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(71) Applicant(s)
Sangamo Therapeutics, Inc.

(72) Inventor(s)
Miller, Jeffrey C.;Paschon, David;Rebar, Edward J.;Wechsler, Thomas;Zhang, Lei

(74) Agent / Attorney
WRAYS PTY LTD, L7 863 Hay St, Perth, WA, 6000, AU

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(71) Applicant: SANGAMO BIOSCIENCES, INC. [US/US];
Point Richmond Tech Center, 501 Canal Blvd., Suite
A100, Richmond, California 94804 (US).

(72) Inventors: MILLER, Jeffrey C.; c/o Sangamo BioSciences, Inc., 501 Canal Blvd., Suite A100, Richmond, California 94804 (US). PASCHON, David; c/o Sangamo BioSciences, Inc., 501 Canal Blvd., Suite A100, Richmond, California 94804 (US). REBAR, Edward J.; c/o Sangamo BioSciences, Inc., 501 Canal Blvd., Suite A100, Richmond, California 94804 (US). WECHSLER, Thomas; c/o Sangamo BioSciences, Inc., 501 Canal Blvd., Suite A100, Richmond, California 94804 (US). ZHANG, Lei; c/o Sangamo BioSciences, Inc., 501 Canal Blvd., Suite A100, Richmond, California 94804 (US).

(74) Agent: PASTERNAK, Dahnna S.; Pasternak Patent Law,
1900 Embarcadero Rd., Suite 211, Palo Alto, California
94303 (US).

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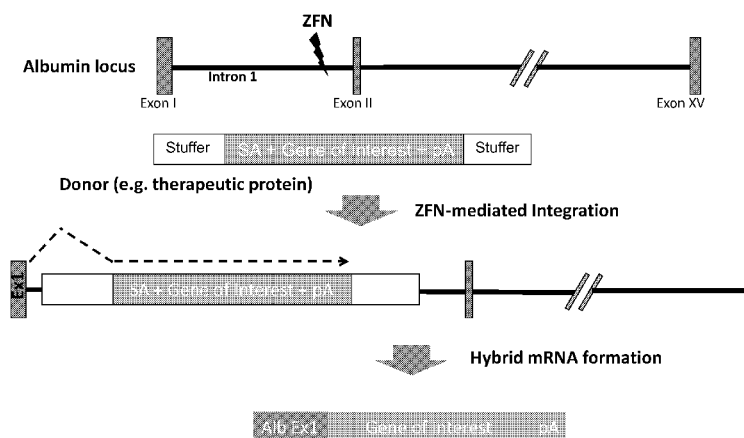
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[Continued on next page]

(54) Title: METHODS AND COMPOSITIONS FOR GENOME ENGINEERING

Figure 1



(57) Abstract: Disclosed herein are methods and compositions for insertion of transgene sequences encoding proteins that is aberrantly expressed in disease or disorder such as a lysosomal storage disease.



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METHODS AND COMPOSITIONS FOR GENOME ENGINEERING

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] The present application claims the benefit of U.S. Provisional Application No. 61/913,838, filed December 9, 2013 and U.S. Provisional Application No. 61/943,884, filed February 24, 2014, the disclosures of which are hereby incorporated by reference in its entirety.

STATEMENT OF RIGHTS TO INVENTIONS

MADE UNDER FEDERALLY SPONSORED RESEARCH

[0002] Not applicable.

TECHNICAL FIELD

[0003] The present disclosure is in the fields of gene modification and treatment of hemophilia.

BACKGROUND

[0004] An especially attractive application of gene therapy involves the treatment of disorders that are either caused by an insufficiency of a secreted gene product or that are treatable by secretion of a therapeutic protein. Such disorders are potentially addressable via delivery of a therapeutic transgene to a modest number of cells, provided that each recipient cell expresses a high level of the therapeutic gene product. In such a scenario, relief from the need for gene delivery to a large number of cells can enable the successful development of gene therapies for otherwise intractable indications. Such applications would require permanent, safe, and very high levels of transgene expression. Thus the development of a safe harbor which exhibits these properties would provide substantial utility in the field of gene therapy.

[0005] Hemophilias such as hemophilia A and hemophilia B, are genetic disorders of the blood-clotting system, characterized by bleeding into joints and soft tissues, and by excessive bleeding into any site experiencing trauma or undergoing surgery. Hemophilia A is clinically indistinguishable from hemophilia B, but factor VIII (FVIII or F8) is deficient or absent in hemophilia A while factor IX (FIX or F.IX) is deficient or absent in patients with hemophilia B. The *F8* gene encodes a plasma glycoprotein that circulates in association with

von Willebrand's factor in its inactive form. Upon surface injury, the intrinsic clotting cascade initiates and factor VIII is released from the complex and becomes activated. The activated form acts with Factor IX to activate Factor X to become the activated Xa, eventually leading to change of fibrinogen to fibrin and clot induction. *See, Levinson et al. (1990) Genomics* 7(1):1-11. 40-50% of hemophilia A patients have a chromosomal inversion involving F8 intron 22 (also known as IVS22). The inversion is caused by an intra-chromosomal recombination event between a 9.6 kb sequence within the intron 22 of the F8 gene and one of the two closely related inversely orientated sequences located about 300 kb distal to the F8 gene, resulting in an inversion of exons 1 to 22 with respect to exons 23 to 26. *See, Textbook of Hemophilia, Lee et al. (eds) 2005, Blackwell Publishing.* Other hemophilia A patients have defects in *F8* including active site mutations, and nonsense and missense mutations. For its part, Factor IX (F.IX) encodes one of the serine proteases involved with the coagulation system, and it has been shown that restoration of even 3% of normal circulating levels of wild type Factor IX protein can prevent spontaneous bleeding. Additional hemophilias are associated with aberrant expression of other clotting factors. For example, Factor VII deficiency is an autosomal recessive trait occurring in approximately 1 in 300,000 to 500,000 people and is associated with inadequate Factor VII levels in the patient. Similarly, Factor X deficiency is also an autosomal recessive trait occurring in 1 in every 500,000 to 1 million people, and is caused by genetic variants of the *FX* gene. Factor X deficiency can have varying degrees of severity in the patient population.

[0006] Current treatments for Hemophilia B rely on chronic, repeated intravenous infusions of purified recombinant Factor IX and suffer from a number of drawbacks. This includes the need for repeated intravenous infusions, is associated with inhibitor formation, and is prophylactic rather than curative.

[0007] Gene therapy for patients with Hemophilia A or B, involving the introduction of plasmid and other vectors (*e.g.*, AAV) encoding a functional F8 or F.IX proteins have been described. *See, e.g.*, U.S. Patent Nos. 6,936,243; 7,238,346 and 6,200,560; Shi *et al.* (2007) *J Thromb Haemost.* (2):352-61; Lee *et al.* (2004) *Pharm. Res.* 7:1229-1232; Graham *et al.* (2008) *Genet Vaccines Ther.* 3:6-9; Manno *et al.* (2003) *Blood* 101(8): 2963-72; Manno *et al.* (2006) *Nature Medicine* 12(3): 342-7; Nathwani *et al.* (2011) *Molecular Therapy* 19(5): 876-85; Nathwani *et al.* (2011); *N Engl J Med.* 365(25): 2357-65. However, in these protocols, the formation of inhibitory anti-factor VIII or IX (anti-F8 or anti-F.IX) antibodies and antibodies against the delivery vehicle remains a major complication of F8 and F.IX

replacement-based treatment for hemophilia. *See, e.g.,* Scott & Lozier (2012) *Br J Haematol.* 156(3):295-302.

[0008] Lysosomal storage diseases (LSDs) are a group of rare metabolic monogenic diseases characterized by the lack of functional individual lysosomal proteins normally involved in the breakdown of waste lipids, glycoproteins and mucopolysaccharides. These diseases are characterized by a buildup of these compounds in the cell since it is unable to process them for recycling due to the mis-functioning of a specific enzyme. The most common examples are Gaucher's (glucocerebrosidase deficiency- gene name: GBA), Fabry's (α galactosidase deficiency- GLA), Hunter's (iduronate-2-sulfatase deficiency-IDS), Hurler's (alpha-L iduronidase deficiency- IDUA), and Niemann-Pick's (sphingomyelin phosphodiesterase 1 deficiency- SMPD1) diseases. When grouped all together, LSDs have an incidence in the population of about 1 in 7000 births. These diseases have devastating effects on those afflicted with them. They are usually first diagnosed in babies who may have characteristic facial and body growth patterns and may have moderate to severe mental retardation. Treatment options include enzyme replacement therapy (ERT) where the missing enzyme is given to the patient, usually through intravenous injection in large doses. Such treatment is only to treat the symptoms and is not curative, thus the patient must be given repeated dosing of these proteins for the rest of their lives, and potentially may develop neutralizing antibodies to the injected protein. Often these proteins have a short serum half-life, and so the patient must also endure frequent infusions of the protein. For example, Gaucher's disease patients receiving the Cerezyme® product (imiglucerase) must have infusions three times per week. Production and purification of the enzymes is also problematic, and so the treatments are very costly (>\$100,000 per year per patient).

[0009] Various methods and compositions for targeted cleavage of genomic DNA have been described. Such targeted cleavage events can be used, for example, to induce targeted mutagenesis, induce targeted deletions of cellular DNA sequences, and facilitate targeted recombination at a predetermined chromosomal locus. *See, e.g.,* U.S. Patent Nos. 8,623,618; 8,034,598; 8,586,526; 6,534,261; 6,599,692; 6,503,717; 6,689,558; 7,067,317; 7,262,054; 7,888,121; 7,972,854; 7,914,796; 7,951,925; 8,110,379; 8,409,861; U.S. Patent Publications 20030232410; 20050208489; 20050026157; 20060063231; 20080159996; 201000218264; 20120017290; 20110265198; 20130137104; 20130122591; 20130177983 and 20130177960 and U.S. Application No. 14/278,903, the disclosures of which are incorporated by reference in their entireties for all purposes. These methods often involve the use of engineered cleavage systems to induce a double strand break (DSB) or a nick in a

target DNA sequence such that repair of the break by an error born process such as non-homologous end joining (NHEJ) or repair using a repair template (homology directed repair or HDR) can result in the knock out of a gene or the insertion of a sequence of interest (targeted integration). This technique can also be used to introduce site specific changes in the genome sequence through use of a donor oligonucleotide, including the introduction of specific deletions of genomic regions, or of specific point mutations or localized alterations (also known as gene correction). Cleavage can occur through the use of specific nucleases such as engineered zinc finger nucleases (ZFN), transcription-activator like effector nucleases (TALENs), or using the CRISPR/Cas system with an engineered crRNA/tracr RNA ('single guide RNA') to guide specific cleavage. Further, targeted nucleases are being developed based on the Argonaute system (*e.g.*, from *T. thermophilus*, known as 'TtAgo', see Swarts *et al* (2014) *Nature* 507(7491): 258-261), which also may have the potential for uses in genome editing and gene therapy.

[0010] This nuclease-mediated targeted transgene insertion approach offers the prospect of improved transgene expression, increased safety and expressional durability, as compared to classic integration approaches, since it allows exact transgene positioning for a minimal risk of gene silencing or activation of nearby oncogenes.

[0011] Targeted integration of a transgene may be into its cognate locus, for example, insertion of a wild type transgene into the endogenous locus to correct a mutant gene. Alternatively, the transgene may be inserted into a non-cognate locus, for example a "safe harbor" locus. Several safe harbor loci have been described, including *CCR5*, *HPRT*, *AAVSI*, *Rosa* and albumin. See, *e.g.*, U.S. Patent Nos. 7,951,925 and 8,110,379; U.S. Publication Nos. 20080159996; 201000218264; 20120017290; 20110265198; 20130137104; 20130122591; 20130177983 and 20130177960 and U.S. Application No. 14/278,903. For example, U.S. Patent Publication No. 20110027235 relates to targeted integration of functional proteins into isolated stem cells and U.S. Publication No. 20120128635 describes methods of treating hemophilia B. In addition, U.S. Publication Nos. 2014-0017212 and 2014-0112896 describe methods of treating lysosomal storage diseases. See also Li *et al* (2011) *Nature* 475 (7355):217-221 and Anguela *et al* (2013) *Blood* 122:3283-3287.

[0012] However, there remains a need for additional compositions and methods of providing therapeutic proteins to a subject with a disease or disorder in which one or more proteins are lacking, deficient and/or aberrantly expressed.

SUMMARY

[0013] Disclosed herein are methods and compositions that can be used to express a transgene under the control of an albumin promoter *in vivo* (e.g., endogenous or exogenous albumin promoter). In some aspects, the transgene may encode a therapeutic protein of interest. The transgene may encode a protein such that the methods of the invention can be used for production of protein that is deficient or lacking (e.g., “protein replacement”). In some instances, the protein may be involved treatment for a lysosomal storage disease. Other therapeutic proteins may be expressed, including protein therapeutics for conditions as diverse as epidermolysis bullosa, diabetes, cancer, clotting disorders or AAT deficient emphysema. In other aspects, the transgene may comprise sequences (e.g., engineered sequences) such that the expressed protein has characteristics which give it novel and desirable features (increased half-life, changed plasma clearance characteristics etc.). Engineered sequences can also include amino acids derived from the albumin sequence. In some aspects, the transgenes encode therapeutic proteins, therapeutic hormones, plasma proteins, antibodies and the like. In some aspects, the transgenes may encode proteins involved in blood disorders such as clotting disorders. In some aspects, the transgenes encode structural nucleic acids (shRNAs, RNAi, miRNAs and the like).

[0014] In one aspect, disclosed here are methods and compositions for targeted integration of a sequence encoding a functional clotting factor protein (e.g., Factor VII, Factor VIII, Factor IX and/or Factor X). Expression of a functional Factor VIII (“F8”) and/or Factor IX (“F.IX” or “FIX”) protein can result, for example, in the treatment and/or prevention of hemophilia A (F8) and/or hemophilia B (F.IX), while expression of a functional Factor VII or Factor X can treat or prevent hemophilias associated with Factor VII and/or Factor X deficiency.

[0015] In another aspect, disclosed herein are methods and compositions for targeted integration of a sequence encoding a functional protein that is lacking in a subject with a lysosomal storage disease. Nucleases, for example engineered meganucleases, zinc finger nucleases (ZFNs), TALE-nucleases (TALENs including fusions of TALE effectors domains with nuclease domains from restriction endonucleases and/or from meganucleases (such as mega TALEs and compact TALENs)), Ttgo system and/or CRISPR/Cas nuclease systems are used to cleave DNA at a ‘safe harbor’ gene locus (e.g. *CCR5*, *AAVSI*, *HPRT*, *Rosa* or albumin) in the cell into which the gene is inserted. Targeted insertion of a donor transgene may be via homology directed repair (HDR) or non-homology repair mechanisms (e.g., NHEJ donor capture). The nuclease can induce a double-stranded (DSB) or single-stranded

break (nick) in the target DNA. In some embodiments, two nickases are used to create a DSB by introducing two nicks. In some cases, the nickase is a ZFN, while in others, the nickase is a TALEN or a CRISPR/Cas nickase.

[0016] In one aspect, described herein is a non-naturally occurring zinc-finger protein (ZFP) that binds to a target site in a region of interest (*e.g.*, an albumin gene) in a genome, wherein the ZFP comprises one or more engineered zinc-finger binding domains. In one embodiment, the ZFP is a zinc-finger nuclease (ZFN) that cleaves a target genomic region of interest, wherein the ZFN comprises one or more engineered zinc-finger binding domains and a nuclease cleavage domain or cleavage half-domain. Cleavage domains and cleavage half domains can be obtained, for example, from various restriction endonucleases and/or homing endonucleases. In one embodiment, the cleavage half-domains are derived from a Type IIS restriction endonuclease (*e.g.*, *Fok I*). In certain embodiments, the zinc finger domain recognizes a target site in an albumin gene, for example a zinc finger protein with the recognition helix domains ordered as shown in a single row of Table 5.

[0016a] In another aspect, there is provided a zinc finger nuclease (ZFN) pair comprising a left ZFN and a right ZFN, each ZFN comprising a wild-type or engineered cleavage domain and a zinc finger protein comprising five or six zinc finger domains designated and ordered F1 to F5 or F1 to F6, as follows: (i) the recognition helix regions of F1 to F5 of SBS#47171 ordered SEQ ID NOs: 23, 18, 24, 20 and 21; (ii) the recognition helix regions of F1 to F6 of SBS#47898 ordered SEQ ID NOs: 14, 28, 25, 15, 16 & 12; (iii) the recognition helix regions of F1 to F6 of SBS#47079 or SBS#47169 ordered SEQ ID NOs: 14, 9, 10, 15, 16 & 12; (iv) the recognition helix regions of F1 to F6 of SBS#47931 ordered SEQ ID NOs: 14, 9, 25, 15, 16 & 12; (v) the recognition helix regions of F1 to F5 of SBS#47863 ordered SEQ ID NOs: 2, 26, 24, 20, & 27; (vi) the recognition helix regions of F1 to F5 of SBS#47192 ordered SEQ ID NOs: 2, 26, 24, 20 & 21; (vii) the recognition helix regions of F1 to F5 of SBS#47864 ordered SEQ ID NOs: 2, 112, 105, 106, & 107; or (viii) the recognition helix regions of F1 to F6 of SBS#40477 ordered SEQ ID NOs: 8, 114, 114, 11, 126 & 12, respectively, wherein the left ZFN comprises the zinc finger recognition helix regions selected from the (i), (v), (vi), and (vii) and the right ZFN comprises the zinc finger recognition helix regions selected from the (ii), (iii), (iv), and (viii).

[0017] In another aspect, described herein is a Transcription Activator Like Effector (TALE) protein that binds to target site in a region of interest (*e.g.*, an albumin gene) in a genome, wherein the TALE comprises one or more engineered TALE binding domains. In one

embodiment, the TALE is a nuclease (TALEN) that cleaves a target genomic region of interest, wherein the TALEN comprises one or more engineered TALE DNA binding domains and a nuclease cleavage domain or cleavage half-domain. Cleavage domains and cleavage half domains can be obtained, for example, from various restriction endonucleases and/or homing endonucleases (meganuclease). In one embodiment, the cleavage half-domains are derived from a Type IIS restriction endonuclease (*e.g.*, *Fok I*). In other embodiments, the cleavage domain is derived from a meganuclease, which meganuclease domain may also exhibit DNA-binding functionality.

[0018] In another aspect, described herein is a CRISPR/Cas system that binds to target site in a region of interest (*e.g.*, an albumin gene) in a genome, wherein the CRISPR/Cas system comprises one or more engineered single guide RNA or a functional equivalent, as well as a Cas9 nuclease.

[0019] The nucleases (*e.g.*, ZFN, CRISPR/Cas system, Ttgo and/or TALEN) as described herein may bind to and/or cleave the region of interest in a coding or non-coding region within or adjacent to the gene, such as, for example, a leader sequence, trailer sequence or intron, or within a non-transcribed region, either upstream or downstream of the

[Text continued on page 7]

coding region. In certain embodiments, the nuclease (*e.g.*, ZFN) binds to and/or cleaves an albumin gene.

[0020] In another aspect, described herein is a polynucleotide encoding one or more nucleases (*e.g.*, ZFNs, CRISPR/Cas systems, Ttago and/or TALENs described herein). The polynucleotide may be, for example, mRNA. In some aspects, the mRNA may be chemically modified (See *e.g.* Kormann *et al.*, (2011) *Nature Biotechnology* 29(2):154-157).

[0021] In another aspect, described herein is a ZFN, CRISPR/Cas system, Ttago and/or TALEN expression vector comprising a polynucleotide, encoding one or more nucleases (*e.g.*, ZFNs, CRISPR/Cas systems, Ttago and/or TALENs) as described herein, operably linked to a promoter. In one embodiment, the expression vector is a viral vector. In one aspect, the viral vector exhibits tissue specific tropism.

[0022] In another aspect, described herein is a host cell comprising one or more nuclease (*e.g.*, ZFN, CRISPR/Cas systems, Ttago and/or TALEN) expression vectors.

[0023] In another aspect, pharmaceutical compositions comprising an expression vector as described herein are provided. In some embodiments, the pharmaceutical composition may comprise more than one expression vector. In some embodiments, the pharmaceutical composition comprises a first expression vector comprising a first polynucleotide, and a second expression vector comprising a second polynucleotide. In some embodiments, the first polynucleotide and the second polynucleotide are different. In some embodiments, the first polynucleotide and the second polynucleotide are substantially the same. The pharmaceutical composition may further comprise a donor sequence (*e.g.*, a transgene encoding a protein lacking or deficient in a disease or disorder such as an LSD or a hemophilia). In some embodiments, the donor sequence is associated with an expression vector.

[0024] In some embodiments, a fusion protein comprising a zinc finger protein and a wild-type or engineered cleavage domain or cleavage half-domain are provided.

[0025] In some embodiments, a pharmaceutical composition is provided comprising: (i) a first polynucleotide (*e.g.*, plasmid, mRNA, Ad vector, AAV vector, etc.) encoding a zinc finger nuclease, the zinc finger nuclease comprising a FokI cleavage domain and a zinc finger protein comprising 5 or 6 zinc finger domains ordered F1 to F5 or F1 to F6, wherein each zinc finger domain comprises a recognition helix region and wherein the recognition helix regions of the zinc finger protein are shown in a single row of Table 1, 2 or 5; (ii) a second polynucleotide (*e.g.*, plasmid, mRNA, Ad vector, AAV vector, etc.) encoding a zinc finger nuclease, the zinc finger nuclease comprising a FokI cleavage domain and a zinc finger

protein comprising 5 or 6 zinc finger domains ordered F1 to F5 or F1 to F6, wherein each zinc finger domain comprises a recognition helix region and wherein the recognition helix regions of the zinc finger protein are shown in a single row of Table 1, 2 or 5; and (iii) a third polynucleotide (*e.g.*, plasmid, mRNA, Ad vector, AAV vector, etc.) vector comprising a donor encoding a protein lacking or deficient in a disease or disorder (*e.g.*, LSD or hemophilia). The ZFPs of the two ZFNs may be the same or different. Similarly, the cleavage domains of the two ZFNs may be the same or different (*e.g.*, may be mutants that form obligate heterodimers). In some embodiments, (i), (ii), and (iii) are provided in a ratio about 1:1:1, about 1:1:2, about 1:1:3, about 1:1:4, about 1:1:5, about 1:1:6, about 1:1:7, about 1:1:8, about 1:1:9, about 1:1:10, about 1:1:11, about 1:1:12, about 1:1:13, about 1:1:14, about 1:1:15, about 1:1:16, about 1:1:17, about 1:1:18, about 1:1:19, or about 1:1:20.

[0026] In one aspect, the methods and compositions of the invention comprise genetically modified cells comprising a transgene expressing a functional version of a protein that is aberrantly expressed in a hemophilia (Factor VII, F8, F.IX and/or Factor X protein), in which the transgene is integrated into an endogenous safe-harbor gene (*e.g.*, albumin gene) of the cell's genome. In another aspect, the methods and compositions of the invention comprise genetically modified cells comprising a transgene expressing a functional version of a protein that is lacking or abnormally expressed in a subject with a lysosomal storage disease. In certain embodiments, the transgene is integrated in a site-specific (targeted) manner using at least one nuclease. In certain embodiments, the nuclease (*e.g.*, ZFNs, TALENs, Ttgo and/or CRISPR/Cas systems) is specific for a safe harbor gene (*e.g.* *CCR5*, *HPRT*, *AAVSI*, *Rosa* or albumin. *See, e.g.*, U.S. Patent Nos. 7,951,925 and 8,110,379; U.S. Publication Nos. 20080159996; 201000218264; 20120017290; 20110265198; 20130137104; 20130122591; 20130177983 and 20130177960 and U.S. Application No. 14/278,903). In some embodiments, the safe harbor is an albumin gene.

[0027] In another aspect, described herein is a method of genetically modifying a cell, *in vitro* and/or *in vivo*, to produce a therapeutic protein (*e.g.*, a protein lacking in a disease or disorder such as a hemophilia (Factor VII, F8, F.IX and/or Factor X) or a lysosomal storage disease (IDS, IDUA, etc.), the method comprising cleaving an endogenous safe harbor gene in the cell using one or more nucleases (*e.g.*, ZFNs, TALENs, CRISPR/Cas) such that a transgene encoding the therapeutic protein is integrated into the safe harbor locus and expressed in the cell. In certain embodiments, the safe harbor gene is a *CCR5*, *HPRT*, *AAVSI*, *Rosa* or albumin gene. In a further aspect, described herein is a method of genetically modifying a cell, *in vitro* and/or *in vivo*, to produce a protein that is lacking in a lysosomal

storage disease. The most common examples of these are glucocerebrosidase deficiency (gene name: GBA), associated with Gaucher's disease, α galactosidase deficiency (gene name: GLA), associated with Fabry's disease, iduronate-2-sulfatase deficiency (gene name: IDS), associated with Hunter's disease, alpha-L iduronidase deficiency (gene name: IDUA), associated with Hurler's disease, and sphingomyelin phosphodiesterase 1 deficiency (gene name: SMPD1), associated with Niemann-Pick's disease. In certain embodiments, the cell is a mammalian cell. In certain embodiments, the cell is a primate cell. In certain embodiments, the cell is a human cell. In one set of embodiments, methods for cleaving an albumin gene in a cell (*e.g.*, a liver cell) are provided comprising introducing, into the cell, one or more expression vectors disclosed herein under conditions such that the one or more proteins are expressed and the albumin gene is cleaved. The albumin gene may be modified, for example, by integration of a donor sequence into the cleaved albumin gene. In certain embodiments, the method comprises genetically modifying a cell to produce a clotting factor or a protein lacking in a lysosomal storage disease, the method comprising administering to the cell the zinc finger nucleases (ZFNs) shown in Table 5 (or polynucleotides encoding these ZFNs) and a donor. The ZFNs and donor may be on the same or different vectors in any combination, for example on 3 separate vectors (*e.g.*, AAV vectors) each carrying one of the components; one vector carrying two of the components and a separate vector carrying the 3rd component; or one vector carrying all 3 components.

[0028] In other aspects, the invention comprises delivery of a donor nucleic acid to a target cell. The donor may be delivered prior to, after, or along with the nucleic acid encoding the nuclease(s). The donor nucleic acid may comprise an exogenous sequence (transgene) to be integrated into the genome of the cell, for example, an endogenous locus. In some embodiments, the donor may comprise a full length gene or fragment thereof flanked by regions of homology with the targeted cleavage site. In some embodiments, the donor lacks homologous regions and is integrated into a target locus through homology independent mechanism (*i.e.* NHEJ). The donor may comprise any nucleic acid sequence, for example a nucleic acid that, when used as a substrate for homology-directed repair of the nuclease-induced double-strand break, leads to a donor-specified deletion to be generated at the endogenous chromosomal locus or, alternatively (or in addition to), novel allelic forms of (*e.g.*, point mutations that ablate a transcription factor binding site) the endogenous locus to be created. In some aspects, the donor nucleic acid is an oligonucleotide wherein integration leads to a gene correction event, or a targeted deletion. In some aspects the donor comprises a therapeutic protein, for example a clotting factor.

[0029] In some embodiments, the polynucleotide encoding the DNA binding protein is a mRNA. In some aspects, the mRNA may be chemically modified (*See e.g.* Kormann *et al.*, (2011) *Nature Biotechnology* 29(2):154-157). In other aspects, the mRNA may comprise an ARCA cap (see U.S. Patents 7,074,596 and 8,153,773). In further embodiments, the mRNA may comprise a mixture of unmodified and modified nucleotides (see U.S. Patent Publication 2012-0195936).

[0030] In another aspect, provided herein are methods for providing one or more functional proteins lacking or deficient in a mammal, or in a primate, such as a human primate, such as a human patient with an LSD and/or a hemophilia, for example for treating the disease by supplying the protein(s) lacking or deficient in the subject. In another aspect, provided herein are methods for providing a functional protein (*e.g.*, F.IX) lacking or deficient in a mammal, or in a primate, such as a human primate, such as a human patient with hemophilia B, for example for treating hemophilia B. In another aspect, provided herein are methods for providing a functional protein (*e.g.* Factor VII) to a mammal, or in a primate, such as a human primate, such as a human patient, for treating hemophilia associated with Factor VII deficiency. In another aspect, provided herein are methods for providing a functional protein (*e.g.* Factor X) for treating hemophilia associated with Factor X deficiency. In certain embodiments, the methods comprise using nucleases to integrate a sequence encoding a functional Factor VII, F8, F.IX and/or Factor X protein in a cell in a subject in need thereof. In other embodiments, the methods comprise using nucleases to integrate a sequence encoding a functional protein lacking or deficient in a lysosomal storage disease. In other embodiments, the method comprises administering a genetically modified cell (expressing a functional version of a protein that is aberrantly expressed in a subject with hemophilia) to the subject. Thus, an isolated cell may be introduced into the subject (*ex vivo* cell therapy) or a cell may be modified when it is part of the subject (*in vivo*). Also provided is the use of the donors and/or nucleases described herein for the treatment of a hemophilia (*e.g.*, hemophilia A with Factor VIII donor, hemophilia B with Factor IX donor, Factor VII deficiency with Factor VII, Factor X deficiency with Factor X, Gaucher's with a GBA donor, Fabry's with a GLA donor, Hunter's with a IDS donor, Hurler's with a IDUA donor, and/or Niemann-Pick's with a SMPD1 donor), for example, in the preparation of medicament for treatment of a disease. In certain embodiments, the F8 protein comprises a B-domain deletion. In certain embodiments, the F8- and/or F.IX-encoding sequence is delivered using a viral vector, a non-viral vector (*e.g.*, plasmid) and/or combinations thereof.

[0031] In any of the compositions and methods described, the nuclease(s) and/or transgene(s) may be carried on an AAV vector, including but not limited to AAV1, AAV3, AAV4, AAV5, AAV6, AAV8, AAV9 and AAVrh10 or pseudotyped AAV such as AAV2/8, AAV8.2, AAV2/5 and AAV2/6 and the like. In certain embodiments, the nucleases and transgene donors are delivered using the same AAV vector types. In other embodiments, the nucleases and transgene donors are delivered using different AAV vector types. The nucleases and transgenes may be delivered using one or more vectors, for example, one vector carries both the transgene and nuclease(s); two vectors where one carries the nuclease(s) (*e.g.*, left and right ZFNs of a ZFN pair, for example with a 2A peptide) and one carries the transgene; or three vectors where one vector carries one nuclease of a nuclease pair (*e.g.*, left ZFN), a separate vector carries the other nuclease of a nuclease pair (*e.g.*, right ZFN) and a third separate vector carries the transgene. *See*, Figure 2. In embodiments in which two or more vectors are used, the vectors may be used at the same concentrations or in different ratios, for example, the transgene donor vector(s) may be administered at 2-fold, 3-fold, 4-fold, 5-fold or more higher concentrations than the nuclease vector(s). In certain embodiments, the nucleases and/or transgene donors are delivered via intravenous (*e.g.*, intra-portal vein) administration into the liver of an intact animal.

[0032] In any of the compositions and methods described herein, the protein encoded by the transgene may comprise a F8 protein, for example a B-Domain Deleted Factor VIII (BDD-F8). In other embodiments, the protein encoded by the transgene comprises a F.IX protein. In other embodiments, the protein encoded by the transgene comprises a Factor VII protein. In other embodiments, the protein encoded by the transgene comprises a Factor X protein. In some embodiments, the protein encoded by the transgene comprises a glucocerebrosidase. In other embodiments, the protein encoded by the transgene comprises an α galactosidase. In further embodiments, the protein encoded by the transgene comprises an iduronate-2-sulfatase. In some embodiments, the protein encoded by the transgene comprises an alpha-L iduronidase. In further embodiments, the protein encoded by the transgene comprises sphingomyelin phosphodiesterase. In any of the compositions or methods described herein, the transgene also comprises a transcriptional regulator while in others, it does not and transcription is regulated by an endogenous regulator. In another aspect, the methods of the invention comprise a composition for therapeutic treatment of a subject in need thereof. In some embodiments, the composition comprises engineered stem cells comprising a safe harbor specific nuclease, and a transgene donor encoding Factor VII, F8, F.IX, Factor X, GBA, GLA, IDS, IDUA and/or SMPD1 protein or a functional fragment

and/or truncation thereof. In other embodiments, the composition comprises engineered stem cells that have been modified and express a transgene donor encoding Factor VII, F8, F.IX, Factor X, GBA, GLA, IDS, IDUA and/or SMPD1 protein or a functional fragment and/or truncation thereof.

[0033] In any of the compositions or methods described herein, the cell may be a eukaryotic cell. Non-limiting examples of suitable cells include eukaryotic cells or cell lines such as secretory cells (*e.g.*, liver cells, mucosal cells, salivary gland cells, pituitary cells, etc.), blood cells (red blood cells), red blood precursory cells, hepatic cells, muscle cells, stem cells (*e.g.*, embryonic stem cells, induced pluripotent stem cells, hepatic stem cells, hematopoietic stem cells (*e.g.*, CD34+)) or endothelial cells (*e.g.*, vascular, glomerular, and tubular endothelial cells). Thus, the target cells may be primate cells, for example human cells, or the target cells may be mammalian cells, (including veterinary animals), for example especially nonhuman primates and mammals of the orders *Rodenta* (mice, rats, hamsters), *Lagomorpha* (rabbits), *Carnivora* (cats, dogs), and *Arteriodactyla* (cows, pigs, sheep, goats, horses). In some aspects, the target cells comprise a tissue (*e.g.* liver). In some aspects, the target cell is a stem cell (*e.g.*, an embryonic stem cell, an induced pluripotent stem cell, a hepatic stem cell, etc.) or animal embryo by any of the methods described herein, and then the embryo is implanted such that a live animal is born. The animal is then raised to sexual maturity and allowed to produce offspring wherein at least some of the offspring comprise the genomic modification. The cell can also comprise an embryo cell, for example, of a mouse, rat, rabbit or other mammal cell embryo. The cell may be from any organism, for example human, non-human primate, mouse, rat, rabbit, cat, dog or other mammalian cells. The cell may be isolated or may be part of an organism (*e.g.*, subject).

[0034] In any of the methods or compositions described herein, the cell containing the engineered locus (*e.g.*, albumin locus) can be a stem cell that may be useful for therapeutic purposes. Specific stem cell types that may be used with the methods and compositions of the invention include embryonic stem cells (ESC), induced pluripotent stem cells (iPSC) and hepatic or liver stem cells. The iPSCs can be derived from patient samples and from normal controls wherein the patient derived iPSC can be mutated to normal gene sequence at the gene of interest, or normal cells can be altered to the known disease allele at the gene of interest. Similarly, the hepatic stem cells can be isolated from a patient. These cells are then engineered to express the transgene of interest, expanded and then reintroduced into the patient.

[0035] In any of the methods and compositions described herein, the transgene may be integrated into the endogenous safe harbor gene such that some, all or none of the endogenous gene is expressed, for example a fusion protein with the integrated transgene. In some embodiments, the endogenous safe harbor gene is an albumin gene and the endogenous sequences are albumin sequences. The endogenous may be present on the amino (N)-terminal portion of the exogenous protein and/or on the carboxy (C)-terminal portion of the exogenous protein. The albumin sequences may include full-length wild-type or mutant albumin sequences or, alternatively, may include partial albumin amino acid sequences. In certain embodiments, the albumin sequences (full-length or partial) serve to increase the serum half-life of the polypeptide expressed by the transgene to which it is fused and/or as a carrier. In other embodiments, the transgene comprises albumin sequences and is targeted for insertion into another safe harbor within a genome. Furthermore, the transgene may include an exogenous promoter (*e.g.*, constitutive or inducible promoter) that drives its expression or its expression may be driven by endogenous control sequences (*e.g.*, endogenous albumin promoter). In some embodiments, the donor includes additional modifications, including but not limited to codon optimization, addition of glycosylation sites and the like.

[0036] In any of the compositions or methods described herein, cleavage can occur through the use of specific nucleases such as engineered zinc finger nucleases (ZFN), transcription-activator like effector nucleases (TALENs), or using the Ttgo or CRISPR/Cas system with an engineered crRNA/tracrRNA ('single guide RNA') to guide specific cleavage. In some embodiments, two nickases are used to create a DSB by introducing two nicks. In some cases, the nickase is a ZFN, while in others, the nickase is a TALEN or a CRISPR/Cas system. Targeted integration may occur via homology directed repair mechanisms (HDR) and/or via non-homology repair mechanisms (*e.g.*, NHEJ donor capture). The nucleases as described herein may bind to and/or cleave the region of interest in a coding or non-coding region within or adjacent to the gene, such as, for example, a leader sequence, trailer sequence or intron, or within a non-transcribed region, either upstream or downstream of the coding region. In certain embodiments, the nuclease cleaves the target sequence at or near the binding site (*e.g.*, the binding site shown in Table 5). Cleavage can result in modification of the gene, for example, via insertions, deletions or combinations thereof. In certain embodiments, the modification is at or near the nuclease(s) binding and/or cleavage site(s), for example, within 1-300 (or any value therebetween) base pairs upstream or downstream of the site(s) of cleavage, more preferably within 1-100 base pairs (or any value therebetween) of either side of the binding and/or cleavage site(s), even more preferably

within 1 to 50 base pairs (or any value therebetween) on either side of the binding and/or cleavage site(s).

[0037] The methods and compositions described may be used to treat or prevent a hemophilia in a subject in need thereof. In some embodiments, the compositions comprise vectors and are used to target liver cells. In other embodiments, the compositions comprise engineered stem cells and are given to a patient as a bone marrow transplant. In some instances, patients are partially or completely immunoablated prior to transplantation. In other instances, patients are treated with one or more immunosuppressive agents before, during and/or after nuclease-mediated modification an endogenous gene (*e.g.*, targeted integration of a transgene into an albumin locus). Furthermore, any of the methods described herein may further comprise additional steps, including partial hepatectomy or treatment with secondary agents that enhance transduction and/or induce hepatic cells to undergo cell cycling. Examples of secondary agents include gamma irradiation, UV irradiation, tritiated nucleotides such as thymidine, cis-platinum, etoposide, hydroxyurea, aphidicolin, prednisolone, carbon tetrachloride and/or adenovirus.

[0038] The methods described herein can be practiced *in vitro*, *ex vivo* or *in vivo*. In certain embodiments, the compositions are introduced into a live, intact mammal. The mammal may be at any stage of development at the time of delivery, *e.g.*, embryonic, fetal, neonatal, infantile, juvenile or adult. Additionally, targeted cells may be healthy or diseased. In certain embodiments, one or more of the compositions are delivered intravenously (*e.g.*, to the liver via the intraportal vein, for example tail vein injection), intra-arterially, intraperitoneally, intramuscularly, into liver parenchyma (*e.g.*, via injection), into the hepatic artery (*e.g.*, via injection), and/or through the biliary tree (*e.g.*, via injection).

[0039] In one particular aspect, the methods and compositions described herein include a therapeutic composition comprising (i) a donor transgene coding for FVIII (ii) a nuclease (*e.g.*, ZFN, TALENs, Ttago or CRISPR/Cas system) targeting a locus of an endogenous gene other than FVIII, respectively, for example, targeting the endogenous albumin gene of a mammal, or primate or human, such as hemophilia patient. In certain embodiments, the therapeutic composition comprises the FVIII donor transgene and the albumin gene-specific nuclease in separate, independent vectors, such as separate AAV vectors, in different amounts, which can be administered together (*e.g.*, mixed into a single solution or administered simultaneously) or, alternatively, which can be administered separately (for example, administered in separate solutions with a substantial delay, *e.g.*, 10 minutes or more, 30 minutes or more, 1 hour or more, 2 hours or more, 3 hours or more, or

longer between respective administrations).). In certain embodiments, the therapeutic composition is administered to provide integration of the FVIII donor transgene into the non-FVIII locus and subsequent expression of the integrated FVIII to achieve a therapeutic level of FVIII in the plasma of the mammal, or primate or human, or hemophilia patient. In certain embodiments, a therapeutic level of FVIII can include, for example, greater than 2%, greater than 4%, greater than 5%, greater than 6%, greater than 8%, greater than 10%, greater than 12%, greater than 15%, greater than 20%, greater than 25%, or more of a clinically-acceptable normal plasma concentration of FVIII. Alternatively or in addition, a therapeutic level of FVIII can include, for example, greater than 2%, greater than 4%, greater than 5%, greater than 6%, greater than 8%, greater than 10%, greater than 12%, greater than 15%, greater than 20%, greater than 25%, greater than 30%, greater than 35%, or more of the plasma concentration of functional FVIII measured in the individual mammal, or primate or human, or hemophilia patient prior to administration of the FVIII donor transgene and the albumin gene nuclease to that individual.

[0040] In one particular aspect, the methods and compositions described herein include a therapeutic composition comprising (i) a donor transgene coding for a protein deficient in a lysosomal storage protein (ii) a nuclease (*e.g.*, ZFN, TALENs, Ttgo or CRISPR/Cas system) targeting a locus of an endogenous gene other than the gene for the protein deficient in a lysosomal storage disease, respectively, for example, targeting the endogenous albumin gene of a mammal, or primate or human, such as subject with a lysosomal storage disease. In certain embodiments, the therapeutic composition comprises the donor transgene selected from GBA, GLA, IDS, IDUA and/or SMPD1 and the albumin gene-specific nuclease in separate, independent vectors, such as separate AAV vectors, in different amounts, which can be administered together (*e.g.*, mixed into a single solution or administered simultaneously) or, alternatively, which can be administered separately (for example, administered in separate solutions with a substantial delay, *e.g.*, 10 minutes or more, 30 minutes or more, 1 hour or more, 2 hours or more, 3 hours or more, or longer between respective administrations). In certain embodiments, the therapeutic composition is administered to provide integration of the GBA, GLA, IDS, IDUA and/or SMPD1 donor transgene into a locus that is not the locus encoding GBA, GLA, IDS, IDUA and/or SMPD1, respectively, and subsequent expression of the integrated GBA, GLA, IDS, IDUA and/or SMPD1 to achieve a therapeutic level of GBA, GLA, IDS, IDUA and/or SMPD1 in the plasma of the mammal, or primate or human, or hemophilia patient. In certain embodiments, a therapeutic level of GBA, GLA, IDS, IDUA and/or SMPD1 can include, for example,

greater than 2%, greater than 4%, greater than 5%, greater than 6%, greater than 8%, greater than 10%, greater than 12%, greater than 15%, greater than 20%, greater than 25%, or more of a clinically-acceptable normal plasma concentration of GBA, GLA, IDS, IDUA and/or SMPD1. Alternatively or in addition, a therapeutic level of GBA, GLA, IDS, IDUA and/or SMPD1 can include, for example, greater than 2%, greater than 4%, greater than 5%, greater than 6%, greater than 8%, greater than 10%, greater than 12%, greater than 15%, greater than 20%, greater than 25%, greater than 30%, greater than 35%, or more of the plasma concentration of functional GBA, GLA, IDS, IDUA and/or SMPD1 measured in the individual mammal, or primate or human, or patient prior to administration of the GBA, GLA, IDS, IDUA and/or SMPD1 donor transgene and the albumin gene nuclease to that individual.

[0041] In another particular aspect, the methods and compositions described herein include a therapeutic composition comprising (i) a donor transgene coding for FIX (ii) a nuclease (*e.g.*, ZFN, TALENs, Ttgo or CRISPR/Cas system) targeting a locus of an endogenous gene other than FIX, respectively, for example, targeting the endogenous albumin gene of a mammal, or primate or human, such as hemophilia patient. In certain embodiments, the therapeutic composition comprises the FIX donor transgene and the albumin gene nuclease in separate, independent vectors, such as separate AAV vectors, in different amounts, which can be administered together (*e.g.*, mixed into a single solution or administered simultaneously) or, alternatively, which can be administered separately ((for example, administered in separate solutions with a substantial delay, *e.g.*, 10 minutes or more, 30 minutes or more, 1 hour or more, 2 hours or more, 3 hours or more, or longer between respective administrations). In certain embodiments, the therapeutic composition is administered to provide integration of the FIX donor transgene into the non-FIX locus and subsequent expression of the integrated FIX to achieve a therapeutic level of FIX in the plasma of the mammal, or primate or human, or hemophilia patient. In certain embodiments, a therapeutic level of FIX can include, for example, greater than 2%, greater than 4%, greater than 5%, greater than 6%, greater than 8%, greater than 10%, greater than 12%, greater than 15%, greater than 20%, greater than 25%, or more of a clinically-acceptable normal plasma concentration of FIX. Alternatively or in addition, a therapeutic level of FIX can include, for example, greater than 2%, greater than 4%, greater than 5%, greater than 6%, greater than 8%, greater than 10%, greater than 12%, greater than 15%, greater than 20%, greater than 25%, greater than 30%, greater than 35%, or more of the plasma concentration of functional

FIX measured in the individual mammal, or primate or human, or hemophilia patient prior to administration of the FIX donor transgene and the albumin gene nuclease to that individual.

[0042] For targeting the compositions to a particular type of cell, *e.g.*, platelets, fibroblasts, hepatocytes, etc., one or more of the administered compositions may be associated with a homing agent that binds specifically to a surface receptor of the cell. For example, the vector may be conjugated to a ligand (*e.g.*, galactose) for which certain hepatic system cells have receptors. The conjugation may be covalent, *e.g.*, a crosslinking agent such as glutaraldehyde, or noncovalent, *e.g.*, the binding of an avidinated ligand to a biotinylated vector. Another form of covalent conjugation is provided by engineering the AAV helper plasmid used to prepare the vector stock so that one or more of the encoded coat proteins is a hybrid of a native AAV coat protein and a peptide or protein ligand, such that the ligand is exposed on the surface of the viral particle.

[0043] A kit, comprising the compositions (*e.g.*, genetically modified cells, ZFPs, CRISPR/Cas system and/or TALENs and optionally transgene donors) of the invention, is also provided. The kit may comprise nucleic acids encoding the nucleases, (*e.g.* RNA molecules or nuclease-encoding genes contained in a suitable expression vector), donor molecules, suitable host cell lines, instructions for performing the methods of the invention, and the like.

[0044] These and other aspects will be readily apparent to the skilled artisan in light of disclosure as a whole.

BRIEF DESCRIPTION OF THE DRAWINGS

[0045] **Figure 1** is a schematic depicting zinc finger nuclease-mediated insertion of a transgene encoding a protein (*e.g.*, therapeutic protein) at the endogenous albumin locus. “SA” refers to a splice acceptor site; “pA” refers to a polyadenylation signal; and “Alb Ex1” refers to exon 1 of the endogenous albumin locus.

[0046] **Figures 2A to 2C** are schematics depicting exemplary nuclease and donor designs for a F8 transgene donor. Figure 2A shows an exemplary donor design and Figure 2B shows another exemplary design (“optimized donor”) that includes codon optimization, use of different polyadenylation signals and/or addition of a putative glycosylation motif (“V3 peptide”). The donors depicted in Figures 2A and 2B lack promoter/enhancer regions and are approximately 4.4 to 4.7 kb in size, which is ideal for packaging into AAVs. Figure 2C is a schematic depicting design of separate vectors for each ZFN of the pair used for safe harbor cleavage for targeted integration of the F8 and/or F9 transgene.

[0047] **Figures 3A to 3D** depict results of zinc finger nuclease-mediated targeted integration of a sequence encoding a human F8 protein (hFVIII) into an endogenous albumin locus by showing hFVIII activity in plasma of HA / CD4^{-/-} mice following ZFN and donor administration using AAV2/8 vectors. Figure 3A is a schematic depicting the AAV2/8 transgene donor. Figure 3B and 3C are graphs depicting plasma levels of hFVIII as a percentage of normal levels in HA/CD4^{-/-} mice injected with both the ZFN vectors and transgene donors (“ZFN+Donor”) or empty vectors (no ZFN sequence) and the transgene donors (“Mock+Donor”). Results are shown 2 weeks post-administration (Figure 3B) or 2 and 8 weeks post-administration (Figure 3C). The vectors and amounts administered are shown. Figure 3D is a graph showing albumin gene modification levels in mice administered either a single vector encoding both left and right ZFNs (“2A fusion”) or separate vectors each encoding one ZFN of the pair as shown in Figure 2C (“individual ZFNs”). Vectors used were AAV2/8 and dose is plotted along the horizontal axis (Viral Genomes (“VGs”) per mouse).

[0048] **Figure 4** is a graph depicting plasma levels of hFVIII as a percentage of normal levels in HA/CD4^{-/-} mice injected with the albumin-targeted ZFNs and optimized (V3) donor construct shown in Figure 2B. AAV2/8-ZFN (5×10^{10} vg of each ZFN) + AAV2/8-Donor (1×10^{11} vg/mouse) was used.

[0049] **Figures 5A and 5B** depict nuclease-mediated integration of a F.IX transgene into the albumin locus. Figure 5A is a schematic of the donor transgene F.IX construct used. The donor comprises donor arms that are homologous to the human F9 locus (“Human Arm Left” and “Human Arm Right”), thus they are not expected to promote HDR in this experiment. Insertion of the donor is therefore dependent upon NHEJ via end capture. Figure 5B shows circulating hFIX levels following administration of ZFNs targeted to an endogenous mouse albumin locus (“mAlb ZFN”) or to the human Factor IX locus (“hF9 ZFN”) and a hF9 donor transgene (“Donor”) to wild-type mice. AAV vectors and amounts administered were as follows: AAV2/8-ZFN at 1×10^{11} vg/mouse and AAV2/8-Donor at 5×10^{11} vg/mouse for mAlb ZFN and F.IX donor and AAV2/8-ZFN at 1×10^{11} vg/mouse and AAV2/8-Donor at 5×10^{11} vg/mouse for hF9 ZFN and F.IX donor. Note that the hF9 ZFN do not cleave the endogenous mouse F9 locus.

[0050] **Figure 6** is a graph depicting hF.IX levels following administration of albumin-targeted ZFNs and an hF.IX donor to mice. AAV2/8-ZFNs were administered at the indicated dose and AAV2/8-Donor at 5x the dose of the ZFNs. Genome editing is proportional to AAV dose over three orders of magnitude.

[0051] **Figures 7A and 7B** are graphs depicting clotting times in HB Mice treated with ZFNs and hF.IX donors. Figure 7A shows plasma hF.IX levels in the indicated animals and Figure 7B shows activated partial thromboplastin time(s) (aPTT(s)). AAV dosages are shown at the bottom.

[0052] **Figures 8A through 8D** are graphs showing ELISPOT data at day 65 post-administration of ZFNs in non-human primates receiving albumin-targeted ZFNs only. “LN” refers to lymph node. As shown, there is no immune response against the AAV8 capsid or the ZFNs.

[0053] **Figure 9** is a graph that shows nuclease-mediated targeting of the albumin locus in primary human hepatocytes. Human primary hepatocytes were transduced in vitro with AAV2/6 hF9 donor (MOI 9×10^5 vg/cell) and 24hrs later with 500ng of hALB ZFN mRNA. Bottom panel: % Indels measured by MiSeq analysis. Supernatants taken at Day 5 (left-most bar of each group), 7 (middle bar of each group) and 9 (right-most bar of each group) were analyzed for hFIX protein levels by ELISA. Error bars=s.e.m. Data are representative of at least 2 independent experiments.

[0054] **Figures 10A through 10D** show characterization of mAlb ZFNs *in vitro* and *in vivo*. Figure 10A shows ZFN activity measured by indels in Hepa 1-6 cells transfected with indicated amount of ZFN or GFP mRNA. Genomic DNA was isolated and the target sequence was PCR amplified for Illumina MiSeq sequencing. Percentages indicate reads containing insertions and/or deletions consistent with cleavage and NHEJ repair. Figure 10B shows levels of hF.IX in treated mice remained stable for more than a year following IV injection with 5×10^{11} vg AAV8-hF9-Donor and 1×10^{11} vg AAV8-mALB-ZFN. Figure 10C shows plasma ALT values following treatment did not deviate from normal range (shaded area). Figure 10D depicts the use of quantitative PCR to determine the relative abundance of “hybrid F9-mAlb” vs. wild type albumin mRNA. Mice were injected with 1:1 ratio of ZFN:Donor at indicated doses. Total RNA was isolated from livers of mice 2 weeks post injection. As a negative control, Luciferase (Mock) + Donor was given at the higher dose of 5×10^{11} vg each. A 2-tailed Mann-Whitney test was used to compare 2 groups. n=6-8 mice/group. Error bars=s.e.m. **P<0.01 vs Mock.

[0055] **Figure 11** is a schematic showing the study design for a non-human primate (Rhesus macaque) study of nuclease-mediated insertion of F.IX transgenes. “ALB ZFN” refers to albumin targeted ZFNs as described in U.S. Publication No. 20130177983. “FokI-eHi/fi” refers to engineered FokI cleavage domains that form obligate heterodimers as used in the ZFNs. *See, e.g.*, U.S. Patent Nos. 7,914,796, 8,034,598; U.S. Publication Nos.

20110201055 and 20120142062. The AAV vectors and serotypes were used as shown either at high dose (single ZFN: 1.5e11 vg/kg; Donor: 1.5e14 vg/kg) or low dose (single ZFN: 5e12 vg/kg; Donor: 5e13 vg/kg).

[0056] Figures 12A and 12B show amino acid sequences of exemplary ZFNs SBS#30724 and SBS# 30725, respectively, used to target intron 1 of the mouse Albumin locus. Figure 12A ZFN^{Left} (SEQ ID NO:29) and Figure 12B ZFN^{Right} (SEQ ID NO:30) amino acid sequences used in the study. 3xFLAG tag is annotated in italics. The SV40 large T antigen nuclear localization sequence is annotated by underlining. The FokI domain is annotated in bold font. The recognition helix regions are shown in double-underlined and italics.

[0057] Figures 13A through 13D show the expression of proteins deficient in lysosomal storage diseases and the presence of enzymatic activity in the supernatant of the cells following modification with albumin-specific ZFN and a donor. Figure 13A shows enzymatic expression of the alpha-L iduronidase (IDUA) protein via Western blot analysis (Figure 13B) and the presence of IDUA enzyme activity in the supernatant of the cells. Cultures were sampled at days 3 and 6 following transfection and either low or high doses of ZFN and IDUA donor. Figures 13C and 13D show a similar set of data measuring the presence of iduronate-2-sulfatase deficiency-(IDS) protein and enzymatic activity following transfection of ZFNs and an IDS donor.

DETAILED DESCRIPTION

[0058] Disclosed herein are compositions and methods for modifying a cell to produce one or more proteins whose expression or gene sequence, prior to modification, is aberrant and is associated with a disease or disorder, for example, a hemophilia or a lysosomal storage disease (LSD). The cell is modified by targeted insertion of a transgene encoding one or more functional proteins into a safe harbor gene (*e.g.*, albumin) of the cell. In some embodiments, the transgene is inserted into an endogenous albumin gene. The transgene can encode any protein or peptide involved in hemophilia, for example Factor VII, F8, F.IX, Factor X, GBA, GLA, IDS, IDUA, SMPD1 and/or functional fragments thereof. Also disclosed are methods of treating a disorder in which one or more proteins or lacking or deficient (*e.g.*, a hemophilia or a lysosomal storage disease) using a cell as described herein and/or by modifying a cell (*ex vivo* or *in vivo*) as described herein. Further described are compositions comprising nucleic acids encoding nucleases and donor molecules for modifying a cell, and methods for modifying the cell *in vivo* or *ex vivo*. Additionally,

compositions comprising cells that have been modified by the methods and compositions of the invention are described.

[0059] The genomically-modified cells described herein are typically modified via nuclease-mediated (ZFN, TALEN and/or CRISPR/Cas) targeted integration to insert a sequence encoding a therapeutic protein (*e.g.*, Factor VII, Factor VIII (F8), Factor IX, Factor X, glucocerebrosidase, α galactosidase, iduronate-2-sulfatase (IDS), alpha-L iduronidase (IDUA) and/or sphingomyelin phosphodiesterase 1), wherein the protein, whose gene in an altered or aberrant state, is associated with a disease, into the genome of one or more cells of the subject (*in vivo* or *ex vivo*), such that the cells produce the protein *in vivo*. In certain embodiments, the methods further comprise inducing cells of the subject, particularly liver cells, to proliferate (enter the cell cycle), for example, by partial hepatectomy and/or by administration of one or more compounds that induce hepatic cells to undergo cell cycling. Subjects include but are not limited to humans, non-human primates, veterinary animals such as cats, dogs, rabbits, rats, mice, guinea pigs, cows, pigs, horses, goats and the like.

General

[0060] Practice of the methods, as well as preparation and use of the compositions disclosed herein employ, unless otherwise indicated, conventional techniques in molecular biology, biochemistry, chromatin structure and analysis, computational chemistry, cell culture, recombinant DNA and related fields as are within the skill of the art. These techniques are fully explained in the literature. *See*, for example, Sambrook *et al.* MOLECULAR CLONING: A LABORATORY MANUAL, Second edition, Cold Spring Harbor Laboratory Press, 1989 and Third edition, 2001; Ausubel *et al.*, CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, John Wiley & Sons, New York, 1987 and periodic updates; the series METHODS IN ENZYMOLOGY, Academic Press, San Diego; Wolffe, CHROMATIN STRUCTURE AND FUNCTION, Third edition, Academic Press, San Diego, 1998; METHODS IN ENZYMOLOGY, Vol. 304, "Chromatin" (P.M. Wassarman and A. P. Wolffe, eds.), Academic Press, San Diego, 1999; and METHODS IN MOLECULAR BIOLOGY, Vol. 119, "Chromatin Protocols" (P.B. Becker, ed.) Humana Press, Totowa, 1999.

Definitions

[0061] The terms "nucleic acid," "polynucleotide," and "oligonucleotide" are used interchangeably and refer to a deoxyribonucleotide or ribonucleotide polymer, in linear or circular conformation, and in either single- or double-stranded form. For the purposes of the

present disclosure, these terms are not to be construed as limiting with respect to the length of a polymer. The terms can encompass known analogues of natural nucleotides, as well as nucleotides that are modified in the base, sugar and/or phosphate moieties (*e.g.*, phosphorothioate backbones). In general, an analogue of a particular nucleotide has the same base-pairing specificity; *i.e.*, an analogue of A will base-pair with T.

[0062] The terms "polypeptide," "peptide" and "protein" are used interchangeably to refer to a polymer of amino acid residues. The term also applies to amino acid polymers in which one or more amino acids are chemical analogues or modified derivatives of a corresponding naturally-occurring amino acids.

[0063] "Binding" refers to a sequence-specific, non-covalent interaction between macromolecules (*e.g.*, between a protein and a nucleic acid). Not all components of a binding interaction need be sequence-specific (*e.g.*, contacts with phosphate residues in a DNA backbone), as long as the interaction as a whole is sequence-specific. Such interactions are generally characterized by a dissociation constant (K_d) of 10^{-6} M⁻¹ or lower. "Affinity" refers to the strength of binding: increased binding affinity being correlated with a lower K_d .

[0064] A "binding protein" is a protein that is able to bind non-covalently to another molecule. A binding protein can bind to, for example, a DNA molecule (a DNA-binding protein), an RNA molecule (an RNA-binding protein) and/or a protein molecule (a protein-binding protein). In the case of a protein-binding protein, it can bind to itself (to form homodimers, homotrimers, *etc.*) and/or it can bind to one or more molecules of a different protein or proteins. A binding protein can have more than one type of binding activity. For example, zinc finger proteins have DNA-binding, RNA-binding and protein-binding activity.

[0065] A "zinc finger DNA binding protein" (or binding domain) is a protein, or a domain within a larger protein, that binds DNA in a sequence-specific manner through one or more zinc fingers, which are regions of amino acid sequence within the binding domain whose structure is stabilized through coordination of a zinc ion. The term zinc finger DNA binding protein is often abbreviated as zinc finger protein or ZFP.

[0066] A "TALE DNA binding domain" or "TALE" is a polypeptide comprising one or more TALE repeat domains/units. The repeat domains are involved in binding of the TALE to its cognate target DNA sequence. A single "repeat unit" (also referred to as a "repeat") is typically 33-35 amino acids in length and exhibits at least some sequence homology with other TALE repeat sequences within a naturally occurring TALE protein. *See, e.g.*, U.S. Patent No. 8,586,526.

[0067] Zinc finger and TALE binding domains can be "engineered" to bind to a predetermined nucleotide sequence, for example via engineering (altering one or more amino acids) of the recognition helix region of a naturally occurring zinc finger or TALE protein. Therefore, engineered DNA binding proteins (zinc fingers or TALEs) are proteins that are non-naturally occurring. Non-limiting examples of methods for engineering DNA-binding proteins are design and selection. A designed DNA binding protein is a protein not occurring in nature whose design/composition results principally from rational criteria. Rational criteria for design include application of substitution rules and computerized algorithms for processing information in a database storing information of existing ZFP and/or TALE designs and binding data. See, for example, US Patents 6,140,081; 6,453,242; 6,534,261; and 8,586,526 see also WO 98/53058; WO 98/53059; WO 98/53060; WO 02/016536 and WO 03/016496.

[0068] A "selected" zinc finger protein or TALE is a protein not found in nature whose production results primarily from an empirical process such as phage display, interaction trap or hybrid selection. See *e.g.*, US 5,789,538; US 5,925,523; US 6,007,988; US 6,013,453; US 6,200,759; WO 95/19431; WO 96/06166; WO 98/53057; WO 98/54311; WO 00/27878; WO 01/60970 WO 01/88197 and WO 02/099084.

[0069] "TtAgo" is a prokaryotic Argonaute protein thought to be involved in gene silencing. TtAgo is derived from the bacteria *Thermus thermophilus*. See, *e.g.*, Swarts *et al*, *ibid*, G. Sheng *et al.*, (2013) *Proc. Natl. Acad. Sci. U.S.A.* 111, 652). A "TtAgo system" is all the components required including, for example, guide DNAs for cleavage by a TtAgo enzyme.

[0070] "Recombination" refers to a process of exchange of genetic information between two polynucleotides, including but not limited to, donor capture by non-homologous end joining (NHEJ) and homologous recombination. For the purposes of this disclosure, "homologous recombination (HR)" refers to the specialized form of such exchange that takes place, for example, during repair of double-strand breaks in cells via homology-directed repair mechanisms. This process requires nucleotide sequence homology, uses a "donor" molecule to template repair of a "target" molecule (*i.e.*, the one that experienced the double-strand break), and is variously known as "non-crossover gene conversion" or "short tract gene conversion," because it leads to the transfer of genetic information from the donor to the target. Without wishing to be bound by any particular theory, such transfer can involve mismatch correction of heteroduplex DNA that forms between the broken target and the donor, and/or "synthesis-dependent strand annealing," in which the donor is used to

resynthesize genetic information that will become part of the target, and/or related processes. Such specialized HR often results in an alteration of the sequence of the target molecule such that part or all of the sequence of the donor polynucleotide is incorporated into the target polynucleotide.

[0071] In the methods of the disclosure, one or more targeted nucleases as described herein create a double-stranded break in the target sequence (*e.g.*, cellular chromatin) at a predetermined site, and a “donor” polynucleotide, having homology to the nucleotide sequence in the region of the break, can be introduced into the cell. The presence of the double-stranded break has been shown to facilitate integration of the donor sequence. The donor sequence may be physically integrated or, alternatively, the donor polynucleotide is used as a template for repair of the break via homologous recombination, resulting in the introduction of all or part of the nucleotide sequence as in the donor into the cellular chromatin. Thus, a first sequence in cellular chromatin can be altered and, in certain embodiments, can be converted into a sequence present in a donor polynucleotide. Thus, the use of the terms “replace” or “replacement” can be understood to represent replacement of one nucleotide sequence by another, (*i.e.*, replacement of a sequence in the informational sense), and does not necessarily require physical or chemical replacement of one polynucleotide by another.

[0072] In any of the methods described herein, additional pairs of zinc-finger proteins or TALEN can be used for additional double-stranded cleavage of additional target sites within the cell.

[0073] In certain embodiments of methods for targeted recombination and/or replacement and/or alteration of a sequence in a region of interest in cellular chromatin, a chromosomal sequence is altered by homologous recombination with an exogenous “donor” nucleotide sequence. Such homologous recombination is stimulated by the presence of a double-stranded break in cellular chromatin, if sequences homologous to the region of the break are present.

[0074] In any of the methods described herein, the first nucleotide sequence (the “donor sequence”) can contain sequences that are homologous, but not identical, to genomic sequences in the region of interest, thereby stimulating homologous recombination to insert a non-identical sequence in the region of interest. Thus, in certain embodiments, portions of the donor sequence that are homologous to sequences in the region of interest exhibit between about 80 to 99% (or any integer therebetween) sequence identity to the genomic sequence that is replaced. In other embodiments, the homology between the donor and

genomic sequence is higher than 99%, for example if only 1 nucleotide differs as between donor and genomic sequences of over 100 contiguous base pairs. In certain cases, a non-homologous portion of the donor sequence can contain sequences not present in the region of interest, such that new sequences are introduced into the region of interest. In these instances, the non-homologous sequence is generally flanked by sequences of 50-1,000 base pairs (or any integral value therebetween) or any number of base pairs greater than 1,000, that are homologous or identical to sequences in the region of interest. In other embodiments, the donor sequence is non-homologous to the first sequence, and is inserted into the genome by non-homologous recombination mechanisms.

[0075] Any of the methods described herein can be used for partial or complete inactivation of one or more target sequences in a cell by targeted integration of donor sequence that disrupts expression of the gene(s) of interest. Cell lines with partially or completely inactivated genes are also provided.

[0076] Furthermore, the methods of targeted integration as described herein can also be used to integrate one or more exogenous sequences. The exogenous nucleic acid sequence can comprise, for example, one or more genes or cDNA molecules, or any type of coding or noncoding sequence, as well as one or more control elements (*e.g.*, promoters). In addition, the exogenous nucleic acid sequence may produce one or more RNA molecules (*e.g.*, small hairpin RNAs (shRNAs), inhibitory RNAs (RNAis), microRNAs (miRNAs), *etc.*).

[0077] "Cleavage" refers to the breakage of the covalent backbone of a DNA molecule. Cleavage can be initiated by a variety of methods including, but not limited to, enzymatic or chemical hydrolysis of a phosphodiester bond. Both single-stranded cleavage and double-stranded cleavage are possible, and double-stranded cleavage can occur as a result of two distinct single-stranded cleavage events. DNA cleavage can result in the production of either blunt ends or staggered ends. In certain embodiments, fusion polypeptides are used for targeted double-stranded DNA cleavage.

[0078] A "cleavage half-domain" is a polypeptide sequence which, in conjunction with a second polypeptide (either identical or different) forms a complex having cleavage activity (preferably double-strand cleavage activity). The terms "first and second cleavage half-domains," "+ and – cleavage half-domains" and "right and left cleavage half-domains" are used interchangeably to refer to pairs of cleavage half-domains that dimerize.

[0079] An "engineered cleavage half-domain" is a cleavage half-domain that has been modified so as to form obligate heterodimers with another cleavage half-domain (*e.g.*,

another engineered cleavage half-domain). *See, also*, U.S. Patent Nos. 7,914,796; 8,034,598; and 8,623,618; incorporated herein by reference in their entireties.

[0080] The term "sequence" refers to a nucleotide sequence of any length, which can be DNA or RNA; can be linear, circular or branched and can be either single-stranded or double stranded. The term "donor sequence" refers to a nucleotide sequence that is inserted into a genome. A donor sequence can be of any length, for example between 2 and 10,000 nucleotides in length (or any integer value therebetween or thereabove), preferably between about 100 and 1,000 nucleotides in length (or any integer therebetween), more preferably between about 200 and 500 nucleotides in length.

[0081] "Chromatin" is the nucleoprotein structure comprising the cellular genome. Cellular chromatin comprises nucleic acid, primarily DNA, and protein, including histones and non-histone chromosomal proteins. The majority of eukaryotic cellular chromatin exists in the form of nucleosomes, wherein a nucleosome core comprises approximately 150 base pairs of DNA associated with an octamer comprising two each of histones H2A, H2B, H3 and H4; and linker DNA (of variable length depending on the organism) extends between nucleosome cores. A molecule of histone H1 is generally associated with the linker DNA. For the purposes of the present disclosure, the term "chromatin" is meant to encompass all types of cellular nucleoprotein, both prokaryotic and eukaryotic. Cellular chromatin includes both chromosomal and episomal chromatin.

[0082] A "chromosome," is a chromatin complex comprising all or a portion of the genome of a cell. The genome of a cell is often characterized by its karyotype, which is the collection of all the chromosomes that comprise the genome of the cell. The genome of a cell can comprise one or more chromosomes.

[0083] An "episome" is a replicating nucleic acid, nucleoprotein complex or other structure comprising a nucleic acid that is not part of the chromosomal karyotype of a cell. Examples of episomes include plasmids and certain viral genomes.

[0084] A "target site" or "target sequence" is a nucleic acid sequence that defines a portion of a nucleic acid to which a binding molecule will bind, provided sufficient conditions for binding exist.

[0085] An "exogenous" molecule is a molecule that is not normally present in a cell, but can be introduced into a cell by one or more genetic, biochemical or other methods. "Normal presence in the cell" is determined with respect to the particular developmental stage and environmental conditions of the cell. Thus, for example, a molecule that is present only during embryonic development of muscle is an exogenous molecule with respect to an

adult muscle cell. Similarly, a molecule induced by heat shock is an exogenous molecule with respect to a non-heat-shocked cell. An exogenous molecule can comprise, for example, a functioning version of a malfunctioning endogenous molecule or a malfunctioning version of a normally-functioning endogenous molecule.

[0086] An exogenous molecule can be, among other things, a small molecule, such as is generated by a combinatorial chemistry process, or a macromolecule such as a protein, nucleic acid, carbohydrate, lipid, glycoprotein, lipoprotein, polysaccharide, any modified derivative of the above molecules, or any complex comprising one or more of the above molecules. Nucleic acids include DNA and RNA, can be single- or double-stranded; can be linear, branched or circular; and can be of any length. Nucleic acids include those capable of forming duplexes, as well as triplex-forming nucleic acids. See, for example, U.S. Patent Nos. 5,176,996 and 5,422,251. Proteins include, but are not limited to, DNA-binding proteins, transcription factors, chromatin remodeling factors, methylated DNA binding proteins, polymerases, methylases, demethylases, acetylases, deacetylases, kinases, phosphatases, integrases, recombinases, ligases, topoisomerases, gyrases and helicases.

[0087] An exogenous molecule can be the same type of molecule as an endogenous molecule, *e.g.*, an exogenous protein or nucleic acid. For example, an exogenous nucleic acid can comprise an infecting viral genome, a plasmid or episome introduced into a cell, or a chromosome that is not normally present in the cell. Methods for the introduction of exogenous molecules into cells are known to those of skill in the art and include, but are not limited to, lipid-mediated transfer (*i.e.*, liposomes, including neutral and cationic lipids), electroporation, direct injection, cell fusion, particle bombardment, calcium phosphate co-precipitation, DEAE-dextran-mediated transfer and viral vector-mediated transfer. An exogenous molecule can also be the same type of molecule as an endogenous molecule but derived from a different species than the cell is derived from. For example, a human nucleic acid sequence may be introduced into a cell line originally derived from a mouse or hamster.

[0088] By contrast, an "endogenous" molecule is one that is normally present in a particular cell at a particular developmental stage under particular environmental conditions. For example, an endogenous nucleic acid can comprise a chromosome, the genome of a mitochondrion, chloroplast or other organelle, or a naturally-occurring episomal nucleic acid. Additional endogenous molecules can include proteins, for example, transcription factors and enzymes.

[0089] A "fusion" molecule is a molecule in which two or more subunit molecules are linked, preferably covalently. The subunit molecules can be the same chemical type of

molecule, or can be different chemical types of molecules. Examples of the first type of fusion molecule include, but are not limited to, fusion proteins (for example, a fusion between a ZFP or TALE DNA-binding domain and one or more activation domains) and fusion nucleic acids (for example, a nucleic acid encoding the fusion protein described *supra*). Examples of the second type of fusion molecule include, but are not limited to, a fusion between a triplex-forming nucleic acid and a polypeptide, and a fusion between a minor groove binder and a nucleic acid.

[0090] Expression of a fusion protein in a cell can result from delivery of the fusion protein to the cell or by delivery of a polynucleotide encoding the fusion protein to a cell, wherein the polynucleotide is transcribed, and the transcript is translated, to generate the fusion protein. Trans-splicing, polypeptide cleavage and polypeptide ligation can also be involved in expression of a protein in a cell. Methods for polynucleotide and polypeptide delivery to cells are presented elsewhere in this disclosure.

[0091] A "gene," for the purposes of the present disclosure, includes a DNA region encoding a gene product (see *infra*), as well as all DNA regions which regulate the production of the gene product, whether or not such regulatory sequences are adjacent to coding and/or transcribed sequences. Accordingly, a gene includes, but is not necessarily limited to, promoter sequences, terminators, translational regulatory sequences such as ribosome binding sites and internal ribosome entry sites, enhancers, silencers, insulators, boundary elements, replication origins, matrix attachment sites and locus control regions.

[0092] "Gene expression" refers to the conversion of the information, contained in a gene, into a gene product. A gene product can be the direct transcriptional product of a gene (*e.g.*, mRNA, tRNA, rRNA, antisense RNA, ribozyme, structural RNA or any other type of RNA) or a protein produced by translation of an mRNA. Gene products also include RNAs which are modified, by processes such as capping, polyadenylation, methylation, and editing, and proteins modified by, for example, methylation, acetylation, phosphorylation, ubiquitination, ADP-ribosylation, myristilation, and glycosylation.

[0093] "Modulation" of gene expression refers to a change in the activity of a gene. Modulation of expression can include, but is not limited to, gene activation and gene repression. Genome editing (*e.g.*, cleavage, alteration, inactivation, random mutation) can be used to modulate expression. Gene inactivation refers to any reduction in gene expression as compared to a cell that does not include a ZFP as described herein. Thus, gene inactivation may be partial or complete.

[0094] A "region of interest" is any region of cellular chromatin, such as, for example, a gene or a non-coding sequence within or adjacent to a gene, in which it is desirable to bind an exogenous molecule. Binding can be for the purposes of targeted DNA cleavage and/or targeted recombination. A region of interest can be present in a chromosome, an episome, an organellar genome (*e.g.*, mitochondrial, chloroplast), or an infecting viral genome, for example. A region of interest can be within the coding region of a gene, within transcribed non-coding regions such as, for example, leader sequences, trailer sequences or introns, or within non-transcribed regions, either upstream or downstream of the coding region. A region of interest can be as small as a single nucleotide pair or up to 2,000 nucleotide pairs in length, or any integral value of nucleotide pairs.

[0095] "Eukaryotic" cells include, but are not limited to, fungal cells (such as yeast), plant cells, animal cells, mammalian cells and human cells (*e.g.*, T-cells).

[0096] The terms "operative linkage" and "operatively linked" (or "operably linked") are used interchangeably with reference to a juxtaposition of two or more components (such as sequence elements), in which the components are arranged such that both components function normally and allow the possibility that at least one of the components can mediate a function that is exerted upon at least one of the other components. By way of illustration, a transcriptional regulatory sequence, such as a promoter, is operatively linked to a coding sequence if the transcriptional regulatory sequence controls the level of transcription of the coding sequence in response to the presence or absence of one or more transcriptional regulatory factors. A transcriptional regulatory sequence is generally operatively linked in *cis* with a coding sequence, but need not be directly adjacent to it. For example, an enhancer is a transcriptional regulatory sequence that is operatively linked to a coding sequence, even though they are not contiguous.

[0097] With respect to fusion polypeptides, the term "operatively linked" can refer to the fact that each of the components performs the same function in linkage to the other component as it would if it were not so linked. For example, with respect to a fusion polypeptide in which a ZFP DNA-binding domain is fused to an activation domain, the ZFP DNA-binding domain and the activation domain are in operative linkage if, in the fusion polypeptide, the ZFP DNA-binding domain portion is able to bind its target site and/or its binding site, while the activation domain is able to upregulate gene expression. When a fusion polypeptide in which a ZFP DNA-binding domain is fused to a cleavage domain, the ZFP DNA-binding domain and the cleavage domain are in operative linkage if, in the fusion polypeptide, the ZFP DNA-binding domain portion is able to bind its target site and/or its

binding site, while the cleavage domain is able to cleave DNA in the vicinity of the target site.

[0098] A "functional fragment" of a protein, polypeptide or nucleic acid is a protein, polypeptide or nucleic acid whose sequence is not identical to the full-length protein, polypeptide or nucleic acid, yet retains the same function as the full-length protein, polypeptide or nucleic acid. A functional fragment can possess more, fewer, or the same number of residues as the corresponding native molecule, and/or can contain one or more amino acid or nucleotide substitutions. Methods for determining the function of a nucleic acid (*e.g.*, coding function, ability to hybridize to another nucleic acid) are well-known in the art. Similarly, methods for determining protein function are well-known. For example, the DNA-binding function of a polypeptide can be determined, for example, by filter-binding, electrophoretic mobility-shift, or immunoprecipitation assays. DNA cleavage can be assayed by gel electrophoresis. See Ausubel *et al.*, *supra*. The ability of a protein to interact with another protein can be determined, for example, by co-immunoprecipitation, two-hybrid assays or complementation, both genetic and biochemical. See, for example, Fields *et al.* (1989) *Nature* **340**:245-246; U.S. Patent No. 5,585,245 and PCT WO 98/44350.

[0099] A "vector" is capable of transferring gene sequences to target cells. Typically, "vector construct," "expression vector," and "gene transfer vector," mean any nucleic acid construct capable of directing the expression of a gene of interest and which can transfer gene sequences to target cells. Thus, the term includes cloning, and expression vehicles, as well as integrating vectors.

[0100] A "safe harbor" locus is a locus within the genome wherein a gene may be inserted without any deleterious effects on the host cell. Most beneficial is a safe harbor locus in which expression of the inserted gene sequence is not perturbed by any read-through expression from neighboring genes. Non-limiting examples of safe harbor loci that are targeted by nuclease(s) include CCR5, HPRT, AAVS1, *Rosa* and albumin. See, *e.g.*, U.S. Patent Nos. 7,951,925 and 8,110,379; U.S. Publication Nos. 20080159996; 201000218264; 20120017290; 20110265198; 20130137104; 20130122591; 20130177983 and 20130177960 and U.S. Provisional Application No. 61/823,689)

Nucleases

[0101] Described herein are compositions, particularly nucleases that are useful in integration of a sequence encoding a functional protein that is lacking, deficient or aberrantly expressed in a subject with a disease or disorder (*e.g.*, a protein that is lacking or deficient in

a subject with an LSD and/or a clotting factor (*e.g.*, F8 and/or F.IX) protein in the genome of a cell from or in a subject with hemophilia A or B). In certain embodiments, the nuclease is naturally occurring. In other embodiments, the nuclease is non-naturally occurring, *i.e.*, engineered in the DNA-binding domain and/or cleavage domain. For example, the DNA-binding domain of a naturally-occurring nuclease may be altered to bind to a selected target site (*e.g.*, a meganuclease that has been engineered to bind to site different than the cognate binding site). In other embodiments, the nuclease comprises heterologous DNA-binding and cleavage domains (*e.g.*, zinc finger nucleases; TAL-effector domain DNA binding proteins; meganuclease DNA-binding domains with heterologous cleavage domains) and/or a CRISPR/Cas system utilizing an engineered single guide RNA).

A. DNA-binding domains

[0102] Any DNA-binding domain can be used in the compositions and methods disclosed herein, including but not limited to a zinc finger DNA-binding domain, a TALE DNA binding domain, the DNA-binding portion of a CRISPR/Cas nuclease, or a DNA-binding domain from a meganuclease.

[0103] In certain embodiments, the nuclease is a naturally occurring or engineered (non-naturally occurring) meganuclease (homing endonuclease). Exemplary homing endonucleases include I-SceI, I-CeuI, PI-PspI, PI-Sce, I-SceIV, I-CsmI, I-PanI, I-SceII, I-PpoI, I-SceIII, I-CreI, I-TevI, I-TevII and I-TevIII. Their recognition sequences are known. *See also* U.S. Patent No. 5,420,032; U.S. Patent No. 6,833,252; Belfort *et al.* (1997) *Nucleic Acids Res.* **25**:3379–3388; Dujon *et al.* (1989) *Gene* **82**:115–118; Perler *et al.* (1994) *Nucleic Acids Res.* **22**, 1125–1127; Jasin (1996) *Trends Genet.* **12**:224–228; Gimble *et al.* (1996) *J. Mol. Biol.* **263**:163–180; Argast *et al.* (1998) *J. Mol. Biol.* **280**:345–353 and the New England Biolabs catalogue. Engineered meganucleases are described for example in U.S. Patent Publication No. 20070117128. The DNA-binding domains of the homing endonucleases and meganucleases may be altered in the context of the nuclease as a whole (*i.e.*, such that the nuclease includes the cognate cleavage domain) or may be fused to a heterologous cleavage domain. DNA-binding domains from meganucleases may also exhibit nuclease activity (*e.g.*, cTALENs).

[0104] In other embodiments, the DNA-binding domain comprises a naturally occurring or engineered (non-naturally occurring) TAL effector DNA binding domain. *See, e.g.*, U.S. Patent No. 8,586,526, incorporated by reference in its entirety herein. The plant pathogenic bacteria of the genus *Xanthomonas* are known to cause many diseases in

important crop plants. Pathogenicity of *Xanthomonas* depends on a conserved type III secretion (T3S) system which injects more than 25 different effector proteins into the plant cell. Among these injected proteins are transcription activator-like (TAL) effectors which mimic plant transcriptional activators and manipulate the plant transcriptome (see Kay *et al* (2007) *Science* 318:648-651). These proteins contain a DNA binding domain and a transcriptional activation domain. One of the most well characterized TAL-effectors is AvrBs3 from *Xanthomonas campestris* pv. *Vesicatoria* (see Bonaset *et al* (1989) *Mol Gen Genet* 218: 127-136 and WO2010079430). TAL-effectors contain a centralized domain of tandem repeats, each repeat containing approximately 34 amino acids, which are key to the DNA binding specificity of these proteins. In addition, they contain a nuclear localization sequence and an acidic transcriptional activation domain (for a review see Schornack S, *et al* (2006) *J Plant Physiol* 163(3): 256-272). In addition, in the phytopathogenic bacteria *Ralstonia solanacearum* two genes, designated brg11 and hpx17 have been found that are homologous to the AvrBs3 family of *Xanthomonas* in the *R. solanacearum* biovar 1 strain GMI1000 and in the biovar 4 strain RS1000 (See Heuer *et al* (2007) *Appl and Envir Micro* 73(13): 4379-4384). These genes are 98.9% identical in nucleotide sequence to each other but differ by a deletion of 1,575 base pairs in the repeat domain of hpx17. However, both gene products have less than 40% sequence identity with AvrBs3 family proteins of *Xanthomonas*. See, e.g., U.S. Patent No. 8,586,526, incorporated by reference in its entirety herein.

[0105] Specificity of these TAL effectors depends on the sequences found in the tandem repeats. The repeated sequence comprises approximately 102 base pairs and the repeats are typically 91-100% homologous with each other (Bonas *et al, ibid*).

Polymorphism of the repeats is usually located at positions 12 and 13 and there appears to be a one-to-one correspondence between the identity of the hypervariable diresidues (the repeat variable diresidue or RVD region) at positions 12 and 13 with the identity of the contiguous nucleotides in the TAL-effector's target sequence (see Moscou and Bogdanove, (2009) *Science* 326:1501 and Boch *et al* (2009) *Science* 326:1509-1512). Experimentally, the natural code for DNA recognition of these TAL-effectors has been determined such that an HD sequence at positions 12 and 13 (Repeat Variable Diresidue or RVD) leads to a binding to cytosine (C), NG binds to T, NI to A, C, G or T, NN binds to A or G, and ING binds to T. These DNA binding repeats have been assembled into proteins with new combinations and numbers of repeats, to make artificial transcription factors that are able to interact with new sequences and activate the expression of a non-endogenous reporter gene in plant cells (Boch *et al, ibid*). Engineered TAL proteins have been linked to a *FokI* cleavage half domain to

yield a TAL effector domain nuclease fusion (TALEN), including TALENs with atypical RVDs. *See, e.g.*, U.S. Patent No. 8,586,526.

[0106] In some embodiments, the TALEN comprises a endonuclease (*e.g.*, FokI) cleavage domain or cleavage half-domain. In other embodiments, the TALE-nuclease is a mega TAL. These mega TAL nucleases are fusion proteins comprising a TALE DNA binding domain and a meganuclease cleavage domain. The meganuclease cleavage domain is active as a monomer and does not require dimerization for activity. (See Boissel *et al.*, (2013) *Nucl Acid Res*: 1-13, doi: 10.1093/nar/gkt1224).

[0107] In still further embodiments, the nuclease comprises a compact TALEN. These are single chain fusion proteins linking a TALE DNA binding domain to a TevI nuclease domain. The fusion protein can act as either a nickase localized by the TALE region, or can create a double strand break, depending upon where the TALE DNA binding domain is located with respect to the TevI nuclease domain (see Beurdeley *et al* (2013) *Nat Comm*: 1-8 DOI: 10.1038/ncomms2782). In addition, the nuclease domain may also exhibit DNA-binding functionality. Any TALENs may be used in combination with additional TALENs (*e.g.*, one or more TALENs (cTALENs or FokI-TALENs) with one or more mega-TALEs.

[0108] In certain embodiments, the DNA binding domain comprises a zinc finger protein. Preferably, the zinc finger protein is non-naturally occurring in that it is engineered to bind to a target site of choice. *See, for example*, Beerli *et al.* (2002) *Nature Biotechnol.* **20**:135-141; Pabo *et al.* (2001) *Ann. Rev. Biochem.* **70**:313-340; Isalan *et al.* (2001) *Nature Biotechnol.* **19**:656-660; Segal *et al.* (2001) *Curr. Opin. Biotechnol.* **12**:632-637; Choo *et al.* (2000) *Curr. Opin. Struct. Biol.* **10**:411-416; U.S. Patent Nos. 6,453,242; 6,534,261; 6,599,692; 6,503,717; 6,689,558; 7,030,215; 6,794,136; 7,067,317; 7,262,054; 7,070,934; 7,361,635; 7,253,273; and U.S. Patent Publication Nos. 2005/0064474; 2007/0218528; 2005/0267061, all incorporated herein by reference in their entireties.

[0109] An engineered zinc finger binding domain can have a novel binding specificity, compared to a naturally-occurring zinc finger protein. Engineering methods include, but are not limited to, rational design and various types of selection. Rational design includes, for example, using databases comprising triplet (or quadruplet) nucleotide sequences and individual zinc finger amino acid sequences, in which each triplet or quadruplet nucleotide sequence is associated with one or more amino acid sequences of zinc fingers which bind the particular triplet or quadruplet sequence. *See, for example*, co-owned U.S. Patents 6,453,242 and 6,534,261, incorporated by reference herein in their entireties.

[0110] Exemplary selection methods, including phage display and two-hybrid systems, are disclosed in US Patents 5,789,538; 5,925,523; 6,007,988; 6,013,453; 6,410,248; 6,140,466; 6,200,759; and 6,242,568; as well as WO 98/37186; WO 98/53057; WO 00/27878; WO 01/88197 and GB 2,338,237. In addition, enhancement of binding specificity for zinc finger binding domains has been described, for example, in co-owned WO 02/077227.

[0111] In addition, as disclosed in these and other references, zinc finger domains and/or multi-fingered zinc finger proteins may be linked together using any suitable linker sequences, including for example, linkers of 5 or more amino acids in length. See, also, U.S. Patent Nos. 6,479,626; 6,903,185; and 7,153,949 for exemplary linker sequences 6 or more amino acids in length. The proteins described herein may include any combination of suitable linkers between the individual zinc fingers of the protein.

[0112] Selection of target sites; ZFPs and methods for design and construction of fusion proteins (and polynucleotides encoding same) are known to those of skill in the art and described in detail in U.S. Patent Nos. 6,140,081; 5,789,538; 6,453,242; 6,534,261; 5,925,523; 6,007,988; 6,013,453; 6,200,759; WO 95/19431; WO 96/06166; WO 98/53057; WO 98/54311; WO 00/27878; WO 01/60970 WO 01/88197; WO 02/099084; WO 98/53058; WO 98/53059; WO 98/53060; WO 02/016536 and WO 03/016496.

[0113] In addition, as disclosed in these and other references, zinc finger domains and/or multi-fingered zinc finger proteins may be linked together using any suitable linker sequences, including for example, linkers of 5 or more amino acids in length. See, also, U.S. Patent Nos. 6,479,626; 6,903,185; and 7,153,949 for exemplary linker sequences 6 or more amino acids in length. The proteins described herein may include any combination of suitable linkers between the individual zinc fingers of the protein.

B. Cleavage Domains

[0114] Any suitable cleavage domain can be operatively linked to a DNA-binding domain to form a nuclease, such as a zinc finger nuclease, a TALEN, or a CRISPR/Cas nuclease system. See, e.g., U.S. Patent Nos. 7,951,925; 8,110,379 and 8,586,526; U.S. Publication Nos. 20080159996; 201000218264; 20120017290; 20110265198; 20130137104; 20130122591; 20130177983 and 20130177960 and U.S. Provisional Application No. 61/823,689.

[0115] As noted above, the cleavage domain may be heterologous to the DNA-binding domain, for example a zinc finger DNA-binding domain and a cleavage domain from a nuclease or a TALEN DNA-binding domain and a cleavage domain, or meganuclease DNA-binding domain and cleavage domain from a different nuclease. Heterologous cleavage domains can be obtained from any endonuclease or exonuclease. Exemplary endonucleases from which a cleavage domain can be derived include, but are not limited to, restriction endonucleases and homing endonucleases. *See*, for example, 2002-2003 Catalogue, New England Biolabs, Beverly, MA; and Belfort *et al.* (1997) *Nucleic Acids Res.* **25**:3379-3388. Additional enzymes which cleave DNA are known (*e.g.*, S1 Nuclease; mung bean nuclease; pancreatic DNase I; micrococcal nuclease; yeast HO endonuclease; *see also* Linn *et al.* (eds.) *Nucleases*, Cold Spring Harbor Laboratory Press, 1993). One or more of these enzymes (or functional fragments thereof) can be used as a source of cleavage domains and cleavage half-domains.

[0116] Similarly, a cleavage half-domain can be derived from any nuclease or portion thereof, as set forth above, that requires dimerization for cleavage activity. In general, two fusion proteins are required for cleavage if the fusion proteins comprise cleavage half-domains. Alternatively, a single protein comprising two cleavage half-domains can be used. The two cleavage half-domains can be derived from the same endonuclease (or functional fragments thereof), or each cleavage half-domain can be derived from a different endonuclease (or functional fragments thereof). In addition, the target sites for the two fusion proteins are preferably disposed, with respect to each other, such that binding of the two fusion proteins to their respective target sites places the cleavage half-domains in a spatial orientation to each other that allows the cleavage half-domains to form a functional cleavage domain, *e.g.*, by dimerizing. Thus, in certain embodiments, the near edges of the target sites are separated by 5-8 nucleotides or by 15-18 nucleotides. However any number of nucleotides or nucleotide pairs can intervene between two target sites (*e.g.*, from 2 to 50 nucleotide pairs or more). In general, the site of cleavage lies between the target sites.

[0117] Restriction endonucleases (restriction enzymes) are present in many species and are capable of sequence-specific binding to DNA (at a recognition site), and cleaving DNA at or near the site of binding. Certain restriction enzymes (*e.g.*, Type IIS) cleave DNA at sites removed from the recognition site and have separable binding and cleavage domains. For example, the Type IIS enzyme *Fok* I catalyzes double-stranded cleavage of DNA, at 9 nucleotides from its recognition site on one strand and 13 nucleotides from its recognition site on the other. *See*, for example, US Patents 5,356,802; 5,436,150 and 5,487,994; as well as Li

et al. (1992) *Proc. Natl. Acad. Sci. USA* **89**:4275-4279; Li *et al.* (1993) *Proc. Natl. Acad. Sci. USA* **90**:2764-2768; Kim *et al.* (1994a) *Proc. Natl. Acad. Sci. USA* **91**:883-887; Kim *et al.* (1994b) *J. Biol. Chem.* **269**:31,978-31,982. Thus, in one embodiment, fusion proteins comprise the cleavage domain (or cleavage half-domain) from at least one Type IIS restriction enzyme and one or more zinc finger binding domains, which may or may not be engineered.

[0118] An exemplary Type IIS restriction enzyme, whose cleavage domain is separable from the binding domain, is *Fok* I. This particular enzyme is active as a dimer. Bitinaite *et al.* (1998) *Proc. Natl. Acad. Sci. USA* **95**: 10,570-10,575. Accordingly, for the purposes of the present disclosure, the portion of the *Fok* I enzyme used in the disclosed fusion proteins is considered a cleavage half-domain. Thus, for targeted double-stranded cleavage and/or targeted replacement of cellular sequences using zinc finger-*Fok* I fusions, two fusion proteins, each comprising a *Fok*I cleavage half-domain, can be used to reconstitute a catalytically active cleavage domain. Alternatively, a single polypeptide molecule containing a zinc finger binding domain and two *Fok* I cleavage half-domains can also be used. Parameters for targeted cleavage and targeted sequence alteration using zinc finger-*Fok* I fusions are provided elsewhere in this disclosure.

[0119] A cleavage domain or cleavage half-domain can be any portion of a protein that retains cleavage activity, or that retains the ability to multimerize (*e.g.*, dimerize) to form a functional cleavage domain.

[0120] Exemplary Type IIS restriction enzymes are described in International Publication WO 07/014275, incorporated herein in its entirety. Additional restriction enzymes also contain separable binding and cleavage domains, and these are contemplated by the present disclosure. *See*, for example, Roberts *et al.* (2003) *Nucleic Acids Res.* **31**:418-420.

[0121] In certain embodiments, the cleavage domain comprises one or more engineered cleavage half-domain (also referred to as dimerization domain mutants) that minimize or prevent homodimerization, as described, for example, in U.S. Patent Publication Nos. 20050064474; 20060188987; 20070305346 and 20080131962, the disclosures of all of which are incorporated by reference in their entireties herein. Amino acid residues at positions 446, 447, 479, 483, 484, 486, 487, 490, 491, 496, 498, 499, 500, 531, 534, 537, and 538 of *Fok*I are all targets for influencing dimerization of the *Fok*I cleavage half-domains.

[0122] Exemplary engineered cleavage half-domains of *Fok*I that form obligate heterodimers include a pair in which a first cleavage half-domain includes mutations at amino

acid residues at positions 490 and 538 of *FokI* and a second cleavage half-domain includes mutations at amino acid residues 486 and 499.

[0123] Thus, in one embodiment, a mutation at 490 replaces Glu (E) with Lys (K); the mutation at 538 replaces Iso (I) with Lys (K); the mutation at 486 replaced Gln (Q) with Glu (E); and the mutation at position 499 replaces Iso (I) with Lys (K). Specifically, the engineered cleavage half-domains described herein were prepared by mutating positions 490 (E→K) and 538 (I→K) in one cleavage half-domain to produce an engineered cleavage half-domain designated “E490K:I538K” and by mutating positions 486 (Q→E) and 499 (I→L) in another cleavage half-domain to produce an engineered cleavage half-domain designated “Q486E:I499L”. The engineered cleavage half-domains described herein are obligate heterodimer mutants in which aberrant cleavage is minimized or abolished. *See, e.g.*, U.S. Patent Nos. 7,914,796 and 8,034,598, the disclosures of which are incorporated by reference in their entireties for all purposes. In certain embodiments, the engineered cleavage half-domain comprises mutations at positions 486, 499 and 496 (numbered relative to wild-type *FokI*), for instance mutations that replace the wild type Gln (Q) residue at position 486 with a Glu (E) residue, the wild type Iso (I) residue at position 499 with a Leu (L) residue and the wild-type Asn (N) residue at position 496 with an Asp (D) or Glu (E) residue (also referred to as a “ELD” and “ELE” domains, respectively). In other embodiments, the engineered cleavage half-domain comprises mutations at positions 490, 538 and 537 (numbered relative to wild-type *FokI*), for instance mutations that replace the wild type Glu (E) residue at position 490 with a Lys (K) residue, the wild type Iso (I) residue at position 538 with a Lys (K) residue, and the wild-type His (H) residue at position 537 with a Lys (K) residue or a Arg (R) residue (also referred to as “KKK” and “KKR” domains, respectively). In other embodiments, the engineered cleavage half-domain comprises mutations at positions 490 and 537 (numbered relative to wild-type *FokI*), for instance mutations that replace the wild type Glu (E) residue at position 490 with a Lys (K) residue and the wild-type His (H) residue at position 537 with a Lys (K) residue or a Arg (R) residue (also referred to as “KIK” and “KIR” domains, respectively). (See U.S. Patent No. 8,623,618). In other embodiments, the engineered cleavage half domain comprises the “Sharkey” and/or “Sharkey’ ” mutations (see Guo *et al.*, (2010) *J. Mol. Biol.* 400(1):96-107).

[0124] Engineered cleavage half-domains described herein can be prepared using any suitable method, for example, by site-directed mutagenesis of wild-type cleavage half-domains (*FokI*) as described in U.S. Patent Nos. 7,888,121; 7,914,796; 8,034,598 and 8,623,618.

[0125] Alternatively, nucleases may be assembled *in vivo* at the nucleic acid target site using so-called “split-enzyme” technology (*see, e.g.* U.S. Patent Publication No. 20090068164). Components of such split enzymes may be expressed either on separate expression constructs, or can be linked in one open reading frame where the individual components are separated, for example, by a self-cleaving 2A peptide or IRES sequence. Components may be individual zinc finger binding domains or domains of a meganuclease nucleic acid binding domain.

[0126] Nucleases can be screened for activity prior to use, for example in a yeast-based chromosomal system as described in WO 2009/042163 and 20090068164. Nuclease expression constructs can be readily designed using methods known in the art. *See, e.g.*, United States Patent Publications 20030232410; 20050208489; 20050026157; 20050064474; 20060188987; 20060063231; and International Publication WO 07/014275. Expression of the nuclease may be under the control of a constitutive promoter or an inducible promoter, for example the galactokinase promoter which is activated (de-repressed) in the presence of raffinose and/or galactose and repressed in presence of glucose.

[0127] In certain embodiments, the nuclease comprises a CRISPR/Cas system. The CRISPR (clustered regularly interspaced short palindromic repeats) locus, which encodes RNA components of the system, and the cas (CRISPR-associated) locus, which encodes proteins (Jansen *et al.*, 2002. *Mol. Microbiol.* 43: 1565-1575; Makarova *et al.*, 2002. *Nucleic Acids Res.* 30: 482-496; Makarova *et al.*, 2006. *Biol. Direct* 1: 7; Haft *et al.*, 2005. *PLoS Comput. Biol.* 1: e60) make up the gene sequences of the CRISPR/Cas nuclease system. CRISPR loci in microbial hosts contain a combination of CRISPR-associated (Cas) genes as well as non-coding RNA elements capable of programming the specificity of the CRISPR-mediated nucleic acid cleavage.

[0128] The Type II CRISPR is one of the most well characterized systems and carries out targeted DNA double-strand break in four sequential steps. First, two non-coding RNA, the pre-crRNA array and tracrRNA, are transcribed from the CRISPR locus. Second, tracrRNA hybridizes to the repeat regions of the pre-crRNA and mediates the processing of pre-crRNA into mature crRNAs containing individual spacer sequences. Third, the mature crRNA:tracrRNA complex directs Cas9 to the target DNA via Watson-Crick base-pairing between the spacer on the crRNA and the protospacer on the target DNA next to the protospacer adjacent motif (PAM), an additional requirement for target recognition. Finally, Cas9 mediates cleavage of target DNA to create a double-stranded break within the protospacer. Activity of the CRISPR/Cas system comprises of three steps: (i) insertion of

alien DNA sequences into the CRISPR array to prevent future attacks, in a process called 'adaptation', (ii) expression of the relevant proteins, as well as expression and processing of the array, followed by (iii) RNA-mediated interference with the alien nucleic acid. Thus, in the bacterial cell, several of the so-called 'Cas' proteins are involved with the natural function of the CRISPR/Cas system and serve roles in functions such as insertion of the alien DNA etc.

[0129] In certain embodiments, Cas protein may be a "functional derivative" of a naturally occurring Cas protein. A "functional derivative" of a native sequence polypeptide is a compound having a qualitative biological property in common with a native sequence polypeptide. "Functional derivatives" include, but are not limited to, fragments of a native sequence and derivatives of a native sequence polypeptide and its fragments, provided that they have a biological activity in common with a corresponding native sequence polypeptide. A biological activity contemplated herein is the ability of the functional derivative to hydrolyze a DNA substrate into fragments. The term "derivative" encompasses both amino acid sequence variants of polypeptide, covalent modifications, and fusions thereof. Suitable derivatives of a Cas polypeptide or a fragment thereof include but are not limited to mutants, fusions, covalent modifications of Cas protein or a fragment thereof. Cas protein, which includes Cas protein or a fragment thereof, as well as derivatives of Cas protein or a fragment thereof, may be obtainable from a cell or synthesized chemically or by a combination of these two procedures. The cell may be a cell that naturally produces Cas protein, or a cell that naturally produces Cas protein and is genetically engineered to produce the endogenous Cas protein at a higher expression level or to produce a Cas protein from an exogenously introduced nucleic acid, which nucleic acid encodes a Cas that is same or different from the endogenous Cas. In some case, the cell does not naturally produce Cas protein and is genetically engineered to produce a Cas protein.

[0130] Exemplary CRISPR/Cas nuclease systems targeted to safe harbor and other genes are disclosed for example, in U.S. Provisional Application No. 61/823,689.

[0131] Thus, the nuclease comprises a DNA-binding domain in that specifically binds to a target site in any gene into which it is desired to insert a donor (transgene).

Target Sites

[0132] As described in detail above, DNA-binding domains can be engineered to bind to any sequence of choice, for example in a safe-harbor locus such as albumin. An engineered DNA-binding domain can have a novel binding specificity, compared to a

naturally-occurring DNA-binding domain. Engineering methods include, but are not limited to, rational design and various types of selection. Rational design includes, for example, using databases comprising triplet (or quadruplet) nucleotide sequences and individual zinc finger amino acid sequences, in which each triplet or quadruplet nucleotide sequence is associated with one or more amino acid sequences of zinc fingers which bind the particular triplet or quadruplet sequence. *See*, for example, co-owned U.S. Patents 6,453,242 and 6,534,261, incorporated by reference herein in their entireties. Rational design of TAL-effector domains can also be performed. *See, e.g.*, U.S. Patent No. 8,586,526.

[0133] Exemplary selection methods applicable to DNA-binding domains, including phage display and two-hybrid systems, are disclosed in US Patents 5,789,538; 5,925,523; 6,007,988; 6,013,453; 6,410,248; 6,140,466; 6,200,759; and 6,242,568; as well as WO 98/37186; WO 98/53057; WO 00/27878; WO 01/88197 and GB 2,338,237. In addition, enhancement of binding specificity for zinc finger binding domains has been described, for example, in co-owned WO 02/077227.

[0134] Selection of target sites; nucleases and methods for design and construction of fusion proteins (and polynucleotides encoding same) are known to those of skill in the art and described in detail in U.S. Patent Application Publication Nos. 20050064474 and 20060188987, incorporated by reference in their entireties herein.

[0135] In addition, as disclosed in these and other references, DNA-binding domains (*e.g.*, multi-fingered zinc finger proteins) may be linked together using any suitable linker sequences, including for example, linkers of 5 or more amino acids. *See, e.g.*, U.S. Patent Nos. 6,479,626; 6,903,185; and 7,153,949 for exemplary linker sequences 6 or more amino acids in length. The proteins described herein may include any combination of suitable linkers between the individual DNA-binding domains of the protein. *See, also*, U.S. Patent No. 8,586,526.

[0136] For treatment of hemophilia via targeted insertion of a sequence encoding a functional F8 and/or F.IX protein, any desired site of insertion in the genome of the subject is cleaved with a nuclease, which stimulates targeted insertion of the donor polynucleotide carrying the F8- and/or F.IX-encoding sequence. DNA-binding domains of the nucleases may be targeted to any desired site in the genome. In certain embodiments, the DNA-binding domain of the nuclease is targeted to an endogenous safe harbor locus, for example an endogenous albumin locus.

Donor Sequences

[0137] Any donor can be inserted via nuclease-mediated targeted integration as described herein. In certain embodiments, the donor comprises a polynucleotide (transgene) that encodes a therapeutic protein, for example a protein is lacking, deficient and/or aberrantly expressed in a subject with a disease or disorder. Non-limiting examples of such disorders include, epidermolysis bullosa, diabetes, cancer, clotting disorders or AAT deficient emphysema, clotting disorders and/or lysosomal storage diseases.

[0138] For treating hemophilia, the donor sequence (also called an “exogenous sequence” or “donor” or “transgene”) comprises a sequence encoding a functional clotting factor protein, or part thereof, to result in a sequence encoding and expressing a functional clotting factor protein following donor integration. Non-limiting examples of clotting factor protein transgenes include Factor VIII and/or Factor IX, including functional fragments of these proteins. In certain embodiments, the B-domain of the F8 protein is deleted. *See, e.g.,* Chuah et al. (2003) 101(5):1734-1743. In other embodiments, the transgene comprises a sequence encoding a functional F.IX protein, or part thereof, to result in a sequence encoding and expressing a function F.IX protein following donor integration. Similarly, for treating an LSD, the donor sequence encodes one or more proteins lacking in a subject with an LSD. Non-limiting examples of such proteins include glucocerebrosidase (GBA), which is deficient in Gaucher’s; α galactosidase (GLA), which is deficient in Fabry’s; iduronate-2-sulfatase deficiency (IDS), which is deficient in Hunter’s; alpha-L iduronidase (IDUA), which is deficient in Hurler’s; sphingomyelin phosphodiesterase 1 (SMPD1), which is deficient in Niemann-Pick’s.

[0139] The donor molecule may be inserted into an endogenous gene such that all, some or none of the endogenous gene is expressed. For example, a transgene comprising functional clotting factor protein (*e.g.*, F8 and/or F.IX) sequences as described herein may be inserted into an endogenous albumin locus such that some or none of the endogenous albumin is expressed with the transgene.

[0140] The donor (transgene) sequence can be introduced into the cell prior to, concurrently with, or subsequent to, expression of the fusion protein(s) (*e.g.*, nucleases). The donor polynucleotide may contain sufficient homology (continuous or discontinuous regions) to a genomic sequence to support homologous recombination (or homology-directed repair) between it and the genomic sequence to which it bears homology or, alternatively, donor sequences can be integrated via non-HDR mechanisms (*e.g.*, NHEJ donor capture), in which case the donor polynucleotide (*e.g.*, vector) need not containing sequences that are

homologous to the region of interest in cellular chromatin. *See, e.g.*, U.S. Patent Nos. 7,888,121 and 7,972,843 and U.S. Patent Publication No. 20110281361; 20100047805 and 20110207221.

[0141] The donor polynucleotide can be DNA or RNA, single-stranded, double-stranded or partially single- and partially double-stranded and can be introduced into a cell in linear or circular (*e.g.*, minicircle) form. *See, e.g.*, U.S. Patent Publication Nos. 20100047805, 20110281361, 20110207221. If introduced in linear form, the ends of the donor sequence can be protected (*e.g.*, from exonucleolytic degradation) by methods known to those of skill in the art. For example, one or more dideoxynucleotide residues are added to the 3' terminus of a linear molecule and/or self-complementary oligonucleotides are ligated to one or both ends. *See, for example*, Chang *et al.* (1987) *Proc. Natl. Acad. Sci. USA* **84**:4959-4963; Nehls *et al.* (1996) *Science* **272**:886-889. Additional methods for protecting exogenous polynucleotides from degradation include, but are not limited to, addition of terminal amino group(s) and the use of modified internucleotide linkages such as, for example, phosphorothioates, phosphoramidates, and O-methyl ribose or deoxyribose residues. A polynucleotide can be introduced into a cell as part of a vector molecule having additional sequences such as, for example, replication origins, promoters and genes encoding antibiotic resistance. Moreover, donor polynucleotides can be introduced as naked nucleic acid, as nucleic acid complexed with an agent such as a liposome or poloxamer, or can be delivered by viruses (*e.g.*, adenovirus, AAV, herpesvirus, retrovirus, lentivirus).

[0142] The donor is generally inserted so that its expression is driven by the endogenous promoter at the integration site (*e.g.*, the endogenous albumin promoter when the donor is integrated into the patient's albumin locus). Thus, the transgene typically lacks control elements (*e.g.*, promoter and/or enhancer) that drive its expression (*e.g.*, also referred to as a "promoterless construct"). Nonetheless, it will be apparent that the donor may comprise a promoter and/or enhancer, for example a constitutive promoter or an inducible or tissue specific (*e.g.*, liver- or platelet-specific) promoter that drives expression of the functional protein upon integration.

[0143] The donor sequence can be integrated specifically into any target site of choice, thereby eliminating the issues associated with random integration in traditional gene therapy.

[0144] When albumin sequences (endogenous or part of the transgene) are expressed with the transgene, the albumin sequences may be full-length sequences (wild-type or mutant) or partial sequences. Preferably the albumin sequences are functional. Non-limiting

examples of the function of these full length or partial albumin sequences include increasing the serum half-life of the polypeptide expressed by the transgene (*e.g.*, therapeutic gene) and/or acting as a carrier.

[0145] Furthermore, although not required for expression, exogenous sequences may also include transcriptional or translational regulatory sequences, for example, promoters, enhancers, insulators, internal ribosome entry sites, sequences encoding 2A peptides and/or polyadenylation signals.

[0146] Any of the donor sequences may include one or more of the following modifications: codon optimization (*e.g.*, to human codons) and/or addition of one or more glycosylation sites. *See, e.g.*, McIntosh *et al.* (2013) *Blood* (17):3335-44. Exogenous sequences may also comprise peptide sequences allowing for targeted delivery of a therapeutic protein. For example, nucleic acid sequences encoding the human p97 polypeptide and/or fragments thereof may be linked to a donor exogenous sequence such that the fusion protein will have the potential to cross the blood brain barrier (*see e.g.* U.S. Provisional Patent application No. 20130183368 and Karkan *et al* (2008) *PLOS One*. DOI: 10.1371/journal.pone.0002469) or other peptides can be used to target a transgene donor encoded protein to intracellular organelles such as mitochondria (*e.g.* Jacotot *et al* (2006) *Biochim Biophys Acta Bioenerg* 1757: 1312-1323).

Delivery

[0147] The nucleases, polynucleotides encoding these nucleases, donor polynucleotides and compositions comprising the proteins and/or polynucleotides described herein may be delivered *in vivo* or *ex vivo* by any suitable means.

[0148] Methods of delivering nucleases as described herein are described, for example, in U.S. Patent Nos. 8,586,526; 6,453,242; 6,503,717; 6,534,261; 6,599,692; 6,607,882; 6,689,558; 6,824,978; 6,933,113; 6,979,539; 7,013,219; and 7,163,824, the disclosures of all of which are incorporated by reference herein in their entireties.

[0149] Nucleases and/or donor constructs as described herein may also be delivered using vectors containing sequences encoding one or more of the zinc finger protein(s), TALEN protein(s) and/or a CRISPR/Cas system. Any vector systems may be used including, but not limited to, plasmid vectors, retroviral vectors, lentiviral vectors, adenovirus vectors, poxvirus vectors; herpesvirus vectors and adeno-associated virus vectors, etc. *See, also*, U.S. Patent Nos. 6,534,261; 6,607,882; 6,824,978; 6,933,113; 6,979,539; 7,013,219; and

7,163,824, incorporated by reference herein in their entireties. Furthermore, it will be apparent that any of these vectors may comprise one or more of the sequences needed. Thus, when one or more nucleases and a donor construct are introduced into the cell, the nucleases and/or donor polynucleotide may be carried on the same vector or on different vectors. When multiple vectors are used, each vector may comprise a sequence encoding one or multiple nucleases and/or donor constructs. In certain embodiments, one vector is used to carry both the transgene and nuclease(s). In other embodiments, two vector are used (the same or different vector types), where one vector carries the nuclease(s) (e.g., left and right ZFNs of a ZFN pair, for example with a 2A peptide) and one carries the transgene. In still further embodiments, three vectors are used where the first vector carries one nuclease of a nuclease pair (e.g., left ZFN), the second vector carries the other nuclease of a nuclease pair (e.g., right ZFN) and the third vector carries the transgene. See, Figure 2.

[0150] The donors and/or nuclease may be used at any suitable concentrations. In certain embodiments, the donor and separate nuclease vector(s) are used the same concentration. In other embodiments, the donor and separate nuclease vector(s) are used at different concentrations, for example, 2-, 3-, 4-, 5-, 10- or more fold of one vector than other (e.g., more donor vector(s) than nuclease vector(s)). When AAV vectors are used for delivery, for example, the donor-and/or nuclease-comprising viral vector(s) are between 1×10^8 and 1×10^{13} particles per dose (e.g., cell or animal).

[0151] Conventional viral and non-viral based gene transfer methods can be used to introduce nucleic acids encoding nucleases and donor constructs in cells (e.g., mammalian cells) and target tissues. Non-viral vector delivery systems include DNA plasmids, naked nucleic acid, and nucleic acid complexed with a delivery vehicle such as a liposome or poloxamer. Viral vector delivery systems include DNA and RNA viruses, which have either episomal or integrated genomes after delivery to the cell. For a review of *in vivo* delivery of engineered DNA-binding proteins and fusion proteins comprising these binding proteins, see, e.g., Rebar (2004) *Expert Opinion Invest. Drugs* 13(7):829-839; Rossi *et al.* (2007) *Nature Biotech.* 25(12):1444-1454 as well as general gene delivery references such as Anderson, *Science* 256:808-813 (1992); Nabel & Felgner, *TIBTECH* 11:211-217 (1993); Mitani & Caskey, *TIBTECH* 11:162-166 (1993); Dillon, *TIBTECH* 11:167-175 (1993); Miller, *Nature* 357:455-460 (1992); Van Brunt, *Biotechnology* 6(10):1149-1154 (1988); Vigne, *Restorative Neurology and Neuroscience* 8:35-36 (1995); Kremer & Perricaudet, *British Medical Bulletin* 51(1):31-44 (1995); Haddada *et al.*, in *Current Topics in Microbiology and Immunology* Doerfler and Böhm (eds.) (1995); and Yu *et al.*, *Gene Therapy* 1:13-26 (1994).

[0152] Methods of non-viral delivery of nucleic acids include electroporation, lipofection, microinjection, biolistics, virosomes, liposomes, immunoliposomes, polycation or lipid:nucleic acid conjugates, naked DNA, artificial virions, and agent-enhanced uptake of DNA. Sonoporation using, *e.g.*, the Sonitron 2000 system (Rich-Mar) can also be used for delivery of nucleic acids.

[0153] Additional exemplary nucleic acid delivery systems include those provided by AmaxaBiosystems (Cologne, Germany), Maxcyte, Inc. (Rockville, Maryland), BTX Molecular Delivery Systems (Holliston, MA) and Copernicus Therapeutics Inc, (*see* for example U.S. 6,008,336). Lipofection is described in *e.g.*, U.S. Patent Nos. 5,049,386; 4,946,787; and 4,897,355) and lipofection reagents are sold commercially (*e.g.*, Transfectam™ and Lipofectin™). Cationic and neutral lipids that are suitable for efficient receptor-recognition lipofection of polynucleotides include those of Felgner, WO 91/17424, WO 91/16024.

[0154] The preparation of lipid:nucleic acid complexes, including targeted liposomes such as immunolipid complexes, is well known to one of skill in the art (*see, e.g.*, Crystal, *Science* 270:404-410 (1995); Blaese *et al.*, *Cancer Gene Ther.* 2:291-297 (1995); Behr *et al.*, *Bioconjugate Chem.* 5:382-389 (1994); Remy *et al.*, *Bioconjugate Chem.* 5:647-654 (1994); Gao *et al.*, *Gene Therapy* 2:710-722 (1995); Ahmad *et al.*, *Cancer Res.* 52:4817-4820 (1992); U.S. Pat. Nos. 4,186,183, 4,217,344, 4,235,871, 4,261,975, 4,485,054, 4,501,728, 4,774,085, 4,837,028, and 4,946,787).

[0155] Additional methods of delivery include the use of packaging the nucleic acids to be delivered into EnGeneIC delivery vehicles (EDVs). These EDVs are specifically delivered to target tissues using bispecific antibodies where one arm of the antibody has specificity for the target tissue and the other has specificity for the EDV. The antibody brings the EDVs to the target cell surface and then the EDV is brought into the cell by endocytosis. Once in the cell, the contents are released (*see* MacDiarmid *et al* (2009) *Nature Biotechnology* 27(7):643).

[0156] The use of RNA or DNA viral based systems for the delivery of nucleic acids encoding engineered nucleases and/or donors take advantage of highly evolved processes for targeting a virus to specific cells in the body and trafficking the viral payload to the nucleus. Viral vectors can be administered directly to patients (*in vivo*) or they can be used to treat cells *in vitro* and the modified cells are administered to patients (*ex vivo*). Conventional viral based systems for the delivery of nucleases and/or donors include, but are not limited to, retroviral, lentivirus, adenoviral, adeno-associated, vaccinia and herpes simplex virus vectors

for gene transfer. Integration in the host genome is possible with the retrovirus, lentivirus, and adeno-associated virus gene transfer methods, often resulting in long term expression of the inserted transgene. Additionally, high transduction efficiencies have been observed in many different cell types and target tissues.

[0157] The tropism of a retrovirus can be altered by incorporating foreign envelope proteins, expanding the potential target population of target cells. Lentiviral vectors are retroviral vectors that are able to transduce or infect non-dividing cells and typically produce high viral titers. Selection of a retroviral gene transfer system depends on the target tissue. Retroviral vectors are comprised of *cis*-acting long terminal repeats with packaging capacity for up to 6-10 kb of foreign sequence. The minimum *cis*-acting LTRs are sufficient for replication and packaging of the vectors, which are then used to integrate the therapeutic gene into the target cell to provide permanent transgene expression. Widely used retroviral vectors include those based upon murine leukemia virus (MuLV), gibbon ape leukemia virus (GaLV), Simian Immunodeficiency virus (SIV), human immunodeficiency virus (HIV), and combinations thereof (*see, e.g.,* Buchscher *et al.*, *J. Virol.* 66:2731-2739 (1992); Johann *et al.*, *J. Virol.* 66:1635-1640 (1992); Sommerfelt *et al.*, *Virol.* 176:58-59 (1990); Wilson *et al.*, *J. Virol.* 63:2374-2378 (1989); Miller *et al.*, *J. Virol.* 65:2220-2224 (1991); PCT/US94/05700).

[0158] In applications in which transient expression is preferred, adenoviral based systems can be used. Adenoviral based vectors are capable of very high transduction efficiency in many cell types and do not require cell division. With such vectors, high titer and high levels of expression have been obtained. This vector can be produced in large quantities in a relatively simple system. Adeno-associated virus ("AAV") vectors are also used to transduce cells with target nucleic acids, *e.g.*, in the *in vitro* production of nucleic acids and peptides, and for *in vivo* and *ex vivo* gene therapy procedures (*see, e.g.,* West *et al.*, *Virology* 160:38-47 (1987); U.S. Patent No. 4,797,368; WO 93/24641; Kotin, *Human Gene Therapy* 5:793-801 (1994); Muzyczka, *J. Clin. Invest.* 94:1351 (1994). Construction of recombinant AAV vectors is described in a number of publications, including U.S. Pat. No. 5,173,414; Tratschin *et al.*, *Mol. Cell. Biol.* 5:3251-3260 (1985); Tratschin, *et al.*, *Mol. Cell. Biol.* 4:2072-2081 (1984); Hermonat & Muzyczka, *PNAS* 81:6466-6470 (1984); and Samulski *et al.*, *J. Virol.* 63:03822-3828 (1989).

[0159] At least six viral vector approaches are currently available for gene transfer in clinical trials, which utilize approaches that involve complementation of defective vectors by genes inserted into helper cell lines to generate the transducing agent.

[0160] pLASN and MFG-S are examples of retroviral vectors that have been used in clinical trials (Dunbar *et al.*, *Blood* 85:3048-305 (1995); Kohn *et al.*, *Nat. Med.* 1:1017-102 (1995); Malech *et al.*, *PNAS* 94:22 12133-12138 (1997)). PA317/pLASN was the first therapeutic vector used in a gene therapy trial. (Blaese *et al.*, *Science* 270:475-480 (1995)). Transduction efficiencies of 50% or greater have been observed for MFG-S packaged vectors. (Ellem *et al.*, *Immunol Immunother.* 44(1):10-20 (1997); Dranoff *et al.*, *Hum. Gene Ther.* 1:111-2 (1997)).

[0161] Recombinant adeno-associated virus vectors (rAAV) are a promising alternative gene delivery systems based on the defective and nonpathogenic parvovirus adeno-associated type 2 virus. All vectors are derived from a plasmid that retains only the AAV 145 base pair inverted terminal repeats flanking the transgene expression cassette. Efficient gene transfer and stable transgene delivery due to integration into the genomes of the transduced cell are key features for this vector system. (Wagner *et al.*, *Lancet* 351:9117 1702-3 (1998), Kearns *et al.*, *Gene Ther.* 9:748-55 (1996)). Other AAV serotypes, including AAV1, AAV3, AAV4, AAV5, AAV6, AAV8, AAV9 and AAVrh10 or pseudotyped AAV such as AAV2/8, AAV8.2, AAV2/5 and AAV2/6 and any novel AAV serotype can also be used in accordance with the present invention.

[0162] Replication-deficient recombinant adenoviral vectors (Ad) can be produced at high titer and readily infect a number of different cell types. Most adenovirus vectors are engineered such that a transgene replaces the Ad E1a, E1b, and/or E3 genes; subsequently the replication defective vector is propagated in human 293 cells that supply deleted gene function *in trans*. Ad vectors can transduce multiple types of tissues *in vivo*, including nondividing, differentiated cells such as those found in liver, kidney and muscle. Conventional Ad vectors have a large carrying capacity. An example of the use of an Ad vector in a clinical trial involved polynucleotide therapy for antitumor immunization with intramuscular injection (Stermann *et al.*, *Hum. Gene Ther.* 7:1083-9 (1998)). Additional examples of the use of adenovirus vectors for gene transfer in clinical trials include Rosenecker *et al.*, *Infection* 24:1 5-10 (1996); Stermann *et al.*, *Hum. Gene Ther.* 9:7 1083-1089 (1998); Welsh *et al.*, *Hum. Gene Ther.* 2:205-18 (1995); Alvarez *et al.*, *Hum. Gene Ther.* 5:597-613 (1997); Topf *et al.*, *Gene Ther.* 5:507-513 (1998); Stermann *et al.*, *Hum. Gene Ther.* 7:1083-1089 (1998).

[0163] Packaging cells are used to form virus particles that are capable of infecting a host cell. Such cells include 293 cells, which package adenovirus, and ψ 2 cells or PA317 cells, which package retrovirus. Viral vectors used in gene therapy are usually generated by a

producer cell line that packages a nucleic acid vector into a viral particle. The vectors typically contain the minimal viral sequences required for packaging and subsequent integration into a host (if applicable), other viral sequences being replaced by an expression cassette encoding the protein to be expressed. The missing viral functions are supplied *trans* by the packaging cell line. For example, AAV vectors used in gene therapy typically only possess inverted terminal repeat (ITR) sequences from the AAV genome which are required for packaging and integration into the host genome. Viral DNA is packaged in a cell line, which contains a helper plasmid encoding the other AAV genes, namely *rep* and *cap*, but lacking ITR sequences. The cell line is also infected with adenovirus as a helper. The helper virus promotes replication of the AAV vector and expression of AAV genes from the helper plasmid. The helper plasmid is not packaged in significant amounts due to a lack of ITR sequences. Contamination with adenovirus can be reduced by, *e.g.*, heat treatment to which adenovirus is more sensitive than AAV.

[0164] In many gene therapy applications, it is desirable that the gene therapy vector be delivered with a high degree of specificity to a particular tissue type. Accordingly, a viral vector can be modified to have specificity for a given cell type by expressing a ligand as a fusion protein with a viral coat protein on the outer surface of the virus. The ligand is chosen to have affinity for a receptor known to be present on the cell type of interest. For example, Han *et al.*, *Proc. Natl. Acad. Sci. USA* 92:9747-9751 (1995), reported that Moloney murine leukemia virus can be modified to express human heregulin fused to gp70, and the recombinant virus infects certain human breast cancer cells expressing human epidermal growth factor receptor. This principle can be extended to other virus-target cell pairs, in which the target cell expresses a receptor and the virus expresses a fusion protein comprising a ligand for the cell-surface receptor. For example, filamentous phage can be engineered to display antibody fragments (*e.g.*, FAB or Fv) having specific binding affinity for virtually any chosen cellular receptor. Although the above description applies primarily to viral vectors, the same principles can be applied to nonviral vectors. Such vectors can be engineered to contain specific uptake sequences which favor uptake by specific target cells.

[0165] Gene therapy vectors can be delivered *in vivo* by administration to an individual patient, typically by systemic administration (*e.g.*, intravenous, intraperitoneal, intramuscular, subdermal, or intracranial infusion) or topical application, as described below. Alternatively, vectors can be delivered to cells *ex vivo*, such as cells explanted from an individual patient (*e.g.*, lymphocytes, bone marrow aspirates, tissue biopsy) or universal

donor hematopoietic stem cells, followed by reimplantation of the cells into a patient, usually after selection for cells which have incorporated the vector.

[0166] Vectors (*e.g.*, retroviruses, adenoviruses, liposomes, etc.) containing nucleases and/or donor constructs can also be administered directly to an organism for transduction of cells *in vivo*. Alternatively, naked DNA can be administered. Administration is by any of the routes normally used for introducing a molecule into ultimate contact with blood or tissue cells including, but not limited to, injection, infusion, topical application and electroporation. Suitable methods of administering such nucleic acids are available and well known to those of skill in the art, and, although more than one route can be used to administer a particular composition, a particular route can often provide a more immediate and more effective reaction than another route.

[0167] Vectors suitable for introduction of polynucleotides (*e.g.* nuclease-encoding and/or donors) described herein include non-integrating lentivirus vectors (IDLV). *See*, for example, Ory *et al.* (1996) *Proc. Natl. Acad. Sci. USA* **93**:11382-11388; Dull *et al.* (1998) *J. Virol.* **72**:8463-8471; Zuffery *et al.* (1998) *J. Virol.* **72**:9873-9880; Follenzi *et al.* (2000) *Nature Genetics* **25**:217-222; U.S. Patent Publication No 2009/054985.

[0168] Pharmaceutically acceptable carriers are determined in part by the particular composition being administered, as well as by the particular method used to administer the composition. Accordingly, there is a wide variety of suitable formulations of pharmaceutical compositions available, as described below (*see, e.g., Remington's Pharmaceutical Sciences*, 17th ed., 1989).

[0169] Delivery *in vitro* and *in vivo* may also be accomplished through the use of nanoparticles. Many nanoparticles currently being investigated are comprised of therapeutic molecules that self-assemble with lipids or polymers into nanostructures. These particles have the potential to deliver therapeutic doses of nucleic acids to target tissues (*e.g.* tumor cells, specific organs etc). *See e.g.* Rink *et al* (2013), *Curr Opin Oncol*: 25(6): p. 646-651.

[0170] It will be apparent that the nuclease-encoding sequences and donor constructs can be delivered using the same or different systems. For example, the nucleases and donors can be carried by the same vector (*e.g.*, AAV). Alternatively, a donor polynucleotide can be carried by a plasmid, while the one or more nucleases can be carried by a different vector (*e.g.*, AAV vector). Furthermore, the different vectors can be administered by the same or different routes (intramuscular injection, tail vein injection, other intravenous injection, intraperitoneal administration and/or intramuscular injection. The vectors can be delivered simultaneously or in any sequential order.

[0171] Thus, the instant disclosure includes *in vivo* or *ex vivo* treatment and/or prevention of a disease or disorder in which a protein is lacking or deficient. For instance, Hemophilia A may be treated, via nuclease-mediated integration of F8-encoding sequence. The disclosure also includes *in vivo* or *ex vivo* treatment of Hemophilia B, via nuclease-mediated integration of a F.IX encoding sequence. Similarly, the disclosure includes the treatment of Factor VII deficiency and Factor X deficiency related hemophilias via nuclease-mediated integration of a Factor VII or Factor X encoding sequence, respectively. In addition, the disclosure includes the treatment of one or more LSDs via nuclease-mediated integration of one or more proteins lacking or deficient in the LSD. The compositions are administered to a human patient in an amount effective to obtain the desired concentration of the therapeutic polypeptide in the serum, the liver or the target cells. Administration can be by any means in which the polynucleotides are delivered to the desired target cells. For example, both *in vivo* and *ex vivo* methods are contemplated. Intravenous injection to the portal vein is a preferred method of administration. Other *in vivo* administration modes include, for example, direct injection into the lobes of the liver or the biliary duct and intravenous injection distal to the liver, including through the hepatic artery, direct injection in to the liver parenchyma, injection via the hepatic artery, and/or retrograde injection through the biliary tree. *Ex vivo* modes of administration include transduction *in vitro* of resected hepatocytes or other cells of the liver, followed by infusion of the transduced, resected hepatocytes back into the portal vasculature, liver parenchyma or biliary tree of the human patient, see *e.g.*, Grossman *et al.* (1994) *Nature Genetics*, 6:335-341. Other modes of administration include the *ex vivo* nuclease-mediated insertion of a Factor VII, F8, F.IX, Factor X, glucocerebrosidase, α galactosidase, iduronate-2-sulfatase, and/or alpha-L iduronidase encoding transgene into a safe harbor location into patient or allogenic stem cells. Following modification, the treated cells are then re-infused into the patient for treatment of the disease or disorder (*e.g.*, LSD and/or a hemophilia).

[0172] The effective amount of nuclease(s) and donor (*e.g.*, Factor VII, F8, F.IX, Factor X, GBA, GLA, IDS, IDUA, or SMPD1) to be administered will vary from patient to patient and according to the therapeutic polypeptide of interest. Accordingly, effective amounts are best determined by the physician administering the compositions and appropriate dosages can be determined readily by one of ordinary skill in the art. After allowing sufficient time for integration and expression (typically 4-15 days, for example), analysis of the serum or other tissue levels of the therapeutic polypeptide and comparison to the initial level prior to administration will determine whether the amount being administered is too low, within the

right range or too high. Suitable regimes for initial and subsequent administrations are also variable, but are typified by an initial administration followed by subsequent administrations if necessary. Subsequent administrations may be administered at variable intervals, ranging from daily to annually to every several years. One of skill in the art will appreciate that appropriate immunosuppressive techniques may be recommended to avoid inhibition or blockage of transduction by immunosuppression of the delivery vectors, see *e.g.*, Vilquin *et al.* (1995) *Human Gene Ther.* 6:1391-1401.

[0173] Formulations for both *ex vivo* and *in vivo* administrations include suspensions in liquid or emulsified liquids. The active ingredients often are mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients include, for example, water, saline, dextrose, glycerol, ethanol or the like, and combinations thereof. In addition, the composition may contain minor amounts of auxiliary substances, such as, wetting or emulsifying agents, pH buffering agents, stabilizing agents or other reagents that enhance the effectiveness of the pharmaceutical composition.

Applications

[0174] The methods and compositions of the invention can be used in any circumstance wherein it is desired to supply a transgene encoding one or more proteins such that the protein(s) is(are) secreted from the targeted cell. Thus, this technology is of use in a condition where a patient is deficient in some protein due to problems (*e.g.*, problems in expression level or problems with the protein expressed as sub- or non-functioning). Additionally, A1AT-deficiency disorders such as COPD or liver damage, or other disorders, conditions or diseases that can be mitigated by the supply of exogenous proteins by a secretory organ may be successfully treated by the methods and compositions of this invention. Lysosomal storage diseases can be treated by the methods and compositions of the invention, as are metabolic diseases such as diabetes.

[0175] Proteins that are useful therapeutically and that are typically delivered by injection or infusion are also useful with the methods and compositions of the invention. By way of non-limiting examples, production of a C-peptide (*e.g.* Ersatta™ by Cebix) or insulin for use in diabetic therapy. A further application includes treatment of Epidermolysis Bullosa via production of collagen VII. Expression of IGF-1 in secretory tissue as described herein can be used to increase levels of this protein in patients with liver cirrhosis and lipoprotein lipase deficiency by expression of lipoprotein lipase. Antibodies may also be secreted for therapeutic benefit, for example, for the treatment of cancers, autoimmune and other diseases.

Examples of therapeutic antibodies include antibodies against TNF- α , EpCAM, CD20, CD19, VEGFR, CD52 and the like. Other proteins related to clotting could be produced in secretory tissue, include fibrinogen, prothrombin, tissue factor, Factor V, Factor XI, Factor XII (Hageman factor), Factor XIII (fibrin-stabilizing factor), von Willebrand factor, prekallikrein, high molecular weight kininogen (Fitzgerald factor), fibronectin, antithrombin III, heparin cofactor II, protein C, protein S, protein Z, protein Z-related protease inhibitor, plasminogen, alpha 2-antiplasmin, tissue plasminogen activator, urokinase, plasminogen activator inhibitor-1, and plasminogen activator inhibitor-2.

[0176] The methods and compositions of the invention can also be used in any circumstance wherein it is desired to supply and express a transgene encoding one or more non-coding or structural nucleic acids (*e.g.* shRNA or RNAi). Such RNAs may form inhibitory structures and be useful in the treatment of diseases such as lipid disorders (targeting *e.g.* ApoB-100, ApoC-III, ANGPTL3, PCSK9); coronary artery disease (targeting *e.g.* CRP, Apo(a)); clotting and blood disorders (targeting *e.g.* F.XI, FVII, antithrombin, TMPRSS6); autoimmune diseases (targeting *e.g.* ICAM-1, GCCR, GCGR, PTP-1B, VLA-4); TTR amyloidosis; muscular diseases (targeting *e.g.* SMN2, GHr, DMPK); inflammatory disease (targeting *e.g.* PKK); obesity (targeting *e.g.* FGFR4); liver disease (targeting *e.g.* DGAT2, ALAS-1, C5, AAT); Cancer (targeting *e.g.* clusterin, eIF-4E, Hsp27, AR); fibrotic disease (targeting *e.g.* CTGF); ocular disease (targeting *e.g.* C-raf kinase); or infectious disease (targeting *e.g.* aminoglycoside, hepcidin, RG-101).

[0177] The following Examples relate to exemplary embodiments of the present disclosure in which the nuclease comprises a zinc finger nuclease (ZFN). It will be appreciated that this is for purposes of exemplification only and that other nucleases can be used, for instance homing endonucleases (meg nucleases) with engineered DNA-binding domains and/or fusions of naturally occurring or engineered homing endonucleases (meg nucleases) DNA-binding domains and heterologous cleavage domains, TALENs and/or a CRISPR/Cas system comprising an engineered single guide RNA.

EXAMPLES

Example 1: Targeted integration of a F8 transgene *in vivo*

[0178] HA / CD4^{-/-} mice were administered either (1) control AAV2/8 vectors and AAV2/8 donor transgenes encoding F8, (“Mock + Donor”) or (2) AAV vectors encoding ZFN pairs targeting the albumin locus (as described in U.S. Patent Publication 20130177983

and in Figure 10A and 10B) and AAV donor transgenes encoding F8 (“ZFN + Donor”), both by injection to the tail vein as described in U.S. Patent Publication No. 20120128635.

Donors used as shown schematically in Figures 1 and 2 and include a promotorless B-Domain Deleted Factor VIII (BDD-F8) cDNA of approximately 4.4 to 4.7 kb in size. Doses were administered to the mice in two dosing levels, a “low” dosing level comprising AAV8-ZFN ($1\text{e}^{11}\text{vg}/\text{mouse}$) + AAV8-Donor ($1\text{e}^{11}\text{vg}/\text{mouse}$): Figure 3B; and a “high” dosing level comprising AAV8-ZFN ($5\text{e}^{11}\text{vg}/\text{mouse}$) + AAV8-Donor ($5\text{e}^{11}\text{vg}/\text{mouse}$): Figure 3C.

[0179] Additionally, F8 donors were optimized by codon optimization for expression in mammalian cells as per standard protocols and by addition of a linker (V3) with glycosylation sites (see McIntosh *et al*, (2013) *Blood* 121:3335). In this experiment, HA / CD4^{-/-} mice were dosed with AAV8-ZFN ($5\text{e}^{10}\text{vg}$ of each ZFN) + AAV8-Donor ($1\text{e}^{11}\text{vg}/\text{mouse}$): Figure 4.

[0180] Plasma levels of F8 were evaluated using standard techniques.

[0181] As shown in Figures 3 and 4, targeted integration of a Factor 8 donor into the mouse albumin locus resulted in activity levels up to 50% of normal in Hemophilia A mice when either donor was used. However, when the optimized F8 donor construct was used, comparable F8 plasma levels were observed using only 20% of the dose. In addition, when two ZFN vectors were used, each comprising one of the two ZFNs needed for the pair, higher levels of cleavage were observed than when the ZFNs were introduced together on one expression vector separated by a 2A site (Figure 3D).

Example 2: Targeted integration of an F9 transgene *in vivo*

A. Human Hepatocytes

[0182] We first transduced human primary hepatocytes with AAV6 vector containing a F9 donor together with transfection of mRNA encoding a ZFN pair targeting a site within the first intron of human albumin.

[0183] As shown in Figure 9, hepatocytes treated with donor and ZFNs exhibited measurable human F.IX in the culture supernatant.

B. Mice

[0184] We next sought to demonstrate this approach *in vivo* in the mouse. To accomplish this, we first engineered a ZFN pair (shown below in Table 1) targeting an analogous location in mouse albumin intron 1 (shown in Figure 12) as shown below and confirmed the pair’s activity *in vitro* in murine hepatoma cells.

Table 1: Mouse Albumin-specific nuclease designs

SBS #,Target	Design					
Mouse- Albumin specific ZFNs						
	F1	F2	F3	F4	F5	F6
SBS#30724 ctGAAGGTgGC AATGGTTcctctet get (SEQ ID NO:31)	TSGSLT R(SEQ ID NO:33)	RSDALS T(SEQ ID NO:34)	QSATRT K(SEQ ID NO:35)	TSGHLS R(SEQ ID NO:36)	QSGNLAR (SEQ ID NO:2)	N/A
SBS#30725 ttTCCTGTAACG ATCGGgaactggca tc (SEQ ID NO:32)	RSDHLS A(SEQ ID NO:37)	TKSNRT K(SEQ ID NO:38)	DRSNLS R(SEQ ID NO:5)	WRSSLR A(SEQ ID NO:39)	DSSDRKK Q(SEQ ID NO:40)	N/A

[0185] As shown in Figure 10, the pair was active in murine hepatoma cells.

[0186] In addition, wild-type mice (3 animals per group) were administered via tail vein injection AAV2/8 donor transgenes encoding F.IX (see, Figure 5A) with either AAV vectors encoding mouse albumin-targeted ZFN pairs (“mAlb ZFN”) as described in U.S. Publication No. 20130177983 or human F.IX-targeted ZFN pairs (“hF9 ZFN”) as described in U.S. Publication No. 20120128635 and shown in Figure 12A and 12B. Vector constructs and dosages were as follows: albumin AAV2/8-ZFN at 1×10^{11} vg/mouse and AAV8-Donor at 5×10^{11} vg/mouse and F.IX AAV8-ZFN at 1×10^{11} vg/mouse and AAV8-Donor at 5×10^{11} vg/mouse.

[0187] Plasma levels of F9 were evaluated using standard commercially available ELISA kits using commercially available antibodies. In addition, Cel-I assays (Surveyor™, Transgenomics) were conducted as described in U.S. Publication No. 20120128635 and Perez et al, (2008) Nat. Biotechnol. 26: 808-816 and Guschin *et al*, (2010) Methods Mol Biol. 649:247-56).

[0188] As shown in Figure 5B, robust circulating hFIX levels were obtained following albumin ZFN and F9 donor delivery. The human F.IX specific ZFNs do not recognize the endogenous mouse F.IX locus, and so there is no appreciable integration of the F9 donor using this nuclease pair. Furthermore, as shown in Figure 6, genome editing is proportional to AAV dose over three orders of magnitude.

[0189] In addition, hemophilic mice (HB mice) were administered the donors and albumin-ZFN as described above (AAV8-mAlb-ZFN at 1×10^{11} vg/mouse and AAV8-F9 donor at 5×10^{11} vg/mouse, also see Li *et al* (2011) *ibid* and Anguela *et al* (2013) *ibid*) and plasma levels of hF.IX and activated partial thromboplastin time(s) (aPTT(s)) were also

determined by standard commercially available kits (*e.g.* Rox Factor IX chromogenic kit from Rossix, and Vitaclot, Vital® Diagnostics).

[0190] As shown in Figure 7, ZFN-mediated integration of a F9 donor transgene into the albumin locus of HB mice resulted in high levels of F.IX in the plasma and in correction of prolonged clotting times.

C. Rhesus macaques

[0191] To test ZFN driven genome modification and transgene insertion in larger animals, two studies were performed. The ZFNs used are shown below in Table 2.

Uppercase in the target sequence denotes bound nucleotides and lowercase denotes unbound nucleotides.

Table 2: Rhesus Albumin-specific nuclease designs

SBS #, Target	Design					
	F1	F2	F3	F4	F5	F6
SBS#36806 (rhesus) ttAGGGACAGT TATGAAttcaa tcttca (SEQ ID NO:1)	QSGNLAR (SEQ ID NO:2)	LMQNRNQ (SEQ ID NO:3)	LKHHLTD (SEQ ID NO:4)	DRSNLSR (SEQ ID NO:5)	RSDHLTQ (SEQ ID NO:6)	N/A
SBS#35396 (human/rhesus) ccTATCCATTG CACTATGCTtt atttaa (SEQ ID NO:7)	QSSDLR (SEQ ID NO:8)	LKWNLR (SEQ ID NO:9)	DQSNLRA (SEQ ID NO:10)	RPYTLRL (SEQ ID NO:11)	QSSDLR (SEQ ID NO:8)	HRSNLNK (SEQ ID NO:12)
SBS#37804 (rhesus) ttAGGGACAGT TATGAAttcaa tcttca (SEQ ID NO:1)	QSGNLAR (SEQ ID NO:2)	LMQNRNQ (SEQ ID NO:3)	LAHHLVE (SEQ ID NO:13)	DRSNLSR (SEQ ID NO:5)	RSDHLTQ (SEQ ID NO:6)	N/A

[0192] All designs shown in Table 2 bound to their target sites.

[0193] Exemplary studies were performed with ZFN pair 36806 and 35396 (Pair 2) as follows. Rhesus monkeys (purpose-bred), ages 2 to 4 years old with weights of 3 to 4.6 kg were prescreened for the presence of rAAV 2/6 and 2/8 neutralizing antibodies, the genotype of the albumin locus, and normal serum chemistry and hematology. The animals were

socially housed (up to 3 animals of same dosing group housed together). Vector administration was performed by IV infusion into a peripheral vein at a rate of 1 mL/min, for a dosing duration ranging from ~10-30 minutes (10 mL each for Study 1, 29 mL each for Study 2). The monkeys were evaluated throughout the study for mortality/moribundity, routine clinical observations, cage side observations and food consumption(daily), body weights (prestudy and weekly), clinical pathology including liver enzyme levels (ALT and AST), clinical chemistry and hematology, and coagulation using routine methodologies. Liver biopsies were performed and tissues were examined for histopathology and the pharmacokinetics of rAAV vectors as well as evaluated for gene modification by miSEQ (Illumina) and ZFN expression by Western analysis. Anti-drug antibody analysis was done throughout the study and PBMCs were isolated from whole blood for EliSpot analysis (see above). Gross and microscopic pathology are performed on tissues evaluation at the end of the study.

[0194] Study #1: Rhesus macaques were administered albumin-targeted ZFNs in AAV2/8 vectors as described in U.S. Publication No. 20130177983. In this study, a variant of the wild type Fok1 cleavage domain was also used wherein the sequence had been optimized for mammalian expression according to standard techniques (DNA 2.0). The dosing groups and animal IDs are shown below in Table 3.

Table 3: NHP Study #1; Dosing groups

Group	Description	Dose	Animal ID
1	Negative control	None	1001
2	ZFN Pair 2, Fok1 WT, ZFN only-	1.5 e+13 each ZFN	7001
		1.5 e+13 each ZFN	7002
3	ZFN Pair 2, Codon optimized Fok1 WT, ZFN only	1.5 e+13 each ZFN	8001
	ZFN Pair 2, Codon optimized Fok1 WT, ZFN only	1.5 e+13 each ZFN	8002

[0195] Enzyme-linked immunosorbent spot assays (ELISPOT, see Markusic *et al* (2013), *EMBO Mol Med* 5:1698-1709) were performed on the spleen and mesenteric lymph node tissue isolated from the animals at day 65 and, as shown in Figure 8, there is no immune response elicited against the AAV8 capsid or the ZFN transgenes in the animals. Animals 7001 and 7002 (Figure 8, panels A and C) as well as animals 8001 and 8002 (Figure 8, panels B and C) were all negative for antibody response.

[0196] Study #2: In a separate study, three groups of two animals each were evaluated for ZFN-mediated insertion of an F9 transgene into the albumin locus of rhesus macaques. See, Figure 11.

[0197] Exemplary results were obtained using Pair 2 as described above, which comprised either wild type FokI nuclease cleavage domains (labeled “FokI WT”) or engineered domains (labeled “FokI eHiFi”, see U.S. Patent No. 8,623,618) as indicated, in either an AAV2/8 or AAV2/6 vector. Animals that received the donor containing AAVs were given the F9 donor (with albumin homology arms) in an AAV2/8 vector. See Table 4 below. In the table “High” and “Low” doses refer to the total amount of AAV given.

Table 4: NHP Study #2; Dosing groups

Group	Description	AAV serotype	Dose	Animal ID
1	Negative control	-	None	1001
6	ZFN Pair 2, FokI-WT, + F9 donor (1:5 ratio ZFNs:donor), High dose	AAV2/6	1.5e+13 each ZFN, 1.5e+14 donor	6101
			1.5e+13 each ZFN, 1.5e+14 donor	6102
7	Donor only	-	1.5e+14	7001

[0198] Animals receiving ZFNs only (no donor) showed robust cleavage (0.4 - 4.1%) at day 14 post-administration.

[0199] Western analysis was performed on the samples to evaluate ZFN expression. In addition, expression of F.IX protein was detected in the plasma in animals that had received both the ZFNs and donor vectors. In the presence of both ZFN and donor, hFIX levels in plasma were detectable and increased over time.

[0200] Taken together, these data show targeted insertion of a Factor 8 or Factor 9 donor into the albumin locus increases activity levels, including up to 50% of normal FVIII in Hemophilia A mice. Furthermore, optimization of both the donor and ZFN constructs (*e.g.*, codon optimization, inclusion of glycosylation sites and/or administration of ZFNs on separate vectors), AAV dose can be reduced while maintaining transgenes activity. Indeed, a single intravenous co-injection of AAV encoding each of the albumin-specific ZFNs with an hF.IX donor resulted in detectable DNA cleavage and hF.IX expression in the plasma of Rhesus macaques.

Example 3: Design, construction and general characterization of human albumin-specific nucleases

[0201] Nucleases (*e.g.*, ZFNs, TALENs, CRISPR/Cas) targeted to albumin are described in U.S. Patent Publication Nos. 20130177983 and 20130177960 and U.S. Application No. 14/278,903). For these experiments, ZFNs comprising the ZFPs (operably linked to the engineered cleavage domains) were used to cleave the endogenous albumin locus in human cells. The human albumin-specific pairs are shown below in Table 5. All nucleases in Table 5 bound to their targets.

Table 5: Human Albumin-specific nuclease designs

SBS #,Target	Design					
Human Albumin specific ZFNs						
	F1	F2	F3	F4	F5	F6
SBS#35396 (human/rhesus) ccTATCCATTGCAC TATGCTttattttaa (SEQ ID NO:7)	QSSDLR (SEQ ID NO:8)	LKWNLRT (SEQ ID NO:9)	DQSNLRA (SEQ ID NO:10)	RPYTLRL (SEQ ID NO:11)	QSSDLR (SEQ ID NO:8)	HRSNLNK (SEQ ID NO:12)
SBS#39330 (human) ttTGGGATAGTTAT GAAttcaatcttca (SEQ ID NO:17)	QSGNLR (SEQ ID NO:2)	LKQNLCM (SEQ ID NO:18)	WQSNLQN (SEQ ID NO:19)	TSGNLTR (SEQ ID NO:20)	RQSHLCL (SEQ ID NO:21)	NA
SBS#43116 (human) ccTATCCATTGCAC TATgctttattttaa (SEQ ID NO:7)	LKWNLRT (SEQ ID NO:9)	DQSNLRA (SEQ ID NO:10)	RNFSLTM (SEQ ID NO:15)	QSSTLDT (SEQ ID NO:22)	HRSNLNK (SEQ ID NO:12)	NA
SBS#47171 (human) ttTGGGATAGTTAT GAAttcaatcttca (SEQ ID NO:17)	QSGNLSR (SEQ ID NO:23)	LKQNLCM (SEQ ID NO:18)	WADNLQN (SEQ ID NO:24)	TSGNLTR (SEQ ID NO:20)	RQSHLCL (SEQ ID NO:21)	NA
SBS#47931 (human)	TPQLLDR (SEQ ID	LKWNLRT (SEQ ID	DQSNLNA (SEQ ID	RNFSLTM (SEQ ID	LRHDLDR	HRSNLNK (SEQ ID

ccTATCCATTGCA CTATGCTttatttta a (SEQ ID NO:7)	NO:14)	NO:9)	NO:25)	NO:15)	(SEQ ID NO:16)	NO:12)
SBS#47863 (human) tttGGGATAGTTAT GAAttcaatcttca (SEQ ID NO:17)	QSGNLR (SEQ ID NO:2)	LIQYLQS (SEQ ID NO:26)	WADNLQN (SEQ ID NO:24)	TSGNLTR (SEQ ID NO:20)	RQSHLSL (SEQ ID NO:27)	NA
SBS#47079 (human) ccTATCCATTGCAC TATGCTttatttta (SEQ ID NO:7)	TPQLLDR (SEQ ID NO:14)	LKWNLR (SEQ ID NO:9)	DQSNLRA (SEQ ID NO:10)	RNFSLT (SEQ ID NO:15)	LRHDLDR (SEQ ID NO:16)	HRSNLNK (SEQ ID NO:12)
SBS#47192 (human) tttGGGATAGTTAT GAAttcaatcttca (SEQ ID NO:17)	QSGNLR (SEQ ID NO:2)	LIQYLQS (SEQ ID NO:26)	WADNLQN (SEQ ID NO:24)	TSGNLTR (SEQ ID NO:20)	RQSHLCL (SEQ ID NO:21)	NA
SBS#47898 (human) ccTATCCATTGCAC TATGCTttatttta (SEQ ID NO:7)	TPQLLDR (SEQ ID NO:14)	LKHNLRT (SEQ ID NO:28)	DQSNLNA (SEQ ID NO:25)	RNFSLT (SEQ ID NO:15)	LRHDLDR (SEQ ID NO:16)	HRSNLNK (SEQ ID NO:12)
SBS#47169 (human) ccTATCCATTGCAC TATGCTttatttta (SEQ ID NO:7)	TPQLLDR (SEQ ID NO:14)	LKWNLR (SEQ ID NO:9)	DQSNLRA (SEQ ID NO:10)	RNFSLT (SEQ ID NO:15)	LRHDL R (SEQ ID NO:16)	HRSNLNK (SEQ ID NO:12)
SBS#47864 (human) tttGGGATAGTTAT GAAttcaatcttca (SEQ ID NO:5)	QSGNLR (SEQ ID NO:2)	LIQYLQS (SEQ ID NO:112)	WQSNLQN (SEQ ID NO:105)	TSGNLTR (SEQ ID NO:106)	RQSHLCL (SEQ ID NO:107)	N/A
SBS#40477 (human) ccTATCCATTGCAC TATGCTttatttta (SEQ ID NO:7)	QSSDLR (SEQ ID NO:8)	LKHNLRT (SEQ ID NO:28)	LKHNLRT (SEQ ID NO:28)	RPYTLRL (SEQ ID NO:11)	LRPDLR (SEQ ID NO:41)	HRSNLNK (SEQ ID NO:12)

[0202] In these experiments, the ZFNs were transfected into the cells in the form of mRNAs and introduced via BTX nucleofection by standard methods. The concentrations of ZFN mRNA varied by experiment. NHEJ activity was measured by MiSeq analysis (Illumina), done according to methods known in the art.

[0203] For testing in human primary hepatocytes, 50 ng of RNA encoding each ZFN of the pair was used. The results are shown below (Table 6) and demonstrate that all the ZFN pairs had activity. For testing in human HepG2, 100 ng of RNA encoding each ZFN of the pair was used. The results are shown below (Table 7) and demonstrate that all the ZFN pairs

had activity. Human K562 cells were also tested using 75 ng (Table 8) in duplicates and demonstrated that the pairs were active.

Table 6: Human Primary Hepatocytes

		Left ZFN					% Indels
Right ZFN		47162	47171	47192	47863	47864	
	40477	8.9	6.5	6.1	11.7	14.0	
	47079	8.2	13.0	12.3	13.7	13.7	
	47169	11.2	9.6	11.6	20.3	14.1	
	47898	12.1	10.8	11.1	16.8	14.6	
	47931	10.1	11.8	11.5	17.2	14.7	

Table 7: Human HepG2 Cells

		Left ZFN					% Indels
Right ZFN		47162	47171	47192	47863	47864	
	40477	20.3	21.3	23.7	22.2	18.4	
	47079	20.8	25.8	22.9	23.5	16.0	
	47169	21.0	22.2	22.2	21.6	17.7	
	47898	21.5	22.0	13.9	22.4	14.3	
	47931	23.1	19.4	23.1	20.9	21.3	

Table 8: K562 Cells

ZFN pair	1	2	AVG%Indels
47171:47931	73.9	75.7	74.8
47171:47079	77.1	81.7	79.4
47171:47898	75.3	68.6	72
47863:47931	76.9	80.9	78.9
47863:47079	86.8	86.7	86.8
47863:47898	91.1	92.7	91.9
47192:47931	69.9	76.1	73
47192:47079	80	75.9	77.9
47192:47898	70.1	72.3	71.2

Example 5: Integration of LSD Donors using human albumin-specific ZFNs.

[0204] For these experiments, the IDS or IDUA cDNA donor was delivered via an AAV2/8 particle, where the cDNA transgenes comprised homology arms for the albumin regions flanking the cut site. In these donor constructs, the therapeutic gene was flanked by sequences homologous to the albumin gene. 5' of the transgene, the donor constructs all

contain sequences homologous to the murine albumin intron 1, while 3' of the gene, the constructs contain sequences homologous to the murine albumin intron 1-exon 2 boundary (as described in U.S. Patent Publication 2014-0017212).

[0205] To integrate the IDS or IDUA cDNA transgenes and assay their expression, albumin specific zinc finger nucleases in the form of mRNA were transfected into human HepG2/C3a cells. Briefly, 100,000 cells were transfected by viral delivery by standard methods, the MOI X1000 for zfn:zfn:donor was 100:100:200 ("low" or "L") or 300:300:600 ("high" or "H"). Expression was analyzed by either assaying enzymatic activity of the protein encoded by the transgene in the cell supernatant or by performing Western blots on the cell pellets after 6 days. The data, shown in Figure 13, demonstrate expression of the donor IDS and IDUA in the cells.

[0206] All patents, patent applications and publications mentioned herein are hereby incorporated by reference in their entirety.

[0207] Although disclosure has been provided in some detail by way of illustration and example for the purposes of clarity of understanding, it will be apparent to those skilled in the art that various changes and modifications can be practiced without departing from the spirit or scope of the disclosure. Accordingly, the foregoing descriptions and examples should not be construed as limiting.

[0208] Throughout the specification and claims, unless the context requires otherwise, the word "comprise" or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

CLAIMS

What is claimed is:

1. A zinc finger nuclease (ZFN) pair comprising a left ZFN and a right ZFN, each ZFN comprising a wild-type or engineered cleavage domain and a zinc finger protein comprising five or six zinc finger domains designated and ordered F1 to F5 or F1 to F6, as follows:

	<u>F1</u>	<u>F2</u>	<u>F3</u>	<u>F4</u>	<u>F5</u>	<u>F6</u>
<u>(i)</u> <u>SBS#47171</u>	<u>QSGNLSR</u> (SEQ ID NO:23)	<u>LKQNLCM</u> (SEQ ID NO:18)	<u>WADNLQN</u> (SEQ ID NO:24)	<u>TSGNLTR</u> (SEQ ID NO:20)	<u>RQSHLCL</u> (SEQ ID NO:21)	<u>NA</u>
<u>(ii)</u> <u>SBS#47898</u>	<u>TPQLLDR</u> (SEQ ID NO:14)	<u>LKHNLIT</u> (SEQ ID NO:28)	<u>DQSNLNA</u> (SEQ ID NO:25)	<u>RNFSLTM</u> (SEQ ID NO:15)	<u>LRHDLDR</u> (SEQ ID NO:16)	<u>HRSNLNK</u> (SEQ ID NO:12)
<u>(iii)</u> <u>SBS#47079</u> <u>or</u> <u>SBS#47169</u>	<u>TPQLLDR</u> (SEQ ID NO:14)	<u>LKWNLRT</u> (SEQ ID NO:9)	<u>DQSNLRA</u> (SEQ ID NO:10)	<u>RNFSLTM</u> (SEQ ID NO:15)	<u>LRHDLDR</u> (SEQ ID NO:16)	<u>HRSNLNK</u> (SEQ ID NO:12)
<u>(iv)</u> <u>SBS#47931</u>	<u>TPQLLDR</u> (SEQ ID NO:14)	<u>LKWNLRT</u> (SEQ ID NO:9)	<u>DQSNLNA</u> (SEQ ID NO:25)	<u>RNFSLTM</u> (SEQ ID NO:15)	<u>LRHDLDR</u> (SEQ ID NO:16)	<u>HRSNLNK</u> (SEQ ID NO:12)
<u>(v)</u> <u>SBS#47863</u>	<u>QSGNLAR</u> (SEQ ID NO:2)	<u>LIQYLQS</u> (SEQ ID NO:26)	<u>WADNLQN</u> (SEQ ID NO:24)	<u>TSGNLTR</u> (SEQ ID NO:20)	<u>RQSHLSL</u> (SEQ ID NO:27)	<u>NA</u>
<u>(vi)</u> <u>SBS#47192</u>	<u>QSGNLAR</u> (SEQ ID NO:2)	<u>LIQYLQS</u> (SEQ ID NO:26)	<u>WADNLQN</u> (SEQ ID NO:24)	<u>TSGNLTR</u> (SEQ ID NO:20)	<u>RQSHLCL</u> (SEQ ID NO:21)	<u>NA</u>
<u>(vii)</u>	<u>QSGNLAR</u>	<u>LIQYLQS</u>		<u>TSGNLTR</u>	<u>RQSHLCL</u>	<u>N/A</u>

<u>SBS#47864</u>	<u>(SEQ ID NO:2)</u>	<u>(SEQ ID NO:26)</u>	<u>WQSNLQN</u> <u>(SEQ ID NO:19)</u>	<u>(SEQ ID NO:20)</u>	<u>(SEQ ID NO:21)</u>	
<u>(viii)</u> <u>SBS#40477</u>	<u>QSSDLSR</u> <u>(SEQ ID NO:8)</u>	<u>LKHNLTT</u> <u>(SEQ ID NO:28)</u>	<u>LKHNLTT</u> <u>(SEQ ID NO:28)</u>	<u>RPYTLRL</u> <u>(SEQ ID NO:11)</u>	<u>LRPDLER</u> <u>(SEQ ID NO:41)</u>	<u>HRSNLNK</u> <u>(SEQ ID NO:12)</u>

wherein the left ZFN comprises the zinc finger recognition helix regions selected from the (i), (v), (vi), and (vii) and the right ZFN comprises the zinc finger recognition helix regions selected from the (ii), (iii), (iv), and (viii).

2. The zinc finger nuclease pair of claim 1, wherein the left ZFN comprises the zinc finger recognition helix regions of (i) and the right recognition helix regions of (ii).

3. One or more polynucleotides encoding of the zinc finger nuclease pair of claim 1 or claim 2.

4. One or more expression vectors comprising the one or more polynucleotides according to claim 3.

5. The one or more expression vectors of claim 4, wherein at least one of the vectors is an AAV vector.

6. The one or more expression vectors of claim 5, wherein the at least one AAV vector is an AAV2/8 vector.

7. A pharmaceutical composition comprising the one or more expression vectors according to any one of claims 4 to 6.

8. The pharmaceutical composition of claim 7, comprising:

(a) an expression vector comprising a transgene encoding a therapeutic protein;

(b) an expression vector encoding the left ZFN of the ZFN pair; and

(c) an expression vector encoding the right ZFN of the ZFN pair.

9. A method of cleaving an albumin gene in a cell, the method comprising: introducing, into the cell, the one or more expression vectors according to any one of claims 4 to 6, or the pharmaceutical composition of claims 7 or claim 8, under conditions such that the one or more proteins are expressed and the albumin gene is cleaved.

10. The method of claim 9, wherein a donor sequence is integrated into the cleaved albumin gene.

11. The method of claim 10, wherein the donor sequence is introduced using an AAV vector.

12. The method of claim 11, wherein the AAV vector is an AAV2/8 vector.

13. The method of any one of claims 9 to 12, wherein the donor sequence encodes a protein lacking or deficient in a lysosomal storage disease (LSD).

14. The method of any one of claims 9 to 13, wherein the cell is a liver cell.

15. A method of treating a patient with a lysosomal storage disease, the method comprising administering to the patient an expression vector according to any of claims 4 to 6 or a pharmaceutical composition according to claim 7 or claim 8 that mediates targeted integration of a transgene encoding a functional protein lacking in a subject with a lysosomal storage disease into an endogenous albumin gene.

16. The method of claim 15, wherein the expression vector(s) or pharmaceutical composition and transgene are administered intravenously.

17. A kit comprising an expression vector according to any of claims 4 to 6 or the pharmaceutical composition of claim 7 or claim 8.

18. Use of a pharmaceutical composition according to claim 7 or claim 8 in treating a patient lacking or deficient in the therapeutic protein encoded by the transgene.

19. Use of the one or more expression vectors according to any one of claims 4 to 6 in the preparation of a medicament for the treatment of a patient lacking or deficient in the therapeutic protein encoded by the transgene.

20. The use of claim 18 or claim 19, wherein the patient has a lysosomal storage disorder.

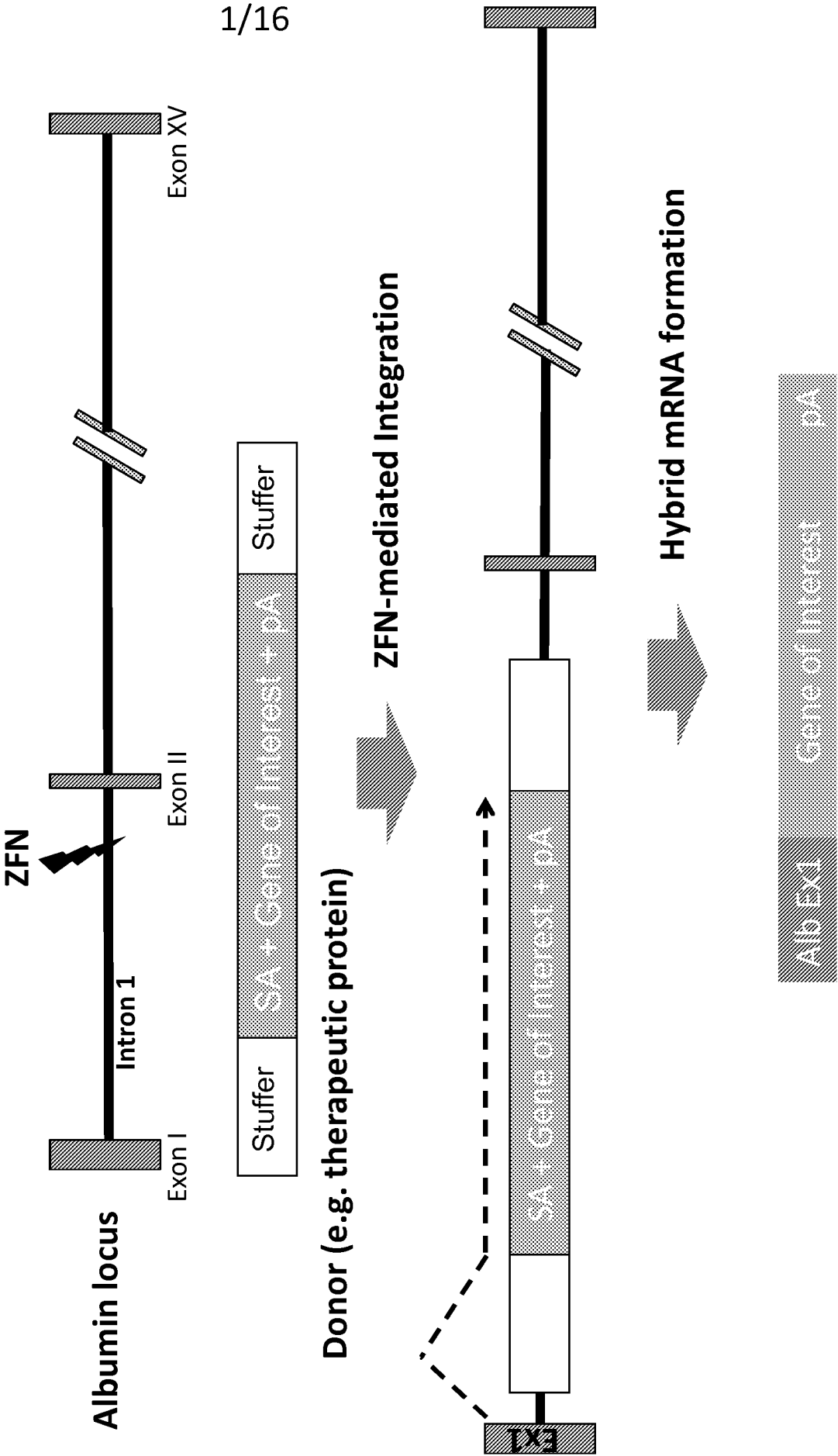
21. The use of any one of claims 18-20, wherein the pharmaceutical composition is administered intravenously.

22. The use of any one of claims 18-21, wherein the pharmaceutical composition does not elicit an immune response in the patient.

23. The use of any one of claims 18-22, wherein the therapeutic protein is expressed at an amount of at least 50% higher than normal expression levels.

24. The method of any one of claims 9-14, wherein the ZFN pair cleaves in between 8.2 and 17.2 percent of cells.

Figure 1



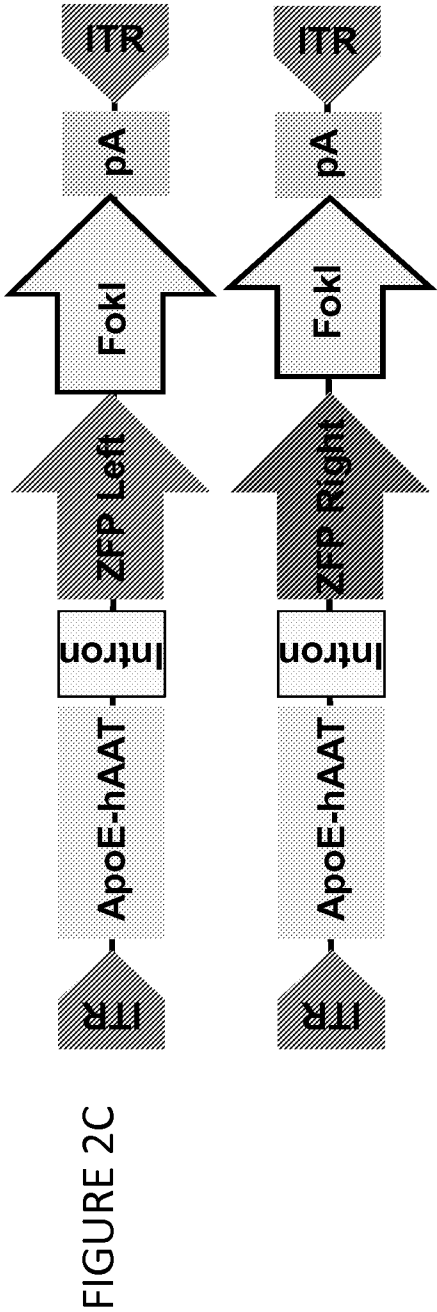
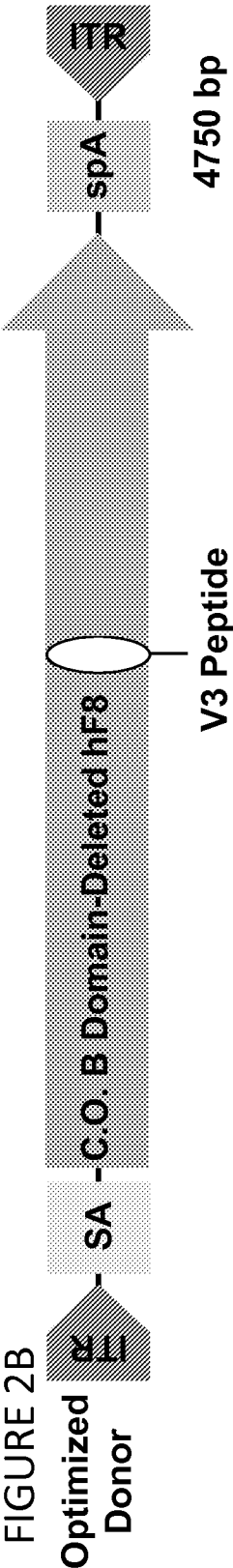
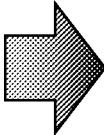
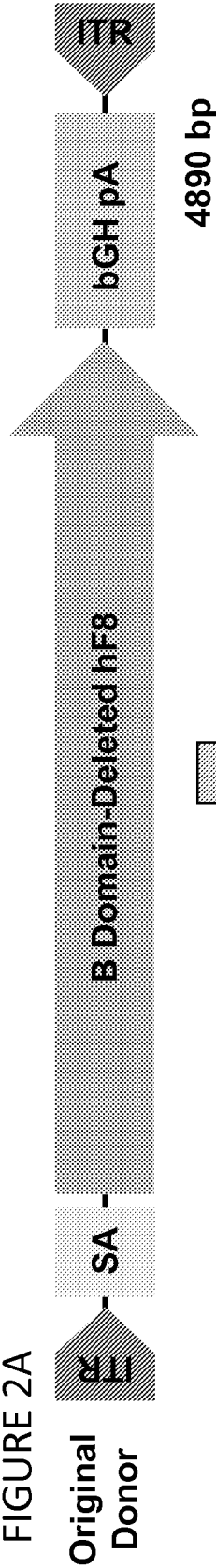
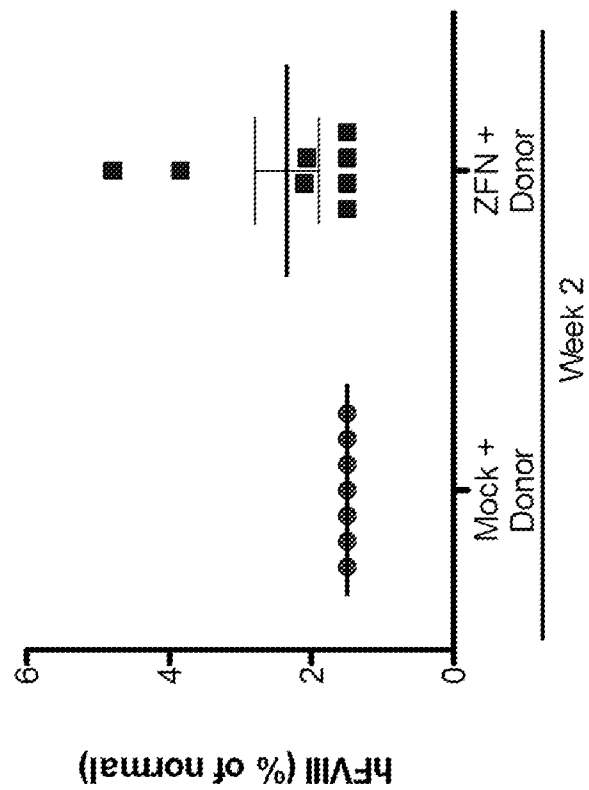


Figure 3A

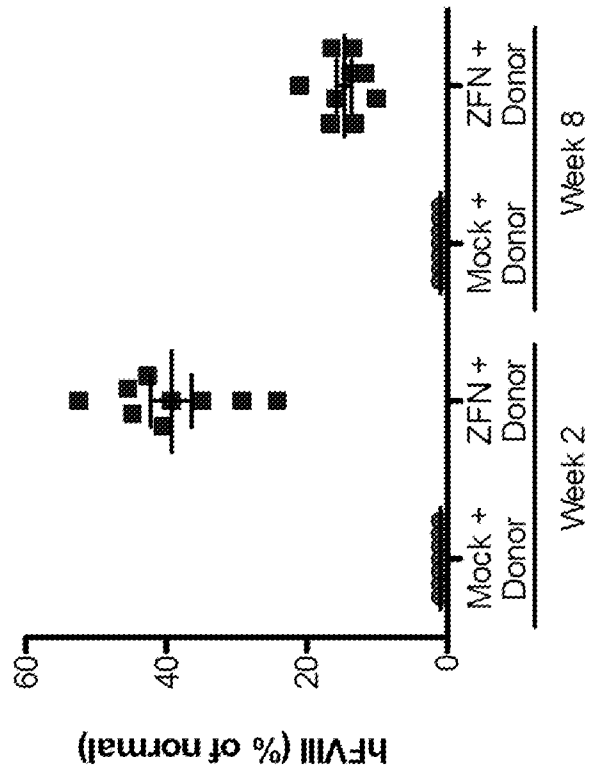


4890bp



AAV8-ZFN ($1e^{11}$ vg/mouse) +
AAV8-Donor ($1e^{11}$ vg/mouse)

Figure 3B



AAV8-ZFN (5e¹¹vg/mouse) +
AAV8-Donor (5e¹¹vg/mouse)

Figure 3C

Figure 3D

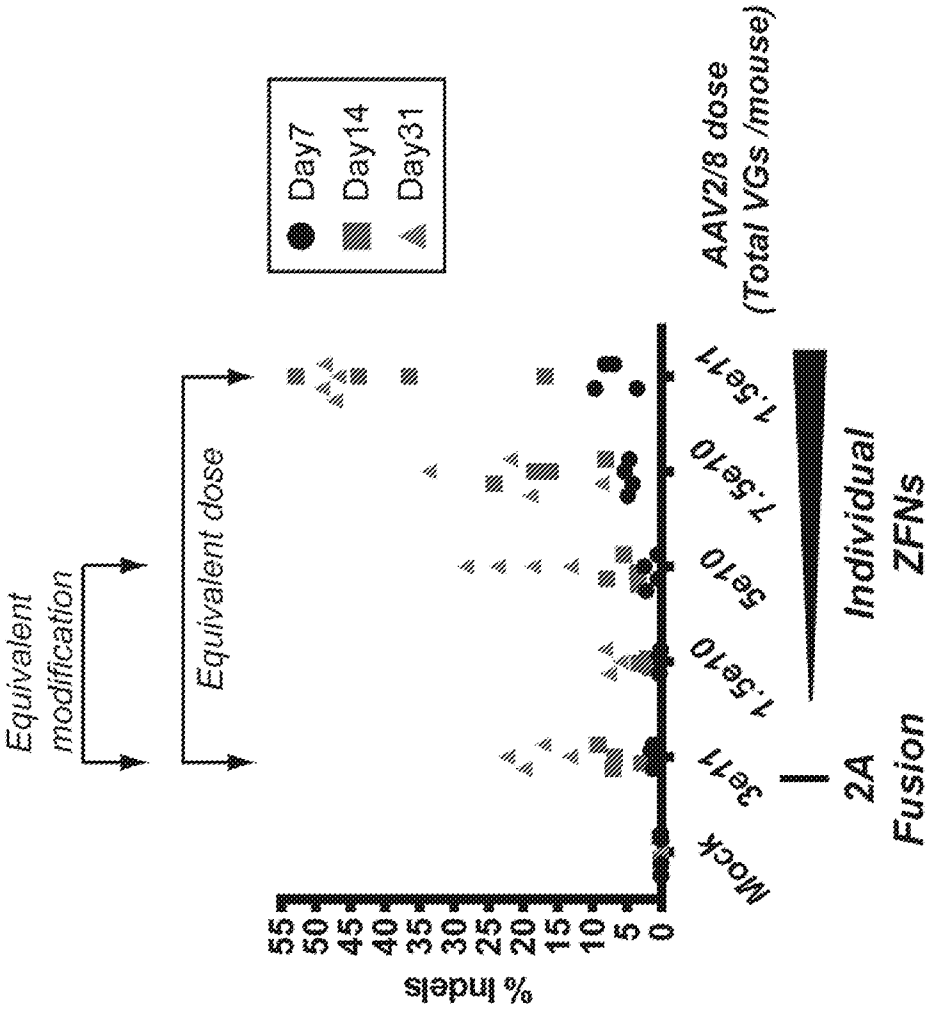


Figure 4

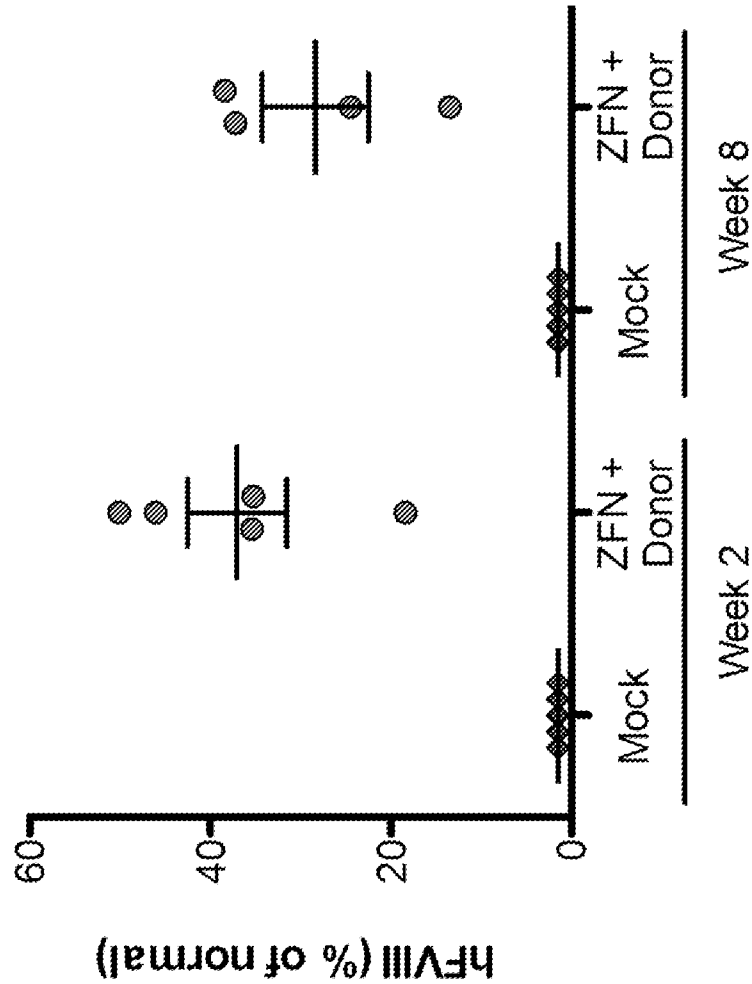


Figure 5A

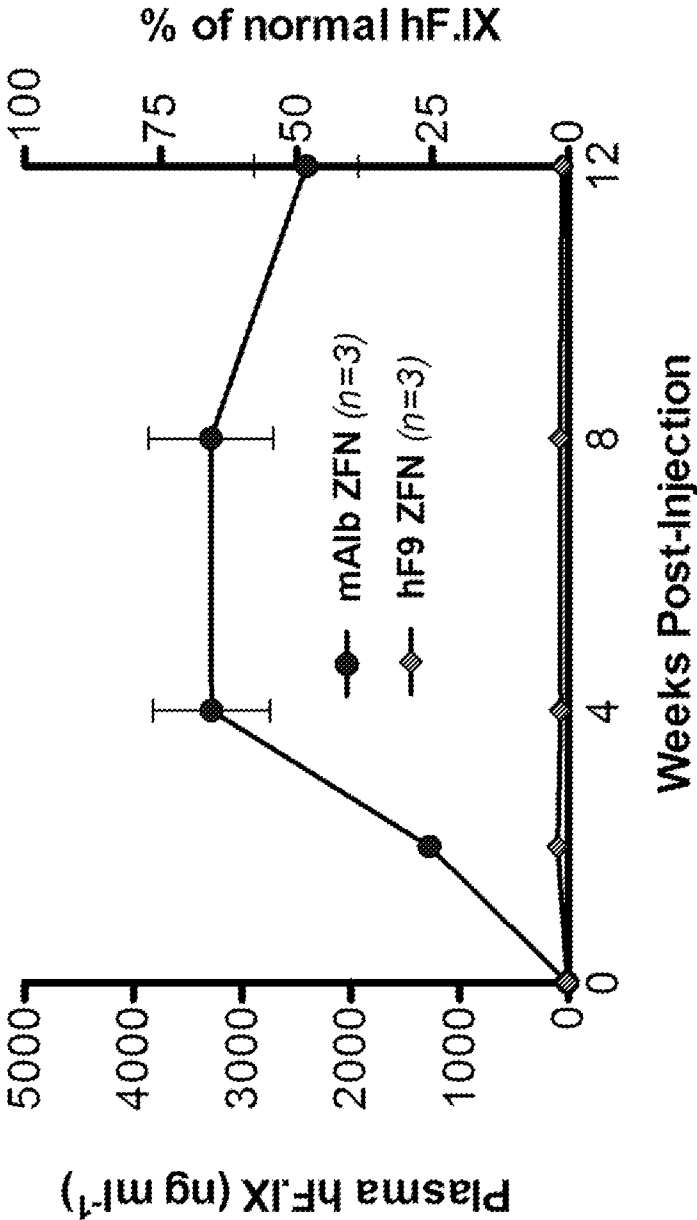
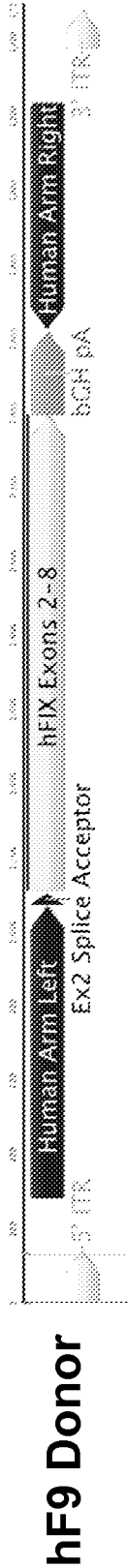
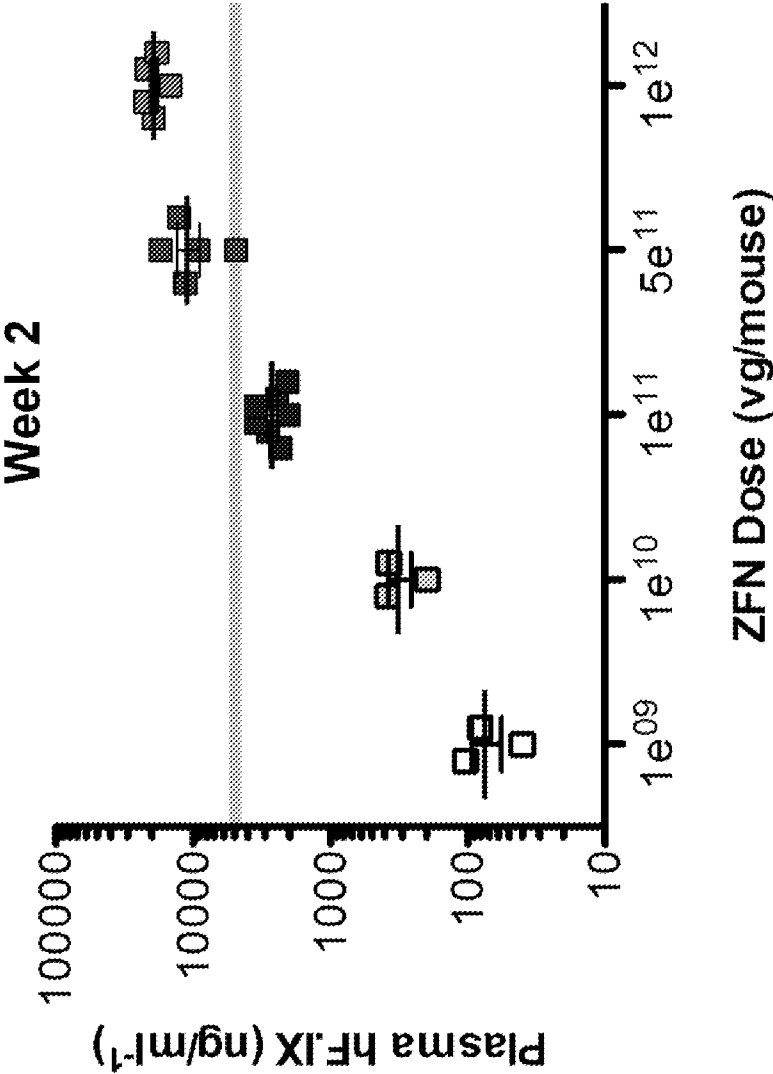
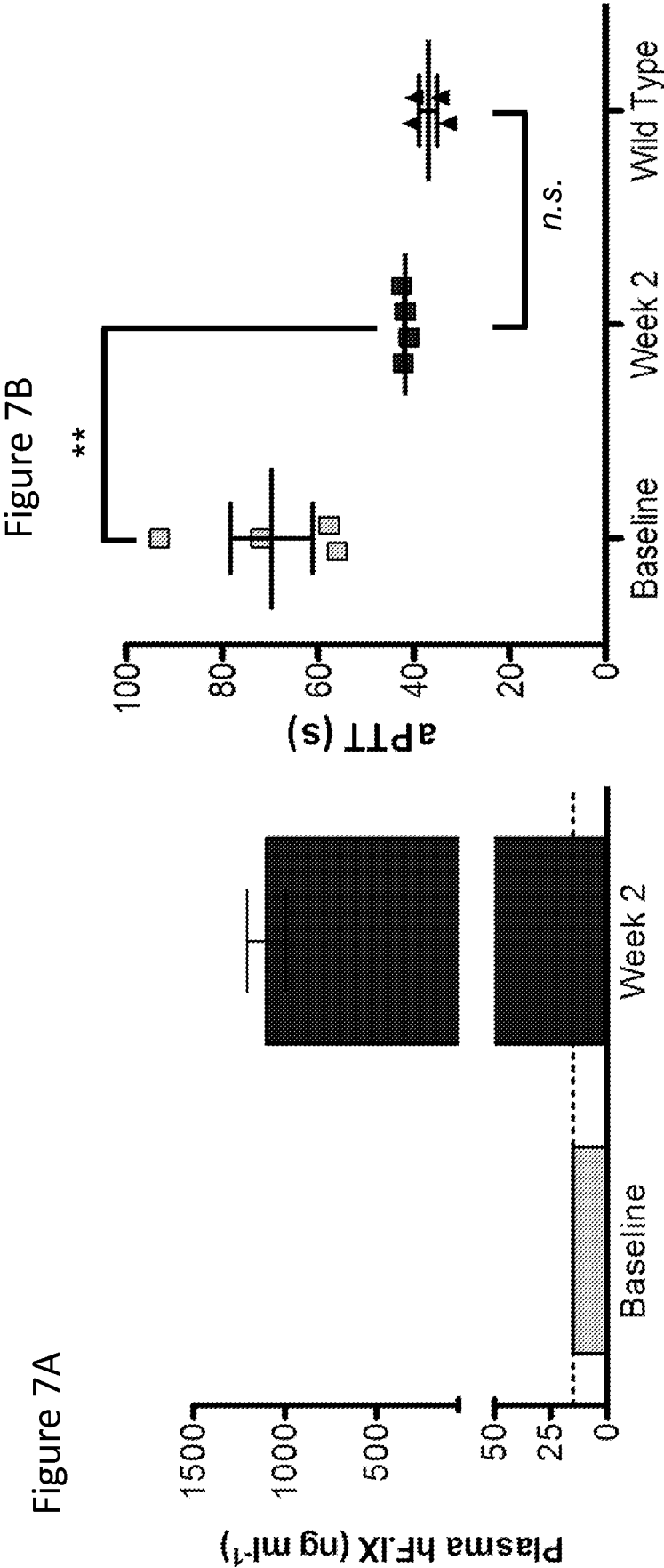


Figure 5B

FIGURE 6





mAlb ZFN + Donor: AAV8-ZFN (1x10¹¹ vg/mouse) + AAV8-Donor (5x10¹¹ vg/mouse)

Figure 8A

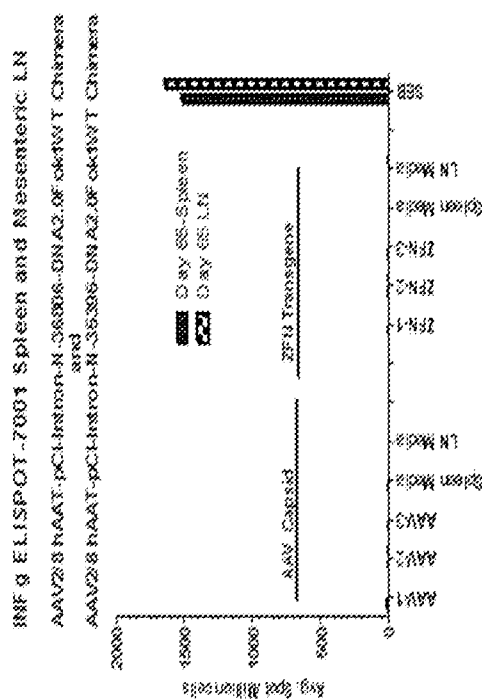


Figure 8C

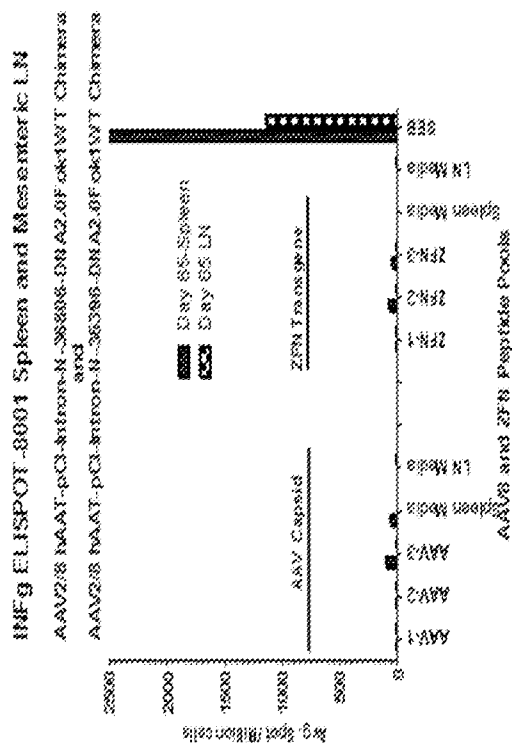


Figure 8B

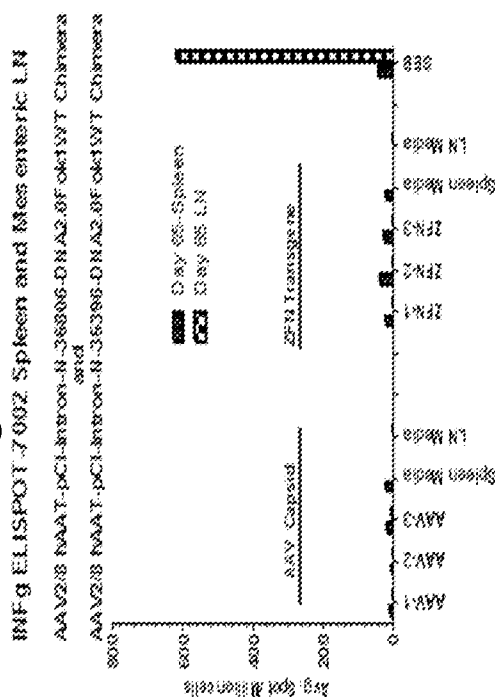


Figure 8D

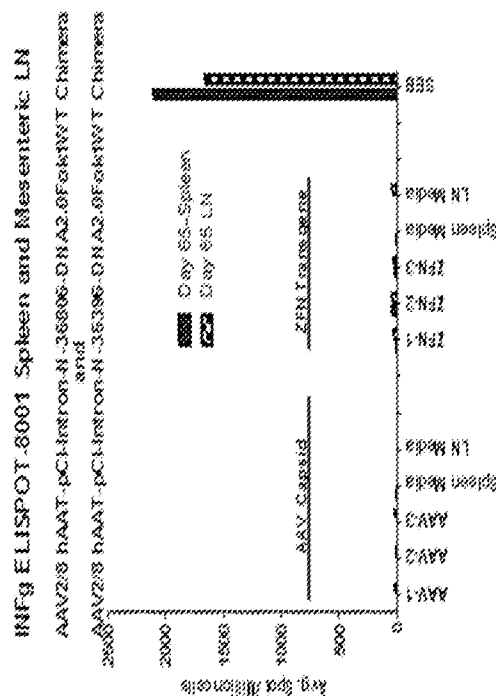
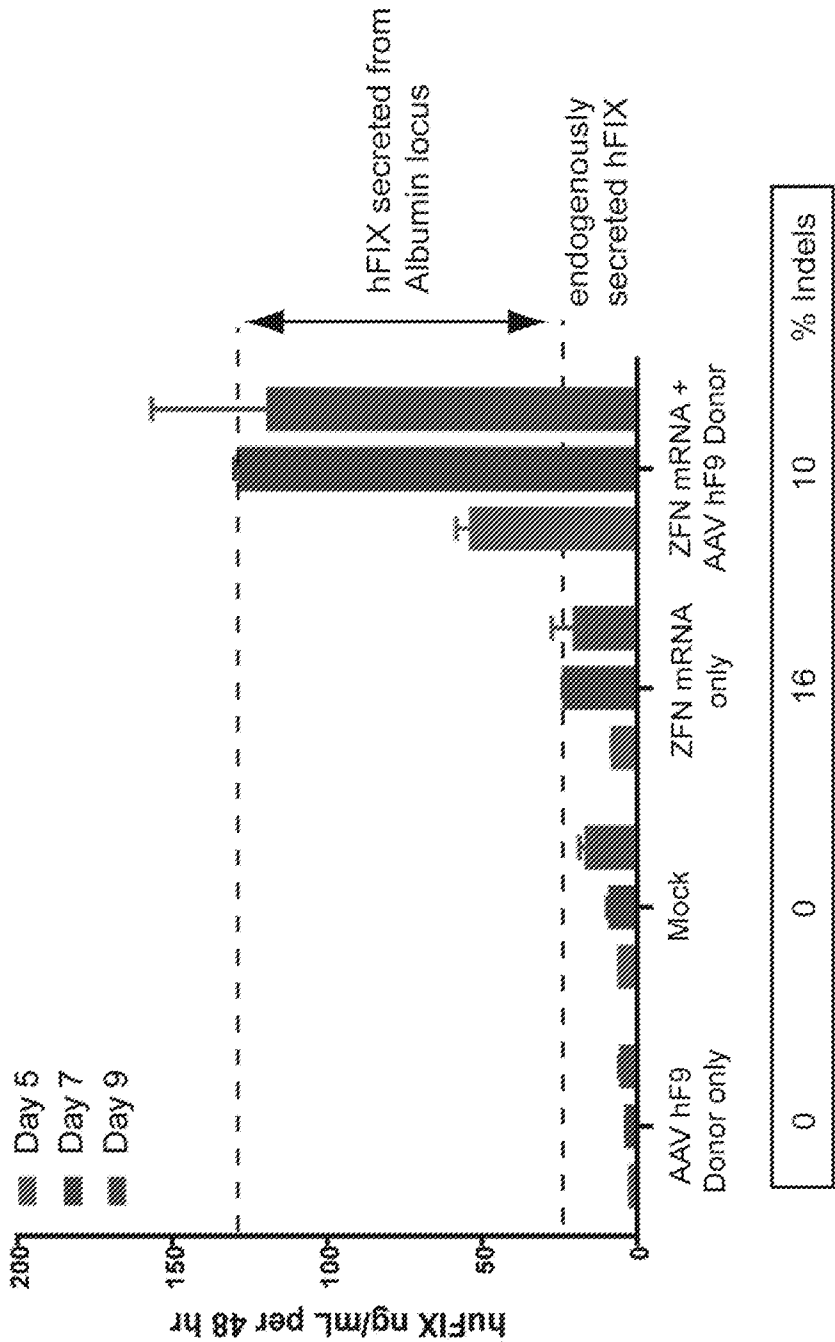


FIGURE 9
Human primary Hepatocytes



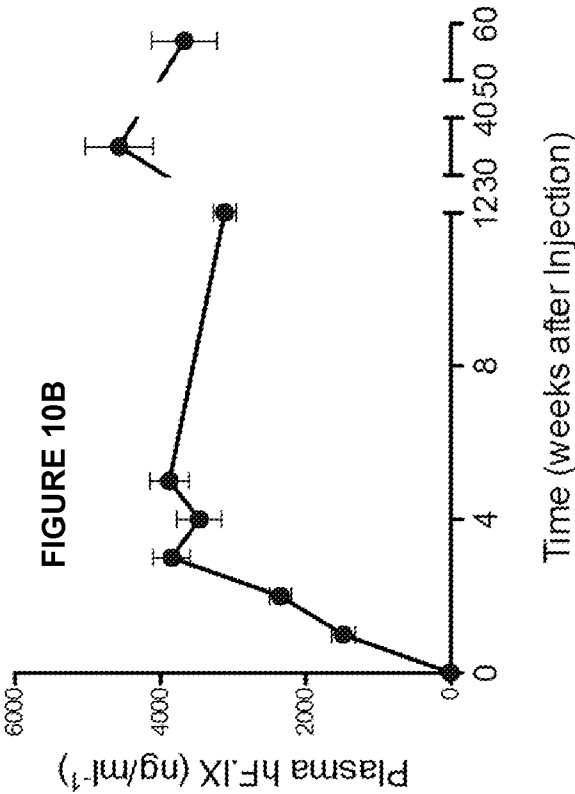
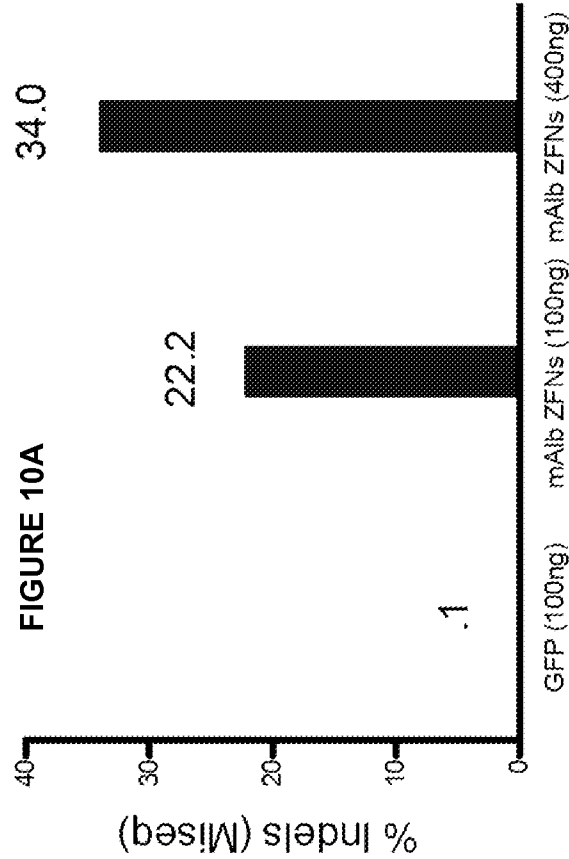


FIGURE 10C

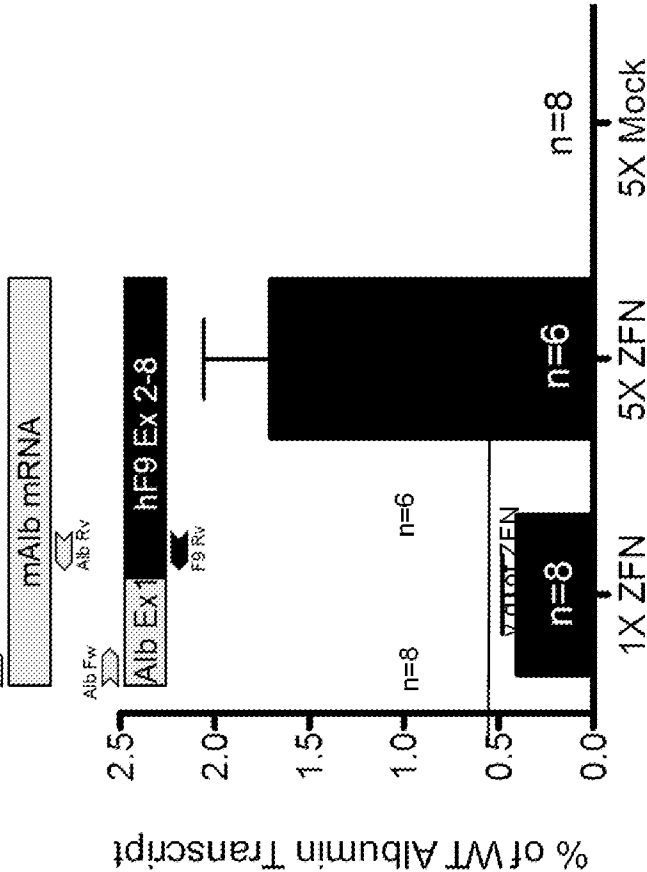
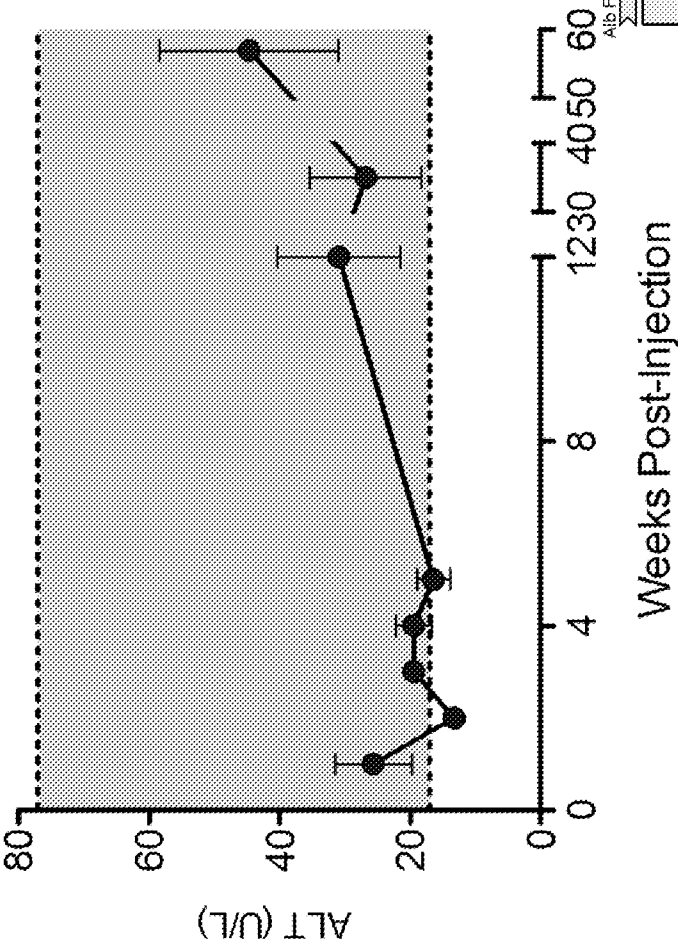
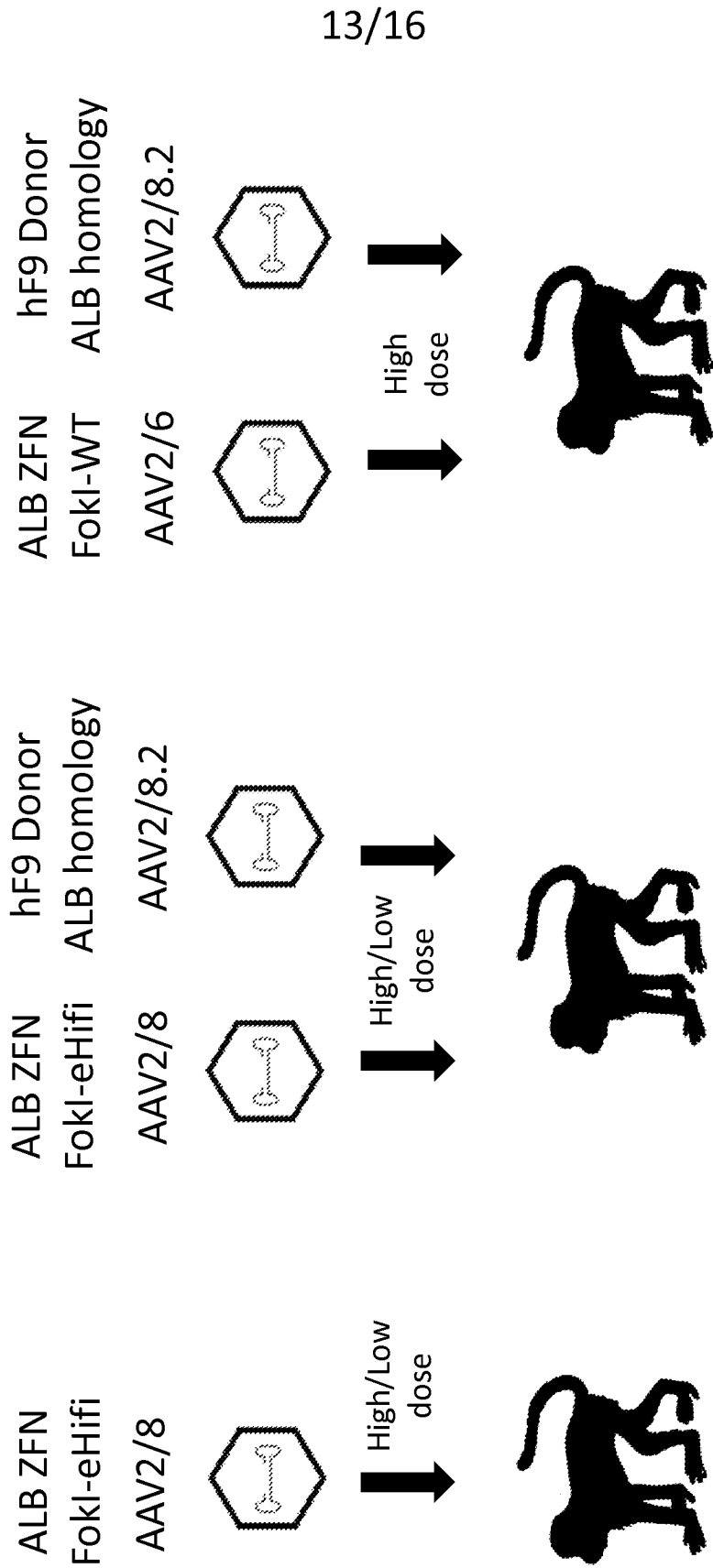


FIGURE 10D

Figure 11



Analysis:

- Liver biopsies for Vector genomes and ZFN cutting (%Indels by miSEQ)
- Plasma collection for hFIX ELISA
- Liver enzyme levels (ALT-AST)
- Liver Histopathology
- ELISPOT from Liver, Spleen and PBMCs

14/16

Figure 12A

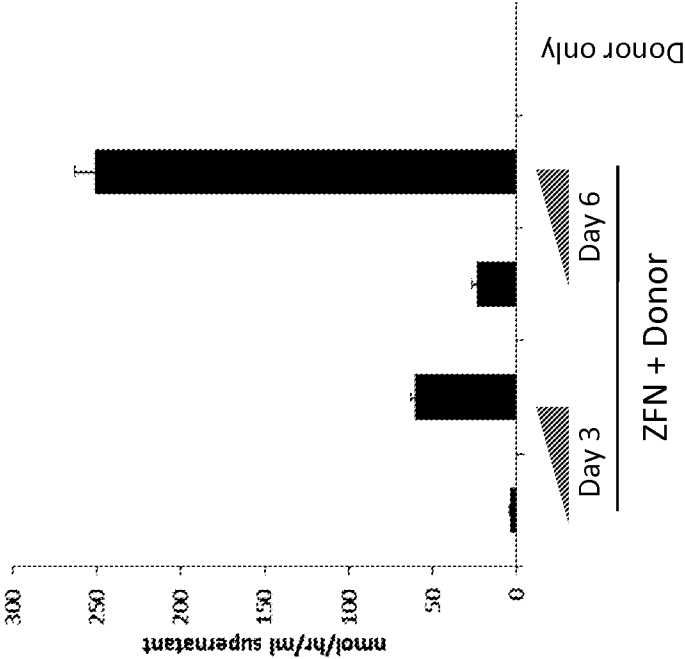
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 PFACDICGRKFAQSATRTKHTKIHTHPRAPIPKPFQCRICMRNFSTSGHL
SRIRTHTEKPFACDICGRKFAQSGNLARHTKIHRLRGSQLVKSELEEK
 SELRHKLKYPHEYIELIEIARNSTQDRILEMKVMEFFMKVGYRGKHLG
 GSRKPDGAIYTVGSPIDYGVIVDTKAYSGGYNLPIGQADEMERYVEENQT
 RDKHLNPNEWKVYPSSVTEFKFLVSGHFKGNYKAQLTRLNHNITNCNGA
 VLSVEELLIGGEMIKAGTLTLEEVRKFNNGEINF

MDYKDHDGDYKDHDIDYKDDDDKMAPKKKRVGIHGVPAAMAERPFCRI
 CMRNFSRSDHLSAHIRTHTEKPFACDICGRKFATKSNRTKHTKIHTGSQ
 KPFQCRICMRNFSDRSNLSRHIRTHTEKPFACDICGRKFAWRSSLRAHT
 KIHTGEKPFQCRICMRKFADSSDRKKHTKIHRLRGSQLVKSELEEKSEL
 HKLKYPHEYIELIEIARNSTQDRILEMKVMEFFMKVGYRGKHLGSRK
 PDGAIYTVGSPIDYGVIVDTKAYSGGYNLPIGQADEMQRYVKENQTRNKH
 INPNEWKVYPSSVTEFKFLVSGHFKGNYKAQLTRLNRKTNCGAVLSV
 EELLIGGEMIKAGTLTLEEVRKFNNGEINF

Figure 12B

Figure 13A

Enzymatic activity
(supernatant)



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Figure 13B

Expression
(western blot, cell pellet)

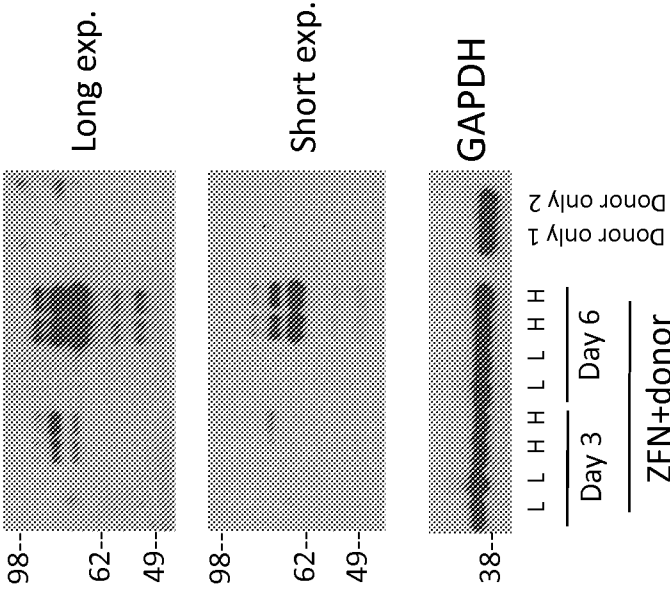


Figure 13C

Enzymatic activity
(supernatant)

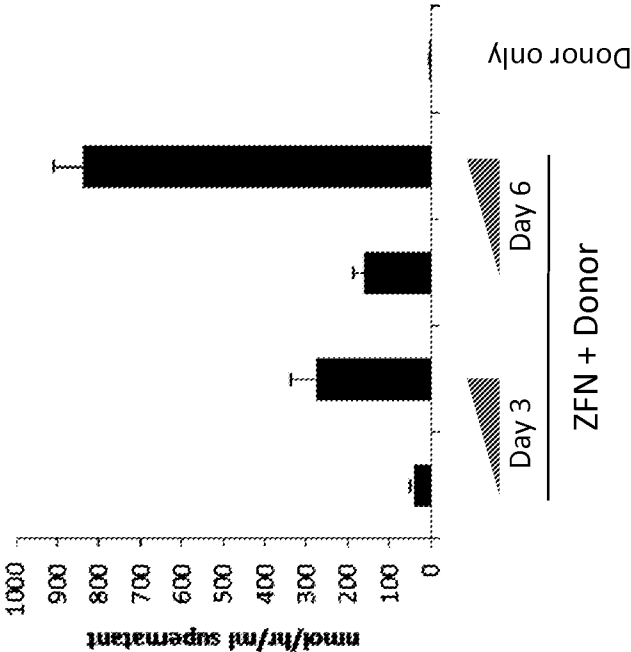
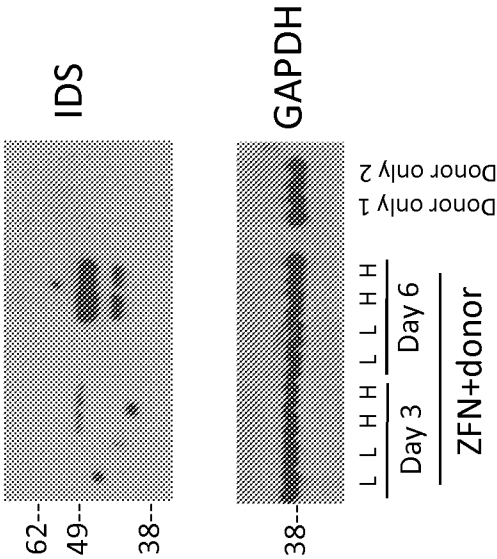


Figure 13D

Expression
(western blot, cell pellet)



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