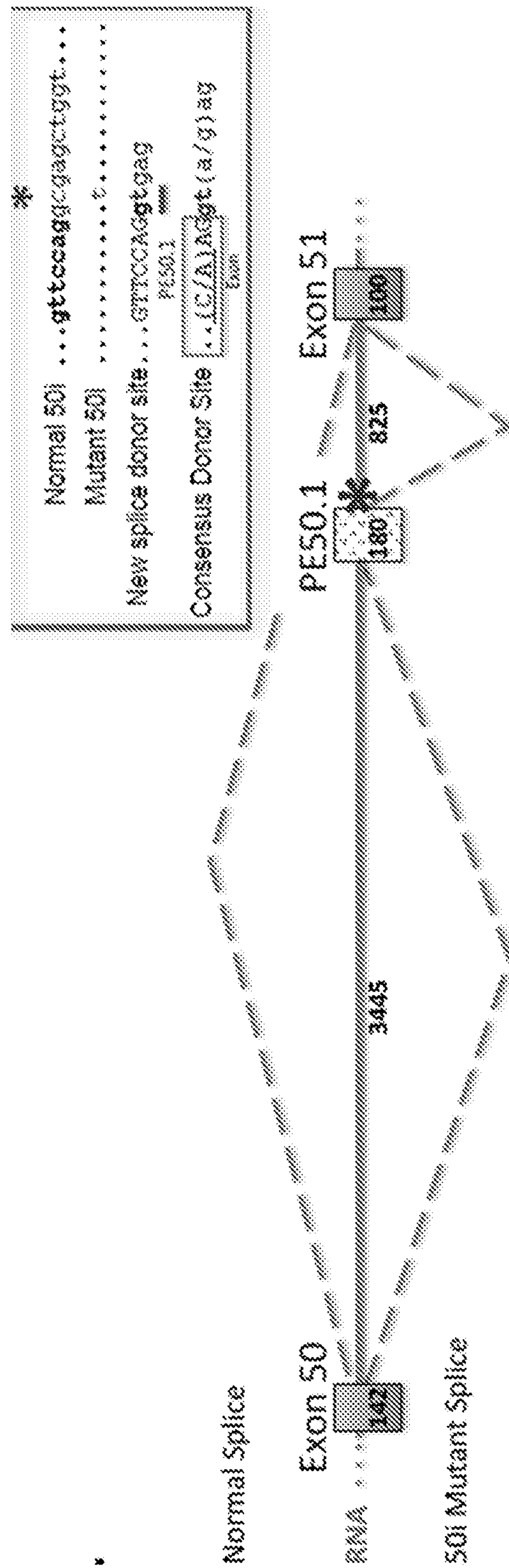


FIG. 2B

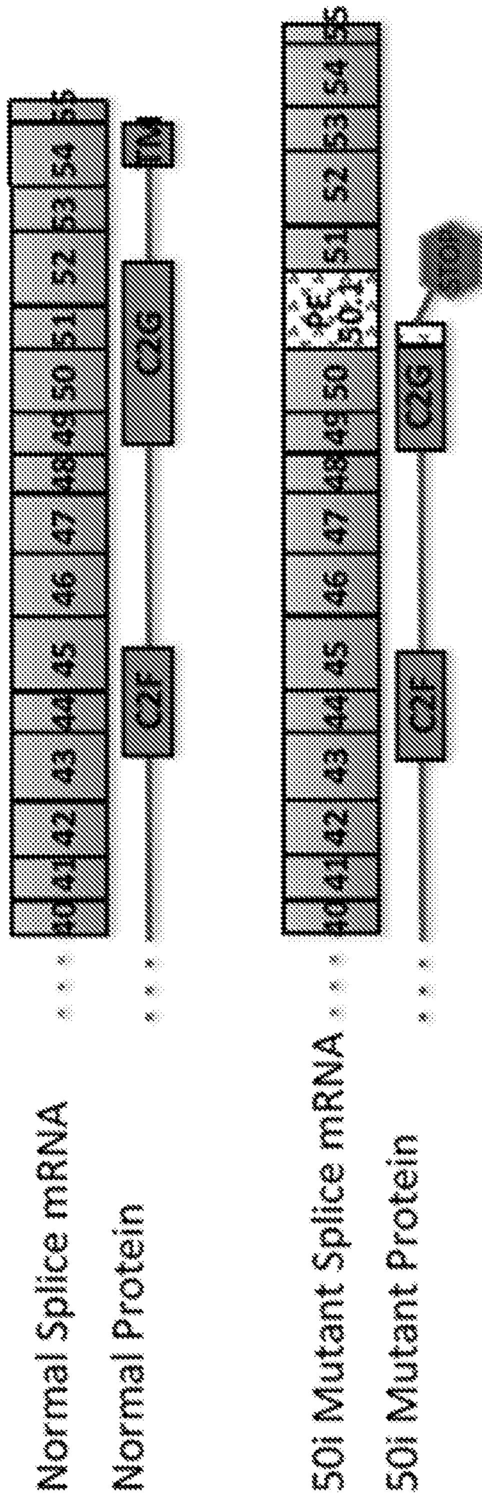


FIG. 2C

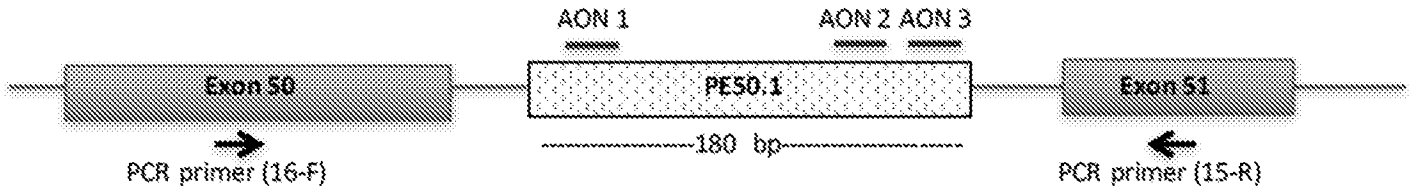


FIG. 3A

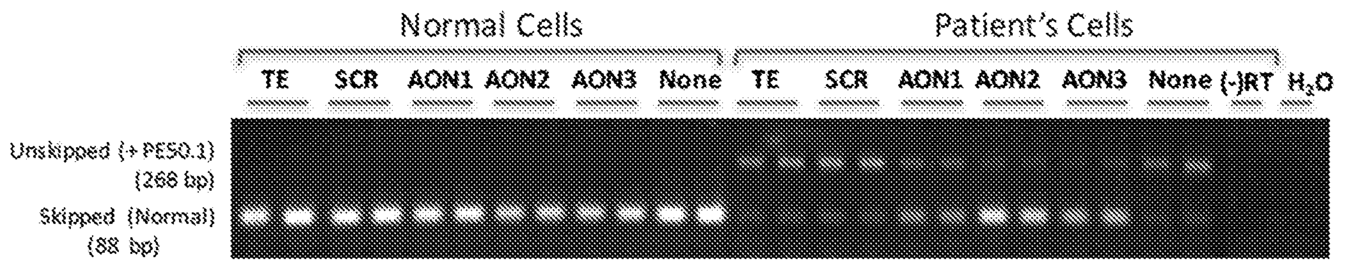


FIG. 3B

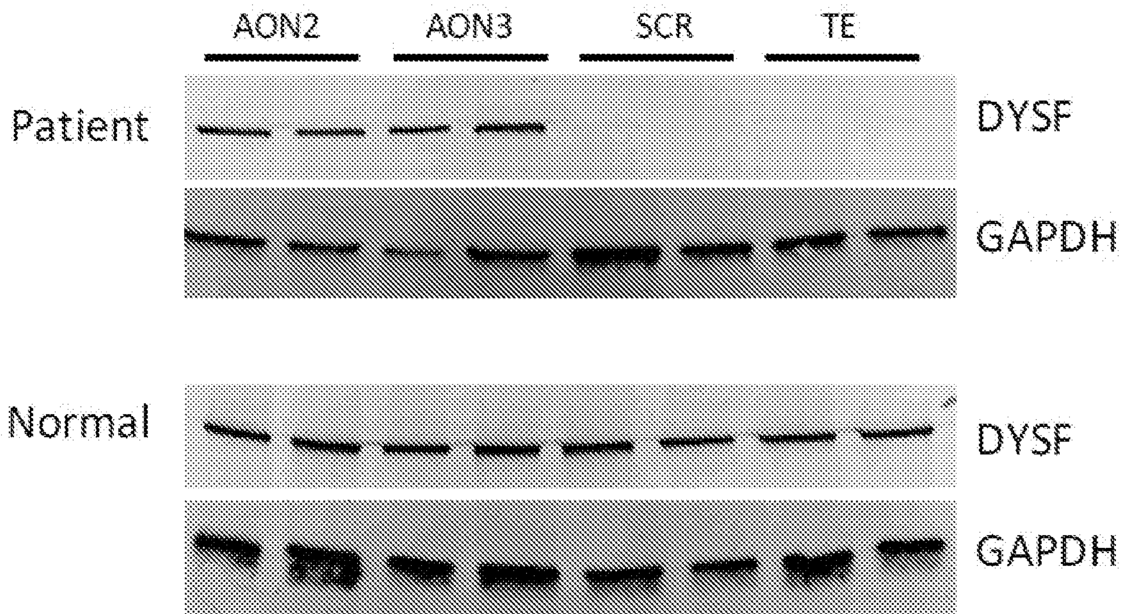


FIG. 4A

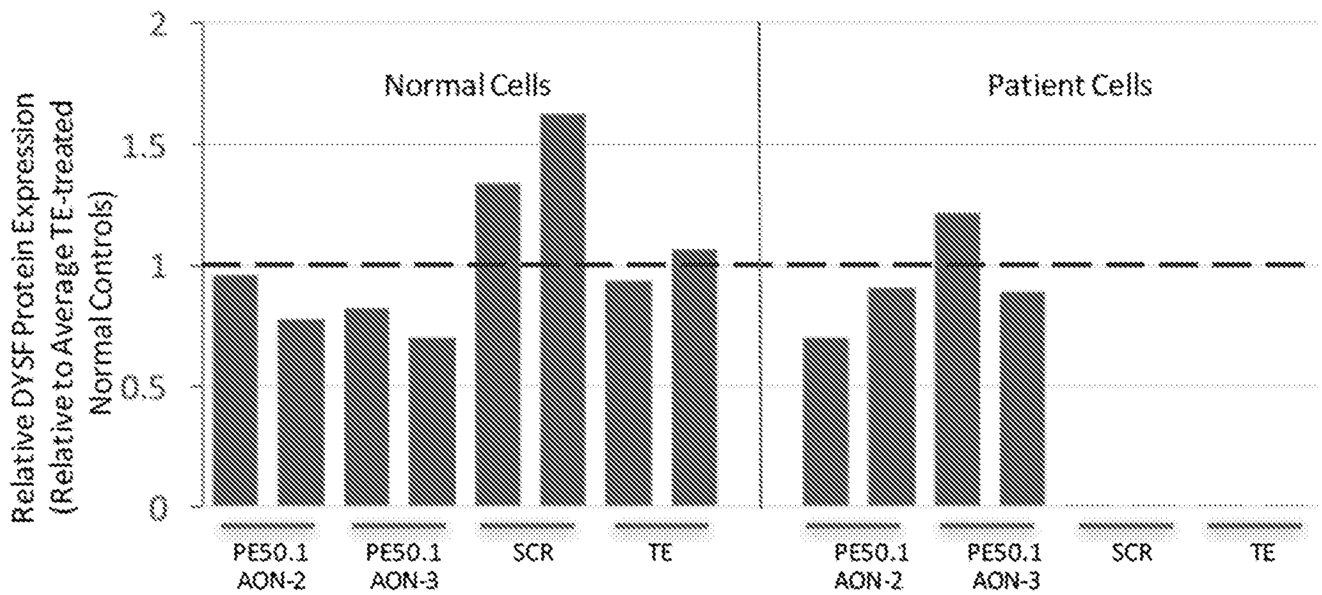


FIG. 4B

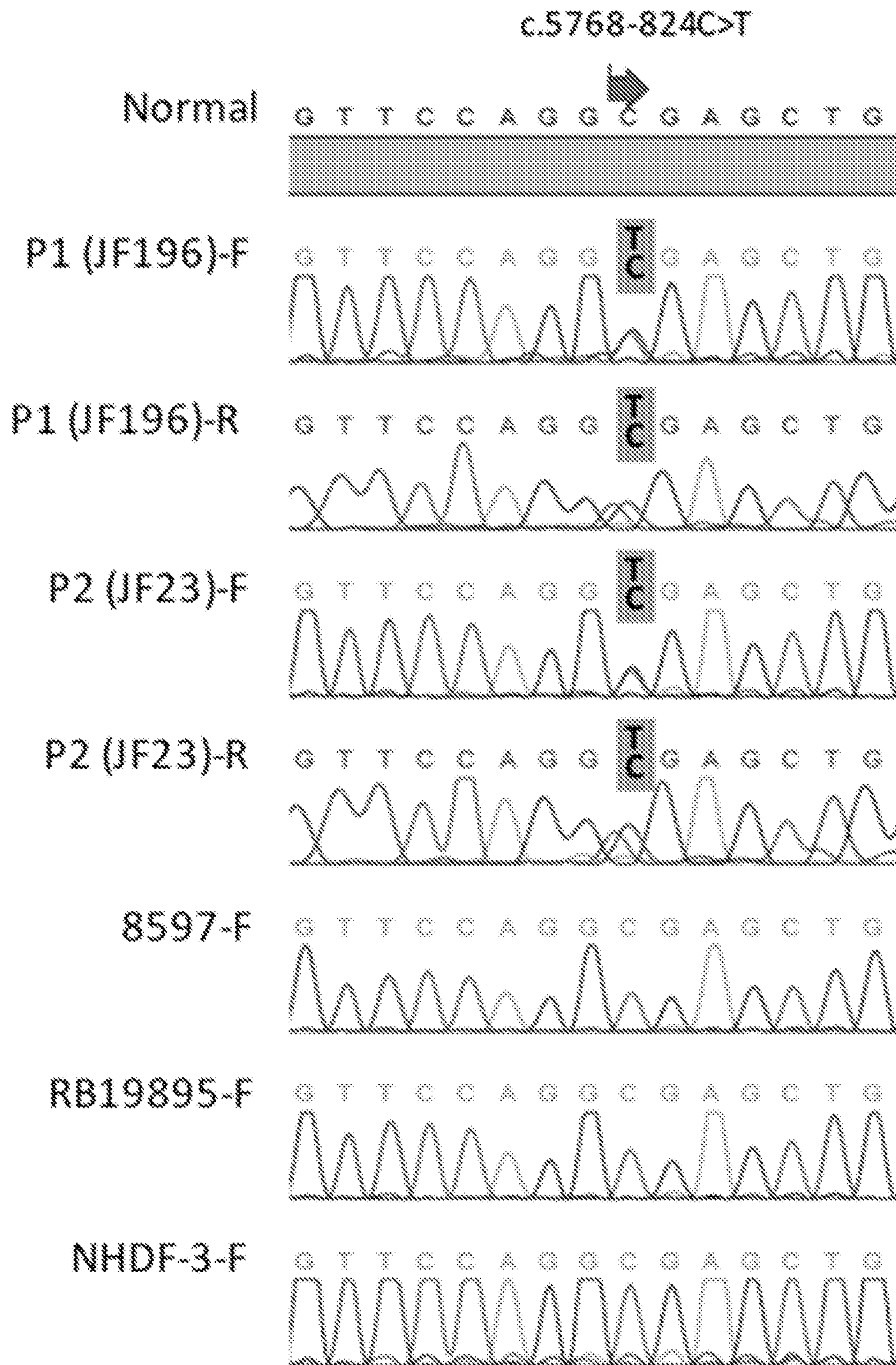


FIG. 5

Potential Branch Points   Pyrimidine-Rich   PE50.1

....acttggcacccaccatctgcagggtggtcAcActgtttcctgtccttgccactcacccagGGGAGAGTT....

-60   -40   -20

FIG. 6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2019/036045

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. ☒ forming part of the international application as filed:

      ☒ in the form of an Annex C/ST.25 text file.

      ☐ on paper or in the form of an image file.

   b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

      ☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

SEQ ID NOs: 1-34, 63-74, and 103-105 were searched.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2019/036045

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 9-13, 16-21, 28, 29, 37-40, 42-55, 64, 65, 71-77, 83
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2019/036045

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12N 15/113; C12Q 1/6883 (2019.01)
CPC - C12N 2310/113; C12N 2320/33; C12Q 2600/156 (2019.08)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 435/6.17; 514/44A (keyword delimited)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	US 2017/0101645 A1 (UNIVERSITY OF MASSACHUSETTS) 13 April 2017 (13.04.2017) entire document	66, 67 ------------- 14, 56-63
X -- Y	US 2004/0219528 A1 (MORRIS et al) 04 November 2004 (04.11.2004) entire document	33, 35, 36, 41, 68, 70 ------------- 14, 56-63
A	US 6,673,909 B1 (BROWN, JR. et al) 06 January 2004 (06.01.2004) entire document	1-8, 14, 15, 22-27, 30-36, 41, 56-63, 66-70, 78-82
A	US 2012/0208865 A1 (LEVY et al) 16 August 2012 (16.08.2012) entire document	1-8, 14, 15, 22-27, 30-36, 41, 56-63, 66-70, 78-82
A	US 2010/0266551 A1 (RICHARD et al) 21 October 2010 (21.10.2010) entire document	1-8, 14, 15, 22-27, 30-36, 41, 56-63, 66-70, 78-82
A	US 2012/0270930 A1 (VAN DER MAAREL et al) 25 October 2012 (25.10.2012) entire document	1-8, 14, 15, 22-27, 30-36, 41, 56-63, 66-70, 78-82

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
06 September 2019	05 NOV 2019

Name and mailing address of the ISA/US	Authorized officer
Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, VA 22313-1450 Facsimile No. 571-273-8300	Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774