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(54) Title: NON-HUMAN ANIMALS COMPRISING A HUMANIZED *KLKB1* LOCUS AND METHODS OF USE

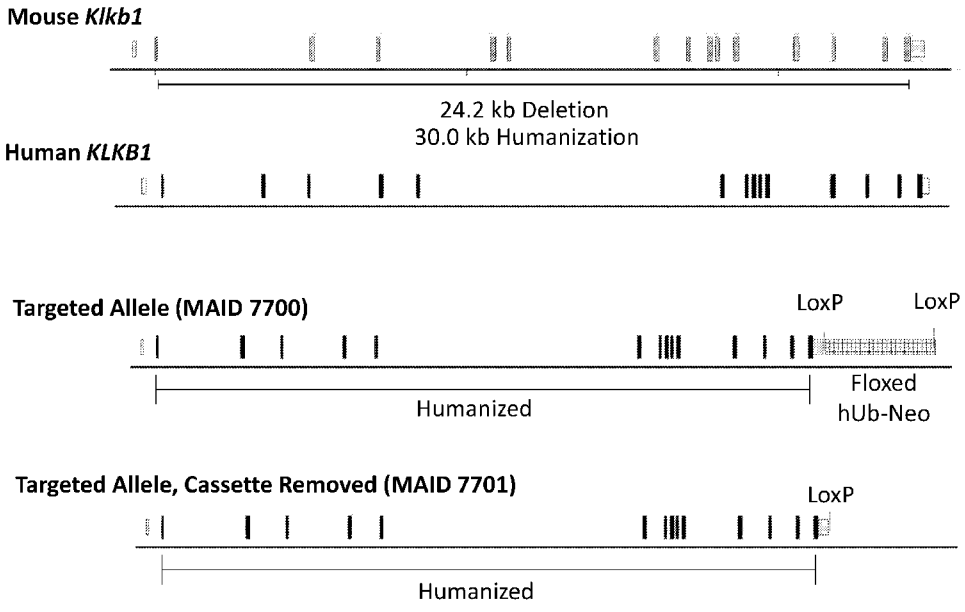

FIG. 1

(57) Abstract: Non-human animal genomes, non-human animal cells, and non-human animals comprising a humanized KLKB1 locus and methods of making and using such non-human animal genomes, non-human animal cells, and non-human animals are provided. Non-human animal cells or non-human animals comprising a humanized KLKB1 locus express a human plasma kallikrein protein or a chimeric plasma kallikrein protein, fragments of which are from human plasma kallikrein. Methods are provided for using such non-human animals comprising a humanized KLKB1 locus to assess in vivo efficacy of human-KLKB 1 -targeting reagents such as nuclease agents designed to target human KLKB1.

[Continued on next page]

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NON-HUMAN ANIMALS COMPRISING A HUMANIZED *KLKB1* LOCUS AND METHODS OF USE

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of US Application No. 62/971,826, filed February 7, 2020, and US Application No. 63/018,978, filed May 1, 2020, each of which is herein incorporated by reference in its entirety for all purposes.

REFERENCE TO A SEQUENCE LISTING SUBMITTED AS A TEXT FILE VIA EFS WEB

[0002] The Sequence Listing written in file 554181SEQLIST.txt is 195 kilobytes, was created on January 27, 2021, and is hereby incorporated by reference.

BACKGROUND

[0003] Hereditary angioedema (HAE) is a rare genetic disorder characterized by recurring and unpredictable severe swelling attacks in various parts of the body. Prekallikrein, encoded by *KLKB1*, is a protein that is produced in the liver and secreted into plasma, where it is converted into its active enzymatic form, plasma kallikrein. Inhibition of plasma kallikrein is one possible approach for treatment of HAE. However, there remains a need for suitable non-human animals providing the true human target or a close approximation of the true human target of human-KLKB1-targeting reagents at the endogenous *KLKB1* locus, thereby enabling testing of the efficacy and mode of action of such agents in live animals as well as pharmacokinetic and pharmacodynamics studies in a setting where the humanized protein and humanized gene are the only version of KLKB1 present.

SUMMARY

[0004] Non-human animals, non-human animal cells, and non-human animal genomes comprising a humanized *KLKB1* locus are provided, as well as methods of making and using such non-human animals, non-human animal cells, and non-human animal genomes. Also provided are humanized non-human animal *KLKB1* genes, nuclease agents and/or targeting vectors for use in humanizing a non-human animal *KLKB1* gene, and methods of making and

using such humanized *KLKB1* genes.

[0005] In one aspect, provided are non-human animals, non-human animal cells, and non-human animal genomes comprising a humanized *KLKB1* locus. In one aspect, provided are non-human animals, non-human animal cells, and non-human animal genomes comprising a humanized *KLKB1* locus, wherein a humanized plasma kallikrein protein is expressed from the humanized *KLKB1* locus. In some such non-human animals, non-human animal cells, and non-human animal genomes, the non-human animal, non-human animal cell, or non-human animal genome comprises in its genome a humanized endogenous *KLKB1* locus in which a segment of the endogenous *KLKB1* locus has been deleted and replaced with a corresponding human *KLKB1* sequence.

[0006] In some such non-human animals, non-human animal cells, and non-human animal genomes, the humanized endogenous *KLKB1* locus encodes a protein comprising a human plasma kallikrein heavy chain. Optionally, the human plasma kallikrein heavy chain comprises the sequence set forth in SEQ ID NO: 23. Optionally, the human plasma kallikrein heavy chain is encoded by a sequence comprising the sequence set forth in SEQ ID NO: 25.

[0007] In some such non-human animals, non-human animal cells, and non-human animal genomes, the humanized endogenous *KLKB1* locus encodes a protein comprising a human plasma kallikrein light chain. Optionally, the human plasma kallikrein light chain comprises the sequence set forth in SEQ ID NO: 24. Optionally, the human plasma kallikrein light chain is encoded by a sequence comprising the sequence set forth in SEQ ID NO: 26.

[0008] In some such non-human animals, non-human animal cells, and non-human animal genomes, the humanized endogenous *KLKB1* locus encodes a protein comprising a human plasma kallikrein signal peptide. Optionally, the human plasma kallikrein signal peptide comprises the sequence set forth in SEQ ID NO: 4. Optionally, the human plasma kallikrein signal peptide is encoded by a sequence comprising the sequence set forth in SEQ ID NO: 8.

[0009] In some such non-human animals, non-human animal cells, and non-human animal genomes, a region of the endogenous *KLKB1* locus comprising both coding sequence and non-coding sequence has been deleted and replaced with a corresponding human *KLKB1* sequence comprising both coding sequence and non-coding sequence. In some such non-human animals, non-human animal cells, and non-human animal genomes, the humanized endogenous *KLKB1* locus comprises an endogenous *KLKB1* promoter, wherein the human *KLKB1* sequence is

operably linked to the endogenous *KLKB1* promoter. In some such non-human animals, non-human animal cells, and non-human animal genomes, at least one intron and at least one exon of the endogenous *KLKB1* locus have been deleted and replaced with the corresponding human *KLKB1* sequence.

[0010] In some such non-human animals, non-human animal cells, and non-human animal genomes, the entire *KLKB1* coding sequence of the endogenous *KLKB1* locus has been deleted and replaced with the corresponding human *KLKB1* sequence. Optionally, a region of the endogenous *KLKB1* locus from the start codon to the stop codon has been deleted and replaced with the corresponding human *KLKB1* sequence.

[0011] In some such non-human animals, non-human animal cells, and non-human animal genomes, the endogenous *KLKB1* 3' untranslated region (3' UTR) has not been deleted and replaced with the corresponding human *KLKB1* sequence. In some such non-human animals, non-human animal cells, and non-human animal genomes, the endogenous *KLKB1* 5' untranslated region (5' UTR) has not been deleted and replaced with the corresponding human *KLKB1* sequence.

[0012] In some such non-human animals, non-human animal cells, and non-human animal genomes, the region of the endogenous *KLKB1* locus from the start codon to the stop codon has been deleted and replaced with the corresponding human *KLKB1* sequence, the endogenous *KLKB1* 3' untranslated region (3' UTR) has not been deleted and replaced with the corresponding human *KLKB1* sequence, the endogenous *KLKB1* 5' untranslated region (5' UTR) has not been deleted and replaced with the corresponding human *KLKB1* sequence, and the humanized endogenous *KLKB1* locus comprises an endogenous *KLKB1* promoter, wherein the human *KLKB1* sequence is operably linked to the endogenous *KLKB1* promoter.

[0013] In some such non-human animals, non-human animal cells, and non-human animal genomes, (i) the human *KLKB1* sequence at the humanized endogenous *KLKB1* locus comprises a sequence at least 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the sequence set forth in SEQ ID NO: 11; and/or (ii) the humanized endogenous *KLKB1* locus encodes a protein comprising a sequence at least 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the sequence set forth in SEQ ID NO: 3; and/or (iii) the humanized endogenous *KLKB1* locus comprises a coding sequence comprising a sequence at least 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the sequence set forth in SEQ ID NO: 7; and/or (iv) the humanized

endogenous *KLKB1* locus comprises a sequence at least 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the sequence set forth in SEQ ID NO: 9 or 10.

[0014] In some such non-human animals, non-human animal cells, and non-human animal genomes, the humanized endogenous *KLKB1* locus does not comprise a selection cassette or a reporter gene. In some such non-human animals, non-human animal cells, and non-human animal genomes, the non-human animal is homozygous for the humanized endogenous *KLKB1* locus. In some such non-human animals, non-human animal cells, and non-human animal genomes, the non-human animal comprises the humanized endogenous *KLKB1* locus in its germline.

[0015] In some such non-human animals, non-human animal cells, and non-human animal genomes, the non-human animal is a mammal. Optionally, the non-human animal is a rat or mouse. Optionally, the non-human animal is a mouse.

[0016] In another aspect, provided are targeting vectors for generating a humanized endogenous *KLKB1* locus in which a segment of the endogenous *KLKB1* locus has been deleted and replaced with a corresponding human *KLKB1* sequence. Some such targeting vectors comprise an insert nucleic acid comprising the corresponding human *KLKB1* sequence flanked by a 5' homology arm targeting a 5' target sequence at the endogenous *KLKB1* locus and a 3' homology arm targeting a 3' target sequence at the endogenous *KLKB1* locus.

[0017] In another aspect, provided are humanized non-human animal *KLKB1* genes. Some such genes are genes in which a segment of the non-human animal *KLKB1* gene has been deleted and replaced with a corresponding human *KLKB1* sequence.

[0018] In another aspect, provided are methods of assessing the activity of a human-KLKB1-targeting reagent *in vivo*. Some such methods comprise: (a) administering the human-KLKB1-targeting reagent to any of the above non-human animals comprising a humanized endogenous *KLKB1* locus; and (b) assessing the activity of the human-KLKB1-targeting reagent in the non-human animal.

[0019] In some such methods, the administering comprises adeno-associated virus (AAV)-mediated delivery, lipid nanoparticle (LNP)-mediated delivery, hydrodynamic delivery (HDD), or injection.

[0020] In some such methods, step (b) comprises assessing the activity of the human-KLKB1-targeting reagent in the liver of the non-human animal. In some such methods, step (b)

comprises measuring expression of an *KLKB1* messenger RNA encoded by the humanized endogenous *KLKB1* locus. In some such methods, step (b) comprises measuring expression of a plasma kallikrein protein encoded by the humanized endogenous *KLKB1* locus. In some such methods, measuring expression of the plasma kallikrein protein comprises measuring serum levels of the plasma kallikrein protein in the non-human animal. In some such methods, measuring expression of the plasma kallikrein protein comprises measuring expression of the plasma kallikrein protein in the liver of the non-human animal.

[0021] In some such methods, the human-KLKB1-targeting reagent is a genome-editing agent, and step (b) comprises assessing modification of the humanized endogenous *KLKB1* locus. In some such methods, step (b) comprises measuring the frequency of insertions or deletions within the humanized endogenous *KLKB1* locus.

[0022] In some such methods, the human-KLKB1-targeting reagent comprises a nuclease agent designed to target a region of a human *KLKB1* gene. Optionally, the nuclease agent comprises a Cas protein and a guide RNA designed to target a guide RNA target sequence in the human *KLKB1* gene. Optionally, the Cas protein is a Cas9 protein.

[0023] In some such methods, the human-KLKB1-targeting reagent comprises an exogenous donor nucleic acid, wherein the exogenous donor nucleic acid is designed to target the human *KLKB1* gene. Optionally, wherein the exogenous donor nucleic acid is delivered via AAV. In some such methods, the human-KLKB1-targeting reagent is an RNAi agent or an antisense oligonucleotide. In some such methods, the human-KLKB1-targeting reagent is an antigen-binding protein. In some such methods, the human-KLKB1-targeting reagent is small molecule.

[0024] In some such methods, assessing the activity of the human-KLKB1-targeting reagent in the non-human animal comprises assessing plasma kallikrein activity. Optionally, assessing plasma kallikrein activity comprises measure captopril-induced vascular permeability *in vivo*. Optionally, assessing plasma kallikrein activity comprises measuring plasma kallikrein activity *in vitro* using a plasma kallikrein substrate linked to a chromogen.

[0025] In another aspect, provided are methods of optimizing the activity of a human-KLKB1-targeting reagent *in vivo*. Some such methods comprise: (I) performing any of the above methods of assessing the activity of a human-KLKB1-targeting reagent *in vivo* a first time in a first non-human animal comprising in its genome a humanized endogenous *KLKB1* locus; (II) changing a variable and performing the method of step (I) a second time with the changed

variable in a second non-human animal comprising in its genome a humanized endogenous *KLKB1* locus; and (III) comparing the activity of the human-KLKB1-targeting reagent in step (I) with the activity of the human-KLKB1-targeting reagent in step (II), and selecting the method resulting in the higher activity.

[0026] In some such methods, the changed variable in step (II) is the delivery method of introducing the human-KLKB1-targeting reagent into the non-human animal. In some such methods, the changed variable in step (II) is the route of administration of introducing the human-KLKB1-targeting reagent into the non-human animal. In some such methods, the changed variable in step (II) is the concentration or amount of the human-KLKB1-targeting reagent introduced into the non-human animal. In some such methods, the changed variable in step (II) is the form of the human-KLKB1-targeting reagent introduced into the non-human animal. In some such methods, the changed variable in step (II) is the human-KLKB1-targeting reagent introduced into the non-human animal.

[0027] In another aspect, provided are methods of making any of the above non-human animals comprising a humanized endogenous *KLKB1* locus.

[0028] Some such methods comprise: (a) introducing into a non-human animal host embryo a genetically modified non-human animal embryonic stem (ES) cell comprising in its genome a humanized endogenous *KLKB1* locus in which a segment of the endogenous *KLKB1* locus has been deleted and replaced with a corresponding human *KLKB1* sequence; and (b) gestating the non-human animal host embryo in a surrogate mother, wherein the surrogate mother produces an F0 progeny genetically modified non-human animal comprising the humanized endogenous *KLKB1* locus.

[0029] Some such methods comprise: (a) modifying the genome of a non-human animal one-cell stage embryo to comprise in its genome a humanized endogenous *KLKB1* locus in which a segment of the endogenous *KLKB1* locus has been deleted and replaced with a corresponding human *KLKB1* sequence, thereby generating a non-human animal genetically modified embryo; and (b) gestating the non-human animal genetically modified embryo in a surrogate mother, wherein the surrogate mother produces an F0 progeny genetically modified non-human animal comprising the humanized endogenous *KLKB1* locus.

[0030] Some such methods comprise: (a) introducing into a non-human animal embryonic stem (ES) cell a targeting vector comprising a nucleic acid insert comprising the human *KLKB1*

sequence flanked by a 5' homology arm corresponding to a 5' target sequence in the endogenous *KLKB1* locus and a 3' homology arm corresponding to a 3' target sequence in the endogenous *KLKB1* locus, wherein the targeting vector recombines with the endogenous *KLKB1* locus to produce a genetically modified non-human ES cell comprising in its genome the humanized endogenous *KLKB1* locus comprising the human *KLKB1* sequence; (b) introducing the genetically modified non-human ES cell into a non-human animal host embryo; and (c) gestating the non-human animal host embryo in a surrogate mother, wherein the surrogate mother produces an F0 progeny genetically modified non-human animal comprising in its genome the humanized endogenous *KLKB1* locus comprising the human *KLKB1* sequence. Optionally, the targeting vector is a large targeting vector at least 10 kb in length or in which the sum total of the 5' and 3' homology arms is at least 10 kb in length.

[0031] Some such methods comprise: (a) introducing into a non-human animal one-cell stage embryo a targeting vector comprising a nucleic acid insert comprising the human *KLKB1* sequence flanked by a 5' homology arm corresponding to a 5' target sequence in the endogenous *KLKB1* locus and a 3' homology arm corresponding to a 3' target sequence in the endogenous *KLKB1* locus, wherein the targeting vector recombines with the endogenous *KLKB1* locus to produce a genetically modified non-human one-cell stage embryo comprising in its genome the humanized endogenous *KLKB1* locus comprising the human *KLKB1* sequence; (b) gestating the genetically modified non-human animal one-cell stage embryo in a surrogate mother to produce a genetically modified F0 generation non-human animal comprising in its genome the humanized endogenous *KLKB1* locus comprising the human *KLKB1* sequence.

[0032] In some such methods, step (a) further comprises introducing a nuclease agent or a nucleic acid encoding the nuclease agent, wherein the nuclease agent targets a target sequence in the endogenous *KLKB1* locus. Optionally, the nuclease agent comprises a Cas protein and a guide RNA. Optionally, the Cas protein is a Cas9 protein. Optionally, step (a) further comprises introducing a second guide RNA or a DNA encoding the second guide RNA, wherein the second guide RNA targets a second target sequence within the endogenous *KLKB1* locus. Optionally, step (a) further comprises introducing a third guide RNA or a DNA encoding the third guide RNA, wherein the third guide RNA targets a third target sequence within the endogenous *KLKB1* locus, and a fourth guide RNA or a DNA encoding the fourth guide RNA, wherein the fourth guide RNA targets a fourth target sequence within the endogenous *KLKB1* locus.

[0033] In some such methods, the non-human animal is a rodent. Optionally, the rodent is a mouse or a rat. Optionally, the rodent is a mouse.

BRIEF DESCRIPTION OF THE FIGURES

[0034] **Figure 1** (not to scale) shows a schematic of the targeting scheme for humanization of the mouse *Klkb1* locus. The top portion of the figure shows the endogenous wild type mouse *Klkb1* locus and the endogenous human *KLKB1* locus, and the bottom portion of the figure shows the humanized *KLKB1* locus with or without the self-deleting selection cassette. Mouse 5' and 3' untranslated regions (UTRs) are designated by light gray boxes, mouse exons (coding sequence) are designated by dark gray boxes, human 5' and 3' UTRs are designated by white boxes, and human exons (coding sequence) are designated by black boxes. The self-deleting ubiquitin puromycin selection cassette is designated by the cross-hatched box.

[0035] **Figure 2** (not to scale) shows a schematic of the TAQMAN® assays for screening humanization of the mouse *Klkb1* locus. Gain-of-allele (GOA) assays include hTU and hTD. Loss-of-allele (LOA) assays include mTU and mTD.

[0036] **Figure 3** shows an alignment of the wild type mouse plasma kallikrein protein and the wild type human plasma kallikrein protein (mKLKB1 and hKLKB1, respectively). The signal peptide is indicated.

[0037] **Figure 4** shows percent editing at the humanized *KLKB1* locus following single administration of lipid nanoparticles comprising various guide RNAs targeting human *KLKB1* together with Cas9 mRNA to humanized *KLKB1* mice.

[0038] **Figure 5** shows plasma kallikrein levels (as measured by ELISA) in the serum following single administration of lipid nanoparticles comprising various guide RNAs targeting human *KLKB1* together with Cas9 mRNA to humanized *KLKB1* mice.

[0039] **Figure 6** shows plasma kallikrein levels (as measured by electrochemiluminescence-based array) in the serum following single administration of lipid nanoparticles comprising various guide RNAs targeting human *KLKB1* together with Cas9 mRNA to humanized *KLKB1* mice.

[0040] **Figure 7** shows the fold change of *KLKB1* mRNA levels following single administration of lipid nanoparticles comprising various guide RNAs targeting human *KLKB1* together with Cas9 mRNA to humanized *KLKB1* mice.

[0041] **Figures 8A-8D** show levels of *KLKB1* editing (**Figure 8A**), serum KLKB1 protein (prekallikrein and kallikrein) (**Figure 8B**), and serum KLKB1 protein (% of basal expression) (**Figure 8C**), and correlation of percent liver editing to percent KLKB1 protein (**Figure 8D**) following single administration of lipid nanoparticles comprising various guide RNAs targeting human *KLKB1* together with Cas9 mRNA to humanized *KLKB1* mice.

[0042] **Figure 9** shows dose-dependent levels of *KLKB1* gene editing and percent knockdown of *KLKB1* mRNA and plasma kallikrein in humanized *KLKB1* mice.

DEFINITIONS

[0043] The terms "protein," "polypeptide," and "peptide," used interchangeably herein, include polymeric forms of amino acids of any length, including coded and non-coded amino acids and chemically or biochemically modified or derivatized amino acids. The terms also include polymers that have been modified, such as polypeptides having modified peptide backbones. The term "domain" refers to any part of a protein or polypeptide having a particular function or structure.

[0044] Proteins are said to have an "N-terminus" and a "C-terminus." The term "N-terminus" relates to the start of a protein or polypeptide, terminated by an amino acid with a free amine group (-NH2). The term "C-terminus" relates to the end of an amino acid chain (protein or polypeptide), terminated by a free carboxyl group (-COOH).

[0045] The terms "nucleic acid" and "polynucleotide," used interchangeably herein, include polymeric forms of nucleotides of any length, including ribonucleotides, deoxyribonucleotides, or analogs or modified versions thereof. They include single-, double-, and multi-stranded DNA or RNA, genomic DNA, cDNA, DNA-RNA hybrids, and polymers comprising purine bases, pyrimidine bases, or other natural, chemically modified, biochemically modified, non-natural, or derivatized nucleotide bases.

[0046] Nucleic acids are said to have "5' ends" and "3' ends" because mononucleotides are reacted to make oligonucleotides in a manner such that the 5' phosphate of one mononucleotide pentose ring is attached to the 3' oxygen of its neighbor in one direction via a phosphodiester linkage. An end of an oligonucleotide is referred to as the "5' end" if its 5' phosphate is not linked to the 3' oxygen of a mononucleotide pentose ring. An end of an oligonucleotide is referred to as the "3' end" if its 3' oxygen is not linked to a 5' phosphate of another

mononucleotide pentose ring. A nucleic acid sequence, even if internal to a larger oligonucleotide, also may be said to have 5' and 3' ends. In either a linear or circular DNA molecule, discrete elements are referred to as being "upstream" or 5' of the "downstream" or 3' elements.

[0047] The term "genomically integrated" refers to a nucleic acid that has been introduced into a cell such that the nucleotide sequence integrates into the genome of the cell. Any protocol may be used for the stable incorporation of a nucleic acid into the genome of a cell.

[0048] The term "targeting vector" refers to a recombinant nucleic acid that can be introduced by homologous recombination, non-homologous-end-joining-mediated ligation, or any other means of recombination to a target position in the genome of a cell.

[0049] The term "viral vector" refers to a recombinant nucleic acid that includes at least one element of viral origin and includes elements sufficient for or permissive of packaging into a viral vector particle. The vector and/or particle can be utilized for the purpose of transferring DNA, RNA, or other nucleic acids into cells *in vitro*, *ex vivo*, or *in vivo*. Numerous forms of viral vectors are known.

[0050] The term "isolated" with respect to cells, tissues (e.g., liver samples), lipid droplets, proteins, and nucleic acids includes cells, tissues (e.g., liver samples), lipid droplets, proteins, and nucleic acids that are relatively purified with respect to other bacterial, viral, cellular, or other components that may normally be present *in situ*, up to and including a substantially pure preparation of the cells, tissues (e.g., liver samples), lipid droplets, proteins, and nucleic acids. The term "isolated" also includes cells, tissues (e.g., liver samples), lipid droplets, proteins, and nucleic acids that have no naturally occurring counterpart, have been chemically synthesized and are thus substantially uncontaminated by other cells, tissues (e.g., liver samples), lipid droplets, proteins, and nucleic acids, or has been separated or purified from most other components (e.g., cellular components) with which they are naturally accompanied (e.g., other cellular proteins, polynucleotides, or cellular components).

[0051] The term "wild type" includes entities having a structure and/or activity as found in a normal (as contrasted with mutant, diseased, altered, or so forth) state or context. Wild type genes and polypeptides often exist in multiple different forms (e.g., alleles).

[0052] The term "endogenous sequence" refers to a nucleic acid sequence that occurs naturally within a rat cell or rat. For example, an endogenous *Klkb3* sequence of a mouse refers to a native *Klkb3* sequence that naturally occurs at the *Klkb3* locus in the mouse.

[0053] "Exogenous" molecules or sequences include molecules or sequences that are not normally present in a cell in that form. Normal presence includes presence with respect to the particular developmental stage and environmental conditions of the cell. An exogenous molecule or sequence, for example, can include a mutated version of a corresponding endogenous sequence within the cell, such as a humanized version of the endogenous sequence, or can include a sequence corresponding to an endogenous sequence within the cell but in a different form (i.e., not within a chromosome). In contrast, endogenous molecules or sequences include molecules or sequences that are normally present in that form in a particular cell at a particular developmental stage under particular environmental conditions.

[0054] The term "heterologous" when used in the context of a nucleic acid or a protein indicates that the nucleic acid or protein comprises at least two segments that do not naturally occur together in the same molecule. For example, the term "heterologous," when used with reference to segments of a nucleic acid or segments of a protein, indicates that the nucleic acid or protein comprises two or more sub-sequences that are not found in the same relationship to each other (e.g., joined together) in nature. As one example, a "heterologous" region of a nucleic acid vector is a segment of nucleic acid within or attached to another nucleic acid molecule that is not found in association with the other molecule in nature. For example, a heterologous region of a nucleic acid vector could include a coding sequence flanked by sequences not found in association with the coding sequence in nature. Likewise, a "heterologous" region of a protein is a segment of amino acids within or attached to another peptide molecule that is not found in association with the other peptide molecule in nature (e.g., a fusion protein, or a protein with a tag). Similarly, a nucleic acid or protein can comprise a heterologous label or a heterologous secretion or localization sequence.

[0055] "Codon optimization" takes advantage of the degeneracy of codons, as exhibited by the multiplicity of three-base pair codon combinations that specify an amino acid, and generally includes a process of modifying a nucleic acid sequence for enhanced expression in particular host cells by replacing at least one codon of the native sequence with a codon that is more frequently or most frequently used in the genes of the host cell while maintaining the native

amino acid sequence. For example, a nucleic acid encoding a plasma kallikrein protein can be modified to substitute codons having a higher frequency of usage in a given prokaryotic or eukaryotic cell, including a bacterial cell, a yeast cell, a human cell, a non-human cell, a mammalian cell, a rodent cell, a mouse cell, a rat cell, a hamster cell, or any other host cell, as compared to the naturally occurring nucleic acid sequence. Codon usage tables are readily available, for example, at the "Codon Usage Database." These tables can be adapted in a number of ways. *See* Nakamura et al. (2000) *Nucleic Acids Research* 28:292, herein incorporated by reference in its entirety for all purposes. Computer algorithms for codon optimization of a particular sequence for expression in a particular host are also available (*see, e.g.*, Gene Forge).

[0056] The term "locus" refers to a specific location of a gene (or significant sequence), DNA sequence, polypeptide-encoding sequence, or position on a chromosome of the genome of an organism. For example, a "*Klkb3* locus" may refer to the specific location of a *Klkb3* gene, *Klkb3* DNA sequence, plasma-kallikrein-encoding sequence, or *Klkb3* position on a chromosome of the genome of an organism that has been identified as to where such a sequence resides. A "*Klkb3* locus" may comprise a regulatory element of a *Klkb3* gene, including, for example, an enhancer, a promoter, 5' and/or 3' untranslated region (UTR), or a combination thereof.

[0057] The term "gene" refers to DNA sequences in a chromosome that may contain, if naturally present, at least one coding and at least one non-coding region. The DNA sequence in a chromosome that codes for a product (e.g., but not limited to, an RNA product and/or a polypeptide product) can include the coding region interrupted with non-coding introns and sequence located adjacent to the coding region on both the 5' and 3' ends such that the gene corresponds to the full-length mRNA (including the 5' and 3' untranslated sequences). Additionally, other non-coding sequences including regulatory sequences (e.g., but not limited to, promoters, enhancers, and transcription factor binding sites), polyadenylation signals, internal ribosome entry sites, silencers, insulating sequence, and matrix attachment regions may be present in a gene. These sequences may be close to the coding region of the gene (e.g., but not limited to, within 10 kb) or at distant sites, and they influence the level or rate of transcription and translation of the gene.

[0058] The term "allele" refers to a variant form of a gene. Some genes have a variety of different forms, which are located at the same position, or genetic locus, on a chromosome. A diploid organism has two alleles at each genetic locus. Each pair of alleles represents the

genotype of a specific genetic locus. Genotypes are described as homozygous if there are two identical alleles at a particular locus and as heterozygous if the two alleles differ.

[0059] A "promoter" is a regulatory region of DNA usually comprising a TATA box capable of directing RNA polymerase II to initiate RNA synthesis at the appropriate transcription initiation site for a particular polynucleotide sequence. A promoter may additionally comprise other regions which influence the transcription initiation rate. The promoter sequences disclosed herein modulate transcription of an operably linked polynucleotide. A promoter can be active in one or more of the cell types disclosed herein (e.g., a mouse cell, a rat cell, a pluripotent cell, a one-cell stage embryo, a differentiated cell, or a combination thereof). A promoter can be, for example, a constitutively active promoter, a conditional promoter, an inducible promoter, a temporally restricted promoter (e.g., a developmentally regulated promoter), or a spatially restricted promoter (e.g., a cell-specific or tissue-specific promoter). Examples of promoters can be found, for example, in WO 2013/176772, herein incorporated by reference in its entirety for all purposes.

[0060] "Operable linkage" or being "operably linked" includes juxtaposition of two or more components (e.g., a promoter and another sequence element) 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. For example, a promoter can be operably linked to a coding sequence if the promoter controls the level of transcription of the coding sequence in response to the presence or absence of one or more transcriptional regulatory factors. Operable linkage can include such sequences being contiguous with each other or acting in trans (e.g., a regulatory sequence can act at a distance to control transcription of the coding sequence).

[0061] The methods and compositions provided herein employ a variety of different components. Some components throughout the description can have active variants and fragments. The term "functional" refers to the innate ability of a protein or nucleic acid (or a fragment or variant thereof) to exhibit a biological activity or function. The biological functions of functional fragments or variants may be the same or may in fact be changed (e.g., with respect to their specificity or selectivity or efficacy) in comparison to the original molecule, but with retention of the molecule's basic biological function.

[0062] The term "variant" refers to a nucleotide sequence differing from the sequence most prevalent in a population (e.g., by one nucleotide) or a protein sequence different from the sequence most prevalent in a population (e.g., by one amino acid).

[0063] The term "fragment," when referring to a protein, means a protein that is shorter or has fewer amino acids than the full-length protein. The term "fragment," when referring to a nucleic acid, means a nucleic acid that is shorter or has fewer nucleotides than the full-length nucleic acid. A fragment can be, for example, when referring to a protein fragment, an N-terminal fragment (i.e., removal of a portion of the C-terminal end of the protein), a C-terminal fragment (i.e., removal of a portion of the N-terminal end of the protein), or an internal fragment (i.e., removal of a portion of each of the N-terminal and C-terminal ends of the protein). A fragment can be, for example, when referring to a nucleic acid fragment, a 5' fragment (i.e., removal of a portion of the 3' end of the nucleic acid), a 3' fragment (i.e., removal of a portion of the 5' end of the nucleic acid), or an internal fragment (i.e., removal of a portion each of the 5' and 3' ends of the nucleic acid).

[0064] "Sequence identity" or "identity" in the context of two polynucleotides or polypeptide sequences refers to the residues in the two sequences that are the same when aligned for maximum correspondence over a specified comparison window. When percentage of sequence identity is used in reference to proteins, residue positions which are not identical often differ by conservative amino acid substitutions, where amino acid residues are substituted for other amino acid residues with similar chemical properties (e.g., charge or hydrophobicity) and therefore do not change the functional properties of the molecule. When sequences differ in conservative substitutions, the percent sequence identity may be adjusted upwards to correct for the conservative nature of the substitution. Sequences that differ by such conservative substitutions are said to have "sequence similarity" or "similarity." Means for making this adjustment are well known. Typically, this involves scoring a conservative substitution as a partial rather than a full mismatch, thereby increasing the percentage sequence identity. Thus, for example, where an identical amino acid is given a score of 1 and a non-conservative substitution is given a score of zero, a conservative substitution is given a score between zero and 1. The scoring of conservative substitutions is calculated, e.g., as implemented in the program PC/GENE (Intelligenetics, Mountain View, California).

[0065] "Percentage of sequence identity" includes the value determined by comparing two optimally aligned sequences (greatest number of perfectly matched residues) over a comparison window, wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison, and multiplying the result by 100 to yield the percentage of sequence identity. Unless otherwise specified (e.g., the shorter sequence includes a linked heterologous sequence), the comparison window is the full length of the shorter of the two sequences being compared.

[0066] Unless otherwise stated, sequence identity/similarity values include the value obtained using GAP Version 10 using the following parameters: % identity and % similarity for a nucleotide sequence using GAP Weight of 50 and Length Weight of 3, and the nwsgapdna.cmp scoring matrix; % identity and % similarity for an amino acid sequence using GAP Weight of 8 and Length Weight of 2, and the BLOSUM62 scoring matrix; or any equivalent program thereof. "Equivalent program" includes any sequence comparison program that, for any two sequences in question, generates an alignment having identical nucleotide or amino acid residue matches and an identical percent sequence identity when compared to the corresponding alignment generated by GAP Version 10.

[0067] The term "conservative amino acid substitution" refers to the substitution of an amino acid that is normally present in the sequence with a different amino acid of similar size, charge, or polarity. Examples of conservative substitutions include the substitution of a non-polar (hydrophobic) residue such as isoleucine, valine, or leucine for another non-polar residue. Likewise, examples of conservative substitutions include the substitution of one polar (hydrophilic) residue for another such as between arginine and lysine, between glutamine and asparagine, or between glycine and serine. Additionally, the substitution of a basic residue such as lysine, arginine, or histidine for another, or the substitution of one acidic residue such as aspartic acid or glutamic acid for another acidic residue are additional examples of conservative substitutions. Examples of non-conservative substitutions include the substitution of a non-polar (hydrophobic) amino acid residue such as isoleucine, valine, leucine, alanine, or methionine for a

polar (hydrophilic) residue such as cysteine, glutamine, glutamic acid or lysine and/or a polar residue for a non-polar residue. Typical amino acid categorizations are summarized below.

[0068] Table 1. Amino Acid Categorizations.

Alanine	Ala	A	Nonpolar	Neutral	1.8
Arginine	Arg	R	Polar	Positive	-4.5
Asparagine	Asn	N	Polar	Neutral	-3.5
Aspartic acid	Asp	D	Polar	Negative	-3.5
Cysteine	Cys	C	Nonpolar	Neutral	2.5
Glutamic acid	Glu	E	Polar	Negative	-3.5
Glutamine	Gln	Q	Polar	Neutral	-3.5
Glycine	Gly	G	Nonpolar	Neutral	-0.4
Histidine	His	H	Polar	Positive	-3.2
Isoleucine	Ile	I	Nonpolar	Neutral	4.5
Leucine	Leu	L	Nonpolar	Neutral	3.8
Lysine	Lys	K	Polar	Positive	-3.9
Methionine	Met	M	Nonpolar	Neutral	1.9
Phenylalanine	Phe	F	Nonpolar	Neutral	2.8
Proline	Pro	P	Nonpolar	Neutral	-1.6
Serine	Ser	S	Polar	Neutral	-0.8
Threonine	Thr	T	Polar	Neutral	-0.7
Tryptophan	Trp	W	Nonpolar	Neutral	-0.9
Tyrosine	Tyr	Y	Polar	Neutral	-1.3
Valine	Val	V	Nonpolar	Neutral	4.2

[0069] A "homologous" sequence (e.g., nucleic acid sequence) includes a sequence that is either identical or substantially similar to a known reference sequence, such that it is, for example, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to the known reference sequence. Homologous sequences can include, for example, orthologous sequence and paralogous sequences. Homologous genes, for example, typically descend from a common ancestral DNA sequence, either through a speciation event (orthologous genes) or a genetic duplication event (paralogous genes). "Orthologous" genes include genes in different species that evolved from a common ancestral gene by speciation. Orthologs typically retain the same function in the course of evolution. "Paralogous" genes include genes related by duplication within a genome. Paralogs can evolve new functions in the course of evolution.

[0070] The term "*in vitro*" includes artificial environments and to processes or reactions that occur within an artificial environment (e.g., a test tube or an isolated cell or cell line). The term "*in vivo*" includes natural environments (e.g., a cell or organism or body) and to processes or reactions that occur within a natural environment. The term "*ex vivo*" includes cells that have been removed from the body of an individual and processes or reactions that occur within such cells.

[0071] The term "reporter gene" refers to a nucleic acid having a sequence encoding a gene product (typically an enzyme) that is easily and quantifiably assayed when a construct comprising the reporter gene sequence operably linked to a heterologous promoter and/or enhancer element is introduced into cells containing (or which can be made to contain) the factors necessary for the activation of the promoter and/or enhancer elements. Examples of reporter genes include, but are not limited, to genes encoding beta-galactosidase (lacZ), the bacterial chloramphenicol acetyltransferase (cat) genes, firefly luciferase genes, genes encoding beta-glucuronidase (GUS), and genes encoding fluorescent proteins. A "reporter protein" refers to a protein encoded by a reporter gene.

[0072] The term "fluorescent reporter protein" as used herein means a reporter protein that is detectable based on fluorescence wherein the fluorescence may be either from the reporter protein directly, activity of the reporter protein on a fluorogenic substrate, or a protein with affinity for binding to a fluorescent tagged compound. Examples of fluorescent proteins include green fluorescent proteins (e.g., GFP, GFP-2, tagGFP, turboGFP, eGFP, Emerald, Azami Green, Monomeric Azami Green, CopGFP, AceGFP, and ZsGreenl), yellow fluorescent proteins (e.g., YFP, eYFP, Citrine, Venus, YPet, PhiYFP, and ZsYellowl), blue fluorescent proteins (e.g., BFP, eBFP, eBFP2, Azurite, mKalamal, GFPuv, Sapphire, and T-sapphire), cyan fluorescent proteins (e.g., CFP, eCFP, Cerulean, CyPet, AmCyanl, and Midoriishi-Cyan), red fluorescent proteins (e.g., RFP, mKate, mKate2, mPlum, DsRed monomer, mCherry, mRFP1, DsRed-Express, DsRed2, DsRed-Monomer, HcRed-Tandem, HcRedl, AsRed2, eqFP611, mRaspberry, mStrawberry, and Jred), orange fluorescent proteins (e.g., mOrange, mKO, Kusabira-Orange, Monomeric Kusabira-Orange, mTangerine, and tdTomato), and any other suitable fluorescent protein whose presence in cells can be detected by flow cytometry methods.

[0073] Repair in response to double-strand breaks (DSBs) occurs principally through two conserved DNA repair pathways: homologous recombination (HR) and non-homologous end

joining (NHEJ). *See* Kasparek & Humphrey (2011) *Semin. Cell Dev. Biol.* 22(8):886-897, herein incorporated by reference in its entirety for all purposes. Likewise, repair of a target nucleic acid mediated by an exogenous donor nucleic acid can include any process of exchange of genetic information between the two polynucleotides.

[0074] The term "recombination" includes any process of exchange of genetic information between two polynucleotides and can occur by any mechanism. Recombination can occur via homology directed repair (HDR) or homologous recombination (HR). HDR or HR includes a form of nucleic acid repair that can require nucleotide sequence homology, uses a "donor" molecule as a template for repair of a "target" molecule (i.e., the one that experienced the double-strand break), and leads to transfer of genetic information from the donor to 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. In some cases, the donor polynucleotide, a portion of the donor polynucleotide, a copy of the donor polynucleotide, or a portion of a copy of the donor polynucleotide integrates into the target DNA. *See* Wang et al. (2013) *Cell* 153:910-918; Mandalos et al. (2012) *PLoS ONE* 7:e45768:1-9; and Wang et al. (2013) *Nat. Biotechnol.* 31:530-532, each of which is herein incorporated by reference in its entirety for all purposes.

[0075] Non-homologous end joining (NHEJ) includes the repair of double-strand breaks in a nucleic acid by direct ligation of the break ends to one another or to an exogenous sequence without the need for a homologous template. Ligation of non-contiguous sequences by NHEJ can often result in deletions, insertions, or translocations near the site of the double-strand break. For example, NHEJ can also result in the targeted integration of an exogenous donor nucleic acid through direct ligation of the break ends with the ends of the exogenous donor nucleic acid (i.e., NHEJ-based capture). Such NHEJ-mediated targeted integration can be preferred for insertion of an exogenous donor nucleic acid when homology directed repair (HDR) pathways are not readily usable (e.g., in non-dividing cells, primary cells, and cells which perform homology-based DNA repair poorly). In addition, in contrast to homology-directed repair, knowledge concerning large regions of sequence identity flanking the cleavage site is not needed, which can be beneficial when attempting targeted insertion into organisms that have genomes for which there is limited

knowledge of the genomic sequence. The integration can proceed via ligation of blunt ends between the exogenous donor nucleic acid and the cleaved genomic sequence, or via ligation of sticky ends (i.e., having 5' or 3' overhangs) using an exogenous donor nucleic acid that is flanked by overhangs that are compatible with those generated by a nuclease agent in the cleaved genomic sequence. *See, e.g.*, US 2011/020722, WO 2014/033644, WO 2014/089290, and Maresca et al. (2013) *Genome Res.* 23(3):539-546, each of which is herein incorporated by reference in its entirety for all purposes. If blunt ends are ligated, target and/or donor resection may be needed to generation regions of microhomology needed for fragment joining, which may create unwanted alterations in the target sequence.

[0076] Compositions or methods "comprising" or "including" one or more recited elements may include other elements not specifically recited. For example, a composition that "comprises" or "includes" a protein may contain the protein alone or in combination with other ingredients. The transitional phrase "consisting essentially of" means that the scope of a claim is to be interpreted to encompass the specified elements recited in the claim and those that do not materially affect the basic and novel characteristic(s) of the claimed invention. Thus, the term "consisting essentially of" when used in a claim of this invention is not intended to be interpreted to be equivalent to "comprising."

[0077] "Optional" or "optionally" means that the subsequently described event or circumstance may or may not occur and that the description includes instances in which the event or circumstance occurs and instances in which the event or circumstance does not.

[0078] Designation of a range of values includes all integers within or defining the range, and all subranges defined by integers within the range.

[0079] Unless otherwise apparent from the context, the term "about" encompasses values ± 5 of a stated value.

[0080] The term "and/or" refers to and encompasses any and all possible combinations of one or more of the associated listed items, as well as the lack of combinations when interpreted in the alternative ("or").

[0081] The term "or" refers to any one member of a particular list and also includes any combination of members of that list.

[0082] The singular forms of the articles "a," "an," and "the" include plural references unless the context clearly dictates otherwise. For example, the term "a protein" or "at least one protein" can include a plurality of proteins, including mixtures thereof.

[0083] Statistically significant means $p \leq 0.05$.

DETAILED DESCRIPTION

I. Overview

[0084] Disclosed herein are non-human animal genomes, non-human animal cells, and non-human animals comprising a humanized *KLKB1* locus and methods of making and using such non-human animal cells and non-human animals. Also disclosed herein are humanized non-human animal *KLKB1* genes comprising a targeted genetic modification that humanizes the non-human animal *KLKB1* genes and nuclease agents and targeting vectors for use in humanizing a non-human animal *KLKB1* gene. Also disclosed herein are isolated liver samples (e.g., fractioned liver samples) prepared from the non-human animals comprising a humanized *KLKB1* locus.

[0085] In some of the non-human animal cells and non-human animals disclosed herein, some or most or all of the human *KLKB1* genomic DNA is inserted into the corresponding orthologous non-human animal *KLKB1* locus. In some of the non-human animal cells and non-human animals disclosed herein, some or most or all of the non-human animal *KLKB1* genomic DNA is replaced one-for-one with corresponding orthologous human *KLKB1* genomic DNA. A humanized *KLKB1* allele resulting from replacing most or all of the non-human animal genomic DNA one-for-one with corresponding orthologous human genomic DNA or inserting human *KLKB1* genomic sequence in the corresponding orthologous non-human *KLKB1* locus will provide the true human target or a close approximation of the true human target of human-KLKB1-targeting reagents (e.g., CRISPR/Cas9 reagents designed to target human *KLKB1*), thereby enabling testing of the efficacy and mode of action of such agents in live animals as well as pharmacokinetic and pharmacodynamics studies in a setting where the humanized protein and humanized gene are the only version of KLKB1 present.

II. Non-Human Animals Comprising a Humanized KLKB1 *Locus*

[0086] The non-human animal genomes, non-human animal cells, and non-human animals disclosed herein comprise a humanized *KLKB1* locus. Cells or non-human animals comprising a

humanized *KLKB1* locus express a human plasma kallikrein protein or a partially humanized, chimeric plasma kallikrein protein in which one or more fragments of the native plasma kallikrein protein have been replaced with corresponding fragments from human plasma kallikrein.

A. *KLKB1*

[0087] The cells and non-human animals described herein comprise a humanized *KLKB1* locus. Plasma kallikrein (also known as Fletcher factor, kininogenin, plasma prekallikrein, PKK, or kallikrein B1) is encoded by the *KLKB1* gene (also known as kallikrein B1, KLK3, PKK, PKKD, or PPK). Prekallikrein, which is encoded by the *KLKB1* gene, is a protein that is produced in the liver and secreted into plasma where it is converted into its active enzymatic form, plasma kallikrein, which acts to release bradykinin. Kallikrein participates in the surface-dependent activation of blood coagulation, fibrinolysis, kinin generation, and inflammation. The encoded preproprotein present in plasma as a non-covalent complex with high molecular weight kininogen undergoes proteolytic processing mediated by activated coagulation factor XII to generate a disulfide-linked, heterodimeric serine protease comprised of heavy and light chains.

[0088] Human *KLKB1* maps to 4q35.2 on chromosome 4 (NCBI RefSeq Gene ID 3818; Assembly GRCh38.p13 (GCF_000001405.39); location NC_000004.12 (186215714..186258477)). The gene has been reported to have 15 exons (including 14 coding exons starting with exon 2). The human plasma kallikrein protein has been assigned UniProt Accession No. P03952. The sequence for the canonical isoform, NCBI Accession No. NP_000883.2, is set forth in SEQ ID NO: 3. The sequence of another isoform, UniProt Accession No. P03952-1, is set forth in SEQ ID NO: 14. An mRNA (cDNA) encoding the canonical isoform is assigned NCBI Accession No. NM_000892.5 and is set forth in SEQ ID NO: 13. An exemplary coding sequence (CDS) encoding the canonical isoform is set forth in SEQ ID NO: 7 (CCDS ID CCDS34120.1). The full-length human plasma kallikrein protein set forth in SEQ ID NO: 3 has 638 amino acids, including a signal peptide (amino acids 1-19), a heavy chain (amino acids 20-390), and a light chain (amino acids 391-638). Delineations between these domains are as designated in UniProt. Reference to human plasma kallikrein includes the canonical (wild type) forms as well as all allelic forms and isoforms. Any other forms of human plasma kallikrein have amino acids numbered for maximal alignment with the

wild type form, aligned amino acids being designated the same number.

[0089] Mouse *Klkb1* maps to 8 B1.1; 8 25.17 cM on chromosome 8 (NCBI RefSeq Gene ID 16621; Assembly GRCm38.p6 (GCF_000001635.26); location NC_000074.6 (45266688..45294835, complement)). The gene has been reported to have 15 exons (including 14 coding exons starting with exon 2). The mouse plasma kallikrein protein has been assigned UniProt Accession No. P26262. The sequence for the canonical isoform, NCBI Accession No. NP_032481.2 and UniProt Accession No. P26262-1, is set forth in SEQ ID NO: 1. An exemplary mRNA (cDNA) isoform encoding the canonical isoform is assigned NCBI Accession No. NM_008455.3 and is set forth in SEQ ID NO: 12. An exemplary coding sequence (CDS) (CCDS ID CCDS22275.1) encoding the canonical isoform is set forth in SEQ ID NO: 5. The canonical full-length mouse plasma kallikrein protein set forth in SEQ ID NO: 1 has 638 amino acids, including a signal peptide (amino acids 1-19), a heavy chain (amino acids 20-390), and a light chain (amino acids 391-638). Delineations between these domains are as designated in UniProt. Reference to mouse plasma kallikrein includes the canonical (wild type) forms as well as all allelic forms and isoforms. Any other forms of mouse plasma kallikrein have amino acids numbered for maximal alignment with the wild type form, aligned amino acids being designated the same number.

[0090] Rat *Klkb1* maps to 16q11on chromosome 16 (NCBI RefSeq Gene ID 25048; Assembly Rnor_6.0 (GCF_000001895.5); location NC_005115.4 (50151127..50175407)). The gene has been reported to have 15 exons (including 14 coding exons). The rat plasma kallikrein protein has been assigned UniProt Accession No. P14272. The sequence for the canonical isoform, NCBI Accession No. NP_036857.2, is set forth in SEQ ID NO: 15. The sequence for another isoform, UniProt Accession No. P14272-1, is set forth in SEQ ID NO: 18. An mRNA (cDNA) encoding the canonical isoform is assigned NCBI Accession No. NM_012725.2 and is set forth in SEQ ID NO: 16. An exemplary coding sequence (CDS) encoding the canonical isoform is set forth in SEQ ID NO: 17. The canonical full-length rat plasma kallikrein protein set forth in SEQ ID NO: 15 has 638 amino acids, including a signal peptide (amino acids 1-19), a heavy chain (amino acids 20-390), and a light chain (amino acids 391-638). Delineations between these domains are as designated in UniProt. Reference to rat plasma kallikrein includes the canonical (wild type) forms as well as all allelic forms and isoforms. Any other forms of rat plasma kallikrein have amino acids numbered for maximal alignment with the wild type form,

aligned amino acids being designated the same number.

B. Humanized *KLKB1* Loci

[0091] Disclosed herein are humanized endogenous *KLKB1* loci in which a segment of an endogenous *KLKB1* locus has been deleted and replaced with a corresponding human *KLKB1* sequence (e.g., a corresponding human *KLKB1* genomic sequence), wherein a humanized plasma kallikrein protein is expressed from the humanized endogenous *KLKB1* locus. A humanized *KLKB1* locus can be a *KLKB1* locus in which the entire *KLKB1* gene is replaced with the corresponding orthologous human *KLKB1* sequence, it can be a *KLKB1* locus in which only a portion of the *KLKB1* gene is replaced with the corresponding orthologous human *KLKB1* sequence (i.e., humanized), it can be a *KLKB1* locus in which a portion of an orthologous human *KLKB1* locus is inserted, or it can be a *KLKB1* locus in which a portion of the *KLKB1* gene is deleted and a portion of the orthologous human *KLKB1* locus is inserted. The portion of the orthologous human *KLKB1* locus that is inserted can, for example, comprise more of the human *KLKB1* locus than is deleted from the endogenous *KLKB1* locus. A human *KLKB1* sequence corresponding to a particular segment of endogenous *KLKB1* sequence refers to the region of human *KLKB1* that aligns with the particular segment of endogenous *KLKB1* sequence when human *KLKB1* and the endogenous *KLKB1* are optimally aligned (greatest number of perfectly matched residues). The corresponding orthologous human sequence can comprise, for example, complementary DNA (cDNA) or genomic DNA. Optionally, a codon-optimized version of the corresponding orthologous human *KLKB1* sequence can be used and is modified to be codon-optimized based on codon usage in the non-human animal. Replaced or inserted (i.e., humanized) regions can include coding regions such as an exon, non-coding regions such as an intron, an untranslated region, or a regulatory region (e.g., a promoter, an enhancer, or a transcriptional repressor-binding element), or any combination thereof. As one example, exons corresponding to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or all 15 exons (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, or all 14 coding exons, which start at exon 2) of the human *KLKB1* gene can be humanized. For example, exons corresponding to exons 2-14 or 2-15 of the human *KLKB1* gene can be humanized. In a specific example, exons corresponding to exons 2-14 and the coding region of exon 15 (i.e., not including the 3' UTR) of the human *KLKB1* gene can be humanized. Alternatively, a region of *KLKB1* encoding an epitope recognized by an anti-human-

plasma-kallikrein antigen-binding protein or a region targeted by human-KLKB1-targeting reagent (e.g., a small molecule) can be humanized. Likewise, introns corresponding to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, or all 14 introns of the human *KLKB1* gene can be humanized or can remain endogenous. In one example, introns corresponding to the introns between all of the coding exons of the human *KLKB1* gene can be humanized. For example, introns corresponding to the introns between exons 2 and 15 (i.e., introns 2-14) of the human *KLKB1* gene can be humanized.

[0092] Flanking untranslated regions including regulatory sequences can also be humanized or remain endogenous. For example, the 5' untranslated region (UTR), the 3'UTR, or both the 5' UTR and the 3' UTR can be humanized, or the 5' UTR, the 3'UTR, or both the 5' UTR and the 3' UTR can remain endogenous. One or both of the human 5' and 3' UTRs can be inserted, and/or one or both of the endogenous 5' and 3' UTRs can be deleted. In a specific example, both the 5' UTR and the 3' UTR remain endogenous. Depending on the extent of replacement by orthologous sequences, regulatory sequences, such as a promoter, can be endogenous or supplied by the replacing human orthologous sequence. For example, the humanized *KLKB1* locus can include the endogenous non-human animal *KLKB1* promoter (i.e., the inserted human *KLKB1* sequence or humanized plasma-kallikrein-coding sequence can be operably linked to the endogenous non-human animal *KLKB1* promoter).

[0093] One or more or all of the regions encoding the signal peptide, the heavy chain, or the light chain can be humanized, or one or more of such regions can remain endogenous. Exemplary coding sequences for a mouse plasma kallikrein signal peptide, heavy chain, and light chain are set forth in SEQ ID NOS: 6, 21, and 22, respectively. Exemplary coding sequences for a human plasma kallikrein signal peptide, heavy chain, and light chain are set forth in SEQ ID NOS: 8, 25, and 26, respectively.

[0094] For example, all or part of the region of the *KLKB1* locus encoding the signal peptide can be humanized, and/or all or part of the region of the *KLKB1* locus encoding the heavy chain can be humanized, and/or all or part of the region of the *KLKB1* locus encoding light chain can be humanized. In one example, all or part of the region of the *KLKB1* locus encoding the signal peptide is humanized. Optionally, the CDS of the human plasma kallikrein signal peptide comprises a sequence, consists essentially of a sequence, or consists of a sequence that is at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at

least about 98%, at least about 99%, or about 100% identical to SEQ ID NO: 8 (or degenerates thereof) (e.g., at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 8 (or degenerates thereof)). The humanized plasma kallikrein protein can retain the activity of the native plasma kallikrein protein and/or the human plasma kallikrein protein (e.g., retains activity as demonstrated by activity assays disclosed elsewhere herein). In another example, all or part of the region of the *KLKB1* locus encoding the heavy chain is humanized. Optionally, the CDS of the human plasma kallikrein heavy chain comprises a sequence, consists essentially of a sequence, or consists of a sequence that is at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or about 100% identical to SEQ ID NO: 25 (or degenerates thereof) (e.g., at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 25 (or degenerates thereof)). The humanized plasma kallikrein protein can retain the activity of the native plasma kallikrein protein and/or the human plasma kallikrein protein (e.g., retains activity as demonstrated by activity assays disclosed elsewhere herein). In another example, all or part of the region of the *KLKB1* locus encoding the light chain is humanized. Optionally, the CDS of the human plasma kallikrein light chain comprises a sequence, consists essentially of a sequence, or consists of a sequence that is at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or about 100% identical to SEQ ID NO: 26 (or degenerates thereof) (e.g., at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 26 (or degenerates thereof)). The humanized plasma kallikrein protein can retain the activity of the native plasma kallikrein protein and/or the human plasma kallikrein protein (e.g., retains activity as demonstrated by activity assays disclosed elsewhere herein). In another example, all or part of the region of the *KLKB1* locus encoding the signal peptide, the heavy chain, and the light chain is humanized. The humanized plasma kallikrein protein can retain the activity of the native plasma kallikrein protein and/or the human plasma kallikrein protein (e.g., retains activity as demonstrated by activity assays disclosed elsewhere herein). For example, the region of the *KLKB1* locus encoding all of the signal peptide, the heavy chain, and the light chain can be humanized such that a fully humanized plasma kallikrein protein is produced with a human signal peptide, a human heavy chain, and a human light chain.

[0095] One or more of the regions encoding the signal peptide, the heavy chain, and the light chain can remain endogenous. For example, the region encoding the signal peptide and/or the heavy chain and/or the light chain can remain endogenous. Optionally, the CDS of the endogenous plasma kallikrein signal peptide comprises a sequence, consists essentially of a sequence, or consists of a sequence that is at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or about 100% identical to SEQ ID NO: 6 (or degenerates thereof) (e.g., at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 6 (or degenerates thereof)). Optionally, the CDS of the endogenous plasma kallikrein heavy chain comprises a sequence, consists essentially of a sequence, or consists of a sequence that is at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or about 100% identical to SEQ ID NO: 21 (or degenerates thereof) (e.g., at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 21 (or degenerates thereof)). Optionally, the CDS of the endogenous plasma kallikrein light chain comprises a sequence, consists essentially of a sequence, or consists of a sequence that is at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or about 100% identical to SEQ ID NO: 22 (or degenerates thereof) (e.g., at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 22 (or degenerates thereof)). In each case, the plasma kallikrein protein can retain the activity of the native plasma kallikrein protein and/or the human plasma kallikrein protein.

[0096] The plasma kallikrein protein encoded by the humanized *KLKB1* locus can comprise one or more domains that are from a human plasma kallikrein protein and/or one or more domains that are from an endogenous (i.e., native) plasma kallikrein protein. Exemplary amino acid sequences for a mouse plasma kallikrein signal peptide, heavy chain, and light chain are set forth in SEQ ID NOS: 2, 19, and 20, respectively. Exemplary amino acid sequences for a human plasma kallikrein signal peptide, heavy chain, and light chain are set forth in SEQ ID NOS: 4, 23, and 24, respectively. An alternative amino acid sequence for a human plasma kallikrein heavy chain is set forth in SEQ ID NO: 27.

[0097] The humanized plasma kallikrein protein can comprise one or more or all of a human

plasma kallikrein signal peptide, a human plasma kallikrein heavy chain and a human plasma kallikrein light chain. As one example, the humanized plasma kallikrein protein can comprise a human plasma kallikrein signal peptide, a human plasma kallikrein heavy chain, and a human plasma kallikrein light chain.

[0098] The humanized plasma kallikrein protein encoded by the humanized *KLKB1* locus can also comprise one or more domains that are from the endogenous (i.e., native) non-human animal plasma kallikrein protein. As one example, the plasma kallikrein protein encoded by the humanized *KLKB1* locus can comprise a signal peptide from the endogenous (i.e., native) non-human animal plasma kallikrein protein and/or a heavy chain from the endogenous (i.e., native) non-human animal plasma kallikrein protein and/or a light chain from the endogenous (i.e., native) non-human animal plasma kallikrein protein.

[0099] Domains in a humanized plasma kallikrein protein that are from a human plasma kallikrein protein can be encoded by a fully humanized sequence (i.e., the entire sequence encoding that domain is replaced with the orthologous human *KLKB1* sequence) or can be encoded by a partially humanized sequence (i.e., some of the sequence encoding that domain is replaced with the orthologous human *KLKB1* sequence, and the remaining endogenous (i.e., native) sequence encoding that domain encodes the same amino acids as the orthologous human *KLKB1* sequence such that the encoded domain is identical to that domain in the human plasma kallikrein protein). For example, part of the region of the *KLKB1* locus encoding the signal peptide (e.g., encoding the N-terminal region of the signal peptide) can remain endogenous *KLKB1* sequence, wherein the amino acid sequence of the region of the signal peptide encoded by the remaining endogenous *KLKB1* sequence is identical to the corresponding orthologous human plasma kallikrein amino acid sequence. As another example, part of the region of the *KLKB1* locus encoding the light chain (e.g., encoding the C-terminal region of the light chain) can remain endogenous *KLKB1* sequence, wherein the amino acid sequence of the region of the light chain encoded by the remaining endogenous *KLKB1* sequence is identical to the corresponding orthologous human plasma kallikrein amino acid sequence.

[00100] Likewise, domains in a humanized protein that are from the endogenous plasma kallikrein protein cay be encoded by a fully endogenous sequence (i.e., the entire sequence encoding that domain is the endogenous *KLKB1* sequence) or can be encoded by a partially humanized sequence (i.e., some of the sequence encoding that domain is replaced with the

orthologous human *KLKB1* sequence, but the orthologous human *KLKB1* sequence encodes the same amino acids as the replaced endogenous *KLKB1* sequence such that the encoded domain is identical to that domain in the endogenous plasma kallikrein protein). For example, part of the region of the *KLKB1* locus encoding the signal peptide (e.g., encoding the C-terminal region of the signal peptide) can be replaced with orthologous human *KLKB1* sequence, wherein the amino acid sequence of the region of the signal peptide encoded by the orthologous human *KLKB1* sequence is identical to the corresponding endogenous amino acid sequence. As another example, part of the region of the *KLKB1* locus encoding the light chain (e.g., encoding the N-terminal region of the light chain) can be replaced with orthologous human *KLKB1* sequence, wherein the amino acid sequence of the region of the light chain encoded by the orthologous human *KLKB1* sequence is identical to the corresponding endogenous amino acid sequence.

[00101] As one example, the plasma kallikrein protein encoded by the humanized *KLKB1* locus can comprise a human plasma kallikrein signal peptide. Optionally, the human plasma kallikrein signal peptide comprises a sequence, consists essentially of a sequence, or consists of a sequence that is at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or about 100% identical to SEQ ID NO: 4 (e.g., at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 4). The humanized plasma kallikrein protein can retain the activity of the native plasma kallikrein protein and/or the human plasma kallikrein protein (e.g., retains activity as demonstrated by activity assays disclosed elsewhere herein). As another example, the plasma kallikrein protein encoded by the humanized *KLKB1* locus can comprise a human plasma kallikrein heavy chain. Optionally, the human plasma kallikrein heavy chain comprises a sequence, consists essentially of a sequence, or consists of a sequence that is at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or about 100% identical to SEQ ID NO: 23 or 27 (e.g., at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 23 or 27). The humanized plasma kallikrein protein can retain the activity of the native plasma kallikrein protein and/or the human plasma kallikrein protein (e.g., retains activity as demonstrated by activity assays disclosed elsewhere herein). As another example, the plasma kallikrein protein encoded by the humanized *KLKB1* locus can comprise a human plasma kallikrein light chain. Optionally, the human plasma kallikrein light chain

comprises a sequence, consists essentially of a sequence, or consists of a sequence that is at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or about 100% identical to SEQ ID NO: 24 (e.g., at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 24). The humanized plasma kallikrein protein can retain the activity of the native plasma kallikrein protein and/or the human plasma kallikrein protein (e.g., retains activity as demonstrated by activity assays disclosed elsewhere herein). For example, the plasma kallikrein protein encoded by the humanized *KLKB1* locus can comprise a sequence, consist essentially of a sequence, or consist of a sequence that is at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or about 100% identical to SEQ ID NO: 3 or 14 (e.g., at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 3 or 14). Optionally, the *KLKB1* CDS encoded by the humanized *KLKB1* locus can comprise a sequence, consist essentially of a sequence, or consist of a sequence that is at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or about 100% identical to SEQ ID NO: 7 (or degenerates thereof) (e.g., at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 7 (or degenerates thereof)). In each case, the humanized plasma kallikrein protein can retain the activity of the native plasma kallikrein protein and/or the human plasma kallikrein protein.

[00102] Optionally, a humanized *KLKB1* locus can comprise other elements. Examples of such elements can include selection cassettes, reporter genes, recombinase recognition sites, or other elements. Alternatively, the humanized *KLKB1* locus can lack other elements (e.g., can lack a selection marker or selection cassette). Examples of suitable reporter genes and reporter proteins are disclosed elsewhere herein. Examples of suitable selection markers include neomycin phosphotransferase (neo_r), hygromycin B phosphotransferase (hyg_r), puromycin-N-acetyltransferase ($puro_r$), blasticidin S deaminase (bsr_r), xanthine/guanine phosphoribosyl transferase (gpt), and herpes simplex virus thymidine kinase (HSV-k). Examples of recombinases include Cre, Flp, and Dre recombinases. One example of a Cre recombinase gene is Crei, in which two exons encoding the Cre recombinase are separated by an intron to prevent its expression in a prokaryotic cell. Such recombinases can further comprise a nuclear

localization signal to facilitate localization to the nucleus (e.g., NLS-Crei). Recombinase recognition sites include nucleotide sequences that are recognized by a site-specific recombinase and can serve as a substrate for a recombination event. Examples of recombinase recognition sites include FRT, FRT11, FRT71, attp, att, rox, and lox sites such as loxP, lox511, lox2272, lox66, lox71, loxM2, and lox5171.

[00103] Other elements such as reporter genes or selection cassettes can be self-deleting cassettes flanked by recombinase recognition sites. *See, e.g.,* US 8,697,851 and US 2013/0312129, each of which is herein incorporated by reference in its entirety for all purposes. As an example, the self-deleting cassette can comprise a Crei gene (comprises two exons encoding a Cre recombinase, which are separated by an intron) operably linked to a mouse *Prm1* promoter and a neomycin resistance gene operably linked to a human ubiquitin promoter. By employing the *Prm1* promoter, the self-deleting cassette can be deleted specifically in male germ cells of F0 animals. The polynucleotide encoding the selection marker can be operably linked to a promoter active in a cell being targeted. Examples of promoters are described elsewhere herein. As another specific example, a self-deleting selection cassette can comprise a hygromycin resistance gene coding sequence operably linked to one or more promoters (e.g., both human ubiquitin and EM7 promoters) followed by a polyadenylation signal, followed by a Crei coding sequence operably linked to one or more promoters (e.g., an *mPrm1* promoter), followed by another polyadenylation signal, wherein the entire cassette is flanked by loxP sites.

[00104] The humanized *KLKB1* locus can also be a conditional allele. For example, the conditional allele can be a multifunctional allele, as described in US 2011/0104799, herein incorporated by reference in its entirety for all purposes. For example, the conditional allele can comprise: (a) an actuating sequence in sense orientation with respect to transcription of a target gene; (b) a drug selection cassette (DSC) in sense or antisense orientation; (c) a nucleotide sequence of interest (NSI) in antisense orientation; and (d) a conditional by inversion module (COIN, which utilizes an exon-splitting intron and an invertible gene-trap-like module) in reverse orientation. *See, e.g.*, US 2011/0104799. The conditional allele can further comprise recombinable units that recombine upon exposure to a first recombinase to form a conditional allele that (i) lacks the actuating sequence and the DSC; and (ii) contains the NSI in sense orientation and the COIN in antisense orientation. *See, e.g.*, US 2011/0104799.

[00105] One exemplary humanized *KLKB1* locus (e.g., a humanized mouse *KLKB1* locus) is

one in which a region from the start codon to the stop codon of the non-human animal *KLKB1* locus is deleted and replaced with the corresponding human sequence. As a specific example, an exemplary humanized *KLKB1* locus (e.g., a humanized mouse *KLKB1* locus) is one in which a region starting in exon 2 (coding exon 1; from amino acid 1) through the stop codon in exon 15, including all the introns from introns 2 through 14, is deleted from the non-human animal *KLKB1* locus and replaced with a region from the human *KLKB1* locus including exon 2/coding exon 1 (from amino acid 1) through the stop codon in exon 15, including all the introns from introns 2 through 14. Endogenous exon 1 (non-coding; 5' UTR) and the endogenous 3' UTR can optionally be retained. *See* **Figure 1**. Exemplary sequences for a humanized *KLKB1* locus are set forth in SED ID NOS: 9 and 10.

[00106] In one specific example, the human *KLKB1* sequence at the humanized endogenous *KLKB1* locus can comprise a sequence, consist essentially of a sequence, or consist of a sequence at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or about 100% identical to the sequence set forth in SEQ ID NO: 11 (e.g., at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to the sequence set forth in SEQ ID NO: 11). In another specific example, the humanized *KLKB1* locus can encode a protein comprising a sequence, consisting essentially of a sequence, or consisting of a sequence at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or about 100% identical to the sequence set forth in SEQ ID NO: 3 or 14 (e.g., at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to the sequence set forth in SEQ ID NO: 3 or 14). In another specific example, the humanized *KLKB1* locus can comprise a coding sequence comprising a sequence, consisting essentially of a sequence, or consisting of a sequence at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or about 100% identical to the sequence set forth in SEQ ID NO: 7 (e.g., at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to the sequence set forth in SEQ ID NO: 7). In another specific example, the humanized *KLKB1* locus can comprise a sequence, consist essentially of a sequence, or consist of a sequence at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or about 100% identical to the sequence set

forth in SEQ ID NO: 9 or 10 (e.g., at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to the sequence set forth in SEQ ID NO: 9 or 10).

C. Non-Human Animal Genomes, Non-Human Animal Cells, and Non-Human Animals Comprising a Humanized *KLKB1* Locus

[00107] Non-human animal genomes, non-human animal cells, and non-human animals comprising a humanized *KLKB1* locus as described elsewhere herein are provided. The genomes, cells, or non-human animals can be male or female. The genomes, cells, or non-human animals can express a humanized plasma kallikrein protein encoded by the humanized *KLKB1* locus. The genomes, cells, or non-human animals can be heterozygous or homozygous for the humanized *KLKB1* locus. A diploid organism has two alleles at each genetic locus. Each pair of alleles represents the genotype of a specific genetic locus. Genotypes are described as homozygous if there are two identical alleles at a particular locus and as heterozygous if the two alleles differ. A non-human animal comprising a humanized *KLKB1* locus can comprise the humanized *KLKB1* locus in its germline.

[00108] The non-human animal genomes or cells provided herein can be, for example, any non-human animal genome or cell comprising a *KLKB1* locus or a genomic locus homologous or orthologous to the human *KLKB1* locus. The genomes can be from or the cells can be eukaryotic cells, which include, for example, animal cells, mammalian cells, non-human mammalian cells, and human cells. The term "animal" includes any member of the animal kingdom, including, for example, mammals, fishes, reptiles, amphibians, birds, and worms. A mammalian cell can be, for example, a non-human mammalian cell, a rodent cell, a rat cell, or a mouse cell. Other non-human mammals include, for example, non-human primates. The term "non-human" excludes humans.

[00109] The cells can also be any type of undifferentiated or differentiated state. For example, a cell can be a totipotent cell, a pluripotent cell (e.g., a human pluripotent cell or a non-human pluripotent cell such as a mouse embryonic stem (ES) cell or a rat ES cell), or a non-pluripotent cell (e.g., a non-ES cell). Totipotent cells include undifferentiated cells that can give rise to any cell type, and pluripotent cells include undifferentiated cells that possess the ability to develop into more than one differentiated cell types. Such pluripotent and/or totipotent cells can be, for

example, ES cells or ES-like cells, such as an induced pluripotent stem (iPS) cells. ES cells include embryo-derived totipotent or pluripotent cells that are capable of contributing to any tissue of the developing embryo upon introduction into an embryo. ES cells can be derived from the inner cell mass of a blastocyst and are capable of differentiating into cells of any of the three vertebrate germ layers (endoderm, ectoderm, and mesoderm).

[00110] The cells provided herein can also be germ cells (e.g., sperm or oocytes). The cells can be mitotically competent cells or mitotically-inactive cells, meiotically competent cells or meiotically-inactive cells. Similarly, the cells can also be primary somatic cells or cells that are not a primary somatic cell. Somatic cells include any cell that is not a gamete, germ cell, gametocyte, or undifferentiated stem cell. For example, the cells can be liver cells, such as hepatoblasts or hepatocytes.

[00111] Suitable cells provided herein also include primary cells. Primary cells include cells or cultures of cells that have been isolated directly from an organism, organ, or tissue. Primary cells include cells that are neither transformed nor immortal. They include any cell obtained from an organism, organ, or tissue which was not previously passed in tissue culture or has been previously passed in tissue culture but is incapable of being indefinitely passed in tissue culture. Such cells can be isolated by conventional techniques and include, for example, hepatocytes.

[00112] Other suitable cells provided herein include immortalized cells. Immortalized cells include cells from a multicellular organism that would normally not proliferate indefinitely but, due to mutation or alteration, have evaded normal cellular senescence and instead can keep undergoing division. Such mutations or alterations can occur naturally or be intentionally induced. A specific example of an immortalized cell line is the HepG2 human liver cancer cell line. Numerous types of immortalized cells are well known. Immortalized or primary cells include cells that are typically used for culturing or for expressing recombinant genes or proteins.

[00113] The cells provided herein also include one-cell stage embryos (i.e., fertilized oocytes or zygotes). Such one-cell stage embryos can be from any genetic background (e.g., BALB/c, C57BL/6, 129, or a combination thereof for mice), can be fresh or frozen, and can be derived from natural breeding or *in vitro* fertilization.

[00114] The cells provided herein can be normal, healthy cells, or can be diseased or mutant-bearing cells.

[00115] Non-human animals comprising a humanized *KLKB1* locus as described herein can

be made by the methods described elsewhere herein. The term "animal" includes any member of the animal kingdom, including, for example, mammals, fishes, reptiles, amphibians, birds, and worms. In a specific example, the non-human animal is a non-human mammal. Non-human mammals include, for example, non-human primates and rodents (e.g., mice and rats). The term "non-human animal" excludes humans. Preferred non-human animals include, for example, rodents, such as mice and rats.

[00116] The non-human animals can be from any genetic background. For example, suitable mice can be from a 129 strain, a C57BL/6 strain, a mix of 129 and C57BL/6, a BALB/c strain, or a Swiss Webster strain. Examples of 129 strains include 129P1, 129P2, 129P3, 129X1, 129S1 (e.g., 129S1/SV, 129S1/Svlm), 129S2, 129S4, 129S5, 129S9/SvEvH, 129S6 (129/SvEvTac), 129S7, 129S8, 129T1, and 129T2. *See, e.g.*, Festing et al. (1999) *Mamm. Genome* 10(8):836, herein incorporated by reference in its entirety for all purposes. Examples of C57BL strains include C57BL/A, C57BL/An, C57BL/GrFa, C57BL/Kal_wN, C57BL/6, C57BL/6J, C57BL/6ByJ, C57BL/6NJ, C57BL/10, C57BL/10ScSn, C57BL/10Cr, and C57BL/Ola. Suitable mice can also be from a mix of an aforementioned 129 strain and an aforementioned C57BL/6 strain (e.g., 50% 129 and 50% C57BL/6). Likewise, suitable mice can be from a mix of aforementioned 129 strains or a mix of aforementioned BL/6 strains (e.g., the 129S6 (129/SvEvTac) strain).

[00117] Similarly, rats can be from any rat strain, including, for example, an ACI rat strain, a Dark Agouti (DA) rat strain, a Wistar rat strain, a LEA rat strain, a Sprague Dawley (SD) rat strain, or a Fischer rat strain such as Fisher F344 or Fisher F6. Rats can also be obtained from a strain derived from a mix of two or more strains recited above. For example, a suitable rat can be from a DA strain or an ACI strain. The ACI rat strain is characterized as having black agouti, with white belly and feet and an $RT1^{av1}$ haplotype. Such strains are available from a variety of sources including Harlan Laboratories. The Dark Agouti (DA) rat strain is characterized as having an agouti coat and an $RT1^{av1}$ haplotype. Such rats are available from a variety of sources including Charles River and Harlan Laboratories. Some suitable rats can be from an inbred rat strain. *See, e.g.*, US 2014/0235933, herein incorporated by reference in its entirety for all purposes.

[00118] The non-human animals disclosed herein can express a human plasma kallikrein protein or a partially humanized, chimeric plasma kallikrein protein. The expressed plasma

kallikrein protein can show activity in a plasma kallikrein activity assay (e.g., in a plasma kallikrein activity assay in plasma samples activated by dextran sulfate).

III. Methods of Making Non-Human Animals Comprising a Humanized KLKB1 *Locus*

[00119] Various methods are provided for making a non-human animal genome, non-human animal cell, or non-human animal comprising a humanized *KLKB1* locus as disclosed elsewhere herein. Likewise, various methods are provided for making a humanized *KLKB1* gene or locus or for making a non-human animal genome or non-human animal cell comprising a humanized *KLKB1* locus as disclosed elsewhere herein. Any convenient method or protocol for producing a genetically modified organism is suitable for producing such a genetically modified non-human animal. *See, e.g.*, Poueymirou et al. (2007) *Nat. Biotechnol.* 25(1):91-99; US 7,294,754; US 7,576,259; US 7,659,442; US 8,816,150; US 9,414,575; US 9,730,434; and US 10,039,269, each of which is herein incorporated by reference in its entirety for all purposes (describing mouse ES cells and the VELOCIMOUSE® method for making a genetically modified mouse). *See also* US 2014/0235933 A1, US 2014/0310828 A1, each of which is herein incorporated by reference in its entirety for all purposes (describing rat ES cells and methods for making a genetically modified rat). *See also* Cho et al. (2009) *Curr. Protoc. Cell. Biol.* 42:19.11.1–19.11.22 (doi: 10.1002/0471143030.cb1911s42) and Gama Sosa et al. (2010) *Brain Struct. Funct.* 214(2-3):91-109, each of which is herein incorporated by reference in its entirety for all purposes. Such genetically modified non-human animals can be generated, for example, through gene knock-in at a targeted *KLKB1* locus.

[00120] For example, the method of producing a non-human animal comprising a humanized *KLKB1* locus can comprise: (1) providing a pluripotent cell (e.g., an embryonic stem (ES) cell such as a mouse ES cell or a rat ES cell) comprising the humanized *KLKB1* locus; (2) introducing the genetically modified pluripotent cell into a non-human animal host embryo; and (3) gestating the host embryo in a surrogate mother.

[00121] As another example, the method of producing a non-human animal comprising a humanized *KLKB1* locus can comprise: (1) modifying the genome of a pluripotent cell (e.g., an embryonic stem (ES) cell such as a mouse ES cell or a rat ES cell) to comprise the humanized *KLKB1* locus; (2) identifying or selecting the genetically modified pluripotent cell comprising the humanized *KLKB1* locus; (3) introducing the genetically modified pluripotent cell into a non-

human animal host embryo; and (4) gestating the host embryo in a surrogate mother. The donor cell can be introduced into a host embryo at any stage, such as the blastocyst stage or the pre-morula stage (i.e., the 4-cell stage or the 8-cell stage). Optionally, the host embryo comprising modified pluripotent cell (e.g., a non-human ES cell) can be incubated until the blastocyst stage before being implanted into and gestated in the surrogate mother to produce an F0 non-human animal. The surrogate mother can then produce an F0 generation non-human animal comprising the humanized *KLKB1* locus (and capable of transmitting the genetic modification through the germline).

[00122] Alternatively, the method of producing the non-human animals described elsewhere herein can comprise: (1) modifying the genome of a one-cell stage embryo to comprise the humanized *KLKB1* locus using the methods described above for modifying pluripotent cells; (2) selecting the genetically modified embryo; and (3) gestating the genetically modified embryo in a surrogate mother. Progeny that are capable of transmitting the genetic modification though the germline are generated.

[00123] Nuclear transfer techniques can also be used to generate the non-human mammalian animals. Briefly, methods for nuclear transfer can include the steps of: (1) enucleating an oocyte or providing an enucleated oocyte; (2) isolating or providing a donor cell or nucleus to be combined with the enucleated oocyte; (3) inserting the cell or nucleus into the enucleated oocyte to form a reconstituted cell; (4) implanting the reconstituted cell into the womb of an animal to form an embryo; and (5) allowing the embryo to develop. In such methods, oocytes are generally retrieved from deceased animals, although they may be isolated also from either oviducts and/or ovaries of live animals. Oocytes can be matured in a variety of well-known media prior to enucleation. Enucleation of the oocyte can be performed in a number of well-known manners. Insertion of the donor cell or nucleus into the enucleated oocyte to form a reconstituted cell can be by microinjection of a donor cell under the zona pellucida prior to fusion. Fusion may be induced by application of a DC electrical pulse across the contact/fusion plane (electrofusion), by exposure of the cells to fusion-promoting chemicals, such as polyethylene glycol, or by way of an inactivated virus, such as the Sendai virus. A reconstituted cell can be activated by electrical and/or non-electrical means before, during, and/or after fusion of the nuclear donor and recipient oocyte. Activation methods include electric pulses, chemically induced shock, penetration by sperm, increasing levels of divalent cations in the oocyte, and reducing phosphorylation of

cellular proteins (as by way of kinase inhibitors) in the oocyte. The activated reconstituted cells, or embryos, can be cultured in well-known media and then transferred to the womb of an animal. *See, e.g.*, US 2008/0092249, WO 1999/005266, US 2004/0177390, WO 2008/017234, and US 7,612,250, each of which is herein incorporated by reference in its entirety for all purposes.

[00124] The modified cell or one-cell stage embryo can be generated, for example, through recombination by (a) introducing into the cell one or more exogenous donor nucleic acids (e.g., targeting vectors) comprising an insert nucleic acid flanked, for example, by 5' and 3' homology arms corresponding to 5' and 3' target sites (e.g., target sites flanking the endogenous sequences intended for deletion and replacement with the insert nucleic acid), wherein the insert nucleic acid comprises a human *KLKB1* sequence to generate a humanized *KLKB1* locus; and (b) identifying at least one cell comprising in its genome the insert nucleic acid integrated at the endogenous *KLKB1* locus (i.e., identifying at least one cell comprising the humanized *KLKB1* locus). Likewise, a modified non-human animal genome or humanized non-human animal *KLKB1* gene can be generated, for example, through recombination by (a) contacting the genome or gene with one or more exogenous donor nucleic acids (e.g., targeting vectors) comprising 5' and 3' homology arms corresponding to 5' and 3' target sites (e.g., target sites flanking the endogenous sequences intended for deletion and replacement with an insert nucleic acid (e.g., comprising a human *KLKB1* sequence to generate a humanized *KLKB1* locus) flanked by the 5' and 3' homology arms), wherein the exogenous donor nucleic acids are designed for humanization of the endogenous non-human animal *KLKB1* locus.

[00125] Alternatively, the modified pluripotent cell or one-cell stage embryo can be generated by (a) introducing into the cell: (i) a nuclease agent, wherein the nuclease agent induces a nick or double-strand break at a target site within the endogenous *KLKB1* locus; and (ii) one or more exogenous donor nucleic acids (e.g., targeting vectors) comprising an insert nucleic acid flanked by, for example, 5' and 3' homology arms corresponding to 5' and 3' target sites (e.g., target sites flanking the endogenous sequences intended for deletion and replacement with the insert nucleic acid), wherein the insert nucleic acid comprises a human *KLKB1* sequence to generate a humanized *KLKB1* locus; and (c) identifying at least one cell comprising in its genome the insert nucleic acid integrated at the endogenous *KLKB1* locus (i.e., identifying at least one cell comprising the humanized *KLKB1* locus). Likewise, a modified non-human animal genome or humanized non-human animal *KLKB1* gene can be generated by contacting the genome or gene

with: (i) a nuclease agent, wherein the nuclease agent induces a nick or double-strand break at a target site within the endogenous *KLKB1* locus or gene; and (ii) one or more exogenous donor nucleic acids (e.g., targeting vectors) comprising an insert nucleic acid (e.g., comprising a human *KLKB1* sequence to generate a humanized *KLKB1* locus) flanked by, for example, 5' and 3' homology arms corresponding to 5' and 3' target sites (e.g., target sites flanking the endogenous sequences intended for deletion and replacement with the insert nucleic acid), wherein the exogenous donor nucleic acids are designed for humanization of the endogenous *KLKB1* locus. Any nuclease agent that induces a nick or double-strand break into a desired recognition site can be used. Examples of suitable nucleases include a Transcription Activator-Like Effector Nuclease (TALEN), a zinc-finger nuclease (ZFN), a meganuclease, and Clustered Regularly Interspersed Short Palindromic Repeats (CRISPR)/CRISPR-associated (Cas) systems (e.g., CRISPR/Cas9 systems) or components of such systems (e.g., CRISPR/Cas9). *See, e.g.*, US 2013/0309670 and US 2015/0159175, each of which is herein incorporated by reference in its entirety for all purposes. In one example, the nuclease comprises a Cas9 protein and a guide RNA. In another example, the nuclease comprises a Cas9 protein and two or more, three or more, or four or more guide RNAs.

[00126] The step of modifying the genome can, for example, utilize exogenous repair templates (e.g., targeting vectors) to modify a *KLKB1* locus to comprise a humanized *KLKB1* locus disclosed herein. As one example, the targeting vector can be for generating a humanized *KLKB1* gene at an endogenous *KLKB1* locus (e.g., endogenous non-human animal *KLKB1* locus), wherein the targeting vector comprises a nucleic acid insert comprising human *KLKB1* sequence to be integrated in the *KLKB1* locus flanked by a 5' homology arm targeting a 5' target sequence at the endogenous *KLKB1* locus and a 3' homology arm targeting a 3' target sequence at the endogenous *KLKB1* locus. Integration of a nucleic acid insert in the *KLKB1* locus can result in addition of a nucleic acid sequence of interest in the *KLKB1* locus, deletion of a nucleic acid sequence of interest in the *KLKB1* locus, or replacement of a nucleic acid sequence of interest in the *KLKB1* locus (i.e., deleting a segment of the endogenous *KLKB1* locus and replacing with an orthologous human *KLKB1* sequence).

[00127] The exogenous repair templates can be for non-homologous-end-joining-mediated insertion or homologous recombination. Exogenous repair templates can comprise deoxyribonucleic acid (DNA) or ribonucleic acid (RNA), they can be single-stranded or double-

stranded, and they can be in linear or circular form. For example, a repair template can be a single-stranded oligodeoxynucleotide (ssODN). Exogenous repair templates can also comprise a heterologous sequence that is not present at an untargeted endogenous *KLKB1* locus. For example, an exogenous repair template can comprise a selection cassette, such as a selection cassette flanked by recombinase recognition sites.

[00128] In cells other than one-cell stage embryos, the exogenous repair template can be a "large targeting vector" or "LTVEC," which includes targeting vectors that comprise homology arms that correspond to and are derived from nucleic acid sequences larger than those typically used by other approaches intended to perform homologous recombination in cells. *See, e.g.*, US 2004/0018626; WO 2013/163394; US 9,834,786; US 10,301,646; WO 2015/088643; US 9,228,208; US 9,546,384; US 10,208,317; and US 2019-0112619, each of which is herein incorporated by reference in its entirety for all purposes. LTVECs also include targeting vectors comprising nucleic acid inserts having nucleic acid sequences larger than those typically used by other approaches intended to perform homologous recombination in cells. For example, LTVECs make possible the modification of large loci that cannot be accommodated by traditional plasmid-based targeting vectors because of their size limitations. For example, the targeted locus can be (i.e., the 5' and 3' homology arms can correspond to) a locus of the cell that is not targetable using a conventional method or that can be targeted only incorrectly or only with significantly low efficiency in the absence of a nick or double-strand break induced by a nuclease agent (e.g., a Cas protein). LTVECs can be of any length and are typically at least 10 kb in length. The sum total of the 5' homology arm and the 3' homology arm in an LTVEC is typically at least 10 kb. Generation and use of large targeting vectors (LTVECs) derived from bacterial artificial chromosome (BAC) DNA through bacterial homologous recombination (BHR) reactions using VELOCIGENE® genetic engineering technology is described, e.g., in US 6,586,251 and Valenzuela et al. (2003) *Nat. Biotechnol.* 21(6):652-659, each of which is herein incorporated by reference in its entirety for all purposes. Generation of LTVECs through *in vitro* assembly methods is described, e.g., in US 2015/0376628 and WO 2015/200334, each of which is herein incorporated by reference in its entirety for all purposes.

[00129] The methods can further comprise identifying a cell or animal having a modified target genomic locus. Various methods can be used to identify cells and animals having a targeted genetic modification. The screening step can comprise, for example, a quantitative assay

for assessing modification-of-allele (MOA) of a parental chromosome. *See, e.g.*, US 2004/0018626; US 2014/0178879; US 2016/0145646; WO 2016/081923; and Frendewey et al. (2010) *Methods Enzymol.* 476:295-307, each of which is herein incorporated by reference in its entirety for all purposes. For example, the quantitative assay can be carried out via a quantitative PCR, such as a real-time PCR (qPCR). The real-time PCR can utilize a first primer set that recognizes the target locus and a second primer set that recognizes a non-targeted reference locus. The primer set can comprise a fluorescent probe that recognizes the amplified sequence. Other examples of suitable quantitative assays include fluorescence-mediated in situ hybridization (FISH), comparative genomic hybridization, isothermic DNA amplification, quantitative hybridization to an immobilized probe(s), INVADER® Probes, TAQMAN® Molecular Beacon probes, or ECLIPSE™ probe technology (*see, e.g.*, US 2005/0144655, incorporated herein by reference in its entirety for all purposes).

[00130] The various methods provided herein allow for the generation of a genetically modified non-human F0 animal wherein the cells of the genetically modified F0 animal comprise the humanized *KLKB1* locus. It is recognized that depending on the method used to generate the F0 animal, the number of cells within the F0 animal that have the humanized *KLKB1* locus will vary. With mice, for example, the introduction of the donor ES cells into a pre-morula stage embryo from the mouse (e.g., an 8-cell stage mouse embryo) via, for example, the VELOCIMOUSE® method allows for a greater percentage of the cell population of the F0 mouse to comprise cells having the targeted genetic modification. For example, at least 50%, 60%, 65%, 70%, 75%, 85%, 86%, 87%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% of the cellular contribution of the non-human F0 animal can comprise a cell population having the targeted modification. The cells of the genetically modified F0 animal can be heterozygous for the humanized *KLKB1* locus or can be homozygous for the humanized *KLKB1* locus.

IV. Methods of Using Non-Human Animals Comprising a Humanized KLKB1 *Locus for Assessing Delivery or Efficacy of Human-KLKB1-Targeting Reagents* In Vivo *or* Ex Vivo

[00131] Various methods are provided for using the non-human animals comprising a humanized *KLKB1* locus as described elsewhere herein for assessing delivery or efficacy of human-KLKB1-targeting reagents *in vivo* or *ex vivo*. Because the non-human animals comprise a humanized *KLKB1* locus, the non-human animals will more accurately reflect the efficacy of a

human-KLKB1-targeting reagent.

A. Methods of Testing Efficacy of Human-KLKB1-Targeting Reagents *In Vivo* or *Ex Vivo*

[00132] Various methods are provided for assessing delivery or efficacy of human-KLKB1-targeting reagents *in vivo* using non-human animals comprising a humanized *KLKB1* locus as described elsewhere herein. Such methods can comprise: (a) introducing into the non-human animal a human-KLKB1-targeting reagent; and (b) assessing the activity of the human-KLKB1-targeting reagent.

[00133] The human-KLKB1-targeting reagent can be a human-KLKB1-targeting antibody or antigen-binding protein or any other large molecule or small molecule that targets human plasma kallikrein protein. Alternatively, the human-KLKB1-targeting reagent can be any biological or chemical agent that targets the human *KLKB1* locus (the human *KLKB1* gene), the human *KLKB1* mRNA, or the human plasma kallikrein protein. Examples of human-KLKB1-targeting reagents are disclosed elsewhere herein.

[00134] Such human-KLKB1-targeting reagents can be administered by any delivery method (e.g., AAV, LNP, HDD, or injection) and by any route of administration. Means of delivering complexes and molecules and routes of administration are disclosed in more detail elsewhere herein. In particular methods, the reagents delivered via AAV-mediated delivery. For example, AAV8 can be used to target the liver. In other particular methods, the reagents are delivered by LNP-mediated delivery. In other particular methods, the reagents are delivered by hydrodynamic delivery (HDD). The dose can be any suitable dose.

[00135] Methods for assessing activity of the human-KLKB1-targeting reagent are well-known and are provided elsewhere herein. Assessment of activity can be in any cell type, any tissue type, or any organ type. In some methods, assessment of activity is in liver cells or in the liver. As one example, assessing activity can comprise using a plasma kallikrein activity assay in plasma samples activated by dextran sulfate.

[00136] If the human-KLKB1-targeting reagent is a genome editing reagent (e.g., a nuclease agent), such methods can comprise assessing modification of the humanized *KLKB1* locus. As one example, the assessing can comprise measuring non-homologous end joining (NHEJ) activity at the humanized *KLKB1* locus. This can comprise, for example, measuring the

frequency of insertions or deletions within the humanized *KLKB1* locus. For example, the assessing can comprise sequencing the humanized *KLKB1* locus in one or more cells isolated from the non-human animal (e.g., next-generation sequencing). Assessment can comprise isolating a target organ or tissue (e.g., liver) from the non-human animal and assessing modification of humanized *KLKB1* locus in the target organ or tissue. Assessment can also comprise assessing modification of humanized *KLKB1* locus in two or more different cell types within the target organ or tissue. Similarly, assessment can comprise isolating a non-target organ or tissue (e.g., two or more non-target organs or tissues) from the non-human animal and assessing modification of humanized *KLKB1* locus in the non-target organ or tissue.

[00137] Such methods can also comprise measuring expression levels of the mRNA produced by the humanized *KLKB1* locus, or by measuring expression levels of the protein encoded by the humanized *KLKB1* locus. For example, protein levels can be measured in a particular cell, tissue, or organ type (e.g., liver), or secreted levels can be measured in the serum. Methods for assessing expression of *KLKB1* mRNA or plasma kallikrein protein expressed from the humanized *KLKB1* locus are provided elsewhere herein and are well-known.

[00138] As one specific example, if the human-KLKB1-targeting reagent is a genome editing reagent (e.g., a nuclease agent), percent editing (e.g., total number of insertions or deletions observed over the total number of sequences read in the PCR reaction from a pool of lysed cells) at the humanized *KLKB1* locus can be assessed (e.g., in liver cells).

[00139] The various methods provided above for assessing activity *in vivo* can also be used to assess the activity of human-KLKB1-targeting reagents *ex vivo* (e.g., in a liver comprising a humanized *KLKB1* locus) or *in vitro* (e.g., in a cell comprising a humanized *KLKB1* locus) as described elsewhere herein.

B. Methods of Optimizing Delivery or Efficacy of Human-KLKB1-Targeting Reagent *In Vivo* or *Ex Vivo*

[00140] Various methods are provided for optimizing delivery of human-KLKB1-targeting reagents to a cell or non-human animal or optimizing the activity or efficacy of human-KLKB1-targeting reagents *in vivo*. Such methods can comprise, for example: (a) performing the method of testing the efficacy of a human-KLKB1-targeting reagents as described above a first time in a first non-human animal or first cell comprising a humanized *KLKB1* locus; (b) changing a

variable and performing the method a second time in a second non-human animal (i.e., of the same species) or a second cell comprising a humanized *KLKB1* locus with the changed variable; and (c) comparing the activity of the human-KLKB1-targeting reagents in step (a) with the activity of the human-KLKB1-targeting reagents in step (b), and selecting the method resulting in the higher activity.

[00141] Methods of measuring delivery, efficacy, or activity of human-KLKB1-targeting reagents are disclosed elsewhere herein. For example, such methods can comprise measuring modification of the humanized *KLKB1* locus. More effective modification of the humanized *KLKB1* locus can mean different things depending on the desired effect within the non-human animal or cell. For example, more effective modification of the humanized *KLKB1* locus can mean one or more or all of higher levels of modification, higher precision, higher consistency, or higher specificity. Higher levels of modification (i.e., higher efficacy) of the humanized *KLKB1* locus refers to a higher percentage of cells is targeted within a particular target cell type, within a particular target tissue, or within a particular target organ (e.g., liver). Higher precision refers to more precise modification of the humanized *KLKB1* locus (e.g., a higher percentage of targeted cells having the same modification or having the desired modification without extra unintended insertions and deletions (e.g., NHEJ indels)). Higher consistency refers to more consistent modification of the humanized *KLKB1* locus among different types of targeted cells, tissues, or organs if more than one type of cell, tissue, or organ is being targeted (e.g., modification of a greater number of cell types within the liver). If a particular organ is being targeted, higher consistency can also refer to more consistent modification throughout all locations within the organ (e.g., the liver). Higher specificity can refer to higher specificity with respect to the genomic locus or loci targeted, higher specificity with respect to the cell type targeted, higher specificity with respect to the tissue type targeted, or higher specificity with respect to the organ targeted. For example, increased genomic locus specificity refers to less modification of off-target genomic loci (e.g., a lower percentage of targeted cells having modifications at unintended, off-target genomic loci instead of or in addition to modification of the target genomic locus). Likewise, increased cell type, tissue, or organ type specificity refers to less modification of off-target cell types, tissue types, or organ types if a particular cell type, tissue type, or organ type is being targeted (e.g., when a particular organ is targeted (e.g., the liver), there is less modification of cells in organs or tissues that are not intended targets).

[00142] Alternatively, such methods can comprise measuring expression of *KLKB1* mRNA or plasma kallikrein protein. In one example, a more effective human-KLKB1-targeting agent results in a greater decrease in *KLKB1* mRNA or plasma kallikrein protein expression. Alternatively, such methods can comprise measuring plasma kallikrein activity. In one example, a more effective human-KLKB1-targeting agent results in a greater decrease in plasma kallikrein activity.

[00143] The variable that is changed can be any parameter. As one example, the changed variable can be the packaging or the delivery method by which the human-KLKB1-targeting reagent or reagents are introduced into the cell or non-human animal. Examples of delivery methods, such as LNP, HDD, and AAV, are disclosed elsewhere herein. For example, the changed variable can be the AAV serotype. Alternatively, the changed variable can be the dose of AAV delivered (e.g., about 10^{11}, about 10^{12}, about 10^{13}, or about 10^{14} vg/kg of body weight). Similarly, the administering can comprise LNP-mediated delivery, and the changed variable can be the LNP formulation. Alternatively, the administering can comprise LNP-mediated delivery, and the changed variable can be the dose of the LNP delivered (e.g., about 0.01 mg/kg, about 0.03 mg/kg, about 0.1 mg/kg, about 0.3 mg/kg, about 1 mg/kg, about 3 mg/kg, or about 10 mg/kg). As another example, the changed variable can be the route of administration for introduction of the human-KLKB1-targeting reagent or reagents into the cell or non-human animal. Examples of routes of administration, such as intravenous, intravitreal, intraparenchymal, and nasal instillation, are disclosed elsewhere herein.

[00144] As another example, the changed variable can be the concentration or amount of the human-KLKB1-targeting reagent or reagents introduced. As another example, the changed variable can be the concentration or the amount of one human-KLKB1-targeting reagent introduced (e.g., guide RNA, Cas protein, exogenous donor nucleic acid, RNAi agent, or ASO) relative to the concentration or the amount another human-KLKB1-targeting reagent introduced (e.g., guide RNA, Cas protein, exogenous donor nucleic acid, RNAi agent, or ASO).

[00145] As another example, the changed variable can be the timing of introducing the human-KLKB1-targeting reagent or reagents relative to the timing of assessing the activity or efficacy of the reagents. As another example, the changed variable can be the number of times or frequency with which the human-KLKB1-targeting reagent or reagents are introduced. As another example, the changed variable can be the timing of introduction of one human-KLKB1-

targeting reagent introduced (e.g., guide RNA, Cas protein, exogenous donor nucleic acid, RNAi agent, or ASO) relative to the timing of introduction of another human-KLKB1-targeting reagent introduced (e.g., guide RNA, Cas protein, exogenous donor nucleic acid, RNAi agent, or ASO).

[00146] As another example, the changed variable can be the form in which the human-KLKB1-targeting reagent or reagents are introduced. For example, a guide RNA can be introduced in the form of DNA or in the form of RNA. A Cas protein (e.g., Cas9) can be introduced in the form of DNA, in the form of RNA, or in the form of a protein (e.g., complexed with a guide RNA). An exogenous donor nucleic acid can be DNA, RNA, single-stranded, double-stranded, linear, circular, and so forth. Similarly, each of the components can comprise various combinations of modifications for stability, to reduce off-target effects, to facilitate delivery, and so forth. Likewise, RNAi agents and ASOs, for example, can comprise various combinations of modifications for stability, to reduce off-target effects, to facilitate delivery, and so forth.

[00147] As another example, the changed variable can be the human-KLKB1-targeting reagent or reagents that are introduced. For example, if the human-KLKB1-targeting reagent comprises a guide RNA, the changed variable can be introducing a different guide RNA with a different sequence (e.g., targeting a different guide RNA target sequence). Similarly, if the human-KLKB1-targeting reagent comprises an RNAi agent or an ASO, the changed variable can be introducing a different RNAi agent or ASO with a different sequence. Likewise, if the human-KLKB1-targeting reagent comprises a Cas protein, the changed variable can be introducing a different Cas protein (e.g., introducing a different Cas protein with a different sequence, or a nucleic acid with a different sequence (e.g., codon-optimized) but encoding the same Cas protein amino acid sequence. Likewise, if the human-KLKB1-targeting reagent comprises an exogenous donor nucleic acid, the changed variable can be introducing a different exogenous donor nucleic acid with a different sequence (e.g., a different insert nucleic acid or different homology arms (e.g., longer or shorter homology arms or homology arms targeting a different region of the human *KLKB1* gene)).

[00148] In a specific example, the human-KLKB1-targeting reagent comprises a Cas protein and a guide RNA designed to target a guide RNA target sequence in a human *KLKB1* gene. In such methods, the changed variable can be the guide RNA sequence and/or the guide RNA target sequence. In some such methods, the Cas protein and the guide RNA can each be administered in

the form of RNA, and the changed variable can be the ratio of Cas mRNA to guide RNA (e.g., in an LNP formulation). In some such methods, the changed variable can be guide RNA modifications (e.g., a guide RNA with a modification is compared to a guide RNA without the modification).

C. Human-KLKB1-Targeting Reagents

[00149] A human-KLKB1-targeting reagent can be any reagent that targets a human plasma kallikrein protein, a human *KLKB1* gene, or a human *KLKB1* mRNA. A human-KLKB1-targeting reagent can be, for example, a known human-KLKB1-targeting reagent, can be a putative human-KLKB1-targeting reagent (e.g., candidate reagents designed to target human *KLKB1*), or can be a reagent being screened for human-KLKB1-targeting activity.

[00150] For example, a human-KLKB1-targeting reagent can be an antigen-binding protein (e.g., agonist antibody) targeting an epitope of a human plasma kallikrein protein. The term "antigen-binding protein" includes any protein that binds to an antigen. Examples of antigen-binding proteins include an antibody, an antigen-binding fragment of an antibody, a multispecific antibody (e.g., a bi-specific antibody), an scFV, a bis-scFV, a diabody, a triabody, a tetrabody, a V-NAR, a VHH, a VL, a F(ab), a $F(ab)_2$, a DVD (dual variable domain antigen-binding protein), an SVD (single variable domain antigen-binding protein), a bispecific T-cell engager (BiTE), or a Davisbody (US Pat. No. 8,586,713, herein incorporated by reference herein in its entirety for all purposes). Other human-KLKB1-targeting reagents include small molecules targeting a human plasma kallikrein protein.

[00151] Other human-KLKB1-targeting reagents can include genome editing reagents such as a nuclease agent (e.g., a Clustered Regularly Interspersed Short Palindromic Repeats (CRISPR)/CRISPR-associated (Cas) (CRISPR/Cas) nuclease, a zinc finger nuclease (ZFN), or a Transcription Activator-Like Effector Nuclease (TALEN)) that cleaves a recognition site within the human *KLKB1* gene. Likewise, a human-KLKB1-targeting reagent can be an exogenous donor nucleic acid (e.g., a targeting vector or single-stranded oligodeoxynucleotide (ssODN)) designed to recombine with the human *KLKB1* gene.

[00152] Other human-KLKB1-targeting reagents can include RNAi agents. An "RNAi agent" is a composition that comprises a small double-stranded RNA or RNA-like (e.g., chemically modified RNA) oligonucleotide molecule capable of facilitating degradation or inhibition of

translation of a target RNA, such as messenger RNA (mRNA), in a sequence-specific manner. The oligonucleotide in the RNAi agent is a polymer of linked nucleosides, each of which can be independently modified or unmodified. RNAi agents operate through the RNA interference mechanism (i.e., inducing RNA interference through interaction with the RNA interference pathway machinery (RNA-induced silencing complex or RISC) of mammalian cells). While it is believed that RNAi agents, as that term is used herein, operate primarily through the RNA interference mechanism, the disclosed RNAi agents are not bound by or limited to any particular pathway or mechanism of action. RNAi agents disclosed herein comprise a sense strand and an antisense strand, and include, but are not limited to: short interfering RNAs (siRNAs), double-stranded RNAs (dsRNA), micro RNAs (miRNAs), short hairpin RNAs (shRNA), and dicer substrates. The antisense strand of the RNAi agents described herein is at least partially complementary to a sequence (i.e., a succession or order of nucleobases or nucleotides, described with a succession of letters using standard nomenclature) in the target RNA.

[00153] Other human-KLKB1-targeting reagents can include antisense oligonucleotides (ASOs). Single-stranded ASOs and RNA interference (RNAi) share a fundamental principle in that an oligonucleotide binds a target RNA through Watson-Crick base pairing. Without wishing to be bound by theory, during RNAi, a small RNA duplex (RNAi agent) associates with the RNA-induced silencing complex (RISC), one strand (the passenger strand) is lost, and the remaining strand (the guide strand) cooperates with RISC to bind complementary RNA. Argonaute 2 (Ago2), the catalytic component of the RISC, then cleaves the target RNA. The guide strand is always associated with either the complementary sense strand or a protein (RISC). In contrast, an ASO must survive and function as a single strand. ASOs bind to the target RNA and block ribosomes or other factors, such as splicing factors, from binding the RNA or recruit proteins such as nucleases. Different modifications and target regions are chosen for ASOs based on the desired mechanism of action. A gapmer is an ASO oligonucleotide containing 2–5 chemically modified nucleotides (e.g. LNA or 2'-MOE) on each terminus flanking a central 8–10 base gap of DNA. After binding the target RNA, the DNA-RNA hybrid acts substrate for RNase H.

D. Administering Human-KLKB1-Targeting Reagents to Non-Human Animals or Cells

[00154] The methods disclosed herein can comprise introducing into a non-human animal or cell various molecules (e.g., human-KLKB1-targeting reagents such as therapeutic molecules or complexes), including nucleic acids, proteins, nucleic-acid-protein complexes, protein complexes, or small molecules. "Introducing" includes presenting to the cell or non-human animal the molecule (e.g., nucleic acid or protein) in such a manner that it gains access to the interior of the cell or to the interior of cells within the non-human animal. The introducing can be accomplished by any means, and two or more of the components (e.g., two of the components, or all of the components) can be introduced into the cell or non-human animal simultaneously or sequentially in any combination. For example, a Cas protein can be introduced into a cell or non-human animal before introduction of a guide RNA, or it can be introduced following introduction of the guide RNA. As another example, an exogenous donor nucleic acid can be introduced prior to the introduction of a Cas protein and a guide RNA, or it can be introduced following introduction of the Cas protein and the guide RNA (e.g., the exogenous donor nucleic acid can be administered about 1, 2, 3, 4, 8, 12, 24, 36, 48, or 72 hours before or after introduction of the Cas protein and the guide RNA). *See, e.g.,* US 2015/0240263 and US 2015/0110762, each of which is herein incorporated by reference in its entirety for all purposes. In addition, two or more of the components can be introduced into the cell or non-human animal by the same delivery method or different delivery methods. Similarly, two or more of the components can be introduced into a non-human animal by the same route of administration or different routes of administration.

[00155] In some methods, components of a CRISPR/Cas system are introduced into a non-human animal or cell. A guide RNA can be introduced into a non-human animal or cell in the form of an RNA (e.g., *in vitro* transcribed RNA) or in the form of a DNA encoding the guide RNA. When introduced in the form of a DNA, the DNA encoding a guide RNA can be operably linked to a promoter active in a cell in the non-human animal. For example, a guide RNA may be delivered via AAV and expressed *in vivo* under a U6 promoter. Such DNAs can be in one or more expression constructs. For example, such expression constructs can be components of a single nucleic acid molecule. Alternatively, they can be separated in any combination among two or more nucleic acid molecules (i.e., DNAs encoding one or more CRISPR RNAs and DNAs

encoding one or more tracrRNAs can be components of a separate nucleic acid molecules).

[00156] Likewise, Cas proteins can be provided in any form. For example, a Cas protein can be provided in the form of a protein, such as a Cas protein complexed with a gRNA. Alternatively, a Cas protein can be provided in the form of a nucleic acid encoding the Cas protein, such as an RNA (e.g., messenger RNA (mRNA)) or DNA. Optionally, the nucleic acid encoding the Cas protein can be codon optimized for efficient translation into protein in a particular cell or organism. For example, the nucleic acid encoding the Cas protein can be modified to substitute codons having a higher frequency of usage in a mammalian cell, a rodent cell, a mouse cell, a rat cell, or any other host cell of interest, as compared to the naturally occurring polynucleotide sequence. When a nucleic acid encoding the Cas protein is introduced into a non-human animal, the Cas protein can be transiently, conditionally, or constitutively expressed in a cell in the non-human animal.

[00157] Nucleic acids encoding Cas proteins or guide RNAs can be operably linked to a promoter in an expression construct. Expression constructs include any nucleic acid constructs capable of directing expression of a gene or other nucleic acid sequence of interest (e.g., a Cas gene) and which can transfer such a nucleic acid sequence of interest to a target cell. For example, the nucleic acid encoding the Cas protein can be in a vector comprising a DNA encoding one or more gRNAs. Alternatively, it can be in a vector or plasmid that is separate from the vector comprising the DNA encoding one or more gRNAs. Suitable promoters that can be used in an expression construct include promoters active, for example, in one or more of a eukaryotic cell, a human cell, a non-human cell, a mammalian cell, a non-human mammalian cell, a rodent cell, a mouse cell, a rat cell, a hamster cell, a rabbit cell, a pluripotent cell, an embryonic stem (ES) cell, an adult stem cell, a developmentally restricted progenitor cell, an induced pluripotent stem (iPS) cell, or a one-cell stage embryo. Such promoters can be, for example, conditional promoters, inducible promoters, constitutive promoters, or tissue-specific promoters. Optionally, the promoter can be a bidirectional promoter driving expression of both a Cas protein in one direction and a guide RNA in the other direction. Such bidirectional promoters can consist of (1) a complete, conventional, unidirectional Pol III promoter that contains 3 external control elements: a distal sequence element (DSE), a proximal sequence element (PSE), and a TATA box; and (2) a second basic Pol III promoter that includes a PSE and a TATA box fused to the 5′ terminus of the DSE in reverse orientation. For example, in the H1

promoter, the DSE is adjacent to the PSE and the TATA box, and the promoter can be rendered bidirectional by creating a hybrid promoter in which transcription in the reverse direction is controlled by appending a PSE and TATA box derived from the U6 promoter. *See, e.g.*, US 2016/0074535, herein incorporated by references in its entirety for all purposes. Use of a bidirectional promoter to express genes encoding a Cas protein and a guide RNA simultaneously allows for the generation of compact expression cassettes to facilitate delivery.

[00158] Molecules (e.g., Cas proteins or guide RNAs or RNAi agents or ASOs) introduced into the non-human animal or cell can be provided in compositions comprising a carrier increasing the stability of the introduced molecules (e.g., prolonging the period under given conditions of storage (e.g., -20°C, 4°C, or ambient temperature) for which degradation products remain below a threshold, such below 0.5% by weight of the starting nucleic acid or protein; or increasing the stability in vivo). Non-limiting examples of such carriers include poly(lactic acid) (PLA) microspheres, poly(D,L-lactic-coglycolic-acid) (PLGA) microspheres, liposomes, micelles, inverse micelles, lipid cochleates, and lipid microtubules.

[00159] Various methods and compositions are provided herein to allow for introduction of molecule (e.g., a nucleic acid or protein) into a cell or non-human animal. Methods for introducing molecules into various cell types are known and include, for example, stable transfection methods, transient transfection methods, and virus-mediated methods.

[00160] Transfection protocols as well as protocols for introducing molecules into cells may vary. Non-limiting transfection methods include chemical-based transfection methods using liposomes; nanoparticles; calcium phosphate (Graham et al. (1973) *Virology* 52 (2): 456–67, Bacchetti et al. (1977) *Proc. Natl. Acad. Sci. USA* 74 (4): 1590–4, and Kriegler, M (1991). Transfer and Expression: A Laboratory Manual. New York: W. H. Freeman and Company. pp. 96–97); dendrimers; or cationic polymers such as DEAE-dextran or polyethylenimine. Non-chemical methods include electroporation, sonoporation, and optical transfection. Particle-based transfection includes the use of a gene gun, or magnet-assisted transfection (Bertram (2006) *Current Pharmaceutical Biotechnology* 7, 277–28). Viral methods can also be used for transfection.

[00161] Introduction of nucleic acids or proteins into a cell can also be mediated by electroporation, by intracytoplasmic injection, by viral infection, by adenovirus, by adeno-associated virus, by lentivirus, by retrovirus, by transfection, by lipid-mediated transfection, or

by nucleofection. Nucleofection is an improved electroporation technology that enables nucleic acid substrates to be delivered not only to the cytoplasm but also through the nuclear membrane and into the nucleus. In addition, use of nucleofection in the methods disclosed herein typically requires much fewer cells than regular electroporation (e.g., only about 2 million compared with 7 million by regular electroporation). In one example, nucleofection is performed using the LONZA® NUCLEOFECTOR™ system.

[00162] Introduction of molecules (e.g., nucleic acids or proteins) into a cell (e.g., a zygote) can also be accomplished by microinjection. In zygotes (i.e., one-cell stage embryos), microinjection can be into the maternal and/or paternal pronucleus or into the cytoplasm. If the microinjection is into only one pronucleus, the paternal pronucleus is preferable due to its larger size. Microinjection of an mRNA is preferably into the cytoplasm (e.g., to deliver mRNA directly to the translation machinery), while microinjection of a Cas protein or a polynucleotide encoding a Cas protein or encoding an RNA is preferable into the nucleus/pronucleus. Alternatively, microinjection can be carried out by injection into both the nucleus/pronucleus and the cytoplasm: a needle can first be introduced into the nucleus/pronucleus and a first amount can be injected, and while removing the needle from the one-cell stage embryo a second amount can be injected into the cytoplasm. If a Cas protein is injected into the cytoplasm, the Cas protein preferably comprises a nuclear localization signal to ensure delivery to the nucleus/pronucleus. Methods for carrying out microinjection are well known. *See, e.g.*, Nagy et al. (Nagy A, Gertsenstein M, Vintersten K, Behringer R., 2003, Manipulating the Mouse Embryo. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press); *see also* Meyer et al. (2010) *Proc. Natl. Acad. Sci. U.S.A.* 107:15022-15026 and Meyer et al. (2012) *Proc. Natl. Acad. Sci. U.S.A.* 109:9354-9359.

[00163] Other methods for introducing molecules (e.g., nucleic acid or proteins) into a cell or non-human animal can include, for example, vector delivery, particle-mediated delivery, exosome-mediated delivery, lipid-nanoparticle-mediated delivery, cell-penetrating-peptide-mediated delivery, or implantable-device-mediated delivery. As specific examples, a nucleic acid or protein can be introduced into a cell or non-human animal in a carrier such as a poly(lactic acid) (PLA) microsphere, a poly(D,L-lactic-coglycolic-acid) (PLGA) microsphere, a liposome, a micelle, an inverse micelle, a lipid cochleate, or a lipid microtubule. Some specific examples of delivery to a non-human animal include hydrodynamic delivery, virus-mediated delivery (e.g.,

adeno-associated virus (AAV)-mediated delivery), and lipid-nanoparticle-mediated delivery.

[00164] Introduction of nucleic acids and proteins into cells or non-human animals can be accomplished by hydrodynamic delivery (HDD). For gene delivery to parenchymal cells, only essential DNA sequences need to be injected via a selected blood vessel, eliminating safety concerns associated with current viral and synthetic vectors. When injected into the bloodstream, DNA is capable of reaching cells in the different tissues accessible to the blood. Hydrodynamic delivery employs the force generated by the rapid injection of a large volume of solution into the incompressible blood in the circulation to overcome the physical barriers of endothelium and cell membranes that prevent large and membrane-impermeable compounds from entering parenchymal cells. In addition to the delivery of DNA, this method is useful for the efficient intracellular delivery of RNA, proteins, and other small compounds *in vivo*. *See, e.g.*, Bonamassa et al. (2011) *Pharm. Res.* 28(4):694-701, herein incorporated by reference in its entirety for all purposes.

[00165] Introduction of nucleic acids can also be accomplished by virus-mediated delivery, such as AAV-mediated delivery or lentivirus-mediated delivery. Other exemplary viruses/viral vectors include retroviruses, adenoviruses, vaccinia viruses, poxviruses, and herpes simplex viruses. The viruses can infect dividing cells, non-dividing cells, or both dividing and non-dividing cells. The viruses can integrate into the host genome or alternatively do not integrate into the host genome. Such viruses can also be engineered to have reduced immunity. The viruses can be replication-competent or can be replication-defective (e.g., defective in one or more genes necessary for additional rounds of virion replication and/or packaging). Viruses can cause transient expression, long-lasting expression (e.g., at least 1 week, 2 weeks, 1 month, 2 months, or 3 months), or permanent expression (e.g., of Cas9 and/or gRNA). Exemplary viral titers (e.g., AAV titers) include about 10^{12}, about 10^{13}, about 10^{14}, about 10^{15}, and about 10^{16} vector genomes/mL. Other exemplary viral titers (e.g., AAV titers) include about 10^{12}, about 10^{13}, about 10^{14}, about 10^{15}, and about 10^{16} vector genomes(vg)/kg of body weight.

[00166] The ssDNA AAV genome consists of two open reading frames, Rep and Cap, flanked by two inverted terminal repeats that allow for synthesis of the complementary DNA strand. When constructing an AAV transfer plasmid, the transgene is placed between the two ITRs, and Rep and Cap can be supplied *in trans*. In addition to Rep and Cap, AAV can require a helper plasmid containing genes from adenovirus. These genes (E4, E2a, and VA) mediate AAV

replication. For example, the transfer plasmid, Rep/Cap, and the helper plasmid can be transfected into HEK293 cells containing the adenovirus gene E1+ to produce infectious AAV particles. Alternatively, the Rep, Cap, and adenovirus helper genes may be combined into a single plasmid. Similar packaging cells and methods can be used for other viruses, such as retroviruses.

[00167] Multiple serotypes of AAV have been identified. These serotypes differ in the types of cells they infect (i.e., their tropism), allowing preferential transduction of specific cell types. Serotypes for CNS tissue include AAV1, AAV2, AAV4, AAV5, AAV8, and AAV9. Serotypes for heart tissue include AAV1, AAV8, and AAV9. Serotypes for kidney tissue include AAV2. Serotypes for lung tissue include AAV4, AAV5, AAV6, and AAV9. Serotypes for pancreas tissue include AAV8. Serotypes for photoreceptor cells include AAV2, AAV5, and AAV8. Serotypes for retinal pigment epithelium tissue include AAV1, AAV2, AAV4, AAV5, and AAV8. Serotypes for skeletal muscle tissue include AAV1, AAV6, AAV7, AAV8, and AAV9. Serotypes for liver tissue include AAV7, AAV8, and AAV9, and particularly AAV8.

[00168] Tropism can be further refined through pseudotyping, which is the mixing of a capsid and a genome from different viral serotypes. For example AAV2/5 indicates a virus containing the genome of serotype 2 packaged in the capsid from serotype 5. Use of pseudotyped viruses can improve transduction efficiency, as well as alter tropism. Hybrid capsids derived from different serotypes can also be used to alter viral tropism. For example, AAV-DJ contains a hybrid capsid from eight serotypes and displays high infectivity across a broad range of cell types *in vivo*. AAV-DJ8 is another example that displays the properties of AAV-DJ but with enhanced brain uptake. AAV serotypes can also be modified through mutations. Examples of mutational modifications of AAV2 include Y444F, Y500F, Y730F, and S662V. Examples of mutational modifications of AAV3 include Y705F, Y731F, and T492V. Examples of mutational modifications of AAV6 include S663V and T492V. Other pseudotyped/modified AAV variants include AAV2/1, AAV2/6, AAV2/7, AAV2/8, AAV2/9, AAV2.5, AAV8.2, and AAV/SASTG.

[00169] To accelerate transgene expression, self-complementary AAV (scAAV) variants can be used. Because AAV depends on the cell's DNA replication machinery to synthesize the complementary strand of the AAV's single-stranded DNA genome, transgene expression may be delayed. To address this delay, scAAV containing complementary sequences that are capable of spontaneously annealing upon infection can be used, eliminating the requirement for host cell

DNA synthesis. However, single-stranded AAV (ssAAV) vectors can also be used.

[00170] To increase packaging capacity, longer transgenes may be split between two AAV transfer plasmids, the first with a 3' splice donor and the second with a 5' splice acceptor. Upon co-infection of a cell, these viruses form concatemers, are spliced together, and the full-length transgene can be expressed. Although this allows for longer transgene expression, expression is less efficient. Similar methods for increasing capacity utilize homologous recombination. For example, a transgene can be divided between two transfer plasmids but with substantial sequence overlap such that co-expression induces homologous recombination and expression of the full-length transgene.

[00171] Introduction of nucleic acids and proteins can also be accomplished by lipid nanoparticle (LNP)-mediated delivery. For example, LNP-mediated delivery can be used to deliver a combination of Cas mRNA and guide RNA or a combination of Cas protein and guide RNA. Delivery through such methods results in transient Cas expression, and the biodegradable lipids improve clearance, improve tolerability, and decrease immunogenicity. Lipid formulations can protect biological molecules from degradation while improving their cellular uptake. Lipid nanoparticles are particles comprising a plurality of lipid molecules physically associated with each other by intermolecular forces. These include microspheres (including unilamellar and multilamellar vesicles, e.g., liposomes), a dispersed phase in an emulsion, micelles, or an internal phase in a suspension. Such lipid nanoparticles can be used to encapsulate one or more nucleic acids or proteins for delivery. Formulations which contain cationic lipids are useful for delivering polyanions such as nucleic acids. Other lipids that can be included are neutral lipids (i.e., uncharged or zwitterionic lipids), anionic lipids, helper lipids that enhance transfection, and stealth lipids that increase the length of time for which nanoparticles can exist *in vivo*. Examples of suitable cationic lipids, neutral lipids, anionic lipids, helper lipids, and stealth lipids can be found in WO 2016/010840 A1, herein incorporated by reference in its entirety for all purposes. An exemplary lipid nanoparticle can comprise a cationic lipid and one or more other components. In one example, the other component can comprise a helper lipid such as cholesterol. In another example, the other components can comprise a helper lipid such as cholesterol and a neutral lipid such as DSPC. In another example, the other components can comprise a helper lipid such as cholesterol, an optional neutral lipid such as DSPC, and a stealth lipid such as S010, S024, S027, S031, or S033.

[00172] The LNP may contain one or more or all of the following: (i) a lipid for encapsulation and for endosomal escape; (ii) a neutral lipid for stabilization; (iii) a helper lipid for stabilization; and (iv) a stealth lipid. *See, e.g.*, Finn et al. (2018) *Cell Reports* 22:1-9 and WO 2017/173054 A1, each of which is herein incorporated by reference in its entirety for all purposes. In certain LNPs, the cargo can include a guide RNA or a nucleic acid encoding a guide RNA. In certain LNPs, the cargo can include an mRNA encoding a Cas nuclease, such as Cas9, and a guide RNA or a nucleic acid encoding a guide RNA.

[00173] The lipid for encapsulation and endosomal escape can be a cationic lipid. The lipid can also be a biodegradable lipid, such as a biodegradable ionizable lipid. One example of a suitable lipid is Lipid A or LP01, which is (9Z,12Z)-3-((4,4-bis(octyloxy)butanoyl)oxy)-2-((((3-(diethylamino)propoxy)carbonyl)oxy)methyl)propyl octadeca-9,12-dienoate, also called 3-((4,4-bis(octyloxy)butanoyl)oxy)-2-((((3-(diethylamino)propoxy)carbonyl)oxy)methyl)propyl (9Z,12Z)-octadeca-9,12-dienoate. *See, e.g.*, Finn et al. (2018) *Cell Reports* 22:1-9 and WO 2017/173054 A1, each of which is herein incorporated by reference in its entirety for all purposes. Another example of a suitable lipid is Lipid B, which is ((5-((dimethylamino)methyl)-1,3-phenylene)bis(oxy))bis(octane-8,1-diyl)bis(decanoate), also called ((5-((dimethylamino)methyl)-1,3-phenylene)bis(oxy))bis(octane-8,1-diyl)bis(decanoate). Another example of a suitable lipid is Lipid C, which is 2-((4-(((3-(dimethylamino)propoxy)carbonyl)oxy)hexadecanoyl)oxy)propane-1,3-diyl(9Z,9'Z,12Z,12'Z)-bis(octadeca-9,12-dienoate). Another example of a suitable lipid is Lipid D, which is 3-(((3-(dimethylamino)propoxy)carbonyl)oxy)-13-(octanoyloxy)tridecyl 3-octylundecanoate. Other suitable lipids include heptatriaconta-6,9,28,31-tetraen-19-yl 4-(dimethylamino)butanoate (also known as Dlin-MC3-DMA (MC3))).

[00174] Some such lipids suitable for use in the LNPs described herein are biodegradable *in vivo*. For example, LNPs comprising such a lipid include those where at least 75% of the lipid is cleared from the plasma within 8, 10, 12, 24, or 48 hours, or 3, 4, 5, 6, 7, or 10 days. As another example, at least 50% of the LNP is cleared from the plasma within 8, 10, 12, 24, or 48 hours, or 3, 4, 5, 6, 7, or 10 days.

[00175] Such lipids may be ionizable depending upon the pH of the medium they are in. For example, in a slightly acidic medium, the lipids may be protonated and thus bear a positive charge. Conversely, in a slightly basic medium, such as, for example, blood where pH is

approximately 7.35, the lipids may not be protonated and thus bear no charge. In some embodiments, the lipids may be protonated at a pH of at least about 9, 9.5, or 10. The ability of such a lipid to bear a charge is related to its intrinsic pKa. For example, the lipid may, independently, have a pKa in the range of from about 5.8 to about 6.2.

[00176] Neutral lipids function to stabilize and improve processing of the LNPs. Examples of suitable neutral lipids include a variety of neutral, uncharged or zwitterionic lipids. Examples of neutral phospholipids suitable for use in the present disclosure include, but are not limited to, 5-heptadecylbenzene-1,3-diol (resorcinol), dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidylcholine (DSPC), phosphocholine (DOPC), dimyristoylphosphatidylcholine (DMPC), phosphatidylcholine (PLPC), 1,2-distearoyl-sn-glycero-3-phosphocholine (DAPC), phosphatidylethanolamine (PE), egg phosphatidylcholine (EPC), dilauryloylphosphatidylcholine (DLPC), dimyristoylphosphatidylcholine (DMPC), 1-myristoyl-2-palmitoyl phosphatidylcholine (MPPC), 1-palmitoyl-2-myristoyl phosphatidylcholine (PMPC), 1-palmitoyl-2-stearoyl phosphatidylcholine (PSPC), 1,2-diarachidoyl-sn-glycero-3-phosphocholine (DBPC), 1-stearoyl-2-palmitoyl phosphatidylcholine (SPPC), 1,2-dieicosenoyl-sn-glycero-3-phosphocholine (DEPC), palmitoyloleoyl phosphatidylcholine (POPC), lysophosphatidyl choline, dioleoyl phosphatidylethanolamine (DOPE), dilinoleoylphosphatidylcholine distearoylphosphatidylethanolamine (DSPE), dimyristoyl phosphatidylethanolamine (DMPE), dipalmitoyl phosphatidylethanolamine (DPPE), palmitoyloleoyl phosphatidylethanolamine (POPE), lysophosphatidylethanolamine, and combinations thereof. For example, the neutral phospholipid may be selected from the group consisting of distearoylphosphatidylcholine (DSPC) and dimyristoyl phosphatidyl ethanolamine (DMPE).

[00177] Helper lipids include lipids that enhance transfection. The mechanism by which the helper lipid enhances transfection can include enhancing particle stability. In certain cases, the helper lipid can enhance membrane fusogenicity. Helper lipids include steroids, sterols, and alkyl resorcinols. Examples of suitable helper lipids suitable include cholesterol, 5-heptadecylresorcinol, and cholesterol hemisuccinate. In one example, the helper lipid may be cholesterol or cholesterol hemisuccinate.

[00178] Stealth lipids include lipids that alter the length of time the nanoparticles can exist in vivo. Stealth lipids may assist in the formulation process by, for example, reducing particle

aggregation and controlling particle size. Stealth lipids may modulate pharmacokinetic properties of the LNP. Suitable stealth lipids include lipids having a hydrophilic head group linked to a lipid moiety.

[00179] The hydrophilic head group of stealth lipid can comprise, for example, a polymer moiety selected from polymers based on PEG (sometimes referred to as poly(ethylene oxide)), poly(oxazoline), poly(vinyl alcohol), poly(glycerol), poly(N- vinylpyrrolidone), polyaminoacids, and poly N-(2-hydroxypropyl)methacrylamide. The term PEG means any polyethylene glycol or other polyalkylene ether polymer. In certain LNP formulations, the PEG, is a PEG-2K, also termed PEG 2000, which has an average molecular weight of about 2,000 daltons. *See, e.g.,* WO 2017/173054 A1, herein incorporated by reference in its entirety for all purposes.

[00180] The lipid moiety of the stealth lipid may be derived, for example, from diacylglycerol or diacylglycamide, including those comprising a dialkylglycerol or dialkylglycamide group having alkyl chain length independently comprising from about C4 to about C40 saturated or unsaturated carbon atoms, wherein the chain may comprise one or more functional groups such as, for example, an amide or ester. The dialkylglycerol or dialkylglycamide group can further comprise one or more substituted alkyl groups.

[00181] As one example, the stealth lipid may be selected from PEG-dilauroylglycerol, PEG-dimyristoylglycerol (PEG-DMG), PEG-dipalmitoylglycerol, PEG-distearoylglycerol (PEG-DSPE), PEG-dilaurylglycamide, PEG- dimyristylglycamide, PEG-dipalmitoylglycamide, and PEG-distearoylglycamide, PEG- cholesterol (l-[8'-(Cholest-5-en-3[beta]-oxy)carboxamido-3',6'-dioxaoctanyl]carbamoyl-[omega]-methyl-poly(ethylene glycol), PEG-DMB (3,4-ditetradecoxylbenzyl-[omega]-methyl-poly(ethylene glycol)ether), 1,2-dimyristoyl-sn- glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (PEG2k- DMG), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (PEG2k-DSPE), 1,2-distearoyl-sn-glycerol, methoxypoly ethylene glycol (PEG2k-DSG), poly(ethylene glycol)-2000-dimethacrylate (PEG2k-DMA), and 1,2- distearyloxypropyl-3-amine-N-[methoxy(polyethylene glycol)-2000] (PEG2k-DSA). In one particular example, the stealth lipid may be PEG2k-DMG.

[00182] The LNPs can comprise different respective molar ratios of the component lipids in the formulation. The mol-% of the CCD lipid may be, for example, from about 30 mol-% to about 60 mol-%, from about 35 mol-% to about 55 mol-%, from about 40 mol-% to about 50

mol-%, from about 42 mol-% to about 47 mol-%, or about 45%. The mol-% of the helper lipid may be, for example, from about 30 mol-% to about 60 mol-%, from about 35 mol-% to about 55 mol-%, from about 40 mol-% to about 50 mol-%, from about 41 mol-% to about 46 mol-%, or about 44 mol-%. The mol-% of the neutral lipid may be, for example, from about 1 mol-% to about 20 mol-%, from about 5 mol-% to about 15 mol-%, from about 7 mol-% to about 12 mol-%, or about 9 mol-%. The mol-% of the stealth lipid may be, for example, from about 1 mol-% to about 10 mol-%, from about 1 mol-% to about 5 mol-%, from about 1 mol-% to about 3 mol-%, about 2 mol-%, or about 1 mol-%.

[00183] The LNPs can have different ratios between the positively charged amine groups of the biodegradable lipid (N) and the negatively charged phosphate groups (P) of the nucleic acid to be encapsulated. This may be mathematically represented by the equation N/P. For example, the N/P ratio may be from about 0.5 to about 100, from about 1 to about 50, from about 1 to about 25, from about 1 to about 10, from about 1 to about 7, from about 3 to about 5, from about 4 to about 5, about 4, about 4.5, or about 5. The N/P ratio can also be from about 4 to about 7 or from about 4.5 to about 6. In specific examples, the N/P ratio can be 4.5 or can be 6.

[00184] In some LNPs, the cargo can comprise Cas mRNA and gRNA. The Cas mRNA and gRNAs can be in different ratios. For example, the LNP formulation can include a ratio of Cas mRNA to gRNA nucleic acid ranging from about 25:1 to about 1:25, ranging from about 10:1 to about 1:10, ranging from about 5:1 to about 1:5, or about 1:1. Alternatively, the LNP formulation can include a ratio of Cas mRNA to gRNA nucleic acid from about 1:1 to about 1:5, or about 10:1. Alternatively, the LNP formulation can include a ratio of Cas mRNA to gRNA nucleic acid of about 1:10, 25:1, 10:1, 5:1, 3:1, 1:1, 1:3, 1:5, 1:10, or 1:25. Alternatively, the LNP formulation can include a ratio of Cas mRNA to gRNA nucleic acid of from about 1:1 to about 1:2. In specific examples, the ratio of Cas mRNA to gRNA can be about 1:1 or about 1:2.

[00185] Exemplary dosing of LNPs includes, for example, about 0.1, about 0.25, about 0.3, about 0.5, about 1, about 2, about 3, about 4, about 5, about 6, about 8, or about 10 mg/kg (mpk) with respect to total RNA (e.g., Cas9 mRNA and gRNA) cargo content. In one example, LNP doses between about 0.01 mg/kg and about 10 mg/kg, between about 0.1 and about 10 mg/kg, or between about 0.01 and about 0.3 mg/kg can be used. For example, LNP doses of about 0.01, about 0.03, about 0.1, about 0.3, about 1, about 3, or about 10 mg/kg can be used.

[00186] In some LNPs, the cargo can comprise exogenous donor nucleic acid and gRNA. The

exogenous donor nucleic acid and gRNAs can be in different ratios. For example, the LNP formulation can include a ratio of exogenous donor nucleic acid to gRNA nucleic acid ranging from about 25:1 to about 1:25, ranging from about 10:1 to about 1:10, ranging from about 5:1 to about 1:5, or about 1:1. Alternatively, the LNP formulation can include a ratio of exogenous donor nucleic acid to gRNA nucleic acid from about 1:1 to about 1:5, about 5:1 to about 1:1, about 10:1, or about 1:10. Alternatively, the LNP formulation can include a ratio of exogenous donor nucleic acid to gRNA nucleic acid of about 1:10, 25:1, 10:1, 5:1, 3:1, 1:1, 1:3, 1:5, 1:10, or 1:25.

[00187] A specific example of a suitable LNP has a nitrogen-to-phosphate (N/P) ratio of 4.5 and contains biodegradable cationic lipid, cholesterol, DSPC, and PEG2k-DMG in a 45:44:9:2 molar ratio. The biodegradable cationic lipid can be (9Z,12Z)-3-((4,4-bis(octyloxy)butanoyl)oxy)-2-((((3-(diethylamino)propoxy)carbonyl)oxy)methyl)propyl octadeca-9,12-dienoate, also called 3-((4,4-bis(octyloxy)butanoyl)oxy)-2-((((3-(diethylamino)propoxy)carbonyl)oxy)methyl)propyl (9Z,12Z)-octadeca-9,12-dienoate. *See, e.g.*, Finn et al. (2018) *Cell Reports* 22:1-9, herein incorporated by reference in its entirety for all purposes. The Cas9 mRNA can be in a 1:1 ratio by weight to the guide RNA. Another specific example of a suitable LNP contains Dlin-MC3-DMA (MC3), cholesterol, DSPC, and PEG-DMG in a 50:38.5:10:1.5 molar ratio.

[00188] Another specific example of a suitable LNP has a nitrogen-to-phosphate (N/P) ratio of 6 and contains biodegradable cationic lipid, cholesterol, DSPC, and PEG2k-DMG in a 50:38:9:3 molar ratio. The biodegradable cationic lipid can be (9Z,12Z)-3-((4,4-bis(octyloxy)butanoyl)oxy)-2-((((3-(diethylamino)propoxy)carbonyl)oxy)methyl)propyl octadeca-9,12-dienoate, also called 3-((4,4-bis(octyloxy)butanoyl)oxy)-2-((((3-(diethylamino)propoxy)carbonyl)oxy)methyl)propyl (9Z,12Z)-octadeca-9,12-dienoate. The Cas9 mRNA can be in a 1:2 ratio by weight to the guide RNA.

[00189] The mode of delivery can be selected to decrease immunogenicity. For example, a Cas protein and a gRNA may be delivered by different modes (e.g., bi-modal delivery). These different modes may confer different pharmacodynamics or pharmacokinetic properties on the subject delivered molecule (e.g., Cas or nucleic acid encoding, gRNA or nucleic acid encoding, or exogenous donor nucleic acid/repair template). For example, the different modes can result in different tissue distribution, different half-life, or different temporal distribution. Some modes of

delivery (e.g., delivery of a nucleic acid vector that persists in a cell by autonomous replication or genomic integration) result in more persistent expression and presence of the molecule, whereas other modes of delivery are transient and less persistent (e.g., delivery of an RNA or a protein). Delivery of Cas proteins in a more transient manner, for example as mRNA or protein, can ensure that the Cas/gRNA complex is only present and active for a short period of time and can reduce immunogenicity caused by peptides from the bacterially-derived Cas enzyme being displayed on the surface of the cell by MHC molecules. Such transient delivery can also reduce the possibility of off-target modifications.

[00190] Administration *in vivo* can be by any suitable route including, for example, parenteral, intravenous, oral, subcutaneous, intra-arterial, intracranial, intrathecal, intraperitoneal, topical, intranasal, or intramuscular. Systemic modes of administration include, for example, oral and parenteral routes. Examples of parenteral routes include intravenous, intraarterial, intraosseous, intramuscular, intradermal, subcutaneous, intranasal, and intraperitoneal routes. A specific example is intravenous infusion. Nasal instillation and intravitreal injection are other specific examples. Local modes of administration include, for example, intrathecal, intracerebroventricular, intraparenchymal (e.g., localized intraparenchymal delivery to the striatum (e.g., into the caudate or into the putamen), cerebral cortex, precentral gyrus, hippocampus (e.g., into the dentate gyrus or CA3 region), temporal cortex, amygdala, frontal cortex, thalamus, cerebellum, medulla, hypothalamus, tectum, tegmentum, or substantia nigra), intraocular, intraorbital, subconjuctival, intravitreal, subretinal, and transscleral routes. Significantly smaller amounts of the components (compared with systemic approaches) may exert an effect when administered locally (for example, intraparenchymal or intravitreal) compared to when administered systemically (for example, intravenously). Local modes of administration may also reduce or eliminate the incidence of potentially toxic side effects that may occur when therapeutically effective amounts of a component are administered systemically.

[00191] Administration *in vivo* can be by any suitable route including, for example, parenteral, intravenous, oral, subcutaneous, intra-arterial, intracranial, intrathecal, intraperitoneal, topical, intranasal, or intramuscular. A specific example is intravenous infusion. Compositions comprising the guide RNAs and/or Cas proteins (or nucleic acids encoding the guide RNAs and/or Cas proteins) can be formulated using one or more physiologically and pharmaceutically

acceptable carriers, diluents, excipients or auxiliaries. The formulation can depend on the route of administration chosen. The term "pharmaceutically acceptable" means that the carrier, diluent, excipient, or auxiliary is compatible with the other ingredients of the formulation and not substantially deleterious to the recipient thereof.

[00192] The frequency of administration and the number of dosages can depend on the half-life of the exogenous donor nucleic acids, guide RNAs, or Cas proteins (or nucleic acids encoding the guide RNAs or Cas proteins) and the route of administration among other factors. The introduction of nucleic acids or proteins into the cell or non-human animal can be performed one time or multiple times over a period of time. For example, the introduction can be performed at least two times over a period of time, at least three times over a period of time, at least four times over a period of time, at least five times over a period of time, at least six times over a period of time, at least seven times over a period of time, at least eight times over a period of time, at least nine times over a period of times, at least ten times over a period of time, at least eleven times, at least twelve times over a period of time, at least thirteen times over a period of time, at least fourteen times over a period of time, at least fifteen times over a period of time, at least sixteen times over a period of time, at least seventeen times over a period of time, at least eighteen times over a period of time, at least nineteen times over a period of time, or at least twenty times over a period of time.

E. Measuring Delivery, Activity, or Efficacy of Human-KLKB1-Targeting Reagents *In Vivo* or *Ex Vivo*

[00193] The methods disclosed herein can further comprise detecting or measuring activity of human-KLKB1-targeting reagents. Measuring the activity of such reagents can comprise, for example, measuring *in vivo* dextran sulfate and captopril-induced vascular permeability (e.g., with increased vascular permeability (i.e., leakage of Evans Blue intravital dye into the GI tract) reflecting increased plasma kallikrein activity) or measuring *in vitro* plasma kallikrein activity (e.g., assessing generation of kallikrein chromogenic substrate that correlates kallikrein activity following plasma prekallikrein activation). As one example, assessing activity can comprise using a plasma kallikrein activity assay in plasma samples activated by dextran sulfate. In the *in vivo* model, for example, dextran sulfate can be administered intravenously with captopril into mice followed by intravenous dosage of Evans Blue intravital dye to assess vascular

permeability in the gastrointestinal tract. In the *in vitro* model, for example, a known plasma kallikrein activity assay can be performed using a plasma-kallikrein-specific substrate linked to a chromogen (e.g., a fluorescence peptide substrate for the detection of plasma kallikrein activity).

[00194] If the human-KLKB1-targeting reagent is a genome editing reagent, the measuring can comprise assessing the humanized *KLKB1* locus for modifications. Various methods can be used to identify cells having a targeted genetic modification. The screening can comprise a quantitative assay for assessing modification-of-allele (MOA) of a parental chromosome. *See, e.g.*, US 2004/0018626; US 2014/0178879; US 2016/0145646; WO 2016/081923; and Frendewey et al. (2010) *Methods Enzymol.* 476:295-307, each of which is herein incorporated by reference in its entirety for all purposes. For example, the quantitative assay can be carried out via a quantitative PCR, such as a real-time PCR (qPCR). The real-time PCR can utilize a first primer set that recognizes the target locus and a second primer set that recognizes a non-targeted reference locus. The primer set can comprise a fluorescent probe that recognizes the amplified sequence. Other examples of suitable quantitative assays include fluorescence-mediated in situ hybridization (FISH), comparative genomic hybridization, isothermic DNA amplification, quantitative hybridization to an immobilized probe(s), INVADER® Probes, TAQMAN® Molecular Beacon probes, or ECLIPSE™ probe technology (*see, e.g.*, US 2005/0144655, herein incorporated by reference in its entirety for all purposes). Next-generation sequencing (NGS) can also be used for screening. Next-generation sequencing can also be referred to as "NGS" or "massively parallel sequencing" or "high throughput sequencing." NGS can be used as a screening tool in addition to the MOA assays to define the exact nature of the targeted genetic modification and whether it is consistent across cell types or tissue types or organ types.

[00195] If the reagent is designed to inactivate the humanized *KLKB1* locus, affect expression of the humanized *KLKB1* locus, or prevent translation of the humanized *KLKB1* mRNA, the measuring can comprise assessing humanized *KLKB1* mRNA or humanized plasma kallikrein protein expression.

[00196] The assessing in a non-human animal can be in any cell type from any tissue or organ. For example, the assessment can be in multiple cell types from the same tissue or organ (e.g., liver) or in cells from multiple locations within the tissue or organ. This can provide information about which cell types within a target tissue or organ are being targeted or which sections of a tissue or organ are being reached by the human-KLKB1-targeting reagent. As

another example, the assessment can be in multiple types of tissue or in multiple organs. In methods in which a particular tissue, organ, or cell type is being targeted, this can provide information about how effectively that tissue or organ is being targeted and whether there are off-target effects in other tissues or organs.

[00197] One example of an assay that can be used are the RNASCOPE™ and BASESCOPE™ RNA *in situ* hybridization (ISH) assays, which are methods that can quantify cell-specific edited transcripts, including single nucleotide changes, in the context of intact fixed tissue. The BASESCOPE™ RNA ISH assay can complement NGS and qPCR in characterization of gene editing. Whereas NGS/qPCR can provide quantitative average values of wild type and edited sequences, they provide no information on heterogeneity or percentage of edited cells within a tissue. The BASESCOPE™ ISH assay can provide a landscape view of an entire tissue and quantification of wild type versus edited transcripts with single-cell resolution, where the actual number of cells within the target tissue containing the edited mRNA transcript can be quantified. The BASESCOPE™ assay achieves single-molecule RNA detection using paired oligo ("ZZ") probes to amplify signal without non-specific background. However, the BASESCOPE™ probe design and signal amplification system enables single-molecule RNA detection with a ZZ probe, and it can differentially detect single nucleotide edits and mutations in intact fixed tissue.

[00198] All patent filings, websites, other publications, accession numbers and the like cited above or below are incorporated by reference in their entirety for all purposes to the same extent as if each individual item were specifically and individually indicated to be so incorporated by reference. If different versions of a sequence are associated with an accession number at different times, the version associated with the accession number at the effective filing date of this application is meant. The effective filing date means the earlier of the actual filing date or filing date of a priority application referring to the accession number if applicable. Likewise, if different versions of a publication, website or the like are published at different times, the version most recently published at the effective filing date of the application is meant unless otherwise indicated. Any feature, step, element, embodiment, or aspect of the invention can be used in combination with any other unless specifically indicated otherwise. Although the present invention has been described in some detail by way of illustration and example for purposes of clarity and understanding, it will be apparent that certain changes and modifications may be

practiced within the scope of the appended claims.

BRIEF DESCRIPTION OF THE SEQUENCES

[00199] The nucleotide and amino acid sequences listed in the accompanying sequence listing are shown using standard letter abbreviations for nucleotide bases, and three-letter code for amino acids. The nucleotide sequences follow the standard convention of beginning at the 5' end of the sequence and proceeding forward (i.e., from left to right in each line) to the 3' end. Only one strand of each nucleotide sequence is shown, but the complementary strand is understood to be included by any reference to the displayed strand. When a nucleotide sequence encoding an amino acid sequence is provided, it is understood that codon degenerate variants thereof that encode the same amino acid sequence are also provided. The amino acid sequences follow the standard convention of beginning at the amino terminus of the sequence and proceeding forward (i.e., from left to right in each line) to the carboxy terminus.

[00200] Table 2. Description of Sequences.

SEQ ID NO	Type	Description
1	Protein	Mouse Plasma Kallikrein (UniProt P26262-1; NCBI NP_032481.2)
2	Protein	Mouse Plasma Kallikrein Signal Peptide
3	Protein	Human Plasma Kallikrein (NCBI NP_000883.2)
4	Protein	Human Plasma Kallikrein Signal Peptide
5	DNA	Mouse Plasma Kallikrein CDS (NCBI CCDS22275.1)
6	DNA	Mouse Plasma Kallikrein Signal Peptide CDS
7	DNA	Human Plasma Kallikrein CDS (NCBI CCDS34120.1)
8	DNA	Human Plasma Kallikrein Signal Peptide CDS
9	DNA	MAID 7700 (*KLKB1* Humanized Region with Neo-Self-Deleting Cassette)
10	DNA	MAID 7701 (*KLKB1* Humanized Region without Neo-Self-Deleting Cassette)
11	DNA	Human *KLKB1* Sequence at Humanized *KLKB1* Locus
12	DNA	Mouse *Klkb1* mRNA (NM_008455.3)
13	DNA	Human *KLKB1* mRNA (NM_000892.5)
14	Protein	Human Plasma Kallikrein (UniProt P03952-1)
15	Protein	Rat Plasma Kallikrein (NCBI NP_036857.2)
16	DNA	Rat *Klkb1* mRNA (NM_012725.2)
17	DNA	Rat Plasma Kallikrein CDS
18	Protein	Rat Plasma Kallikrein (UniProt P14272-1)
19	Protein	Mouse Plasma Kallikrein Heavy Chain
20	Protein	Mouse Plasma Kallikrein Light Chain
21	DNA	Mouse Plasma Kallikrein Heavy Chain CDS
22	DNA	Mouse Plasma Kallikrein Light Chain CDS
23	Protein	Human Plasma Kallikrein Heavy Chain
24	Protein	Human Plasma Kallikrein Light Chain
25	DNA	Human Plasma Kallikrein Heavy Chain CDS
26	DNA	Human Plasma Kallikrein Light Chain CDS
27	Protein	Human Plasma Kallikrein Heavy Chain v2
28-39	DNA	Primers and Probes for LOA and GOA Assays

EXAMPLES

Example 1. Generation of Mice Comprising a Humanized *KLKB1* Locus

[00201] A large targeting vector (LTVEC) comprising a 5' homology arm comprising 120.3 kb of the mouse *Klkb1* locus and 3' homology arm comprising 51.4 kb of the mouse *Klkb1* locus was generated to replace a region of 24.2 kb from the mouse *Klkb1* gene encoding the mouse plasma kallikrein protein with 30.0 kb of the corresponding sequence of the human *KLKB1* gene. Information on mouse and human *KLKB1* genes is provided in **Table 3**. A description of the generation of the large targeting vector is provided in **Table 4**. Generation and use of large targeting vectors (LTVECs) derived from bacterial artificial chromosome (BAC) DNA through bacterial homologous recombination (BHR) reactions using VELOCIGENE® genetic engineering technology is described, e.g., in US 6,586,251 and Valenzuela et al. (2003) *Nat. Biotechnol.* 21(6):652-659, each of which is herein incorporated by reference in its entirety for

all purposes. Generation of LTVECs through *in vitro* assembly methods is described, e.g., in US 2015/0376628 and WO 2015/200334, each of which is herein incorporated by reference in its entirety for all purposes.

[00202] Table 3. Mouse and Human *KLKB1*.

	Gene Symbol	NCBI Gene ID	RefSeq mRNA ID	UniProt ID	Genomic Assembly	Chromosomal Location
Mouse	Klkb1	16621	NM008455	P26262	RGCm38/mm10	chr8:45,266,689-45,294,859 (-)
Human	KLKB1	3818	NM000892	P03952	GRCh38/hg38	chr4:186,227,507-186,258,471 (+)

[00203] Table 4. Mouse *Klkb1* Large Targeting Vector.

	Genome Build	Start	End	Length (bp)
5' Mouse Arm	RGCm38/mm10	chr8:45,368196	Chr8: 45,294,132	120,340
Human Insert	GRCh38/hg38	Chr4: 186,228,196	Chr9: 186,258,212	30,017
3' Mouse Arm	RGCm38/mm10	Chr8: 45,269,341	Chr8: 45,217,948	51,394

[00204] Specifically, a region starting in exon 2 (coding exon 1; from amino acid 1) through the stop codon in exon 15, including all the introns from introns 2 through 14, was deleted from the mouse *Klkb1* locus. A region from the human *KLKB1* locus including exon 2/coding exon 1 (from amino acid 1) through the stop codon in exon 15, including all the introns from introns 2 through 14, was inserted in place of the deleted mouse region. Mouse exon 1 (non-coding; 5' UTR) and the mouse 3' UTR were retained. A loxP-mPrm1-Crei-pA-hUb1-em7-Neo-pA-loxP cassette was inserted downstream of the mouse 3' UTR. This is the MAID 7700 allele (SEQ ID NO: 9). *See* **Figure 1**. After cassette deletion, loxP and cloning sites remained downstream of the mouse 3' UTR. This is the MAID 7701 allele (SEQ ID NO: 10). *See* **Figure 1**.

[00205] Sequences for the mouse plasma kallikrein signal peptide, heavy chain, and light chain are set forth in SEQ ID NOS: 2, 19, and 20, respectively, with the corresponding coding sequence set forth in SEQ ID NOS: 6, 21, and 22, respectively. Sequences for the human plasma kallikrein signal peptide, heavy chain, and light chain are set forth in SEQ ID NOS: 4, 23, and 24, respectively, with the corresponding coding sequences set forth in SEQ ID NOS: 8, 25, and 26, respectively. The expected encoded humanized plasma kallikrein protein has a human signal peptide, a human heavy chain, and a human light chain. *See* **Figure 1**. An alignment of the mouse and human plasma kallikrein proteins is provided in **Figure 3**. The mouse and human *KLKB1* coding sequences are set forth in SEQ ID NOS: 5 and 7, respectively. The mouse and human plasma kallikrein protein sequences are set forth in SEQ ID NOS: 1 and 3, respectively.

The sequences for the expected humanized *KLKB1* coding sequence and the expected humanized plasma kallikrein protein are set forth in SEQ ID NOS: 7 and 3, respectively.

[00206] To generate the mutant allele, the large targeting vector described above was introduced into F1H4 mouse embryonic stem (ES) cells. F1H4 mouse ES cells were derived from hybrid embryos produced by crossing a female C57BL/6NTac mouse to a male 129S6/SvEvTac mouse. *See, e.g.*, US 2015-0376651 and WO 2015/200805, each of which is herein incorporated by reference in its entirety for all purposes. Specifically, 1.8 x 10^6 mouse ES cells (line F1H4) were electroporated with 0.4 mg Klkb1 LTVEC. The electroporation conditions were: 400 V voltage; 100 mF capacitance; and 0 W resistance. Antibiotic selection was performed using G418 at a concentration of 75 mg/mL. Following antibiotic selection, colonies were picked, expanded, and screened by TAQMAN®. *See* **Figure 2**. Loss-of-allele assays were performed to detect loss of the endogenous mouse allele, and gain-of-allele assays were performed to detect gain of the humanized allele using the primers and probes set forth in **Table 5**.

[00207] Table 5. Screening Assays.

Assay	Description	Primer/ Probe	Sequence	SEQ ID NO
mTU	Upstream LOA	Fwd	ACCTGCTTTGGGTTTCACA	28
		Probe (FAM)	ATAGTATCCCTTTGGCAGTCTGGAGGG	29
		Rev	GCACTGACATCGAGTGTTGA	30
mTD	Downstream LOA	Fwd	AGGAGGATGCCTGAGATCATAGA	31
		Probe (Cal-Orange)	AACAAGTCTGCAGAGGCTTGGGTGC	32
		Rev	CGTGCTGCCTTCCTTCTAGTG	33
hTU	Upstream Human Insertion	Fwd	GTCCCTCAACCCTGATTTCTC	34
		Probe (FAM)	AAACCGTAATTTACAAACCCATGTGCAA	35
		Rev	CTCTGGCTTATGCTCCTTCTCA	36
hTD	Downstream Human Insertion	Fwd	CCACCCGCTCCTCAGTGTT	37
		Probe (Cal-Orange)	AGTAGCGTTCCCGTCTCCCAAA	38
		Rev	TCCCGGCCATTAGCATCAAG	39

[00208] Modification-of-allele (MOA) assays including loss-of-allele (LOA) and gain-of-allele (GOA) assays are described, for example, in US 2014/0178879; US 2016/0145646; WO 2016/081923; and Frendewey et al. (2010) *Methods Enzymol.* 476:295-307, each of which is herein incorporated by reference in its entirety for all purposes. The loss-of-allele (LOA) assay inverts the conventional screening logic and quantifies the number of copies in a genomic DNA sample of the native locus to which the mutation was directed. In a correctly targeted heterozygous cell clone, the LOA assay detects one of the two native alleles (for genes not on the X or Y chromosome), the other allele being disrupted by the targeted modification. The same

principle can be applied in reverse as a gain-of-allele (GOA) assay to quantify the copy number of the inserted targeting vector in a genomic DNA sample.

[00209] F0 mice were generated from the modified ES cells using the VELOCIMOUSE® method. Specifically, mouse ES cell clones comprising the humanized *KLKB1* locus described above that were selected by the MOA assay described above were injected into 8-cell stage embryos using the VELOCIMOUSE® method. *See, e.g.*, US 7,576,259; US 7,659,442; US 7,294,754; US 2008/0078000; and Poueymirou et al. (2007) *Nat. Biotechnol.* 25(1):91-99, each of which is herein incorporated by reference in its entirety for all purposes. In the VELOCIMOUSE® method, targeted mouse ES cells are injected through laser-assisted injection into pre-morula stage embryos, e.g., eight-cell-stage embryos, which efficiently yields F0 generation mice that are fully ES-cell-derived. In the VELOCIMOUSE® method, the injected pre-morula stage embryos are cultured to the blastocyst stage, and the blastocyst-stage embryos are introduced into and gestated in surrogate mothers to produce the F0 generation mice. When starting with mouse ES cell clones homozygous for the targeted modification, F0 mice homozygous for the targeted modification are produced. When starting with mouse ES cell clones heterozygous for the targeted modification, subsequent breeding can be performed to produce mice homozygous for the targeted modification.

[00210] The humanized *KLKB1* mice that were generated expressed humanized plasma kallikrein as shown by ELISA. *See, e.g.*, **Figures 5** , **6**, and **8B**. The humanized *KLKB1* mice were further validated using a plasma kallikrein activity assay in plasma samples activated by dextran sulfate. The assay confirmed plasma kallikrein activity (data not shown).

Example 2. Testing Human *KLKB1* Guide RNAs *In Vivo* in Humanized *KLKB1* Mice

[00211] The cassette-deleted humanized *KLKB1* mice generated in Example 1 (MAID 7701) were used to test several different guide RNAs targeting human *KLKB1 in vivo*. Animals were weighed and dosed at volumes specific to individual body weight. There were 5 groups total (N=4 with 2 male and 2 female mice). For each different guide RNA, lipid nanoparticles comprising Cas9 mRNA and the guide RNA targeting human *KLKB1* were administered to the humanized *KLKB1* mice via the lateral tail vein at 0.3 mg/kg based on total RNA cargo in a volume of 10 mL per kilogram body weight. At day 10 post-treatment, mice were euthanized and liver tissue was collected for DNA extraction. The tissues were lysed, and DNA was extracted.

The extracted DNA was subject to PCR to be submitted for sequencing. Blood was collected into serum separator tubes and allowed to clot for 2 hours at room temperature followed by centrifugation. Percent editing at the humanized *KLKB1* locus in the liver was measured, and serum levels of plasma kallikrein were measured.

[00212] To quantitatively determine the efficiency of editing at the target location in the genome, deep sequencing was utilized to identify the presence of insertions and deletions introduced by gene editing. PCR primers were designed around the target site within the humanized *KLKB1* locus, and the genomic area of interest was amplified. The editing percentage (e.g., the editing efficiency or percent editing) is the total number of sequence reads with insertions or deletions ("indels") over the total number of sequence reads, including wild type. Percent editing at the humanized *KLKB1* locus is shown in **Figure 4** and **Table 6**.

[00213] **Table 6. *In Vivo* Editing Data in Humanized *KLKB1* Mice.**

Guide	% Editing	Editing SD	N
1	32.9	10.96	4
2	72.83	1.17	4
3	43.05	5.59	4
4	14.38	5.60	4
5	35.53	11.11	4

[00214] Serum kallikrein levels in humanized *KLKB1* mice pre-dose and post-dose were measured using an ELISA assay. Total secreted KLKB1 protein levels were determined using a prekallikrein ELISA kit (Abcam, Cat. ab202405), which detects prekallikrein and kallikrein (total kallikrein). The results are shown in **Figure 5** and **Table 7**.

[00215] **Table 7. Secreted KLKB1 Protein Levels in Humanized *KLKB1* Mice.**

Guide	Dose (mpk)	Pre-Dose (µg/mL)	SD	Post-Dose (µg/mL)	SD	N
1	0.3	19.04	6.20	13.10	7.96	4
2	0.3	22.48	9.32	1.42	0.41	4
3	0.3	18.54	5.41	7.59	2.10	4
4	0.3	21.21	9.98	23.11	8.58	4
5	0.3	18.07	5.21	11.22	4.51	4

[00216] Serum kallikrein levels in humanized *KLKB1* mice were also measured by an immunoassay using an electrochemiluminescence detection platform by MesoScale Discovery (MSD) and compared to baseline or basal levels. The results are shown in **Figure 6** and **Table 8**.

[00217] Table 8. Secreted KLKB1 Protein Levels in Humanized *KLKB1* Mice.

Guide	Dose (mpk)	Pre-Dose (µg/mL)	SD	Post-Dose (µg/mL)	SD	% Serum KD	N
1	0.3	10.44	0.43	6.84	0.09	38	4
2	0.3	10.59	0.37	BLOD*	-	97**	2
3	0.3	10.74	0.24	3.92	0.04	64	4
4	0.3	13.86	0.34	7.78	0.35	35**	4
5	0.3	8.38	0.10	3.92	0.16	55	4

* Below limit of detection; ** approximate

[00218] *KLKB1* mRNA levels for each sample were measured by quantitative PCR and are shown in **Figure 7** and **Table 9**. Protein reduction was confirmed by western blot analysis.

[00219] Table 9. qPCR Results.

Guide	Fold Change	SD	N
1	1.20	0.35	4
2	0.51	0.41	4
3	0.73	0.22	4
4	1.10	0.23	4
5	1.18	0.41	4
TSS Control	1.01	0.17	2

[00220] The cassette-deleted humanized *KLKB1* mice generated in Example 1 (MAID 7701) were used again to test several different guide RNAs targeting human *KLKB1 in vivo*. Animals were weighed and dosed at volumes specific to individual body weight. There were 5 groups total (N=5 with 2 male and 3 female mice). For each different guide RNA, lipid nanoparticles comprising Cas9 mRNA and the guide RNA targeting human *KLKB1* were administered to the humanized *KLKB1* mice via the lateral tail vein at 0.3 mg/kg based on total RNA cargo in a volume of 10 mL per kilogram body weight. At day 13 post-treatment, mice were euthanized and liver tissue was collected for DNA extraction. The tissues were lysed, and DNA was extracted. The extracted DNA was subject to PCR to be submitted for sequencing. Blood was collected into serum separator tubes and allowed to clot for 2 hours at room temperature followed by centrifugation. Percent editing at the humanized *KLKB1* locus in the liver was measured, and serum levels of plasma kallikrein were measured. **Figures 8A-8D** and **Table 10** show editing data, serum kallikrein levels, and serum kallikrein levels as a percentage of control (TSS) levels.

[00221] Table 10. Percent Editing and Serum Kallikrein Levels in Humanized *KLKB1* Mice.

Dose (mpk)	Guide	Sample	% Editing	Serum Kallikrein (µg/mL)	Serum Kallikrein (% TSS Mean)
0	TSS	Mean	0.1	19.49	100
		Animal 1	0.1	14.77	76
		Animal 2	0.1	18.29	94
		Animal 3	0.1	14.82	76
		Animal 4	0.1	26.82	138
		Animal 5	0.1	22.76	117
0.3	6	Mean	21.9	12.4	64
		Animal 1	24.7	9.53	49
		Animal 2	21.0	9.88	51
		Animal 3	29.3	7.94	41
		Animal 4	18.8	20.32	104
		Animal 5	15.9	14.34	74
	7	Mean	26.0	10.58	54
		Animal 1	20.6	9.09	47
		Animal 2	32.5	8.85	45
		Animal 3	27.9	8.13	42
		Animal 4	22.7	14.08	72
		Animal 5	26.5	12.75	65
	10	Mean	24.2	10.88	56
		Animal 1	41.0	5.83	30
		Animal 2	20.3	9.96	51
		Animal 3	27.6	7.42	38
		Animal 4	9.4	17.83	91
		Animal 5	22.8	13.36	69
	11	Mean	24.0	10.91	56
		Animal 1	29.8	7.15	37
		Animal 2	35.2	6.99	36
		Animal 3	28.6	6.16	32
		Animal 4	18.2	17.21	88
		Animal 5	8.0	17.07	88
	12	Mean	13.8	14.71	75
		Animal 1	21.4	10.70	55
		Animal 2	24.0	8.92	46
		Animal 3	4.8	20.46	105
		Animal 4	8.9	17.34	89
		Animal 5	9.7	16.11	83
	13	Mean	15.9	15.4	79
		Animal 1	21.1	10.38	53
		Animal 2	15.9	9.69	50
		Animal 3	15.2	15.37	79
		Animal 4	14.9	19.13	98
		Animal 5	12.4	22.44	115
	14	Mean	36.9	9.05	46
		Animal 1	40.8	4.99	26
		Animal 2	46.6	5.26	27
		Animal 3	44.9	5.38	28
		Animal 4	25.3	15.01	77
		Animal 5	26.9	14.61	75

Example 3. *In Vivo* Dose Response of *KLKB1* Gene Editing in Humanized *KLKB1* Mice

[00222] The cassette-deleted humanized *KLKB1* mice generated in Example 1 (MAID 7701) were used to test dose response of *KLKB1* gene editing *in vivo*. There were 5 groups total (N=5 with 2 male and 3 female mice or vice versa). LNPs containing guide RNA 2 and mRNA encoding the Cas9 protein were dosed at 0.3, 0.1, 0.03 and 0.01 mg per kg bodyweight and characterized as described in Example 2.

[00223] At day 13 post-treatment, mice were euthanized. Liver tissue was processed as described in Example 4 for DNA sequencing. Blood was processed as described and secreted human prekallikrein was measured via ELISA as described in Example 2.

[00224] For RNA analysis, liver tissue was lysed using a Zymo Research Bashing Bead Lysis Rack, and RNA was extracted using the Qiagen RNeasy Mini Kit (Qiagen, Cat. 74106) according to the manufacturer's protocol. RNA was quantified using a Nanodrop 8000 (ThermoFisher Scientific, Cat. ND-8000-GL). RNA samples were stored at -20°C prior to use.

[00225] The SuperScript III Platinum One-Step qRT-PCR Kit (Invitrogen, Cat. 11732-088) was used to create the PCR reactions. Quantitative PCR probes targeting human *KLKB1* and internal control Ms *PPIA* were used in the reactions. The quantitative PCR assay was performed according to the manufacturer's specifications, scaled to the appropriate reaction volume, as well as using the human *KLKB1* and Ms *PPIA* probes specified above. The StepOnePlus Real-Time PCR System (Thermo Fisher Scientific, Cat. 4376600) was used to perform the real-time PCR reaction and transcript quantification according to the manufacturer's protocol.

[00226] Human *KLKB1* mRNA was quantified using a standard curve starting at 20 ng/μL of pooled mRNA from the vehicle control group, with five further 3-fold dilutions ending at 0.06 ng/μL. Ct values were determined from the StepOnePlus Real-Time PCR System. Reduction of total secreted human prekallikrein protein for cells treated with KLKB1 reagents was determined by ELISA as described above.

[00227] **Table 11** and **Figure 9** show editing data, serum prekallikrein levels as a percent of TSS vehicle control treated mice, and mRNA transcript levels as a percent of TSS vehicle control treated animals.

[00228] Table 11. Percent Editing, *KLKB1* mRNA (% of Basal Level), and Plasma Kallikrein Protein Levels (% of Basal Level) in Humanized *KLKB1* Mice.

Guide	Dose (mpk)	Sample	% Editing	% TSS Protein	% TSS mRNA	SD
TSS	0	Mean	0.1	100	100.5	9.7
		Animal 1	0.1	75.8		
		Animal 2	0.1	93.8		
		Animal 3	0.1	76.0		
		Animal 4	0.1	137.6		
		Animal 5	0.1	116.8		
2	0.01	Mean	3.9	91.9	100.1	9.5
		Animal 1	4.4	55.3		
		Animal 2	3.8	57.3		
		Animal 3	4.5	126.2		
		Animal 4	4.2	122.2		
		Animal 5	2.6	98.6		
	0.03	Mean	19.0	64.2	69.3	13.8
		Animal 1	22.1	38.6		
		Animal 2	0.3	51.0		
		Animal 3	26.9	78.9		
		Animal 4	21.3	80.5		
		Animal 5	24.3	72.2		
	0.1	Mean	55.4	23.3	48	11.4
		Animal 1	52.1	17.6		
		Animal 2	52.7	19.6		
		Animal 3	56.5	25.3		
		Animal 4	57.5	25.6		
		Animal 5	58.0	28.3		
	0.3	Mean	72.9	3.1	23	13
		Animal 1	73.9	2.7		
		Animal 2	70.4	2.9		
		Animal 3	72.7	3.1		
		Animal 4	73.1	3.4		
		Animal 5	74.3	3.4		

We claim:

1. A non-human animal comprising in its genome a humanized endogenous *KLKB1* locus in which a segment of the endogenous *KLKB1* locus has been deleted and replaced with a corresponding human *KLKB1* sequence.

2. The non-human animal of claim 1, wherein the humanized endogenous *KLKB1* locus encodes a protein comprising a human plasma kallikrein heavy chain.

3. The non-human animal of claim 2, wherein the human plasma kallikrein heavy chain comprises the sequence set forth in SEQ ID NO: 23, and optionally wherein the human plasma kallikrein heavy chain is encoded by a sequence comprising the sequence set forth in SEQ ID NO: 25.

4. The non-human animal of any preceding claim, wherein the humanized endogenous *KLKB1* locus encodes a protein comprising a human plasma kallikrein light chain.

5. The non-human animal of claim 4, wherein the human plasma kallikrein light chain comprises the sequence set forth in SEQ ID NO: 24, and optionally wherein the human plasma kallikrein light chain is encoded by a sequence comprising the sequence set forth in SEQ ID NO: 26.

6. The non-human animal of any preceding claim, wherein the humanized endogenous *KLKB1* locus encodes a protein comprising a human plasma kallikrein signal peptide.

7. The non-human animal of claim 6, wherein the human plasma kallikrein signal peptide comprises the sequence set forth in SEQ ID NO: 4, and optionally wherein the human plasma kallikrein signal peptide is encoded by a sequence comprising the sequence set forth in SEQ ID NO: 8.

8. The non-human animal of any preceding claim, wherein a region of the endogenous *KLKB1* locus comprising both coding sequence and non-coding sequence has been deleted and replaced with a corresponding human *KLKB1* sequence comprising both coding sequence and non-coding sequence.

9. The non-human animal of any preceding claim, wherein the humanized endogenous *KLKB1* locus comprises an endogenous *KLKB1* promoter, wherein the human *KLKB1* sequence is operably linked to the endogenous *KLKB1* promoter.

10. The non-human animal of any preceding claim, wherein at least one intron and at least one exon of the endogenous *KLKB1* locus have been deleted and replaced with the corresponding human *KLKB1* sequence.

11. The non-human animal of any preceding claim, wherein the entire *KLKB1* coding sequence of the endogenous *KLKB1* locus has been deleted and replaced with the corresponding human *KLKB1* sequence.

12. The non-human animal of claim 11, wherein a region of the endogenous *KLKB1* locus from the start codon to the stop codon has been deleted and replaced with the corresponding human *KLKB1* sequence.

13. The non-human animal of any preceding claim, wherein the endogenous *KLKB1* 3' untranslated region (3' UTR) has not been deleted and replaced with the corresponding human *KLKB1* sequence.

14. The non-human animal of any preceding claim, wherein the endogenous *KLKB1* 5' untranslated region (5' UTR) has not been deleted and replaced with the corresponding human *KLKB1* sequence.

15. The non-human animal of any preceding claim, wherein a region of the endogenous *KLKB1* locus from the start codon to the stop codon has been deleted and replaced with the corresponding human *KLKB1* sequence,

wherein the endogenous *KLKB1* 3' untranslated region (3' UTR) has not been deleted and replaced with the corresponding human *KLKB1* sequence,

wherein the endogenous *KLKB1* 5' untranslated region (5' UTR) has not been deleted and replaced with the corresponding human *KLKB1* sequence, and

wherein the humanized endogenous *KLKB1* locus comprises an endogenous *KLKB1* promoter, wherein the human *KLKB1* sequence is operably linked to the endogenous *KLKB1* promoter.

16. The non-human animal of any preceding claim, wherein:

(i) the human *KLKB1* sequence at the humanized endogenous *KLKB1* locus comprises a sequence at least 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the sequence set forth in SEQ ID NO: 11; and/or

(ii) the humanized endogenous *KLKB1* locus encodes a protein comprising a sequence at least 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the sequence set forth in SEQ ID NO: 3; and/or

(iii) the humanized endogenous *KLKB1* locus comprises a coding sequence comprising a sequence at least 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the sequence set forth in SEQ ID NO: 7; and/or

(iv) the humanized endogenous *KLKB1* locus comprises a sequence at least 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the sequence set forth in SEQ ID NO: 9 or 10.

17. The non-human animal of any preceding claim, wherein the humanized endogenous *KLKB1* locus does not comprise a selection cassette or a reporter gene.

18. The non-human animal of any preceding claim, wherein the non-human animal is homozygous for the humanized endogenous *KLKB1* locus.

19. The non-human animal of any preceding claim, wherein the non-human animal comprises the humanized endogenous *KLKB1* locus in its germline.

20. The non-human animal of any preceding claim, wherein the non-human animal is a mammal.

21. The non-human animal of claim 20, wherein the non-human animal is a rat or mouse.

22. The non-human animal of claim 21, wherein the non-human animal is a mouse.

23. A non-human animal cell comprising in its genome a humanized endogenous *KLKB1* locus in which a segment of the endogenous *KLKB1* locus has been deleted and replaced with a corresponding human *KLKB1* sequence.

24. A non-human animal genome comprising a humanized endogenous *KLKB1* locus in which a segment of the endogenous *KLKB1* locus has been deleted and replaced with a corresponding human *KLKB1* sequence.

25. A targeting vector for generating a humanized endogenous *KLKB1* locus in which a segment of the endogenous *KLKB1* locus has been deleted and replaced with a corresponding human *KLKB1* sequence, wherein the targeting vector comprises an insert nucleic acid comprising the corresponding human *KLKB1* sequence flanked by a 5' homology arm targeting a 5' target sequence at the endogenous *KLKB1* locus and a 3' homology arm targeting a 3' target sequence at the endogenous *KLKB1* locus.

26. A humanized non-human animal *KLKB1* gene in which a segment of the non-human animal *KLKB1* gene has been deleted and replaced with a corresponding human *KLKB1* sequence.

27. A method of assessing the activity of a human-KLKB1-targeting reagent *in vivo*, comprising:

(a) administering the human-KLKB1-targeting reagent to the non-human animal of any one of claims 1-22; and

(b) assessing the activity of the human-KLKB1-targeting reagent in the non-human animal.

28. The method of claim 27, wherein the administering comprises adeno-associated virus (AAV)-mediated delivery, lipid nanoparticle (LNP)-mediated delivery, hydrodynamic delivery (HDD), or injection.

29. The method of claim 27 or 28, wherein step (b) comprises assessing the activity of the human-KLKB1-targeting reagent in the liver of the non-human animal.

30. The method of any one of claims 27-29, wherein step (b) comprises measuring expression of an *KLKB1* messenger RNA encoded by the humanized endogenous *KLKB1* locus.

31. The method of any one of claims 27-30, wherein step (b) comprises measuring expression of a plasma kallikrein protein encoded by the humanized endogenous *KLKB1* locus.

32. The method of claim 31, wherein measuring expression of the plasma kallikrein protein comprises measuring serum levels of the plasma kallikrein protein in the non-human animal.

33. The method of claim 31 or 32, wherein measuring expression of the plasma kallikrein protein comprises measuring expression of the plasma kallikrein protein in the liver of the non-human animal.

34. The method of any one of claims 27-33, wherein the human-KLKB1-targeting reagent is a genome-editing agent, and step (b) comprises assessing modification of the humanized endogenous *KLKB1* locus.

35. The method of claim 34, wherein step (b) comprises measuring the frequency of insertions or deletions within the humanized endogenous *KLKB1* locus.

36. The method of any one of claims 27-35, wherein the human-KLKB1-targeting reagent comprises a nuclease agent designed to target a region of a human *KLKB1* gene.

37. The method of claim 36, wherein the nuclease agent comprises a Cas protein and a guide RNA designed to target a guide RNA target sequence in the human *KLKB1* gene.

38. The method of claim 37, wherein the Cas protein is a Cas9 protein.

39. The method of any one of claims 27-38, wherein the human-KLKB1-targeting reagent comprises an exogenous donor nucleic acid, wherein the exogenous donor

nucleic acid is designed to target the human *KLKB1* gene, and optionally wherein the exogenous donor nucleic acid is delivered via AAV.

40. The method of any one of claims 27-33, wherein the human-KLKB1-targeting reagent is an RNAi agent or an antisense oligonucleotide.

41. The method of any one of claims 27-33, wherein the human-KLKB1-targeting reagent is an antigen-binding protein.

42. The method of any one of claims 27-33, wherein the human-KLKB1-targeting reagent is small molecule.

43. The method of any one of claims 27-42, wherein assessing the activity of the human-KLKB1-targeting reagent in the non-human animal comprises assessing plasma kallikrein activity.

44. The method of claim 43, wherein assessing plasma kallikrein activity comprises measure captopril-induced vascular permeability *in vivo* or comprises measuring plasma kallikrein activity *in vitro* using a plasma kallikrein substrate linked to a chromogen.

45. A method of optimizing the activity of a human-KLKB1-targeting reagent *in vivo*, comprising:

(I) performing the method of any one of claims 27-44 a first time in a first non-human animal comprising in its genome a humanized endogenous *KLKB1* locus;

(II) changing a variable and performing the method of step (I) a second time with the changed variable in a second non-human animal comprising in its genome a humanized endogenous *KLKB1* locus; and

(III) comparing the activity of the human-KLKB1-targeting reagent in step (I) with the activity of the human-KLKB1-targeting reagent in step (II), and selecting the method resulting in the higher activity.

46. The method of claim 45, wherein the changed variable in step (II) is the delivery method of introducing the human-KLKB1-targeting reagent into the non-human animal.

47. The method of claim 45, wherein the changed variable in step (II) is the route of administration of introducing the human-KLKB1-targeting reagent into the non-human animal.

48. The method of claim 45, wherein the changed variable in step (II) is the concentration or amount of the human-KLKB1-targeting reagent introduced into the non-human animal.

49. The method of claim 45, wherein the changed variable in step (II) is the form of the human-KLKB1-targeting reagent introduced into the non-human animal.

50. The method of claim 45, wherein the changed variable in step (II) is the human-KLKB1-targeting reagent introduced into the non-human animal.

51. A method of making the non-human animal of any one of claims 1-22, comprising:

(a) introducing into a non-human animal host embryo a genetically modified non-human animal embryonic stem (ES) cell comprising in its genome a humanized endogenous *KLKB1* locus in which a segment of the endogenous *KLKB1* locus has been deleted and replaced with a corresponding human *KLKB1* sequence; and

(b) gestating the non-human animal host embryo in a surrogate mother, wherein the surrogate mother produces an F0 progeny genetically modified non-human animal comprising the humanized endogenous *KLKB1* locus.

52. A method of making the non-human animal of any one of claims 1-22, comprising:

(a) modifying the genome of a non-human animal one-cell stage embryo to comprise in its genome a humanized endogenous *KLKB1* locus in which a segment of the endogenous *KLKB1* locus has been deleted and replaced with a corresponding human *KLKB1* sequence, thereby generating a non-human animal genetically modified embryo; and

(b) gestating the non-human animal genetically modified embryo in a surrogate mother, wherein the surrogate mother produces an F0 progeny genetically modified non-human animal comprising the humanized endogenous *KLKB1* locus.

53. A method of making the non-human animal of any one of claims 1-22, comprising:

(a) introducing into a non-human animal embryonic stem (ES) cell a targeting vector comprising a nucleic acid insert comprising the human *KLKB1* sequence flanked by a 5' homology arm corresponding to a 5' target sequence in the endogenous *KLKB1* locus and a 3' homology arm corresponding to a 3' target sequence in the endogenous *KLKB1* locus,

wherein the targeting vector recombines with the endogenous *KLKB1* locus to produce a genetically modified non-human ES cell comprising in its genome the humanized endogenous *KLKB1* locus comprising the human *KLKB1* sequence;

(b) introducing the genetically modified non-human ES cell into a non-human animal host embryo; and

(c) gestating the non-human animal host embryo in a surrogate mother, wherein the surrogate mother produces an F0 progeny genetically modified non-human animal comprising in its genome the humanized endogenous *KLKB1* locus comprising the human *KLKB1* sequence.

54. The method of claim 53, wherein the targeting vector is a large targeting vector at least 10 kb in length or in which the sum total of the 5' and 3' homology arms is at least 10 kb in length.

55. A method of making the non-human animal of any one of claims 1-22, comprising:

(a) introducing into a non-human animal one-cell stage embryo a targeting vector comprising a nucleic acid insert comprising the human *KLKB1* sequence flanked by a 5' homology arm corresponding to a 5' target sequence in the endogenous *KLKB1* locus and a 3' homology arm corresponding to a 3' target sequence in the endogenous *KLKB1* locus,

wherein the targeting vector recombines with the endogenous *KLKB1* locus to produce a genetically modified non-human one-cell stage embryo comprising in its genome the humanized endogenous *KLKB1* locus comprising the human *KLKB1* sequence;

(b) gestating the genetically modified non-human animal one-cell stage embryo in a surrogate mother to produce a genetically modified F0 generation non-human

animal comprising in its genome the humanized endogenous *KLKB1* locus comprising the human *KLKB1* sequence.

56. The method of any one of claims 53-55, wherein step (a) further comprises introducing a nuclease agent or a nucleic acid encoding the nuclease agent, wherein the nuclease agent targets a target sequence in the endogenous *KLKB1* locus.

57. The method of claim 56, wherein the nuclease agent comprises a Cas protein and a guide RNA.

58. The method of claim 57, wherein the Cas protein is a Cas9 protein.

59. The method of claim 57 or 58, wherein step (a) further comprises introducing a second guide RNA or a DNA encoding the second guide RNA, wherein the second guide RNA targets a second target sequence within the endogenous *KLKB1* locus.

60. The method of claim 59, wherein step (a) further comprises introducing a third guide RNA or a DNA encoding the third guide RNA, wherein the third guide RNA targets a third target sequence within the endogenous *KLKB1* locus, and a fourth guide RNA or a DNA encoding the fourth guide RNA, wherein the fourth guide RNA targets a fourth target sequence within the endogenous *KLKB1* locus.

61. The method of any one of claims 51-60, wherein the non-human animal is a mouse or a rat.

62. The method of claim 61, wherein the non-human animal is a mouse.

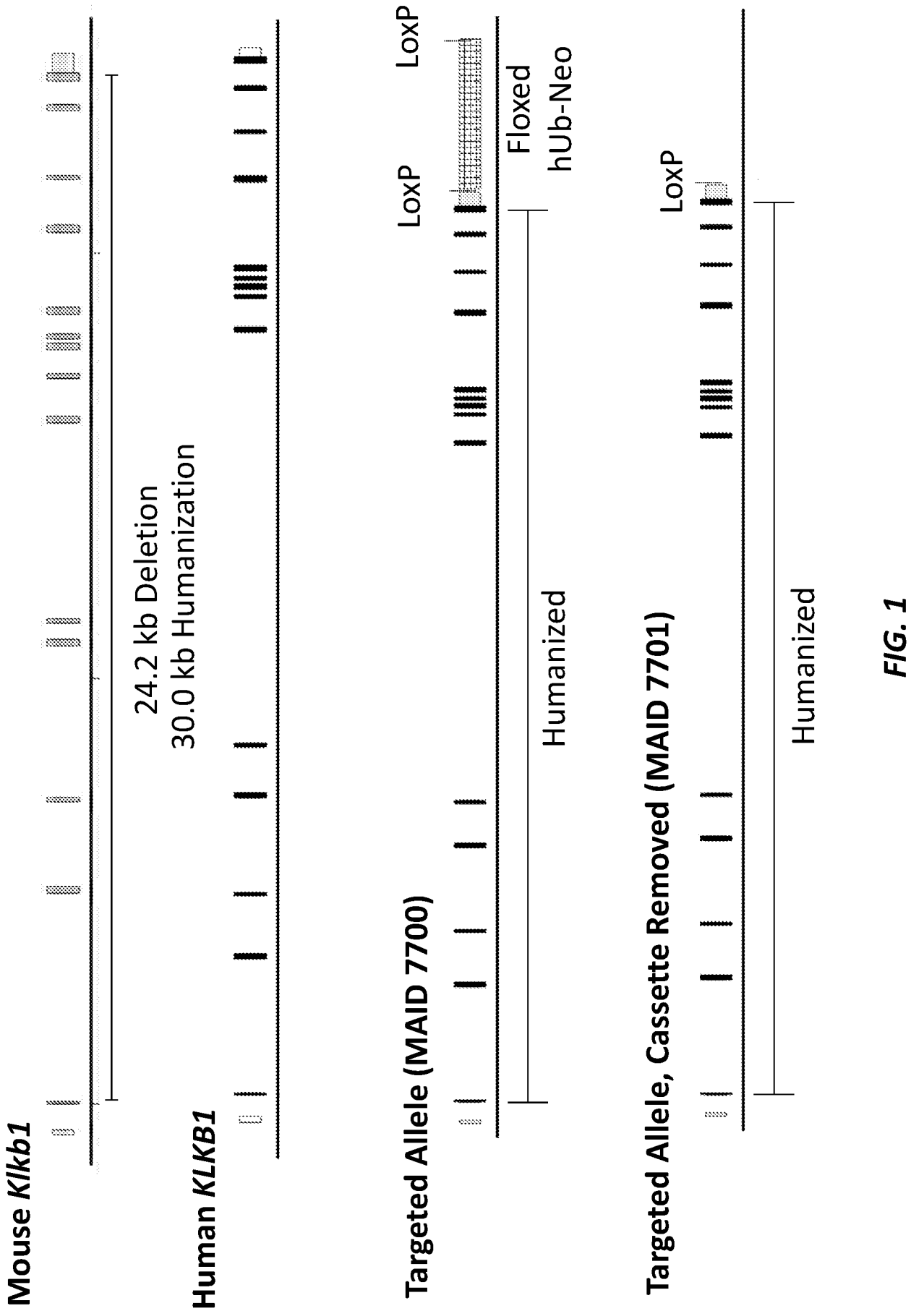
Mouse Klkb1
24.2 kb Deletion
30.0 kb Humanization
Human KLKB1
Targeted Allele (MAID 7700)
LoxP
LoxP
Floxed
hUb-Neo
Humanized
Targeted Allele, Cassette Removed (MAID 7701)
LoxP
Humanized

FIG. 1

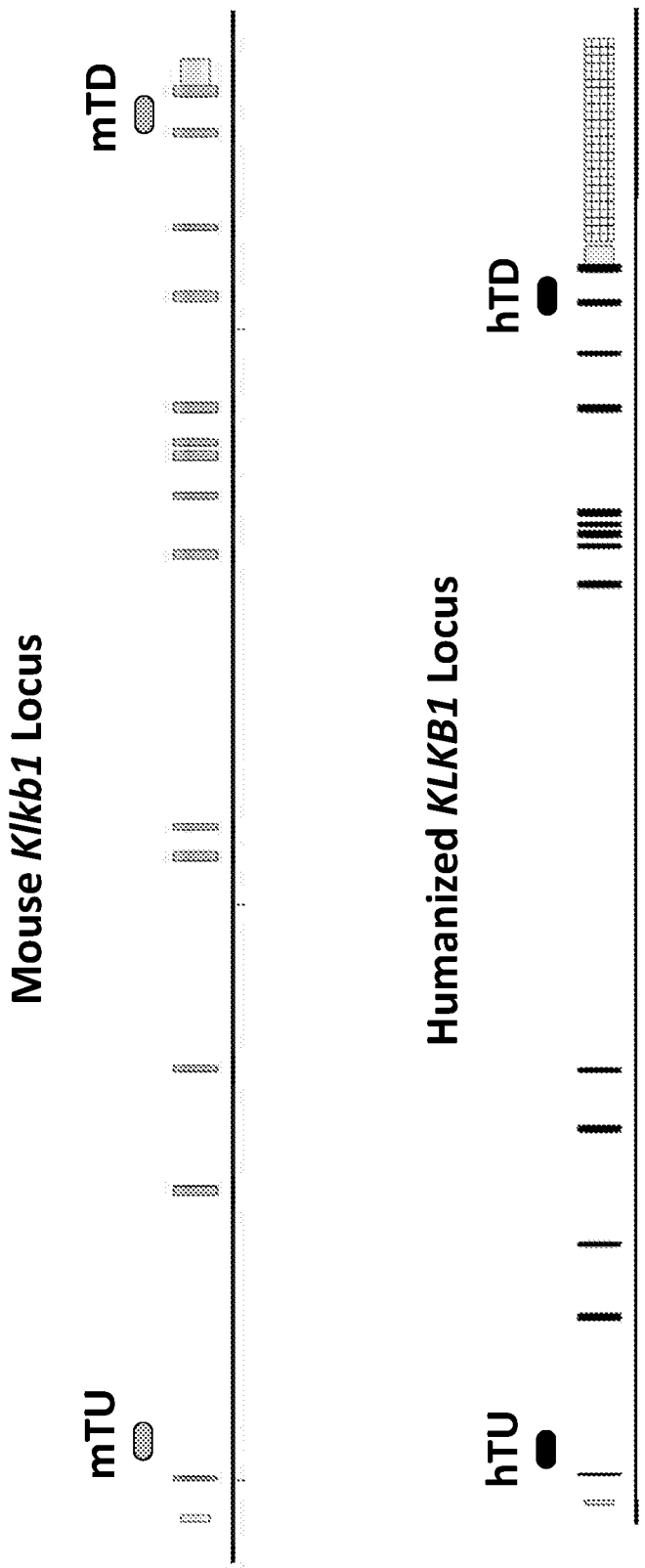

FIG. 2

```
                  Signal Peptide
mKLKB1      1   MILFNRVGYFVSLFATVSCGCMTQLYKNTFFRGGDLAAIYTPDAQYCQKMCTFHPRCLLF   60
hKLKB1      1   MILFKQATYFISLFATVSCGCLTQLYENAFFRGGDVASMYTPNAQYCQMRCTFHPRCLLF   60

mKLKB1     61   SFLAVTPPKETNKRFGCFMKESITGTLPRIHRTGAISGHSLKQCGHQISACHRDIYKGLD   120
hKLKB1     61   SFLPASSINDMEKRFGCFLKDSVTGTLPKVHRTGAVSGHSLKQCGHQISACHRDIYKGVD   120

mKLKB1    121   MRGSNFNISKTDNIEECQKLCTNNFHCQFFTYATSAFYRPEYRKKCLLKHSASGTPTSIK   180
hKLKB1    121   MRGVNFNVSKVSSVEECQKRCTSNIRCQFFSYATQTFHKAEYRNNCLLKYSPGGTPTAIK   180

mKLKB1    181   SADNLVSGFSLKSCALSEIGCPMDIFQHSAFADLNVSQVITPDAFVCRTICTFHPNCLFF   240
hKLKB1    181   VLSNVESGFSLKPCALSEIGCHMNIFQHLAFSDVDVARVLTPDAFVCRTICTYHPNCLFF   240

mKLKB1    241   TFYTNEWETESQRNVCFLKTSKSGRPSPPIPQENAISGYSLLTCRKTRPEPCHSKIYSGV   300
hKLKB1    241   TFYTNVWKIESQRNVCLLKTSESGTPSSSTPQENTISGYSLLTCKRTLPEPCHSKIYPGV   300

mKLKB1    301   DFEGEELNVTFVQGADVCQETCTKTIRCQFFIYSLLPQDCKEEGCKCSLRLSTDGSPTRI   360
hKLKB1    301   DFGGEELNVTFVKGVNVCQETCTKMIRCQFFTYSLLPEDCKEEKCKCFLRLSMDGSPTRI   360

mKLKB1    361   TYGMQGSSGYSLRLCKLVDSPDCTTKINARIVGGTNASLGEWPWQVSLQVKLVSQTHLCG   420
hKLKB1    361   AYGTQGSSGYSLRLCNTGDNSVCTTKTSTRIVGGTNSSWGEWPWQVSLQVKLTAQRHLCG   420

mKLKB1    421   GSIIGRQWVLTAAHCFDGIPYPDVWRIYGGILSLSEITKETPSSRIKELIIHQEYKVSEG   480
hKLKB1    421   GSLIGHQWVLTAAHCFDGLPLQDVWRIYSGILNLSDITKDTPFSQIKEIIIHQNYKVSEG   480

mKLKB1    481   NYDIALIKLQTPLNYTEFQKPICLPSKADTNTIYTNCWVTGWGYTKEQGETQNILQKATI   540
hKLKB1    481   NHDIALIKLQAPLNYTEFQKPICLPSKGDTSTIYTNCWVTGWGFSKEKGEIQNILQKVNI   540

mKLKB1    541   PLVPNEECQKKYRDYVINKQMICAGYKEGGTDACKGDSGGPLVCKHSGRWQLVGITSWGE   600
hKLKB1    541   PLVTNEECQKRYQDYKITQRMVCAGYKEGGKDACKGDSGGPLVCKHNGMWRLVGITSWGE   600

mKLKB1    601   GCARKDQPGVYTKVSEYMDWILEKTQSSDVRALETSSA  638  (SEQ ID NO: 1)
hKLKB1    601   GCARREQPGVYTKVAEYMDWILEKTQSSDGKAQMQSPA  638  (SEQ ID NO: 3)
```

FIG. 3

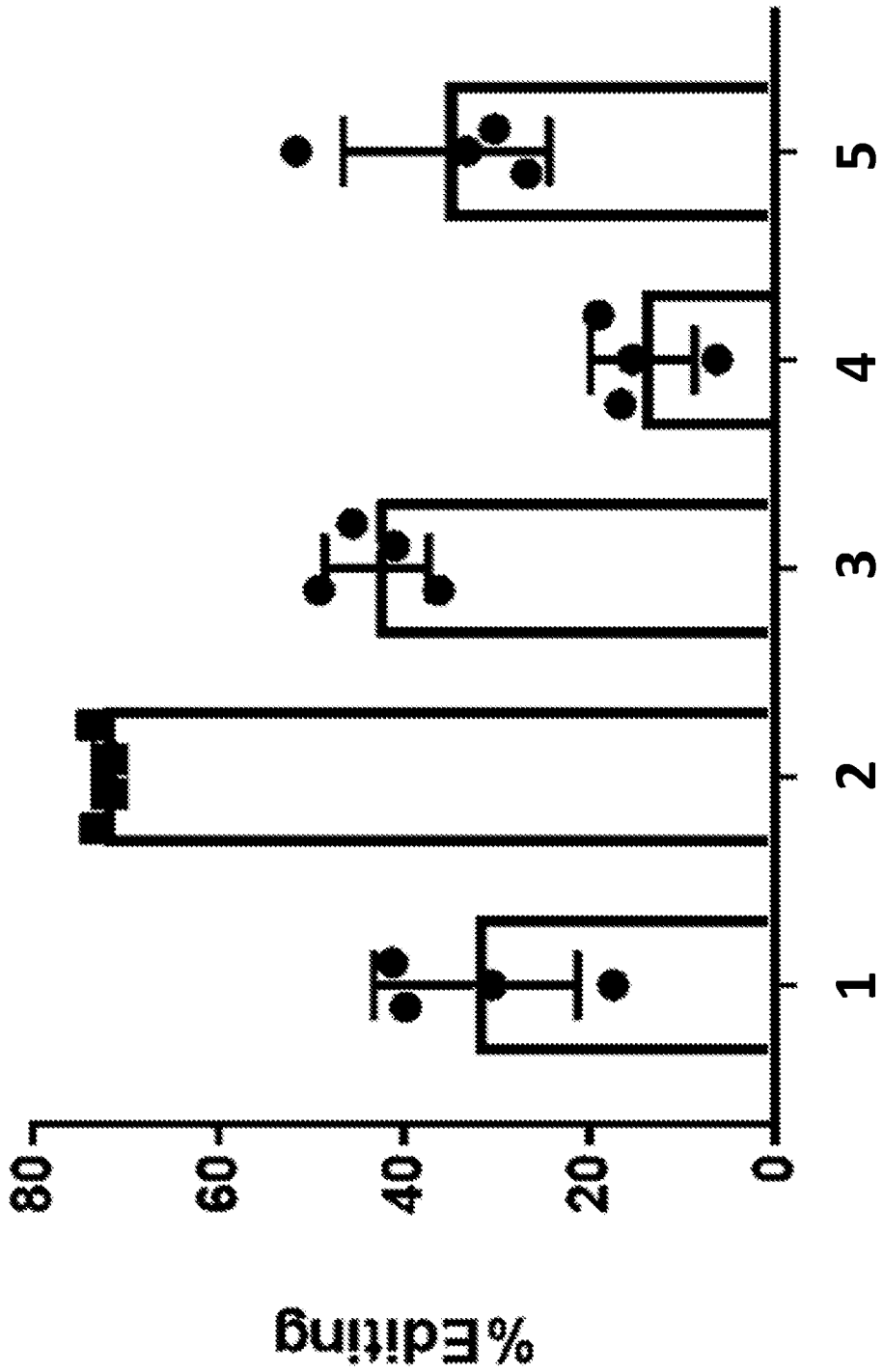

FIG. 4

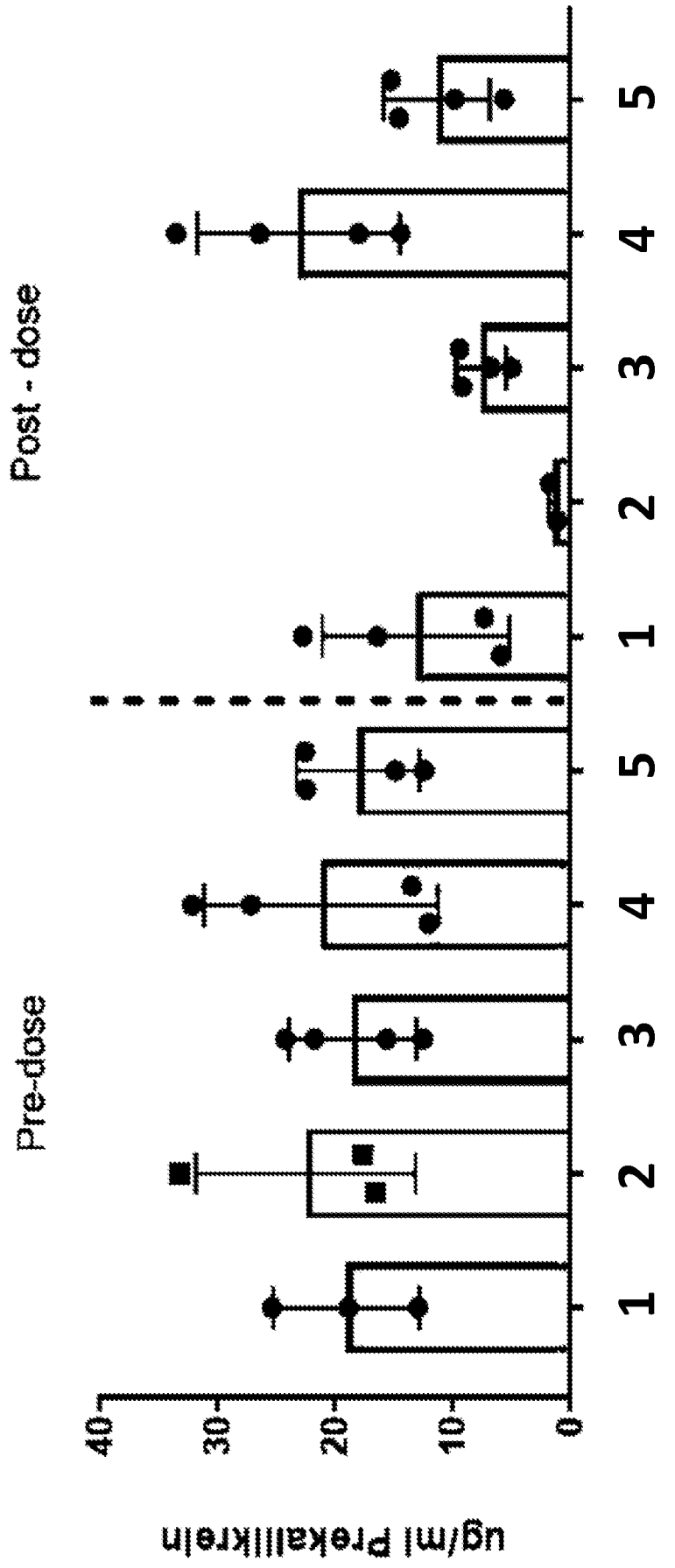
ug/ml Prekallikrein
40
30
20
10
0
Pre-dose
Post - dose
1
2
3
4
5
1
2
3
4
5

FIG. 5

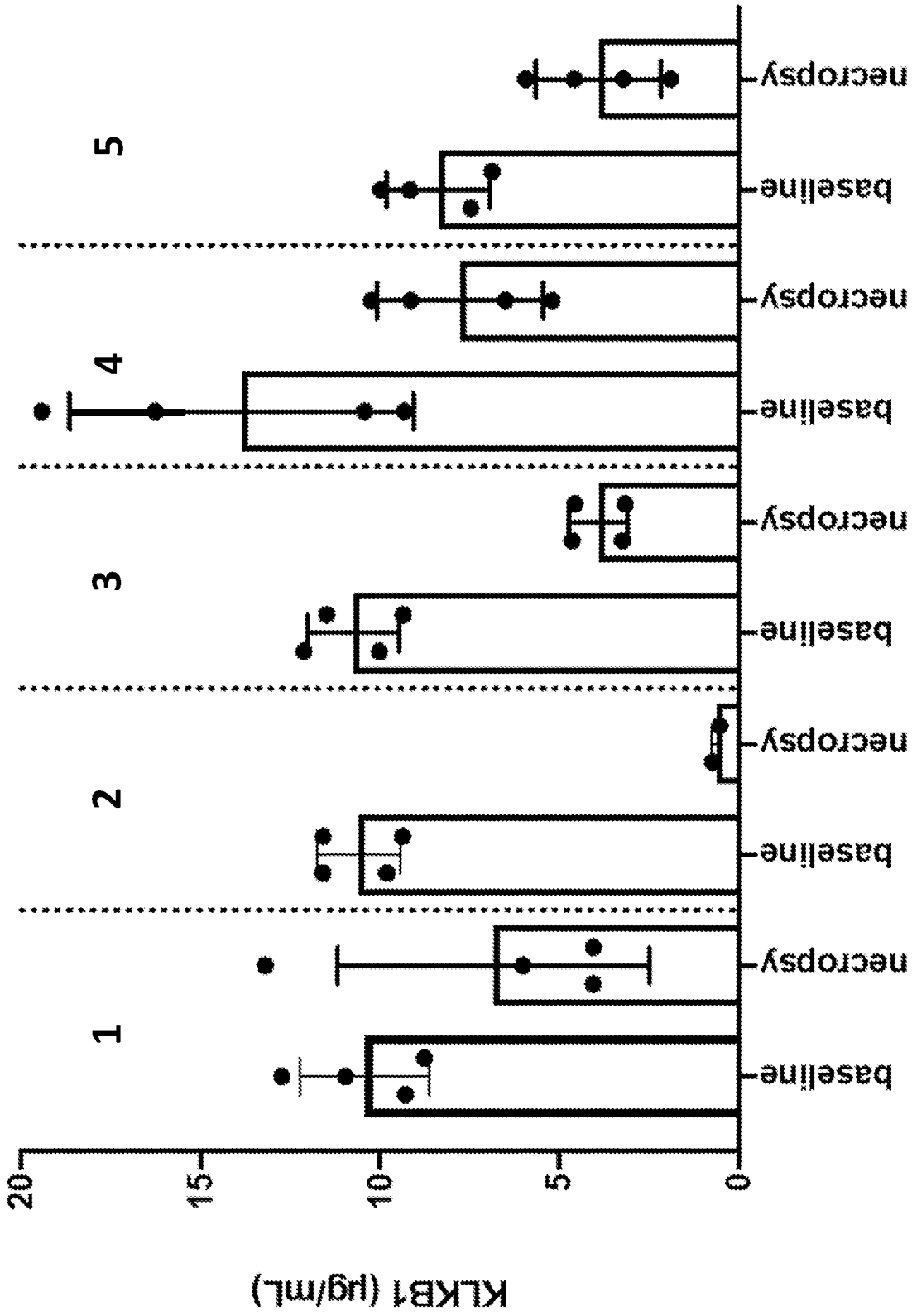
KLKB1 (µg/mL)
20
15
10
5
0
1
2
3
4
5
baseline
necropsy
baseline
necropsy
baseline
necropsy
baseline
necropsy
baseline
necropsy

FIG. 6

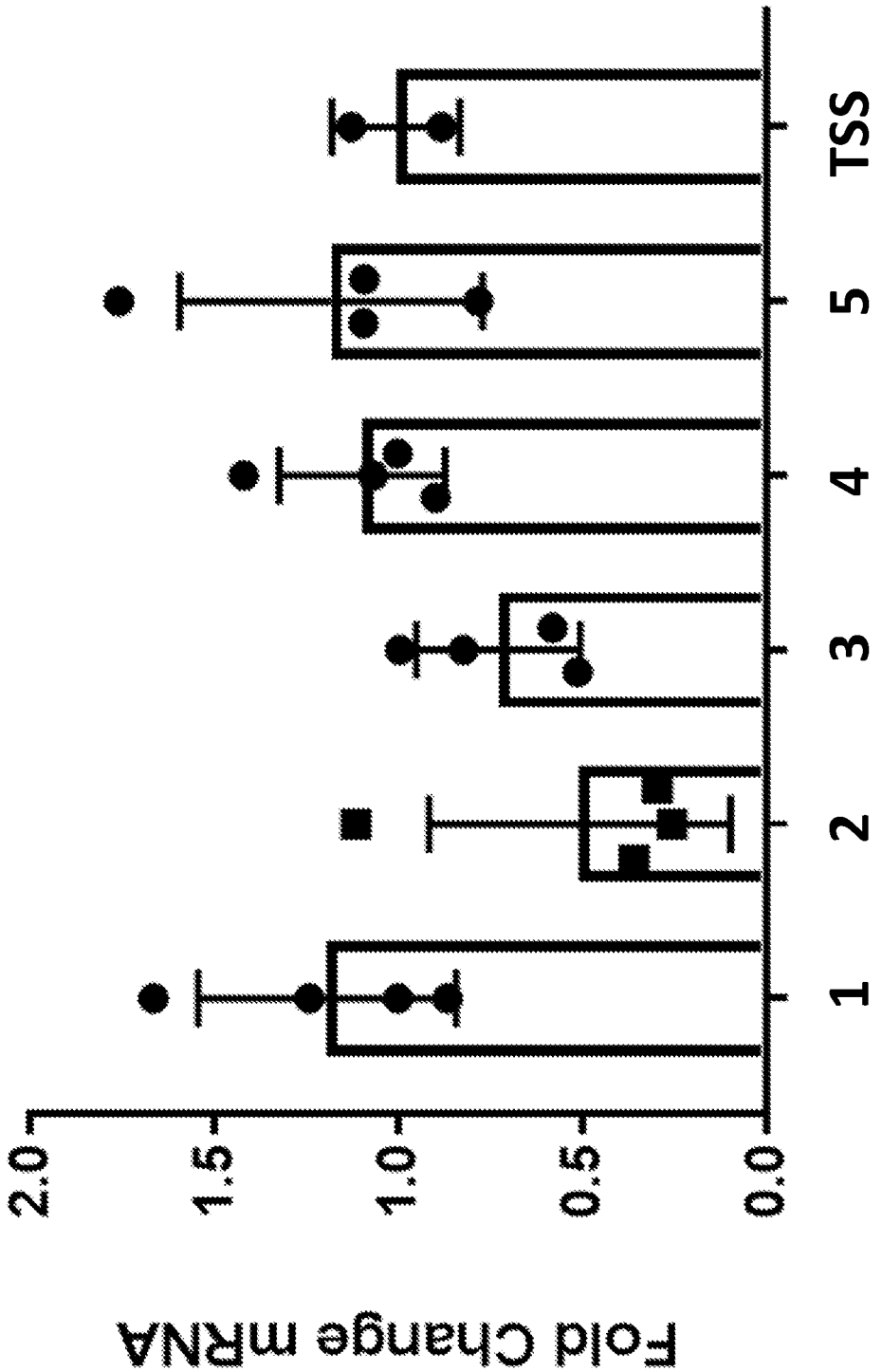

FIG. 7

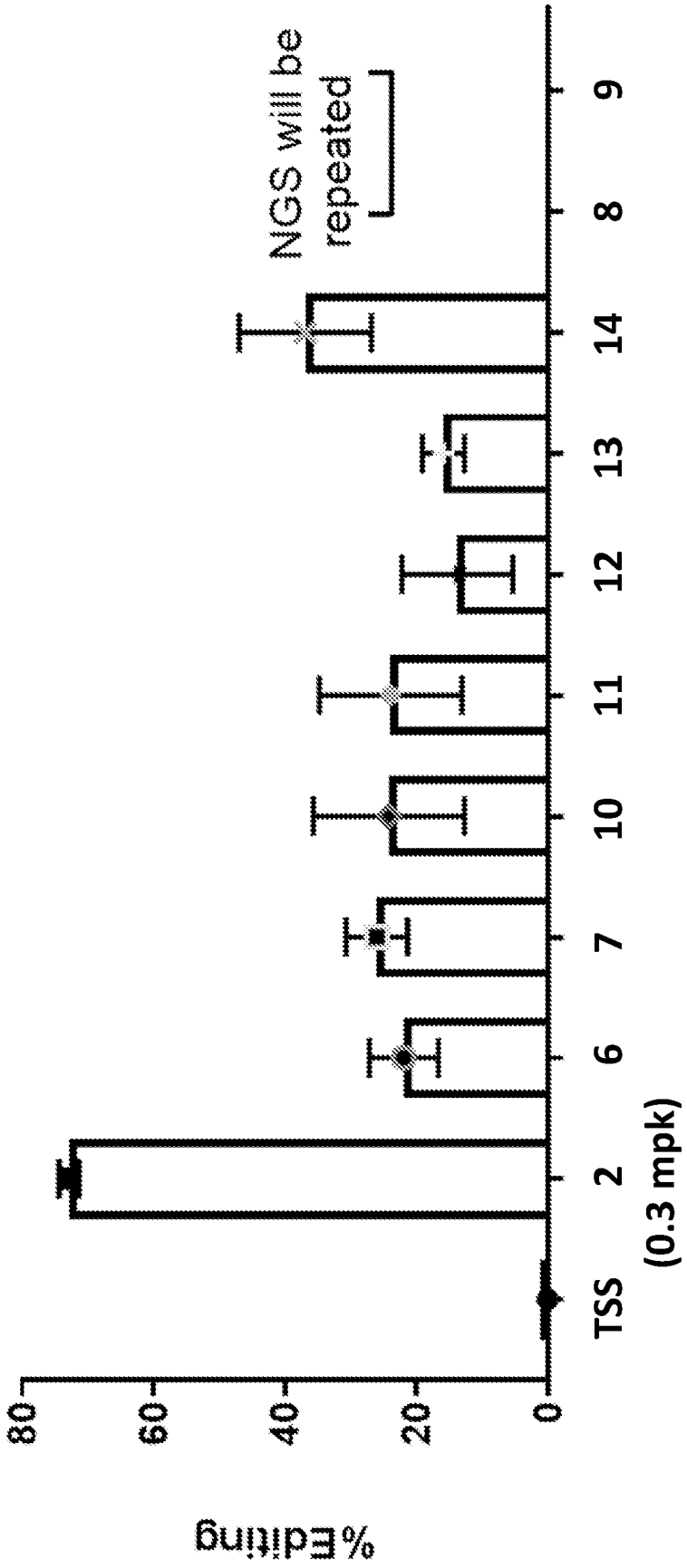
%Editing
80
60
40
20
0
TSS
2
6
7
10
11
12
13
14
8
9
(0.3 mpk)
NGS will be repeated

FIG. 8A

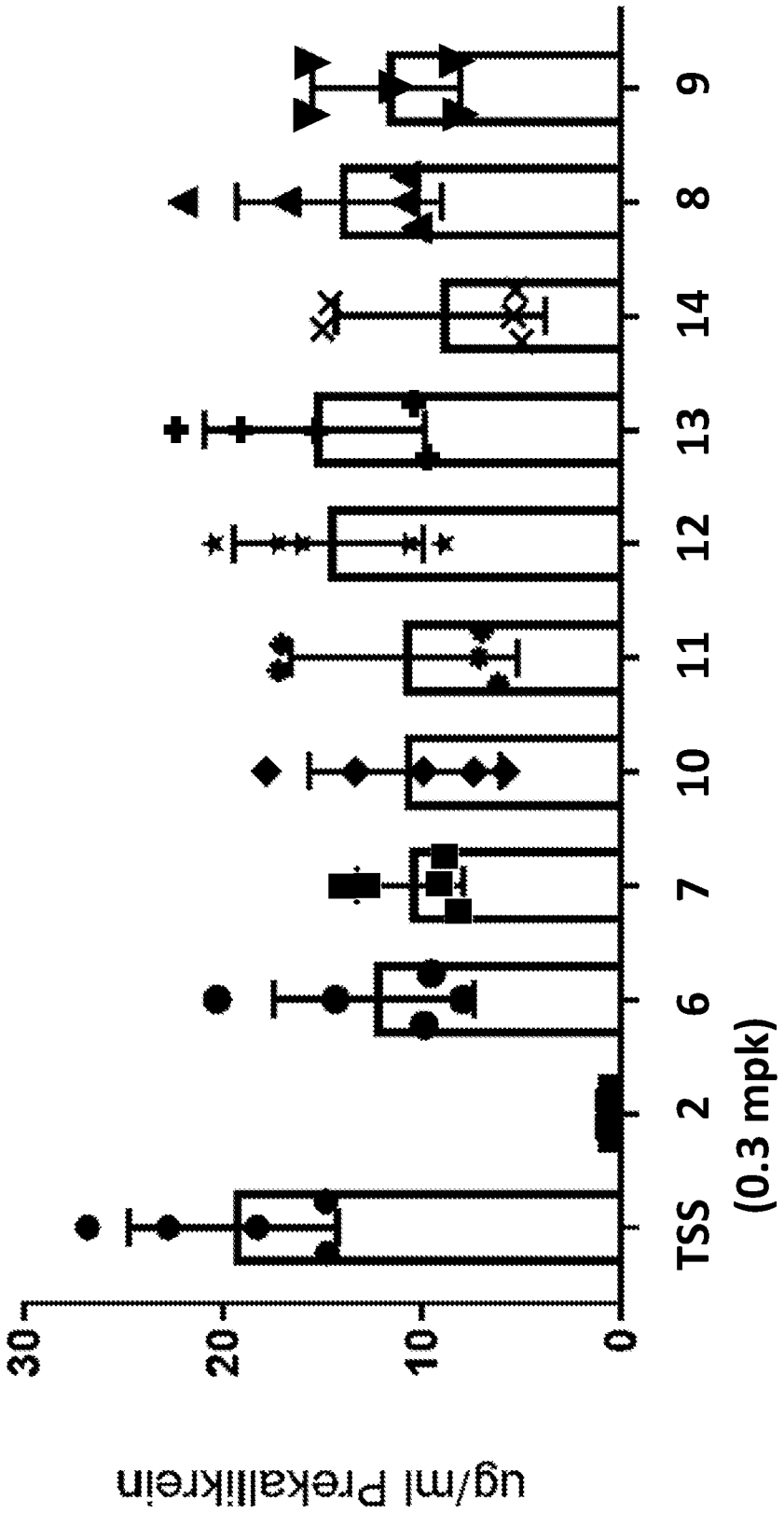

FIG. 8B

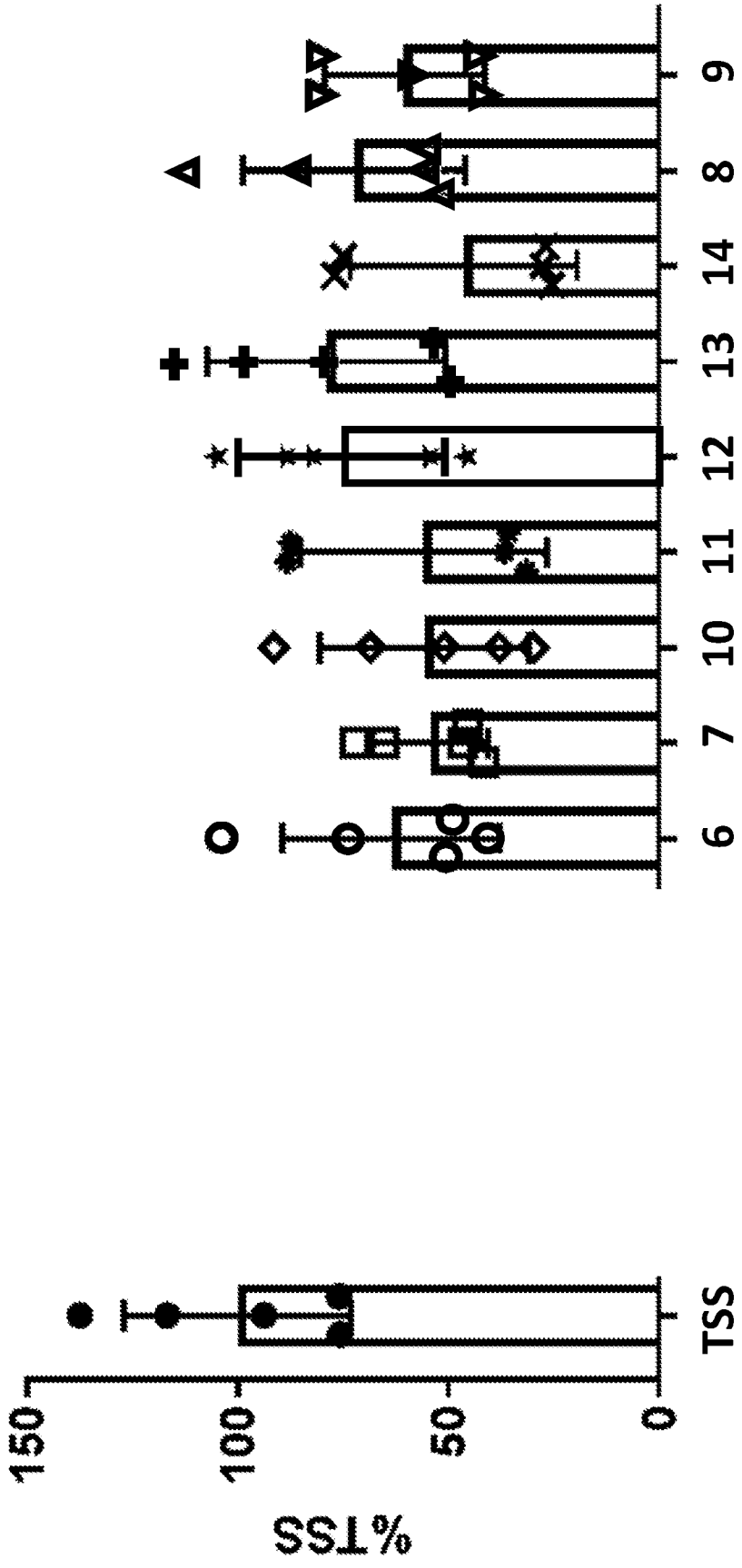

FIG. 8C

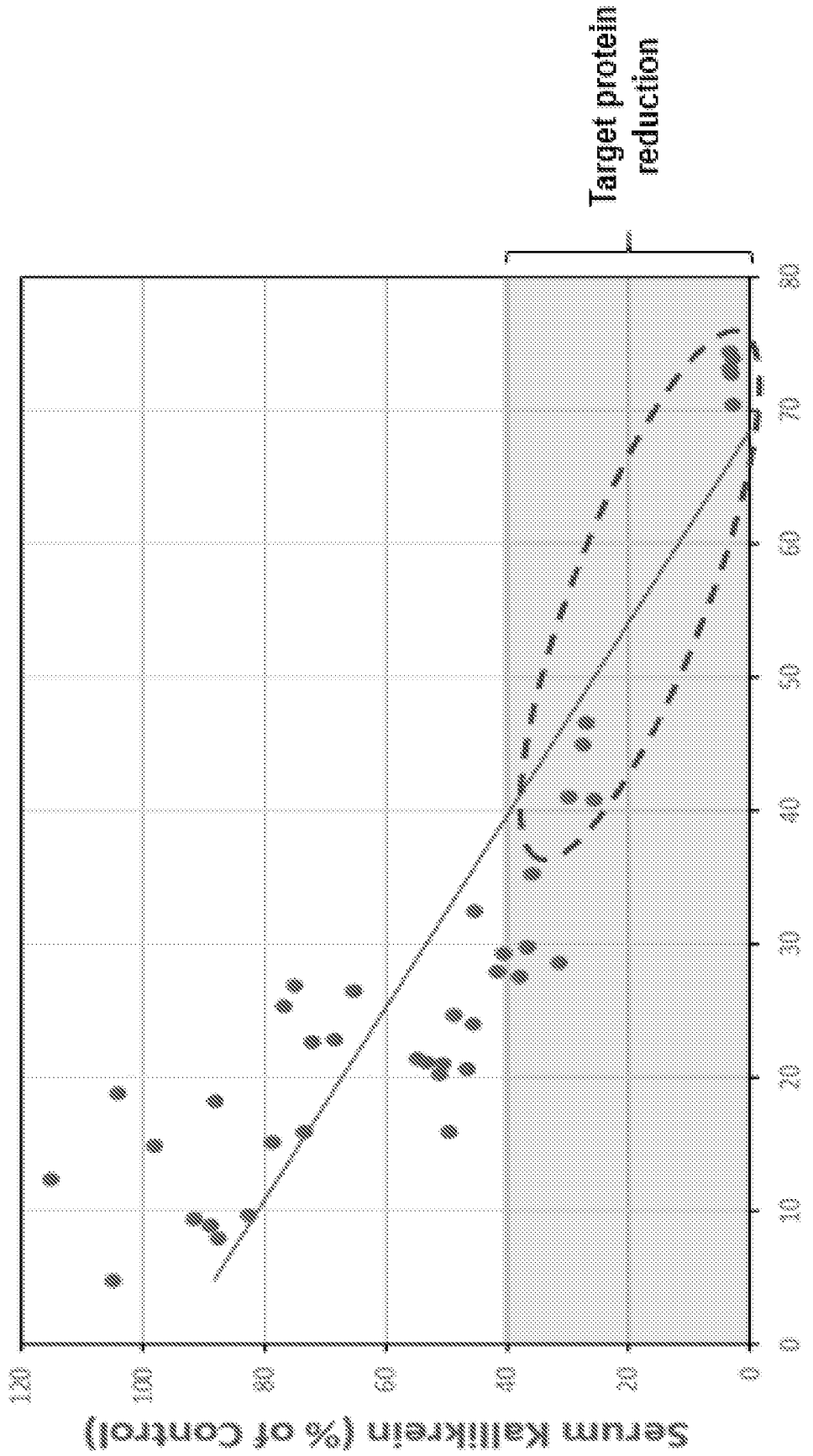
Target protein reduction
Serum Kallikrein (% of control)
120
100
80
60
40
20
0
0
10
20
30
40
50
60
70
80
% Liver Editing

FIG. 8D

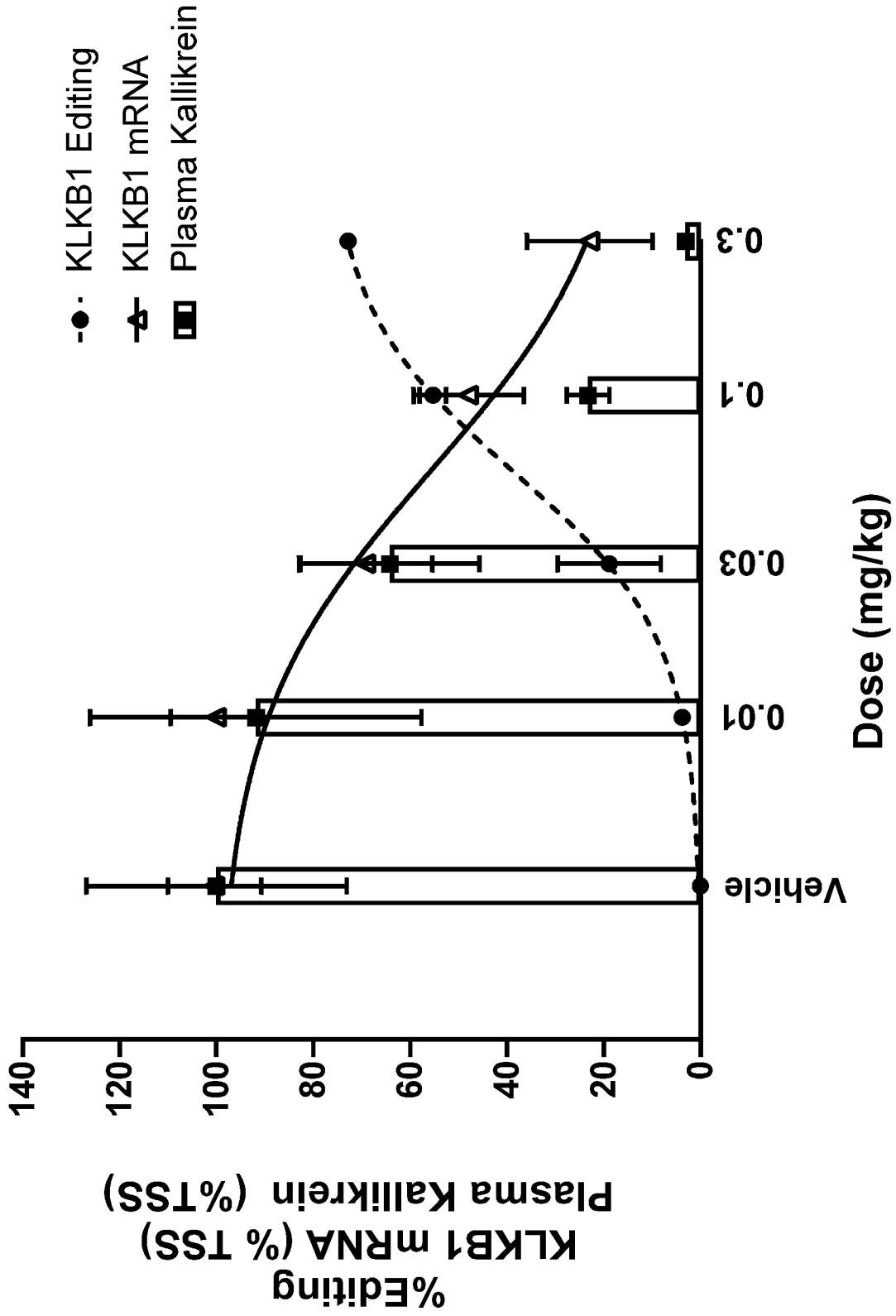
KLKB1 Editing
KLKB1 mRNA
Plasma Kallikrein
%Editing
KLKB1 mRNA (% TSS)
Plasma Kallikrein (%TSS)
140
120
100
80
60
40
20
0
Vehicle
0.01
0.03
0.1
0.3
Dose (mg/kg)

FIG. 9

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2021/016762

A. CLASSIFICATION OF SUBJECT MATTER
INV. A01K67/027 A61K48/00 C07K14/47 C12N9/22 C12N15/10
C12N15/11 C12N15/90
ADD.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A01K C40B C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2005/075665 A2 (BAYER HEALTHCARE AG [DE]; GOLZ STEFAN [DE] ET AL.) 18 August 2005 (2005-08-18) example 13	1-62
A	US 2019/098879 A1 (DRUMMOND-SAMUELSON MEGHAN [US] ET AL) 4 April 2019 (2019-04-04) abstract; [002], [0300] ff.; claims 23, 41, 52	27-62
A	US 2019/159436 A1 (MUJICA ALEXANDER O [US] ET AL) 30 May 2019 (2019-05-30) abstract; [0186], [0199], claims 32, 47, 52	27-62

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 May 2021	28/05/2021

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Brero, Alessandro

Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2021/016762

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2018/064827 A1 (CONWAY ANTHONY [US] ET AL) 8 March 2018 (2018-03-08) [0044] ff., [0046], [0207] ff.	27-62
A	SOTIROPOULOU GEORGIA ET AL: "Targeting the kallikrein-related peptidases for drug development", TRENDS IN PHARMACOLOGICAL SCIENCES., vol. 33, no. 12, 1 December 2012 (2012-12-01), pages 623-634, XP055803517, GB ISSN: 0165-6147, DOI: 10.1016/j.tips.2012.09.005 the whole document	1-62
X,P	SEITZER JESSICA: "NTLA-2002: CRISPR/Cas9-mediated gene knockout of KLKB1 to treat hereditary angioedema", JOURNAL OF ALLERGY AND CLINICAL IMMUNOLOGY, ELSEVIER, AMSTERDAM, NL, vol. 147, no. 2, 1 February 2021 (2021-02-01), XP086479755, ISSN: 0091-6749, DOI: 10.1016/J.JACI.2020.12.531 [retrieved on 2021-02-01] abstract	1-62

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2021/016762

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. [X] forming part of the international application as filed:

      [X] in the form of an Annex C/ST.25 text file.

      [ ] on paper or in the form of an image file.

   b. [ ] furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. [ ] furnished subsequent to the international filing date for the purposes of international search only:

      [ ] in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      [ ] on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. [ ] In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2021/016762

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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