mediated by cis acting elements in the 3' UTR (Mignone *et al* (2002) *Genome Biol* 3(3): PMCID: PMC139023).

[0009] However, there remains a need for additional methods and compositions to engineered nuclease cleavage systems to provide enhanced transcription/translation efficiency and to increase nuclease activity and/or specificity.

SUMMARY

[0010] The present disclosure provides methods and compositions to increase expression of an artificial nuclease, as well as increase the efficiency (activity) and/or specificity of a nuclease (*e.g.,* nuclease pair) for its intended target. Thus, described herein are polynucleotides (*e.g.,* DNA expression vector or mRNA) for expressing artificial nucleases (*e.g.,* zinc finger nucleases (ZFNs), TALENs, CRISPR/Cas nucleases) comprising engineered promoters comprising elements in the 5' and/or 3' untranslated sequences that enhance expression of the artificial nucleases. Optionally, the nuclease-encoding polynucleotides further comprise sequences encoding small peptides (including but not limited to polycationic peptides such as peptide tags and/or nuclear localization sequences), and/or comprise mutations in one or more of the DNA binding domain regions (*e.g.,* the backbone of a zinc finger protein or TALE) and/or one or more mutations in a *FokI* nuclease cleavage domain or cleavage half domain. When these polynucleotide components are used individually or in any combination (*e.g.,* peptide sequence such as FLAG (*e,g,,* 3X FLAG), NLS, WPRE and/or poly A signal in any combination), the methods and compositions of the disclosure provide surprising and unexpected increases in expression of artificial nucleases with increased efficiency of cleavage and/or targeted integration of a transgene (*e.g.,* 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or more fold cleavage as compared to nucleases without the sequences/modifications described herein) *in vitro* or *in vivo* and/or targeting specificity (reduced off-target effects). The disclosure also provides methods of using these compositions for targeted cleavage of cellular chromatin in a region of interest and/or integration of a transgene via targeted integration at a predetermined region of interest in cells.

[0010a] In one aspect, the present disclosure refers to a zinc finger nuclease (ZFN) comprising first and second ZFNs, the first ZFN comprising the ZFN designated 71557 and the second ZFN comprising the ZFN designated 71728.

[0010b] In another aspect, the present disclosure refers to one or more polynucleotides encoding the first and second ZFNs as disclosed herein, optionally wherein the one or more polynucleotides are carried on AAV vectors.

[0010c] In another aspect, the present disclosure refers to an AAV vector as disclosed herein, wherein the AAV vector encoding the first ZFN comprises the sequence shown in SEQ ID NO:43.

[0010d] In another aspect, the present disclosure refers to a cell comprising one or more ZFNs, one or more polynucleotides, and/or one or more AAV vectors as disclosed herein, optionally a stem cell or precursor cell.

[0010e] In another aspect, the present disclosure refers to a pharmaceutical composition comprising one or more ZFNs, one or more polynucleotides, one or more AAV vectors and/or one or more cells as disclosed herein.

[0010f] In another aspect, the present disclosure refers to the one or more ZFNs, one or more polynucleotides, one or more AAV vectors, one or more cells and/or one or more pharmaceutical composition as disclosed herein for use in therapy, wherein the one or more ZFNs, one or more polynucleotides, one or more AAV vectors, one or more cells and/or one or more pharmaceutical composition as disclosed herein cleaves an endogenous albumin gene in a subject.

[0010g] In another aspect, the present disclosure refers to use of one or more ZFNs, one or more polynucleotides, one or more AAV vectors, one or more cells and/or one or more pharmaceutical composition as disclosed herein in the manufacture of a medicament for cleaving an endogenous albumin gene in a subject.

[0010h] In another aspect, the present disclosure refers to a kit comprising one or more ZFNs, one or more polynucleotides, one or more AAV vectors, one or more cells and/or one or more pharmaceutical compositions as disclosed herein.

[0010i] In another aspect, the present disclosure refers to a composition comprising: (a) a first polynucleotide encoding a first ZFN designated 71557, the first polynucleotide optionally comprising an AAV vector comprising the sequences as shown in Table 4 or SEQ ID NO:43; (b) a second polynucleotide encoding a second ZFN designated 71728, the second polynucleotide optionally comprising an AAV vector comprising the sequences as shown in Table 5 or SEQ ID NO:56; and (c) a donor polynucleotide comprising a sequence encoding a Factor IX (FIX) sequence.

[0010j] In another aspect, the present disclosure refers to use of the composition as disclosed herein in the manufacture of a medicament for treating

hemophilia in a subject in need thereof, wherein the composition is to be administered to the subject such that the ZFN cleaves an endogenous albumin gene in the subject, the FIX sequence is integrated into the cleaved albumin gene and a FIX protein is expressed in the subject.

[0010k] In another aspect, the present disclosure refers to use of one or more compositions as disclosed herein in the manufacture of a medicament for treating and/or preventing hemophilia in a subject in need thereof, wherein the composition is to be administered to the subject such that that a FIX protein is expressed in a cell of the subject.

[0010l] In another aspect, the present disclosure refers to a composition comprising: (a) a first polynucleotide encoding a first ZFN designated 71557, the first polynucleotide optionally comprising an AAV vector comprising the sequences as shown in Table 4 or SEQ ID NO:43; (b) a second polynucleotide encoding a second ZFN designated 71728, the second polynucleotide optionally comprising an AAV vector comprising the sequences as shown in Table 5 or SEQ ID NO:56; and (c) a donor polynucleotide comprising a sequence encoding an iduronate-2-sulfatase (IDS) sequence.

[0010m] In another aspect, the present disclosure refers to use of the composition as disclosed herein in the manufacture of a medicament for treating Mucopolysaccharidosis II (MPS II) disease in a subject in need thereof, wherein the composition is to be administered to the subject such that the ZFN cleaves an endogenous albumin gene in the subject, the IDS sequence is integrated into the cleaved albumin gene and an IDS protein is expressed in the subject.

[0010n] In another aspect, the present disclosure refers to use of one or more compositions as disclosed herein in the manufacture of a medicament for treating and/or preventing MPS II disease in a subject in need thereof, wherein the composition is to be such that that an IDS protein is expressed in a cell of the subject.

[0010o] In another aspect, the present disclosure refers to a kit comprising one or more compositions as disclosed herein.

[0011] Thus, described herein is a polynucleotide (mRNA, plasmid, viral vector such as AAV) encoding a nuclease (*e.g.,* ZFN, TALEN, CRISPR/Cas nuclease, etc.), the polynucleotide further comprising at least one, two, three, four, five, six, seven or eight of any combination of the following elements: (i) a sequence encoding

a polycationic sequence (*e.g.,* a 3X FLAG sequence), optionally 5' to the nuclease-encoding sequence; (ii) a 5'UTR sequence (*e.g.,* a *Xenopus* beta-globin sequence such as shown in SEQ ID NO:1), optionally 5' to the nuclease-encoding sequence; (iii) a WPRE sequence, 3' and/or 5' to the nuclease coding sequence; (iv) modifications to the sequence encoding backbone (non-DNA binding residues) of the nuclease domain (*e.g.,* phosphate backbone residues of a ZFN); (v) modifications to the cleavage domain sequence of the nuclease (*e.g.,* engineered FokI domains); (vi) a tissue specific promoter and/or enhancer (*e.g.,* hAAT, ApoE, etc.), optionally operably linked to the sequence encoding the nuclease; (vii) a NLS sequence (5' or 3' to the nuclease encoding sequence); and/or (viii) a polyA sequence. In certain examples, the polynucleotide is mRNA. In other examples, the polynucleotide is an AAV vector, further optionally comprising an ITR, for example an AAV vector as shown in any of the constructs the appended Figures and/or Tables. A single polynucleotide may encode some or all components of the nuclease, *e.g*., a pair of ZFNs, a single guide RNA, etc. Alternatively, separate polynucleotides (of the same or different types) may encode components of the nuclease, for example, separate nucleotides each encoding one ZFN or TALEN of a ZFN or TALEN pair. Thus, provided herein are one or more polynucleotides (*e.g.,* AAV vectors) encoding one or more nucleases (*e.g.,* ZFNs). The polynucleotides described herein can be used for *in vitro, ex vivo* and/or *in vivo* methods for targeted cleavage and/or integration of at least one donor and may increase nuclease activity (cleavage and/or integration) and/or specificity (on-target as compared to off-target activity) by 1-50 fold (or any value therebetween, including 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18. 19, 20, etc. fold).

[0012] In one example, the disclosure describes a polynucleotide comprising a tissue specific promoter for expressing an artificial nuclease in a desired tissue. In some examples, the tissue specific promoter is a liver-specific promoter. In further examples, the liver specific promoter is a human-α1 anti-trypsin promoter (hAAT) or a transthyretin minimal promoter (see U.S. Patent publication 20170119906). In some instances, the liver specific promoter comprises an ApoE enhancer sequence (Shachter *et al.* (1993) *J. Lipid Res* 34(10):1699-707). In some examples, the liver specific promoter comprises one or more ApoE enhancer sequences (*e.g*., 1, 2, 3 and/or 4; *see* Okuyama *et al.* (1996) *Hum Gen Ther* 7(5):637-45). In further examples, the promoter is linked to an intron. In preferred

examples, the intron is an HGG-IGG chimeric intron comprising the 5' donor site from the first intron of the human β-globin gene and the branch and 3' acceptor site from the intron of an immunoglobulin gene heavy chain variable region. The polynucleotides described herein may be cDNA constructs (for example carried on a viral vector such as AAV), mRNA, plasmid DNA, or part of an expression cassette for insertion into a genome.

[0013] Thus, in one example, described herein is an mRNA or AAV vector encoding a nuclease, the mRNA or AAV vector comprising elements for increasing transcriptional and translational efficiency. In some examples, the elements comprise untranslated sequences such as natural or artificial 5' and/or 3' UTR sequences. In some examples, a 5' UTR sequence is included in an expression cassette, while in others, a 3' UTR sequence is used. In preferred examples, an mRNA or AAV encoding an artificial nuclease comprises both a 5' UTR and a 3' UTR. In one example, the 5' UTR is a Xenopus β-globin UTR (*see* Falcone and Andrews (1991) Mol Cell Bio 11(5): 2656-2664; Krieg and Melton (1994) *Nuc Acid Res* 12(18):7057). In preferred examples, the DNA sequence encoding the Xenopus β-globin UTR is 5' [TG]CTTGTTCTTTTTGCAGAAGCTCAGAATAAACGCTCAACTTTGGCAGAT (SEQ ID NO:1) (where TG is optional). In some examples, the mRNA or AAV encoding the nuclease comprises a 3' WPRE sequence (*see* U.S. Patent publication 20160326548). In further examples, the WPRE element is a mutated in the 'X' region to prevent expression of Protein X (see U.S. Patent 7,419,829). In some examples, the mutated WPRE sequence is a truncated WPRE element. In some examples the mutated WPRE sequence is mutated in the X region of the J02442 or J04514 woodchuck hepatitis virus (Galibert *et al* (1982) *J. Virol* 41(1): 51-65; Zanta-Boussif *et al.* (2009) *Gene Ther* 16(5):605-619). Non-limiting examples of WPRE sequences that may be used in the polynucleotides described herein are shown below in the Examples. In further examples, the 3' UTR comprises a poly A signal sequence. The poly A signal may be 3' or 5' to the WPRE sequence when these elements are used in combination. In preferred examples, the poly A signal sequence is the bovine Growth Hormone signal sequence (*see* Woychik *et al* (1984) *Proc Natl Acad Sci* 81(13):3944-8). The nuclease-encoding polynucleotides (mRNA, AAV vectors) as described herein may further include modifications to the nuclease encoding sequence, for example modifications to the backbone region of a ZFP DNA-

binding domain of the nuclease and/or modifications to the cleavage domain (or cleavage half-domain) of the nuclease(s).

[0014] In another example, described herein is an engineered nuclease cleavage half domain comprising one or more mutations as compared to a parental (*e.g.,* wild-type) cleavage domain from which these mutants are derived and/or a polynucleotide (mRNA) comprising these cleavage domains. Mutations as described herein, include but are not limited to, mutations that change the charge of the cleavage domain, for example mutations of positively charged residues to non-positively charged residues (*e.g.,* mutations of K and R residues (*e.g.,* mutated to S); N residues (*e.g.,* to D), and Q residues (*e.g.,* to E); mutations to residues that are predicted to be close to the DNA backbone based on molecular modeling and that show variation in *Fok*I homologs; and/or mutations at other residues (*e.g.,* U.S. Patent No. 8,623,618 and Guo *et al*, (2010) *J. Mol. Biol*. 400(1):96-107).

[0015] In certain examples, the engineered cleavage half domains are derived from *Fok*I or *Fok*I homologues and comprise a mutation in one or more of amino acid residues 416, 422, 447, 448, and/or 525, numbered relative to the wild-type full length *Fok*I as shown in SEQ ID NO:2 or corresponding residues in *Fok*I homologues In other examples, the cleavage half domains derived from *FokI* comprises a mutation in one or more of amino acid residues 414-426, 443-450, 467-488, 501-502, and/or 521-531, including one or more of 387, 393, 394, 398, 400, 416, 418, 422, 427, 434, 439, 441, 442, 444, 446, 448, 472, 473, 476, 478, 479, 480, 481, 487, 495, 497, 506, 516, 523, 525, 527, 529, 534, 542, 559, 569, 570, and/or 571, numbered relative to wild-type FokI or corresponding residues in any FokI homologue. The mutations may include mutations to residues found in natural restriction enzymes homologous to *Fok*I at the corresponding positions. In certain examples, the mutations are substitutions, for example substitution of the wild-type residue with any different amino acid, for example alanine (A), cysteine (C), aspartic acid (D), glutamic acid (E), histidine (H), phenylalanine (F), glycine (G), asparagine (N), serine (S) or threonine (T). In certain examples, the *Fok*I nuclease domain comprises a mutation at one or more of 416, 422, 447, 479 and/or 525 (numbered relative to wild-type, SEQ ID NO:2). The nuclease domains may also comprise one or more mutations at positions 418, 432, 441, 448, 476, 481, 483, 486, 487, 490, 496, 499, 523, 527, 537, 538 and 559, including but not limited to ELD, KKR, ELE, KKS. *See, e.g.,* U.S. Patent No. 8,623,618. In still further examples,

the cleavage domain includes mutations at one or more of the residues (*e.g.,* 419, 420, 425, 446, 447, 470, 471, 472, 475, 478, 480, 492, 500, 502, 521, 523, 526, 530, 536, 540, 545, 573 and/or 574). In certain examples, the variant cleavage domains described herein include mutations to the residues involved in nuclease dimerization (dimerization domain mutations), and one or more additional mutations; for example to phosphate contact residues: *e.g*. dimerization mutants (such as ELD, KKR, ELE, KKS, etc.) in combination with one, two, three, four, five, six or more mutations at amino acid positions outside of the dimerization domain, for example in amino acid residues that may participate in phosphate contact. In a preferred example, the mutation at positions 416, 422, 447, 448 and/or 525 comprise replacement of a positively charged amino acid with an uncharged or a negatively charged amino acid. In other examples, mutations at positions 446, 472 and/or 478 (and optionally additional residues for example in the dimerization or catalytic domains) are made. In certain examples, the engineered cleavage half-domain comprises a mutation at position 542 (*e.g.,* N542D) and/or 478 (*e.g.,* P478S). Also described are heterodimers of engineered cleavage domains, for example, a first (left) nuclease comprising one engineered cleavage domain (*e.g.,* N542D) and a second nuclease comprising a different engineered cleavage domain (*e.g.,* P478S).

[0016] Any of the engineered cleavage half domains described above may be incorporated into artificial nucleases (and polynucleotides expressing these artificial nucleases), for example by associating them with a DNA-binding domain, including but not limited to zinc finger nucleases, TALENs, CRISPR/Cas nucleases, and the like. The zinc finger proteins of the zinc finger nucleases may comprise non-canonical zinc-coordinating residues (*e.g*. CCHC rather than the canonical C2H2 configuration, see United States Patent 9,234,187).

[0017] In another example, fusion molecules comprising a DNA binding domain and an engineered FokI or homologue thereof cleavage half-domain as described herein that produce an artificial nuclease are provided. In certain examples, the DNA-binding domain of the fusion molecule is a zinc finger binding domain (*e.g.,* an engineered zinc finger binding domain). In other examples, the DNA-binding domain is a TALE DNA-binding domain. In still further examples, the DNA binding domain comprises a DNA binding molecule (*e.g*. guide RNA) and a catalytically inactive Cas9 or Cfp1 protein (dCas9 or dCfp1). In some examples, the engineered fusion molecules form a nuclease complex with a catalytically inactive

engineered cleavage half-domain such that the dimeric nuclease is only capable of cleaving only one strand of a double-stranded DNA molecule, forming a nickase (*see* U.S. Patent 9,200,266).

[0018] The methods and compositions of the disclosure can also include mutations to one or more amino acids within the DNA binding domain outside the residues that recognize the nucleotides of the target sequence (*e.g.,* one or more mutations to the 'ZFP backbone' (outside the DNA recognition helix region) or to the 'TALE backbone' (outside of the RVDs)) that can interact non-specifically with phosphates on the DNA backbone. Thus, in certain examples, the disclosure includes mutations of cationic amino acid residues in the ZFP backbone that are not required for nucleotide target specificity. In some examples, these mutations in the ZFP backbone comprise mutating a cationic amino acid residue to a neutral or anionic amino acid residue. In some examples, these mutations in the ZFP backbone comprise mutating a polar amino acid residue to a neutral or non-polar amino acid residue. In preferred examples, mutations at made at position (-5), (-9) and/or position (-14) relative to the DNA binding helix. In some examples, a zinc finger may comprise one or more mutations at (-5), (-9) and/or (-14). In further examples, one or more zinc fingers in a multi-finger zinc finger protein may comprise mutations in (-5), (-9) and/or (-14). In some examples, the amino acids at (-5), (-9) and/or (-14) (*e.g*. an arginine (R) or lysine (K)) are mutated to an alanine (A), leucine (L), Ser (S), Asp (N), Glu (E), Tyr (Y) and/or glutamine (Q). *See, e.g.,* U.S. Publication No. US-2018-0087072.

[0019] In another example, polynucleotides encoding any of the engineered cleavage half-domains or fusion molecules (including artificial nucleases) as described herein are provided. Non-limiting examples of suitable polynucleotides include mRNA, cDNA, viral vectors (AAV, Ad, LV), and/or non-viral vectors (plasmid vectors).

[0020] In some examples, the methods and compositions of the disclosure include the use of sequences encoding exogenous peptide sequences fused to eukaryotic transgene sequences. In some examples, exogenous peptides are fused to protein sequences post-translationally, and in other examples, the sequences encoding the exogenous peptides are linked in frame (3' and/or 5') to sequences encoding the artificial nuclease (*e.g*., a fusion protein). The exogenous peptides may encode sequences useful for purification or labeling, *e.g*. affinity purification or

immunohistochemistry. Examples of such peptides are polyhistidine tags ("His tag", Hochuli *et al* (1988), *Bio/Technol* 6(11):1321-5) or cationic peptide tags such as Flag tags (Hopp *et al* (1988) *Bio/Technol* 6(10):1204-10; Hernan *et al*. (2000) *BioTechniques* 28(4), 789-793). One or more (1, 2, 3, 4, 5 or more) of these peptide tag sequences can be used in any combinations. In some examples, the sequence encoding an exogenous Flag peptide comprising the sequence N-term DYKDDDK (SEQ ID NO:3) is fused in frame at the C-terminus or N-terminus of a sequence encoding an artificial nuclease. In preferred examples, a sequence encoding 3 FLAG sequences (3x FLAG peptide) is used (see U.S. Patent 6,379,903), wherein the amino acid sequence is N-term (M)DYKDHDG-DYKDHDI-DYKDDDDK (SEQ ID NO:4), where an N-terminal methionine (M) is optional. Inclusion of one or more of such peptide sequences (*e.g.,* polycationic sequences such as 3X FLAG) can increase nuclease (cleavage) activity by 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 or more fold) as compared to nucleases without the peptide sequences.

[0021] In some examples, the mRNA encoding an artificial nuclease comprises a nuclear localization peptide sequence (NLS). In some examples, the NLS comprises the sequence PKKKRKV (SEQ ID NO:5) from the SV40 virus large T gene (*see* Kalderon *et al* (1984) *Nature* 311(5981):33-8) while in others, the NLS comprises the sequence PAAKRVKLD (SEQ ID NO:6) from the c-myc protein (*see* Dang and Lee (1988) *Mol Cell Biol* 8(10):4048-54). In some examples, the NLS comprises the sequence EGAPPAKRAR (SEQ ID NO:7) from the hepatitis delta virus (*see* Alves *et al* (2008) Virology 370: 12-21) or VSRKRPRP (SEQ ID NO:8) from the polyoma T protein (Richardson *et al* (1986) *Cell* 44(1):77-85). In other examples, the NLS comprises the sequence KRPAATKKAGQAKKKKLD (SEQ ID NO:9), derived from the nucleoplasmin carboxy tail (*see* Dingwall (1988) *J Cell Biol* 107:841-849 and Robbins *et al* (1991) *Cell* 64(3):615-23), while in some examples, the NLS comprises the sequence NQSSNFGPMKGGNFGGRSSGPYGGGGQYFAKPRNQGGY (SEQ ID NO:10) first described by Siomi and Dreyfuss (Siomi and Dreyfus (1995) *J Cell Biol* 129(3):551-560). In further examples, the NLS comprises the sequence PKTRRRPRRSQRKRPPT (SEQ ID NO:11) from the Rex protein in HTLV-1 (Siomi *et al* (1988) *Cell* 55(2):197-209). Inclusion of one or more of NLS sequences as described herein can increase nuclease (cleavage) activity by 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 or more fold) as compared to nucleases without the peptide sequences.

[0022] In yet another example, cells comprising any of the nucleases, polypeptides (*e.g.,* fusion molecules or fusion polypeptides) and/or polynucleotides as described herein are also provided. In one example, the cells comprise a pair of fusion polypeptides, one fusion polypeptide comprising, in addition to one or more mutations in amino acid residues 393, 394, 398, 416, 421, 422, 442, 444, 447, 448, 473, 480, 530 and/or 525, an ELD or ELE cleavage half-domain and one fusion polypeptide comprising, in addition to one or more mutations at residues 393, 394, 398, 416, 421, 422, 442, 444, 446, 447, 448, 472, 473, 478, 480, 530 and/or 525, a KKK or KKR cleavage half-domain (see U.S. Patent 8,962,281). In some examples, one fusion protein comprises a mutation in residue 542 of FokI (residue 159 of the cleavage domain) such as N542D and one fusion polypeptide comprises a mutation in residue 478 of FokI (residue 95 of the cleavage domain) such as P478S).

[0023] In any of these fusion polypeptides described herein, the ZFP partners may further comprise mutations in the zinc finger DNA binding domain in the (-5), (-9) and/or (-14) positions. In some examples, the Arg (R) at position -5 is changed to a Tyr (Y), Asp (N), Glu (E), Leu (L), Gln (Q), or Ala (A). In other examples, the Arg (R) at position (-9) is replaced with Ser (S), Asp (N), or Glu (E). In further examples, the Arg (R) at position (-14) is replaced with Ser (S) or Gln (Q). In other examples, the fusion polypeptides can comprise mutations in the zinc finger DNA binding domain where the amino acids at the (-5), (-9) and/or (-14) positions are changed to any of the above listed amino acids in any combination.

[0024] Also provided herein are cells that have been modified by the polypeptides and/or polynucleotides of the disclosure, including cells descended and/or differentiated from cells comprising one or more artificial nucleases as described herein. In some examples, the cells comprise a nuclease-mediated insertion of a transgene, or a nuclease-mediated knock out of a gene. The modified cells, and any cells derived from the modified cells do not necessarily comprise the nucleases of the disclosure more than transiently, but the genomic modifications mediated by such nucleases remain.

[0025] In yet another example, methods for targeted cleavage of cellular chromatin in a region of interest; methods of causing homologous recombination to occur in a cell; methods of treating infection; and/or methods of treating disease are provided. These methods maybe practiced *in vitro, ex vivo* or *in vivo* or a combination thereof. The methods involve cleaving cellular chromatin at a

predetermined region of interest in cells using one or more artificial nucleases as described herein. In certain examples, a pair of fusion polypeptides as described herein (*i.e.,* a pair of fusion polypeptides in which one or both fusion polypeptide(s) comprises the engineered cleavage half-domains as described herein). In certain examples, the targeted cleavage of the on-target site is increased by at least 50 to 200% (or any value therebetween) or more, including 50%-60% (or any value therebetween), 60%-70% (or any value therebetween), 70%-80% (or any value therebetween), 80%-90% (or any value therebetween, 90% to 200% (or any value therebetween), as compared to cleavage domains without the mutations as described herein. Similarly, using the methods and compositions as described herein, off-target site cleavage is reduced by 1-100 or more-fold, including but not limited to 1-50-fold (or any value therebetween). In certain examples, the targeted cleavage of the nuclease activity is increased by at least 50 to 200% (or any value therebetween) or more, including 50%-60% (or any value therebetween), 60%-70% (or any value therebetween), 70%-80% (or any value therebetween), 80%-90% (or any value therebetween, 90% to 200% (or any value therebetween) or by1-100 or more-fold, including but not limited to 1-50-fold (or any value therebetween), as compared to when constructs encoding the nucleases described herein do not include the modifications (enhancements).

[0026] The artificial nucleases (and polynucleotides encoding the same) described herein can be used in methods for targeted cleavage of cellular chromatin in a region of interest and/or homologous recombination at a predetermined region of interest in cells. Cells include cultured cells, cell lines, cells in an organism, cells that have been removed from an organism for treatment in cases where the cells and/or their descendants will be returned to the organism after treatment, and cells removed from an organism, modified using the fusion molecules of the disclosure, and then returned to the organism in a method of treatment (cell therapy). A region of interest in cellular chromatin can be, for example, a genomic sequence or portion thereof. Compositions include fusion molecules or polynucleotides encoding fusion molecules that comprise a DNA binding molecule (*e.g.,* an engineered zinc finger or TALE binding domain or an engineered CRISPR guide RNA) and a cleavage half domain as described.

[0027] A fusion molecule can be expressed in a cell, *e.g.*, by delivering the fusion molecule to the cell as a polypeptide, or by delivering a polynucleotide

encoding the fusion molecule to a cell, wherein the polynucleotide, if DNA, is transcribed and is translated, to generate the fusion molecule. Further, if the polynucleotide is an mRNA encoding the fusion molecule (or component thereof), following delivery of the mRNA to the cell, the mRNA is translated, thus generating the fusion molecule.

[0028] In other examples of the disclosure are provided methods and compositions for increasing engineered nuclease specificity. In one example, methods are provided for increasing overall on-target cleavage specificity by decreasing off-target cleavage activity. In some examples, the component engineered cleavage half-domain-containing partners of an engineered nuclease complex are used to contact a cell, where each partner of the complex is given in a ratio to the other partner other than one to one. In some examples, the ratio of the two partners (half cleavage domains) is given at a 1:2, 1:3, 1:4, 1:5, 1:6, 1:8, 1:9, 1:10 or 1:20 ratio, or any value therebetween. In other examples, the ratio of the two partners is greater than 1:30. In other examples, the two partners are deployed at a ratio that is chosen to be different from 1:1. In some examples, each partner is delivered to the cell as an mRNA or is delivered in a viral or non-viral vector where different quantities of mRNA or vector encoding each partner are delivered. In further examples, each partner of the nuclease complex may be comprised on a single viral or non-viral vector, but is deliberately expressed such that one partner is expressed at a higher or lower value that the other, ultimately delivering the cell a ratio of cleavage half domains that is other than one to one. In some examples, each cleavage half domain is expressed using different promoters with different expression efficiencies. In other examples, the two cleavage domains are delivered to the cell using a viral or non-viral vector where both are expressed from the same open reading frame, but the genes encoding the two partners are separated by a sequence (*e.g.* self-cleaving 2A sequence or IRES) that results in the 3' partner being expressed at a lower rate, such that the ratios of the two partners are 1:2, 1:3, 1:4, 1:5, 1:6, 1:8, 1:9, 1:10 or 1:20 ratio, or any value therebetween. In other examples, the two partners are deployed at a ratio that is chosen to be different from 1:1.

[0029] Accordingly, in another example, a method for cleaving cellular chromatin in a region of interest can comprise (a) selecting a first sequence in the region of interest; (b) engineering a first DNA-binding molecule to specifically bind

to the first sequence; (c) expressing a first fusion molecule in the cell, the first fusion molecule comprising the first DNA-binding molecule (*e.g.*, zinc finger, TALE, sgRNA), and a cleavage domain (or half-domain); and (d) expressing a second fusion protein in the cell, the second fusion molecule comprising a second DNA-binding domain, and a second cleavage domain (or half-domain), wherein at least one of the fusion molecules comprises a linker as described herein, and further wherein the first fusion molecule binds to the first sequence, and the second fusion molecule binds to a second sequence located between 2 and 50 nucleotides from the first sequence, such that an active nuclease complex can form and cellular chromatin is cleaved in the region of interest. In certain examples, both fusion molecules comprise a linker as described herein between the DNA binding domain and the catalytic nuclease domain.

[0030] Also provided are methods of altering a region of cellular chromatin (*e.g.,* an endogenous gene), for example to introduce targeted mutations. In certain examples, methods of altering cellular chromatin comprise introducing into the cell one or more targeted nucleases to create a double-stranded break in cellular chromatin at a predetermined site, and a donor polynucleotide, having homology to the nucleotide sequence of the cellular chromatin in the region of the break. Cellular DNA repair processes are activated by the presence of the double-stranded break and the donor polynucleotide is used as a template for repair of the break, resulting in the introduction of all or part of the nucleotide sequence of the donor into the cellular chromatin. Thus, a sequence in cellular chromatin can be altered and, in certain examples, can be converted into a sequence present in a donor polynucleotide. One or more targets may be altered using the methods and compositions described herein.

[0031] Targeted alterations include, but are not limited to, point mutations (*i.e.*, conversion of a single base pair to a different base pair), substitutions (*i.e.*, conversion of a plurality of base pairs to a different sequence of identical length), insertions or one or more base pairs, deletions of one or more base pairs and any combination of the aforementioned sequence alterations. Alterations can also include conversion of base pairs that are part of a coding sequence such that the encoded amino acid is altered.

[0032] The donor polynucleotide can be DNA or RNA, can be linear or circular, and can be single-stranded or double-stranded. It can be delivered to the cell

as naked nucleic acid, as a complex with one or more delivery agents (*e.g.*, liposomes, nanoparticles, poloxamers) or contained in a viral delivery vehicle, such as, for example, an adenovirus, lentivirus or an Adeno-Associated Virus (AAV). Donor sequences can range in length from 10 to 1,000 nucleotides (or any integral value of nucleotides therebetween) or longer. In some examples, the donor comprises a full-length gene flanked by regions of homology with the targeted cleavage site. In some examples, the donor lacks homologous regions and is integrated into a target locus through homology independent mechanism (*i.e.* NHEJ). In other examples, the donor comprises a smaller piece of nucleic acid flanked by homologous regions for use in the cell *(i.e.* for gene correction). In some examples, the donor comprises a gene encoding a functional or structural component such as a shRNA, RNAi, miRNA or the like. In other examples, the donor comprises sequences encoding a regulatory element that binds to and/or modulates expression of a gene of interest. In other examples, the donor is a regulatory protein of interest (*e.g.* ZFP TFs, TALE TFs or a CRISPR/Cas TF) that binds to and/or modulates expression of a gene of interest.

[0033] In certain of the methods and compositions described herein, the nuclease and donor are delivered using one or more mRNAs and/or AAV vectors. Any dose of mRNA (ng) or AAV vector (vg/dose) can be used. In examples in which mRNA is deliver the nuclease(s) and/or optional donor, dosages of mRNA typically range between 10 and 5000 ng/cell or subject (*e.g.,* 2000 ng, 62.5 ng, 31.3 ng, 15.6 ng). In examples in which AAV vectors are used to carry the nucleases and/or optional donor, dosages typically range between 1.00E+9 to 1.00E+13 vg/subject or cell for each nuclease (*e.g.,* left and right ZFNs) and the optional donor is given at 1.00E+10 to 1.00E+13. In certain examples, each nuclease of a pair is carried on a separate AAV vector and is given at 2.00E+10, 6.00E+10 or 2.00E+11 vg/cell or subject and the donor is carried on another AAV vector and is given at 1.60E+11, 4.8E+11 or 1.6E+12 vg/cell or subject.

[0034] For any of the aforementioned methods, the cellular chromatin can be in a chromosome, episome or organellar genome. Cellular chromatin can be present in any type of cell including, but not limited to, prokaryotic and eukaryotic cells, fungal cells, plant cells, animal cells, mammalian cells, primate cells and human cells.

[0035] In one example, described herein is a zinc finger nuclease comprising first and second (also referred to as left and right or ZFN partners) ZFNs, the first

ZFN comprising the ZFN designated 71557 (comprising a ZFP having the recognition helix regions shown in Table 1 for SBS 42875 and having additional features (*e.g.,* mutations in the FokI sequences and ZFP backbone, 5' UTR sequence, etc.) shown in Table 3 and Table 4) and the second ZFN comprising the designated 71728 (comprising a ZFP having the recognition helix regions shown in Table 1 for SBS 47874 and other features (*e.g.,* mutations in the FokI sequences and ZFP backbone, 5' UTR sequence, etc.) shown in Table 3 and Table 5) and/or one or more polynucleotides encoding one or both of the left and right ZFNs. In certain examples, the first and second (left and right) ZFNs are encoded by separate polynucleotides, which separate polynucleotides may be of the same or different types (*e.g.,* 2 AAV vectors in which one AAV comprises sequences encoding the left ZFN and one AAV comprises sequences encoding the right ZFN, 2 mRNAs where one mRNA encodes the left ZFN and the other encodes the right ZFN, 1 AAV comprising both ZFNs linked together by a self-cleaving peptide sequence (for example 2A) and 1 mRNA encoding one ZFN used together with one AAV comprising sequences encoding the other ZFN, etc.). In certain examples, the vectors are AAV vectors comprising the elements (sequences) as shown in Table 4 and/or Table 5, including the complete AAV sequences designated "71557 AAV" or "SB71557 AAV" (SEQ ID NO:43) and "71728 AAV" or "SB71728 AAV" (SEQ ID NO:56) as shown herein. In other examples, one or more of the elements shown in Tables 4 and 5 are replaced with any analogous sequence, for example the WPRE sequence of these Tables may be replaced with WPRE sequences known in the art or set forth herein in Example 4 (*e.g.,* SEQ ID NO:68 or SEQ ID NO:69 or other WPRE sequence in place of SEQ ID NO:53). In some examples, many amino acid modifications can be made to the ZFNs. In some examples, 3, 6, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or more amino acid substitutions are made. In some examples, 13 combined amino acid substitutions to six of the fingers on the right and left sides are made.

[0036] Thus, described herein is a zinc finger nuclease comprising first and second ZFNs, the first ZFN comprising the ZFN designated 71557 and the second ZFN comprising the ZFN designated 71728. One or more polynucleotides comprising sequences encoding the first and second ZFNs as described herein are also provided. In certain examples, a single polynucleotide encodes the first and second ZFNs and in other examples, two separate polynucleotides comprise sequences encoding the first and second ZFNs. The one or more polynucleotides

encoding the ZFN may be carried on the same or different AAV vectors. In certain examples, provided herein are two polynucleotides, the first polynucleotide (*e.g.,* AAV vector) comprising sequences as shown in Table 4 or the sequence as shown in SEQ ID NO:43 and the second polynucleotide (*e.g.,* AAV vector) comprising sequences as shown in Table 5 or the sequence as shown in SEQ ID NO:56. In some examples, the AAV comprising the left ZFN (SB-71557) is referred to as SB-A6P-ZL2. In some examples, the AAV comprising the right ZFN (SB-71728) is referred to as SB-A6P-ZR2.

[0037] In another example, described herein is a cell (*e.g.,* stem cell, precursor cell or hepatic cells of a subject) comprising one or more ZFNs and/or polynucleotides (*e.g.,* AAV vectors) as described herein. Any cell or cell line can be used, including but not limited to a stem cell, a precursor cell, a liver cell, a blood cell, or the like. Cells may further include a donor polynucleotide, typically a polynucleotide encoding an exogenous sequence such as a transgene encoding a therapeutic protein or fragment thereof, which exogenous sequence is integrated into the genome of the cell following cleavage of the endogenous albumin gene. The donor may be carried on the same vector as one or both of the ZFN partners or, alternatively, may be administered using a separate vector, which may be the same or different type than the vector(s) carrying one or both of the ZFN partners. In certain examples, the cells comprise 3 separate AAV vectors, the first comprising sequences encoding the left ZFN, the second comprising sequences encoding the right ZFN and the third comprising the donor polynucleotide. Daughter cells descended from cells comprising the ZFNs and donor polynucleotide, which daughter cells include genetic modifications made by the ZFNs (*e.g.,* integrated donor polynucleotide), are also described. Such genetic modifications may be identified through standard methods known in the art, including next generation sequencing of the genomic DNA of the daughter cells wherein such sequence results are compared to wildtype cells that have not been treated with the ZFN and donor polynucleotides.

[0038] In another example, described herein are pharmaceutical compositions comprising one or more ZFNs, one or more polynucleotides (*e.g.,* AAV vectors) and/or one or more cells as described herein. In certain examples, the pharmaceutical composition comprises 3 separate AAV vectors: a first AAV comprising ZFN 71557 (*e.g.,* "71557 AAV"); a second AAV comprising ZFN 71728 (*e.g.,* "71728 AAV"); and third AAV comprising a donor polynucleotide.

[0039] In another example, methods of using one or more of the ZFNs, one or more of the polynucleotides (*e.g.,* AAV vectors), one or more of the cells and/or one or more of the pharmaceutical compositions (*e.g.,* a pharmaceutical composition comprising 3 separate AAV vectors) as described herein for cleaving an endogenous albumin gene, optionally wherein the method (use) further comprises administering a donor polynucleotide comprising an exogenous sequence (*e.g.,* carried by an AAV vector) such that the exogenous sequence is integrated into the cleaved albumin gene in the isolated cell or in a cell of a subject. In some examples, such one or more of the ZFNs, one or more of the polynucleotides, one or more of the cells and/or one or more of the pharmaceutical compositions described herein are used to prevent or treat a human disease.

[0040] Kits comprising one or more zinc finger nucleases, one or more polynucleotides, one or more cells and/or one or more pharmaceutical compositions as described herein as well as optional instructions for their use are also provided.

[0041] In yet another example, described herein is a composition (also referred to as a "FIX composition") comprising: (a) a first polynucleotide (*e.g.,* AAV) comprising sequences encoding a first ZFN designated 71557, the first polynucleotide optionally comprising the sequences as shown in Table 4 or the sequence designated SB71557 AAV (SEQ ID NO:43); (b) a second polynucleotide (*e.g.,* AAV) comprising sequences encoding a second ZFN designated 71728, the second polynucleotide optionally comprising sequences as shown in Table 5 or the sequence designated SB71728 AAV (SEQ ID NO:56); and (c) a donor polynucleotide (*e.g.,* AAV) comprising a sequence encoding a Factor IX (FIX) protein. In certain examples, the donor comprises the sequences as shown in Table 6, optionally the sequence as shown in SEQ ID NO:59. In any of the FIX compositions described herein, the first, second and donor polynucleotides may be carried on three separate AAV vectors. Methods of using the compositions as described herein for expressing FIX in a subject in need thereof are also provided. In certain examples, the composition is administered to the subject such that the ZFN (71557 and 71728) cleaves an endogenous albumin gene in the subject, the FIX sequence is integrated into the cleaved albumin gene and a FIX protein is expressed in the subject. The methods and compositions described herein can be used to treat and/or prevent a hemophilia in a subject in need thereof. Kits comprising one or more of the FIX compositions and optionally instructions for their use are also provided.

[0042] In a still further example, described herein is a composition (also referred to as "MPS II composition" or "IDS composition") comprising: (a) a first polynucleotide (*e.g.,* AAV) comprising sequences encoding a first ZFN designated 71557, the first polynucleotide optionally comprising the sequences as shown in Table 4 or the sequence designated SB 71557 AAV (SEQ ID NO:43); (b) a second polynucleotide (*e.g.,* AAV) comprising sequences encoding a second ZFN designated 71728, the second polynucleotide optionally comprising sequences as shown in Table 5 or the sequence designated SB 71728 (SEQ ID NO:56); and (c) a donor polynucleotide (*e.g.,* AAV) comprising a sequence encoding an iduronate-2-sulfatase (IDS) sequence. In certain examples, the donor comprises the sequences as shown in Table 7, optionally the sequence as shown in SEQ ID NO:65. In any of the MPS II compositions described herein, the first, second and donor polynucleotides may be carried on three separate AAV vectors. Methods of using the compositions as described herein for expressing IDS in a subject in need thereof are also provided. In certain examples, the composition is administered to the subject such that the ZFN (71557 and 71728) cleaves an endogenous albumin gene in the subject, the IDS sequence is integrated into the cleaved albumin gene and an IDS protein is expressed in the subject. The methods and compositions described herein can be used to treat and/or prevent MPS II in a subject in need thereof. Kits comprising one or more of the MPS II compositions and optionally instructions for their use are also provided.

[0043] In a still further example, described herein is a composition (also referred to as "MPS I composition" or "IDUA composition") comprising: (a) a first polynucleotide (*e.g.,* AAV) comprising sequences encoding a first ZFN designated 71557, the first polynucleotide optionally comprising the sequences as shown in Table 4 or the sequence designated SB 71557 AAV (SEQ ID NO:43); (b) a second polynucleotide (*e.g.,* AAV) comprising sequences encoding a second ZFN designated 71728, the second polynucleotide optionally comprising sequences as shown in Table 5 or the sequence designated SB SEQ ID NO:56; and (c) a donor polynucleotide (*e.g.,* AAV) comprising a sequence encoding an alpha-L iduronidase (IDUA) sequence. In certain examples, the donor comprises the sequences as shown in Table 8, optionally the sequence as shown in SEQ ID NO:72. In any of the MPS I compositions described herein, the first, second and donor polynucleotides may be carried on three separate AAV vectors. Methods of using the compositions as described herein for expressing IDUA in a subject in need thereof are also provided.

In certain examples, the composition is administered to the subject such that the ZFN (71557 and 71728) cleaves an endogenous albumin gene in the subject, the IDUA sequence is integrated into the cleaved albumin gene and an IDUA protein is expressed in the subject. The methods and compositions described herein can be used to treat and/or prevent MPS I in a subject in need thereof. Kits comprising one or more of the MPS I compositions and optionally instructions for their use are also provided.

[0044] In some examples, any of the compositions disclosed herein are administered to the subject in need thereof in a single dose. In other examples, the compositions are administered in more than one dose. In some examples, the compositions are administered in more than one dose with a time period in between the doses. In some examples, the time period comprises 1, 2, 3, 4, 5, or 6 months. In some examples, the time period comprises half a year, a year, two years, three years, four years, 5 years or more.

[0045] In yet another example, cells comprising any of the polypeptides (*e.g.,* fusion molecules) and/or polynucleotides as described herein are also provided. In one example, the cells comprise a pair of fusion molecules, each comprising a cleavage domain as disclosed herein. Cells include cultured cells, cells in an organism and cells that have been removed from an organism for treatment in cases where the cells and/or their descendants will be returned to the organism after treatment. A region of interest in cellular chromatin can be, for example, a genomic sequence or portion thereof.

[0046] In another example, described herein is a kit comprising a fusion protein as described herein or a polynucleotide encoding one or more zinc finger proteins, cleavage domains and/or fusion proteins as described herein; ancillary reagents; and optionally instructions and suitable containers. The kit may also include one or more nucleases or polynucleotides encoding such nucleases.

[0047] These and other examples will be readily apparent to the skilled artisan in light of disclosure as a whole.

BRIEF DESCRIPTION OF THE DRAWINGS

[0048] **Figures 1A and 1B** depict partial sequence of a human albumin genomic sequence (SEQ ID NO:41) and show the binding sites of exemplary ZFNs 47171-FLAG and 47898-FLAG (target sites shown by underline or overline). Figure

CLAIMS

What is claimed is:

1. A zinc finger nuclease (ZFN) comprising first and second ZFNs, the first ZFN comprising the ZFN designated 71557 and the second ZFN comprising the ZFN designated 71728.

2. One or more polynucleotides encoding the first and second ZFNs of claim 1, optionally wherein the one or more polynucleotides are carried on AAV vectors.

3. The one or more polynucleotides of claim 2, wherein a first polynucleotide encoding the first ZFN comprises an AAV vector comprising the sequences as shown in Table 4 and a second polynucleotide encoding the second ZFN comprises an AAV vector comprising the sequences as shown in Table 5.

4. An AAV vector of claim 2 or 3, wherein the AAV vector encoding the first ZFN comprises the sequence shown in SEQ ID NO:43.

5. The AAV vector of claim 2 or 3, wherein the AAV vector encoding the second ZFN comprises the sequence shown in SEQ ID NO:56.

6. A cell comprising one or more ZFNs of claim 1, one or more polynucleotides of any of claims 2 to 3, and/or one or more AAV vectors of claims 4 and 5, optionally a stem cell or precursor cell.

7. A pharmaceutical composition comprising one or more ZFNs of claim 1, one or more polynucleotides of any of claims 2 to 3, one or more AAV vectors of claims 4 and 5 and/or one or more cells of claim 6.

8. The one or more ZFNs, one or more polynucleotides, one or more AAV vectors, one or more cells and/or one or more pharmaceutical composition of any of the preceding claims for use in therapy, wherein the one or more ZFNs, one or more polynucleotides, one or more AAV vectors, one or more cells and/or one or more pharmaceutical composition of

any of the preceding claims cleaves an endogenous albumin gene in a subject.

9. Use of one or more ZFNs, one or more polynucleotides, one or more AAV vectors, one or more cells and/or one or more pharmaceutical composition of any of the preceding claims in the manufacture of a medicament for cleaving an endogenous albumin gene in a subject.

10. The one or more ZFNs, one or more polynucleotides, one or more AAV vectors, one or more cells and/or one or more pharmaceutical composition for use of claim 8, further comprising that a donor is to be administered, optionally carried on an AAV vector, to the subject such that the donor is integrated into the cleaved albumin gene in the subject.

11. A kit comprising one or more ZFNs of claim 1, one or more polynucleotides of any of claims 2 to 3, one or more AAV vectors of claims 4 and 5, one or more cells of claim 6 and/or one or more pharmaceutical compositions according to claim 7.

12. A composition comprising:

(a) a first polynucleotide encoding a first ZFN designated 71557, the first polynucleotide optionally comprising an AAV vector comprising the sequences as shown in Table 4 or SEQ ID NO:43;

(b) a second polynucleotide encoding a second ZFN designated 71728, the second polynucleotide optionally comprising an AAV vector comprising the sequences as shown in Table 5 or SEQ ID NO:56; and

(c) a donor polynucleotide comprising a sequence encoding a Factor IX (FIX) sequence.

13. The composition of claim 12, wherein the donor polynucleotide is an AAV vector comprising the sequences as shown in Table 6, optionally the sequence as shown in SEQ ID NO:59.

14. The composition of claim 12 or 13, wherein the first, second and donor polynucleotides are carried on separate AAV vectors.

15. Use of the composition of any of claims 12 to 14 in the manufacture of a medicament for treating hemophilia in a subject in need thereof, wherein the composition is to be administered to the subject such that the ZFN cleaves an endogenous albumin gene in the subject, the FIX sequence is integrated into the cleaved albumin gene and a FIX protein is expressed in the subject.

16. Use of one or more compositions of any of claims 12 to 14 in the manufacture of a medicament for treating and/or preventing hemophilia in a subject in need thereof, wherein the composition is to be administered to the subject such that that a FIX protein is expressed in a cell of the subject.

17. A kit comprising one or more compositions of any of claims 12-14.

18. A composition comprising:

(a) a first polynucleotide encoding a first ZFN designated 71557, the first polynucleotide optionally comprising an AAV vector comprising the sequences as shown in Table 4 or SEQ ID NO:43;

(b) a second polynucleotide encoding a second ZFN designated 71728, the second polynucleotide optionally comprising an AAV vector comprising the sequences as shown in Table 5 or SEQ ID NO:56; and

(c) a donor polynucleotide comprising a sequence encoding an iduronate-2-sulfatase (IDS) sequence.

19. The composition of claim 17, wherein the donor polynucleotide is an AAV vector comprising the sequences as shown in Table 7, optionally the sequence as shown in SEQ ID NO:65.

20. The composition of claim 18 or 19, wherein the first, second and donor polynucleotides are carried on separate AAV vectors.

21. Use of the composition of any of claims 18 to 20 in the manufacture of a medicament for treating Mucopolysaccharidosis II (MPS II) disease in a subject in need thereof, wherein the composition is to be administered to the subject such that the ZFN

cleaves an endogenous albumin gene in the subject, the IDS sequence is integrated into the cleaved albumin gene and an IDS protein is expressed in the subject.

22. Use of one or more compositions of any of claims 18 to 20 in the manufacture of a medicament for treating and/or preventing MPS II disease in a subject in need thereof, wherein the composition is to be such that that an IDS protein is expressed in a cell of the subject.

23. A kit comprising one or more compositions of any of claims 18 to 20.

24. A composition comprising:

(a) a first polynucleotide encoding a first ZFN designated 71557, the first polynucleotide optionally comprising an AAV vector comprising the sequences as shown in Table 4 or SEQ ID NO:43;

(b) a second polynucleotide encoding a second ZFN designated 71728, the second polynucleotide optionally comprising an AAV vector comprising the sequences as shown in Table 5 or SEQ ID NO:56; and

(c) a donor polynucleotide comprising a sequence encoding an alpha-L iduronidase (IDUA) sequence.

25. The composition of claim 24, wherein the donor comprises the sequences as shown in Table 8, optionally the sequence as shown in SEQ ID NO:72.

26. The composition of claim 24 or claim 25, wherein the first, second and donor polynucleotides are carried on separate AAV vectors.

27. Use of the composition of any of claims 24 to 26 in the manufacture of a medicament for treating Mucopolysaccharidosis type I (MPS I) disease, wherein the composition is to be administered to a subject in need thereof such that the ZFN cleaves an endogenous albumin gene in the subject, the IDUA sequence is integrated into the cleaved albumin gene and an IDUA protein is expressed in the subject.

28. Use of one or more compositions of any of claims 24 to 26 in the manufacture of a medicament for treating and/or preventing MPS I disease in a subject in need thereof,

wherein the composition is to be administered to the subject such that that a IDUA protein is expressed in a cell of the subject.

29. A kit comprising one or more compositions of any of claims 24 to 26.