[0004] FVIII is initially expressed as a protein having the domain structure A1-A2-B-A3-C1-C2. The protein is activated by proteolytic cleavage of the bulky, heavily glycosylated B domain, leaving a heavy chain (A1-A2) and light chain (A3-C1-C2) heterodimer. The B domain of the FVIII protein is not required for biological activity. Removal of the large B domain from the FVIII coding sequence is essential to enable reliable packaging into AAV vectors used for *in vivo* delivery. However, removal of the B domain, which contains up to 18 N-linked glycosylation sites, results in impaired secretion of FVIII protein. Thus, there is a critical need for improved forms of FVIII which can be efficiently and effectively expressed.

SUMMARY

[0005] Applicants have discovered compositions and methods of gene editing that can be used to supplement a defective F8 gene, resulting in expression of a functional FVIII protein. Accordingly, disclosures provided herein include systems and compositions for altering a host cell DNA sequence, methods for altering a host cell genome, methods and systems for inserting a synthetic Factor VIII coding sequence that provides for improved expression, a cell having a synthetic Factor VIII coding sequence that provides for improved expression that can be administered to a subject, methods for treating hemophilia A, and kits that embody any of the foregoing.

[0006] In one aspect, the present disclosure provides a system for altering a host cell DNA sequence, comprising: a deoxyribonucleic acid (DNA) endonuclease or a nucleic acid encoding the DNA endonuclease; a guide RNA (gRNA) comprising a spacer sequence complementary to a genetic locus of a host cell or a nucleic acid encoding the gRNA; optionally wherein the genetic locus is in a gene expressed in liver; and a donor template comprising a nucleic acid sequence encoding a synthetic FVIII protein, wherein the synthetic FVIII protein comprises a B domain substitute, wherein the B domain substitute comprises from zero to nine N-linked glycosylation sites and from three to about 40 amino acids in length; optionally wherein the B domain substitute comprises from zero to six N-linked glycosylation sites, or wherein the B domain substitute comprises from zero to three N-linked glycosylation sites. In one example, provided herein is a system for altering a host cell DNA sequence, having: a DNA endonuclease or a nucleic acid encoding the DNA endonuclease; a guide RNA (gRNA) having a spacer sequence complementary to a host cell locus or a nucleic acid encoding the gRNA; and a donor template having a nucleic acid sequence encoding a synthetic FVIII protein, where the synthetic FVIII protein comprises a B domain substitute,

where the B domain substitute has from zero to nine N-linked glycosylation sites and from three to about 40 amino acids in length.

[0007] In another aspect, the present disclosure provides an *in vitro* method of editing a genome in a host cell, the method comprising providing to the cell a system as described herein. In another example, provided is a method of editing a genome in a host cell, which includes providing to the cell: a gRNA having a spacer sequence complementary to a host cell locus or a nucleic acid encoding the gRNA; a DNA endonuclease or a nucleic acid encoding the DNA endonuclease; and a donor template having a nucleic acid sequence encoding a synthetic FVIII protein, where the synthetic FVIII protein has a B domain substitute, the B domain substitute having from zero to nine N-linked glycosylation sites and from three to about 40 amino acids in length.

[0008] In yet another aspect, the present disclosure provides a cell, wherein the genome of the cell comprises DNA encoding a synthetic FVIII protein, wherein the synthetic FVIII protein comprises a B domain substitute, wherein the B domain substitute comprises from zero to nine N-linked glycosylation sites and from three to about 40 amino acids in length; optionally wherein the synthetic FVIII protein is operably linked to an endogenous albumin promoter, an endogenous transferrin promoter, or an endogenous fibrinogen alpha promoter. In another example, provided is a cell, where the genome of the cell includes DNA encoding a synthetic FVIII protein, the synthetic FVIII protein having a B domain substitute, where the B domain substitute has from zero to nine N-linked glycosylation sites and from three to about 40 amino acids in length.

[0009] In another example, provided is a method of treating hemophilia A in a subject, by administering a cell having DNA encoding a synthetic FVIII protein as described above to the subject.

[0010] In another example, provided is a method of treating hemophilia A in a subject, by providing the following to a cell in the subject: a gRNA having a spacer sequence complementary to a host cell locus or a nucleic acid encoding the gRNA; a DNA endonuclease or a nucleic acid encoding the DNA endonuclease; and a donor template having a nucleic acid sequence encoding a synthetic FVIII protein, where the synthetic FVIII protein has a B domain substitute, the B domain substitute having from zero to nine N-linked glycosylation sites and from three to about 40 amino acids in length.

[0011] In another aspect, provided herein is a kit comprising one or more elements of a system described above, and further comprising instructions for use.

[0012] In yet another aspect, the present disclosure provides a nucleic acid comprising a polynucleotide sequence encoding a synthetic FVIII protein, wherein the synthetic FVIII protein comprises a B domain substitute, wherein the B domain substitute comprises from zero to nine N-linked glycosylation sites and from three to about 40 amino acids in length; optionally wherein the B domain substitute comprises from zero to six N-linked glycosylation sites; or wherein the B domain substitute comprises from zero to three N-linked glycosylation sites. In another example, provided herein is a nucleic acid having a polynucleotide sequence encoding a synthetic FVIII protein, where the synthetic FVIII protein has a B domain substitute, the B domain substitute having from zero to nine N-linked glycosylation sites and from three to about 40 amino acids in length.

[0013] In another example, provided herein is a method of increasing the amount of FVIII in a subject, by providing the following to a cell in the subject, where the subject has a first serum level of FVIII: a gRNA having a spacer sequence complementary to a host cell locus or a nucleic acid encoding the gRNA; a DNA endonuclease or a nucleic acid encoding the DNA endonuclease; and a donor template having a nucleic acid sequence encoding a synthetic FVIII protein, where the synthetic FVIII protein has a B domain substitute, the B domain substitute having from zero to nine N-linked glycosylation sites and from three to about 40 amino acids in length.

[0013a] In a further aspect, the present disclosure provides a system as described herein for use in treating hemophilia A. In a further aspect, the present disclosure provides use of the system as described herein in the manufacture of a medicament for the treatment of hemophilia A. In a further aspect, the present disclosure provides a cell as described herein for use in treating hemophilia A in a subject. In a further aspect, the present disclosure provides use of the cell as described herein in the manufacture of a medicament for the treatment of hemophilia A. In a further aspect, the present disclosure provides a nucleic acid as described herein for use in treating hemophilia A. In a further aspect, the present disclosure provides use of the nucleic acid as described herein in the manufacture of a medicament for the treatment of hemophilia A.

[0013b] In still another aspect, the present disclosure provides a synthetic FVIII protein, wherein the synthetic FVIII protein comprises a B domain substitute, wherein the B domain substitute comprises from zero to nine N-linked glycosylation sites and from three to about 40 amino acids in length.

BRIEF DESCRIPTION OF THE DRAWINGS

[0014] An understanding of certain features and advantages of the present disclosure will be obtained by reference to the following detailed description that sets forth illustrative embodiments, in which the principles of the disclosure are utilized, and the accompanying drawings of which:

WHAT IS CLAIMED IS:

1. A system for altering a host cell DNA sequence, comprising:

a deoxyribonucleic acid (DNA) endonuclease or a nucleic acid encoding the DNA endonuclease;

a guide RNA (gRNA) comprising a spacer sequence complementary to a genetic locus of a host cell or a nucleic acid encoding the gRNA; optionally wherein the genetic locus is in a gene expressed in liver; and

a donor template comprising a nucleic acid sequence encoding a synthetic FVIII protein, wherein the synthetic FVIII protein comprises a B domain substitute, wherein the B domain substitute comprises from zero to nine N-linked glycosylation sites and from three to about 40 amino acids in length; optionally

wherein the B domain substitute comprises from zero to six N-linked glycosylation sites, or

wherein the B domain substitute comprises from zero to three N-linked glycosylation sites.

2. The system of claim 1, wherein the B domain substitute comprises the amino acid sequence of any one of SEQ ID NOs: 362-369, 371, and 373, or a variant thereof having at least 80% identity to any one of SEQ ID NOs: 362-369, 371, and 373.

3. The system of claim 2, wherein the B domain substitute comprises the amino acid sequence of SEQ ID NO:364.

4. The system of any one of claims 1-3, wherein the genetic locus is in a gene encoding an acute phase protein, which optionally

is an albumin, a transferrin, or a fibrinogen; or wherein the genetic locus is in

a safe harbor locus.

5. The system of any one of claims 1-4, wherein the DNA endonuclease is a Cas9.

6. The system of any one of claims 1-5, wherein the nucleic acid encoding the DNA endonuclease is codon-optimized for expression in the host cell; optionally wherein

the nucleic acid encoding the DNA endonuclease is a deoxyribonucleic acid (DNA); or

wherein the nucleic acid encoding the DNA endonuclease is a ribonucleic acid (RNA), which preferably

is an mRNA.

7. The system of any one of claims 1-6, wherein the donor template nucleic acid sequence is codon optimized for expression in the host cell.

8. The system of any one of claims 1-7, wherein the donor template nucleic acid sequence comprises a reduced content of CpG di-nucleotides as compared to a wild type nucleic acid sequence encoding a FVIII protein; optionally

wherein the donor template nucleic acid sequence does not comprise CpG di-nucleotides.

9. The system of any one of claims 1-8, wherein the donor template is encoded in an Adeno Associated Virus (AAV) vector.

10. The system of any one of claims 1-9, wherein the donor template comprises a donor cassette comprising the nucleic acid sequence encoding a synthetic FVIII protein, and wherein the donor cassette is flanked on one or both sides by a gRNA target site; optionally

wherein the donor cassette is flanked on both sides by a gRNA target site; or

wherein the donor cassette is flanked on its 5’ side by a gRNA target site; optionally

wherein the gRNA target site is a target site for a gRNA in the system; preferably

wherein the gRNA target site of the donor template is the reverse complement of a genomic gRNA target site for a gRNA in the system.

11. The system of any one of claims 1-10, wherein the DNA endonuclease or nucleic acid encoding the DNA endonuclease is contained in a liposome or lipid nanoparticle; optionally

wherein the liposome or lipid nanoparticle also comprises the gRNA; preferably wherein

the DNA endonuclease is complexed with the gRNA, thereby providing a Ribonucleoprotein (RNP) complex.

12. An *in vitro* method of editing a genome in a host cell, the method comprising providing to the cell a system set forth in any one of claims 1-11.

13. The *in vitro* method of claim 12, wherein the cell is a liver cell, which optionally is a human hepatocyte or human sinusoidal epithelial cell.

14. A cell, wherein the genome of the cell comprises DNA encoding a synthetic FVIII protein, wherein the synthetic FVIII protein comprises a B domain substitute, wherein the B domain substitute comprises from zero to nine N-linked glycosylation sites and from three to about 40 amino acids in length; optionally

wherein the synthetic FVIII protein is operably linked to an endogenous albumin promoter, an endogenous transferrin promoter, or an endogenous fibrinogen alpha promoter.

15. The cell of claim 14, wherein the nucleic acid sequence encoding the synthetic FVIII protein is codon-optimized for expression in the cell.

16. The cell of claim 14 or claim 15, wherein the cell is a human liver cell, which optionally is a human hepatocyte or a human sinusoid epithelial cell.

17. The cell of any one of claims 14-16, wherein the cell is prepared by the method of claim 12 or claim 13, or the progeny of a cell prepared by the method of claim 12 or claim 13.

18. A kit comprising one or more elements of the system of any one of claims 1-11, further comprising instructions for use.

19. A nucleic acid comprising a polynucleotide sequence encoding a synthetic FVIII protein, wherein the synthetic FVIII protein comprises a B domain substitute,

wherein the B domain substitute comprises from zero to nine N-linked glycosylation sites and from three to about 40 amino acids in length; optionally wherein

the B domain substitute comprises from zero to six N-linked glycosylation sites; or

wherein the B domain substitute comprises from zero to three N-linked glycosylation sites.

20. The nucleic acid of claim 19, wherein the B domain substitute comprises the amino acid sequence of any one of SEQ ID NOs: 362-369, 371, and 373, or a variant thereof having at least 80% identity to any one of SEQ ID NOs: 362-369, 371, and 373.

21. The nucleic acid of claim 20, wherein the B domain substitute comprises the amino acid sequence of SEQ ID NO: 364.

22. The nucleic acid of any one of claims 19-21, wherein the polynucleotide sequence encoding a synthetic FVIII protein is codon optimized for expression in a host cell; or

wherein the polynucleotide sequence encoding a synthetic FVIII protein comprises a reduced content of CpG di-nucleotides as compared to a wild type nucleic acid sequence encoding FVIII; optionally

wherein the polynucleotide sequence encoding a synthetic FVIII protein does not comprise CpG di-nucleotides.

23. The nucleic acid of any one of claims 19-22, wherein the nucleic acid is a viral vector, which optionally is an AAV vector.

24. A system of any one of claims 1-11 for use in treating hemophilia A.

25. Use of the system of any one of claims 1-11 in the manufacture of a medicament for the treatment of hemophilia A.

26. A cell of any one of claims 14-17 for use in treating hemophilia A in a subject.

27. Use of the cell of any one of claims 14-17 in the manufacture of a medicament for the treatment of hemophilia A.

28. The cell of claim 26 or the use of claim 27, wherein the cell is autologous to the subject.

29. A nucleic acid of any one of claims 19-23 for use in treating hemophilia A.

30. Use of the nucleic acid of any one of claims 19-23 in the manufacture of a medicament for the treatment of hemophilia A.

31. A synthetic FVIII protein, wherein the synthetic FVIII protein comprises a B domain substitute, wherein the B domain substitute comprises from zero to nine N-linked glycosylation sites and from three to about 40 amino acids in length.