Exons 45-55 skipping using mutation-tailored cocktails of antisense morpholinos in the *DMD* gene

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Abstract

Mutations in the *dystrophin* (*DMD*) gene and consequent loss of dystrophin cause Duchenne muscular dystrophy (DMD). A promising therapy for DMD, single-exon skipping using antisense phosphorodiamidate morpholino oligomers (PMOs), currently confronts major issues that an antisense drug induces the production of functionally undefined dystrophin and may not be similarly efficacious among patients with different mutations. Accordingly, the applicability of this approach is limited to out-of-frame mutations. Here, using an exon-skipping efficiency predictive tool, we designed three different PMO-cocktail sets for exons 45-55 skipping aiming to produce a dystrophin form with preserved functionality as seen in milder/asymptomatic individuals with an in-frame exons 45-55 deletion. Of them, the most effective set was composed of select PMOs of which each efficiently skips an assigned exon in cell-based screening. Its combinational PMOs fitted to different deletions of immortalized DMD patient-muscle cells significantly induced exons 45-55-skipped transcripts with removing three, eight or ten exons and dystrophin restoration as represented by Western blotting. *In vivo* skipping of the maximum eleven human *DMD* exons was confirmed in humanized mice. The finding indicates that our PMO set can be used as mutation-tailored cocktails for exons 45-55 skipping and treat over 65% DMD patients carrying out-of- or in-frame deletions.

Introduction

Duchenne muscular dystrophy (DMD), a lethal X-linked recessive neuromuscular disorder, is caused by mutations in the *dystrophin* (*DMD*) gene and the absence of dystrophin for maintaining muscle membrane integrity.[1] Although the *DMD* gene is the largest known in humans consisting of 79 exons in 2.4 Mb, there exists a mutational hotspot ranging from exon 43 to 55.[2] Deletions are the most frequent mutations to occur and account for approx. 68% of cases.[3] Of them, severe DMD results from mostly out-of-frame deletions that do not allow for the production of dystrophin. In contrast, in-frame deletions, which permits the production of internally-truncated dystrophins, mostly give rise to the mild counterpart, Becker muscular dystrophy (BMD).[4]

These *DMD* genotype-phenotype associations provide the rationale of a promising therapy, exon skipping using synthetic nucleic acid analogs called antisense oligonucleotides (AOs). The current approach targets a single exon and aims to transform DMD-related out-of-frame mRNAs into in-frame ones, enabling the expression of truncated dystrophin as seen in BMD. In 2016, the first exon 51-skipping AO drug with the phosphorodiamidate morpholino oligomer (PMO) chemistry, though conditional, has been approved by the US Food and Drug Association (FDA)[5] and clinical trials with other PMO-based AOs that target exon 45 or 53 are currently ongoing.[6, 7] As such, PMO-mediated single-exon skipping has great promise for treating DMD.

In this approach, however, a couple of major issues are raised. Single-exon skipping has not been fully correlated with functional correction of dystrophin. Dystrophin functionality

fluctuates in response to the structural difference between the resulting products.[8] Indeed, it has been reported that clinical severity of BMD patients who have truncated dystrophin as seen following single-exon skipping vary depending on their in-frame patterns.[9-12] This creates a serious concern that an AO drug may not necessarily provide its expected therapeutic benefit equally across patients even though the same target exon is removed. Due to this uncertainty, the applicability is currently limited to DMD patients with out-of-frame deletions despite the fact that DMD is found at 22% of those with in-frame deletions.[2]

Exons 45-55 skipping using AO cocktails is expected to overcome these limitations in single-exon skipping therapies.[13] This multi-exon skipping strategy intends to produce a consistent dystrophin form with preserved functionality as seen in exceptionally milder or asymptomatic subjects carrying an exons 45-55 deletion.[11,13-18] The exons 45-55-deleted dystrophin supposedly provides a favorable outcome among patients with different mutations. As demonstrated in pre-clinical studies, the strategy is achieved by excluding all the target exons from one mRNA at the same time and thus, success in treatment largely relies on the ability of respective AOs in a cocktail to skip a given exon within the region.[19-21] A ready-to-use cocktail set composed of such effective AOs could serve as tailored medication to different deletions for treating DMD patients.

In this study, for the first time, we demonstrated using the Leiden DMD database, that the exons 45-55 deletion is statistically associated with the occurrence of the mild BMD phenotype. The database analysis also revealed that a variety of AO combinations, in particular, those to skip ten and eight exons, are needed in exons 45-55 skipping therapy. Accordingly, the

applicability was shown to reach to more than 65% of DMD patients with out-of- and in-frame deletions. Given the need for tailored cocktail treatment, we designed three different cocktail sets composed of PMO-based AOs using an exon-skipping efficiency predictive tool we developed previously.[22] Of them, the most effective cocktail set was one formulated with select PMOs which each efficiently skipped an assigned exon in *in vitro* screening. Derivative PMO cocktails from this set significantly skipped up to ten exons in immortalized DMD muscle cell lines, accompanied by dystrophin restoration as represented by Western blotting. In a mouse model having the normal human *DMD* gene, we demonstrated the feasibility of simultaneous skipping of all eleven exons from exon 45 to 55 using the PMOs in the most effective cocktail set. This work represents the first step toward clinical application of PMO-mediated exons 45-55 skipping using a mutation-tailored cocktail approach for treating DMD.

Results

Overview of clinical presentation in patients with an exons 45-55 deletion

We first ensured their clinical profile by summarizing literature published so far, using 52 patients of which the exons 45-55 deletion was determined by Multiplex Ligation-dependent Probe Amplification (MLPA) or a combination of multiplex PCR and Southern blotting (**Table S1**). For profiling, five cases of patients were newly obtained from the Canadian Neuromuscular Disease Registry (CNDR). The clinical data confirmed that those with this large deletion consistently exhibit mild to asymptomatic phenotypes and retain walking ability up to the late seventies. In all patients referred, elevated serum creatine kinase levels were present. Some patients were reported to manifest cardiac involvement but not respiratory symptoms.

Association between exons 45-55 deletion and BMD phenotype

We analyzed the *DMD* genotype-phenotype associations using the registries of 4,929 patients with deletions determined by MLPA or equivalently accurate methods, and consequent phenotypes from the Leiden DMD database. The analyses revealed that more than 67% of deletion mutations occur within exons 45-55 (**Figure S1A**). More BMD phenotype and in-frame-type deletions were found in this region compared to those in other regions ranging from exon 2 to 44 or from 56 to 78 (**Figure S1B and C**). In the exons 45-55 region, in-frame deletions were statistically more associated with BMD, and the reading frame rule held at a higher 97% in BMD compared to other regions (**Figure S1D and E**).

Phenotypes found in patients with in-frame deletions involving a frame-shifting exon as the first or last one in the region partially explain therapeutic outcome from a single-exon

skipping.[11, 17, 23] An analysis of the proportion of BMD/DMD in in-frame deletions within the region first statistically revealed that an in-frame exons 45-55 deletion is more associated with the onset of BMD compared to in-frame deletion types starting or ending at an exon 46, 50, 51, 52 or 55 (**Figure 1**). In the group of deletions that start or end at exon 45 or 53, no statistical difference was found (proportions in individual deletions are available in **Table S2**). In exon 55-related in-frame deletions, the exons 45-55 deletion involved more than 90% patients as being BMD (75 out of 83), while in other deletions ending at exon 55, 3 out of 5 patients were diagnosed with DMD. The result emphasizes the therapeutic relevance of exons 45-55 removal.

Applicability of exons 45-55 skipping therapy using combinational AO cocktails

Table 1 and S2 represent the applicability of AO cocktails for exons 45-55 skipping therapy to *DMD* deletion types and phenotypes from the Leiden DMD database. It was revealed that this approach can be applied to ~65% of all patients having deletions (*n*=4,929). Approx. 69% and 45% of DMD patients carrying out-of- and in-frame deletions, respectively, are amenable to exons 45-55 skipping. In DMD with out-of-frame deletions, cocktails of 10 AOs in combination permit treatment of the largest population (18% of cases), followed by that of 8 AOs (11%). In DMD with in-frame deletions, cocktail 7 AOs were the most required (9%). In terms of the phenotypes, ~65% and ~70% of DMD and BMD patients having deletions are treatable with exons 45-55 skipping.

Design of cocktail sets with PMO-modified AOs

To establish a therapeutic set of AOs that can be used as mutation-tailored cocktails, we designed and compared three different cocktail sets composed of PMO-based AOs, each of which

contained PMOs assigned to an exon in the exons 45-55 region (**Table 2**). Individual PMOs composing these sets were optimized through a screening method using *in silico* and *in vitro* approaches, i.e., predicted and actual exon skipping efficiencies, respectively. Cocktail set no. 1 consisted of 11 30-mer PMOs that were selected to prevent dimerization between PMOs which may affect the therapeutic activity and safety in use. Set no. 2 consisted of 11 25-mer PMOs that are mostly the human analog versions of sequences used in our previous studies involving mouse vivo-PMOs that showed efficient exons 45-55 skipping of the mouse *Dmd* gene.[20, 24] Cocktail set no. 3 is composed of 12 30-mer PMOs, including 2 PMOs for exon 48 skipping, of which each was found to be the most effective for skipping an assigned AO in cell-based screening using RT-PCR. The screening process is described in the following sections:

***In silico* screening of AO sequences:** First, we designed 151 to 413 AO sequences against each exon in the exon 45-55 region, covering all possible target sites in individual exons (**Table S3**). According to our AO screening model,[22, 25] AO length was determined with 30- and 25-mer for PMO modification. Exon skipping efficiencies of all sequences were predicted using robust algorithms we have previously developed,[22] providing us with a final ranking that can be used for the selection of AO sequences (**Table S3**). In all exons tested, predicted skipping efficiencies of 30-mer AOs were higher than those of 25-mer AOs.

We also calculated the dimerization potential between AO sequences using a formula for the Gibbs free energy of binding (dG) (**Table S4**). The dimer formation relates to lowered exon skipping efficiency and an increase in potential side effects.[26-28] Along with AO ranking, the composition of set no. 1 was determined with 30-mer PMOs having potentially less chances

of dimerization, as represented by a higher integration value of dG -363 kcal/mole than that of -504 kcal/mole in set no. 3. Using the NCBI BLAST, the theoretical specificity of selected AO sequences to a target *DMD* exon was confirmed by the absence of mRNA sequences of other genes identical to the entire AO sequences in the results; 100% identity was found with less than 56% and 84% of the query covering for 30- and 25-mer sequences, respectively. Sequence searching with the GGGenome server revealed fewer genome sites similar to AO sequences with an increase in the length (**Table S5**), indicating that longer 30-mer AOs can work in a more sequence-specific manner and have less potential for affecting untargeted transcripts including non-coding RNAs that mostly exist in nuclei where AOs work.

***In vitro* screening of PMO-based AOs:** We next evaluated the actual exon skipping efficiencies of AO sequences selected through *in silico* screening. All the AOs tested here were prepared as PMOs that are a promising chemistry as to effectiveness and safety in patients.[5, 7] In *in vitro* screening, a DMD patient-derived immortalized skeletal muscle cell line carrying an exon 52 deletion (ID: KM571) was used for testing single-exon skipping except exon 52 skipping, for which that with an exons 48-50 deletion (ID: 6594) was used. PMO-mediated single-exon skipping as represented by RT-PCR was efficiently induced in all the target exons (**Figures 2 and S2**). PMOs that resulted in greater than 20% exon skipping efficiency when tested at 5 or 10 μM were selected to compose cocktail set no. 3, according to our previous studies, i.e., *in vitro* PMO activity can increase up to 10 μM and such skipping levels can be considered associated with dystrophin production as detected by Western blotting.[22, 25] Effective 30-mer PMOs in each exon were found within the top 17 in the ranking of exon skipping efficiencies. While efficient

exon skipping was found using a single PMO in most exons, exon 48 skipping was remarkably induced with 2 different PMOs. Thus, for cocktail set no. 3, we included 2 PMOs for skipping exon 48. Such a synergistic effect was also observed for the skipping of exons 46 and 47. PMOs with 25-mer that were previously optimized with vivo-PMOs[20] were not as effective to induce exon skipping efficiencies over 20%, except one for exon 46 skipping and one for exon 52 skipping that was first in the ranking.

Exons 45-55 skipping by tailored PMO cocktail approach in DMD muscle cells

To assess the therapeutic potential of cocktail set nos. 1, 2, and 3 in exons 45-55 skipping, we tested its derivative combinational PMO cocktails tailored to treat the different *DMD* deletions of exon(s) 45-52, 48-50, and 52 in immortalized DMD muscle cell lines referred to as 6311, 6594, and KM571, respectively (**Figure 3**). In RT-PCR analyses, as represented by the expression of exons 45-55-skipped transcripts, all the derivative cocktails prepared from set no. 1, 2 or 3 induced 3-, 8-, and 10-exon skipping at doses of 1, 3, and 10 μM per PMO (**Figures S3A-C** for the set nos. 1 and 2; **Figure 4A-C** for the set no. 3). In all the cocktail sets/combinations, the efficiency of exons 45-55 skipping was increased in a dose-dependent manner. PMO cocktail set no. 3 was significantly effective at skipping multiple exons in DMD cells, compared to the other two sets (**Figure 4D-F**); using the cocktails at 10 μM each, levels of exons 45-55-skipped mRNA reached up to 61%, 43% and 27% on average in 3-, 8-, and 10-exon skipping applications, respectively. In the course of testing all the cocktail sets and combinations used, various intermediate transcripts that included in-frame and out-of-frame species were produced. The expression patterns of these intermediates, however, were unchanged between different

concentrations, indicating that the activity of respective PMOs in a cocktail still proportionately increases depending on the dose.

Consistent with the RT-PCR result, dystrophin restoration was induced in DMD muscle cells treated with derivative PMO cocktails prepared from set no. 3 when tested at a dose of 10 μM per PMO (**Figure 5A-C**). In the treatment of DMD cells with set no.3 PMO cocktails for 3-, 8-, and 10-exon skipping, 14%, 7% and 3% dystrophin of normal levels were induced, respectively (**Figure 5D-F**). For set no. 1 (**Figure S3D-F**), appreciable dystrophin bands were found only in 6311 cells treated with the 3-PMO cocktail, while 8- and 10-exon skipping using this set produced very small amounts of dystrophin in 6594 and KM571 cells, having less than 2% of normal levels. Using set no. 2, no substantial dystrophin bands were detected in any of the three DMD cells. Compared to set nos. 1 and 2, the significant effect of set no. 3 on skipping 3, 8, or 10 exons was confirmed.

***In vivo* efficacy of the cocktail PMOs to skip 11-human *DMD* exons in a mouse model**

Finally, we tested the *in vivo* efficacy of exons 45-55 skipping using PMO set no. 3 in a humanized mouse model called the *hDMD/Dmd-null* mouse that has the normal human *DMD* gene and lacks the entire mouse *Dmd* gene.[25] In this model, to induce exons 45-55-skipped transcripts, all eleven exons need to be simultaneously skipped from the *DMD* mRNA, which allows for evaluating the maximum capability of set no. 3 in *in vivo* exons 45-55 skipping. In this test, we intramuscularly injected 12 PMOs composing set no. 3 as a cocktail at the dose of 20 or 100 μg in total (1.67 and 8.33 μg of each PMO) into tibialis anterior muscles. One week after injection, muscles were harvested for analyses of exon skipping using RT-PCR and of truncated

dystrophin production by Western blotting. The result showed exons 45-55 skipping efficiency of 15% and 22% on average at the low and high dose, respectively (**Figure 6**). Although skipping levels were variable between PMO-treated samples, the dose-dependent effect of the 12-PMO cocktail on skipping exons 45-55 *in vivo* was confirmed. Consistent with a previous report,[29] spontaneous *DMD* exons 45-55-skipped transcripts were detected in saline-treated control muscles. In Western blotting, the dystrophin of the treated *hDMD/Dmd-null* mice was detected only at the expected molecular size of the full-length protein as confirmed using samples from saline-treated mice and transgenic mice expressing the truncated dystrophin protein lacking the exons 45-55 region [30] (**Figure S4**).

Discussion

As shown through analyses of clinical overview and genotype-phenotype association (**Table S1** and **Figure 1**), skipping of the entire exons 45-55 region possesses strong rationale to be applied for DMD therapy. An important finding from the analysis is that the in-frame deletion of the entire exon 45-55 region is statistically associated with the milder BMD when compared to other in-frame deletions arising within the region. Given this clinical relevance of the exons 45-55 deletion, here, we have successfully developed the complete set of PMO-based AOs for exons 45-55 skipping from which the PMOs can be used in combination tailored to different *DMD* mutations. One key feature of our cocktail set is a use of the PMO chemistry that has been deemed sufficiently safe for human use.[5] Accordingly, the present study outlined a screening model for success in developing multi-exon skipping PMOs. Our model involves a series of *in*

silico pre-screening allowing for the rational selection of PMO sequences, which uses the prediction analyses of exon skipping efficiency and potential off-target effects (**Table S3-5**), followed by an *in vitro* screening with immortalized DMD muscle cells that determines PMOs to be included in a cocktail set (**Figure 2**). With the substantial activity of individual PMOs to skip a given exon, the feasibility of the tailored cocktail approach has been proved by the successful skipping of 3, 8, and 10 exons (**Figure 4**), accompanied with dystrophin rescue (**Figure 5**), in three different DMD muscle cells having acceptable mutations. Importantly, while PMO-based AOs are typically incompetent for *in vivo* application, in particular, multi-exon skipping,[31-33] our cocktail PMOs achieved in a humanized mouse model the removal of the maximum 11 exons from the normal human *DMD* mRNA (**Figure 6**).

The present results revealed that the effect of cocktail PMOs is largely dependent on the sequence/target RNA position of each, highlighting the need for a rigorous selection of respective PMOs to compose a cocktail set as done here. For the selection process, a reliable *in silico* pre-screening is indispensable to reasonably narrow down the options of AO sequences moving on to a subsequent cell-based screening, out of a few hundred candidates designed as encompassing an entire exon region (**Table S3**). Here, this pre-screening allowed for the selection of highly effective PMOs against all the exons in the exons 45-55 region, except exon 48, using the ranking of predicted exon skipping efficiencies with our *in silico* tool, [22, 25] as validated by the actual efficiencies in DMD cells (**Figure 2**). Although useful to find effective PMOs for individual exons in the region of interest, the current tool has some issues including that the use is limited to 30- and 25-mer PMO sequences and that the synergistic effect of AOs

on the removal of an exon, as found in exon 48 skipping, cannot be predicted. With the improvement of the predictive algorithms, *in silico* pre-screening will increase the opportunity to discover more effective PMOs not only for exons 45-55 skipping but also for different multi-exon skipping strategies.[2] Such advanced algorithms are also expected to enable the optimization of AO sequences used with other AO chemistries that have greater bioavailability in multi-exon skipping, e.g., peptide-conjugated PMOs.[34]

Along with the optimal design of PMO sequences, appropriate patient cell models in the subsequent *in vitro* screening are an essential tool to evaluate and develop multi-exon skipping PMOs. Because rescued dystrophin levels are a primary biomarker of therapeutic benefits from exon skipping therapies, cell models need to allow for the quantification of the protein by Western blotting that is suggested by the FDA in clinical trials with eteplirsen.[35] We have previously shown in DMD patient fibroblast-converted myotubes, the induction of exons 45-55 skipped transcripts using 5- and 6-exon skipping PMO cocktails,[21] but this transdifferentiated cell model was not enough to quantify the efficiency at exons 45-55 skipping and dystrophin rescue due to low differentiation ability of the cells. In contrast, immortalized DMD muscle cells enabled the quantification of dystrophin restoration by Western blotting in the test with exons 45-55 skipping PMOs. Because such DMD muscle cell lines available are currently limited, the development of those with different mutations amenable to exons 45-55 skipping are required to further confirm the application of tailored approaches with a cocktail set.

Following cell-based screening, the *in vivo* efficacy of the selected AOs needs to be examined in an appropriate animal model, such as the humanized mouse model used in this study.

Our *hDMD/Dmd-null* mouse model has the advantage of allowing for the assessment of the activity of human-specific AOs *in vivo* without being confounded by expression of homologous mRNA derived from the mouse *Dmd* gene. In this model, however, treatment effects such as dystrophin rescue, histological amelioration, and functional recovery cannot be examined because of the lack of dystrophic pathology. The *hDMD/Dmd-null* mouse model also holds normal muscle membrane permeability that can be associated with the lowered efficiency of AO uptake. Another concern is that the reactivity of the normal *DMD* transcript to AOs may be different from the mutated versions found in patients. These conditions may affect the estimation of the effectiveness of human AOs in patient muscles. Indeed, the dose-dependent effect of the PMO cocktail was unclear in the healthy mouse model (**Figure 6**). As a possible solution to these limitations, dystrophic *hDMD* mouse models having a mutation in the human *DMD* gene have been developed by crossing with *mdx* mice that have a nonsense mutation in the mouse *Dmd* gene. However, murine dystrophin transcripts are still present in these mice, which may pose difficulties in skipping evaluation as described previously.[36, 37] To assess the potential benefit of AOs designed for patients, and in particular, dystrophin rescue levels, the development of dystrophic humanized mouse models, in which mutations in the human *DMD* gene cause dystrophic phenotypes and the mouse *Dmd* gene is absent, will be required.

With the database analysis, we revealed that cocktail AOs for skipping 10 exons are the most required combination to treat DMD deletions, accounted for approx. 17% of those (**Table 1**). In this study, we have demonstrated the 10-exon skipping in a DMD muscle cell line with the fourth most common single deletion, an exon 52 deletion (**Figures 4 and 5**).[2] Based on

the definite effect of individual PMOs in the cocktail set no. 3 on skipping an assigned exon (**Figure 2**), the PMO set has the potential for being adapted to other 10-exon skipping approaches targeting different single exon deletions, in particular, an exon 45 deletion that creates the largest population of DMD (approx. 6%) (**Table S2**). This possibility can be further supported by the 11-exon skipping *in vivo* shown in a mouse model with the human *DMD* gene (**Figure 6**). As such, an exons 45-55-skipping cocktail set is versatile in that it can treat more than 65% of DMD patients with deletions (**Table 1**), whether they are single (e.g. Δ45, Δ51, Δ52) or multiple (e.g. Δ45-50, Δ45-52, Δ48-50) exon deletions, and whether they are out-of-frame or in-frame. In this study, theoretical applicability of this approach to BMD with deletions was also shown, opening a potential avenue of treatment for 70% of the cases, in particular, those with severe phenotypes and cardiac impairment that is a leading cause of death.[38]

While exons 45-55 skipping is expected to lead to similar therapeutic outcomes among patients regardless of mutation patterns within amenable boundaries, a concern regarding the truncated dystrophin produced from exon skipping is the potential structural change it may create in the binding site of neuronal nitric oxide synthase (nNOS) encoded by exons 42-45 (**Figure 3B**). nNOS and its metabolite NO play a crucial function in directing numerous physiological activities of muscle, such as contractile force and blood flow regulation.[39] In BMD patients, reduced expression of nNOS and its mislocalization from the sarcolemma to the cytoplasm have been identified.[16, 40] A recent study with a transgenic *mdx* mouse model that carries the human *DMD* gene with a deletion of the exons 45-55 region demonstrated normalized activity of nNOS

in muscles expressing truncated dystrophin as seen following exons 45-55 skipping therapy despite nNOS remaining mislocalized in the cytoplasm.[30] In this humanized mouse, muscle histology and function were also comparable to wild-type mice. The observed rescue effect with the truncated dystrophin may be partially associated with the amino acid sequence similarity of the hybrid rod domain 17/22 encoded by exons 44/56 to the native rod domain 17 by exon 45 in the nNOS binding site.[41] In addition, the binding sites of F-actin and the sarcolemmal lipid layer are partially affected by the exons 45-55 deletion,[42, 43] which suggests that the resulting dystrophin can alter sarcolemmal stability. A hybrid rod similar to the native rod domain 17 composed of three α-helices has been computationally predicted in some in-frame deletions such as the deletions of exons 45-48, 45-51, and 45-55.[44] Of them, the exons 45-55 deleted dystrophin has a structural resemblance to the native protein with 16 rod domains, from the hinge 2 to the next hinge 4 (**Figure 3B**). A future challenge will be to address how the truncation of dystrophin impacts interactions with its binding partners and, consequently, on muscle function. This will help in better understanding the possible effects of exons 45-55 skipping as a therapy.

An issue in PMO cocktail approaches is that the efficiency of exons 45-55 skipping is lowered with an increase in the number of target exons or AOs in a cocktail. In the test using both cocktail set nos. 1 and 3 comprising 30-mer PMOs, 3- and 10-exon skipping induced the highest and lowest efficiencies, respectively (**Figure 4**). This event did not occur with the 25-mer PMO set, probably due to low activity in exons 45-55 skipping. To skip the entirety of exons 45 to 55, all AOs in a cocktail have to simultaneously bind their target exons of the same pre-mRNA but such will not always be the case. In the current cocktail approach using

one-to-one interaction of an AO with an exon of a target, the unequable binding of multiple AOs to a pre-mRNA is unavoidable, decreasing the efficacy of the intended multi-exon skipping. Although the dose escalation of PMOs can improve the chance of simultaneous binding of different PMOs, this also increases that of off-target effects *in vivo*. A possible solution to this issue may be to remove the exons 45-55 region as one or a few exon blocks from the pre-mRNA. Encouragingly, endogenous exons 45-55 skipped mRNAs have been identified in the normal *DMD* gene.[29] By revealing a mechanism for this spontaneous multi-exon skipping phenomena, exon-block skipping using minimal PMOs can become a practical approach in exons 45-55 skipping therapy. The strategy will also reduce a concern associated with the formation of unintended intermediately skipped transcripts, as found after multi-exon skipping (**Figures 4 and 6**) that may have unexpected impacts on therapeutic efficacy.

Finally, drug development regulation is another challenge to surmount for the clinical translation of tailored cocktail approaches with exons 45-55 skipping AOs. Currently, there is no specific regulatory guidance for the development of cocktail drugs using multiple AOs targeting different RNA positions in a gene. In this context, an FDA guidance, Codevelopment of Two or More New Investigational Drugs for Use in Combination, has been issued on June 2013,[45] which may partially provide some leads for the cocktail AO drug development. Referring to this guidance, it is desired to demonstrate that the greater efficacy and better toxicity profile of exons 45-55 skipping AO cocktails to single-exon skipping AOs in an *in vivo* (preferable) or *in vitro* model with mutations amenable to both strategies. Second, if the exons 45-55 skipping AOs in the cocktail set were to be adapted for patients with different mutation types, clinical trials would

need to be respectively performed to separate cocktail compositions, i.e., to the number of mutation patterns, which can count 62 of the combination cocktails for 36 out-of-frame and 26 in-frame deletion patterns found in the region (**Table S2**). However, it is in practice difficult to design such clinical trials with sufficient subjects. One significant issue is that some cocktail compositions induce harmful out-of-frame transcripts in healthy volunteers. One solution to these is to simply use the complete exons 45-55 skipping cocktail as a single agent regardless of mutation type in the region. However, compared to such a cocktail that inevitably contains non-therapeutic AOs targeting exons deleted in the patient, it is evident that tailored cocktail approaches using only AOs targeting exons that patients retain have a lower risk of side effects.

In this study, we conclude that PMO-mediated exons 45-55 skipping is doable in tailored cocktail approaches and has a potential for treating patients with DMD arising from out-of- and in-frame deletion mutations. The approach, however, still needs to overcome certain challenges. These include, among others, determining the functional superiority of exons 45-55 skipped dystrophin, and the efficacy and safety profile in *in vivo* models such as transgenic animals with dystrophic pathology arising from human *DMD* mutations, as well as dealing with current drug development regulations.[2, 45] It is also to be noted that patients with other mutation types, e.g., duplication and point mutations, require this methodology as some of those can be corrected only by skipping multiple exons.[46, 47] With more research on the approach, we expect that mutation-tailored AO cocktails will become a treatment modality not only for DMD but also other genetic disorders such as dysferlinopathy with which patients can receive more therapeutic benefit from the functional correction of a causative protein.[48]

Materials and Methods

Ethics statement

Experiments using human cells and animals in this study were performed with approval from the Ethics Committee for the Animal Care and Use Committee (ACUC) of the University of Alberta and National Center of Neurology and Psychiatry (NCNP). Clinical data of patients enrolled in the Canadian Neuromuscular Disease Registry (CNDR) were reviewed with the approval of the Health Research Ethics Board of the University of Alberta (Pro00059937).

Patients

Five new Canadian cases with *DMD* exons 45-55 deletion were obtained from the CNDR for this study. The information of the new cases: date at an examination, ambulatory ability, and cardiac involvement, were summarized together with that of cases previously published (**Table S1**).

Genotype-Phenotype associations and applicability of cocktail treatment

A total of 16,032 patients in the Leiden Open Variation Database (LOVD v.3.0, https://databases.lovd.nl/shared/genes/DMD) were reviewed (accessed June 22nd, 2018). Of all these patients, 4,929 cases with large exonic deletions (≥ 1 exon) determined with accurate and sensitive diagnostic methods were extracted for analyses. These methods include: Multiplex Ligation-dependent Probe Amplification (MLPA), Multiplex Amplifiable Probe Hybridization (MAPH), array Comparative Genomic Hybridization (array CGH), Next Generation Sequencing (NGS), or a combination of multiplex PCR and Southern blotting. In frame type-based analyses,

a total of 4,843 cases were used: 3,232 and 1,611 with out-of- and in-frame deletions, respectively; 86 cases with deletions starting and/or ending at exon 1 and/or 79, which are not applicable to the definition of a frameshift, were excluded from the analyses (**Figure S1**). In phenotype-based analyses, a total of 3,712 data were analyzed: 2,688 of DMD and 1,024 of BMD. Registrations without a diagnosis of DMD or BMD were omitted from the analyses. Applicability of combinational AO cocktails was analyzed with these populations (**Table 1 and S2**).

Design of antisense sequences

All possible AO sequences 30- or 25-mer in length were designed for each of the eleven exons within exons 45-55 (**Table S3**). Exon skipping efficiencies of the designed sequences were quantitatively predicted using the computational tool we developed previously.[22]

Dimerization potential of AO sequences

The lowest free energy (dG) of binding of between AOs or individual AOs was predicted with RNAstructure web servers (version 6.0.1) (https://rna.urmc.rochester.edu/RNAstructureWeb/). Dimerization potential of AO pairs, as shown in **Table S4**, was formulated as follows: dG of an AO pair – (dG of an AO + dG of the other AO). Integrated values of dimerization dG were represented as the potential risk of using an AO cocktail.

Specificity of AO sequences

The specificity of AO sequences was analyzed with both plus and minus strands of the human genome (reference ID: GRCh38/hg38) in GGGenome (http://gggenome.dbcls.jp/en/hg38/); the parameter was set to explore genomic sequences that differ in 5 or 4 nucleotides with mismatches/gaps from given 30- or 25-mer AOs, respectively, which considered >16.7% difference from a given AO sequence that may lead to unexpected, off-target effects.[49]

Antisense morpholinos and PMO cocktails

All AO sequences experimentally tested in this study were synthesized with the PMO chemistry by Gene Tools. PMO cocktails were prepared just before use in experiments; respective PMO stock vials at 1 mM were heated at 65ºC for 10 min in order to dissociate aggregations and only PMOs required to induce exons 45-55 skipping were mixed in transfection media or saline.

Immortalized patient-derived skeletal muscle cells

Human-derived skeletal muscle cell lines were obtained with the help of Dr. Francesco Muntoni of the MRC Centre for Neuromuscular Diseases Biobank (NHS Research Ethics Committee reference 06/Q0406/33, HTA license number 12198) in the context of Myobank, affiliated with Eurobiobank (European certification). Healthy and DMD patient-derived skeletal muscle cell lines were immortalized with *CDK4* and *Telomerase*-expressing pBABE retroviral vectors as described previously.[50] The immortalized DMD muscle cell lines tested were 6311, 6594, and KM571 which have deletions of *DMD* ex45-52, ex48-50, and ex52, respectively. The

immortalized healthy muscle cell lines KM155 and 8220 were used as controls.

Transfection of individual and cocktail PMOs

Immortalized healthy and DMD skeletal muscle cells were grown and differentiated as described previously.[25] Briefly, cells were seeded at 1.7 x 10^4/cm^2 in collagen type I-coated culture plates, then cultured in a growth medium (GM): DMEM/F12 with skeletal muscle supplement mix (Promocell), 20% fetal bovine serum (Gibco), and antibiotics (50 U penicillin and 50 mg/ml streptomycin). At 80-90% confluence, media were replaced with a differentiation medium (DM): DMEM/F12 supplemented with 2% horse serum (GE Healthcare), 1x insulin-transferrin-sodium selenite (ITS) solution (Sigma-Aldrich), and antibiotics. After 3 days in DM, myotube-differentiated DMD cells were transfected with a single PMO or multiple PMOs as a cocktail at 1, 3, 5, or 10 μM, each containing 6 μM Endo-porter transfection reagent (Gene Tools). The same amount of transfection reagent was used regardless of PMO amount according to the company's suggestion. Cocktails of combinational PMOs were prepared just before the transfection following the heating procedure described previously. Following the incubation with PMOs for 2 days, PMO-containing DM was replaced with regular DM. Three days later, cells were harvested for subsequent experiments.

Humanized transgenic mice

Male transgenic *hDMD* mice with the full-length normal human *DMD* gene on mouse chromosome 5 (Jackson Laboratory)[51] were cross-bred with female *Dmd-null* mice that lack the

entire mouse gene in the X-chromosome.[52] The resulting male offspring, called *hDMD/Dmd-null* mice (*hDMD*$^{+/-}$; *Dmd-null*$^{-/Y}$), accordingly expresses full-length dystrophin protein derived from the human *DMD* gene but not from the mouse *Dmd* gene, which imposes a limitation in assessing exon skipping treatment efficacy. The *hDMD/Dmd-null* mice were used at the age of 6–16 weeks for testing the *in vivo* efficacy of a 12-PMO cocktail at skipping 11 exons from exons 45 to 55. A humanized *mdx* mouse model that has an exons 45-55 deletion in the *DMD* gene and expresses exons 45-55 deleted human dystrophin was used as a positive control in Western blotting analysis with the muscle samples of *hDMD/Dmd-null* mice.[30]

PMO cocktail injections

PMO cocktails with total doses of 20 or 100 μg (1.67 or 8.33 μg per PMO, respectively) in 36 μL of saline were injected into the tibialis anterior (TA) muscles of *hDMD/Dmd-null* mice under anesthesia with sodium pentobarbital (Kyoritsu Seiyaku). The same amount of saline was intramuscularly injected into the TA muscles as a negative control. One week after the injection, mice were euthanized by cervical dislocation, and then the TA muscles injected were collected. Muscle samples were snap-frozen as described previously,[24] and stored at -80ºC until use.

RT-PCR

Total RNA from cells and frozen TA muscle sections was extracted with Trizol reagent (Invitrogen) as described previously.[25] RT-PCR was performed in a 25-μL mixture containing 200 ng RNA and 0.2 μM of each primer with the SuperScript III One-Step RT-PCR System

(Invitrogen), following manufacturer's instructions. Primer sequences are listed in **Table S6**. The cycling conditions were optimized depending on the amplicon size of native *DMD* mRNA in each DMD cell line, and it is as follows: 50°C for 5-15 min; 94°C for 2 min; 35-40 cycles at 94°C for 15 sec, 60°C for 30 sec, and 68°C for 33-118 sec; and 68°C for 5 min. *GAPDH* or *Gapdh* mRNA was detected as an internal control. PCR products were separated on a 1.5% agarose gel and visualized by SYBR Safe DNA Gel Stain (Invitrogen). Skipping percentage was calculated as $\frac{\text{Skipped transcript}}{\text{Native + Skipped transcript}} \times 100$ for single exon skipping or $\frac{\text{Exons 45–55 skipped transcript}}{\text{Native + Intermediates + Skipped transcripts}} \times 100$ for multiple exon skipping using ImageJ (NIH). Bands with the expected size of the transcript were excised and purified with a gel extraction kit (Promega). Sequencing reactions were performed with Big Dye Terminator v3.1 **(**Applied Biosystems).

Western blotting

Total protein from cells was extracted with RIPA buffer (Pierce Biotechnology) containing protease inhibitors (complete mini EDTA-free, Roche), and concentrations were measured by BCA assay (Pierce Biotechnology). Total protein from frozen muscle sections was prepared as previously described.[24] Total protein extracts were loaded onto wells of a NuPAGE Novex 3–8% Tris-Acetate Midi Gel (Invitrogen) and separated by SDS-PAGE at 150 V for 75 min for cell samples and 150 min for tissues samples. Proteins were transferred onto a PVDF membrane (Millipore) by semidry blotting at 20 V for 70 min. The membrane was blocked with PBS

containing 0.05% Tween 20 and 2% ECL advance blocking reagent (GE Healthcare) overnight at 4 ºC. The membrane was incubated with anti-dystrophin C-terminal domain antibody (1:2500, ab15277; Abcam) or NCL-DYS1 (1:200, Leica Biosystems) for 1 hour at room temperature. The primary antibody was detected with HRP-conjugated IgG H+L secondary antibody (1:10000, Invitrogen). Blots were visualized by electrochemiluminescence (GE Healthcare). Expression levels of the dystrophin protein induced by PMO cocktails were calculated using a calibration curve from 0.12 to 1.8 μg protein of immortalized healthy skeletal muscle cell lines, KM155 or 8220 (**Figure S3H**). As a loading control and differentiation marker, α-actinin was detected using a primary antibody (Sigma-Aldrich). Myosin heavy chain (MyHC) on the post-transferred gel was stained by Coomassie Brilliant Blue as a loading control and as another indicator of muscle cell differentiation.

Statistical analysis

For association analyses between genotypes and phenotypes shown in **Figure S1**, two-tailed Fisher's exact test (2x2 contingency table) was used with a p-value < 0.05 considered to be statistically significant. Differences in phenotype proportions between exons 45-55 deletion and other in-frame deletions that start and end at exon(s) within the exons 45-55 region (**Figure 1**) were computed using a two-tailed Fisher's exact test, and then the resulting p values were adjusted for multiple comparisons using the Benjamini-Hochberg procedure: false discovery rates (FDRs) of 0.05 or 0.01 were considered as a significant difference. Odds ratios (odds of BMD with other in-frame deletions/odds of that with exons 45-55 deletion) and 95% confidence

intervals were calculated to quantify differences in the association between BMD and in-frame deletion mutations. Statistical tests for efficiency at skipping exons and rescuing dystrophin expression were performed using the Tukey-Kramer's or Dunnett's test. All statistical analyses were conducted with R (version 3.5.1).

Conflicts of interest

The authors have no conflicts of interest.

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Author contributions

Conceptualization, T.Y.; Methodology, Y.E., K.R.Q.L., K.Y.A., V.M., and T.Y.; Software, Y.E., W.D., and T.Y.; Investigation, Y.E., K.R.Q.L., M.D., B.B., N.T., Y.M., and R.M.; Resources, Y.E.,

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Figure legends

Figure 1. Associations between in-frame deletion (del.) mutations arising within the exons (ex) 45-55 region and consequent phenotypes

The functionality of different dystrophin forms can partially be explained with the proportion of two distinct phenotypes: DMD and BMD in an in-frame deletion. Exon 45, 46, 50, 51, 52, 53, and 55 are a frame-shifting one targeted by single-exon skipping therapies. The phenotype ratio in in-frame deletions that start/end at a concerned exon and are complete within the exons 45-55 region are considered associated with therapeutic outcome after exon skipping therapies, enabling the comparison of the estimated efficacy between exons 45-55 skipping and single-exon skipping strategies. A total of 897 patients carrying acceptable deletions that are determined by MLPA or equivalent methods were extracted from the Leiden DMD database. Patients having an exons 45-55 del. was not included in the group with ex45 or ex55. FDR-adjusted p values of 0.05 (*) or 0.01 (**) were considered to be statistically significant compared to ex45-55 del. (Fisher's exact test, Benjamini-Hochberg procedure). Odds ratio (OR) for BMD (odds of other in-frame del./odds of ex45-55 del.) and the 95% confidence intervals (CI) were calculated using the unconditional Maximum Likelihood Estimate. Statistically significant differences were set at *$p < 0.05$ or **$p < 0.01$. The information of individual sample sizes are shown in **Table S2**.

Figure 2. *In vitro* screening of antisense PMOs for skipping individual *DMD* exons in the exons 45-55 region using RT-PCR

Efficiencies of exon skipping were tested in an immortalized DMD muscle cell line with an exon 52 deletion (KM571) except exon 52 skipping for which a DMD muscle cell line with an exons 48-50 deletion (6594) was utilized. Most PMOs were tested at 5 μM. 10 μM was used for a single or combinational PMOs when less than 20% skipping efficiency was found at 5 μM. Black and gray bars indicate efficiency at skipping an exon using one and two kinds of PMOs, respectively. Data represent mean (SD) from three or four experiments in each. hAc, the human version of 25-mer mouse antisense oligos identified in our previous study.[20] R, a rank with 30-mer AOs in an exon; r, a rank with 25-mer AOs in an exon; NA, not available; Ete, a PMO with eteplirsen sequence. § and # indicate values adapted from our previous reports using an identical method to the present study.[22, 25] All the DNA electrophoresis images and individual skipping values used here are shown in **Figure S2**.

Figure 3. Schemes of exons 45-55 skipping using antisense PMO cocktails and the resulting truncated dystrophin structure

(A) Dystrophin mRNA structures in immortalized DMD muscle cell lines (6311, 6594, and KM571) and a humanized mouse model, *hDMD/Dmd-null*, which has the normal human *DMD* gene, and the strategy of exons 45-55 skipping by cocktail PMOs. Boxes indicate exons. The shapes denote phase of triplet codons. Exon 48 can be skipped using 2 PMOs from the cocktail set 3. (B) A semi-functional dystrophin isoform found in patients with an exons 45-55 deletion or following exons 45-55 skipping treatment. In a schematic of wild-type dystrophin, binding domains that can partially be affected in the truncated dystrophin are shown: nNOS, the binding domain of neuronal nitric oxide synthase; ABD2, actin-binding domain 2; Lipid binding domain 2, a domain of binding to a phospholipid membrane bilayer. H, hinge region.

Figure 4. Efficiencies of exons 45-55 skipping in immortalized DMD-patient derived skeletal muscle cells treated with cocktails of combinational PMOs at 1, 3, and 10 μM each tailored to their deletion mutations

(A-C) *DMD* exons 45-55-skipping efficiencies using combinational PMOs from the cocktail set no. 3; (A) 3-exon skipping in DMD-6311 cells with ex45-52 del., (B) 8-exon skipping in 6594 cells with ex48-50 del., and (C) 10-exon skipping in KM571 cells with ex52 del. The images of tests using the PMO set nos. 1 and 2 are available in **Figure S3A-C**. M, 100 bp marker; NT, non-treated; Mock, a mock 31-mer PMO at 10 μM. (D-F) Quantification of exons 45-55-skipping induced by combinational PMOs from the cocktail set nos. 1, 2, and 3; (D) 3-exon skipping against ex45-52 del., (E) 8-exon skipping against ex48-50 del., and (F) 10-exon skipping against ex52 del. Efficiency (%) of exons 45-55 skipping following treatment was normalized by that of spontaneous one observed in non-treated cells. Data represent the mean (SD) from three independent experiments. * $p < 0.05$, ** $p < 0.01$ compared to the next lower PMO dosage in the same cocktail set. †† $p < 0.01$ compared to the cocktail set 1 at the same PMO dosage. ‡ $p < 0.05$, ‡‡ $p < 0.01$ compared to the cocktail set 2 at the same dosage (Tukey–Kramer test).

Figure 5. Dystrophin restoration in DMD muscle cells treated with 3-, 8- or 10-exon skipping using cocktail PMOs

Rescued dystrophin in (A) DMD-6311 cells treated with 3 PMOs, (B) 6594 cells with 8 PMOs, and (C) KM571 cells with 11 PMOs (10 μM each) from the cocktail set no. 3 was measured by Western blotting with the anti-dystrophin C-terminal domain antibody. Total protein of 9 μg from 6311 cells and 18 μg from 6594 or KM571 cells was loaded. The band images with the cocktail set nos. 1 and 2 are available in **Figure S3D-F**. To calculate the expression levels in DMD cells, healthy muscle cell lines, KM155 and 8220 were used for a standard curve in the range from 1.3% to 20% protein of that of DMD cells (averaged R^2 = 0.97, SD 0.028, representatives are shown in **Figure S3G**). Total protein amount of KM155 cells was adjusted to the same amount of DMD cells using the total protein of non-treated DMD cells. (D-F) Quantification of dystrophin induced by combinational PMOs from the cocktail set no. 1, 2 or 3 in (D) 6311 cells with ex45-52 del., (E) 6594 with ex48-50 del., and (F) KM571 with ex52 del. Expression levels of rescued dystrophin were normalized by that of spontaneous one observed in non-treated DMD cells and were calculated with a standard curve using the 8220 healthy muscle cells for the comparison. Data represent the mean (SD) from three independent experiments. **, $p < 0.01$ compared to the set 1; ††, $p < 0.01$ compared to the set 2 (Tukey–Kramer test).

Figure 6. *In vivo* exons 45-55 skipping using 12 PMOs of the cocktail set no. 3 by the intramuscular (i.m.) injection into tibialis anterior (TA) muscles of a humanized mouse model with the normal human *DMD* gene and without the entire mouse *Dmd* gene (*hDMD/Dmd-null* mouse)

A cocktail of 12 PMOs at 20 and 100 μg in total (1.67 and 8.33 μg each PMO, respectively) was injected once into left and right TA muscles of mice, respectively. One week after the injection, the muscles were harvested. The efficiency (%) of exons 45-55 skipping was analyzed by RT-PCR as shown in the bottom of the image. M, 100 bp marker. (A) Representative images of *in vivo* exons 45-55 skipping in individual TA muscles of *hDMD/Dmd-null* mice. (B) Quantification of exons 45-55 skipped mRNA levels as represented by the mean (SEM). n = 5 in injected TA muscles, n = 4 in control TA muscles. The statistical significance was set at * $p < 0.05$ (Dunnett's test).

Table 1. Applicability of exons 45-55 skipping to patients with deletion mutaions.

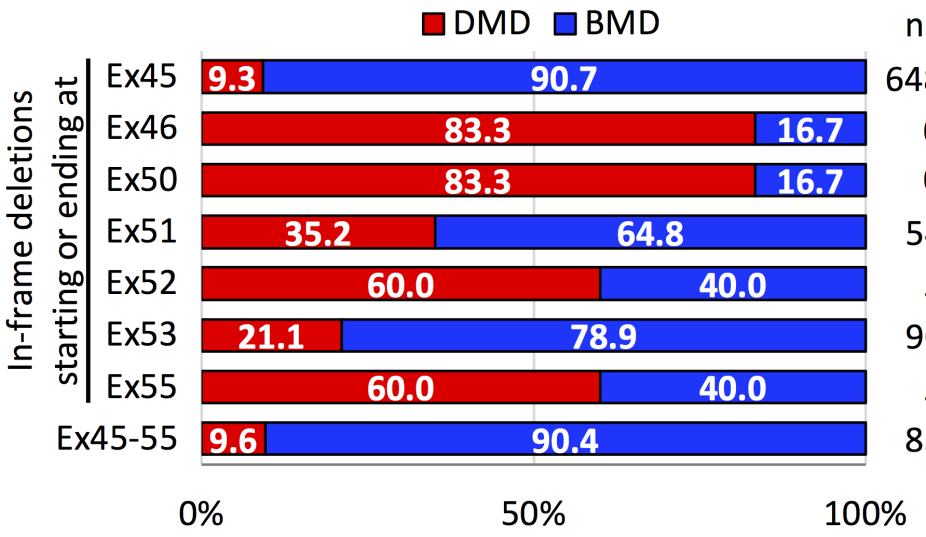
Exon no. to be skipped	% applicability of exons 45-55 skipping to:				
	Del. total (n=4929)	DMD			BMD (n=1030)
		Out-of-frame del. (n=2425)	In-frame del. (n=263)	DMD total (n=2744)	
10	14.1	18.4	5.7	16.8	5.3
9	6.9	8.0	8.4	7.9	4.8
8	13.8	10.9	7.3	10.3	26.8
7	8.7	4.3	9.2	4.7	19.8
6	6.2	7.1	5.0	6.7	5.1
5	6.0	9.1	2.3	8.3	0.5
4	2.3	2.5	2.3	2.4	1.4
3	3.7	6.1	0.4	5.4	0.1
2	1.8	0.0	4.2	0.4	6.5
1	1.8	2.6	0.0	2.3	0.1
Total	65.2	69.0	44.8	65.3	70.4

Deletion (del.) total includes patients diagnosed with DMD or BMD, and those not determined with either. Deletion types in DMD consist of deletions in the region from exon 2 to 78 where the reading frame rule is applied. DMD total and BMD include patients carrying deletions in exons 1-79. The applicability to individual deletions is shown in **Table S2**.

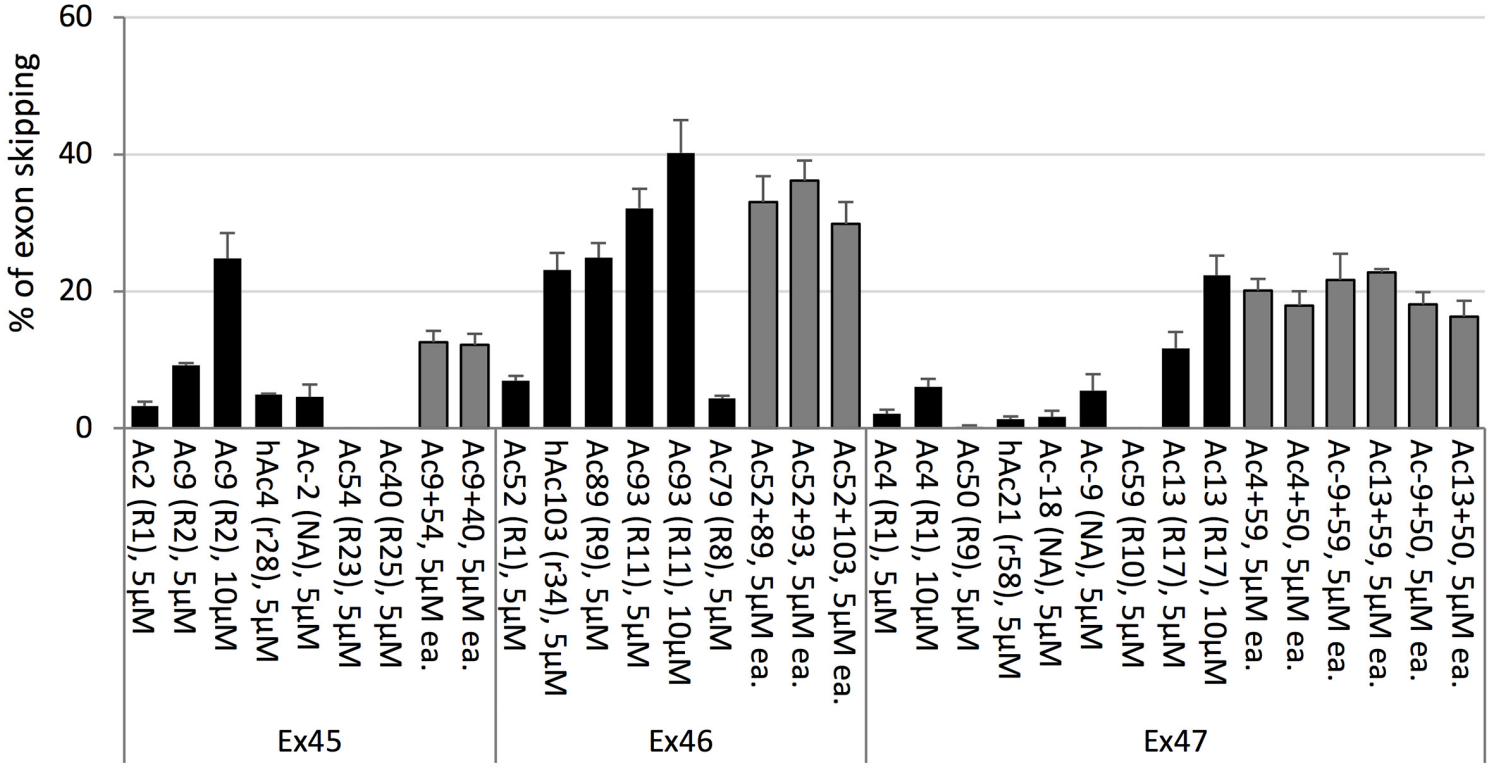
Table 2. PMO sequences composing cocktail sets and its rank with exon skipping efficiency predicted in a computational tool

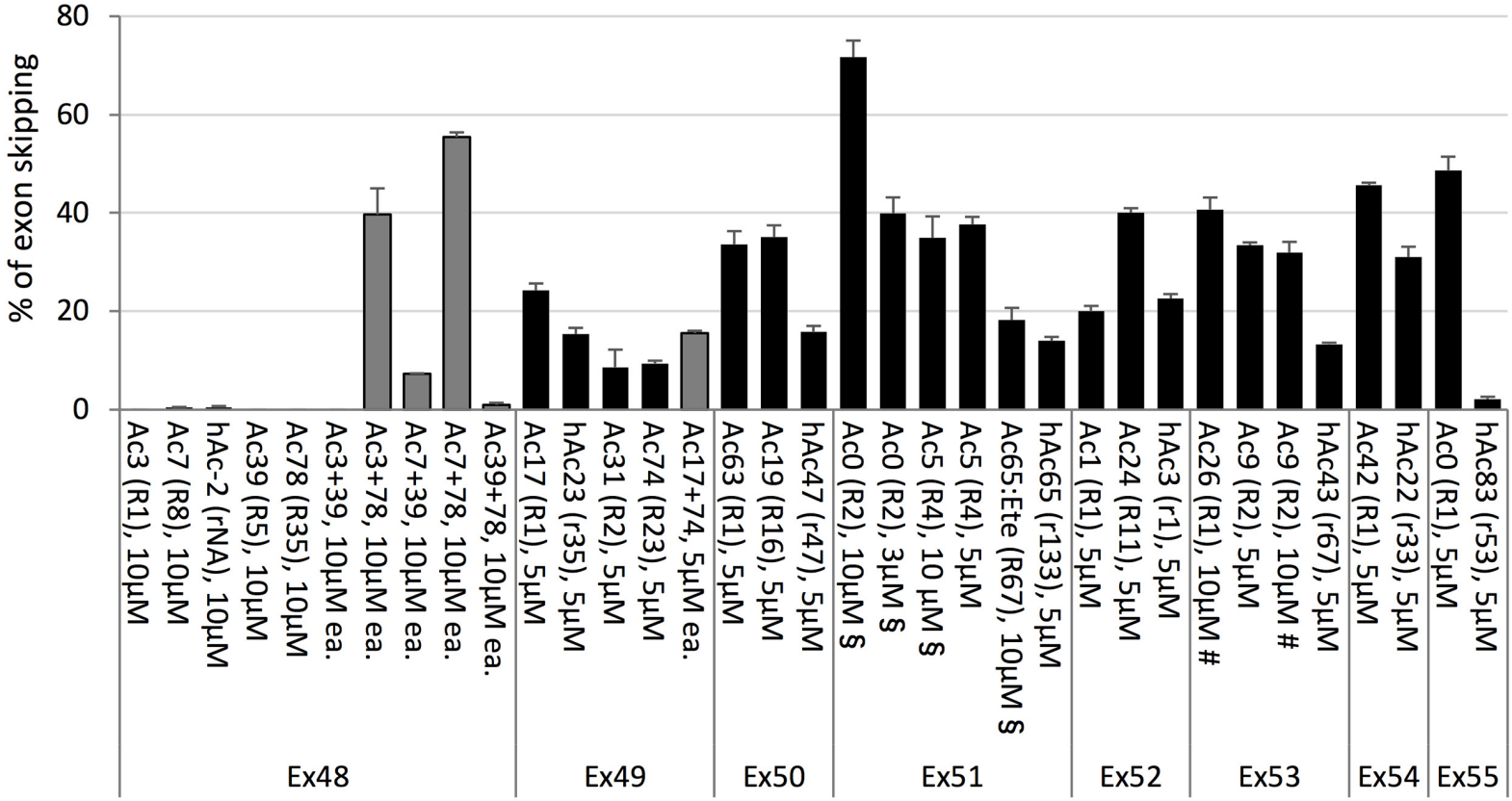
Cocktail	Name	AO sequence (5' to 3')	Rank within an exon	Predicted skipping %
Set no. 1	Ex45_Ac9_30mer	GACAACAGTTTGCCGCTGCCCAATGCCATC	2	76.2
	Ex46_Ac52_30mer	GTTATCTGCTTCCTCCAACCATAAAACAAA	1	66.7
	Ex47_Ac50_30mer	GCACTTACAAGCACGGGTCCTCCAGTTTCA	9	53.0
	Ex48_Ac7_30mer	CAATTTCTCCTTGTTTCTCAGGTAAAGCTC	8	65.0
	Ex49_Ac17_30mer	ATCTCTTCCACATCCGGTTGTTTAGCTTGA	1	90.0
	Ex50_Ac19_30mer	GTAAACGGTTTACCGCCTTCCACTCAGAGC	20	76.6
	Ex51_Ac5_30mer	AGGTTGTGTCACCAGAGTAACAGTCTGAGT	4	73.0
	Ex52_Ac24_30mer	GGTAATGAGTTCTTCCAACTGGGGACGCCT	25	90.1
	Ex53_Ac9_30mer	GTTCTTGTACTTCATCCCACTGATTCTGAA	2	73.9
	Ex54_Ac42_30mer	GAGAAGTTTCAGGGCCAAGTCATTTGCCAC	1	62.0
	Ex55_Ac0_30mer	TCTTCCAAAGCAGCCTCTCGCTCACTCACC	1	120.4
Set no. 2	hEx45_Ac4_25mer	TGCCGCTGCCCAATGCCATCCTGGA	4	42.7
	hEx46_Ac103_25mer	CTTTTAGTTGCTGCTCTTTTCCAGG	34	32.8
	hEx47_Ac21_25mer	ATTGTTTGAGAATTCCCTGGCGCAG	58	8.2
	hEx48_Ac-2_25mer	TTCTCAGGTAAAGCTCTGGAAACCT	NA	NA
	hEx49_Ac23_25mer	AATCTCTTCCACATCCGGTTGTTTA	31	41.9
	hEx50_Ac47_25mer	CTGCTTTGCCCTCAGCTCTTGAAGT	44	36.4
	hEx51_Ac65_25mer	ACATCAAGGAAGATGGCATTTCTAG	133	-5.4
	hEx52_Ac3_25mer	GCCTCTGTTCCAAATCCTGCATTGT	1	74.6
	hEx53_Ac43_25mer	ATTCAACTGTTGCCTCCGGTTCTGA	67	7.3
	hEx54_Ac22_25mer	GCCACATCTACATTTGTCTGCCACT	33	12.8
	hEx55_Ac83_25mer	GCAGTTGTTTCAGCTTCTGTAAGCC	53	32.7
Set no. 3	Ex45_Ac9_30mer	The same as the AO in the set 1	2	76.2
	Ex46_Ac93_30mer	AGTTGCTGCTCTTTTCCAGGTTCAAGTGGG	11	60.4
	Ex47_Ac13_30mer	GTTTGAGAATTCCCTGGCGCAGGGGCAACT	17	49.2
	Ex48_Ac7_30mer	The same as the AO in the set 1	8	65.0
	Ex48_Ac78_30mer	CAGATGATTTAACTGCTCTTCAAGGTCTTC	35	44.5
	Ex49_Ac17_30mer	The same as the AO in the set 1	1	90.0
	Ex50_Ac19_30mer	The same as the AO in the set 1	16	76.6
	Ex51_Ac0_30mer	GTGTCACCAGAGTAACAGTCTGAGTAGGAG	2	80.1
	Ex52_Ac24_30mer	The same as the AO in the set 1	11	90.1
	Ex53_Ac26_30mer	CCTCCGGTTCTGAAGGTGTTCTTGTACTTC	1	75.2
	Ex54_Ac42_30mer	The same as the AO in the set 1	1	62.0
	Ex55_Ac0_30mer	The same as the AO in the set 1	1	120.4

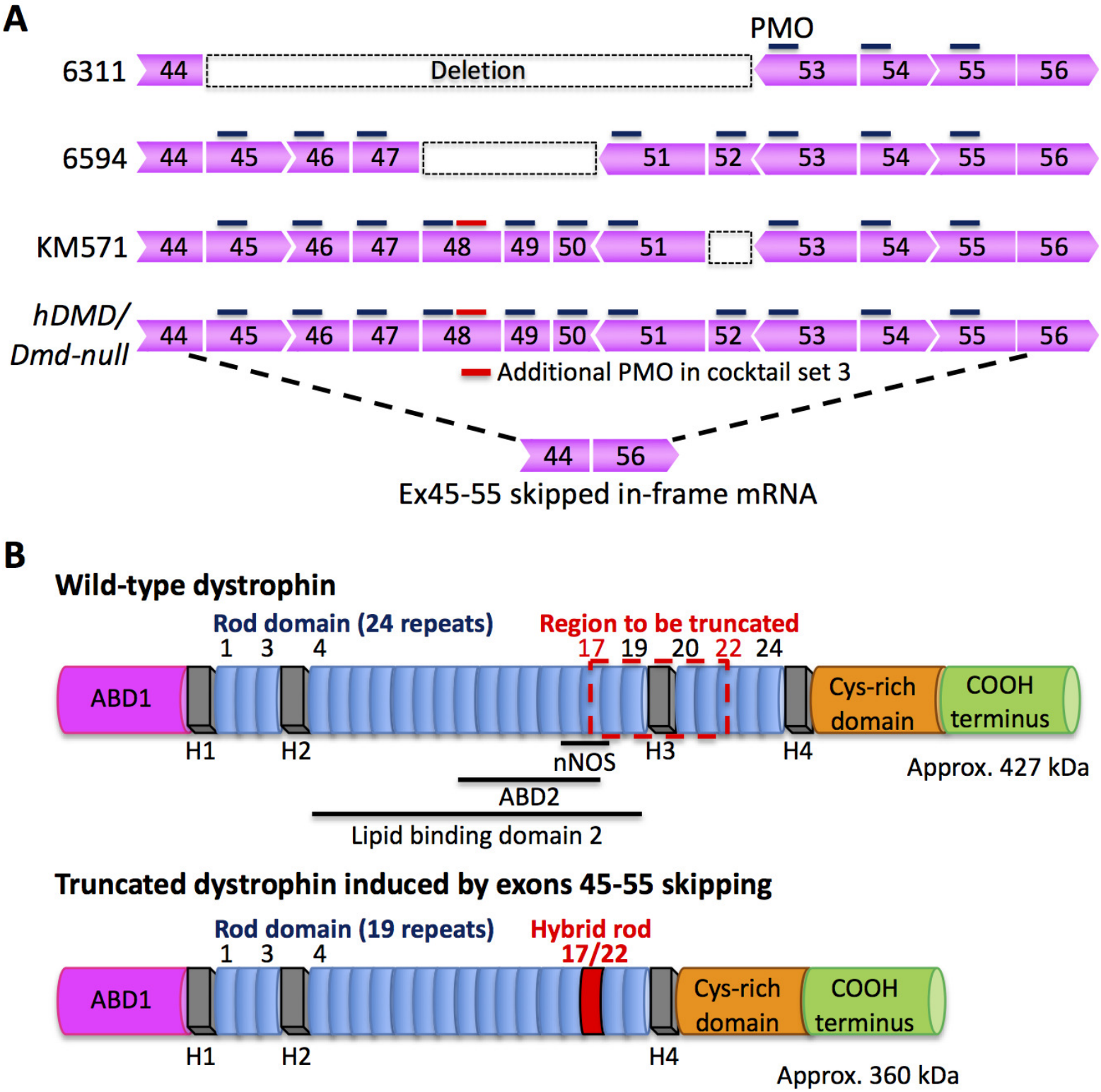
The cocktail set no. 2 was composed of human PMOs that are analog ones of mouse vivo-PMOs developed in our previous study,[20] except hEx52_Ac3_25mer. Underlined bases indicate human nucleotides substituted from mouse ones. The number following Ac indicates a distance from an acceptor splice site. NA, not available in the predictive tool used here.

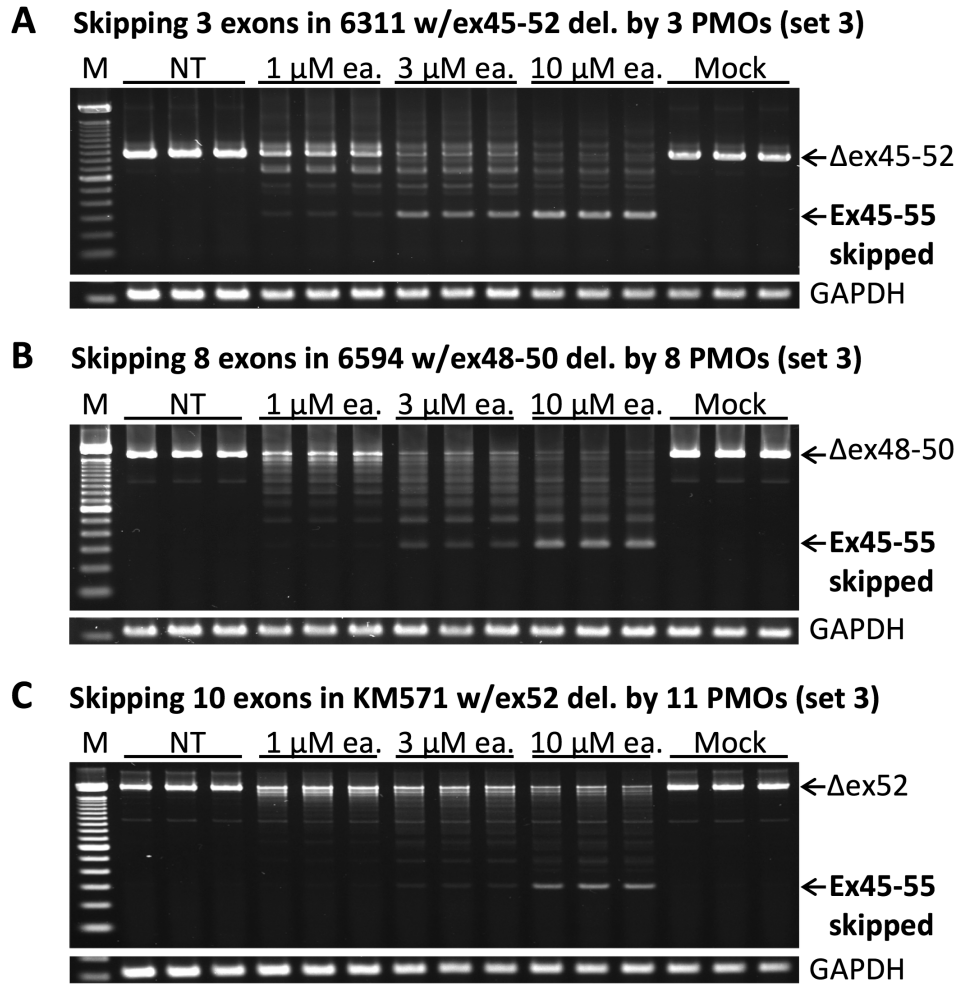


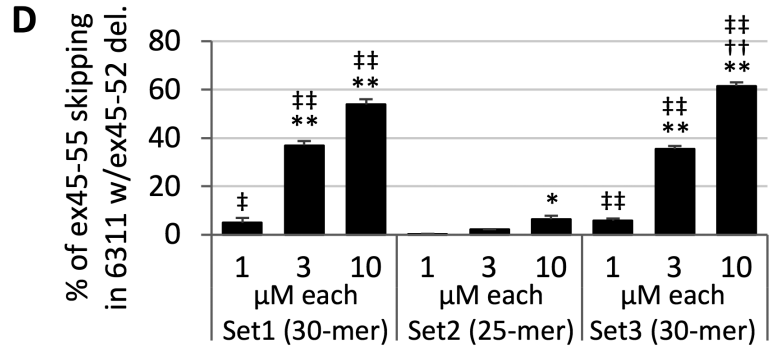
	n	Adj. p value	BMD OR (95%CI)
Ex45	648	0.907	0.96 (0.437 – 2.326)
Ex46	6	0.001**	0.02 (0.001 – 0.213)**
Ex50	6	0.001**	0.02 (0.001 – 0.213)**
Ex51	54	0.002**	0.20 (0.078 – 0.527)**
Ex52	5	0.028*	0.08 (0.008 – 0.551)*
Ex53	90	0.101	0.40 (0.160 – 1.007)
Ex55	5	0.028*	0.08 (0.008 – 0.551)*
Ex45-55	83	-	1.00

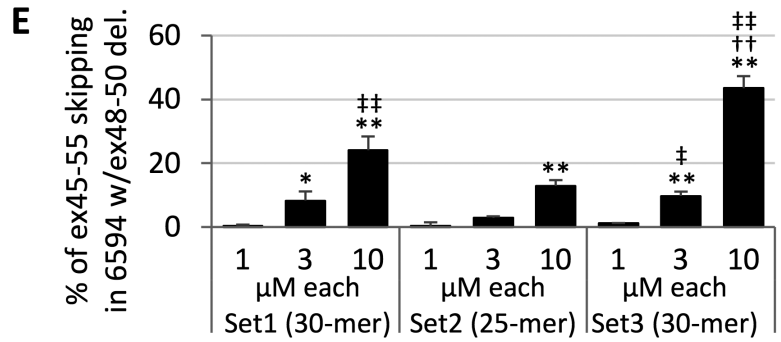

% of exon skipping
60
40
20
0
Ac2 (R1), 5µM
Ac9 (R2), 5µM
Ac9 (R2), 10µM
hAc4 (r28), 5µM
Ac-2 (NA), 5µM
Ac54 (R23), 5µM
Ac40 (R25), 5µM
Ac9+54, 5µM ea.
Ac9+40, 5µM ea.
Ac52 (R1), 5µM
hAc103 (r34), 5µM
Ac89 (R9), 5µM
Ac93 (R11), 5µM
Ac93 (R11), 10µM
Ac79 (R8), 5µM
Ac52+89, 5µM ea.
Ac52+93, 5µM ea.
Ac52+103, 5µM ea.
Ac4 (R1), 5µM
Ac4 (R1), 10µM
Ac50 (R9), 5µM
hAc21 (r58), 5µM
Ac-18 (NA), 5µM
Ac-9 (NA), 5µM
Ac59 (R10), 5µM
Ac13 (R17), 5µM
Ac13 (R17), 10µM
Ac4+59, 5µM ea.
Ac4+50, 5µM ea.
Ac-9+59, 5µM ea.
Ac13+59, 5µM ea.
Ac-9+50, 5µM ea.
Ac13+50, 5µM ea.
Ex45
Ex46
Ex47

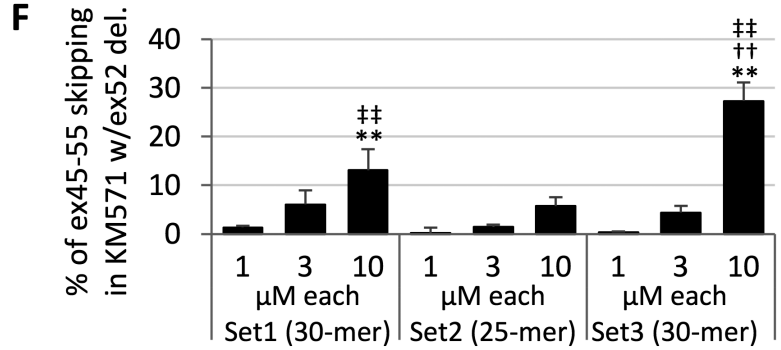

% of exon skipping
80
60
40
20
0
Ac3 (R1), 10µM
Ac7 (R8), 10µM
hAc-2 (rNA), 10µM
Ac39 (R5), 10µM
Ac78 (R35), 10µM
Ac3+39, 10µM ea.
Ac3+78, 10µM ea.
Ac7+39, 10µM ea.
Ac7+78, 10µM ea.
Ac39+78, 10µM ea.
Ac17 (R1), 5µM
hAc23 (r35), 5µM
Ac31 (R2), 5µM
Ac74 (R23), 5µM
Ac17+74, 5µM ea.
Ac63 (R1), 5µM
Ac19 (R16), 5µM
hAc47 (r47), 5µM
Ac0 (R2), 10µM §
Ac0 (R2), 3µM §
Ac5 (R4), 10 µM §
Ac5 (R4), 5µM
Ac65:Ete (R67), 10µM §
hAc65 (r133), 5µM
Ac1 (R1), 5µM
Ac24 (R11), 5µM
hAc3 (r1), 5µM
Ac26 (R1), 10µM #
Ac9 (R2), 5µM
Ac9 (R2), 10µM #
hAc43 (r67), 5µM
Ac42 (R1), 5µM
hAc22 (r33), 5µM
Ac0 (R1), 5µM
hAc83 (r53), 5µM
Ex48
Ex49
Ex50
Ex51
Ex52
Ex53
Ex54
Ex55

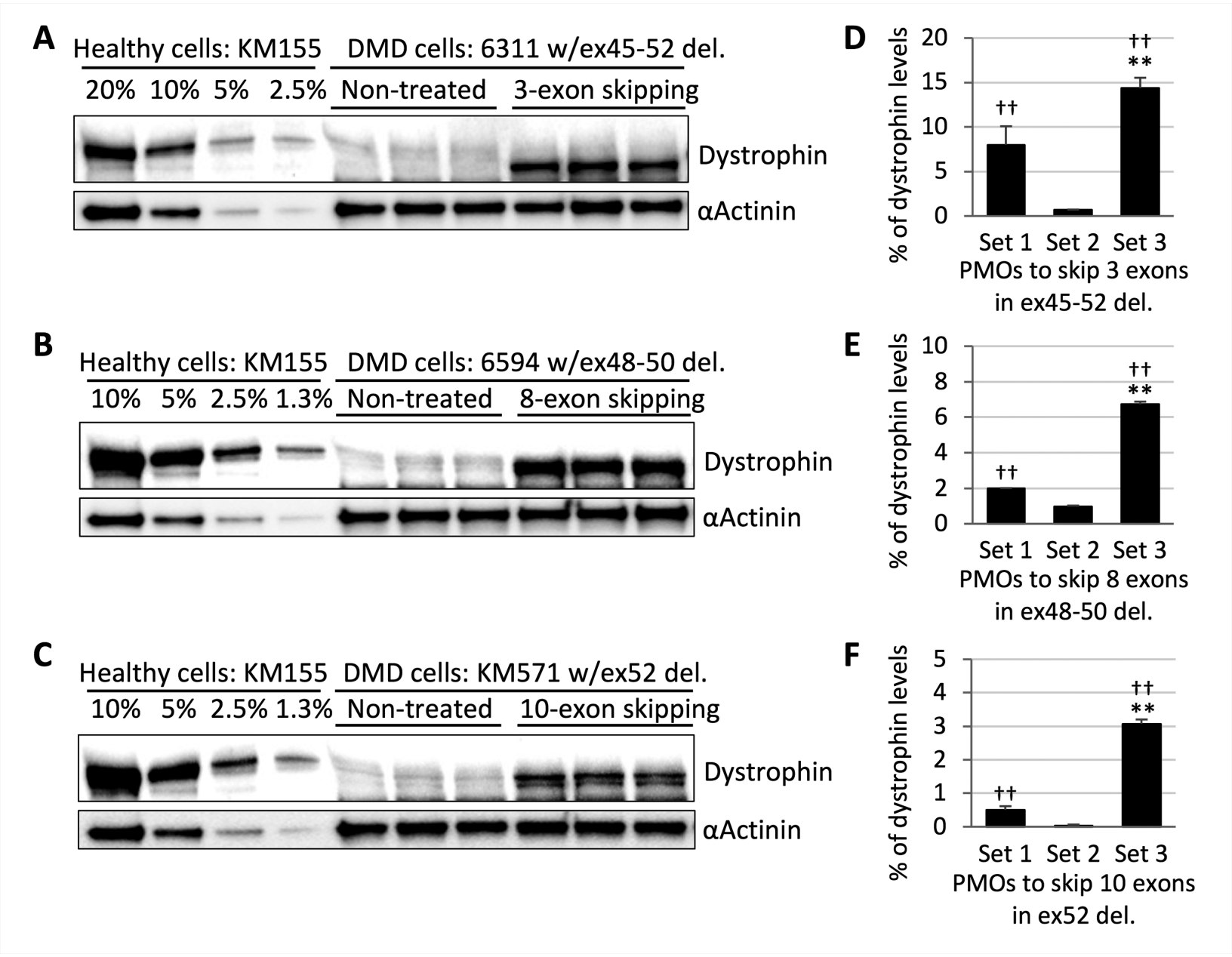

A
PMO
6311
44
Deletion
53
54
55
56
6594
44
45
46
47
51
52
53
54
55
56
KM571
44
45
46
47
48
49
50
51
53
54
55
56
hDMD/
Dmd-null
44
45
46
47
48
49
50
51
52
53
54
55
56
Additional PMO in cocktail set 3
44
56
Ex45-55 skipped in-frame mRNA
B
Wild-type dystrophin
Rod domain (24 repeats)
Region to be truncated
1
3
4
17
19
20
22
24
ABD1
Cys-rich domain
COOH terminus
H1
H2
nNOS
H3
H4
Approx. 427 kDa
ABD2
Lipid binding domain 2
Truncated dystrophin induced by exons 45-55 skipping
Rod domain (19 repeats)
Hybrid rod 17/22
1
3
4
ABD1
Cys-rich domain
COOH terminus
H1
H2
H4
Approx. 360 kDa


A
Skipping 3 exons in 6311 w/ex45-52 del. by 3 PMOs (set 3)
M
NT
1 µM ea.
3 µM ea.
10 µM ea.
Mock
←Δex45-52
←Ex45-55 skipped
GAPDH
B
Skipping 8 exons in 6594 w/ex48-50 del. by 8 PMOs (set 3)
M
NT
1 µM ea.
3 µM ea.
10 µM ea.
Mock
←Δex48-50
←Ex45-55 skipped
GAPDH
C
Skipping 10 exons in KM571 w/ex52 del. by 11 PMOs (set 3)
M
NT
1 µM ea.
3 µM ea.
10 µM ea.
Mock
←Δex52
←Ex45-55 skipped
GAPDH

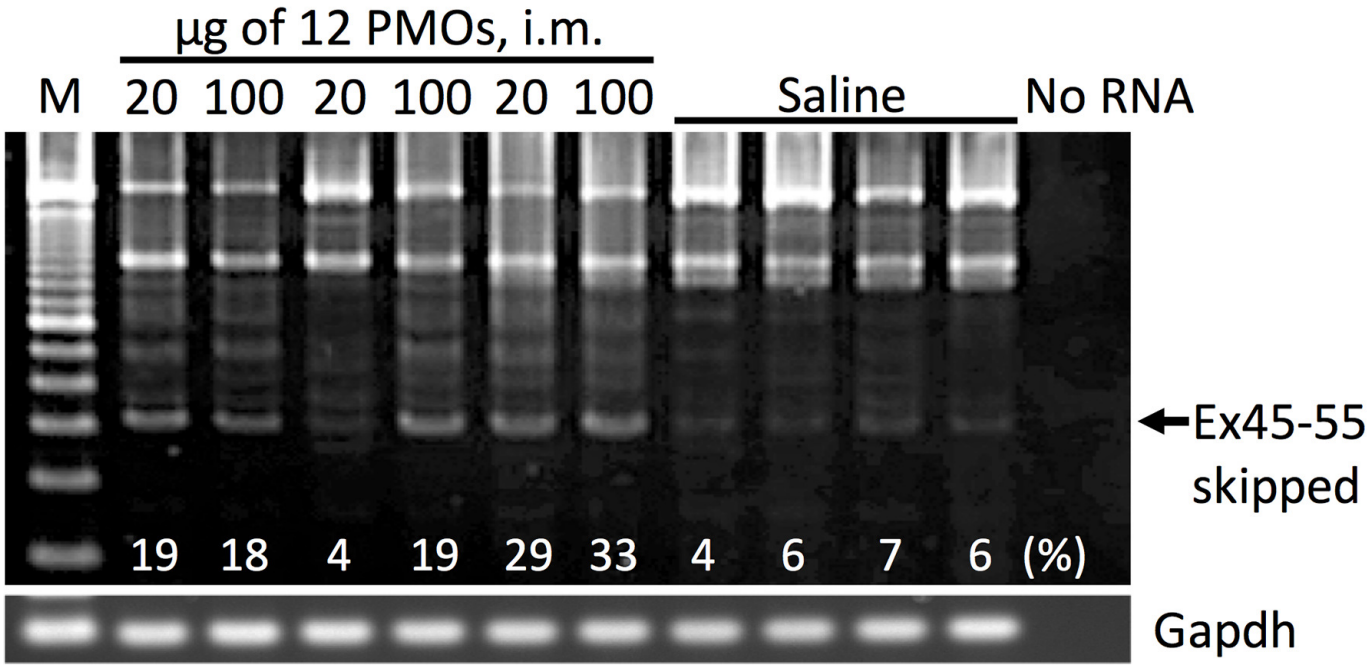

D
% of ex45-55 skipping in 6311 w/ex45-52 del.
80
60
40
20
0
1 3 10
µM each
Set1 (30-mer)
Set2 (25-mer)
Set3 (30-mer)


E
% of ex45-55 skipping in 6594 w/ex48-50 del.
60
40
20
0
1 3 10
µM each
Set1 (30-mer)
Set2 (25-mer)
Set3 (30-mer)

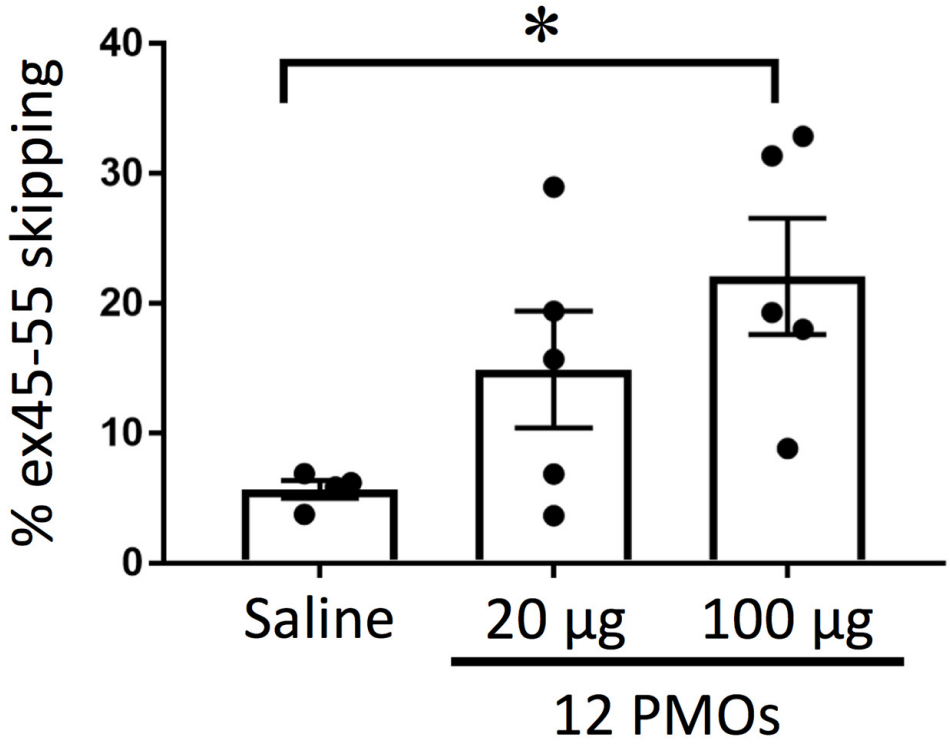

F
% of ex45-55 skipping in KM571 w/ex52 del.
40
30
20
10
0
1 3 10
µM each
Set1 (30-mer)
Set2 (25-mer)
Set3 (30-mer)


A
Healthy cells: KM155
DMD cells: 6311 w/ex45-52 del.
20% 10% 5% 2.5%
Non-treated
3-exon skipping
Dystrophin
αActinin
B
Healthy cells: KM155
DMD cells: 6594 w/ex48-50 del.
10% 5% 2.5% 1.3%
Non-treated
8-exon skipping
Dystrophin
αActinin
C
Healthy cells: KM155
DMD cells: KM571 w/ex52 del.
10% 5% 2.5% 1.3%
Non-treated
10-exon skipping
Dystrophin
αActinin
D
% of dystrophin levels
20
15
10
5
0
††
††
**
Set 1 Set 2 Set 3
PMOs to skip 3 exons
in ex45-52 del.
E
% of dystrophin levels
10
8
6
4
2
0
††
††
**
Set 1 Set 2 Set 3
PMOs to skip 8 exons
in ex48-50 del.
F
% of dystrophin levels
5
4
3
2
1
0
††
††
**
Set 1 Set 2 Set 3
PMOs to skip 10 exons
in ex52 del.

A


μg of 12 PMOs, i.m.
M
20
100
20
100
20
100
Saline
No RNA
19
18
4
19
29
33
4
6
7
6
(%)
←Ex45-55 skipped
Gapdh

B


*
% ex45-55 skipping
40
30
20
10
0
Saline
20 μg
100 μg
12 PMOs