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(54) **Title**: MEGANUCLEASE REAGENTS OF USES THEREOF FOR TREATING GENETIC DISEASES CAUSED BY FRAME SHIFT/NON SENSE MUTATIONS

(57) **Abstract**: The present invention relates to a method to treat a genetic disease in an individual caused by at least one frame shift or at least one non sense mutation in the human dystrophin gene comprising at least the step of bringing into contact at least one meganuclease enzyme, which recognizes and cuts a target site in the human dystrophin gene, with the genome of said individual under conditions wherein said at least one meganuclease recognizes and cleaves its target site in the human dystrophin gene. Said method applies also to a set of meganuclease enzymes, which each recognizes and cuts a different target site. The present invention also relates to a kit comprising, at least one meganuclease enzyme as defined above and medicament comprising said meganuclease.

Meganuclease reagents of uses thereof for treating genetic diseases caused by frame shift/non sense mutations

The present application claims the benefit of US provisional application serial n°61/333,987, filed on May 12, 2010, and of US provisional application serial n°61/272,434, filed on September 24, 2009.

The present invention relates to methods which use one or more meganuclease enzymes which can recognise and cleave a target in a gene in which a frame shift and/or non sense mutation exists which causes a human genetic disease, to correct the frame shift mutation in the gene and so cure the genetic disease. In particular the present invention relates to meganucleases which can cleave targets in the human dystrophin gene.

Duchenne Muscular Dystrophy (DMD) is a hereditary disease caused by mutations of the dystrophin gene, which leads to a premature termination of the dystrophin protein due to the presence of a nonsense mutation or a frame shift mutation, which results in a premature stop codon. These truncated dystrophin proteins cannot integrate into the dystrophin complex under the sarcolemma [1] leading to the absence of this protein [2] which in turn leads to severe muscle wastage meaning most patients are confined to a wheel chair by their teens and mortality usually occurs before the age of 40. By contrast, deletions within the dystrophin gene, which maintain the reading-frame, give rise either to asymptomatic subjects or to a Becker dystrophy in which the internally deleted form of dystrophin (with its amino- and carboxy-terminal ends intact) is present under the sarcolemma. Becker dystrophy is usually less severe than DMD, with some patients reportedly able to walk with a cane until age 65 [3].

There are currently several therapies being pursued for DMD, each with certain advantages and disadvantages:

(1) *In vivo* gene therapy with adeno-associated virus (AAV) vectors [4-8]. This approach uses AAV to introduce a truncated version of the dystrophin cDNA called micro-dystrophin or mini-dystrophin. Experiments in *mdx* mice, the standard DMD animal model, have shown that this micro-dystrophin can protect the muscle fibers and

prevent the development of the disease [9]. Nevertheless, there are some potential drawbacks to this therapeutic approach including: the limited size of the possible insert due to packaging limit of the vector, this can mean that the truncated micro-dystrophin or mini-dystrophin gene included in the vector can not fully functionally replace the full length dystrophin in the patient ; a further problem is the immune response against the AAV capsids and risks of random integration of the vector leading to further pathologies associated with this insertional mutagenesis.

(2) Cell transplantation therapy of muscle precursor cells (myoblasts, satellite cells, muscle-derived stem cells, mesoangioblasts or pericytes) [10-15]. In this approach, transplanted cells fuse with the host muscle fibers to introduce a few normal nuclei containing the normal dystrophin gene. A recent clinical trial demonstrated that myoblast transplantation does indeed restore the expression of dystrophin in up to 34% of the muscle fibers [10, 11]. However, potential limitations of this strategy include: the need to perform injections every mm of muscle (due to inefficient migration of myoblasts and the need to maintain sustained immunosuppression in patients due to the use of allogeneic cells for transplant.

(3) Pharmacologic rescue of a nonsense mutation in dystrophin using a drug such as PTC124 [16, 17]. This appears to be a promising approach but will likely be useful for less than 13-15% of DMD patients as it appears these drugs may work better for some types of non-sense mutations than others. Moreover, this drug would have to be used throughout the life of the patients and at present its long-term toxicity has not yet been evaluated.

(4) "Exon skipping" strategies, which aim to restore translation of carboxy-terminally truncated dystrophin mutants. This strategy is a promising approach for treating a large fraction of the DMD patients with a non-sense mutation, a micro-deletion or a deletion of one or several exons [18-20]. The main objective of exon-skipping strategies is to bypass one or more exons containing a frame-shifting alteration or a stop codon and to thereby restore production of a dystrophin protein, which contains wild-type amino- and carboxy-terminal sequences. Depending upon the part of the protein, which is missing, the resulting dystrophin proteins might still be able to incorpo-

rate in the dystrophin complex, essentially converting DMD patients into Becker-type patients with a less severe phenotype. Exon skipping can be induced with a short anti-sense oligonucleotide directed by complementarity to a splice donor or a splice acceptor seqeunce. To avoid rapid degradation of the oligonucleotides, they are synthesized using chemically modified 2'-O-methyl modified bases on a phosphorothioate backbone and phosphorodiamidate morpholino oligomers [21]. Nonetheless, a drawback of this approach is that, even if successful, it will require life-long administration of the exon skipping oligos. Another potential issue is that at present the long-term effects of repetitively administrating such non-degradable oligonucleotides to patients have not been investigated.

Specific gene targeting is the ultimate tool for making beneficial genetic modifications to treat a variety of genetic diseases, but its use is often limited by its low efficiency. In a number of recent studies, site-specific DNA double-strand breaks (DSBs) have been used to induce efficient gene targeting in chosen genes [22] [23, 24]. Meganucleases, also called homing endonucleases are sequence-specific endonucleases, which recognize and cleave unique large (>12 bp) target sites in living cells [25]. They can induce site-specific DSBs and thereby stimulate homologous recombination (HR) up to 10 000-fold in cultured cells [26, 27] in comparison to homologous recombination at a non-cleaved site.

Meganucleases have been used to induce HR in a variety of cell types and organisms (for review, see [28]) including mammalian cells, mice, plants, Drosophila, *E. coli* and trypanosome [29].

A meganuclease induced DSB can also be repaired by non-homologous end-joining (NHEJ), an error prone process, which frequently results in micro-insertions or micro-deletions (indels) at the site of the break [30].

Engineering highly specific, dedicated DNA endonucleases is the key to a wider usage of this technology. Several groups have developped methods to locally engineer natural meganucleases [31-33] and a combinatorial approach allowing for the complete redesign of the meganuclease DNA binding interface has been described [34].

These recently developments provide the potential to create reagents which target any chromosomal locus with engineered meganucleases.

The Applicants seeing the limitations with existing and proposed treatments for DMD and other genetic diseases associated with frame shift/nonsense mutations, have developed a new set of therapeutic materials and methods of using these to reverse the effects of the mutations causing DMD and other genetic diseases. In particular the inventors have shown that it is possible using a meganuclease to induce non-homologous end-joining (NHEJ) in the coding sequence of a gene of interest either *ex vivo* (upon an isolated tissue sample) or *in vivo*.

According to a first aspect of the present invention therefore there is provided a meganuclease enzyme which recognizes and cuts a target site in a gene of interest, wherein said gene of interest comprises at least one frame shift or nonsense mutation, for use in treating a disease caused by said at least one mutation.

In the present Patent Application a frameshift mutation is a genetic mutation caused by indels, ie. an insertion or deletion of a number of nucleotides that is not evenly divisible by three from a DNA coding sequence leading to an alteration in the codons of the following sequence and hence the final gene product.

In the present Patent Application a non sense mutation is a nucleotide change which changes a codon that specified an amino acid to one of the STOP codons (TAA, TAG, or TGA) and hence leading to a truncated final gene product.

There are a number of advantages of the approach developed by the inventors over other therapeutic strategies currently under active investigation. These include:

(1) No need for repeated long-term administration of treatment, because this therapeutic approach using meganucleases will induce permanent changes in the targeted gene of the affected progenitor cell lines and so, this treatment does not need to be given multiple times.

(2) This treatment will also avoid the use of viral vectors, which can have undesirable side effects due to uncontrolled integration events and/or adverse immune responses.

(3) This treatment will avoid the administration of non-degradable oligonucleotides.

(4) This treatment utilizes well-established and cost-effective technologies for producing pure recombinant proteins under Good Manufacturing Practice conditions.

In particular the genetic disease is one caused by a recessive mutation.

The Applicants have shown that meganucleases according to the present invention can be used for two novel strategies to correct different mutations in a variety of genes responsible for various genetic diseases, including dystrophin.

These strategies are:

(1) Deletion of nonsense mutations. Meganucleases may be used to induce a DSB at or within a few base pairs of a nonsense mutation in the targeted gene. Error-prone NHEJ-mediated repair of such a DSB leads to the introduction of micro-deletions at the DSB thereby eliminating the mutant stop codon. Assuming that the number of bases deleted for any given imperfect repair event is random, on average one out of three micro-deletions removes a number of base pairs that is a multiple of three. These deletions therefore not only eliminate the nonsense codon but also maintain the reading frame of the targeted gene. In the case of the dystrophin gene, the resulting dystrophin protein has a deletion of a few amino acids, which, by analogy with dystrophin variants found in Becker Muscular Dystrophy patients, might be expected to retain at least partial function.

(2) Restoration of reading frame for frame-shift mutations. As on average two-thirds of the indels introduced by meganuclease induced error-prone NHEJ will shift the reading frame of the coding sequence (i.e.—these alterations will be of lengths that are not multiples of 3 bps), 1 out of 3 indels lead to restoration of the reading frame for out of frame deletions. These indels, however, have to be induced at the end of the exon that precedes the out of frame deletion so that they do not induce a new stop codon within the modified exon. Alternatively, the indel could be induced at the beginning of the exon that follows the out of frame deletion, in the sequence that precedes the first stop codon induced by the frame shift deletion. As with exon-skipping strategies

currently being pursued, the resulting dystrophin mRNA encodes a short altered amino acid sequence in the middle of the protein but have intact amino- and carboxy-terminal sequences. Such variants might therefore be expected to have at least partial activity. The Applicants note that in contrast to exon-skipping strategies, dystrophin alterations induced by meganucleases are permanent because the DNA rather than the mRNA is targeted and therefore does not require repeated treatment.

In particular the present invention relates to a meganuclease enzyme which recognizes and cuts a target site in the human dystrophin gene, for use in the treatment of a genetic disease caused by at least one frame shift or nonsense mutation in the human dystrophin gene.

In accordance with another aspect of the present invention there is provided a set of meganuclease enzymes which each recognise and cut a different target site in the human dystrophin gene, for use in the treatment of a genetic disease caused by at least one frame shift or nonsense mutation in the human dystrophin gene.

In accordance with another aspect of the present invention there is provided a method to treat a genetic disease in an individual caused by at least one frame shift or at least one non sense mutation in the human dystrophin gene comprising at least the step of:

bringing into contact at least one meganuclease enzyme, which recognizes and cuts a target site in the human dystrophin gene, with the genome of said individual under conditions wherein said at least one meganuclease recognizes and cleaves its target site in the human dystrophin gene.

In particular the method may involve a set of meganuclease enzymes, which each recognise and cut a different target site in the human dystrophin gene, being brought into contact with the genome of said individual.

The inventors have in the present Patent Application demonstrated that a DMD phenotype can be rescued using a meganuclease to correct a frame shift or nonsense mutation in the coding sequence of the dystrophin gene.

This novel therapeutic approach can be used for not only DMD but for many genetic diseases that are due to a non-sense mutation(s), a frame shift mutation(s)

or to an out of frame deletion(s). Although specific meganucleases will need to be engineered for individual stop codon mutations, it is also likely that a single meganuclease will be able to re-establish the reading frame of multiple frame-shift mutations.

Although the dystrophin gene is not corrected in all of the nuclei of the muscle fibers, the correction of the dystrophin gene in only one nucleus is enough to restore the expression over several hundred microns of a muscle fiber. The inventors have previously shown that in *mdx* muscle, the introduction of one normal nucleus capable of producing dystrophin was able to restore the expression of dystrophin over a 400 μm length of the fiber despite the presence of several hundreds of nuclei that still harbored the mutated dystrophin gene [51].

It is known in the art that there are exons, which are more frequently deleted than other in DMD. A table of the frequency of these deletions is available on the Center for Human and Clinical Genetics, Leiden University web site (http://www.dmd.nl/). Using this information and the sequences of the exons that precede or follow these deletions, it is possible to identify the sequences to be targeted by meganucleases to treat a high percentage of the DMD patients (see Tables 1A and 1B).

Tables 1A and 1B list the sequences which could be targeted by meganucleases, upstream and downstream of the frame shift mutation respectively, so as to restore the reading frame of the most frequent deletions observed in DMD patients from the Netherlands (http://www.dmd.nl/). The sequence to be targeted may be located at the end of the exon, which precedes the deletion.The number of bp to be deleted has to take into account the reading frame switch. For a one reading frame shift, n+2 bp have to be deleted and for a two reading frame shift, n+1 bp is be deleted. However, the deletion must not induce a stop codon in the exon, which precedes the deletion. The sequence to be targeted by a meganuclease to restore the reading frame may also be located in the exon, which follows the deletion. However, this sequence has to be located before the first stop codon induced by the patient deletion. The sequence to be targeted to restore the reading frame for some frequent deletion, e.g., deletion of exon 44, may be the same as the sequence to be targeted for other less frequent deletion, e.g. deletion of exons 44 to

47. For the deletion of exons 46 and 47, which induces a frame shift of one, the complete sequence of the preceeding exon (exon 45) can be targeted. However, for deletion of exons 46 to 50, which induces a frame shift of two, only the end of exon 45 can be targeted (sequence (6)). Sequence (6) can be targeted by the same meganuclease and could correct all deletions, which start at exon 46. Similarly, in patients, which have a deletion of exon 44, the sequence (4) at the beginning of exon 45 may be targeted by a meganuclease. The same meganuclease could be used to restore the reading frame of patients with a deletion of exons 46 to 47, or 46 to 48 or 46 to 49. Thus using this logic, the production of only a limited number of meganucleases targeting the sequences listed in Tables 1A and 1B, could restore the reading frame of approximately 50% of the DMD deletions.

TABLE 1A

First Exon Deleted	Last Exon Deleted	Number of DMD Patients	% of DMD Patients	Number of Base Pairs Deleted	Frame Shift	Downstream Exon Targeted	Target Sequence*
44		10	4.52	148	1	43	(1)AAGTTAACAAAA TGTACAAGGACCG ACAAGG
44	47	1	0.45	622	1	43	(1)AAGTTAACAAAA TGTACAAGGACCG ACAAGG
44	50	1	0.45	1019	2	43	(2)AGGGTGAAGCTA CAGGAAGCTCTCTC CCAGCTTGATTTCC AATGGGAAA (1)AAGTTAACAAAA TGTACAAGGACCG ACAAGG
44	52	1	0.45	1370	2	43	(2)AGGGTGAAGCTA CAGGAAGCTCTCTC CCAGCTTGATTTCC AATGGGAAA (1)AAGTTAACAAAA TGTACAAGGACCG ACAAGG
45		16	7.24	176	2	44	(3)TGGCTAACAGAA GCTGAACAGTTTCT CAGAAAGACACAA ATTCCTGAGAATTG GGAACATGCTAAAT ACAAATGGTATCTT AAG

45	54	3	1.36	1589	2	44	[3]TGGCTAACAGAA GCTGAACAGTTTCT CAGAAAGACACAA ATTCCTGAGAATTG GGAACATGCTAAAT ACAAATGGTATCTT AAG
46	47	6	2.71	298	1	45	[4]GAACTCCAGGAT GGCATTGGGCAGC GGCAAACTGTTGTC AGAACATTGAATGC [5]AACTGGGGAAGA AATAATTCAGCAAT CCTCAAAAACAGAT GCCAGTATTCTACA GGAAAAATTGGGA [6]AGCCTGAATCTGC GGTGGCAGGAGGT CTGCAAACAGCTGT CAGACAGAAAAAA GAG
46	48	3	1.36	484	1	45	[4]GAACTCCAGGAT GGCATTGGGCAGC GGCAAACTGTTGTC AGAACATTGAATGC [5]AACTGGGGAAGA AATAATTCAGCAAT CCTCAAAAACAGAT GCCAGTATTCTACA GGAAAAATTGGGA [6]AGCCTGAATCTGC GGTGGCAGGAGGT CTGCAAACAGCTGT CAGACAGAAAAAA GAG
46	49	3	1.36	586	1	45	[4]GAACTCCAGGAT GGCATTGGGCAGC GGCAAACTGTTGTC AGAACATTGAATGC [5]AACTGGGGAAGA AATAATTCAGCAAT CCTCAAAAACAGAT GCCAGTATTCTACA GGAAAAATTGGGA [6]AGCCTGAATCTGCG GTGGCAGGAGGTCTG CAAACAGCTGTCAGA CAGAAAAAAGAG

46	50	6	2.71	695	2	45	[6]AGCCTGAATCTGC GGTGGCAGGAGGT CTGCAAACAGCTGT CAGACAGAAAAAA GAG
47	50	2	0.90	547	1	46	[12]AGCTTGAGCAAG TCAAG
47	52	3	1.36	898	1	46	[12]AGCTTGAGCAAG TCAAG
47	54	1	0.45	1265	2	46	[13]CAACTAAAAGAA AAGCTTGAGCAAGT CAAG
48	50	8	3.62	397	1	47	[14]AAAATAAGCTCA AGCAGACAAATCTC CAGTGGATAAAG
48	52	6	2.71	748	1	47	[14]AAAATAAGCTCA AGCAGACAAATCTC CAGTGGATAAAG
49	50	10	4.52	211	1	48	[15]CATTTGACGTTC AG
49	52	4	1.81	562	1	48	[15]CATTTGACGTTC AG
49	54	1	0.45	929	2	48	[16]CAGTTAAATCAT CTGCTGCTGTGGTT ATCTCCTATTAGGA ATCAGTTGGAAATT TATAACCAACCAAA CCAAGAAGGACCA TTTGACGTTCAG
50		6	2.71	109	1	49	[17]TGTCTAAAGGGC AGCATTTGTACAAG GAAAAACCAGCCA CTCAGCCAGTGAAG
50	52	2	0.90	460	1	49	[17]TGTCTAAAGGGC AGCATTTGTACAAG GAAAAACCAGCCA CTCAGCCAGTGAAG
51		6	2.71	233	2	50	[18]GGACTGACCACT ATTGGAGCCT
51	53	2	0.90	563	2	50	[18]GGACTGACCACT ATTGGAGCCT
51	60	1	0.45	1775	2	50	[18]GGACTGACCACT ATTGGAGCCT
52		8	3.62	118	1	51	[19]TTACTAAGGAAA CTGCCATCTCCAAA CTAGAAATGCCATC TTCCTTGATGTTGG AGGTACCTGCTCTG GCA
Total			**49.77**				

TABLE 1B

First Exon Deleted	Last Exon Deleted	Number of DMD Patients	% of DMD Patients	Number of Base Pairs Deleted	Frame Shift	Upstream Exon Targeted	Target Sequence*
44		10	4.52	148	1	45	(4)GAACTCCAGGA TGGCATTGGGCA GCGGCAAACTGT TGTCAGAACATT GAATGC
45		16	7,24	176	2	46	(10)GCTAGAAGAA CAAAAGAATATC TTGTCAGAATTT CAAAGAGATTTA AATGAATTT
44	47	1	0,45	622	1	48	(7)GTTTCCAGAGC TTTACCTGAGAA ACAAGGAGAAAT TGAAGCTCAAAT AAAAGA
46	47	6	2,71	298	1	48	(7)GTTTCCAGAGC TTTACCTGAGAA ACAAGGAGAAAT TGAAGCTCAAAT AAAAGA
44	50	1	0,45	1019	2	51	(8)CTCCTACTCAG ACTGTTACTCTG GTGACACA (9)ACCTGTGGTTA CTAAGGAA
45	50	10	4,52	871	1	51	(8)CTCCTACTCAG ACTGTTACTCTG GTGACACA
46	50	6	2,71	695	2	51	(8)CTCCTACTCAG ACTGTTACTCTG GTGACACA (9)ACCTGTGGTTA CTAAGGAA
48	50	14	6,33	397	1	51	(8)CTCCTACTCAG ACTGTTACTCTG GTGACACA
49	50	10	2,54	211	1	51	(8)CTCCTACTCAG ACTGTTACTCTG GTGACACA
46	51	3	0,76	928	1	52	(20)GCAACAATGC AGGATTTGGAAC AGAGGCGTCCCC AGTTGGAAGAAC TCATTACCGCTG CCCAAAATTTGA AAAA

	51	6	1,52	233	2	52	[20]GCAACAATGC AGGATTTGGAAC AGAGGCGTCCCC AGTTGGAAGAAC TCATTACCGCTG CCCAAAATTTGA AAAA [21]CAAGACCAGC AATCAAGAGGCT AGAACA
48	52	5	2,26	748	1	53	[11]TTGAAAGAAT TCAGAATCAGTG GGATGAAGTACA AGAACACCTTCA GAACCGGAGGCA ACAGTTGAATGA
45	52	7	3,17	1222	1	53	[11]TTGAAAGAAT TCAGAATCAGTG GGATGAAGTACA AGAACACCTTCA GAACCGGAGGCA ACAGTTGAATGA
Total			**39,21**				

*Wherein : (1) – SEQ ID NO : 1; (2) – SEQ ID NO : 2; (3) – SEQ ID NO : 3; (4) – SEQ ID NO : 4; (5) – SEQ ID NO : 5; (6) – SEQ ID NO : 6; (7) – SEQ ID NO : 7; (8) – SEQ ID NO : 8; (9) – SEQ ID NO : 9; (10) – SEQ ID NO : 10; (11) – SEQ ID NO : 11; (12) – SEQ ID NO: 28; (13) – SEQ ID NO: 29; (14) – SEQ ID NO: 30; (15) – SEQ ID NO: 31; (16) – SEQ ID NO: 32; (17) – SEQ ID NO: 33; (18) – SEQ ID NO: 34; (19) – SEQ ID NO: 35; (20) – SEQ ID NO: 36; (21) – SEQ ID NO: 37.

In particular the target site is located at the end of the exon preceding said at least one frame shift/non sense mutation.

The present invention therefore can use a meganuclease which targets a sequence upstream of the frame shift/non sense mutation in the genome.

Alternatively the target site is located after said at least one frame shift mutation in the exon.

The present invention therefore can use a meganuclease which targets a sequence downstream of the frame shift/non sense mutation in the genome.

In particular the target site is selected from the group consisting of: SEQ ID NO : 1; SEQ ID NO : 2; SEQ ID NO : 3; SEQ ID NO : 4; SEQ ID NO : 5; SEQ ID

NO : 6; SEQ ID NO : 7; SEQ ID NO : 8; SEQ ID NO : 9; SEQ ID NO : 10; SEQ ID NO :11; SEQ ID NO: 28; SEQ ID NO: 29; SEQ ID NO: 30; SEQ ID NO: 31; SEQ ID NO: 32; SEQ ID NO: 33; SEQ ID NO: 34; SEQ ID NO: 35; SEQ ID NO: 36; SEQ ID NO: 37.

Alternatively the target site may be selected from any suitable target in the human dystrophin gene (SEQ ID NO: 27).

In accordance with a further aspect of the present invention the meganuclease is a LAGLIDADG endonuclease, such as *I-Sce I, I-Chu I, I-Cre I, I-Csm I, PI-Sce I, PI-Tli I, PI-Mtu I, I-Ceu I, I-Sce II, I-Sce III, HO, PI-Civ I, PI-Ctr I, PI-Aae I, PI-Bsu I, PI-Dha I, PI-Dra I, PI-Mav I, PI-Mch I, PI-Mfu I, PI-Mfl I, PI-Mga I, PI-Mgo I, PI-Min I, PI-Mka I, PI-Mle I, PI-Mma I, PI-Msh I, PI-Msm I, PI-Mth I, PI-Mtu I, PI-Mxe I, PI-Npu I, PI-Pfu I, PI-Rma I, PI-Spb I, PI-Ssp I, PI-Fac I, PI-Mja I, PI-Pho I, PI-Tag I, PI-Thy I, PI-Tko I, I-MsoI, and PI-Tsp I*; preferably, *I-Sce I, I-Chu I, I-Dmo I, I-Csm I, PI-Sce I, PI-Pfu I, PI-Tli I, PI-Mtu I*, and *I-Ceu I*.

The LAGLIDADG family is the largest of the homing endonucleases families. This family is characterized by a conserved tridimensional structure, but displays very poor conservation at the primary sequence level, but for a short peptide above the catalytic center. This family has been called LAGLIDADG, after a consensus sequence for this peptide, found in one or two copies in each LAGLIDADG protein.

Homing endonucleases with one LAGLIDADG (L) are around 20 kDa in molecular mass and act as homodimers. Those with two copies (LL) range from 25 kDa (230 amino acids) to 50 kDa (HO, 545 amino acids) with 70 to 150 residues between each motif and act as a monomer. Cleavage of the target sequence occurs inside the recognition site, leaving a 4 nucleotide staggered cut with 3'OH overhangs.

The inventors prefer meganucleases selected from the LAGLIDADG family as members of this family have previously been shown to be very amenable to engineering so as to alter their specificity and activity [31-34].

According to a further aspect of the present invention, the gene may also be specifically targeted by a pair of Zinc Finger Nucleases (ZFNs) [52-56]. Thus the approach that the inventors have demonstrated feasible with meganucleases could also be

put into practice using pairs of ZFNs that target the same genomic sequences or any other suitable means.

In accordance with a further aspect of the present invention the meganucleases are coupled to protein transduction domains.

An attractive method for delivering dystrophin-targeted meganuclease *in vivo* is the use of protein transduction domains (PTDs) that can penetrate directly into muscle fibers. PTDs harbor a high density of basic amino acid residues (Arg and Lys), which are critical for their transduction function (recently extensively reviewed by Chauhan et al. [40]). Proteins as large as 110 kDa coupled to a PTD have been transduced into different cells [41] and systemic injection of such fusion proteins has demonstrated the effectiveness PTD-mediated protein delivery *in vivo* [41, 42]. Various active PTDs have been described including Penetratin, Polylysine, Polyarginine, Tat, VP22, [43] Syn B1 [44] FGF-4 [45, 46], anthrax toxin derivative 254-amino acids (aa) peptide segment, diphtheria toxin'R ' binding domain, MPG (HIV gp41/SV40 Tag NLS), pep-1, WR peptide, and exotoxin A. The protein transduction domain of the HIV Tat protein (11 amino acids: YGRKKRRQRRR SEQ ID NO : 12) has been used to efficiently transduce a coupled plasmid into skeletal muscles [47]. It has been shown that a Tat-EGFP fusion protein can penetrate muscle fibers [48]. Finally, VP22 fused with micro-dystrophin has been transduced into cells [49] while Tat-utrophin has been transduced directly into muscle fibers [50].

According to a further aspect of the present invention therefore there are provided meganuclease-PTD fusion proteins.

Such meganuclease-PTD fusion proteins efficiently deliver meganucleases in not only muscle fibers but potentially into satellite cells.

In particular the meganuclease or set of meganucleases, comprise a HIV TAT PTD.

It is possible that meganuclease-PTDs or ZFN-PTDs may be immunogenic but this may not be an issue as the proteins only need to be administered one time to effect permanent changes in the targeted gene. Another possibility might be to use transient immunosuppression when the proteins are administered.

According to a further aspect of the present invention the meganuclease or set of meganucleases as defined above, are encoded at least one purified nucleic acid molecule.

The present invention may be implemented using purified peptide versions of the meganucleases or alternatively the present invention may be implemented using purified nucleic acid molecules encoding these meganucleases.

According to a further aspect of the present invention there is provided a kit comprising, a purified meganuclease peptide or a set of purified meganuclease peptides or at least one purified nucleic acid encoding a meganuclease or a set of meganucleases as defined above and instructions for there use.

According to a further aspect of the present invention there is provided a medicament comprising:

a purified meganuclease peptide or a set of purified meganuclease peptides or at least one purified nucleic acid encoding a meganuclease or a set of meganucleases as defined above, or a pharmaceutically acceptable salt thereof; and further comprising at least one of

a preservative;

a stabiliser;

an excipient;

a vehicle.

In particular wherein the meganuclease or set of meganucleases comprise a protein transduction domain.

For a better understanding of the invention and to show how the same may be carried into effect, there will now be shown by way of example only, specific embodiments, methods and processes according to the present invention with reference to the accompanying drawings in which:

Figure 1: Schematic representation of Meganucleases RAG1 and I-SceI (A) used for episomal gene repair of mutated plasmid dog micro-dystrophin/V5 (target) (B). (A). The expression of both meganucleases RAG1 and I-SceI is drived by translation elongation factor 1 alpha (EF-1a) promoter. The transcription unit is composed

mainly by the presence of exon1 and intron 1 of EF-1a gene fused to MGN cDNA harboring HA-tag and a nuclear localization signal (NLS) at the N-terminal while SV40 polyadenylation site (SV40 polyA) is present in C-terminal following stop codon. (B). Plasmids containing the dog micro-dystrophin/V5 are represented under three different constructs. One of these constructs is the wild type micro-dystrophin/V5 (without target insertion) and the other two constructs show respectively insertion of specific target for RAG1 and I-SceI (nucleotide box) near of the Nhe1 resulting in an out of frame expression of micro-dystrophin/V5 and creating stop codons (TAG underlined). The capital letters GCTAGC represent Nhe1 restriction enzyme site coding for amino acids alanine (A) and serine (S) in wild type micro-dystrophin/V5 construct, while HCSQVPQPAC-HGKTKQFT-SSNGSPE (SEQ ID NO: 59) and HARDNRVIC-HESRI (SEQ ID NO: 60) represent respectively amino acid changes for mutated dog micro-dystrophin/V5 constructs containing a target for RAG1 or I-SceI.

Figure 2: Western blot analysis of dog micro-dystrophin/V5 expression in 293FT cells showing that episomal gene repair by Meganucleases RAG1 and I-SceI is able to restore micro-dystrophin/V5 protein expression from the mutated dystrophin constructs. 293FT cells were co-transfected in 6 well plates with meganucleases RAG1 (A) or I-SceI (B) and mutated dog micro-dystrophin/V5 containing specific target for MGNs. (A). Dog micro-dystrophin/V5 expression shown in lanes 1 to 4 results from co-transfection in 293FT cells of different amounts of MGN RAG1 plasmid with the plasmid containing mutated dog micro-dystrophin/V5 containing RAG1. Lanes 1 and 2 contain 3.8 mg MGN RAG1 + 200 ng plasmid dystrophin target RAG1; lanes 3 and 4 contain 2.8 mg MGN RAG1 + 1200 ng plasmid micro-dystrophin/V5 with the RAG1 target. Lanes 5, 6 and 7 correspond to the following co-transfections: lane 5 (negative control): 1200 ng plasmid micro-dystrophin/V5 with the RAG1 target + 2.8 mg of a plasmid containing EGFP; lanes 6 and 7 (positive controls) respectively: 200 ng or 1200 ng of wild type dog micro-dystrophin/V5 + 3.8 mg or 2.8 mg of a plasmid containing EGFP. (B) As for section A, the co-transfection mixes were as follows: lanes 1 and 2: 3.8 mg MGN I-SceI + 200 ng of micro-dystrophin/V5 plasmid containing the I-SceI target; lanes 3 and 4: 2.8 mg MGN ISce1 + 1200 ng of the micro-dystrophin/V5 plasmid

containing the ISce1 target; lane 5 (negative control): 1200 ng of the micro-dystrophin/V5 plasmid containing the ISce1target + 2.8 mg of a plasmid containing EGFP; lanes 6 and 7 (positive controls) respectively: 600 ng or 1200 ng of the wild type dog micro-dystrophin/V5 plasmid + 3.4 mg or 2.8 mg of a plasmid containing EGFP.

Figure 3: Surveyor® Nuclease digestion products of amplicons derived from mutated dog micro-dystrophin/V5 DNA treated with Meganucleases RAG1 or I-SceI in 293FT cells. In panels A and B, the 681-bp (lanes 1, 2 and 3) and the 657-681 amplicons (lanes 4 and 5) were obtained by PCR amplification with Phusion™ High-Fidelity DNA Polymerase and purified as described in the material and method section. These amplicons were generated from genomic DNA extracted from 293FT co-transfected with the mutated dog micro-dystrophin/V5 plasmid containing a specific target and the meganuclease plasmid specific for that target as described for western blot (figure 2A and B), The 293FT cells from a given well were detached from the plate and divided in two parts: one for protein analysis by Western blot (figure 2) and the other for genomic DNA analysis. Amplicons obtained from genomic DNA were assessed for the presence of mismatches by digestion with the Surveyor® nuclease (Figure 3A and 3B, lanes 1, 2 and 3). In both panels A and B, the lanes 1 represent negative controls, i.e., amplicons originating from genomic DNA extracted from 293FT cells co-transfected with 1200 ng of a mutated micro-dystrophin/V5 plasmid containing either the target RAG1 or ISce1+ 2.8 mg and with a plasmid containing EGFP (this DNA originates from the same cells as those used in lanes 5 in Western blot, figure 2A and B). These amplicons (homoduplex) were digested with the Surveyor® nuclease. This resulted in no specific cleavage as shown in the lanes 1 of the two panels. The lanes 2 and 3 of panels A and B represent products digestion by the Surveyor® nuclease of amplicon mixtures (homoduplex (negative control amplicon) and heteroduplex (amplicons produced from genomic DNA of 293FT cells co-transfected with a plasmid target and its specific MGN). The presence of of amplicon heteroduplexes in lanes 2 and 3 for panels A and B was confirmed by the cleavage of the amplicon mixtures by the Surveyor® nuclease in specific fragments of 400 and 320 bp (white arrows). For panels A and B, the lanes 4 show the absence of specific cleavage by Surveyor® nuclease for amplicon (homoduplex)

provided by genomic DNA from 293FT cells co-transfected with the wild type dog micro-dystrophine/V5 plasmid and with an EGFP plasmid. Lanes 5 represent specific cleavage by Surveyor® nuclease of the homoduplex mixture of amplicons originating from wild type micro-dystrophin/V5 plasmid and the mutated micro-dystrophin/V5 plasmid. In lanes 5 of both panels, the sizes of specific fragments (370 and 300 bp) following digestion by the Surveyor® nuclease were lower than those observed in lanes 2 and 3. The schema in C comparing the mutated dog micro-dystrophin/V5 with the wild type dog micro-dystrophin/V5 explains that the size differences of the fragments generated by the Surveyor® nuclease are due to the presence of the specific target for MGNs (black box) located near of the NheI site. Note the correspondence between the restoration of the expression of dystrophin observed by Western Blot (Figure 2A and B, lanes 1 and 2 or 3 and 4) and the cleavage by Surveyor® nuclease of a hetero/homoduplex in figure 3 (Panels A and B, lanes 2 or 3). These observations confirm the specificity of MGNs RAG1 and I-SceI for their own target present in mutated micro-dystrophin/V5 constructs.

Figure 4: Amino acid and DNA sequences of the mutated dog micro-dystrophin/V5 showing modification by the MGN RAG1 on its specific target leading to the restoration of the dystrophin expression. Two days after the co-transfection of 293FT cells with the a dog micro-dystrophin/V5 plasmid containing the RAG1 target and with after the RAG1 MGN, the region containing the RAG1 sequence was amplified, cloned and sequenced to confirm that the MGN cleavage was the molecular basis of the restoration of dystrophin expression observed in figure 2. (A) Amino acids sequence revealed that four clones (2, 3, 4 and 5) showed corrections of the micro-dystrophin/V5 expression but each of them differs in their amino acids (underlined). Two other clones (6 and 7) produced amino acid sequences that did not correspond to those of micro-dystrophin/V5 because the correct reading frame was not restored by the micro-deletion (lane 6) or the micro-insertion (lane 7). (B) Nucleotide sequence alignments revealed distinct MGN-induced insertions and deletions within the target region of RAG1 except for clone 7 showing more complex NHEJ mechanism. This

figure illustrates that deletions or insertions induced by NHEJ are able to restore the normal reading frame of a mutated dystrophin gene.

Figure 5: Direct intramuscular co-electroporation in muscle fibers of a mutated dog micro-dystrophin/V5 plasmid containing a MGN target and of a MGN plasmid restored micro-dystrophin/V5 expression *in vivo*. In A) the micro-dystrophin/V5 plasmid with a MGN target sequence was electroporated alone in the muscle (as a negative control), only rare weaky labeled muscle fibers were detected 2 weeks later by immunohistochemistry (in red) using an anti-V5 mAb. In B) the micro-dystrophin/V5 plasmid without a MGN target sequence was electroporated alone in the muscle (as a positive control), abundant muscle fibers expressed the V5 epitope. In C) the micro-dystrophin/V5 plasmid with a RAG1 target sequence was co-electroporated with a plasmid coding for RAG1, abundant muscle fibers expressed the V5 epitope. In D) the micro-dystrophin/V5 plasmid with an I-SceI target sequence was co-electroporated with a plasmid coding for I-SceI, abundant muscle fibers expressed the V5 epitope. Figure E, summarizes for both MGNs at 2 different concentrations the total numbers of dystrophin/V5 muscle fibers observed in 10 sections collected throughout the muscles at 150 mm intervals.

Figure 6 : The restoration of micro-dystrophin expression in the previous experiment is due to mutation of the targeted plasmids. Various plasmids were electroporated into mouse muscles: in lane 1 plasmids coding for the mutated micro-dystrophin/V5 containing a sequence targeted by I-*Sce*I (m-dyst/$V5_{I\text{-}SceI}$) and for the I-*Sce*I meganuclease; in lane 2 the plasmid coding for wild type micro-dystrophin (no target for the I-*Sce*I meganuclease); in lane 3 the m-dyst/$V5_{I\text{-}SceI}$ electroporated alone. The DNA was extracted from these muscles 15 days later and the region targeted by the meganuclease I-*Sce*I was amplified. The amplicons were then digested with the Surveyor® enzyme. The presence of the two bands at 300 and 370 bp are due to the presence of some hetero-duplexes that were cut by the Surveyor® enzyme confirming that the I-*Sce*I meganuclease had mutated the micro-dystrophin target plasmid. Lane 4 is a positive control for the Surveyor® enzyme reaction. The DNA of myoblasts electroporated with a plasmid coding for mutated micro-dystrophin containing a sequence

targeted by I-*Sce*I (m-dyst/$V5_{I\text{-}SceI}$) was mixed with the DNA of myoblasts electroporated with a plasmid coding for mutated micro-dystrophin containing a sequence targeted by Rag1 (m-dyst/$V5_{Rag1}$). The targeted regions were amplified and digested with the Surveyor® enzyme. As heterodimers were formed the Surveyor® enzyme cut the amplicons.

Figure 7 Mutations by meganucleases of targeted micro-dystrophin genes integrated in myoblasts. The lentivirus used contain, under a CMV promoter, either a mutated micro-dystrophin/V5 gene with a inserted target sequence for Rag1 (m-dyst/$V5_{Rag1}$) or a mutated micro-dystrophin/V5 gene with a inserted target sequence for I-*Sce*I (m-dyst/$V5_{I\text{-}SceI}$). These lentivirus also contained a puromycin resistance gene under a SV40 promoter. These lentivirus were used to infect human myoblasts. The day before the infection, 250 000 myoblasts were seeded per well in a 6 wells plate. For the infection, the medium was removed and replaced by 3 ml of 0,45 µl filtered supernatant from 293T lentivirus producing cells. The human myoblasts were obtained from a muscle biopsy of a healthy cadaveric donor. After an overnight incubation, the medium was changed by 3 ml of MB1 medium and cells were proliferated 48h. The infected cells were than selected with puromycin at 2 µg/ml. After 48h with the selection agent (time required to kill all control cells without virus), the medium was changed and cells were proliferated until they reached confluence. Cells were than ready to perform nucleofection experiment with a meganuclease plasmid. Some selected myoblasts were than nucleofected with a plasmid coding either for the Rag1 meganuclease or the I-*Sce*I meganuclease. Control myoblasts were not nucleofected with a meganuclease. Three days later the DNA was extracted from all myoblasts. The region coding for the targeted sequences for Rag1 or I-*Sce*I were amplified by PCR. These amplicons were then digested with the Surveyor® enzyme to verify the presence of hetero-dimers due to insertions or deletions produced by the meganuclease in the genome integrated micro-dystrophin V5 gene. The figure illustrates the results of the Surveyor® reactions. Lane 1 represents the Surveyor® product of amplicons obtained from myoblasts containing the m-dyst/$V5_{Rag1}$ but not nucleofected with a meganuclease. Lane 2 represents the

Surveyor® product of amplicons obtained from myoblasts containing the m-dyst/$V5_{I\text{-}SceI}$ but not nucleofected with a meganuclease. In lanes 1 and 2, the highest band at 670 bp is the amplicon. The lowest band is the primer dimers. There are no bands at 300 and 370 bp because no meganuclease was nucleofected to mutate the m-dyst/V5 genes. Lane 3 represents the Surveyor® product of amplicons obtained from myoblasts containing the m-dyst/$V5_{Rag1}$ and nucleofected with the Rag1 meganuclease. Lane 4 represents the Surveyor® product of amplicons obtained from myoblasts containing the m-dyst/$V5_{I\text{-}SceI}$ and nucleofected with the I-*Sce*I meganuclease. In lanes 3 and 4, there are bands at 300 and 370 bp because the adequate meganuclease was nucleofected to induce indels in the m-dyst/V5 genes. The presence of mutated amplicons led to a cut of the amplicons by the Surveyor® enzyme. Lanes 5, 6 and 7 are positive controls for the Surveyor® enzyme, e.i., the amplicons obtained from cells containing the m-dyst/$V5_{Rag1}$ were mixed in different proportions (respectively 1 to 1, 5 to 1 and 10 to 1) with the amplicons obtained from cells containing the m-dyst/$V5_{I\text{-}SceI}$ gene. As different inserts were present, heteroduplex were formed and were cut by the Surveyor® enzyme. In lanes 5, 6 and 7 as the DNA mixtures were 1 to 1, 5 to 1 and 10 to 1, there were respectively roughly 50%, 20% and 10% of hetero-duplexes that were formed and thus cut by the Surveyor® enzyme. Note than in lane 3, the intensity of the bands at 300 and 370 bp were slightly more than in lane 7 but less than lane 6, thus the Rag1 meganuclease (lane 3) as mutated between 10% and 20% of the target m-dyst/$V5_{Rag1}$.

Figure 8: Expression of meganucleases DMD21, DMD31 and DMD33 in 293FT cells.

Cells were washed twice in HBSS and were lysed in 200 µl of lysis buffer containing 20mM Tris PH7.5, 1 mM DTT, 1 mM PMSF and 1% SDS. Protein samples were prepared as follows: In microtubes containing the 200µl of lysed cells, 600 µl of methanol , 200 µl of chloroform, 500 µl of water were added. After each liquid addition, microtubes were vortexed. Microtubes were centrifuged one minute at 14800 RPM and the solid white phase at the interphase was recuperated and washed with 300 µl of methanol. White pellet were lyophilized. Pellets were boiled in 40 µl of loading buffer containing Tris pH6.8 0.25M, 10% SDS, 7.5% glycerol and 0.5% beta-mercaptoethanol.

10 µl were loaded on 10% gel SDS-page electrophoresis. After protein electrotransfert on nitrocellulose membrane, the membrane was blocked in PBS-Tween (0.05%) containing 5 % milk for one hour. The membrane was incubated overnight at 4 ^{0}C with a rabbit anti-I-Cre antibody (1/20000). The membrane was washed 3 times 10 minutes in PBS-Tween (0.05%). After, the membrane was incubated one hour with a goat anti-rabbit-HRP antibody (1/2000), washed 3 times in PBS-Tween (0.05%) and revealed with a chemiluminescence kit. Lane CTL represents a control experiment (no meganuclease expression), lane RAG represents RAG expression, lanes 2874 and 3387 respectively represent DMD21 2874 and 3387expression, lanes 3631 and 3633 respectively represent DMD31 3631 and 3633 expression, and lanes 3326 and 3330 respectively represent DMD33 3326 and 3330 expression.

There will now be described by way of example a specific mode contemplated by the Inventors. In the following description numerous specific details are set forth in order to provide a thorough understanding. It will be apparent however, to one skilled in the art, that the present invention may be practiced without limitation to these specific details. In other instances, well known methods and structures have not been described so as not to unnecessarily obscure the description.

Example 1: Material and methods

Meganucleases RAG1 and I-*Sce*I.

Functional meganucleases (MGNs) RAG1(SEQ ID NO : 18) and *I-Sce-I* (SEQ ID NO : 13) were used as plasmids containing a transcriptional unit as described in the figure 1A.

The nucleotide sequences of these constructs are provided as SEQ ID NO: 19 and SEQ ID NO: 21; the nucleotide sequences of the plasmids encoding these meganucleases are also provided, SEQ ID NO: 20 (pCLS2262 encoding RAG1) and SEQ ID NO: 22 (pCLS2209 encoding I-*Sce*I).

Plasmid vectors containing the dog micro-dystrophin fused to V5 with insertion of the RAG1 or I-SceI target sequence

Dog micro-dystrophin cDNA (3.8kb) (SEQ ID NO: 23) contained in an adeno-associated viral plasmid (gift from Dr Xiao Xiao [37], University of Pittsburgh,

Pittsburgh, PA) was amplified by polymerase chain reaction (PCR) with Phusion™ High-Fidelity DNA Polymerase (New England Biolabs, Pickering, Canada). The amplification was performed using Forward 5'-gacagttatcaaacagctttggaag-3' (pos 1027-1051 dog microdystrophin cDNA) (SEQ ID NO: 25) and Reverse 5'-gtaatctgtgggtgtcttgtaaaaga-3' (pos 1684-1659 dog microdystrophin cDNA) (SEQ ID NO: 26). Amplification products were then treated 10 min at 72°C with *Taq* DNA Polymerase (New England Biolabs) and cloned in a TA cloning vector, i.e., pDrive (Qiagen, Mississauga, Canada).

The resulting clones were sequenced to confirm the integrity of dystrophin nucleotide sequence. The micro-dystrophin cDNA was introduced in a directional TOPO vector (Invitrogen, Carlsbad, CA) in phase with epitope V5 present in the plasmid. The blasticidin resistance gene in the original vector has been replaced by a puromycin resistance gene. The final construct of dog micro-dystrophin cDNA was fused in C-terminal with the V5 epitope making the WILD TYPE micro-dystrophin/V5 (figure 1B). A unique SalI restriction site has been added to the 5' of this dog micro-dystrophin/V5 cDNA. The presence of another unique NheI site present at position 1313 within the micro-dystrophin cDNA permitted us to introduce specific target sites for MGN RAG1: 5'-ttgttctcaggtacctcagccagca-3' (SEQ ID NO : 14) or for MGN I-*Sce*I: 5'-cacgctagggataacagggtaata-3' (SEQ ID NO : 15) by PCR. For this PCR, the reverse primer contained the target sequence for either RAG1 or I-*Sce*I and a NheI restriction site and the forward primer contained a SalI restriction enzyme site. After amplification of the WILD TYPE micro-dystrophin/V5 plasmid with the previous primers and cloning in the pDrive vector (Qiagen), the fragment SalI/NheI (1300 bp) containing the target sequence for one of the specific meganucleases was sequenced and cloned in the WILD TYPE micro-dystrophin/V5 plasmid also cut with SalI/NheI (removing the original fragment and replacing it by the mutated fragment) and making final constructs MUTATED micro-dystrophin/V5 containing the RAG1 or I-*Sce*I target (see figure 2B). These mutated micro-dystrophin/V5 constructs resulted in an out of frame micro-dystrophin/V5 expression and create stop codons which made it impossible to express the V5 epitope peptide as shown in figure 1B.

293FT cells culture

For the present studies, the inventors used 293FT cells, purchased from Invitrogen, as recipient cells for co-transfection of mutated dog micro-dystrophin/V5 constructs with one the MGN plasmids. The transfections were made with lipofectamine 2000 (Invitrogen) since these cells are easily transfected with this reagent as indicated by the manufacturer. Moreover, these cells are weakly attached on the bottom of plates in culture avoiding the use of trypsin to detach the cells making the possibility to easily divide the cells in two parts for analysis of proteins and of DNA from the same well of a 6 well plate. The 293FT cells were grown in DMEM high glucose, 10% bovine serum, 4 mM glutamine and 1X penicillin/streptomycin. All of these components were purchased from Wisent, Montreal, Canada. To confirm the occurrence of NHEJ in the endogenous dystrophin gene of 293FT cells, 500000 293FT cells were plated per well in a 6 wells plate. The day after, cells were transfected with the lipofectamine 2000 transfection agent. 4 µg of each meganuclease plasmid, namely pCLS2874 (SEQ ID NO: 53), pCLS3387 (SEQ ID NO: 54), pCLS3631 (SEQ ID NO: 55), pCLS3633 (SEQ ID NO: 56), pCLS3326 (SEQ ID NO: 57), and pCLS3330 (SEQ ID NO: 58), were used. 72 hours after transfection, cells were detached from the well and split in two for proteins and genomic DNA extraction.

Episomal micro-dystrophin/V5 gene repair in 293FT cells

For the studies of the episomal gene repair, 293FT cells (Invitrogen) in 6-well plates were co-transfected in the presence of lipofectamine 2000 (Invitrogen) with 200 ng or 1200 ng of pLenti6/V5 MUTATED dog micro-dystrophin/V5 containing the RAG1 or I-*Sce*I target. Some cells were co-transfected with the plasmid coding for the meganuclease RAG1 or I-*Sce*I (Cellectis). The transfection with lipofectamine 2000 was done according to the manufacturer protocols (Invitrogen) in which the total final amount of plasmid was 4 mg, completing with specific MGNs RAG1 or I-*Sce*I or in absence of MGNs with pLenti6/V5 EGFP. The transfection efficiency was qualitatively estimated to be near 100% in control wells transfected with a similar sized EGFP plasmid.

Two days after the transfection, the 293FT cells were harvested by simply detaching them by pipetting up and down followed by several washings in

phosphate buffer saline (PBS). The cells were then divided into two pools in order to extract the proteins and the genomic DNA separately from the same well. Proteins were analyzed by Western blotting using specific antibodies for the V5 epitope or for the HA tag fused with the meganucleases. The genomic DNA was PCR amplified for the identification of heteroduplex formation with the Surveyor® nuclease and for confirmation that Non-Homologous End Joining (NHEJ) was induced by the meganucleases in the 293FT cells.

Mismatch selective endonuclease assay for evaluation of meganuclease-mediated gene disruption

The meganucleases (MGNs) RAG1 or I-*Sce*I are able to produce mutations of their specific target located in the mutated dog micro-dystrophin/V5. These mutations were evaluated by PCR amplification of their specific target present in the mutated micro-dystrophin/V5 constructs described above. DNA was extracted from 293FT cells transfected with the target mutated micro-dystrophin/V5 with and without co-transfection with one of the MGNs. Amplicons from cells transfected with the MGNs were mixed in equal amount with amplicons obtained from cells transfected with the target without a MGN. The amplicon mix was then denaturated and re-annealed allowing modified target amplicon and non-modified target amplicon to re-anneal together to create heteroduplexes. The re-annealed PCR products were then digested with the Surveyor® nuclease (Transgenomic, Omaha, NE) that preferentially cuts DNA at sites of duplex distortions. Briefly, PCR (50 ml reactions) was done with the Phusion™ High-Fidelity DNA Polymerase (New England Biolabs) from 100 ng of genomic DNA extracted from 293FT in which the mutated micro-dystrophin/V5 and one of the MGNs were present and a control (mutated micro-dystrophin/V5 alone without a MGN). The amplification reaction with Phusion™ polymerase was performed as follows: 1 cycle: 98°C - 1 min; 35 cycles: 98°C - 10 sec, 60°C – 30 sec, 72°C – 30 sec; 1 cycle: 72°C – 10 min) using the following primers :forward 5'-gacagttatcaaacagctttggaag-3' SEQ ID NO : 16) (pos 1027-1051 dystrophin cDNA) and reverse 5'-gtaatctgtgggtgtcttgtaaaaga-3' SEQ ID NO : 17) (pos 1684-1673 dystrophin cDNA).

The PCR products (amplicons) were extracted after electrophoresis on agarose gel 1.4% and purified with a Qiaquick gel extraction kit (Qiagen). A mix of equal amount of two PCR products generated from genomic DNA extracted from 293FT cells was done in 9 µl containing annealing buffer 1X (10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$). Heteroduplexe formation was realized with a block heating bath set at 95°C for 5 min and the block was then removed from bath and cooled by itself to <30°C. After re-annealing, 0.5 ml of the Surveyor® Enhancer S and 0.5 ml Surveyor® nuclease (Transgenomic) were added to a total volume of 10 ml. The reaction was incubated at 42°C for 20 min to digest heteroduplexes and the cleaved products were analyzed on a 2% agarose gel containing TBE 1X and ethidium bromide (1 mg/ml). To estimate approximately the expected fragment size generated by the Surveyor® nuclease, the Applicants mixed amplicons of the wild type of micro-dystrophin/V5 (without target) with the mutated micro-dystrophin/V5 produced from genomic DNA extracted of the 293FT cells.

Protein extraction and Western blot analysis of the micro-dystrophin/V5 and meganucleases from 293FT cells

Protein extraction from 293FT cells was performed according to the previously published protocol [38]. The presence of the micro-dystrophin/V5 and MGN RAG1 or MGN I-*Sce*I was confirmed by Western blot of proteins extracted from the 293FT cells. Usually, an aliquot of 20 µg of proteins extracted from 293FT cells treated in different conditions was loaded in each lane and electrophoresed in 8% acrylamide gel (SDS-PAGE). The proteins were then electrotransferred onto a 0.45 mm nitrocellulose membrane (Bio-Rad, CA, USA) and cut in two sections: the upper part (proteins range over 85 kDa) for the detection of the micro-dystrophin/V5 and the lower part for the detection of HA tag fused with the MGN RAG1 or the I-*Sce*I (proteins range under 85 kDa). Membrane sections were blocked in 5% (w/v) non-fat dry milk (Blotto) resuspended in PBS containing 0.05% Tween-20 for 1 hour and then incubated overnight at 4°C in presence of a primary antibody, either a monoclonal antibody directed against the V5 epitope (Invitrogen) diluted 1/5000 to detect the micro-dystrophin/V5 or a goat polyclonal antibody against the HA-tag (GenScript, Piscataway, NJ) diluted 0.5 mg/ml to

detect the HA-MGNs. After incubation of the membrane sections, three consecutive washings were done for 10 min in PBS-Tween 0.05% and then membranes were incubated for 1 hour with a specific secondary antibody for V5 flag, which was a rabbit anti-goat coupled to peroxidase (1/10000) or a rabbit anti-goat coupled to peroxidase (1/10000) for the HA tag both in PBS-Tween 0.05% containing 5 % Blotto. After incubation of membrane sections with their specific secondary antibody, three washings (10 min each) were done in PBS-Tween. The membranes were then treated for 1 min with the enhanced chemiluminescent substrate (Perkin-Elmer, Woodbridge, Canada) and exposed to a Bio-Max film (Perkin-Elmer).

Protein extraction and Western blot analysis to confirm the occurrence of NHEJ in the endogenous dystrophin gene of 293FT cells

Cells were washed twice in HBSS and were lysed in 200 µl of lysis buffer containing 20mM Tris PH7.5, 1 mM DTT, 1 mM PMSF and 1% SDS. Protein samples were prepared as follows: In microtubes containing the 200µl of lysed cells, 600 µl of methanol , 200 µl of chloroform, 500 µl of water were added. After each liquid addition, microtubes were vortexed. Microtubes were centrifuged one minute at 14800 RPM and the solid white phase at the interphase was recuperated and washed with 300 µl of methanol. White pellet were lyophilized. Pellets were boiled in 40 µl of loading buffer containing Tris pH6.8 0.25M, 10% SDS, 7.5% glycerol and 0.5% beta-mercaptoethanol. 10 µl were loaded on 10% gel SDS-page electrophoresis. After protein electrotransfert on nitrocellulose membrane, the membrane was blocked in PBS-Tween (0.05%) containing 5 % milk for one hour. The membrane was incubated overnight at 4 ^{0}C with a C-terminal 6-His tagged polyclonal anti-I-CreI rabbit antibody (1/20000). The membrane was washed 3 times 10 minutes in PBS-Tween (0.05%). After, the membrane was incubated one hour with a goat anti-rabbit-HRP antibody (1/2000), washed 3 times in PBS-Tween (0.05%) and revealed with a chemiluminescence kit.

Gene sequencing of mutated episomal DNA to confirm the occurrence of NHEJ in 293FT cells

Genomic DNA was extracted from 293FT cells (in a six well plate) co-transfected with 1200 ng of the mutated micro-dystrophin/V5 plasmid containing the

RAG1 target and 2.8 mg of the MGN RAG1 plasmid. A protocol to extract genomic DNA based on the use of proteinase K, RNase, phenol/chloroform procedure was used [39]. The plasmid coding for the dog micro-dystrophin/V5 including the RAG1 target was co-precipitated with the genomic DNA since it was amplified with Phusion™ DNA polymerase using specific primers to dog dystrophin (SEQ ID NO : 16 and 17). This amplicon was then treated with TAQ DNA polymerase (New England Biotechnology) to add A at 3'end of the PCR fragments to permit direct cloning in pDrive cloning vector (Qiagen) with the Qiagen PCR cloning kit. Following transformation of ligation products in competent bacteria DH5a, several clones were picked randomly to prepare plasmids (mini-prep) and sequenced with the T7 primer. A total of 15 clones were sequenced and six of them showed deletion/insertion (indel) due to NHEJ.

Gene sequencing to confirm the occurrence of NHEJ in the endogenous dystrophin gene of 293FT cells

Genomic DNA was first extracted by washing the cells twice in HBSS. Cells were then lysed in 100 µl of lysis buffer containing 0.45M EDTA and 1% sarkosyl. 10 µl of proteinase K (20mg/ml) were added and incubated at 50 ^{0}C for 10 minutes. 400 µl of tris(50mM pH 8) solution were then added, followed by phenol/chloroform extraction. DNA were ethanol precipitated and pellets were resuspended in water.

100 ng of genomic DNA was then amplified for only 5 cycles with the PCR parameters as follow: one step at 98 °C for 1 min and 30 cycles of 98 °C, 30 sec, 60 °C, 30 sec, 72 °C 30 sec.

Primers sequences are as follow:

DMD 21 FWD : TCTTGCAGCCTAAAGGAACAAA (SEQ ID NO: 38)

REV: TCCTCTCGCTTTCTCTCATCTG (SEQ ID NO: 39)

DMD 31 FWD : GAACAGGTGGTATTACTAGCCA (SEQ ID NO: 40)

REV: GGTTGCAGTGAGCTGAGATCAT (SEQ ID NO: 41)

DMD 33 FWD : GCAGAGCTAGAGAAGAATGAGAAA (SEQ ID NO: 42)

REV: TTTGTTATTGGTTGAGGTTTGCTG (SEQ ID NO: 43)

Nested PCR were made for each meganuclease target with primers containing sequences required for Illumina procedure and tag for amplicons

identification. For the nested PCR, 5 µl of the first PCR step were used that were treated as follow: 98 °C 1 min, 98 °C 5 sec, 55 °C 10 sec, 72 °C 10 sec for 5 cycles and others 28 cycles as 98 °C 5 sec, 65 °C 10 sec, 72 °C 10 sec. Amplicons were agarose gel purified with the Qiagen gel extraction kit following the manufacturer's instructions. All amplicons from different DMD targets were assayed with nanodrop and were pooled in the same proportion (final DNA concentration is 10 ng/µl) before deep sequencing analysis.

Animals

Rag-*Mdx* mice were from the Laval University colony. All the experiments made on these animals were approved by the Animal Protection Committee of Laval University.

Electroporation

40 µg total of plasmids, i.e., 20 µg of the target plasmid and 20 µg of the meganuclease or 30 µg of the target plasmid and 10 µg of the meganuclease (either I-*Sce*I or RagI) were injected to a final volume of 40 µL in the mouse muscle for electroporation. As a negative control, the target plasmid (20 µg) was injected alone and as positive control, 40 µg of the original micro-dystrophin/V5 plasmid (without target) was injected alone. A single longitudinal injection was made into the *Tibialis anterior* (Ta) and the "Electrode Electrolyte" cream (Teca Corporation, Pleasantville, NY) was applied on the skin to induce the spreading of the electric current between two metal plaques. The parameters were: 10 pulses of 200 V/cm, duration of 25 ms and delay of 300 ms. The electrotransfered muscles were harvested two weeks after the experiment and were rapidly frozen in liquid nitrogen. Serial 12 µm cryostat sections were prepared throughout the entire muscle.

Immunohistochemical detection of micro-dystrophin/V5 in mouse Tibialis anterior muscle

Immunohistological analyses were performed with mouse anti-V5 antibody (1:200, Invitrogen) followed by incubation with a biotinylated anti-mouse antibody (1:300, Dako, Mississauga, Canada) and Streptavidin-Cy3 (1:300, Sigma). Afterwards, the sections were mounted in PBS-Glycerol (1:1). The presence of GFP

proteins and the immunohistological staining were observed under fluorescence using an Axiophot microscope (Zeiss, Oberkochen, Germany).

Example 2: Results

Meganucleases RAG1, I-*Sce*I design and their specific target within the dog micro-dystrophin/V5

As shown in figure 1A, the meganucleases (MGNs) RAG1 and I-*Sce*I were under control of elongation EF1 alpha gene promoter fused to exon1 and intron1 of EF1 gene. The MGN cDNA were followed at their 5' end by nucleotides coding for the HA-tag and for a nuclear localization signal (NLS). The expected molecular weight (MW) for MGN RAG1 and I-*Sce*I with the HA-tag and the NLS were respectively 42.3 kDa and 31.24 kDa. The transcription unit of MGN RAG1 and I-*Sce*I were contained respectively in a plasmid size of 5.51 kb and 5.15 kb.

A sequence of 29 nucleotides including the specific target (24 nucleotides) for RAG1 or I-*Sce*I MGN was inserted within the dog micro-dystrophin/V5 cDNA near of the NheI site as described in figure 1B. The insertion of this sequence in the micro-dystrophin/V5 cDNA changed the reading frame of the part of the dystrophin gene located after the inserted sequence; this resulted in the presence of premature stop codons. The wild type and the mutated micro-dystrophin/V5 were under a CMV promoter and introduced in a plasmid pLenti6/V5 containing a puromycin gene resistance. A wild type dog micro-dystrophin/V5 (figure 1B) without a MGN target insertion was used as control to evaluate the efficiency of micro-dystrophin expression in the Applicants analysis system.

Expression of micro-dystrophin/V5 following co-transfection of an appropriate meganuclease plasmid with the corresponding micro-dystrophin-V5 target plasmid

The inventors developed a plasmid based gene repair assay involving the two components (a plasmid target and a meganuclease plasmid) described in figure 1 A and B, to verify the capacity of a meganuclease to modify the reading frame of the mutated micro-dystrophin/V5 containing specific target for RAG1 or I-*Sce*I leading to some expression of the mutated micro-dystrophin/V5. The micro-dystrophin/V5 containing a MGN target (mutated dystrophin) was co-transfected with the corresponding

MGN plasmid in 293FT cells and 48 hours after transfection, the total proteins were extracted from the cells to be analyzed by Western blot using an anti-V5 antibody to detect the expression of micro-dystrophin/V5 and a goat antibody against the HA-tag to detect the expression of the meganuclease protein. As expected, the presence of micro-dystrophin/V5 (MW of 175 kDa) and of the MGN RAG1 (figure 2A, lanes 1-4)) or of the MGN I-*Sce*I (figure 2B, lanes 1-4) were detected in the same protein extracts. In both experiments in which MGN RAG1 or I-*Sce*I were co-transfected with micro-dystrophin/V5 containing their specific target, western blot analysis for the presence of HA-tag showed the expression of the MGN protein with the expected MW of 42 kDa for MGN RAG1 and 31 kDa for MGN I-*Sce*I (figure 2A and B). As expected, micro-dystrophin/V5 was more strongly expressed when the MGN co-transfection was done with a higher amount of target plasmid (1200 ng) (figure 2A and B, lanes 3 and 4) than with a lower amount of target plasmid (200 ng)(figure 2A and B, lanes 1 and 2). However, a reduced amount of MGN had no effect on the micro-dystrophin/V5 expression (lanes1 and 2 vs lanes 3 and 4 in figures 2A and B). As shown in figure 2 A and B, lanes 5, no micro-dystrophin/V5 expression was detected in the cells co-transfected with a EGFP plasmid (instead of a MGN plasmid) and the plasmid containing micro-dystrophin/V5 inserted with a target for RAG1 or for I-*Sce*I. From this last observation, co-transfection with a EGFP plasmid resulted in high transfection efficiency as 100% 293FT cells were fluorescent green (results not shown). This indicates that the co-transfection did not prevent us from obtaining good transfection efficiency in these cells. Finally, the transfection in 293FT cells of 2 different amounts of the Wild type micro-dystrophin/V5 protein plasmid (positive control without a MGN target) produced different expression levels in function of the amount of plasmid used (figure 2A, lanes 5 and 6 and figure 2B, lanes 6 and 7). Interestingly by Western blot analysis, the expression levels observed for the corrected mutated dystrophin in lanes 3 and 4 (Figure 2A and B) were a substantial proportion of the positive controls in lanes 6 and 7 (Figure 2A and B) suggesting that the correction of the micro-dystrophin/V5 gene was substantial.

Mutation detection by Surveyor® nuclease of episomal plasmid mutated micro-dystrophin/V5 in 293FT cells treated with MGNs RAG1 and I-*Sce*I

The inventors then tried to confirm that the expression of the micro-dystrophin/V5 following co-transfection of the target plasmid and of a MGN plasmid was really due to the modification by the MGN endonuclease activity of the target sequence inserted in the mutated micro-dystrophin/V5. A DSB induced by a MGN can be repaired by an error-prone non-homologous end joining (NHEJ) and the resulting DNA often contains small insertions or deletions ("indel" mutations) near of the DSB site. To confirm the presence of indels in the episomal targeted plasmids, the genomic DNA of the transfected cells was extracted (containing also the episomal target plasmid) and amplified by PCR using primers located within the micro-dystrophin/V5 gene before and after the meganuclease target nucleotide sequence, as described in materials and methods section. *In vitro*, the indel mutations can be detected by treatment of amplified DNA fragments (amplicons) with mismatch-sensitive Surveyor® enuclease according to the protocol described in the method section. Amplicons treated with Surveyor® nuclease were analyzed by agarose gel electrophoresis. Only one band of 681 bp (lane 1) and 657 bp (Figure 3 A and B, lane 4) were observed in the cells transfected respectively only with the target plasmid or only with the wild type plasmid, indicating that the Surveyor® nuclease did not cut these amplicons because there were no heteroduplex. However, two fragments of ~400 and ~320 bp (Figure 3A, lanes 2 and 3) for RAG1 or I-*Sce*I (Figure 3B, lanes 2 and 3) were generated when the Surveyor® nuclease was used to cleave amplicons obtained from the cells transfected with the target plasmid with the right meganuclease mixed with amplicons derived from cells transfected with the target plasmid alone (without MGNs). This indicated that the Surveyor® nuclease cut some amplicons because their 2 nucleotide strands were different due to the presence of some indels induced by the meganucleases. As positive control, a mixing of equal amount of amplicons from wild type plasmid and mutated plasmid without MGN treatment was digested with the Surveyor® nuclease giving a similar digestion profile showing two fragments of ~370 and~300 bp (Figure 3A and B, lane 5). The slight differences in the size of the fragments observed in lanes 1, 2 and 3 in comparison to lanes 4 and 5 as

shown in figure 3A and B are the results of the presence of the insertion target (Figure 3C, black box) within the mutated micro-dystrophin. All the observations done from 293FT cells co-transfected with the target plasmid and the right MGN indicate that both MGNs, RAG1 and I-*Sce*I, induced sequence changes in the target of mutated dystrophin, leading to the restoration of the dystrophin expression.

Sequencing of the targeted area confirmed the presence of indels and the restoration of the reading frame for MGN RAG1

The Applicants next wanted to confirm not only the presence of indels but also the presence of indels that restored the normal reading frame of the micro-dystrophin-Rag1/V5 plasmid. The preparation of genomic DNA from 293FT cells co-transfected with 1200 ng of target micro-dystrophin-Rag1/V5 and 2.8 mg of MGN RAG1 has been described in materials and methods section. The amplicons generated by PCR amplification of this DNA were cloned in DH5a and sequenced. As presented in the figure 4B, indels were detected in 6 of the 15 clones randomly picked. Moreover, four of these 6 clones had indels that restored the reading frame (amino acids sequence (Figure 4A) of the micro-dystrophin-Rag1target/V5 transgene. This demonstrates that NHEJ was able to efficiently restore the expression of an out of frame-mutated dystrophin within 293FT cells.

Restoration of micro-dystrophin reading frame in muscle fibers

All of the previous experiments have been done in 293FT cells because they are easy to transfect. However, for an eventual clinical application, it is the dystrophin gene located in muscle fibers that will have to be targeted. Will meganuclease induce indels in muscle fibers? To answer this question, the Applicants electroporated the micro-dystrophin-/V5 plasmid with or without an insertion with or without an appropriate meganuclease plasmid in the *Tibialis anterior* (TA) muscles of Rag-*mdx* mice. As negative control, the Applicants have only electroporated the target plasmid with an inserted sequence that changed the reading frame. Two weeks later, the mice were sacrificed and the TA muscles were prepared for immunohistochemistry analysis of the micro-dystrophin/V5 expression as described in materials and methods section. The expression of the V5 flag was detected in the membrane of rare weakly labeled muscle

fibers (Figure 5A). As a positive control, other muscles were electroporated with the micro-dystrophin/V5 plasmid, not containing any sequence that changed the reading frame. The expression of micro-dystrophin was detected in abundant muscle fibers (Figure 5B). Finally, muscles were co-electroporated with the micro-dystrophin/V5 plasmid containing the target sequence of either Rag1 or I-*Sce*I and with the plasmid coding for the appropriate meganuclease. Figures 5C and D illustrate the expression of the recombinant protein in fibers of muscles co-electroporated respectively with the micro-dystrophin/V5 with a target for Rag1 or I-*Sce*I and with the appropriate meganuclease. These co-electroporations led to the restoration of the normal micro-dystrophin reading frame and thus to its presence in many muscle fibers 5C for Rag1 and Figure 5D for I-*Sce*I). Figure 5E summarizes the results obtained with the 2 meganucleases at 2 concentrations. For both MGNs there were more V5 positive fibers when the ratio of MGN plasmid to the targeted plasmid was higher.

This restoration of the normal micro-dystrophin open reading frame and the restoration of functional micro-dystrophin expression is due to mutation of the targeted plasmids as illustrated in figure 6. DNA was extracted from the electroporated muscles 15 days after the electroporation and the plasmid region targeted by the meganuclease I-*Sce*I was amplified. The amplicons were then digested with the Surveyor® enzyme. The presence of the two bands at 300 and 370 bp seen in Figure 6 are due to the presence of some hetero-duplexes that were cut by the Surveyor® enzyme confirming that the I-SceI meganuclease had caused NHEJ in the micro-dystrophin target plasmids.

Restoration of micro-dystrophin reading frame in myoblasts containing an integrated micro-dystrophin gene with a target sequence for Rag1 or I-SceI

Lentivirus vectors were made as specified in figure 7 legend; said lentivirus vectors contained under a CMV promoter, either a mutated micro-dystrophin/V5 gene with a inserted target sequence for Rag1 (m-dyst/$V5_{Rag1}$) or a mutated micro-dystrophin/V5 gene with a inserted target sequence for I-*Sce*I (m-dyst/$V5_{I\text{-}SceI}$). These lentiviruses also contained a puromycin resistance gene under a SV40 promoter. These lentivirus were used to infect human myoblasts. The infected cells were

selected with puromycin and allowed to propagate. Some selected myoblasts were than nucleofected with a plasmid coding either for the Rag1 meganuclease or the I-*Sce*I meganuclease. Control myoblasts were not nucleofected with a meganuclease. Three days later DNA was extracted from all myoblasts. The region coding of the micro-dystrophin construct targeted by Rag1 or I-*Sce*I were amplified by PCR with the same primers used in the experiments in 293FT cells/mice. These amplicons were then digested with the Surveyor® enzyme to verify the presence of heterodimers due to insertions or deletions produced by NHEJ following the creation of a DSB by the meganuclease in the genome integrated micro-dystrophin V5 gene. Figure 7 illustrates the results of the Surveyor® reactions. These results confirm that the meganucleases are able to mutate the micro-dystrophin gene integrated in the cell genome so as to restore function.

Inducement of NHEJ in the endogenous dystrophin gene of 293FT cells by meganucleases

Six meganucleases derived from I-*Cre*I and targeting three different introns of dystrophin (two variants for each of the targeted site) were used to demonstrate that meganucleases could induce NHEJ in the endogenous, *i.e.* chromosomal, dystrophin gene of 293FT cells.

Meganuclease name or reference	Target sequence	Position on dystrophin gene
DMD21 2874 (SEQ ID NO: 44) DMD21 3387 (SEQ ID NO: 45)	GAAACCTCAAGTACCAAATGTAAA (SEQ ID NO: 50)	Intron38 nt 993350-993373
DMD31 3631 (SEQ ID NO: 46) DMD31 3633 (SEQ ID NO: 47)	AATGTCTGATGTTCAATGTGTTGA (SEQ ID NO: 51)	Intron44 nt1125314-1125337
DMD33 3326 (SEQ ID NO: 48) DMD33 3330 (SEQ ID NO: 49)	AAATCCTGCCTTAAAGTATCTCAT (SEQ ID NO: 52)	Intron42 nt1031834-10931857

Table 1: Meganuclease targets

The plasmids coding for these meganucleases, namely pCLS2874 (SEQ ID NO: 53), pCLS3387 (SEQ ID NO: 54), pCLS3631 (SEQ ID NO: 55), pCLS3633 (SEQ ID NO: 56), pCLS3326 (SEQ ID NO: 57), and pCLS3330 (SEQ ID NO: 58), were

transfected in 293FT human cells. The expression of the meganucleases was detected by Western blot using a C-terminal 6-His tagged polyclonal rabbit antibody against the I-*Cre*I meganuclease. This antibody reacts with the constant part of the meganucleases and thus reacts with DMD21, DMD31 and DMD33 meganucleases. All three meganucleases proteins were detected by Western blot (see Figure 8: DMD21 (lanes 2874 and 3387), DMD31 (lanes 3631 and 3633), DMD33 (lanes 3326 and 3330)).

The presence of mutations was detected by using the Deep sequencing technique: INDELs were detected with all six meganucleases targeting the endogenous dystrophin gene in 293FT cells (Table 2). Between 30,000 and 50,000 amplicons were sequenced for each meganuclease. The frequency of INDELs varied between 0.14 to 1.60% depending on the MGN. A meganuclease targeting Rag was taken as a control and mutates 6.40% of the target gene.

	DMD21			DMD31			DMD33				
	Ctrl	2874	3387	Ctrl	3631	3633	Ctrl	3326	3330	Ctrl	RAG
Deletion	0.03%	1.30%	1%	0.00%	1.60%	1%	0.02%	0.20%	0.1%	0.00%	6.40%
Insertion	0.00%	0.11%	0.015%	0%	0.19%	0.09%	0.02%	0.14%	0.04%	0.00%	0.15%

Table 2: Percentage of deletion or insertion obtained by meganucleases

These results thus demonstrate that meganucleases can mutate the real dystrophin gene in its real location on the X chromosome.

References

1. Trimarco, A, Torella, A, Piluso, G, Maria Ventriglia, V, Politano, L, and Nigro, V (2008). Log-PCR: a new tool for immediate and cost-effective diagnosis of up to 85% of dystrophin gene mutations. *Clin Chem* 54: 973-981.

2. Hoffman, EP, Brown, RH, Jr., and Kunkel, LM (1987). Dystrophin: the protein product of the Duchenne muscular dystrophy locus. *Cell* 51: 919-928.

3. England, SB, *et al.* (1990). Very mild muscular dystrophy associated with the deletion of 46% of dystrophin. *Nature* 343: 180-182.

4. Odom, GL, Gregorevic, P, Allen, JM, Finn, E, and Chamberlain, JS (2008). Microutrophin delivery through rAAV6 increases lifespan and improves muscle function in dystrophic dystrophin/utrophin-deficient mice. *Mol Ther* 16: 1539-1545.

5. Wang, Z, *et al.* (2007). Sustained AAV-mediated dystrophin expression in a canine model of Duchenne muscular dystrophy with a brief course of immunosuppression. *Mol Ther* 15: 1160-1166.

6. Lai, Y, Yue, Y, Liu, M, and Duan, D (2006). Synthetic intron improves transduction efficiency of trans-splicing adeno-associated viral vectors. *Hum Gene Ther* 17: 1036-1042.

7. Liu, M, Yue, Y, Harper, SQ, Grange, RW, Chamberlain, JS, and Duan, D (2005). Adeno-associated virus-mediated microdystrophin expression protects young mdx muscle from contraction-induced injury. *Mol Ther* 11: 245-256.

8. Ohshima, S, *et al.* (2009). Transduction efficiency and immune response associated with the administration of AAV8 vector into dog skeletal muscle. *Mol Ther* 17: 73-80.

9. Harper, SQ, *et al.* (2002). Modular flexibility of dystrophin: Implications for gene therapy of Duchenne muscular dystrophy. *Nat Med* 8: 253-261.

10. Skuk, D, *et al.* (2007). First test of a "high-density injection" protocol for myogenic cell transplantation throughout large volumes of muscles in a Duchenne muscular dystrophy patient: eighteen months follow-up. *Neuromuscul Disord* 17: 38-46.

11. Skuk, D, *et al.* (2006). Dystrophin expression in muscles of duchenne muscular dystrophy patients after high-density injections of normal myogenic cells. *J Neuropathol Exp Neurol* 65: 371-386.

12. Sampaolesi, M, *et al.* (2006). Mesoangioblast stem cells ameliorate muscle function in dystrophic dogs. *Nature* 444: 574-579.

13. Ikemoto, M, *et al.* (2007). Autologous Transplantation of SM/C-2.6(+) Satellite Cells Transduced with Micro-dystrophin CS1 cDNA by Lentiviral Vector into mdx Mice. *Mol Ther.*

14. Deasy, BM, Jankowski, RJ, and Huard, J (2001). Muscle-derived stem cells: characterization and potential for cell- mediated therapy. *Blood Cells Mol Dis* 27: 924-933.

15. Peault, B, *et al.* (2007). Stem and progenitor cells in skeletal muscle development, maintenance, and therapy. *Mol Ther* 15: 867-877.

16. Wilton, S (2007). PTC124, nonsense mutations and Duchenne muscular dystrophy. *Neuromuscul Disord* 17: 719-720.

17. Welch, EM, *et al.* (2007). PTC124 targets genetic disorders caused by nonsense mutations. *Nature* 447: 87-91.

18. Yokota, T, Duddy, W, and Partridge, T (2007). Optimizing exon skipping therapies for DMD. *Acta Myol* 26: 179-184.

19. Jearawiriyapaisarn, N, *et al.* (2008). Sustained dystrophin expression induced by peptide-conjugated morpholino oligomers in the muscles of mdx mice. *Mol Ther* 16: 1624-1629.

20. Williams, JH, Schray, RC, Sirsi, SR, and Lutz, GJ (2008). Nanopolymers improve delivery of exon skipping oligonucleotides and concomitant dystrophin expression in skeletal muscle of mdx mice. *BMC Biotechnol* 8: 35.

21. Adams, AM, Harding, PL, Iversen, PL, Coleman, C, Fletcher, S, and Wilton, SD (2007). Antisense oligonucleotide induced exon skipping and the dystrophin gene transcript: cocktails and chemistries. *BMC Mol Biol* 8: 57.

22. Urnov, FD, *et al.* (2005). Highly efficient endogenous human gene correction using designed zinc-finger nucleases. *Nature* 435: 646-651.

23. Lombardo, A, *et al.* (2007). Gene editing in human stem cells using zinc finger nucleases and integrase-defective lentiviral vector delivery. *Nature biotechnology* 25: 1298-1306.

24. Grizot, S, *et al.* (2009). Efficient targeting of a SCID gene by an engineered single-chain homing endonuclease. *Nucleic Acids Res.*

25. Stoddard, BL (2005). Homing endonuclease structure and function. *Q Rev Biophys* 38: 49-95.

26. Rouet, P, Smih, F, and Jasin, M (1994). Introduction of double-strand breaks into the genome of mouse cells by expression of a rare-cutting endonuclease. *Mol Cell Biol* 14: 8096-8106.

27. Choulika, A, Perrin, A, Dujon, B, and Nicolas, JF (1995). Induction of homologous recombination in mammalian chromosomes by using the I-SceI system of Saccharomyces cerevisiae. *Mol Cell Biol* 15: 1968-1973.

28. Paques, F, and Duchateau, P (2007). Meganucleases and DNA double-strand break-induced recombination: perspectives for gene therapy. *Curr Gene Ther* 7: 49-66.

29. Boothroyd, CE, *et al.* (2009). A yeast-endonuclease-generated DNA break induces antigenic switching in Trypanosoma brucei. *Nature* 459: 278-281.

30. Paques, F, and Haber, JE (1999). Multiple pathways of recombination induced by double-strand breaks in Saccharomyces cerevisiae. *Microbiol Mol Biol Rev* 63: 349-404.

31. Arnould, S, *et al.* (2006). Engineering of large numbers of highly specific homing endonucleases that induce recombination on novel DNA targets. *Journal of molecular biology* 355: 443-458.

32. Ashworth, J, *et al.* (2006). Computational redesign of endonuclease DNA binding and cleavage specificity. *Nature* 441: 656-659.

33. Doyon, JB, Pattanayak, V, Meyer, CB, and Liu, DR (2006). Directed evolution and substrate specificity profile of homing endonuclease I-SceI. *Journal of the American Chemical Society* 128: 2477-2484.

34. Smith, J, *et al.* (2006). A combinatorial approach to create artificial homing endonucleases cleaving chosen sequences. *Nucleic Acids Res* 34: e149.

35. Arnould, S, *et al.* (2007). Engineered I-CreI derivatives cleaving sequences from the human XPC gene can induce highly efficient gene correction in mammalian cells. *Journal of molecular biology* 371: 49-65.

36. Redondo, P, *et al.* (2008). Molecular basis of xeroderma pigmentosum group C DNA recognition by engineered meganucleases. *Nature* 456: 107-111.

37. Wang, B, *et al.* (2008). A canine minidystrophin is functional and therapeutic in mdx mice. *Gene Ther* 15: 1099-1106.

38. Chapdelaine, P, Vignola, K, and Fortier, MA (2001). Protein estimation directly from SDS-PAGE loading buffer for standardization of samples from cell lysates or tissue homogenates before Western blot analysis. *Biotechniques* 31: 478, 480, 482.

39. Chapdelaine, P, Delahaye, S, Gauthier, E, Tremblay, RR, and Dube, JY (1993). A one-hour procedure for the preparation of genomic DNA from frozen tissues. *Biotechniques* 14: 163-164.

40. Chauhan, A, Tikoo, A, Kapur, AK, and Singh, M (2007). The taming of the cell penetrating domain of the HIV Tat: myths and realities. *J Control Release* 117: 148-162.

41. Schwarze, SR, Ho, A, Vocero-Akbani, A, and Dowdy, SF (1999). In vivo protein transduction: delivery of a biologically active protein into the mouse [see comments]. *Science* 285: 1569-1572.

42. Dietz, GP, and Bahr, M (2004). Delivery of bioactive molecules into the cell: the Trojan horse approach. *Mol Cell Neurosci* 27: 85-131.

43. Zhao, Y, Lou, D, Burkett, J, and Kohler, H (2001). Chemical engineering of cell penetrating antibodies. *J Immunol Methods* 254: 137-145.

44. Kokryakov, VN, *et al.* (1993). Protegrins: leukocyte antimicrobial peptides that combine features of corticostatic defensins and tachyplesins. *FEBS Lett* 327: 231-236.

45. Jans, DA (1994). Nuclear signaling pathways for polypeptide ligands and their membrane receptors? *Faseb J* 8: 841-847.

46. Chang, M, Zhang, L, Tam, JP, and Sanders-Bush, E (2000). Dissecting G protein-coupled receptor signaling pathways with membrane-permeable blocking peptides. Endogenous 5-HT(2C) receptors in choroid plexus epithelial cells. *J Biol Chem* 275: 7021-7029.

47. Lavigne, MD, Yates, L, Coxhead, P, and Gorecki, DC (2008). Nuclear-targeted chimeric vector enhancing nonviral gene transfer into skeletal muscle of Fabry mice in vivo. *Faseb J* 22: 2097-2107.

48. Caron, NJ, *et al.* (2001). Intracellular delivery of a Tat-eGFP fusion protein into muscle cells. *Mol Ther* 3: 310-318.

49. Xiong, F, *et al.* (2007). Enhanced effect of microdystrophin gene transfection by HSV-VP22 mediated intercellular protein transport. *BMC Neurosci* 8: 50.

50. Sonnemann, KJ, Heun-Johnson, H, Turner, AJ, Baltgalvis, KA, Lowe, DA, and Ervasti, JM (2009). Functional substitution by TAT-utrophin in dystrophin-deficient mice. *PLoS Med* 6: e1000083.

51. Kinoshita, I, Vilquin, JT, Asselin, I, Chamberlain, J, and Tremblay, JP (1998). Transplantation of myoblasts from a transgenic mouse overexpressing dystrophin prduced only a relatively small increase of dystrophin- positive membrane. *Muscle Nerve* 21: 91-103.

52. Cathomen, T, and Joung, JK (2008). Zinc-finger nucleases: the next generation emerges. *Mol Ther* 16: 1200-1207.

53. Bock, HH, Herz, J, and May, P (2007). Conditional animal models for the study of lipid metabolism and lipid disorders. *Handb Exp Pharmacol*: 407-439.

54. Spillmann, L (2006). From perceptive fields to Gestalt. *Prog Brain Res* 155: 67-92.

55. May, P, Herz, J, and Bock, HH (2005). Molecular mechanisms of lipoprotein receptor signalling. *Cell Mol Life Sci* 62: 2325-2338.

56. Durai, S, Mani, M, Kandavelou, K, Wu, J, Porteus, MH, and Chandrasegaran, S (2005). Zinc finger nucleases: custom-designed molecular scissors for genome engineering of plant and mammalian cells. *Nucleic Acids Res* 33: 5978-5990.

Claims

1. A meganuclease enzyme which recognizes and cuts a target site in the human dystrophin gene, for use in the treatment of a genetic disease caused by at least one frame shift or nonsense mutation in the human dystrophin gene in the genome of an individual.

2. The meganuclease enzyme for use according to claim 1, wherein a set of meganuclease enzymes, which each recognises and cuts a different target site in the human dystrophin gene, is brought into contact with the genome of said individual.

3. The meganuclease enzyme for use according to claim 1 or 2, wherein said target site is located at the end of the exon preceding said at least one frame shift mutation.

4. The meganuclease enzyme for use according to claim 1 or 2, wherein said target site is located after said at least one frame shift mutation in the exon.

5. The meganuclease enzyme for use according to any of claims 1 to 4, wherein said target site is selected from the group consisting of: SEQ ID NO : 1; SEQ ID NO : 2; SEQ ID NO : 3; SEQ ID NO : 4; SEQ ID NO : 5; SEQ ID NO : 6; SEQ ID NO : 7; SEQ ID NO : 8; SEQ ID NO : 9; SEQ ID NO : 10; SEQ ID NO : 11; SEQ ID NO: 28; SEQ ID NO: 29; SEQ ID NO: 30; SEQ ID NO: 31; SEQ ID NO: 32; SEQ ID NO: 33; SEQ ID NO: 34; SEQ ID NO: 35; SEQ ID NO: 36; SEQ ID NO: 37.

6. The meganuclease enzyme for use according to claim 1 or 2, wherein said target site is located near a non-sense mutation.

7. The meganuclease enzyme for use according to any of claims 1 to 6, wherein said meganuclease or said set of meganucleases comprise at least one protein transduction domain.

8. The meganuclease enzyme for use according to claim 7, wherein said at least one protein transduction domains comprises SEQ ID NO: 12.

9. The meganuclease enzyme for use according to any of claims 1 to 8, wherein said meganuclease or said set of meganucleases are encoded by at least one purified nucleic acid molecule.

10. A kit comprising, at least one meganuclease enzyme, which recognizes and cuts a target site in the human dystrophin gene, or a set of meganuclease enzymes, which each recognizes and cuts a different target site in the human dystrophin gene, or at least one purified nucleic acid molecule encoding said meganucleases or said set of meganuclease; and instructions for their use.

11. A medicament comprising:

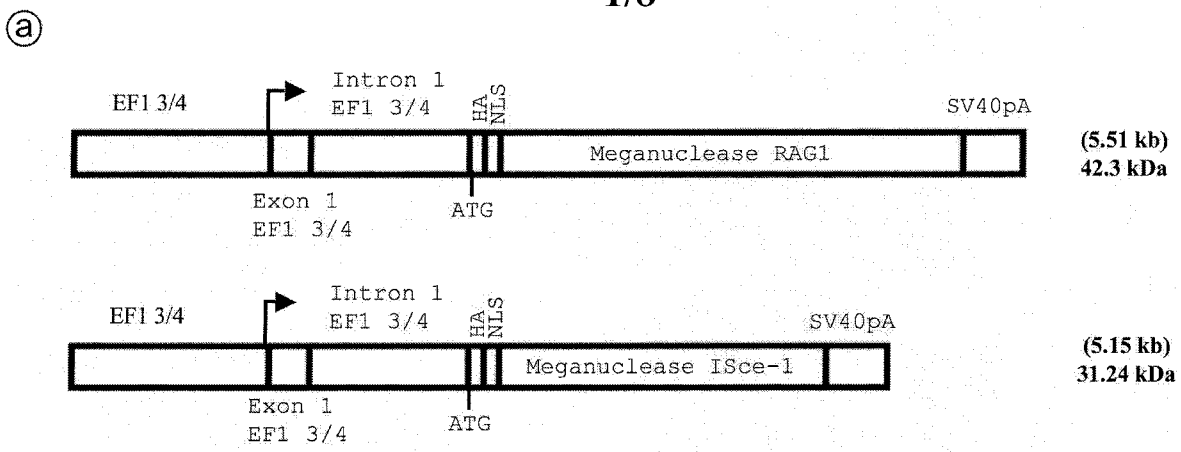
at least one meganuclease enzyme, which recognizes and cuts a target site in the human dystrophin gene, or a set of meganuclease enzymes, which each recognizes and cuts a different target site in the human dystrophin gene, or at least one nucleic acid molecule encoding said at least one meganuclease or said set of meganucleases; and at least one of the following:

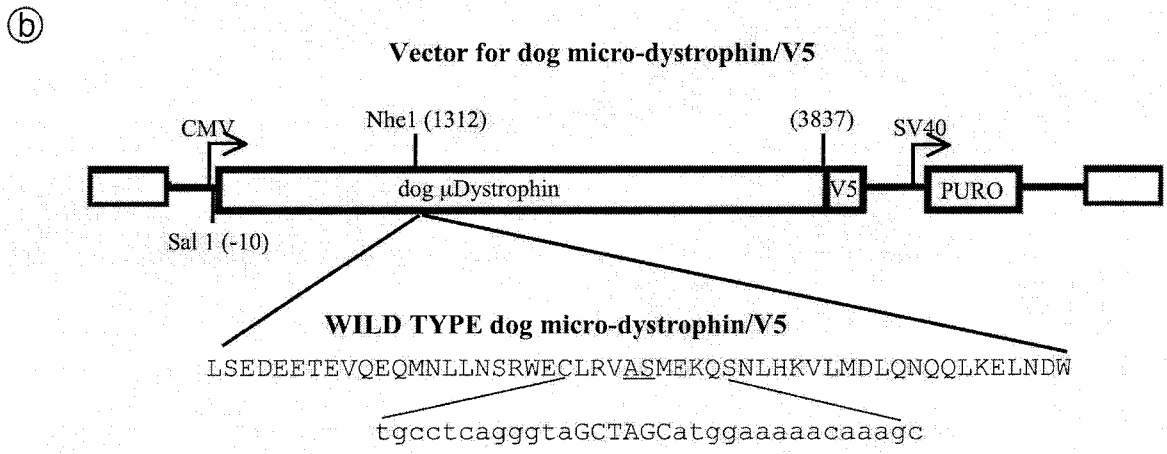
a preservative;

a stabiliser;

an excipient;

a vehicle.





MUTATED dog micro-dystrophin/V5 by insertion of the RAG1 or ISce-1 TARGET

MUTATED dog micro-dystrophin/V5 RAG1 or ISce-1

LSEDEETEVQEQMNLLNSRWECLRVHCSQVPQPAC-HGKTKQFT-SSNGSPE

TARGET RAG1

tgcctcagggtacattgttctcaggtacctcagccagcatGCTAGCatggaaaaacaaagc

LSEDEETEVQEQMNLLNSRWECLRVHARDNRVIC-HGKTK

TARGET ISce-1

tgcctcagggtacacgctagggataacagggtaatatGCTAGCatggaaaaacaaagc

FIGURE 1

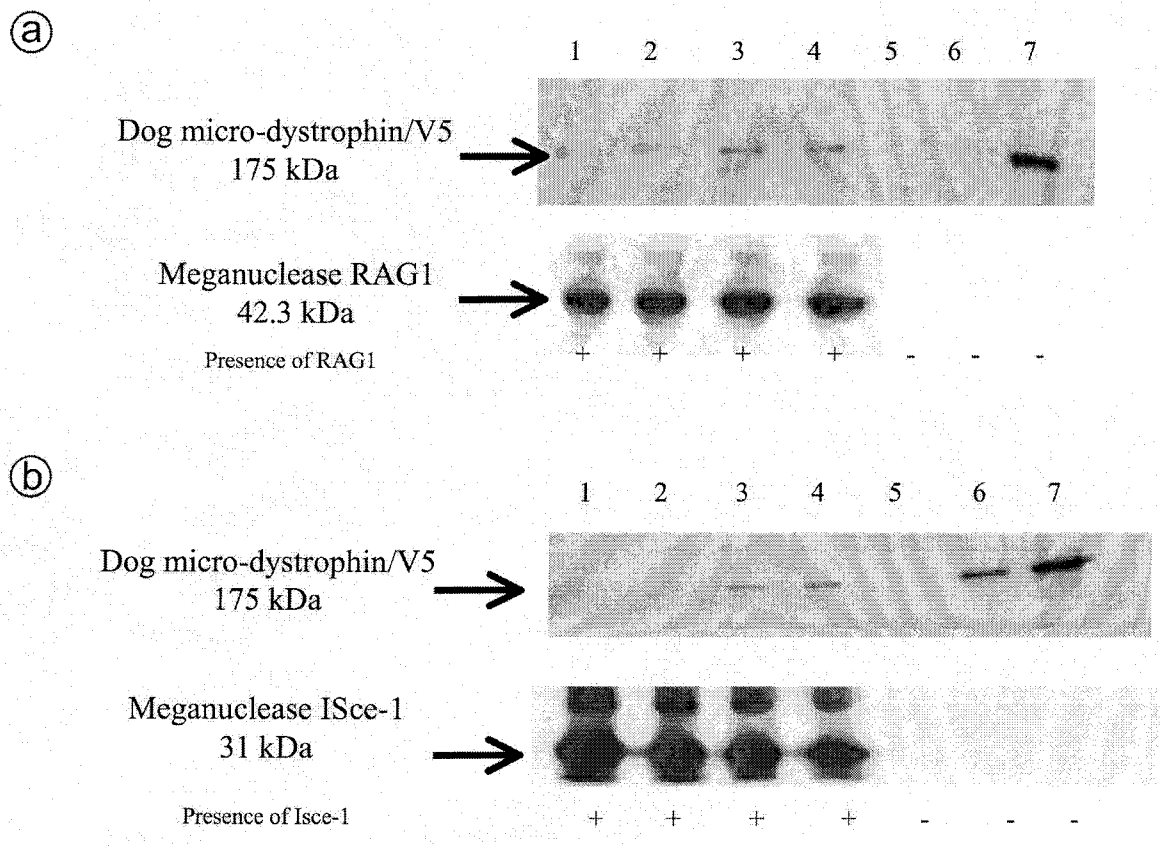

a
1 2 3 4 5 6 7
Dog micro-dystrophin/V5
175 kDa
Meganuclease RAG1
42.3 kDa
Presence of RAG1
+ + + + - - -
b
1 2 3 4 5 6 7
Dog micro-dystrophin/V5
175 kDa
Meganuclease ISce-1
31 kDa
Presence of Isce-1
+ + + + - - -

FIGURE 2

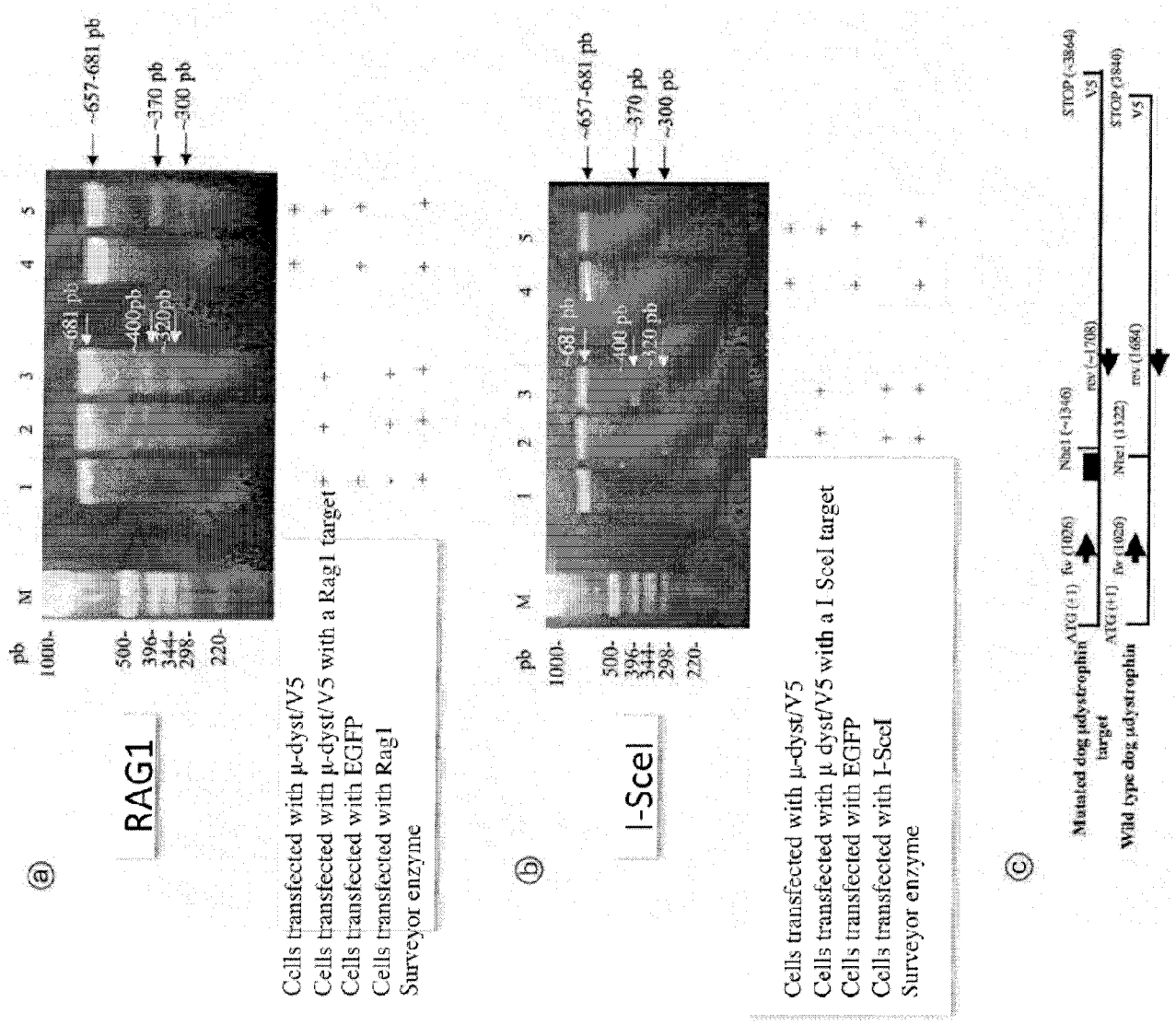

a
RAG1
pb
M
1
2
3
4
5
1000-
500-
396-
344-
298-
220-
~657-681 pb
~370 pb
~300 pb
Cells transfected with μ-dyst/V5
Cells transfected with μ-dyst/V5 with a Rag1 target
Cells transfected with EGFP
Cells transfected with Rag1
Surveyor enzyme
b
I-SceI
pb
M
1
2
3
4
5
1000-
500-
396-
344-
298-
220-
~657-681 pb
~370 pb
~300 pb
Cells transfected with μ-dyst/V5
Cells transfected with μ dyst/V5 with a I SceI target
Cells transfected with EGFP
Cells transfected with I-SceI
Surveyor enzyme
c
Mutated dog μdystrophin target
Wild type dog μdystrophin
ATG (+1)
fw (1026)
NheI (~1346)
rev (~1708)
ATG (+1)
fw (1026)
NheI (1322)
rev (1684)
STOP (~3864)
V5
STOP (3840)
V5

FIGURE 3

ⓐ

LSEDEETEVQEQMNLLNSRWECLRVASMEKQSNLHKVLMDLQNQQLKELNDWL	µdystr wild type
LSEDEETEVQEQMNLLNSRWECLRVHCSQVPQPAC-HGKTKQFT-SSNGSPE	1) µdystr RAG1
LSEDEETEVQEQMNLLNSRWECLRVHCSHLSQHASMEKQSNLHKVLMDLQNQQLKELNDWL	2) µdystr RAG1+MGN
LSEDEETEVQEQMNLLNSRWECLRVHCS QSQHASMEKQSNLHKVLMDLQNQQLKELNDWL	3) µdystr RAG1+MGN
LSEDEETEVQEQMNLLNSRWECLRVHCSQLSQHASMEKQSNLHKVLMDLQNQQLKELNDWL	4) µdystr RAG1+MGN
LSEDEETEVQEQMNLLNSRWECLRVHCSH SQHASMEKQSNLHKVLMDLQNQQLKELNDWL	5) µdystr RAG1+MGN
LSEDEETEVQEQMNLLNSRWECLRVHCSQVLAWKNKAIYIKF-WISRISN-KS-MTG-PKQ	6) µdystr RAG1+MGN
LSEDEETEVQEQMNLLNSRWECLRVHCSQVQSNKFKHQLSLTFAVSASMLAWKNKAIYIK-	7) µdystr RAG1+MGN

ⓑ

RAG1 TARGET

cattgttctcaggtacctcagccagcatgct	µdystr RAG1 (µdystr mutated)	1
cattgttctca....cctcagccagcatgct	µdystr RAG1 (deletion) (µdystr corrected)	2
cattgttctcag.......agccagcatgct	µdystr RAG1 (deletion) (µdystr corrected)	3
cattgttctcag....ctcagccagcatgct	µdystr RAG1 (deletion) (µdystr corrected)	4
cattgttctca.......cagccagcatgct	µdystr RAG1 (deletion) (µdystr corrected)	5
cattgttctcaggta..............ct	µdystr RAG1 (deletion) (µdystr not corrected)	6
cattgttctcaggtacctcagccagcatgct	µdystr RAG1 (insertion) (µdystr not corrected)	7

aatctaacaagttcaaacaccagctctccttgactttgcagt

FIGURE 4

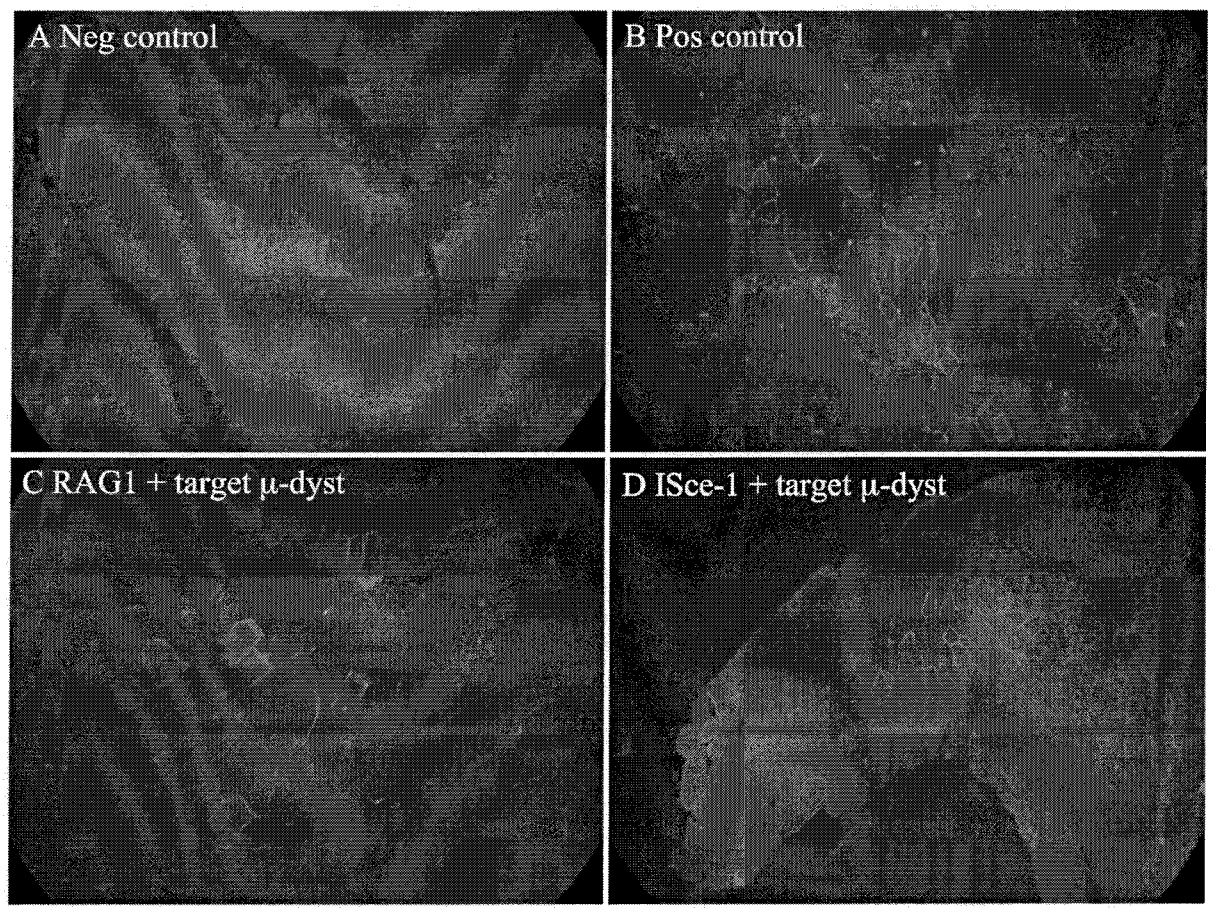

A Neg control
B Pos control
C RAG1 + target μ-dyst
D ISce-1 + target μ-dyst

FIGURE 5

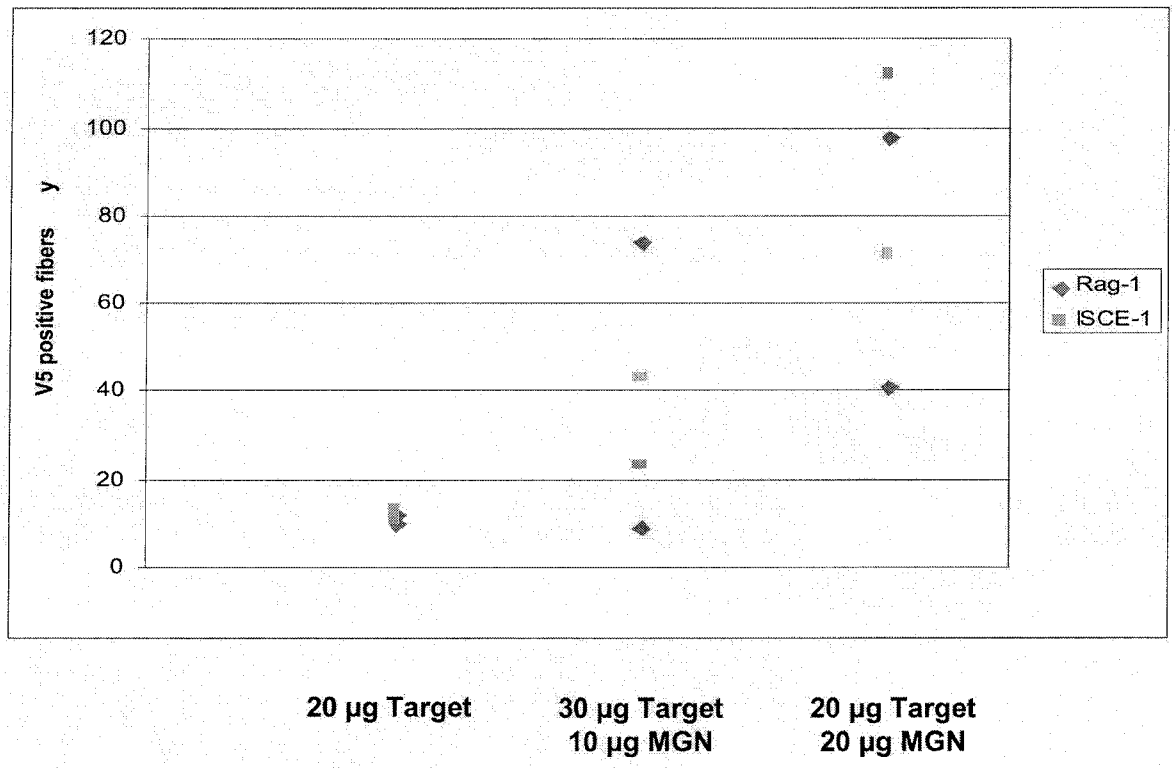
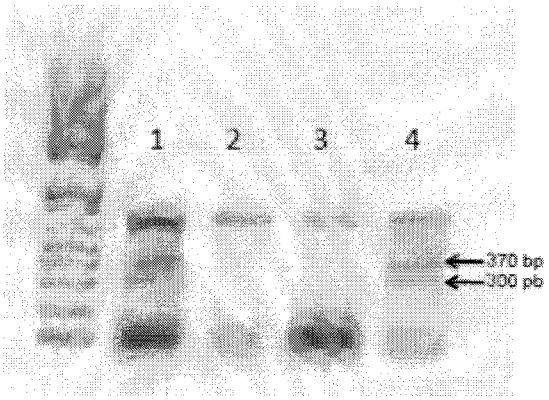

120
100
80
60
40
20
0
V5 positive fibers y
Rag-1
ISCE-1
20 µg Target
30 µg Target
10 µg MGN
20 µg Target
20 µg MGN

FIGURE 5E



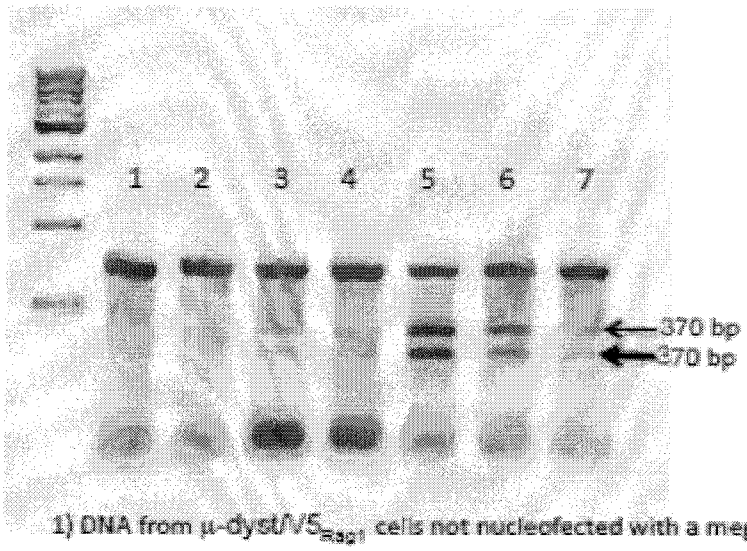
1) μ-dyst/V5$_{I\text{-}SceI}$ + Mega _I-SceI

2) wild type μ-dystro/V5 (no target sequence)

3) μ-dyst/V5$_{I\text{-}SceI}$ alone

4) μ-dyst/V5$_{I\text{-}SceI}$ mixed with μ-dyst/V5$_{Rag1}$

Figure 6



1) DNA from μ-dyst/V5$_{Rag1}$ cells not nucleofected with a meganuclease

2) DNA from μ-dyst/V5$_{I\text{-}SceI}$ cells not nucleofected with a meganuclease

3) DNA from μ-dyst/V5$_{Rag1}$ cells nucleofected with Rag1 meganuclease

4) DNA from μ-dyst/V5$_{I\text{-}SceI}$ cells nucleofected with I-SceI meganuclease

5) DNA from μ-dyst/V5$_{Rag1}$ + DNA from μ-dyst/V5$_{I\text{-}SceI}$ cells in a ratio of 1/1

6) DNA from μ-dyst/V5$_{Rag1}$ cells + DNA from μ-dyst/V5$_{I\text{-}SceI}$ cells in a ratio of 5/1

7) DNA from μ-dyst/V5$_{Rag1}$ cells + DNA from μ-dyst/V5$_{I\text{-}SceI}$ cells in a ratio of 10/1

Figure 7

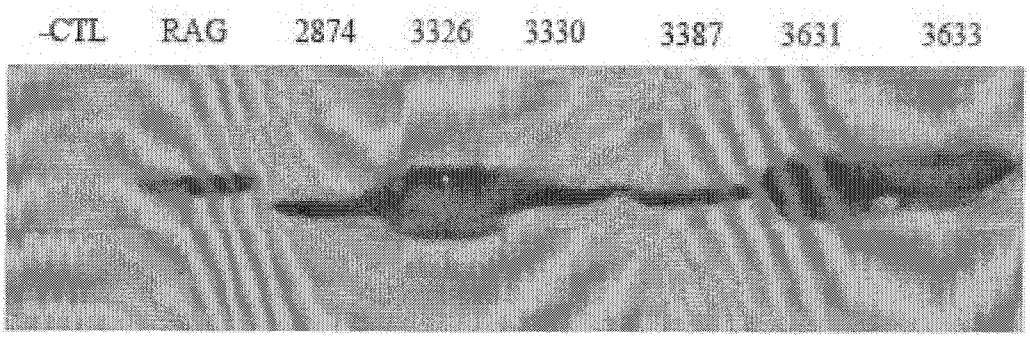


Figure 8