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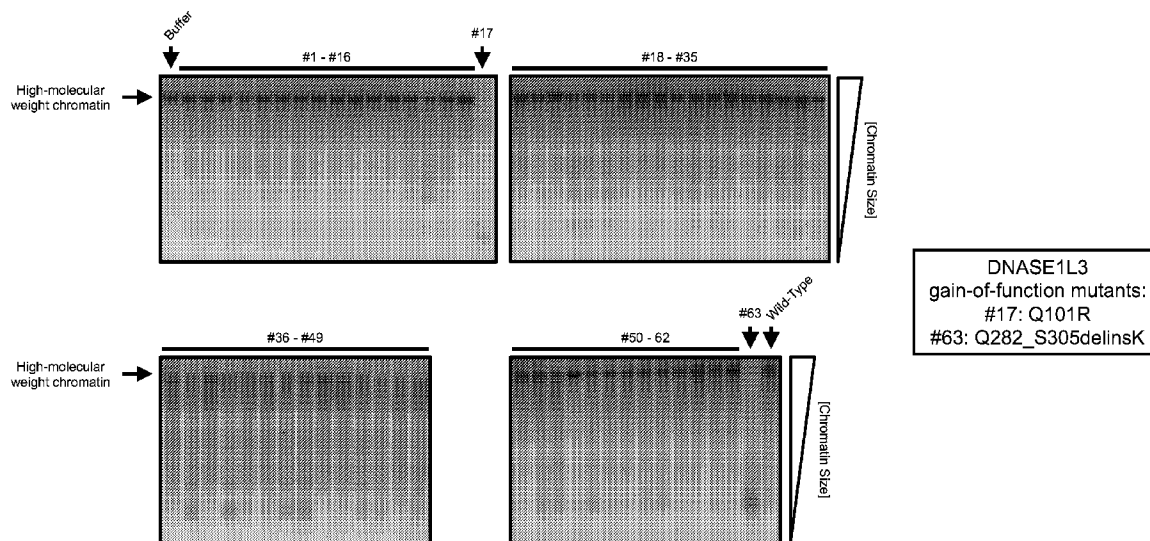
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(54) Titre : INGENIERIE D'ENZYMES D'ADNASE POUR LA FABRICATION ET LA THERAPIE  
(54) Title: ENGINEERING OF DNASE ENZYMES FOR MANUFACTURING AND THERAPY

**FIG. 1**



(57) **Abrégé/Abstract:**

The present disclosure provides engineered human extracellular DNASE proteins (e.g., variants of DNASE1 (D1), DNASE1-LIKE 1 (D1L1), DNASE1-LIKE 2 (D1L2), DNASE1-LIKE 3 Isoform 1 (D1L3), DNASE1-LIKE 3 Isoform 2 (D1L3-2), DNASE2A (D2A), and DNASE2B (D2B)) that are useful for treating conditions characterized by neutrophil extracellular trap (NET) accumulation and/or release. In accordance with the invention, the DNase variant has advantages for therapy and/or large-scale manufacturing.

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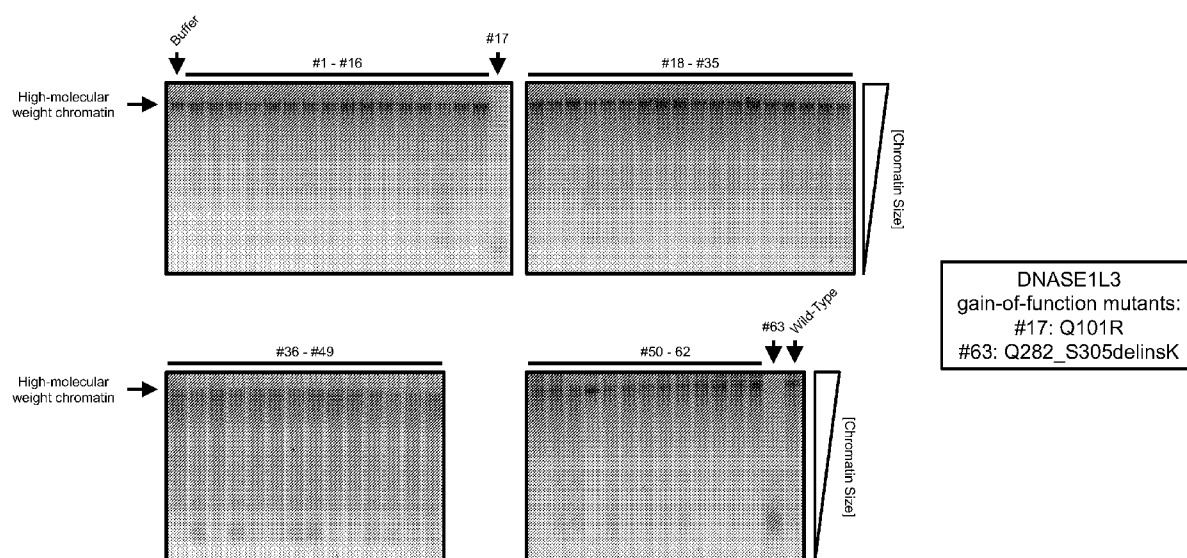
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**FIG. 1**



(57) Abstract: The present disclosure provides engineered human extracellular DNASE proteins (e.g., variants of DNASE1 (D1), DNASE1-LIKE 1 (D1L1), DNASE1-LIKE 2 (D1L2), DNASE1-LIKE 3 Isoform 1 (D1L3), DNASE1-LIKE 3 Isoform 2 (D1L3-2), DNASE2A (D2A), and DNASE2B (D2B)) that are useful for treating conditions characterized by neutrophil extracellular trap (NET) accumulation and/or release. In accordance with the invention, the DNase variant has advantages for therapy and/or large-scale manufacturing.

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for clinical applications. Thus, there is a need for defining a manufacturing process for D1L1, D1L2, and D1L3 enzymes, and for engineering DNases for use in therapy, including for degrading NETs.

### SUMMARY OF THE DISCLOSURE

5           The present invention provides engineered human extracellular DNASE proteins (e.g., variants of DNASE1 (D1), DNASE1-LIKE 1 (D1L1), DNASE1-LIKE 2 (D1L2), DNASE1-LIKE 3 Isoform 1 (D1L3), DNASE1-LIKE 3 Isoform 2 (D1L3-2), DNASE2A (D2A), and DNASE2B (D2B)) that are useful for treating conditions characterized by extracellular DNA, extracellular chromatin, and neutrophil extracellular trap (NET)  
10 accumulation and/or release. In accordance with aspects of the invention, the DNase variants described herein are more suitable for therapy and/or more amenable to large-scale manufacturing. In some embodiments, the DNase variants described herein have benefits for medical therapy, including systemic therapy. Such benefits include slower drug elimination, e.g., increased circulatory half-life (e.g., serum half-life), an extended  
15 duration of pharmacodynamic activity, high chromatin-degrading activity, and protease resistance.

In some aspects, the invention provides a D1L3 variant, wherein the D1L3 variant has one or more of increased protein stability, slower drug elimination and increased duration of pharmacodynamic activity, resistance to proteolytic degradation, higher  
20 production levels with *in vitro* expression systems, better suitability for purification, and not substantially less, the same, or better chromatin and/or NET-degrading activity as compared to wild-type D1L3 Isoform 1 enzyme of SEQ ID NO:4 or wild-type D1L3 Isoform 2 enzyme of SEQ ID NO:5.

In some embodiments, the D1L3 variant is a fusion protein that comprises an  
25 amino acid sequence that is at least 80% identical to the mature enzyme defined by SEQ ID NO:4 or SEQ ID NO:5, an albumin amino acid sequence at the N-terminus of the mature enzyme, and optionally a linking amino acid sequence between the albumin amino acid sequence (the albumin domain) and the D1L3 amino acid sequence (the D1L3 domain). In these embodiments, the D1L3 exhibits slower elimination (e.g., improved  
30 circulatory half-life or serum half-life) and an extended duration of pharmacodynamic activity, including for systemic therapy. In some embodiments, the fusion of albumin with linking sequence to the D1L3 domain does not substantially impact chromatin-

degrading activity of the enzyme (e.g., a measured using an in vitro assay) as compared to the enzyme without an albumin fusion.

In these embodiments, the D1L3 domain of the fusion protein has a deletion of all or part of the C-terminal basic domain that is present in the wild-type D1L3 enzyme.

- 5 Deletion or inactivation of the C-terminal basic domain substantially improves chromatin degrading activity. That is, removal of the C-terminal basic domain (BD) activates the wild-type D1L3 enzyme for degrading chromatin.

- 10 In some embodiments, the D1L3 variant has one or more building block substitutions from D1. For example, the D1L3 variant may have the building block substitution of Q282\_S305delinsK, which includes a deletion of the C-terminal basic domain, which domain is absent in D1. In some embodiments, the D1L3 variant has an amino acid substitution at the position corresponding to position 101 of SEQ ID NO:4. The substitution can be Arg based on the corresponding building block from D1, or in some embodiments is Lys. Substitutions at this position can enhance chromatin-degrading activity of a D1L3 variant.
- 15

- The linker where present may be a flexible linker, a rigid linker, or a physiologically-cleavable linker, such as a protease-cleavable linker. For example, the linker may be a hydrophilic amino acid sequence, and may be predominately constructed from amino acids selected from Gly, Ala, Ser, Thr, and Pro. In some embodiments, the variant is a flexible linker that is predominately glycine and serine residues (e.g., (G<sub>y</sub>S)<sub>n</sub> linkers, where y is from 1 to 5, and n is from 1 to 20). In some embodiments, the linker is an  $\alpha$ -helical linker. In some embodiments, the linker has at least 15 amino acids, or at least 25 amino acids. In various embodiments, longer linkers of at least 15 amino acids can provide improvements in yield upon expression in mammalian and non-mammalian expression systems, such as CHO cells or *Pichia pastoris*. Further, and surprisingly, longer linker sequences showed improved chromatin-degrading activity in an in vitro chromatin-degrading assay, as compared to shorter linker sequences.
- 20
- 25

- In various embodiments, the D1L3 variant comprises the amino acid sequence of any one of SEQ ID NOS: 17 to 30, in each case optionally having from one to twenty amino acid modifications independently selected from insertions, deletions, or substitutions. These sequences provide exemplary fusion proteins between D1L3 (or D1L3 variants) with albumin sequences, including with various linker designs. In some
- 30

embodiments, the amino acid modifications are in the D1L3 domain, the albumin domain, or both domains. In some embodiments, the variant has the amino acid sequence of SEQ ID NO: 19, SEQ ID NO: 22, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, or SEQ ID NO: 30. In these embodiments, the D1L3 variant comprises,  
5 in order from N-terminus to C-terminus: an albumin amino acid sequence, an intermediate or long flexible linker, and a D1L3 amino acid sequence (i.e., including D1L3 variants). SEQ ID NO: 28 further comprises an albumin fusion at the C-terminus through a long flexible peptide linker.

In other embodiments, the linker is cleavable by a protease, such as a coagulation  
10 pathway protease, such as activated Factor XII. In certain embodiments, the linker contains amino acid sequence of Factor XI and/or prekallikrein. In other embodiments, the linker includes a peptide sequence that is targeted for cleavage by a neutrophil specific protease, such as neutrophil elastase, cathepsin G, or proteinase 3.

In some aspects, the invention provides variants of extracellular DNASE  
15 enzymes engineered to have advantages in manufacturing, providing for production of the recombinant enzyme suitable for use in therapy. In various embodiments, the invention provides a recombinant D1, D1L1, D1L2, and D1L3 variant comprising one or more amino acid substitutions in cysteine residues (or PEGylation of Cys residues) resulting in reduced intra- and inter-molecular cross-linking via disulfide bridges during  
20 protein expression.

In other aspects, the invention provides variants of extracellular DNASE enzymes engineered to have advantages in protease resistance, for improving *in vivo* exposure, e.g., slowing elimination, e.g. extending half-life (e.g., serum half-life), and extending duration of pharmacodynamic activity, as well as reducing proteolysis during  
25 recombinant enzyme production. This disclosure identifies, for example, D1L3 residues that are sensitive to proteolysis by plasmin, thrombin, and/or trypsin, as well as residues (e.g., paired basic amino acids) that are sensitive to proteases produced by mammalian and non-mammalian cell lines. Engineered mutation of these residues can confer these advantages in protease resistance.

In other aspects, the invention provides a method for recombinant production of  
30 extracellular DNASE proteins, including variants thereof described herein. In some embodiments, the method employs a non-mammalian expression system, e.g., a

eukaryotic non-mammalian expression system, such as *Pichia pastoris*. In some embodiments, the *Pichia pastoris* encodes the DNase enzyme with its native signal peptide allowing for secretion from host cells. In some embodiments, the expression system is a mammalian cell expression system, such as Chinese Hamster Ovary (CHO) cells.

In some embodiments, the recombinant expression system has a deletion or inactivation of one or more proteases that cleave at paired basic amino acids. Exemplary enzymes include Furin (expressed by CHO cells) and Aspartic proteinase 3 (Ysp1) and Kexin (Kex2) expressed by *Pichia pastoris*. In some embodiments, these enzymes are not genetically deleted or inactivated, but their activity is inhibited with a protease inhibitor during recombinant protein production.

In some embodiments, the growth medium for the non-mammalian expression system or mammalian expression system is supplemented with polyanions such as dextran sulfate, heparins, ferric citrate, and EDTA. In further embodiments, the growth medium of *Pichia pastoris* or other expression system is supplemented with dextran sulfate that has an average molecular weight of between 5 kDa and 100 kDa. For example, the polyanion may be added to the culture in an amount sufficient to complex with the recombinant protein produced. In some embodiments, the recombinant extracellular DNASE proteins and variants thereof from the culture medium of non-mammalian expression system or mammalian expression system, are purified through a method that includes the dissociation of recombinant extracellular DNASE proteins and variants from polyanions such as dextran sulfate, heparins, and EDTA.

In other aspects, the invention provides isolated polynucleotides encoding the D1, D1L1, D1L2, or D1L3 variants, as well as vectors and host cells. Polynucleotides may be encoding mRNA or DNA. Host cells can be cells of a recombinant expression system, including bacterial or eukaryotic, whether non-mammalian such as *Pichia pastoris*, or mammalian such as CHO cells. In other embodiments, the host cell can be delivered for DNASE therapy. For example, the invention in some embodiments provides host cells, e.g., human cells, e.g., white blood cells, modified to secrete one or more of the extracellular DNASE proteins described herein, and intended for administration as a therapeutic agent.

The invention further provides pharmaceutical compositions comprising the extracellular DNASE protein or variant thereof as described herein, or optionally the polynucleotide or the vector as described, and a pharmaceutically acceptable carrier. The pharmaceutical composition may be formulated for any administration route.

5 In other aspects, the invention provides a method for treating a subject in need of extracellular DNA degradation, extracellular chromatin degradation, extracellular trap (ET) degradation and/or neutrophil extracellular trap (NET) degradation, by administering a therapeutically effective amount of the extracellular DNASE or variant thereof or composition described herein.

10 Other aspects and embodiments of the invention will be apparent from the following detailed description.

### DESCRIPTION OF FIGURES

**FIG. 1** illustrates that the mutations Q101R and Q282\_S305delinsK in SEQ ID NO: 4 increase the activity to degrade high-molecular weight chromatin of DNASE1L3. 15 CHO cells were transiently transfected with wild-type DNASE1L3 or DNASE1L3 with building block substitutions. Supernatants of transfected cells were incubated with purified nuclei (high-molecular weight chromatin) or buffer. DNA was isolated and analyzed by agarose gel electrophoresis. The figure shows the agarose gel stained with a DNA dye.

20 **FIG. 2** shows that the characterization of two DNASE1L3 variants. Different concentrations of supernatants of CHO cell that were transfected with wild-type DNASE1L3 or DNASE1L3 with a Q101R or Q282\_S305delinsK mutation were analyzed by Western Blot (WB) using an anti-DNASE1L3 antibody. A larger (variant 1) and a smaller (variant 2) bands were detected in samples with wild-type DNASE1L3 and 25 the Q101R mutant. Only the smaller band (variant 2) was shown in samples with the Q282\_S305delinsK mutant. In parallel, the chromatin degrading activity in the different concentrations of supernatants was analyzed. The figure shows DNA analyzed by agarose gel electrophoresis. Both, Q101R or Q282\_S305delinsK mutations, increased the chromatin degrading activity compared to wild-type DNASE1L3.

30 **FIG. 3** illustrates the presence of DNASE1L3 variant 1 and 2 in supernatants of CHO cell that were stably transfected with wild-type DNASE1L3. Samples were

analyzed by Western Blot (WB) using an anti-DNASE1L3 antibody. A larger (variant 1) and a smaller (variant 2) bands were detected in 5 clones.

**FIG. 4** shows C-terminal amino acid sequences of recombinantly expressed wild-type D1L3 in *Pichia pastoris* to identify frequent cleavage sites. Amino acid sequencing of purified wild-type D1L3 identified three C-terminal deletion mutants: K291\_S305del, K292\_S305del, and S293\_S305del. The C-terminus of wild-type D1L3 was not detected. In parallel, the chromatin degrading activity in the different concentrations of purified protein was analyzed and compared to purified DNASE1 (D1) and the Basic Domain Deleted DNASE1L3 (BDD-D1L3) with a F275Y/F279\_K280delinsVM/Q282\_S305delinsK mutation. The figure shows DNA analyzed by agarose gel electrophoresis.

**FIG. 5** shows that the addition of dextran sulfate to CHO medium improves protein yield. Stable pools of CHO cells expressing wild-type D1L3 were incubated in standard CHO medium or CHO medium supplemented with dextran sulfate. Supernatants were analyzed by Western Blot (WB) using an anti-DNASE1L3 antibody. The figure shows that D1L3 expresses poorly in CHO cells with low yield. Addition of dextran sulfate increases the yield, but does not prevent production fragmentation.

**FIG. 6** illustrates the use of anion exchange surface and cation exchange surface for affinity purification of dextran sulfate-complexed D1L3.

**FIG. 7** lists trypsin cleavage site mutation strategies to limit D1L3 degradation.

**FIG. 8** is an alignment of human D1 (SEQ ID NO: 1) and human D1L3 (SEQ ID NO: 4) amino acid sequences, with plasmin sensitive KR residues shown.

**FIG. 9** illustrates plasmin cleavage site mutation strategies to limit D1L3 degradation.

**FIG. 10** shows that D1L3 with plasmin cleavage sites mutated retains enzymatic activity. Supernatants from cells that were transiently transfected DNASE1L3 containing mutations in four putative plasmin cleavage sites (K180\_A181delinsGL, P198\_A201delinsRPSQ, K259A, R285A) were incubated with purified nuclei (high-

molecular weight chromatin) or buffer. DNA was isolated and analyzed by agarose gel electrophoresis. The figure shows the agarose gel stained with a DNA dye.

**FIG. 11** lists plasmin cleavage sites based on plasmin digestion and shows mutation strategies to limit D1L3 degradation.

5 **FIG. 12** shows that D1L3 has a propensity to misfold when expressed in CHO cells. Panel A illustrates a simple expression vector for D1L3 expression using the native secretory signal peptide. Supernatants of stable pools were analyzed by Western Blot using an anti-DNASE1L3 antibody Panel B shows presence of high molecular weight aggregates under non-reducing conditions, which are resolved under reducing  
10 conditions.

**FIG. 13** is an alignment of human D1 (SEQ ID NO: 1) and human D1L3 (SEQ ID NO: 4) amino acid sequences, with conserved and non-conserved cysteine residues shown.

**FIG. 14** lists the cysteine residues in D1L3, and shows mutation strategies to  
15 limit high molecular weight aggregates during protein expression.

**FIG. 15** shows that C68A and C194A mutation in D1L3 does not impact chromatin degrading activity. The mutations C24A and C52A abrogated chromatin-degrading activity. Supernatants from cells that were transiently transfected with mutated DNASE1L3 variants were incubated with purified nuclei or buffer. DNA was isolated  
20 and analyzed by agarose gel electrophoresis. The figure shows the agarose gel stained with a DNA dye.

**FIG. 16A** illustrates expression of D1L3 in *Pichia pastoris* using either the native secretory signal or  $\alpha$ -mating factor from *Saccharomyces cerevisiae* ( $\alpha$ MF).  $\alpha$ MF resulted in glycosylation and non-processing of the signal peptide (**FIG. 16B**).

25 **FIG. 17A** illustrates a fusion construct with  $\alpha$ MF, human serum albumin (HSA), linker sequence, and D1L3. This fusion construct is not glycosylated in *P. pastoris* expression system (**FIG. 17B**), and retains chromatin-degrading activity (**FIG. 17C**).

**FIG. 18** illustrates the expression levels human serum albumin (HSA) fusion constructs of Basic Domain Deleted-DNASE1L3 (BDD-D1L3) or wild-type DNASE1L3 (D1L3) in *Pichia pastoris*. The HSA is fused either to the N- or C-terminus of BDD-D1L3 or D1L3. Two linker sequences, L1 and L2, were placed between HSA and BDD-D1L3 or D1L3.

**FIG. 19** illustrates the expression levels human serum albumin (HSA) fusion constructs of wild-type DNASE1L3 (D1L3) in *Pichia pastoris*. The HSA is fused to the N- terminus of D1L3. Three different linker sequences (L2, L3, L4) were placed between HSA and D1L3.

**FIG. 20** illustrates the expression levels and chromatin degrading activity of human serum albumin (HSA) fusion constructs of Basic Domain Deleted-DNASE1L3 (BDD-D1L3) produced in *Pichia pastoris*. The HSA is fused to the N- terminus of BDD-D1L3. Three different linker sequences (L5, L6, L7) were placed between HSA and D1L3.

**FIG. 21A** shows that *Dnase1<sup>-/-</sup>Dnase1l3<sup>-/-</sup>* mice injected with SED ID NO: 14 and SEQ ID NO: 19 show similar chromatin degrading activity in serum. **FIG. 21B** shows that SEQ ID NO: 19 has a circulation half-life of 3.3 days in mice expressing the human FcRn receptor.

**FIG. 22** illustrates the expression levels and chromatin degrading activity of human serum albumin (HSA) fusion constructs of Basic Domain Deleted-DNASE1L3 (BDD-D1L3) produced in *Pichia pastoris*. The HSA is fused to the N- terminus and C-terminus of BDD-D1L3. Two different linker sequences (L7 and L8) were placed between HSA and BDD-D1L3.

**FIG. 23** illustrates a fusion construct with HSA and a linker cleavable by Factor XIIa. A linker containing a human Factor XI sequence (SEQ ID NO: 42) and a linker containing a human prekallekrein (SEQ ID NO: 44) sequence are shown.

**FIG. 24** illustrates other constructs that employ Factor XIIa cleavable linkers for half-life extended fusion proteins, including for human extracellular DNases, human coagulation factors, and human complement factors.

## DESCRIPTION OF THE INVENTION

The present invention provides candidates of engineered human extracellular DNASE proteins (e.g., variants of DNASE1 (D1), DNASE1-LIKE 1 (D1L1), DNASE1-LIKE 2 (D1L2), DNASE1-LIKE 3 Isoform 1 (D1L3), DNASE1-LIKE 3 Isoform 2 (D1L3-2), DNASE2A (D2A), and DNASE2B (D2B)) that are useful for treating conditions characterized by extracellular DNA, extracellular chromatin, and neutrophil extracellular trap (NET) accumulation and/or release. In accordance with aspects of the invention, the DNase variants described herein are more suitable and/or effective for therapy and/or are more amenable to large-scale manufacturing. In some embodiments, the DNase variants described herein have benefits for systemic therapy. Such benefits include longer exposure (e.g., slower elimination, longer circulatory half-life), extended duration of pharmacodynamic action, improved chromatin-degrading activity, and protease resistance.

### Definitions

As used herein and in the claims, the singular forms “a,” “an,” and “the” include the singular and the plural reference unless the context clearly indicates otherwise. Thus, for example, a reference to “an agent” includes a single agent and a plurality of such agents.

The term “chromatinase” refers to a class of deoxyribonuclease enzyme that exhibits more than a negligible ability to cut, cleave or digest chromatin, i.e., DNA associated with one or more histone proteins. Human DNASE1L3 is a chromatinase. Generally, the various DNASE1L3 variants disclosed herein are chromatinases. Not all DNASE enzymes are chromatinases. For example, human DNASE1 has essentially no ability to cut, cleave, or digest chromatin and is not a chromatinase.

As used herein with reference to a drug, “half-life” refers to the elimination half-life of the concentration of the drug in an animal, as measured in a matrix of interest, e.g., serum or plasma. The skilled person will understand that not all drugs exhibit first-order kinetics or do so during all phases of elimination. In such cases, the skilled person will understand that the terms “half-life extension” or “extended half-life” are expressions that refer to a slower rate of elimination.

“Isolated” means altered or removed from the natural state. For example, a nucleic acid or a peptide naturally present in a living animal is not “isolated,” but the same nucleic acid or peptide partially or completely separated from the coexisting materials of its natural state is “isolated.” An isolated nucleic acid or protein can exist in substantially purified form, or can exist in a non-native environment such as, for example, a host cell.

As used herein, “neutrophil extracellular trap” and the acronym “NET” refer to a network of extracellular fibers comprising nuclear contents, e.g., DNA bound to histone proteins that are released from an immune cell, typically a neutrophil, in a programmed fashion.

Unless otherwise specified, a “nucleotide sequence or nucleic acid encoding an amino acid sequence” includes all nucleotide sequences that are degenerate versions of each other and that encode the same amino acid sequence. The phrase nucleotide sequence that encodes a protein or an RNA may also include introns to the extent that the nucleotide sequence encoding the protein may in some version contain an intron(s).

The terms “about” and “approximately” include an amount that is  $\pm 10\%$  of an associated numerical value.

The term “extracellular DNASE” refers to extracellular DNASE proteins of the DNASE1- and DNASE2-family (*e.g.*, DNASE1 (D1), DNASE1-LIKE 1 (D1L1), DNASE1-LIKE 2 (D1L2), DNASE1-LIKE 3 Isoform 1 (D1L3), DNASE1-LIKE 3 Isoform 2 (D1L3-2), DNASE2A (D2A), and DNASE2B (D2B)).

In some aspects and embodiments, the extracellular DNASE or variant thereof is fused, optionally by means of an interposed linker, to a half-life extending moiety, such as albumin, transferrin, an Fc, or elastin-like protein, or a variant thereof. See, *e.g.*, US 9,458,218, which is hereby incorporated by reference in its entirety. In some embodiments, the extracellular DNASE or variant thereof is dimerized by an immunoglobulin hinge region. For example, the engineered enzymes described herein may also include an Fc-fusion domain (*e.g.*, a hinge and CH2 domains and CH3 domains of an immunoglobulin). In some embodiments, the DNASE (*e.g.*, D1L3 variant) is fused to an albumin amino acid sequence or domain, *e.g.*, human albumin or a fragment or variant thereof. See, for example, WO 2015/066550 and US 9,221,896, which are hereby

incorporated by reference in their entirety. Albumin can be joined to the DNASE, optionally with an interposed linker, at the N-terminus and/or the C-terminus of the engineered extracellular DNASE or variant thereof. An exemplary albumin amino acid sequence is provided by SEQ ID NO: 39. In some embodiments, D1L3 and D1, or  
5 variants as described herein, are together dimerized by an Fc hinge region, creating a dimeric molecule with synergistic functional properties for degrading NETs. In some embodiments, the extracellular DNASE or variant thereof is fused at the N-terminus to an albumin amino acid sequence, through a peptide linker. The peptide linker may be a flexible linker, a rigid linker, or in some embodiments a physiologically-cleavable linker  
10 (e.g., a protease-cleavable linker). In some embodiments, the linker is 5 to 100 amino acids in length, or is 5 to 50 amino acids in length. In still other embodiments, the linker is an organic molecule, group, polymer (e.g., PEG), or chemical moiety that is covalently coupled to the extracellular DNASE and half-life extending moiety (e.g., albumin).

In some aspects, the invention provides a D1L3 variant, wherein the D1L3 variant  
15 has one or more of increased protein stability, increased pharmacokinetic exposure and duration of pharmacodynamic activity, resistance to proteolytic degradation, higher production levels with *in vitro* expression systems, better suitability for purification, and not substantially less, the same, or better chromatin and/or NET-degrading activity as compared to wild-type D1L3 Isoform 1 enzyme of SEQ ID NO:4 or wild-type D1L3  
20 Isoform 2 enzyme of SEQ ID NO:5. As used herein, unless stated to the contrary, the term “D1L3” includes either Isoform 1 or Isoform 2.

The DNA- and/or chromatin- and/or NET-degrading activity of an enzyme, e.g. a D1L3 variant, can be measured *in vitro*, for example by incubation of the enzyme with DNA, chromatin, or NETs, obtained, e.g., from purified nuclei, DNA, or *ex vivo* blood  
25 or neutrophils induced to form NETs. Alternatively, the DNA- and/or chromatin- and/or NET-degrading activity of an enzyme, e.g. a D1L3 variant, can be measured *in vivo*, for example by administering the enzyme to a subject, wherein the subject produces or is induced to produce extracellular DNA, chromatin, or NETs, and measuring the effect of the enzyme on concentrations of DNA, chromatin, or NET levels in a matrix, e.g. serum,  
30 preferably with a parallel negative control, or by temporally comparing the concentrations before and after administration of the enzyme.

In some embodiments, the D1L3 variant has approximately the same chromatin- and/or NET-degrading activity as compared to wild-type D1L3 Isoform 1 enzyme of

SEQ ID NO:4 or wild-type D1L3 Isoform 2 enzyme of SEQ ID NO:5. In some embodiments, D1L3 variant has higher chromatin- and/or NET-degrading activity as compared to wild-type D1L3 Isoform 1 enzyme of SEQ ID NO:4 or wild-type D1L3 Isoform 2 enzyme of SEQ ID NO:5.

5 In some embodiments, the D1L3 variant is a fusion protein comprising an albumin domain, an optional linker, and a D1L3 domain. In some embodiments, the albumin domain and optional linker are located on the N-terminal side of the D1L3 domain. In some embodiments, the albumin domain and optional linker are located on the C-terminal side of the D1L3 domain. In all such embodiments, the optional linker is  
10 interposed between the albumin domain and the D1L3 domain.

In some embodiments, the albumin amino acid sequence or domain of the fusion protein is at least about 75%, or at least about 80%, or at least about 85%, at least 90%, at least 95%, at least 97%, at least 98%, or at least 99% identical to the reference albumin sequence defined by SEQ ID NO: 39. In some embodiments, the albumin amino acid  
15 sequence or domain comprises or consists of the reference albumin sequence defined by SEQ ID NO:39. In various embodiments, the albumin amino acid sequence binds to the neonatal Fc receptor (FcRn), e.g., human FcRn. The albumin amino acid sequence may be a variant of wild-type HSA (e.g., as represented by SEQ ID NO: 39). In various embodiments, albumin variants may have from one to twenty, or from one to ten amino  
20 acid modifications independently selected from deletions, substitutions, and insertions with respect to SEQ ID NO: 39. In some embodiments, the albumin amino acid sequence is any mammalian albumin amino acid sequence.

In some embodiments, the albumin amino acid sequence or domain is a fragment of full-length albumin, as represented by SEQ ID NO: 39. The term “fragment,” when  
25 used in the context of albumin, refers to any fragment of full-length albumin or a variant thereof (as described above) that extends the half-life of a DNASE enzyme to which it is fused or conjugated, relative to the corresponding non-fused DNASE. In some embodiments, a fragment of an albumin can refer to an amino acid sequence comprising a fusion of multiple domains of albumin (see, e.g., WO2011/124718), such as domains I  
30 and III, and domains II and III. Generally, a fragment of albumin has at least about 100 amino acids or at least about 200 or at least about 300 amino acids of the full-length sequence. In various embodiments, the albumin fragment maintains the ability to bind human FcRn.

In some embodiments, the D1L3-like domain of the fusion protein is at least about 85%, or at least about 90%, or at least about 95%, at least about 97%, or at least about 98%, or at least about 99% identical to the mature D1L3 enzyme reference sequence defined by SEQ ID NO:4 or SEQ ID NO:5. In some embodiments, the D1L3 domain comprises or consists of the reference sequence defined by SEQ ID NO:4 or SEQ ID NO:5. In some embodiments, the reference sequence does not include the C-terminal basic domain of SEQ ID NO: 4 or 5 defined by the C-terminal 23 amino acids.

In some embodiments, the fusion protein comprises an D1L3 domain, wherein the amino acid sequence of the D1L3 domain is at least about 80% identical to the mature enzyme defined by SEQ ID NO:4 or SEQ ID NO:5. The fusion protein can further comprise the albumin amino acid sequence or domain at the N-terminus of the mature enzyme, and a linking amino acid sequence between the albumin amino acid sequence and the amino acid sequence of the mature enzyme. In some embodiments, the D1L3 domain comprises an amino acid sequence that is at least about 90% identical to the mature enzyme reference sequence defined by SEQ ID NO:4 or SEQ ID NO:5. In some embodiments, the reference sequence does not include the C-terminal basic domain of SEQ ID NO: 4 or 5 defined by the C-terminal 23 amino acids. The fusion protein comprising the D1L3 domain exhibits improved circulatory half-life and duration of pharmacodynamic effect, including for systemic therapy. In addition, the fusion of albumin with linking sequence does not substantially impact (or in some embodiments does not have any negative impact on) chromatin-degrading activity as determined using an in vitro chromatin-degrading assay, as compared to the variant without an albumin fusion.

When referring to sequence identity with wild-type DNase enzymes, and unless stated otherwise, sequences refer to mature enzymes lacking the signal peptide. Further, unless stated otherwise, amino acid positions are numbered with respect to the full-translated DNase sequence, including signal peptide, for clarity. Accordingly, for example, reference to sequence identity to the enzyme of SEQ ID NO:4 (human D1L3, Isoform 1) refers to a percent identity with the mature enzyme having M21 at the N-terminus. Similarly, reference to sequence identity to the enzyme of SEQ ID NO:1 (human D1) refers to a percent identity with the mature enzyme having L23 at the N-terminus.

In some embodiments, the D1L3 has a deletion of all or part of the C-terminal basic domain. The C-terminal basic domain is defined as the C-terminal 23 amino acids of SEQ ID NO:4 or SEQ ID NO:5. Deletion or inactivation of the C-terminal basic domain of D1L3 substantially improves chromatin degrading activity. See **FIGS. 1, 2,**  
 5 **and 4.** In some embodiments, the D1L3 variant has a deletion of C-terminal basic domain amino acids, such as at least 5 amino acids, or in some embodiments at least 10 amino acids, or in some embodiments at least 15 amino acids, or in some embodiments at least 20 amino acids of the C-terminal basic domain. In some embodiments, the D1L3 variant has a deletion of the entire C-terminal basic domain defined by the C-terminal 23 amino  
 10 acids of SEQ ID NO:4 or SEQ ID NO:5. Exemplary BD deletions include Q282\_S305delinsK (see SEQ ID NO: 9), S305delinsK (see SEQ ID NO: 10), K292\_S305del (see SEQ ID NO: 11), and S293\_S305del (see SEQ ID NO: 12). In some embodiments, the C-terminus of the D1L3 domain (having a BD deletion) has from 1 to 10 or from 1 to 5 amino acids at the C-terminus that do not align with the C-terminal BD,  
 15 and which do not negatively impact chromatin degrading activity in an in vitro assay.

In some embodiments, the D1L3 variant is an engineered fusion protein comprising: a DNASE1L3 domain of a sequence selected from SEQ ID NO:8 through SEQ ID NO:16; a linker of a sequence selected from SEQ ID NO:31 through SEQ ID NO:38; and an albumin domain having the sequence of SEQ ID NO:39 or a variants or  
 20 fragment as described. In some embodiments, the D1L3 variant has one or more building block substitutions from D1, which are described in PCT/US2018/04708, which is hereby incorporated by reference.

In some embodiments, the D1L3 sequence or domain contains a building block substitution from D1, which can be selected from one or more of: M21\_R22delinsLK,  
 25 C24\_S25delinsAA, V28\_S30delinsIQT, S34T, Q36\_V44delinsMSNATLVSY, K47\_K50delinsQILS, C52Y, I55\_M58delinsIALVQE, I60\_K61delinsVR, N64\_I70delinsHLTAVGK, M72\_K74delinsLDN, R77\_I83delinsQDAPD, N86H, I89V, S91\_R92delinsEP, T97S, Q101R, A103L, L105V, K107\_L110delinsRPDQ, V113\_R115delinsAVD, H118Y, H120D, Y122\_A127delinsGCEPCGN, V129T,  
 30 S131N, F135\_V136delinsAI, W138R, Q140\_H143delinsFSRF, A145\_D148delinsEVRE, V150A, I152V, T156\_T157delinsAA, E159\_S161delinsGDA, K163A, E167A, V169\_E170delinsYD, T173L, K176\_R178delinsQEK, K180\_A181delinsGL, N183\_F186delinsDVML,

P198\_A201delinsRPSQ, K203\_N204delinsSS, R208W, D210S, R212T, V214Q, G218P, Q220\_E221delinsSA, V225\_S228delinsATP, N230H, L238\_R239delinsVA, Q241\_S246delinsMLLRGA, K250D, N252\_V254delinsALP, D256N, K259A, K262G, T264\_E267delinsSDQL, L269\_V271delinsQAI, F275Y, F279\_K280delinsVM,  
 5 Q282\_S205delinsK, wherein each of the foregoing substitutions is numbered with respect to SEQ ID NO: 4.

For example, the D1L3 variant may have the building block substitution from D1 of Q282\_S305delinsK, which includes a deletion of the C terminal basic domain, which is absent in D1. In some embodiments, the D1L3 enzyme has an amino acid substitution  
 10 at the position corresponding to position 101 of SEQ ID NO:4. The substitution can be Arg based on the corresponding building block from D1, or in some embodiments is Lys. Substitutions at this position can enhance chromatin-degrading activity of D1L3. Other substitutions at this position will likely show similar properties.

Linkers where present can be selected from flexible, rigid, and cleavable peptide  
 15 linkers. Flexible linkers are predominately or entirely composed of small, non-polar or polar residues such as Gly, Ser and Thr. An exemplary flexible linker comprises (Gly<sub>y</sub>Ser)<sub>n</sub> linkers, where y is from 1 to 10 (e.g., from 1 to 5), and n is from 1 to about 10, and in some embodiments, is from 3 to about 6. In exemplary embodiments, y is from 2 to 4, and n is from 3 to 8. Due to their flexibility, these linkers are unstructured. More  
 20 rigid linkers include polyproline or poly Pro-Ala motifs and  $\alpha$ -helical linkers. An exemplary  $\alpha$ -helical linker is A(EAAAK)<sub>n</sub>A, where n is as defined above (e.g., from 1 to 10, or 2 to 6). Generally, linkers can be predominately composed of amino acids selected from Gly, Ser, Thr, Ala, and Pro. Exemplary linker sequences contain at least 10 amino acids, and may be in the range of 15 to 35 amino acids. Exemplary linker designs are  
 25 provided as SEQ ID NOS: 31 to 38.

In some embodiments, the variant comprises a linker, wherein the amino acid sequence of the linker is predominately glycine and serine residues, or consists essentially of glycine and serine residues. In some embodiments, the ratio of Ser and Gly in the linker is, respectively, from about 1:1 to about 1:10, from about 1:2 to about 1:6,  
 30 or about 1:4. Exemplary linker sequences comprise S(GGS)<sub>4</sub>GSS (SEQ ID NO: 36), S(GGS)<sub>9</sub>GS (SEQ ID NO: 37), (GGS)<sub>9</sub>GS (SEQ ID NO: 39). In some embodiments, the linker has at least 10 amino acids, or at least 15 amino acids, or at least 20 amino acids, or at least 25 amino acids. For example, the linker may have a length of from 15 to 30

amino acids. In various embodiments, longer linkers of at least 15 amino acids can provide improvements in yield upon expression in *Pichia pastoris*. See **FIG. 20**. Further, and surprisingly, longer linker sequences showed improved chromatin-degrading activity, as compared to shorter linker sequences. See **FIG. 20**.

5 In various embodiments, D1L3 variant is a fusion protein comprising the amino acid sequence of any one of SEQ ID NOS: 17 to 30. In other embodiments, the D1L3 variant is a fusion protein comprising the amino acid sequence of any one of SEQ ID NOS: 17 to 30 and having from one to twenty or from one to ten, or from one to five amino acid modifications independently selected from amino acid insertions, deletions, 10 or substitutions with respect to the reference sequence selected from SEQ ID NOS: 17 to 30. In some embodiments, the amino acid modifications are in the D1L3 domain, the albumin domain, or in both domains of the fusion protein. In some embodiments, the variant has the amino acid sequence of SEQ ID NO: 19, SEQ ID NO: 22, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, or SEQ ID NO: 30. In these 15 embodiments, the albumin amino acid sequence is fused at the N-terminus or N-terminal side of D1L3 (or variant) through an intermediate or long flexible linker.

In other embodiments, the linker is a physiologically-cleavable linker, such as a protease-cleavable linker. For example, the protease may be a coagulation pathway protease, such as activated Factor XII. In certain embodiments, the linker comprises the 20 amino acid sequence of Factor XI (SEQ ID NO: 42) and/or prekallikrein (SEQ ID NO: 44 or 45) or a physiologically cleavable fragment thereof. In selected embodiments, the linker amino acid sequence from Factor XI contains all or parts of SEQ ID NO: 42 (e.g., parts of SEQ ID NO:42, including modifications of SEQ ID NO:42 that allow for cleavage by Factor XIIa). In some embodiments, the linker amino acid sequence from 25 prekallikrein contains all or parts of SEQ ID NO: 44 (e.g., parts of SEQ ID NO: 44, including modifications of SEQ ID NO: 44 that allow for cleavage by Factor XIIa). In other embodiments, the linker includes a peptide sequence that is targeted for cleavage by a neutrophil specific protease, such as neutrophil elastase, cathepsin G, and proteinase 3.

30 Some exemplary embodiments of D1L3 fusion proteins comprise a combination of three amino acid sequences that can be independently selected from sequences disclosed herein, and such sequences arranged in order from N-terminus to C-terminus:

Fusion 1: SEQ ID NO:4, SEQ ID NO:31, SEQ ID NO:39;  
 Fusion 2: SEQ ID NO:5, SEQ ID NO:31, SEQ ID NO:39;  
 Fusion 3: SEQ ID NO:8, SEQ ID NO:31, SEQ ID NO:39;  
 Fusion 4: SEQ ID NO:9, SEQ ID NO:31, SEQ ID NO:39;  
 5 Fusion 5: SEQ ID NO:10, SEQ ID NO:31, SEQ ID NO:39;  
 Fusion 6: SEQ ID NO:11, SEQ ID NO:31, SEQ ID NO:39;  
 Fusion 7: SEQ ID NO:12 SEQ ID NO:31, SEQ ID NO:39;  
 Fusion 8: SEQ ID NO:13, SEQ ID NO:31, SEQ ID NO:39;  
 Fusion 9: SEQ ID NO:14, SEQ ID NO:31, SEQ ID NO:39;  
 10 Fusion 10: SEQ ID NO:15, SEQ ID NO:31, SEQ ID NO:39;  
 Fusion 11: SEQ ID NO:16, SEQ ID NO:31, SEQ ID NO:39;  
 Fusion 12: SEQ ID NO:4, SEQ ID NO:32, SEQ ID NO:39;  
 Fusion 13: SEQ ID NO:5, SEQ ID NO:32, SEQ ID NO:39;  
 Fusion 14: SEQ ID NO:8, SEQ ID NO:32, SEQ ID NO:39;  
 15 Fusion 15: SEQ ID NO:9, SEQ ID NO:32, SEQ ID NO:39;  
 Fusion 16: SEQ ID NO:10, SEQ ID NO:32, SEQ ID NO:39;  
 Fusion 17: SEQ ID NO:11, SEQ ID NO:32, SEQ ID NO:39;  
 Fusion 18: SEQ ID NO:12 SEQ ID NO:32, SEQ ID NO:39;  
 Fusion 19: SEQ ID NO:13, SEQ ID NO:32, SEQ ID NO:39;  
 20 Fusion 20: SEQ ID NO:14, SEQ ID NO:32, SEQ ID NO:39;  
 Fusion 21: SEQ ID NO:15, SEQ ID NO:32, SEQ ID NO:39;  
 Fusion 22: SEQ ID NO:16, SEQ ID NO:32, SEQ ID NO:39;  
 Fusion 23: SEQ ID NO:4, SEQ ID NO:33, SEQ ID NO:39;  
 Fusion 24: SEQ ID NO:5, SEQ ID NO:33, SEQ ID NO:39;  
 25 Fusion 25: SEQ ID NO:8, SEQ ID NO:33, SEQ ID NO:39;  
 Fusion 26: SEQ ID NO:9, SEQ ID NO:33, SEQ ID NO:39;  
 Fusion 27: SEQ ID NO:10, SEQ ID NO:33, SEQ ID NO:39;  
 Fusion 28: SEQ ID NO:11, SEQ ID NO:33, SEQ ID NO:39;  
 Fusion 29: SEQ ID NO:12 SEQ ID NO:33, SEQ ID NO:39;  
 30 Fusion 30: SEQ ID NO:13, SEQ ID NO:33, SEQ ID NO:39;  
 Fusion 31: SEQ ID NO:14, SEQ ID NO:33, SEQ ID NO:39;  
 Fusion 32: SEQ ID NO:15, SEQ ID NO:33, SEQ ID NO:39;  
 Fusion 33: SEQ ID NO:16, SEQ ID NO:33, SEQ ID NO:39;  
 Fusion 34: SEQ ID NO:4, SEQ ID NO:34, SEQ ID NO:39;

Fusion 35: SEQ ID NO:5, SEQ ID NO:34, SEQ ID NO:39;  
 Fusion 36: SEQ ID NO:8, SEQ ID NO:34, SEQ ID NO:39;  
 Fusion 37: SEQ ID NO:9, SEQ ID NO:34, SEQ ID NO:39;  
 Fusion 38: SEQ ID NO:10, SEQ ID NO:34, SEQ ID NO:39;  
 5 Fusion 39: SEQ ID NO:11, SEQ ID NO:34, SEQ ID NO:39;  
 Fusion 40: SEQ ID NO:12 SEQ ID NO:34, SEQ ID NO:39;  
 Fusion 41: SEQ ID NO:13, SEQ ID NO:34, SEQ ID NO:39;  
 Fusion 42: SEQ ID NO:14, SEQ ID NO:34, SEQ ID NO:39;  
 Fusion 43: SEQ ID NO:15, SEQ ID NO:34, SEQ ID NO:39;  
 10 Fusion 44: SEQ ID NO:16, SEQ ID NO:34, SEQ ID NO:39;  
 Fusion 45: SEQ ID NO:4, SEQ ID NO:35, SEQ ID NO:39;  
 Fusion 46: SEQ ID NO:5, SEQ ID NO:35, SEQ ID NO:39;  
 Fusion 47: SEQ ID NO:8, SEQ ID NO:35, SEQ ID NO:39;  
 Fusion 48: SEQ ID NO:9, SEQ ID NO:35, SEQ ID NO:39;  
 15 Fusion 49: SEQ ID NO:10, SEQ ID NO:35, SEQ ID NO:39;  
 Fusion 50: SEQ ID NO:11, SEQ ID NO:35, SEQ ID NO:39;  
 Fusion 51: SEQ ID NO:12 SEQ ID NO:35, SEQ ID NO:39;  
 Fusion 52: SEQ ID NO:13, SEQ ID NO:35, SEQ ID NO:39;  
 Fusion 53: SEQ ID NO:14, SEQ ID NO:35, SEQ ID NO:39;  
 20 Fusion 54: SEQ ID NO:15, SEQ ID NO:35, SEQ ID NO:39;  
 Fusion 55: SEQ ID NO:16, SEQ ID NO:35, SEQ ID NO:39;  
 Fusion 56: SEQ ID NO:4, SEQ ID NO:36, SEQ ID NO:39;  
 Fusion 57: SEQ ID NO:5, SEQ ID NO:36, SEQ ID NO:39;  
 Fusion 58: SEQ ID NO:8, SEQ ID NO:36, SEQ ID NO:39;  
 25 Fusion 59: SEQ ID NO:9, SEQ ID NO:36, SEQ ID NO:39;  
 Fusion 60: SEQ ID NO:10, SEQ ID NO:36, SEQ ID NO:39;  
 Fusion 61: SEQ ID NO:11, SEQ ID NO:36, SEQ ID NO:39;  
 Fusion 62: SEQ ID NO:12 SEQ ID NO:36, SEQ ID NO:39;  
 Fusion 63: SEQ ID NO:13, SEQ ID NO:36, SEQ ID NO:39;  
 30 Fusion 64: SEQ ID NO:14, SEQ ID NO:36, SEQ ID NO:39;  
 Fusion 65: SEQ ID NO:15, SEQ ID NO:36, SEQ ID NO:39;  
 Fusion 66: SEQ ID NO:16, SEQ ID NO:36, SEQ ID NO:39;  
 Fusion 67: SEQ ID NO:4, SEQ ID NO:37, SEQ ID NO:39;  
 Fusion 68: SEQ ID NO:5, SEQ ID NO:37, SEQ ID NO:39;

Fusion 69: SEQ ID NO:8, SEQ ID NO:37, SEQ ID NO:39;  
 Fusion 70: SEQ ID NO:9, SEQ ID NO:37, SEQ ID NO:39;  
 Fusion 71: SEQ ID NO:10, SEQ ID NO:37, SEQ ID NO:39;  
 Fusion 72: SEQ ID NO:11, SEQ ID NO:37, SEQ ID NO:39;  
 5 Fusion 73: SEQ ID NO:12 SEQ ID NO:37, SEQ ID NO:39;  
 Fusion 74: SEQ ID NO:13, SEQ ID NO:37, SEQ ID NO:39;  
 Fusion 75: SEQ ID NO:14, SEQ ID NO:37, SEQ ID NO:39;  
 Fusion 76: SEQ ID NO:15, SEQ ID NO:37, SEQ ID NO:39;  
 Fusion 77: SEQ ID NO:16, SEQ ID NO:37, SEQ ID NO:39;  
 10 Fusion 78: SEQ ID NO:4, SEQ ID NO:38, SEQ ID NO:39;  
 Fusion 79: SEQ ID NO:5, SEQ ID NO:38, SEQ ID NO:39;  
 Fusion 80: SEQ ID NO:8, SEQ ID NO:38, SEQ ID NO:39;  
 Fusion 81: SEQ ID NO:9, SEQ ID NO:38, SEQ ID NO:39;  
 Fusion 82: SEQ ID NO:10, SEQ ID NO:38, SEQ ID NO:39;  
 15 Fusion 83: SEQ ID NO:11, SEQ ID NO:38, SEQ ID NO:39;  
 Fusion 84: SEQ ID NO:12 SEQ ID NO:38, SEQ ID NO:39;  
 Fusion 85: SEQ ID NO:13, SEQ ID NO:38, SEQ ID NO:39;  
 Fusion 86: SEQ ID NO:14, SEQ ID NO:38, SEQ ID NO:39;  
 Fusion 87: SEQ ID NO:15, SEQ ID NO:38, SEQ ID NO:39;  
 20 Fusion 88: SEQ ID NO:16, SEQ ID NO:38, SEQ ID NO:39;  
 Fusion 89: SEQ ID NO:4, SEQ ID NO:42, SEQ ID NO:39;  
 Fusion 90: SEQ ID NO:5, SEQ ID NO:42, SEQ ID NO:39;  
 Fusion 91: SEQ ID NO:8, SEQ ID NO:42, SEQ ID NO:39;  
 Fusion 92: SEQ ID NO:9, SEQ ID NO:42, SEQ ID NO:39;  
 25 Fusion 93: SEQ ID NO:10, SEQ ID NO:42, SEQ ID NO:39;  
 Fusion 94: SEQ ID NO:11, SEQ ID NO:42, SEQ ID NO:39;  
 Fusion 95: SEQ ID NO:12 SEQ ID NO:42, SEQ ID NO:39;  
 Fusion 96: SEQ ID NO:13, SEQ ID NO:42, SEQ ID NO:39;  
 Fusion 97: SEQ ID NO:14, SEQ ID NO:42, SEQ ID NO:39;  
 30 Fusion 98: SEQ ID NO:15, SEQ ID NO:42, SEQ ID NO:39;  
 Fusion 99: SEQ ID NO:16, SEQ ID NO:42, SEQ ID NO:39;  
 Fusion 100: SEQ ID NO:4, SEQ ID NO:43, SEQ ID NO:39;  
 Fusion 101: SEQ ID NO:5, SEQ ID NO:43, SEQ ID NO:39;  
 Fusion 102: SEQ ID NO:8, SEQ ID NO:43, SEQ ID NO:39;

Fusion 103: SEQ ID NO:9, SEQ ID NO:43, SEQ ID NO:39;  
 Fusion 104: SEQ ID NO:10, SEQ ID NO:43, SEQ ID NO:39;  
 Fusion 105: SEQ ID NO:11, SEQ ID NO:43, SEQ ID NO:39;  
 Fusion 106: SEQ ID NO:12 SEQ ID NO:43, SEQ ID NO:39;  
 5 Fusion 107: SEQ ID NO:13, SEQ ID NO:43, SEQ ID NO:39;  
 Fusion 108: SEQ ID NO:14, SEQ ID NO:43, SEQ ID NO:39;  
 Fusion 109: SEQ ID NO:15, SEQ ID NO:43, SEQ ID NO:39;  
 Fusion 110: SEQ ID NO:16, SEQ ID NO:43, SEQ ID NO:39;  
 Fusion 111: SEQ ID NO:4, SEQ ID NO:44, SEQ ID NO:39;  
 10 Fusion 112: SEQ ID NO:5, SEQ ID NO:44, SEQ ID NO:39;  
 Fusion 113: SEQ ID NO:8, SEQ ID NO:44, SEQ ID NO:39;  
 Fusion 114: SEQ ID NO:9, SEQ ID NO:44, SEQ ID NO:39;  
 Fusion 115: SEQ ID NO:10, SEQ ID NO:44, SEQ ID NO:39;  
 Fusion 116: SEQ ID NO:11, SEQ ID NO:44, SEQ ID NO:39;  
 15 Fusion 117: SEQ ID NO:12 SEQ ID NO:44, SEQ ID NO:39;  
 Fusion 118: SEQ ID NO:13, SEQ ID NO:44, SEQ ID NO:39;  
 Fusion 119: SEQ ID NO:14, SEQ ID NO:44, SEQ ID NO:39;  
 Fusion 120: SEQ ID NO:15, SEQ ID NO:44, SEQ ID NO:39;  
 Fusion 121: SEQ ID NO:16, SEQ ID NO:44, SEQ ID NO:39;  
 20 Fusion 122: SEQ ID NO:4, SEQ ID NO:45, SEQ ID NO:39;  
 Fusion 123: SEQ ID NO:5, SEQ ID NO:45, SEQ ID NO:39;  
 Fusion 124: SEQ ID NO:8, SEQ ID NO:45, SEQ ID NO:39;  
 Fusion 125: SEQ ID NO:9, SEQ ID NO:45, SEQ ID NO:39;  
 Fusion 126: SEQ ID NO:10, SEQ ID NO:45, SEQ ID NO:39;  
 25 Fusion 127: SEQ ID NO:11, SEQ ID NO:45, SEQ ID NO:39;  
 Fusion 128: SEQ ID NO:12 SEQ ID NO:45, SEQ ID NO:39;  
 Fusion 129: SEQ ID NO:13, SEQ ID NO:45, SEQ ID NO:39;  
 Fusion 130: SEQ ID NO:14, SEQ ID NO:45, SEQ ID NO:39;  
 Fusion 131: SEQ ID NO:15, SEQ ID NO:45, SEQ ID NO:39;  
 30 Fusion 132: SEQ ID NO:16, SEQ ID NO:45, SEQ ID NO:39;

In some embodiments, the fusion protein is synthesized with a signal peptide.  
 The signal peptide may be removed during secretion from the host cell. Exemplary signal

peptides are shown SEQ ID NOS: 4 to 16 and SEQ ID NOS: 44 to 46. In some embodiments, the fusion protein is the mature protein, that is, lacking a signal peptide.

In various embodiments, the fusion protein is selected from fusion proteins 1 to 132, and the selected fusion protein may optionally have up to 20 (or up to 10) amino acid modifications independently selected from amino acid deletions, insertions, and substitutions.

In some aspects, the invention provides variants of extracellular DNASE enzymes engineered to have advantages in manufacturing, providing for production of the recombinant enzyme suitable for use in therapy, and which can optionally be used in connection with fusion protein embodiments (including albumin fusion embodiments) as already described. In various embodiments, the invention provides a recombinant D1, D1L1, D1L2, and D1L3 variant comprising one or more amino acid substitutions or deletions of cysteine residues resulting in reduced intra- and inter-molecular cross-linking via disulfide bridges during protein expression. For example, the DNase variant may lack one, two, or three cysteine residues present in the wild-type sequence (e.g., one, two, or three cysteine residues are deleted), or has one or more of such cysteine(s) substituted with other amino acid(s). In some embodiments, the one or more cysteine residues are substituted with an amino acid independently selected from Ala, Gly, and Ser, or one or more of the cysteine residues are substituted as part of a building block substitution. In some embodiments, the one or more cysteine residues that are substituted is/are not conserved between other members of the D1 protein family (e.g., D1, D1L1, D1L2, and D1L3). In some embodiments, the engineered enzyme comprises or further comprises at least one building block substitution from another member of the D1 protein family and/or other point mutation that results in increased protein stability, increased resistance towards degradation by proteases, increased bioavailability, and substantially the same or better DNA and/or chromatin and/or NET-degrading activity (*in vitro* or *in vivo*) as compared to the wild-type enzyme. In some embodiments, the substitutions and/or modifications include, among other modifications, only a single modification in cysteine residues. In some embodiments, removal of a single cysteine residue is sufficient for significant advantages in manufacturing.

In other aspects, the invention provides variants of extracellular DNASE enzymes engineered to have advantages in protease resistance, for improving *in vivo* half-life as well as reducing proteolysis during recombinant enzyme production. This disclosure

identifies, for example, D1L3 residues that are sensitive to proteolysis by plasmin, thrombin, and/or trypsin, as well as residues (e.g., paired basic amino acids) that are sensitive to proteases produced by mammalian and non-mammalian cell lines.

5 The recombinant extracellular DNASE variants described herein may have a combination of point mutations including substitutions in cysteine residues, substitutions in protease-sensitive residues, and/or may comprise one or more block substitutions. Building Block Protein Engineering (BBPE) is described in PCT/US18/47084 and US 62/800,790, the disclosures of which are hereby incorporated by reference. BBPE involves providing a protein-protein alignment of donor and recipient extracellular  
10 DNASE enzyme and identifying variable amino acid sequences for transfer (“building block”). The variable amino acid(s) are flanked by one or more conserved amino acids in the donor and recipient extracellular DNASE enzymes (upstream and downstream of the building block). These building blocks can be swapped between recipient and donor proteins, to produce a chimeric enzyme.

15 In other aspects, the invention provides a method for recombinant production of extracellular DNASE proteins, including variants thereof described herein. In some embodiments, the method employs a non-mammalian expression system, such as *Pichia pastoris*. In some embodiments, the *Pichia pastoris* encodes the DNase enzyme with the native signal peptide allowing for secretion from host cells. In some embodiments, the  
20 expression system is a mammalian cell expression system, such as Chinese Hamster Ovary (CHO) cells. In some embodiments, by removing cysteine residues that are unnecessary for activity, the invention avoids inter-molecular and intra-molecular disulfide bonds that otherwise form and hinder recombinant production. In some embodiments, substantial reductions in erroneous inter-molecular and intra-molecular  
25 disulfide bonds can be achieved with the substitution of a single cysteine residue.

In some embodiments, the recombinant expression system has a deletion or inactivation of one or more proteases that cleave at paired basic amino acids. Exemplary enzymes include Furin (expressed by CHO cells) and Aspartic proteinase 3 (Ysp1) and Kexin (Kex2) expressed by *Pichia pastoris*. In some embodiments, these enzymes are  
30 not genetically deleted or inactivated, but their activity is inhibited with a protease inhibitor during recombinant protein production.

In some embodiments, the growth medium for the non-mammalian expression system or mammalian expression system is supplemented with polyanions such as dextran sulfate, heparins, ferric citrate, and EDTA. In further embodiments, the growth medium of *Pichia pastoris* or other expression system is supplemented with dextran sulfate that has an average molecular weight of between 5 kDa and 100 kDa. In some  
5       embodiments, the dextran sulfate has an average molecular weight that is about 10 kDa or less, or about 20 kDa or less, or about 30 kDa or less, or about 40 kDa or less, or about 50 kDa or less, or about 75 kDa or less, or about 100 kDa or less. In various embodiments, the polyanion is added to the culture in an amount sufficient to complex with the  
10       recombinant protein produced.

In some embodiments, the recombinant extracellular DNASE proteins and variants thereof from the culture medium of non-mammalian expression system or mammalian expression system, are purified through a method that includes the dissociation of recombinant extracellular DNASE proteins and variants from polyanions  
15       such as dextran sulfate, heparins, EDTA. In certain embodiments, the purification method includes strong anion exchange resins such as triethylaminoethyl. In some embodiments, the extracellular DNASE protein produced according to the method is D1L3 or a variant thereof.

Accordingly, in some embodiments the invention provides a D1L3 variant  
20       comprising an amino acid sequence that is at least 80% identical to the enzyme defined by SEQ ID NO: 4 (human D1L3, Isoform 1) or SEQ ID NO: 5 (human D1L3, Isoform 2), and having one or more substitutions of cysteine residues and/or one or more substitutions of amino acids that are sensitive to proteolysis, e.g., *in vivo* proteolysis. In some embodiments, the D1L3 protein variant comprises one or more additional  
25       modifications that result in increased protein stability (e.g., protease resistance), higher production levels with *in vitro* expression systems, and/or not substantially less, the same, or better DNA and/or chromatin and/or NET-degrading activity as compared to wild-type D1L3 protein of SEQ ID NO:4 or SEQ ID NO: 5. For example, the D1L3 variant may comprise at least one additional building block substitution or point mutation  
30       disclosed in PCT/US2018/47084 (which is hereby incorporated by reference in its entirety), or may include one or more substitutions described herein for increasing protease resistance.

In some embodiments, the D1L3 variant has a substitution of Cys 68, which is optionally substituted with an amino acid selected from Ala, Ser, and Gly. In some embodiments, the variant comprises the substitution N64\_I70delinsHLTAVGK. In some embodiments, the sequence HLTAVGK can be further modified by one, two, or three  
 5 substitutions, deletions, and/or insertions (collectively), with the proviso that a Cys residue is not included. In some embodiments, the D1L3 variant comprises an amino acid sequence that has at least about 85%, at least about 90%, at least about 95%, or at least about 98% identity to the reference SEQ ID NO:4 or SEQ ID NO: 5.

In some embodiments, the invention provides a D1L3 enzyme having a  
 10 polyethylene glycol (PEG) moiety conjugated at the position corresponding to Cys 68, which is believed to be an unpaired cysteine. In some embodiments, the D1L3 variant has a PEG conjugation to the amino acid corresponding to C194. In these embodiments, the PEG moiety will provide a half-life extension property, while avoiding disulfide scrambling and/or protein misfolding. In some embodiments, the PEG moiety is  
 15 conjugated through maleimide chemistry, which can be conducted under mild conditions. Other conjugation chemistries are known and may be used, such as vinyl sulfone, dithiopyridine, and iodoacetamide activation chemistries. The PEG moiety can be linear or branched, and can be generally in the range of 10 kDa to 40 kDa, or in the range of 20 to 30 kDa.

Alternatively, or in addition, the invention provides a D1L3 variant comprising  
 20 one or more substituted arginine and/or lysine residues resulting in increased protease resistance. In some embodiments, the D1L3 variant has a substitution at one or more positions corresponding to K180, K200, K259, and/or R285 of SEQ ID NO:4. In accordance with this disclosure, such lysine and arginine residues are identified as  
 25 potential protease-sensitive sites. Thus, one or more (e.g., 1, 2, 3, or 4) of these residues may be modified with a non-charged residue, such as a residue independently selected from Ala, Gly, Leu, Ile, Val, Thr, Ser, and Pro. In some embodiments, protease-sensitive lysine or arginine residues are substituted as part of a building block substitution. For example, the D1L3 variant may comprise one or more substitutions selected from:  
 30 K180\_A181delinsGL, P198\_A201delinsRPSQ, and K259A. In some embodiments, the D1L3 variant comprises one or both substitutions: K180\_A181delinsGL, and/or P198\_A201delinsRPSQ, either of which are optionally modified by one or two amino acid substitutions, deletions, or insertions, with the proviso that the building block

substitution is not modified by substitution or insertion with an R or K residue. In some embodiments, the D1L3 variant has increased resistance to proteolysis by one or more proteases selected from plasmin, thrombin, and/or trypsin.

Alternatively or in addition, the D1L3 variant comprises one or more mutations  
5 of a paired basic residue. In some embodiments, the paired basic residue corresponds to a position selected from K50/R51, R80/R81, K114/R115, K199/K200, K226/K227, K291/K292, R297/K298/K299, and K303/R304 of SEQ ID NO:4. In some  
10 embodiments, the D1L3 variant has one or more substitutions selected from a substitution corresponding to R114T, R114A, R114D, R114Q, K227S, and K227E of  
SEQ ID NO:4. In some embodiments, the one or more mutations of a paired basic residue include an amino acid substitution corresponding to R51K, R81K, R115K, and R304K.  
In some embodiments, the paired basic residue is substituted using a corresponding building block substitution. In accordance with these embodiments, the D1L3 variant  
15 will be more resistant to proteases expressed by the recombinant protein expression system (e.g., CHO and *Pichia pastoris*).

In some aspects, the invention provides a DNase 1 (D1) variant comprising an amino acid sequence that is at least 80% identical to the enzyme defined by SEQ ID NO: 1, with one or more substitutions of cysteine residues. In some embodiments, the D1  
20 protein variant has one or more additional modifications resulting in increased protein stability, higher production levels with *in vitro* expression systems, and/or not substantially less, the same, or better DNA and/or chromatin and/or NET-degrading activity as compared to wild-type D1 protein of SEQ ID NO:1. For example, the D1  
variant may comprise at least one additional building block substitution or point mutation disclosed in PCT/US2018/47084, which is hereby incorporated by reference in its  
25 entirety.

In some embodiments, the D1 variant has a substitution of one or both of C123 and C126, and which is/are optionally substituted with Ala, Ser, and Gly. In some  
embodiments, the D1 variant comprises the substitution G122\_N128delinsYQGDA. In some embodiments, the D1 variant comprises an amino acid sequence that has at least  
30 85%, at least 90%, at least 95%, or at least 98% identity to SEQ ID NO:1.

In some embodiments, the invention provides a D1 enzyme having a PEG moiety conjugated at the position corresponding to C123 and/or C126. In these embodiments,

the PEG moiety will provide a half-life extension property, while avoiding disulfide scrambling and/or protein misfolding. In some embodiments, the PEG moiety is conjugated through maleimide chemistry, which can be conducted under mild conditions. Other conjugation chemistries are known and may be used, such as vinyl sulfone, dithiopyridine, and iodoacetamide activation chemistries. The PEG moiety can be linear or branched, and can be generally in the range of 10 kDa to 40 kDa, or in the range of 20 to 30 kDa.

In other aspects, the invention provides a D1L1 variant comprising an amino acid sequence that is at least 80% identical to the enzyme defined by SEQ ID NO: 2, with one or more substituted cysteine residues. The cysteine residue(s) are optionally non-conserved within the D1 family (e.g., C22 and/or C50), and are optionally substituted with Gly, Arg, or Ser, or are substituted as part of a building block substitution. In some embodiments, the D1L1 variant comprises an amino acid sequence that has at least 85%, at least 90%, at least 95%, or at least 98% identity to SEQ ID NO: 2.

In some embodiments, the invention provides a D1L1 enzyme having a PEG moiety conjugated at the position corresponding to C22 and/or C50. In these embodiments, the PEG moiety will provide a half-life extension property, while avoiding disulfide scrambling and/or protein misfolding. In some embodiments, the PEG moiety is conjugated through maleimide chemistry, which can be conducted under mild conditions. Other conjugation chemistries are known and may be used, such as vinyl sulfone, dithiopyridine, and iodoacetamide activation chemistries. The PEG moiety can be linear or branched, and can be generally in the range of 10 kDa to 40 kDa, or in the range of 20 to 30 kDa.

In some aspects, the invention provides a D1L2 variant comprising an amino acid sequence that is at least 80% identical to the enzyme defined by SEQ ID NO: 3, with one or more substituted cysteine residues. The cysteine residues may be non-conserved within the D1 family (e.g., C43), and is/are optionally substituted with Gly, Arg, or Ser, or are substituted as part of a building block substitution. In some embodiments, the D1L2 variant comprises an amino acid sequence that has at least 85%, at least 90%, at least 95%, or at least 98% identity to SEQ ID NO: 3.

In some embodiments, the invention provides a D1L2 enzyme having a PEG moiety conjugated at the position corresponding to C43. In these embodiments, the PEG

moiety will provide a half-life extension property, while avoiding disulfide scrambling and/or protein misfolding. In some embodiments, the PEG moiety is conjugated through maleimide chemistry, which can be conducted under mild conditions. Other conjugation chemistries are known and may be used, such as vinyl sulfone, dithiopyridine, and iodoacetamide activation chemistries. The PEG moiety can be linear or branched, and can be generally in the range of 10 kDa to 40 kDa, or in the range of 20 to 30 kDa.

In other aspects, the invention provides isolated polynucleotides encoding the D1, D1L1, D1L2, or D1L3 variants disclosed herein, as well as vectors and host cells. Host cells can be cells of any expression system, including bacterial or eukaryotic, whether non-mammalian such as *Pichia pastoris*, or mammalian such as CHO cells.

In some embodiments, delivery of polynucleotides is used for therapy. Encoding polynucleotides can be delivered as mRNA or as DNA constructs using known procedures, e.g., electroporation or cell squeezing, and/or vectors (including viral vectors). mRNA polynucleotides can include known modifications (mmRNA) to avoid activation of the innate immune system. See WO 2014/028429, which is hereby incorporated by reference in its entirety. In some embodiments, the polynucleotide is delivered to the body of a subject. In some embodiments, the polynucleotides is delivered into a cell in vitro, and the cell is delivered to the body of a subject. The cell can be, for example, a white blood cell (e.g., a T cell or macrophage), an endothelial cell, an epithelial cell, a hepatocyte, or a stem cell.

In other aspects, the invention provides a method for producing an extracellular DNASE variant described herein. The method comprises culturing cells expressing a polynucleotide encoding the extracellular DNASE, and recovering the recombinant DNase protein. The cells may be prokaryotic or eukaryotic. In some embodiments, the DNase is expressed using a non-mammalian expression system, which is optionally *Pichia pastoris* or *Saccharomyces spp.* In some embodiments, a mammalian expression system, such as CHO cells, is employed.

The invention further provides pharmaceutical compositions comprising the extracellular DNASE or variant thereof as described herein, or optionally the polynucleotide or the vector as described, and a pharmaceutically acceptable carrier.

A vector generally comprises an isolated nucleic acid and which can be used to deliver the isolated nucleic acid to the interior of a cell. Numerous vectors are known in

the art including, but not limited to, linear polynucleotides, polynucleotides associated with ionic or amphiphilic compounds, plasmids, and viruses. Exemplary vectors include autonomously replicating plasmids or a virus. The term should also be construed to include non-plasmid and non-viral compounds which facilitate transfer of nucleic acid  
5 into cells, such as, for example, polylysine compounds, liposomes, and the like. Examples of viral vectors include, but are not limited to, adenoviral vectors, adeno-associated virus vectors, retroviral vectors, and the like.

The pharmaceutical composition may be formulated for any administration route, including topical, parenteral, or pulmonary administration. In various embodiments, the  
10 composition is formulated for intradermal, intramuscular, intraperitoneal, intraarticular, intravenous, subcutaneous, intraarterial, oral, sublingual, pulmonary, or transdermal administration. In some embodiments, the composition is formulated for intravenous or subcutaneous administration.

In other aspects, the invention provides a method for treating a subject in need of  
15 extracellular DNA degradation, extracellular chromatin degradation, extracellular trap (ET) degradation and/or neutrophil extracellular trap (NET) degradation. The method comprises administering a therapeutically effective amount of the extracellular DNASE or variant thereof or composition described herein. Exemplary indications where a subject is in need of extracellular DNA or chromatin degradation (including ET or NET  
20 degradation) are disclosed in PCT/US18/47084, the disclosure of which is hereby incorporated by reference. In some embodiments, the invention provides a method for treating a subject in need thereof, the method comprising administering a therapeutically effective amount of a protein that is represented by any one of the sequences SEQ ID NO:8 to SEQ ID NO:30.

25 In each instance where a method for treating a subject is described, the invention likewise provides the use of one or more of the extracellular DNASE proteins for the treatment or prevention of diseases associated with ETs and/or NETs.

In various embodiments, the present invention provides a method for treating, preventing, or managing diseases or conditions characterized by the presence or  
30 accumulation of NETs. Such diseases or conditions include, but are not limited to, diseases associated with chronic neutrophilia, neutrophil aggregation and leukostasis, thrombosis and vascular occlusion, ischemia-reperfusion injury, surgical and traumatic

tissue injury, an acute or chronic inflammatory reaction or disease, an autoimmune disease, cardiovascular disease, metabolic disease, systemic inflammation, inflammatory diseases of the respiratory tract, renal inflammatory diseases, inflammatory diseases related to transplanted tissue (e.g. graft-versus-host disease) and cancer (including  
5 leukemia).

In certain embodiments, the present invention pertains to the treatment of diseases or conditions characterized by deficiency of D1L3, or a deficiency of D1. In some cases, the subject has a mutation (e.g., a loss of function mutation) in the Dnase1l3 gene or the Dnase1 gene. Such subjects can manifest with an autoimmune disease (e.g.,  
10 systemic lupus erythematosus (SLE) (including lupus nephritis), scleroderma or systemic sclerosis, rheumatoid arthritis, inflammatory bowel disease (including Crohn's disease and ulcerative colitis), and urticarial vasculitis). In some cases, the subject has an acquired inhibitor of D1 (e.g., anti-DNase1-antibody and actin) and/or D1L3 (e.g., anti-Dnase1l3-antibody). Such subjects can also have an autoimmune or inflammatory  
15 disease (e.g., SLE, systemic sclerosis).

In some embodiments, the subject has or is at risk of NETs occluding ductal systems. For example, the DNASE enzymes disclosed herein can be administered to a subject to treat pancreatitis, cholangitis, conjunctivitis, mastitis, dry eye disease, obstructions of vas deferens, or renal diseases.

20 In some embodiments, the subject has or is at risk of NETs accumulating on endothelial surfaces (e.g. surgical adhesions), the skin (e.g. wounds/scarring), or in synovial joints (e.g. gout and arthritis, e.g., rheumatoid arthritis). The DNASE enzymes described herein can be administered to a subject to treat a condition characterized by an accumulation of NETs on an endothelial surface such as, but not limited to, a surgical  
25 adhesion.

Other diseases and conditions associated with NETs, which the DNASE enzymes disclosed herein may be used to treat or prevent, include: ANCA-associated vasculitis, asthma, chronic obstructive pulmonary disease, a neutrophilic dermatosis, dermatomyositis, burns, cellulitis, meningitis, encephalitis, otitis media, pharyngitis,  
30 tonsillitis, pneumonia, endocarditis, cystitis, pyelonephritis, appendicitis, cholecystitis, pancreatitis, uveitis, keratitis, disseminated intravascular coagulation, acute kidney injury, acute respiratory distress syndrome, shock liver, hepatorenal syndrome,

myocardial infarction, stroke, ischemic bowel, limb ischemia, testicular torsion, preeclampsia, eclampsia, and solid organ transplant (e.g., kidney, heart, liver, and/or lung transplant). Furthermore, the DNASE enzymes disclosed herein can be used to prevent a scar or contracture, e.g., by local application to skin, in an individual at risk thereof,  
 5 e.g., an individual with a surgical incision, laceration, or burn.

In various embodiments, the subject has a disease that is or has been treated with wild-type Dnases, including D1 and streptodornase. Such diseases or conditions include thrombosis, stroke, sepsis, lung injury, atherosclerosis, viral infection, sickle cell disease, myocardial infarction, ear infection, wound healing, liver injury, endocarditis, liver  
 10 infection, pancreatitis, primary graft dysfunction, limb ischemia reperfusion, kidney injury, blood clotting, alum-induced inflammation, hepatorenal injury, pleural exudations, hemothorax, intrabiliary blood clots, post pneumatic anemia, ulcers, otolaryngological conditions, oral infections, minor injuries, sinusitis, post-operative rhinoplasties, infertility, bladder catheter, wound cleaning, skin reaction test,  
 15 pneumococcal meningitis, gout, leg ulcers, cystic fibrosis, Kartegener's syndrome, asthma, lobar atelectasis, chronic bronchitis, bronchiectasis, lupus, primary ciliary dyskinesia, bronchiolitis, empyema, pleural infections, cancer, dry eyes disease, lower respiratory tract infections, chronic hematomas, Alzheimer's disease, and obstructive pulmonary disease.

20 Other aspects and embodiments of the invention will be apparent from the following examples.

### **EXAMPLES**

Nearly 70% of all biologics are produced using Chinese Hamster Ovary (CHO) cells. Indeed, wild-type DNASE1 (D1; dornase alpha) is typically produced in CHO  
 25 cells. Despite significant advantages in cell line development and large-scale production using CHO cells, there still remains a significant challenge in the production of Dnase enzymes due to a considerable degree of variability and no reliable methods for predicting or modeling cell growth characteristics. Importantly, CHO cells were not able to stably produce hyperactive variants of D1, which prevented their clinical  
 30 manufacturing, and prior to the present disclosure, the manufacturing properties of other DNASE1-protein family members, including DNASE1-LIKE 3 (D1L3), were unknown.

Using CHO and microbial expression systems, several challenges were identified in manufacturing of D1L3, including low production yield, proteolytic degradation, protein misfolding, and erroneous or undesired glycosylation. This disclosure provides technical solutions to these and other challenges in manufacturing, which also can improve the therapeutic properties of D1L3.

Example 1: Expression and Characterization of D1L3 with Basic Domain Deletion (BDD) in Chinese Hamster Ovarian (CHO) Cells and in *Pichia pastoris*

DNASE1 and DNASE1L3 preferentially cleave protein-free DNA and DNA-histone-complexes (i.e. chromatin), respectively. Previous studies suggest that a basic domain (BD) at the C-terminus of DNASE1L3, which is absent in DNASE1, is responsible for the distinct substrate specificities of both enzymes (Sisrak et al., Cell, 2016; Keyel, Developmental Biology, 2017).

A protein engineering technology, termed Building Block Protein Engineering is described in PCT/US18/47084 and US 62/800,790, the disclosures of which are hereby incorporated by reference in their entireties. This approach can be applied to members of the DNASE1 and DNASE2-protein family. The method is based on the following steps: providing a protein-protein alignment of donor and recipient Dnase enzymes; identifying variable amino acid sequences for transfer, the variable amino acids being flanked by one or more conserved amino acids in the donor and recipient Dnase enzymes; substituting the variable amino acids of the recipient Dnase with the variable amino acids of the donor Dnase to create a chimeric Dnase; and recombinantly producing the chimeric Dnase.

To characterize the amino acids that are responsible for chromatin-degrading activity (“chromatinase” activity), wild-type D1L3 was substituted with building block substitutions from D1, as disclosed in PCT/US2018/047084. The building block substitutions to D1L3 are selected from human D1 and result in variants of human D1L3, which feature the following mutations: M21\_R22delinsLK, C24\_S25delinsAA, V28\_S30delinsIQT, S34T, Q36\_V44delinsMSNATLVSY, K47\_K50delinsQILS, C52Y, I55\_M58delinsIALVQE, I60\_K61delinsVR, N64\_I70delinsHLTAVGK, M72\_K74delinsLDN, R77\_I83delinsQDAPD, N86H, I89V, S91\_R92delinsEP, T97S, Q101R, A103L, L105V, K107\_L110delinsRPDQ, V113\_R115delinsAVD, H118Y, H120D, Y122\_A127delinsGCEPCGN, V129T, S131N, F135\_V136delinsAI, W138R,

Q140\_H143delinFSRF, A145\_D148delinsEVRE, V150A, I152V, T156\_T157delinsAA, E159\_S161delinsGDA, K163A, E167A, V169\_E170delinsYD, T173L, K176\_R178delinsQEK, K180\_A181delinsGL, N183\_F186delinsDVML, P198\_A201delinsRPSQ, K203\_N204delinsSS, R208W, D210S, R212T, V214Q, G218P, Q220\_E221delinsSA, V225\_S228delinsATP, N230H, L238\_R239delinsVA, Q241\_S246delinsMLLRGA, K250D, N252\_V254delinsALP, D256N, K259A, K262G, T264\_E267delinsSDQL, L269\_V271delinsQAI, F275Y, F279\_K280delinsVM, Q282\_S205delinsK with respect to SEQ ID NO: 4.

These 63 D1L3 variants were screened for loss or gain of chromatin-degrading activity. In brief, D1L3 variants were transiently expressed in CHO cells using an *in vitro* expression vector. Culture supernatants were collected and tested for chromatin-degrading activity using purified nuclei as a source of chromatin. As shown in **FIG. 1**, the building block substitutions #17 and #63 from D1 significantly improved the degradation of high-molecular weight (HMW) chromatin to small fragments, when compared to wild-type D1L3. Building block substitution #7 causes a missense mutation Q101R, which replaces glutamine at position 101 with arginine (SEQ ID NO: 8). Building block substitution #63 causes the mutation Q282\_S305delinsK, which deletes the full C-terminal BD of D1L3 from amino acid position 283 to 305 and replaces glutamine (Q) at position 282 with lysine (SEQ ID NO: 9). Next, we performed Western Blot analysis of the supernatants to detect the expression levels of wild-type D1L3 and both mutants (**FIG. 2**). To our surprise, we detected two D1L3 variants of different size in samples with wild-type D1L3 and the Q101R mutant. Samples with the Q282\_S305delinsK contained only the smaller D1L3 variant. The data suggest that the BD of wild-type D1L3 is spontaneously removed (e.g., proteolyzed) during expression or post-secretion in CHO cells. The two D1L3 variants were also detected in supernatants from CHO cells that stably express WT-D1L3 (**FIG. 3**). Of note, the Basic Domain Deleted-D1L3 (BDD-D1L3) showed substantially increased chromatinase activity, when compared to wild-type D1L3.

Next, we tested *Pichia pastoris* as an alternative, microbial expression system to CHO cells. We generally observed higher expression levels with BDD-D1L3, when compared to wild-type D1L3. Here, we purified and characterized wild-type D1L3 and BDD-D1L3 from *Pichia pastoris* fermentation supernatants (**FIG. 4**). Unexpectedly, we observed that wild-type D1L3 was proteolytically truncated within the BD at the amino

acid positions K291, K291, or S293, leading to a heterogenous mix of D1L3 variants after purification. Unlike wild-type D1L3, expression of BDD-D1L3 due to three building block substitutions (F275Y, F279\_K280delinsVM, Q282\_S205delinsK) generated a pure protein.

5           Next, we compared the chromatinase activity of both D1L3 purifications. We observed that the heterogenous mix of D1L3 variants with BD truncations at positions K291, K291, or S293 had approximately 10-fold lower chromatinase activity compared to the D1L3 variant with a full BD deletion due to F275Y/F279\_K280delinsVM/Q282\_S205delinsK. Collectively, the data illustrate that  
10       the proteolytic cleavage of the BD can occur naturally in microbial and mammalian expression systems (i.e. CHO and *P. pastoris*), and removal of the BD appears to activate D1L3 activity to degrade chromatin.

#### Example 2: Expression of D1L3 in CHO Cells in Bioreactors

Disclosed herein is the development of stable CHO cell lines producing wild-type  
15       D1L3 (SEQ ID NO: 4). The cell lines were cultured in bioreactors using standard CHO culture medium. Specifically, **FIG. 5** shows a Western Blot of human D1L3 expressed and secreted by CHO cells in a bioreactor under cGMP-compatible conditions. Samples were collected at different time points (t1 – t3). Only minor levels of D1L3 and D1L3 fragments were detected. The data suggest that low production yield of D1L3 is a  
20       challenge in manufacturing of D1L3.

As disclosed herein, high production levels of wild-type D1L3 were achieved by the addition of polyanions to the culture medium. Such polyanions can comprise one or more of heparin, dextran sulfate, ferric citrate, and ethylenediaminetetraacetic acid, and represent the biologically active ingredient in “anti-cell clumping reagents”. Specifically,  
25       we added dextran sulfate to the CHO culture medium and observed a strong increase in D1L3 as well as D1L3 fragments (**FIG. 5**). The data illustrate that polyanions increased production yield of D1L3, but did not prevent proteolytic degradation.

**FIG. 6A** shows that polyanions, such as dextran sulfate (DS), form a complex with D1L3. The D1L3-DS-complex prevents the interaction and scavenging of D1L3 by  
30       negatively charged surfaces during the production process. Such negatively charged surfaces include, but are not limited to, the cell surface of production cells (e.g. CHO

cells, *Pichia pastoris*, *Saccharomyces spp.*), DNA exposed by dying cells, and bioreactor surfaces. **FIG. 6B** and **FIG. 6C** show the two-step purification process of D1L3 from DS-D1L3-complexes. As shown in **FIG. 6**, the first step aims to dissociate the DS-D1L3 complex. The dissociation can be achieved by incubating the DS-D1L3 complex with strong anion exchange surfaces, which bind DS and thus liberate D1L3. Specifically, the purification process can include the passage of culture medium containing the DS-D1L3 through a chromatography column that is filled with a strong anion exchange resin followed by the collection of the flow through, which contains the DS-free D1L3. The second step of the purification process is shown in **FIG. 6C** and includes the affinity purification of D1L3 from the DS-free flow through via the application of a strong cation exchange resin. In conclusion, the production yield of D1L3 can be substantially increased through the addition of polyanions, such as dextran sulfate.

### Example 3: Engineering D1L3 for Protease Resistance

Wild-type D1L3 contains 50 arginine and lysine residues, which makes the enzyme particularly susceptible to proteases like trypsin, thrombin, and plasmin. In this example, trypsin and plasmin cleavage sites were identified in D1L3. The sites can be mutated to generate protease-resistance variants of D1L3.

In brief, purified D1L3 was digested with trypsin. D1L3 fragments were isolated, and the amino acid sequence of the fragments determined using combinations of liquid chromatography (LC) and mass spectrometry (MS). It was identified that trypsin cleaved D1L3 at the following arginine and lysine residues: R22, R29, R51, R66, R80, R81, R95, K99, R115, K147, K163, K180, R208, R212, R235, R239, K250, and K262. These arginine and lysine residues can be substituted with small amino acids such as alanine, valine, and serine or with amino acids that have similar properties according to the Grantham's distance score (e.g. histidine, glutamine, and glutamate; **FIG. 7**). D1, which is protease resistant, features arginine and lysine residues corresponding to R51, R95, K99, and R235, suggesting that these residues are not primarily responsible for proteolytic degradation of D1L3.

Building Block Protein Engineering was applied to transfer the following Building Blocks from D1 to replace Building Blocks of D1L3 that contain the trypsin cleavage sites (**FIG. 7**): R22 (Mutation: M21\_R22delinsLK), R29 (V28\_S30delinsIQT), R66 (N64\_I70delinsHLTAVGK), R80 (R77\_I83delinsQDAPD), R81

(R77\_I83delinsQDAPD), R115 (V113\_R115delinsAVD), K163 (K163A), K180 (K180\_A181delinsGL), R208 (R208W) MR212 (R212T), R239 (L238\_R239delinsVA), K250 (K250D), and K262 (K262G).

Plasmin is a plasma protease that is generated by activation of its zymogen plasminogen. Plasminogen activator inhibitor 1 (PAI-1) inhibits the activation of plasmin. Interestingly, PAI-1 increases the enzymatic activity of D1L3 in serum, suggesting that plasmin may proteolytically inactivate D1L3. However, the plasmin cleavage sites in D1L3 have not been identified.

*In silico* analysis showed that the amino acid combination lysine-alanine (KA) or arginine-alanine (RA) is believed to be preferably cleaved by the protease plasmin or proteases that have plasmin-like activity. D1L3 contains a total of four putative plasmin-cleavage sites (**FIG. 8**): (Site 1) K180/A181 (K160/A161 without signal peptide), (Site 2) K200/A201 (K180/A181 without signal peptide), (Site 3) K259/A260 (K239/A240 without signal peptide), and (Site 4) R285/A286 (R270/A250 without signal peptide). Using a paired alignment of D1 and D1L3, we found that none of the plasmin cleavage sites are present in D1 (**FIG. 8**). The data are in line with the fact that D1 activity is resistant to inactivation by serum proteases, such as thrombin and plasmin. Building Block Protein Engineering was applied to transfer the following Building Blocks from D1 to replace Building Blocks of D1L3 that contain the plasmin cleavage sites (**FIG. 9**): (Site 1) K180\_A181delinsGL, (Site 2) P198\_A201delinsRPSQ, and (Site 3) K259A. R285/A286 (Site 4) is located in a C-terminal extension that is absent in D1. Consequently, we generated a D1L3 variant in which all four putative plasmin cleavage sites were mutated: K180\_A181delinsGL, P198\_A201delinsRPSQ, K259A, and R285A. Next, we analyzed chromatin degradation by the D1L3 variant and observed potent chromatin degrading activity in the mutated D1L3 (**FIG. 10**). Collectively, the data show that four arginine and lysine residues, K180, K200, K259, and R285, can be mutated to reduce the risk of proteolytic degradation without compromising enzymatic activity.

Next, purified D1L3 was digested with purified plasmin. D1L3 fragments were isolated, and the amino acid sequence of the fragments determined using combinations of LC and MS. We identified that plasmin cleaved D1L3 at the following arginine and lysine residues: R22, R29, K45, K47, K74, R81, R92, K107, K176, R212, R226, R227, K250, K259, and K262. These arginine and lysine residues can be substituted with small amino acids such as alanine, valine, and serine or with amino acids that have similar

properties according to the Grantham's distance score (e.g. histidine, glutamine, and glutamate; **FIG. 11**). D1, which is protease resistant, features a lysine residue corresponding to K45, suggesting that this residue is not primarily responsible for proteolytic degradation of D1L3 by plasmin. Building Block Protein Engineering was applied to transfer the following Building Blocks from D1 to replace Building Blocks of D1L3 that contain the trypsin cleavage sites *in silico* (**FIG. 11**): R22 (Mutation: M21\_R22delinsLK), R29 (V28\_S30delinsIQT), K47 (K47\_K50delinsQILS), K74 (M72\_K74delinsLDN), R81 (R77\_I83delinsQDAPD), R92 (S91\_R92delinsEP), K107 (K107\_L110delinsRPDQ), K176 (K176\_R178delinsQEK), R212 (R212T), K226 (V225\_S228delinsATP), K227 (V225\_S228delinsATP), K250 (K250D), K259 (K259A), and K262 (K262G).

Finally, recombinantly expressed wild-type D1L3 was isolated and its C-terminus sequenced. Three different amino acid sequences were identified ending in S290 (SEQ ID NO: 10), K291 (SEQ ID NO: 11), and K292 (SEQ ID NO: 12), respectively (**FIG. 4**, Example 1). The data identify lysine residues 291 and 292 as prominent proteolytic cleavage sites of D1L3 during large-scale manufacturing.

#### Example 4: Engineering D1L3 to Avoid Degradation

We observed fragmentation of D1L3 after heterologous expression in *Pichia pastoris*. Analysis of the fragments characterized paired basic amino acids, arginine (R) and lysine (K) residues, as proteolytic cleavage sites. A similar degradation pattern was observed after expressing D1L3 in CHO cells. These observations suggest that *Pichia pastoris* and CHO cells share homologous proteases that cleave D1L3 at paired basic amino acids, and although the effect was more significant in CHO cells.

It was determined that the paired basic amino acid cleaving enzyme (PACE) contributed to the DNASE1L3 fragmentation. PACE, also known as Furin (Uniprot ID: P09958), is expressed in humans and mammals. *Pichia pastoris* expresses two enzymes, which target paired basic amino acids, namely Aspartic proteinase 3 (Gene: Ysp1; Uniprot ID: P32329) and Kexin (Gene: Kex2; Uniprot ID: P13134). Thus, DNASE1L3 and DNASE1L3 variants can be expressed in *Pichia pastoris* and in CHO cells in which Furin, Aspartic proteinase 3, and Kexin is pharmacologically inhibited or genetically depleted.

In addition, mutations of paired basic amino acids in DNASE1L3 and DNASE1L3 variants enable their expression in CHO and *Pichia pastoris* with reduced fragmentation. Analysis of DNASE1L3 fragments identified feature paired basic amino acid at positions: K50/R51, R80/R81, K114/R115, K199/K200, K226/K227,  
 5 K291/K292, R297/K298/K299, and K303/R304 in SEQ ID NO: 2.

As disclosed US Provisional Patent Application No. 62/800,790 (which is hereby incorporated by reference in its entirety), DNASE1L3 from other species feature amino acid substitutions at these cleavage sites, including R114T (Mouse), R114A (Rat), R114D (Guinea pig), R114Q (Cow), K227S (Dog), and K227E (Elephant). These amino  
 10 acid substitutions can be applied to human DNASE1L3 to render the enzyme resistant to proteolytic degradation, including during expression in CHO cells and *Pichia pastoris*.

Kexin preferably cleaves after KR and RR residues. DNASE1L3 features at K50/R51, R80/R81, K114/R115, and K303/R304 are 4 KEX2-cleavage sites. Amino acid substitutions of these residues render DNASE1L3 resistant to KEX2 and enable the  
 15 expression of DNASE1L3 and DNASE1L3 variants in *Pichia pastoris* and in CHO cells. These amino acid substitutions can be conservative, e.g. R51K, R81K, R115K, and R304K.

#### Example 5: Engineering D1L3 Variants to Prevent High-Molecular Weight Aggregates

During cGMP-compatible expression of D1L3 in CHO cells (**FIG. 12A**), the  
 20 accumulation of high-molecular weight aggregates of D1L3 was observed, pointing towards an additional challenge for clinical manufacturing D1L3. The high molecular weight aggregates were observed by a much lower extent in *Pichia pastoris*.

The application of reducing conditions to proteins of bioreactor material dissolved D1L3 aggregates. The data illustrate that D1L3 aggregate formation is caused  
 25 by intra- and/or inter-molecular cross-linking via disulfide bridges during protein expression. Specifically, as shown in **FIG. 12B**, the gel was run under non-reducing conditions and shows the accumulation of high-molecular weight aggregates of D1L3 over time. The gel was run under reducing conditions and no aggregates were detected. The data illustrate that erroneous intra- and inter-molecular disulfide bonds cause  
 30 misfolding of human D1L3 under manufacturing conditions.

**FIG. 13** shows an amino acid sequence alignment of human D1 (SEQ ID NO: 1) and human D1L3 (SEQ ID NO: 4). The signal peptide, conserved amino acids, variable

amino acids, non-conserved cysteine residues, and conserved cysteine residues are highlighted. Mutations in non-conserved cysteine residues will reduce the possibilities of intra- and inter-molecular disulfide bonds during protein expression. Analysis of the amino acid sequence of D1L3 (SEQ ID NO: 4) showed the presence of five cysteine (C) residues: C24, C52, C68, C194, and C231 (**FIG. 14**). The cysteine residues C194 and C231 are conserved among all members of the DNASE1-protein family and form disulfide bonds that are required for enzymatic activity of DNASE1. The function of cysteine residues in D1L3 were not known prior to the present disclosure. Accordingly, as disclosed herein, mutation of these cysteine residues reduces the cross-linking via disulfide bridges and thus increases the yield of protein production.

Cysteine residues can be substituted with other small amino acids, namely alanine (A), serine (S), and glycine (G), among others. Such substitutions cause the following amino acid mutations C24A/S/G, C52A/S/G, C68A/S/G, C194A/S/G, and C231A/S/G. In addition, Building Blocks that comprise the conserved cysteine residues can be replaced by Building Blocks from a donor DNase of the DNASE1-protein family (e.g. D1 and D1L3). The following Building Blocks from D1 were used to replace the Building Blocks of D1L3 that contain the non-conserved cysteine residues C24, C52, and C68: C24\_S25delinsAA, C52Y, and N64\_I70delinsHLTAVGK. The chromatin degrading activity of D1L3 variants was quantified, as described in PCT/US18/4708. Both conventional amino acids substitutions (C24A, C52A) and building block substitutions (C24\_S25delinsAA, C52Y) caused a complete absence of chromatin degradation, indicating that C24 and C52 are required for D1L3 activity (**FIG. 15**). Importantly, mutation of cysteine C68, either by conventional amino acid substitution [C68A, (SEQ ID NO: 13)] or by BB mutation (N64\_I70delinsHLTAVGK), resulted in a D1L3 variant with chromatin degrading activity (**FIG. 15**). Amino acid sequence alignment showed that cysteine C68 is not conserved among other DNASE1-protein family members, supporting the notion that C68 is not required for enzymatic activity. Furthermore, it was observed that the amino acid substitution of highly conserved cysteine C194 with alanine (C194A), but not the mutation of the highly conserved cysteine C231 with alanine (C231A), resulted in an enzymatically active D1L3 variant (**FIG. 15**). Thus, cysteine C68 and C194 can be mutated to reduce the risk of erroneous disulfide bonds during D1L3 production.

A similar approach can be applied to mutate the non-conserved cysteine residues in the other members of the DNase1 protein family: D1, DNase1-like 1 (D1L1) and DNase1-like 2 (D1L2). D1 has two non-conserved cysteine: C123 and C126. D1L1 shows two non-conserved cysteine residues (C22, C50) that correspond to C24 and C52 in D1L2 has only one non-conserved cysteine residues: C43. Mutation of non-conserved cysteine residues of members of the DNASE1 protein family will reduce cross-linking via erroneous disulfide bridges during protein expression and thus allow for manufacturing of D1, D1L1, D1L2, and D1L3 for therapeutic applications.

#### Example 6: Construction and Expression of D1L3 and Albumin-D1L3 Fusion Proteins

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##### in *Pichia pastoris*

*Pichia pastoris* expression of recombinant human extracellular DNASES, including D1L3, was tested. As shown in **FIG. 16A**, the N-terminus of D1L3 was led by the alpha-mating factor (aMF) pre-pro secretion leader from *Saccharomyces cerevisiae* (SEQ ID NO: 46), a common tool for heterologous protein expression in *Pichia pastoris*. As disclosed herein, the combination of aMF with D1L3 caused the unexpected non-processing of aMF due to glycosylation (**FIG. 16B**). The glycosylation of the D1L3 protein prevents the use of *P. pastoris* for clinical manufacturing of D1L3. D1L3 was properly processed, when N-terminus was led by native secretory signal peptide of D1L3 [**FIG. 16B**, (SEQ ID NO: 48)]. Importantly, aMF increased D1L3 expression 3-5-fold, when compared to the native signal peptide of D1L3. We therefore tested the processing of D1L3-fusion proteins. In pilot studies, an N-terminal fusion of aMF and human serum albumin [HSA, (SEQ ID NO: 39)] to D1L3 was generated (**FIG. 17A**). Some variants contained linker peptide [e.g. (GSSSS)<sub>3</sub>] between HSA and D1L3. As shown in **FIG. 17B** and **FIG. 17C**, expression of the fusion protein in *P. pastoris* generated a non-glycosylated and enzymatically active D1L3. Furthermore, the expression levels were 5-10-fold increased, when compared to native secretory signal peptide-driven expression of D1L3. Collectively, the data illustrate that fusion of D1L3 to albumin enables manufacturing in *Pichia pastoris*.

Based on these pilot studies, various HSA fusion constructs of wild-type D1L3 and BDD-D1L3 were designed and screened for expression levels of target protein (SEQ ID NOS: 17 to 28). As shown in **FIG. 18**, we observed that the N-terminal fusion of human serum albumin (SEQ ID NO: 17) to a BDD-D1L3 variant (SEQ ID NO: 16) did not substantially increase the expression levels. However, of note, we did detect a strong

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albumin sterically hinders the interaction with substrates and ligands. Thus, peptide linkers can be used to increase the distance between albumin and the fusion protein or peptide. However, the observation that insertion of a linker sequence between HSA and D1L3 simultaneously improves enzymatic activity and expression levels was  
5 unexpected.

We compared the chromatin degrading activity of BDD-D1L3 (SEQ ID NO: 14) with its albumin-fusion counterpart (SEQ ID NO: 19). In brief, *Dnase1<sup>-/-</sup>Dnase1l3<sup>-/-</sup>* mice were injected with SEQ ID NO:4 or SEQ ID NO:19. Serum was collected 15 minutes post injection. As shown in FIG. 21A, we observed similar serum chromatin  
10 degrading activity in both animals. Importantly, the fusion of albumin to the N-terminus of D1L3 and other human extracellular DNASES provides a half-life extended DNASE therapeutics. As disclosed herein, we determined the half-life of SEQ ID NO: 19, an HSA-BDD-D1L3 fusion protein with a flexible, GS-linker of 15 amino acids, in a commercially available rodent model. The animal model is characterized by the  
15 transgenic expression of the human FcRn, which is responsible for long half-life of albumin in circulation. While unconjugated D1L3 (e.g. SEQ ID NO: 4) has a very short half-life in circulation (< 30 minutes), the albumin fusion extended the half-life to 3.3 days, thereby substantially improving systemic exposure, while also conferring rapid absorption with a  $t_{max}$  of 5 minutes (**FIG. 21B**). Collectively, the data demonstrate the N-  
20 terminal fusion of HSA to D1L3 via a linker sequence not only facilitates the manufacturing, but also improves the *in vivo* pharmacokinetic properties of D1L3.

Finally, we tested the dual fusion of HSA to the N- and C-terminus of D1L3. First, we analyzed the C-terminus of D1L3 for potential attachment sites. We identified two serine residues at position 283 and 284, which provide a flexible connection of the  
25 BD (RAFTNSKKSVTLRKKTKSKRS) to the core body of D1L3. Thus, we deleted the BD and chose to attach HSA via a flexible GS-linker (SEQ ID NO: 38) to S284. As shown in **FIG. 22**, fusion of HSA to the N- and C-terminus of BDD-D1L3 (SEQ ID NO: 28) maintained the high expression levels that were observed with N-terminal HSA fusion (SEQ ID NO: 27).

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#### Example 7: Design of Cleavable Linker Sequences

The findings disclosed herein have implications beyond manufacturing. For example, D1L3 variants with C-terminal amino acid deletions, which retain their enzymatic activity to degrade chromatin and/or NETs, as exemplified by SEQ ID NO: 9 to SEQ ID NO: 12, can be used for D1L3 therapy. In addition, the site-specific alkylation of an unpaired cysteine thiol is commonly used to generate half-life extended biologics for therapeutic applications. Specifically, the non-essential cysteines C68 and C194 of D1L3 can be used for site specific PEGylation (PEG, polyethylene glycol). Furthermore, D1L3 variants that are resistant to inactivation by plasmin, due to mutations such as K180\_A181delinsGL, P198\_A201delinsRPSQ, K259A, and R285A, are expected to have an improved half-life and thus efficacy in therapeutic applications.

Importantly, the fusion of albumin to the N-terminus of D1L3 and other human extracellular DNASES provides a half-life extended DNASE therapeutic (**FIG. 23A**). Several linker sequences were used to reduce the steric inhibition of D1L3 by albumin. In addition, a physiologically cleavable peptide linker was developed. The linker peptide was designed to be cleaved when the fusion protein is in close proximity to neutrophil extracellular traps (NETs). Peptide sequences that are targeted by neutrophil specific proteases, such as neutrophil elastase, cathepsin G, and proteinase 3, are candidates for the cleavable linker sequence.

A cleavable linker sequence was developed that is cleaved intravascularly and thus optimal for intravenously and intraarterially applied DNASE therapeutics. To design the peptide, we considered that NETs have the capacity to activate blood clotting factors, in particular the clotting factor XII (FXII). Activated FXII (FXIIa) has two major substrates: clotting factor XI (FXI, SEQ ID NO: 40) and prekallikrein (PK, SEQ ID NO: 41). An amino acid sequence alignment showed that the FXIIa cleavage site is conserved in FXI and PK (**FIG. 23B**). In FXI, the cleavage site is between arginine 387 and isoleucine 388. In PK, the cleavage site is between arginine 390 and isoleucine 391. Indeed, FXI and PK are homologous proteins. As disclosed herein, we designed several linker peptides that contain all or parts of the FXI sequence position 380 to position 403 (SEQ ID NO: 42, SEQ ID NO: 43) or of the PK sequence position 383 to position 406 (SEQ ID NO: 44).

Finally, the FXIIa-cleavable linker can be used for manufacturing half-life extended version of other biologics (**FIG. 24**), including, but not limited to, variants of

other extracellular DNASE, human coagulation factors (e.g. Factor VII, Factor VIII, and Factor IX), and complement factors (e.g. Factor H).

All patents and patent publications cited herein are hereby incorporated by reference in their entireties.

## Wild-Type Human DNASES

### SEQ ID NO: 1

DNASE1 (NP\_005212.2): Signal Peptide, Mature Protein:

5 MRGMKLLGALLALAALLQGAVSLKIAAFNIQTFGETKMSNATLVSYIVQILSRDIAL  
 VQEVDRDShLTAVGKLLDNLNQDAPDTHYHYVSEPLGRNSYKERYLFVYRPDQVSAVDS  
 YYYDDGCEPCGNDTFNREPAIVRFFSRFTEVREFAIVPLHAAPGDAVAEIDALYDVYL  
 DVQEKWGLEDVMLMGDFNAGCSYVRPSQWSSIRLWTSPTFQWLIPDSADTTATPTHCA  
 YDRIVVAGMLLRGAVVPDSALPFNFQAAAYGLSDQLAQAI SDHYPVEVMLK

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### SEQ ID NO: 2

DNASE1-LIKE 1 (NP\_006721.1): Signal Peptide; Mature Protein:

MHYPTALLFLILANGAQAFRICAFNAQRLTLAKVAREQVMDTLVRILARCDIMVLQEV  
 VDSSGSAIPLLLRELNRFDGSGPYSTLSSPQLGRSTYMETYVYFYRSHKTQVLSSYVY  
 15 NDEDDVFAREFPFAQFSLPSNVLP SLVLVPLHTTPKAVEKELNALYDVFLEVSQHWQS  
 KDVILLGDFNADCASLTKKRLDKLELRTEPGFHWVIADGEDTTVRASTHCTYDRVVLH  
 GERCSRLLHTAAAFDFPTS FQLTEEEALNISDHYPVEVELKLSQAHSVQPLSLTVLLL  
 LSLLSPQLCPAA

### 20 SEQ ID NO: 3

DNASE1-LIKE 2 (NP\_001365.1): Signal Peptide, Mature Protein:

MGGPRALLAALWALEAAGTAALRIGAFNIQSFSGDSKVSDPACGSIIAKILAGYDLALV  
 QEVDPDL SAVSALMEQINSVSEHEYSFVSSQPLGRDQYKEMYLFVYRKDAVS VVDY  
 LYPDPEDVFSREPFVVKFSAPGTGERAPPLPSRRALTPPPLPAAAQNLVLIPLHAAPH  
 25 QAVAEIDALYDVYLDVIDKWGTDDMLFLGDFNADCSYVRAQDWAAIRLSSEVFKWLI  
 PDSADTTVGNSDCAYDRIVACGARLRRLKPKQSATVHDFQEEFGLDQTQALAI SDHFP  
 VEVT LKFHR

**SEQ ID NO: 4**DNASE1-LIKE 3; Isoform 1 (NP\_004935.1): Signal Peptide; Mature Protein:

MSRELAPLLLLLLLSIHSALAMRICSFNVRSFGESKQEDKNAMDVIVKVIKRCDIILVM  
 EIKDSNNRICPILMEKLNRRNSRRGITYNYVISSRLGRNTYKEQYAFLYKEKLVSVKRS  
 5 YHYHDYQDGDADVFSREPFVWFQSPHTAVKDFVIIPLHTTPETSVKEIDELVEVYTD  
 VKHRWKAENFI FMGDFNAGCSYVPPKAWKNIRLRTDPRFVWLIGDQEDTTVKKSTNCA  
 YDRIVLRGQEIVSSVVPKSNVDFDFQKAYKLTEEEALDVSDHFPVEFKLQSSRAFTNS  
 KKSVTLRKKT KSKRS

10 **SEQ ID NO: 5**DNASE1-LIKE 3, Isoform 2 (NP\_001243489.1): Signal Peptide; Mature Protein:

MSRELAPLLLLLLLSIHSALAMRICSFNVRSFGESKQEDKNAMDVIVKVIKRCDIILVM  
 EIKDSNNRICPILMEKLNREKLVSVKRSYHYHDYQDGDADVFSREPFVWFQSPHTAV  
 KDFVIIPLHTTPETSVKEIDELVEVYTDVKHRWKAENFI FMGDFNAGCSYVPPKAWKN  
 15 IRLRTDPRFVWLIGDQEDTTVKKSTNCAYDRIVLRGQEIVSSVVPKSNVDFDFQKAYK  
 LTEEEALDVSDHFPVEFKLQSSRAFTNSKKSVTLRKKT KSKRS

**SEQ ID NO: 6**DNASE2A (O00115): Signal Peptide; Mature Protein:

MIPLLLAALLCVPAGALT CYGDSGQPVDFVYKLPALRGSGEAAQRGLQYKYLDESS  
 GGWRDGRALINSPEGAVGRSLQPLYRSNTSQLAFLLYNDQPPQPSKAQDSSMRGHTKG  
 VLLLDHDGGFWLVHSPNFPFPASSAAYS WPHSACTYGQTLLCVSFPPAQFSKMGKQL  
 TYTYPWVYNYQLEGIFAQEFPDLENVVKGHHVSQEPWNSSITLTSQAGAVFQSFQKFS  
 KFGDDL YSGWLAAAALGTNLQVQFWHKT VGILPSNCS DIWQVLNVNQIAFP GPAGPSFN  
 25 STEDHSKWCVSPKGPWTCVGD MNRNQGEEQRGGTLC AQLPALWKAFQPLVKNYQPCN  
 GMARKPSRAYKI

**SEQ ID NO: 7**DNASE2B (Q8WZ79): Signal Peptide; Mature Protein:

MKQKMMARLLRTSFALLFLGLFGVLGAATISCRNEEGKAVDWFTFYKLPKRQNKESGE  
 TGLEYLYLDSTTRSWRKSEQLMNDTKSVLGRTLQQLYEAYASKSNNTAYLIYNDGVPK  
 5 PVNYSRKYGHTKGLLLWNRVQGFVLIHSIPQFPPIPEEGYDYPPTGRRNGQSGICITF  
 KYNQYEAIDSQLLVCNPNVYSCSIPATFHQELIHMPQLCTRASSEIPGRLLTTLQSA  
 QGQKFLHFAKSDSFLDDIFAAWMAQRLKTHLLTETWQRKRQELPSNCSLPYHVYNIKA  
 IKLSRHSYFSSYQDHAKWCISQKGTKNRWTCIGDLNRS PHQAFRSGGFICTQNWQIYQ  
 AFQGLVLYYESCK

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**Human DNASE1L3 variants****SEQ ID NO: 8**DNASE1-LIKE 3, Q101R (Signal Peptide; Mature Protein)

MSRELAPLLLLLLLSIHSALAMRICSFNVRSFGESKQEDKNAMDVIVKVIKRCDIILVM  
 EIKDSNNRICPILMEKLNRRNSRRGITYNYVISSRLGRNTYKERYAFLYKEKLVSVKRS  
 15 YHYHDYQDGDADVFSREPFVWFQSPHTAVKDFVVIPLHTTPETSVKEIDELVEVYTD  
 VKHRWKAENFI FMGDFNAGCSYVPPKAWKNIRLRTDPRFVWLIGDQEDTTVKKSTNCA  
 YDRIVLRGQEIVSSVVPKSNVSFDFQKAYKLTEEEALDVSDHFPVEFKLQSSRAFTNS  
 KKSVTLRKKTKSKRS

**SEQ ID NO: 9**DNASE1L3, Q282\_S305delinksK (Signal Peptide; Mature Protein):

MSRELAPLLLLLLLSIHSALAMRICSFNVRSFGESKQEDKNAMDVIVKVIKRCDIILVM  
 EIKDSNNRICPILMEKLNRRNSRRGITYNYVISSRLGRNTYKEQYAFLYKEKLVSVKRS  
 YHYHDYQDGDADVFSREPFVWFQSPHTAVKDFVVIPLHTTPETSVKEIDELVEVYTD  
 25 VKHRWKAENFI FMGDFNAGCSYVPPKAWKNIRLRTDPRFVWLIGDQEDTTVKKSTNCA  
 YDRIVLRGQEIVSSVVPKSNVSFDFQKAYKLTEEEALDVSDHFPVEFKLK

**SEQ ID NO: 10**DNASE1L3, S305delinsK (Signal Peptide; Mature Protein):

MSRELAPLLLLLLLSIHSALAMRICSFNVRSFGESKQEDKNAMDVIVKVIKRCDIILVM  
 EIKDSNNRICPILMEKLNRRNSRRGITYNYVISSRLGRNTYKEQYAFLYKEKLVSVKRS  
 30 YHYHDYQDGDADVFSREPFVWFQSPHTAVKDFVVIPLHTTPETSVKEIDELVEVYTD

VKHRWGLENFI FMGDFNAGCSYVRPSQWKNIRLRTDPRFVWLIGDQEDTTVKKSTNCA  
YDRIVLRGQEIVSSVVPKSN SVFDFQAAYKLTEEEALDVSDHFPVEFKLQSSRAFTNS

**SEQ ID NO: 11**

5 DNASE1L3, K292\_S305del (Signal Peptide; Mature Protein):

MSRELAPLLLLLLLSIHSALAMRICS FNVRSFGESKQEDKNAMDVIVKVIKRCDIILVM  
EIKDSNNRICPILMEKLN RNSRRGITYNYVISSRLGRNTYKEQYAFLYKEKLVSVKRS  
YHYHDYQDGDADVFSREP FVVWFQSPHTAVKDFV IIPLHTTPETSVKEIDELVEVYTD  
VKHRWGLENFI FMGDFNAGCSYVRPSQWKNIRLRTDPRFVWLIGDQEDTTVKKSTNCA  
10 YDRIVLRGQEIVSSVVPKSN SVFDFQAAYKLTEEEALDVSDHFPVEFKLQSSRAFTNS  
K

**SEQ ID NO: 12**

DNASE1L3, S293\_S305del (Signal Peptide; Mature Protein):

15 MSRELAPLLLLLLLSIHSALAMRICS FNVRSFGESKQEDKNAMDVIVKVIKRCDIILVM  
EIKDSNNRICPILMEKLN RNSRRGITYNYVISSRLGRNTYKEQYAFLYKEKLVSVKRS  
YHYHDYQDGDADVFSREP FVVWFQSPHTAVKDFV IIPLHTTPETSVKEIDELVEVYTD  
VKHRWGLENFI FMGDFNAGCSYVRPSQWKNIRLRTDPRFVWLIGDQEDTTVKKSTNCA  
YDRIVLRGQEIVSSVVPKSN SVFDFQAAYKLTEEEALDVSDHFPVEFKLQSSRAFTNS  
20 KK

**SEQ ID NO: 13**

DNASE1L3, C68A (Signal Peptide; Mature Protein):

MSRELAPLLLLLLLSIHSALAMRICS FNVRSFGESKQEDKNAMDVIVKVIKRCDIILVM  
25 EIKDSNNRI A PILMEKLN RNSRRGITYNYVISSRLGRNTYKEQYAFLYKEKLVSVKRS  
YHYHDYQDGDADVFSREP FVVWFQSPHTAVKDFV IIPLHTTPETSVKEIDELVEVYTD  
VKHRWKAENFI FMGDFNAGCSYVPPKAWKNIRLRTDPRFVWLIGDQEDTTVKKSTNCA  
YDRIVLRGQEIVSSVVPKSN SVFDFQKAYKLTEEEALDVSDHFPVEFKLQSSRAFTNS  
KKSVTLRKKT KSKRS

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**SEQ ID NO: 14**

DNASE1L3, F275Y/F279\_K280delinsVM/Q282\_S305delinsK (Signal Peptide;  
Mature Protein):

MSRELAPLLLLLLLSIHSALAMRICSFNVRSFGESKQEDKNAMDVIVKVIKRCDIILVM  
 EIKDSNNRICPILMEKLNRRNSRRGITYNYVISSRLGRNTYKEQYAFLYKEKLVSVKRS  
 YHYHDYQDGDADVFSREPFVWFQSPHTAVKDFVIIPLHTTPETSVKEIDELVEVYTD  
 VKHRWKAENFI FMGDFNAGCSYVPPKAWKNIRLRTDPRFVWLIGDQEDTTVKKSTNCA  
 5 YDRIVLRGQEIVSSVVPKSNVDFQKAYKLTEEEALDVSDHYPVEVMLK

**SEQ ID NO: 15**

DNASE1L3, S283\_S305del (**Signal Peptide**; Mature Protein):

MSRELAPLLLLLLLSIHSALAMRICSFNVRSFGESKQEDKNAMDVIVKVIKRCDIILVM  
 10 EIKDSNNRICPILMEKLNRRNSRRGITYNYVISSRLGRNTYKEQYAFLYKEKLVSVKRS  
 YHYHDYQDGDADVFSREPFVWFQSPHTAVKDFVIIPLHTTPETSVKEIDELVEVYTD  
 VKHRWKAENFI FMGDFNAGCSYVPPKAWKNIRLRTDPRFVWLIGDQEDTTVKKSTNCA  
 YDRIVLRGQEIVSSVVPKSNVDFQKAYKLTEEEALDVSDHFPVEFKLQ

**15 SEQ ID NO: 16**

DNASE1L3, R285\_S305del (**Signal Peptide**; Mature Protein):

MSRELAPLLLLLLLSIHSALAMRICSFNVRSFGESKQEDKNAMDVIVKVIKRCDIILVM  
 EIKDSNNRICPILMEKLNREKLVSVKRSYHYHDYQDGDADVFSREPFVWFQSPHTAV  
 KDFVIIPLHTTPETSVKEIDELVEVYTDVKHRWKAENFI FMGDFNAGCSYVPPKAWKN  
 20 IRLRTDPRFVWLIGDQEDTTVKKSTNCAYDRIVLRGQEIVSSVVPKSNVDFQKAYK  
 LTEEEALDVSDHFPVEFKLQSS

**Albumin Fusions with DNASE1L3 and Variants****SEQ ID NO: 17**

25 Albumin – DNASE1L3 Variant – Fusion Protein. (**Albumin**, **DNASE1L3 Variant**):

DAHKSEVAHRFKDLGEENFKALVLIAFAQYLQQCPFEDHVKL VNEVTEFAKTCVADES  
 AENCCKSLHTLFGDKLCTVATLRETYGEMADCCAKQEPERNECFLQHKDDNP NLPRLV  
 RPEVDVMCTAFHDNEETFLKKYLYEIARRHPYFYAPELLFFAKRYKAAFTECCQAADK  
 AACLLPKLDEL RDEGKASSAKQRLKASLQKFGERAFAKAWAVARLSQRFPKAEFAEVS  
 30 KLVTDLTKVHTECCHGDLLECADDRADLAKYICENQDSISSKLKECCEKPLEKSHCI  
 AEVENDEMPADLPSLAADFVESKDVCKNYAEAKDVFLGMFLY EYARRHPDYSVVL LRLR  
 LAKTYETTLEKCCAAADPHECYAKVFDEFKPLVEEPQNLIKQNC ELFQGEYKFQNA  
 LLVRYTKKVPQVSTPTLVEVSRNLGKVGSKCKKHPEAKRMPCAEDYLSVVLNQLCVLH

EKT<sup>1</sup>PVSDR<sup>2</sup>VT<sup>3</sup>KCC<sup>4</sup>TES<sup>5</sup>LVNRR<sup>6</sup>PCF<sup>7</sup>SALE<sup>8</sup>VD<sup>9</sup>ET<sup>10</sup>YVP<sup>11</sup>KEF<sup>12</sup>NA<sup>13</sup>ET<sup>14</sup>FT<sup>15</sup>FHAD<sup>16</sup>ICT<sup>17</sup>LSE<sup>18</sup>KER<sup>19</sup>Q  
 IKK<sup>20</sup>QTAL<sup>21</sup>VEL<sup>22</sup>VKHK<sup>23</sup>PKAT<sup>24</sup>KE<sup>25</sup>QLKAV<sup>26</sup>MDD<sup>27</sup>FAAF<sup>28</sup>VEK<sup>29</sup>CCK<sup>30</sup>ADD<sup>31</sup>KET<sup>32</sup>CFAE<sup>33</sup>EGK<sup>34</sup>KLVA<sup>35</sup>AS<sup>36</sup>Q  
 AAL<sup>37</sup>GL<sup>38</sup>MR<sup>39</sup>ICS<sup>40</sup>FN<sup>41</sup>VR<sup>42</sup>SF<sup>43</sup>GES<sup>44</sup>KQ<sup>45</sup>ED<sup>46</sup>KNAM<sup>47</sup>DV<sup>48</sup>IV<sup>49</sup>KV<sup>50</sup>IK<sup>51</sup>RCD<sup>52</sup>IIL<sup>53</sup>VE<sup>54</sup>IK<sup>55</sup>DS<sup>56</sup>NN<sup>57</sup>RIC<sup>58</sup>PIL<sup>59</sup>ME  
 KLN<sup>60</sup>RNS<sup>61</sup>RRG<sup>62</sup>ITY<sup>63</sup>NY<sup>64</sup>VI<sup>65</sup>SS<sup>66</sup>RL<sup>67</sup>GR<sup>68</sup>NTY<sup>69</sup>KE<sup>70</sup>QYAF<sup>71</sup>LY<sup>72</sup>KE<sup>73</sup>KL<sup>74</sup>SV<sup>75</sup>KRSY<sup>76</sup>HY<sup>77</sup>HDY<sup>78</sup>QD<sup>79</sup>GD<sup>80</sup>AD<sup>81</sup>VF<sup>82</sup>S  
 5 REP<sup>83</sup>FVV<sup>84</sup>WF<sup>85</sup>QSP<sup>86</sup>HTAV<sup>87</sup>KDF<sup>88</sup>VI<sup>89</sup>IPL<sup>90</sup>HTT<sup>91</sup>PET<sup>92</sup>SV<sup>93</sup>KE<sup>94</sup>IDEL<sup>95</sup>VE<sup>96</sup>VY<sup>97</sup>TD<sup>98</sup>VK<sup>99</sup>HR<sup>100</sup>WKA<sup>101</sup>EN<sup>102</sup>FI<sup>103</sup>MG<sup>104</sup>D  
 FNAG<sup>105</sup>CSY<sup>106</sup>VPK<sup>107</sup>KAW<sup>108</sup>KNIR<sup>109</sup>LRTD<sup>110</sup>PR<sup>111</sup>FV<sup>112</sup>WL<sup>113</sup>IGD<sup>114</sup>QED<sup>115</sup>TT<sup>116</sup>VK<sup>117</sup>KSTN<sup>118</sup>CAY<sup>119</sup>DRIV<sup>120</sup>LR<sup>121</sup>GQ<sup>122</sup>EIV<sup>123</sup>SS<sup>124</sup>V  
 VPK<sup>125</sup>SNS<sup>126</sup>V<sup>127</sup>FD<sup>128</sup>FQ<sup>129</sup>KAY<sup>130</sup>KL<sup>131</sup>TEEE<sup>132</sup>AL<sup>133</sup>DV<sup>134</sup>SD<sup>135</sup>HY<sup>136</sup>PVE<sup>137</sup>VML<sup>138</sup>K

### SEQ ID NO: 18

10 Albumin – DNASE1L3 Variant – Fusion Protein. (Albumin, **DNASE1L3 Variant**):  
 DAHK<sup>1</sup>SEVA<sup>2</sup>HR<sup>3</sup>FKDL<sup>4</sup>GEEN<sup>5</sup>FKAL<sup>6</sup>VLI<sup>7</sup>IAFA<sup>8</sup>QYL<sup>9</sup>QQCP<sup>10</sup>FED<sup>11</sup>HV<sup>12</sup>KLV<sup>13</sup>NEV<sup>14</sup>TEFA<sup>15</sup>KTC<sup>16</sup>VAD<sup>17</sup>ES  
 AENC<sup>18</sup>DKSL<sup>19</sup>HTL<sup>20</sup>FGD<sup>21</sup>KLCT<sup>22</sup>VAT<sup>23</sup>LRET<sup>24</sup>YGEM<sup>25</sup>ADCC<sup>26</sup>AKQ<sup>27</sup>EPER<sup>28</sup>NEC<sup>29</sup>FLQ<sup>30</sup>HKDD<sup>31</sup>NPNL<sup>32</sup>PRL<sup>33</sup>V  
 RPE<sup>34</sup>VDVM<sup>35</sup>CTAF<sup>36</sup>HDNE<sup>37</sup>ET<sup>38</sup>FL<sup>39</sup>KKY<sup>40</sup>LYE<sup>41</sup>IA<sup>42</sup>RRHP<sup>43</sup>YFYA<sup>44</sup>PELL<sup>45</sup>FFA<sup>46</sup>KRY<sup>47</sup>KAA<sup>48</sup>FTE<sup>49</sup>CCQ<sup>50</sup>AAD<sup>51</sup>K  
 AAC<sup>52</sup>LLPK<sup>53</sup>LDEL<sup>54</sup>RDEG<sup>55</sup>KASS<sup>56</sup>AKQ<sup>57</sup>RLK<sup>58</sup>CAS<sup>59</sup>LQ<sup>60</sup>KFGER<sup>61</sup>AFK<sup>62</sup>AWA<sup>63</sup>VAR<sup>64</sup>LSQ<sup>65</sup>RF<sup>66</sup>PKAE<sup>67</sup>FAE<sup>68</sup>VS  
 15 KLVT<sup>69</sup>DLTK<sup>70</sup>VHTE<sup>71</sup>CCHG<sup>72</sup>DLLE<sup>73</sup>CAD<sup>74</sup>DRAD<sup>75</sup>LAKY<sup>76</sup>ICEN<sup>77</sup>QDS<sup>78</sup>ISS<sup>79</sup>KL<sup>80</sup>ECCE<sup>81</sup>KPLLE<sup>82</sup>KSH<sup>83</sup>CI  
 AEVEN<sup>84</sup>DEMP<sup>85</sup>ADL<sup>86</sup>PSLA<sup>87</sup>ADF<sup>88</sup>VES<sup>89</sup>KDV<sup>90</sup>CKNYA<sup>91</sup>EAK<sup>92</sup>DV<sup>93</sup>FLGM<sup>94</sup>FLY<sup>95</sup>EYARR<sup>96</sup>HPD<sup>97</sup>YSV<sup>98</sup>VLLLR  
 LAK<sup>99</sup>TYET<sup>100</sup>TLEK<sup>101</sup>CCAA<sup>102</sup>ADP<sup>103</sup>HECY<sup>104</sup>AKV<sup>105</sup>FDEF<sup>106</sup>KPL<sup>107</sup>VEEP<sup>108</sup>QNL<sup>109</sup>IKQ<sup>110</sup>NCEL<sup>111</sup>FEQ<sup>112</sup>LGEY<sup>113</sup>KFQ<sup>114</sup>NA  
 LLV<sup>115</sup>RYTK<sup>116</sup>KVP<sup>117</sup>QVST<sup>118</sup>P<sup>119</sup>TLVE<sup>120</sup>VSRL<sup>121</sup>GK<sup>122</sup>VGS<sup>123</sup>KCK<sup>124</sup>HP<sup>125</sup>EAK<sup>126</sup>RMPC<sup>127</sup>AED<sup>128</sup>YLSV<sup>129</sup>VLN<sup>130</sup>QLC<sup>131</sup>VLH  
 EKT<sup>132</sup>PVSDR<sup>133</sup>VT<sup>134</sup>KCC<sup>135</sup>TES<sup>136</sup>LVNRR<sup>137</sup>PCF<sup>138</sup>SALE<sup>139</sup>VD<sup>140</sup>ET<sup>141</sup>YVP<sup>142</sup>KEF<sup>143</sup>NA<sup>144</sup>ET<sup>145</sup>FT<sup>146</sup>FHAD<sup>147</sup>ICT<sup>148</sup>LSE<sup>149</sup>KER<sup>150</sup>Q  
 20 IKK<sup>151</sup>QTAL<sup>152</sup>VEL<sup>153</sup>VKHK<sup>154</sup>PKAT<sup>155</sup>KE<sup>156</sup>QLKAV<sup>157</sup>MDD<sup>158</sup>FAAF<sup>159</sup>VEK<sup>160</sup>CCK<sup>161</sup>ADD<sup>162</sup>KET<sup>163</sup>CFAE<sup>164</sup>EGK<sup>165</sup>KLVA<sup>166</sup>AS<sup>167</sup>Q  
 AAL<sup>168</sup>GL<sup>169</sup>GGG<sup>170</sup>SM<sup>171</sup>RIC<sup>172</sup>SF<sup>173</sup>NVR<sup>174</sup>SF<sup>175</sup>GES<sup>176</sup>KQ<sup>177</sup>ED<sup>178</sup>KNAM<sup>179</sup>DV<sup>180</sup>IV<sup>181</sup>KV<sup>182</sup>IK<sup>183</sup>RCD<sup>184</sup>IIL<sup>185</sup>VE<sup>186</sup>IK<sup>187</sup>DS<sup>188</sup>NN<sup>189</sup>RIC  
 PIL<sup>190</sup>MEK<sup>191</sup>LN<sup>192</sup>RNS<sup>193</sup>RRG<sup>194</sup>ITY<sup>195</sup>NY<sup>196</sup>VI<sup>197</sup>SS<sup>198</sup>RL<sup>199</sup>GR<sup>200</sup>NTY<sup>201</sup>KE<sup>202</sup>QYAF<sup>203</sup>LY<sup>204</sup>KE<sup>205</sup>KL<sup>206</sup>SV<sup>207</sup>KRSY<sup>208</sup>HY<sup>209</sup>HDY<sup>210</sup>QD<sup>211</sup>GD  
 ADV<sup>212</sup>FSRE<sup>213</sup>PVV<sup>214</sup>WF<sup>215</sup>QSP<sup>216</sup>HTAV<sup>217</sup>KDF<sup>218</sup>VI<sup>219</sup>IPL<sup>220</sup>HTT<sup>221</sup>PET<sup>222</sup>SV<sup>223</sup>KE<sup>224</sup>IDEL<sup>225</sup>VE<sup>226</sup>VY<sup>227</sup>TD<sup>228</sup>VK<sup>229</sup>HR<sup>230</sup>WKA<sup>231</sup>EN<sup>232</sup>F  
 IFM<sup>233</sup>GD<sup>234</sup>FNAG<sup>235</sup>CSY<sup>236</sup>VPK<sup>237</sup>KAW<sup>238</sup>KNIR<sup>239</sup>LRTD<sup>240</sup>PR<sup>241</sup>FV<sup>242</sup>WL<sup>243</sup>IGD<sup>244</sup>QED<sup>245</sup>TT<sup>246</sup>VK<sup>247</sup>KSTN<sup>248</sup>CAY<sup>249</sup>DRIV<sup>250</sup>LR<sup>251</sup>GQ<sup>252</sup>E  
 25 IVSS<sup>253</sup>V<sup>254</sup>PK<sup>255</sup>SNS<sup>256</sup>V<sup>257</sup>FD<sup>258</sup>FQ<sup>259</sup>KAY<sup>260</sup>KL<sup>261</sup>TEEE<sup>262</sup>AL<sup>263</sup>DV<sup>264</sup>SD<sup>265</sup>HY<sup>266</sup>PVE<sup>267</sup>VML<sup>268</sup>K

### SEQ ID NO: 19

Albumin – DNASE1L3 Variant – Fusion Protein. (Albumin, **DNASE1L3 Variant**):  
 DAHK<sup>1</sup>SEVA<sup>2</sup>HR<sup>3</sup>FKDL<sup>4</sup>GEEN<sup>5</sup>FKAL<sup>6</sup>VLI<sup>7</sup>IAFA<sup>8</sup>QYL<sup>9</sup>QQCP<sup>10</sup>FED<sup>11</sup>HV<sup>12</sup>KLV<sup>13</sup>NEV<sup>14</sup>TEFA<sup>15</sup>KTC<sup>16</sup>VAD<sup>17</sup>ES  
 30 AENC<sup>18</sup>DKSL<sup>19</sup>HTL<sup>20</sup>FGD<sup>21</sup>KLCT<sup>22</sup>VAT<sup>23</sup>LRET<sup>24</sup>YGEM<sup>25</sup>ADCC<sup>26</sup>AKQ<sup>27</sup>EPER<sup>28</sup>NEC<sup>29</sup>FLQ<sup>30</sup>HKDD<sup>31</sup>NPNL<sup>32</sup>PRL<sup>33</sup>V  
 RPE<sup>34</sup>VDVM<sup>35</sup>CTAF<sup>36</sup>HDNE<sup>37</sup>ET<sup>38</sup>FL<sup>39</sup>KKY<sup>40</sup>LYE<sup>41</sup>IA<sup>42</sup>RRHP<sup>43</sup>YFYA<sup>44</sup>PELL<sup>45</sup>FFA<sup>46</sup>KRY<sup>47</sup>KAA<sup>48</sup>FTE<sup>49</sup>CCQ<sup>50</sup>AAD<sup>51</sup>K  
 AAC<sup>52</sup>LLPK<sup>53</sup>LDEL<sup>54</sup>RDEG<sup>55</sup>KASS<sup>56</sup>AKQ<sup>57</sup>RLK<sup>58</sup>CAS<sup>59</sup>LQ<sup>60</sup>KFGER<sup>61</sup>AFK<sup>62</sup>AWA<sup>63</sup>VAR<sup>64</sup>LSQ<sup>65</sup>RF<sup>66</sup>PKAE<sup>67</sup>FAE<sup>68</sup>VS  
 KLVT<sup>69</sup>DLTK<sup>70</sup>VHTE<sup>71</sup>CCHG<sup>72</sup>DLLE<sup>73</sup>CAD<sup>74</sup>DRAD<sup>75</sup>LAKY<sup>76</sup>ICEN<sup>77</sup>QDS<sup>78</sup>ISS<sup>79</sup>KL<sup>80</sup>ECCE<sup>81</sup>KPLLE<sup>82</sup>KSH<sup>83</sup>CI  
 AEVEN<sup>84</sup>DEMP<sup>85</sup>ADL<sup>86</sup>PSLA<sup>87</sup>ADF<sup>88</sup>VES<sup>89</sup>KDV<sup>90</sup>CKNYA<sup>91</sup>EAK<sup>92</sup>DV<sup>93</sup>FLGM<sup>94</sup>FLY<sup>95</sup>EYARR<sup>96</sup>HPD<sup>97</sup>YSV<sup>98</sup>VLLLR

LAKTYETTLKCCAAADPHECYAKVFDEFKPLVEEPQNLIKQNCLEFEQLGEYKFQNA  
LLVRYTKKVPQVSTPTLVEVSRNLGKVGSKCKKHPEAKRMPCAEDYLSVVLNQLCVLH  
EKTPVSDRVTKCCTESLVNRRPCFSALEVDETYVPKEFNAETFTFHADICTLSEKERQ  
IKKQTALVELVKHKPKATKEQLKAVMDDFAAFVEKCKADDKETCFAEEGKKLVAASQ  
 5 AALGLGGGGSGGGGSGGGGSMRICESFNVSFGESEKQEDKNAMDVIVKVIKRCDIILVM  
EIKDSNNRICPILMEKLNRRNSRRGITYNYVISSRLGRNTYKEQYAFLYKEKLVSVKRS  
YHYHDYQDGDADVFSREPFVWFQSPHTAVKDFVIIPLHTTPETSVKEIDELVEVYTD  
VKHRWKAENFIIFMGDFNAGCSYVPPKAWKNIRLRTDPRFVWLIGDQEDTTVKKSTNCA  
YDRIVLRGQEIIVSSVVPKSNVSFDFQKAYKLTEEEALDVSDHYPVEVMLK

10

**SEQ ID NO: 20**

DNASE1L3 Variant – Albumin – Fusion Protein. (Albumin, **DNASE1L3 Variant**):

MRICESFNVSFGESEKQEDKNAMDVIVKVIKRCDIILVMEIKDSNNRICPILMEKLNRRN  
 SRRGITYNYVISSRLGRNTYKEQYAFLYKEKLVSVKRSYHYHDYQDGDADVFSREPFV  
 15 VWFQSPHTAVKDFVIIPLHTTPETSVKEIDELVEVYTDVKHRWKAENFIIFMGDFNAGC  
SYVPPKAWKNIRLRTDPRFVWLIGDQEDTTVKKSTNCAYDRIVLRGQEIIVSSVVPKSN  
VSFDFQKAYKLTEEEALDVSDHYPVEVMLKGGGGSDAHKSEVAHRFKDLGEENFKALV  
LIAFAQYLQQCPFEDHVKLNEVTEFAKTCVADESAENCCKSLHTLFGDKLCTVATLR  
ETYGEMADCCAKQEPERNECFLOHKDDNPPLRLVRPEVDVMCTAFHDNEETFLKKYL  
 20 YEIARRHPYFYAPELLFFAKRYKAAFTCECCQAADKAACLLPKLDELDEGKASSAKQR  
LKASLQKFGERAFAKAWAVARLSQRFPAEFAEVSKLVTDLTKVHTECCHGDLLECAD  
DRADLAKYICENQDSISSKLKECCEKPLLEKSHCIAEVENDEMPADLPSLAADFVESK  
DVCKNYAEAKDVFLGMFLYFYARRHPDYSVVLRLAKTYETTLKCCAAADPHECYA  
KVFDEFKPLVEEPQNLIKQNCLEFEQLGEYKFQNAALLVRYTKKVPQVSTPTLVEVSRN  
 25 LGKVGSKCKKHPEAKRMPCAEDYLSVVLNQLCVLHEKTPVSDRVTKCCTESLVNRRPC  
FSALEVDETYVPKEFNAETFTFHADICTLSEKERQIKKQTALVELVKHKPKATKEQLK  
AVMDDFAAFVEKCKADDKETCFAEEGKKLVAASQAALGL

**SEQ ID NO: 21**

30 DNASE1L3 Variant – Albumin – Fusion Protein. (Albumin, **DNASE1L3 Variant**):

MRICESFNVSFGESEKQEDKNAMDVIVKVIKRCDIILVMEIKDSNNRICPILMEKLNRRN  
 SRRGITYNYVISSRLGRNTYKEQYAFLYKEKLVSVKRSYHYHDYQDGDADVFSREPFV  
 VWFQSPHTAVKDFVIIPLHTTPETSVKEIDELVEVYTDVKHRWKAENFIIFMGDFNAGC  
 SYVPPKAWKNIRLRTDPRFVWLIGDQEDTTVKKSTNCAYDRIVLRGQEIIVSSVVPKSN

SVFDFQKAYKLTEEEALDVSDHYPVEVMLKGGGSGGGGSGGGGSDAHKSEVAHRFKD  
 LGEENFKALVLI AFAQYLQQCPFEDHVKL VNEVTEFAKTCVADESAENCDKSLHTLFG  
 DKLCTVATLRETYGEMADCCAKQEPERNECFLOHKDDNP NLPRLVRPEVDVMCTAFHD  
 NEETFLKKYLYE IARRHPYFYAPELLFFAKRYKAAFTTECCQAADKAACLLPKLDEL RD  
 5 EGKASSAKQRLK CASLQKFGERAFKAWAVARLSQRF PKAEFAEVSKLVTDLTKVHTEC  
 CHGDLLECADDRADLAKY ICENQDSISSKLKECCEKPLLEKSHCIAEVENDEMPADLP  
 SLAADFVESKDVCKNYAEAKDVFLGMFLY EYARRHPDYSVVL LRLAKTYETTLEKCC  
 AAADPHECYAKVFDEFKPLVEEPQNL IKQNCELFEQLGEYKFQNAL LVRYTKKVPQVS  
 TPTLVEVSRNLGKVGSKCKHPEAKRMPCAEDYLSVVLNQLCVLHEKT PVSDRVTKCC  
 10 TESLVNRRPCFSALEVDETYVPKEFNAETFTFHADICTLSEKERQIKKQTALVELVKH  
 KPKATKEQLKAVMDDFAAFVEKCKADDKETCFAEEGKKLVAASQAALGL

**SEQ ID NO: 22**

Albumin – DNASE1L3 – Fusion Protein. (Albumin, **DNASE1L3**):

15 DAHKSEVAHRFKDLGEENFKALVLI AFAQYLQQCPFEDHVKL VNEVTEFAKTCVADES  
 AENCDKSLHTLFGDKLCTVATLRETYGEMADCCAKQEPERNECFLOHKDDNP NLPRLV  
 RPEVDVMCTAFHDNEETFLKKYLYE IARRHPYFYAPELLFFAKRYKAAFTTECCQAADK  
 AACLLPKLDEL RDDEGKASSAKQRLK CASLQKFGERAFKAWAVARLSQRF PKAEFAEVS  
 KLVTDLT KVHTECCHGDLLECADDRADLAKY ICENQDSISSKLKECCEKPLLEKSHCI  
 20 AEVENDEMPADLP SLAADFVESKDVCKNYAEAKDVFLGMFLY EYARRHPDYSVVL LLLR  
 LAKTYETTLEKCCAAADPHECYAKVFDEFKPLVEEPQNL IKQNCELFEQLGEYKFQNA  
 LLVRYTKKVPQVSTPTLVEVSRNLGKVGSKCKHPEAKRMPCAEDYLSVVLNQLCVLH  
 EKT PVSDRVTKCCTESLVNRRPCFSALEVDETYVPKEFNAETFTFHADICTLSEKERQ  
 IKKQTALVELVKHKPKATKEQLKAVMDDFAAFVEKCKADDKETCFAEEGKKLVAASQ  
 25 AALGLGGGSGGGGSGGGGSMR IC SFNVSFGESKQEDKNAMDVIVKVIKRCDIILVM  
 EIKDSNNRICPILMEKLN RNSRRGITYNYVISSRLGRNTYKEQYAFLYKEKLVSVKRS  
 YHYHDYQGDADVFSREPFV VWFQSPHTAVKDFVI IPLHTTPETSVKEIDELVEVYTD  
 VKHRWKAENFI FMGDFNAGCSYVPKKAWKNIRLRTDPRFVWLIGDQEDTTVKKSTNCA  
 YDRIVLRGQEIVSSVVPKSNSVFDFQKAYKLTEEEALDVSDHFPVEFKLQSSRAFTNS  
 30 KKSVTLRKKT SKRS

**SEQ ID NO: 23**

Albumin – DNASE1L3 – Fusion Protein. (Albumin, **DNASE1L3**):

DAHKSEVAHRFKDLGEENFKALVLI AFAQYLQQCPFEDHVKLVNEVTEFAKTCVADES  
 AENCCKSLHTLFGDKLCTVATLRETYGEMADCCAKQEPERNECFLQHKDDNPNLPRLV  
 RPEVDVMCTAFHDNEETFLKKYLYEIIARRHPYFYAPELLFFAKRYKAAFTECCQAADK  
 AACLLPKLDEL RDEGKASSAKQRLK CASLQKFGERAFAKAWAVARLSQRFPKAEFAEVS  
 5 KLVTDLT KVHTECCHGDLLECADDRADLAKYICENQDSISSKLKECCEKPLLEKSHCI  
 AEVENDEMPADLPSLAADFVESKDVCCKNYAEAKDVFLGMFLY EYARRHPDYSVVL LLLR  
 LAKTYET TLEKCCAAADPHECYAKVFDEFKPLVEEPQNLIKQNC ELFQ LGEYKFQNA  
 LLVRYTKKVPQVSTPTLVEVSRNLGKVGSKCKKHPEAKRMPCAEDYLSVVLNQLCVLH  
 EKT PVSDRVTKCCTESLVNRRPCFSALEVDETYVPKEFNAETFTFHADICTLSEKERQ  
 10 IKKQTALVELVKHKPKATKEQLKAVMDDFAAFVEKCKCKADDKETCFAEEGKKLVAA SQ  
 AALGLAPAPAPAPAPAPAPMRICSFNVR SFGESKQEDKNAMDVIVKVIKRCDIILVME  
 IKDSNNRICPILMEKLNRSRRGITYNYVISSRLGRNTYKEQYAFLYKEKLVSVKRSY  
 HYHDYQDGDADVFSREPFVWFQSPHTAVKDFV I I PLHTTPETSVKEIDELVEVYTDV  
 KHRWKAENFI FMGDFNAGCSYVPKKAWKNIRLRTDPRFVWLIGDQEDTTVKKSTNCAY  
 15 DRIVLRGQEI VSSVVPKSN SVFDFQKAYKLTEEEALDVSDHFPVEFKLQSSRAFTNSK  
 KSVTLRKKTKSKRS

**SEQ ID NO: 24**

Albumin – DNASE1L3 – Fusion Protein. (Albumin, **DNASE1L3**):

20 DAHKSEVAHRFKDLGEENFKALVLI AFAQYLQQCPFEDHVKLVNEVTEFAKTCVADES  
 AENCCKSLHTLFGDKLCTVATLRETYGEMADCCAKQEPERNECFLQHKDDNPNLPRLV  
 RPEVDVMCTAFHDNEETFLKKYLYEIIARRHPYFYAPELLFFAKRYKAAFTECCQAADK  
 AACLLPKLDEL RDEGKASSAKQRLK CASLQKFGERAFAKAWAVARLSQRFPKAEFAEVS  
 KLVTDLT KVHTECCHGDLLECADDRADLAKYICENQDSISSKLKECCEKPLLEKSHCI  
 25 AEVENDEMPADLPSLAADFVESKDVCCKNYAEAKDVFLGMFLY EYARRHPDYSVVL LLLR  
 LAKTYET TLEKCCAAADPHECYAKVFDEFKPLVEEPQNLIKQNC ELFQ LGEYKFQNA  
 LLVRYTKKVPQVSTPTLVEVSRNLGKVGSKCKKHPEAKRMPCAEDYLSVVLNQLCVLH  
 EKT PVSDRVTKCCTESLVNRRPCFSALEVDETYVPKEFNAETFTFHADICTLSEKERQ  
 IKKQTALVELVKHKPKATKEQLKAVMDDFAAFVEKCKCKADDKETCFAEEGKKLVAA SQ  
 30 AALGLAEAAAKEAAAKAMRICSFNVR SFGESKQEDKNAMDVIVKVIKRCDIILVMEIK  
 DSNNRICPILMEKLNRSRRGITYNYVISSRLGRNTYKEQYAFLYKEKLVSVKRSYHY  
 HDYQDGDADVFSREPFVWFQSPHTAVKDFV I I PLHTTPETSVKEIDELVEVYTDVKH  
 RWKAENFI FMGDFNAGCSYVPKKAWKNIRLRTDPRFVWLIGDQEDTTVKKSTNCAYDR

IVLRGQEIVSSVVPKNSVFDQKAYKLTEEEALDVSDHFPVEFKLQSSRAFTNSKKS  
VTLRKKTKSKRS

# SEQ ID NO: 25

- 5 Albumin – DNASE1L3 Variant – Fusion Protein. (Albumin, **DNASE1L3 Variants**):  
DAHKSEVAHRFKDLGEENFKALVLI AFAQYLQQCPFEDHVKLVNEVTEFAKTCVADES  
AENCDKSLHTLFGDKLCTVATLRETYGEMADCCAKQEPERNECFLQHKDDNPNLPRLV  
RPEVDVMCTAFHDNEETFLKKYLYEIIARRHPYFYAPELLFFAKRYKAAFTECCQAADK  
AACLLPKLDEL RDEGKASSAKQRLKCASLQKFGERAFAKAWAVARLSQRFPKAEFAEVS  
10 KLVTDLTKVHTECCHGDLLECADDRADLAKYICENQDSISSKLKECCEKPLLEKSHCI  
AEVENDEMPADLPSLAADFVESKDVCCKNYAEAKDVFLGMFLY EYARRHPDYSVVL LLLR  
LAKTYETTLEKCCAAADPHECYAKVFDEFKPLVEEPQNLIKQNC ELF EQLGEYKFQNA  
LLVRYTKKVPQVSTPTLVEVSRNLGKVGSKCKKHPEAKRMPCAEDYLSVVLNQLCVLH  
EKTPVSDRVT KCCTESLVNRRPCFSALEVDETYVPKEFNAETFTFHADICTLSEKERQ  
15 IKKQTALVELVKHKPKATKEQLKAVMDDFAAFVEKCKADDKETCFAEEGKKLVAASQ  
AALGLSGGSGSSMRICSFNVR SFGESKQEDKNAMDVIVKVIKRCDIILVMEIKDSNNR  
ICPILMEKLNRSRRGITYNYVISSRLGRNTYKEQYAFLYKEKLVSVKRSYHYHDYQD  
GDADVFSREP FVVWFQSPHTAVKDFV IIPLHTT PETS VKEIDELVEVYTDVKHRWKA E  
NFI FMGDFNAGCSYVPKKAWKNIRLRTDPRFVWLIGDQEDTTVKKSTNCAYDRIVLRG  
20 QEIVSSVVPKNSVFDQKAYKLTEEEALDVSDHFPVEFKLQ

# SEQ ID NO: 26

- Albumin – DNASE1L3 Variant – Fusion Protein. (Albumin, **DNASE1L3 Variant**):  
DAHKSEVAHRFKDLGEENFKALVLI AFAQYLQQCPFEDHVKLVNEVTEFAKTCVADES  
25 AENCDKSLHTLFGDKLCTVATLRETYGEMADCCAKQEPERNECFLQHKDDNPNLPRLV  
RPEVDVMCTAFHDNEETFLKKYLYEIIARRHPYFYAPELLFFAKRYKAAFTECCQAADK  
AACLLPKLDEL RDEGKASSAKQRLKCASLQKFGERAFAKAWAVARLSQRFPKAEFAEVS  
KLVTDLTKVHTECCHGDLLECADDRADLAKYICENQDSISSKLKECCEKPLLEKSHCI  
AEVENDEMPADLPSLAADFVESKDVCCKNYAEAKDVFLGMFLY EYARRHPDYSVVL LLLR  
30 LAKTYETTLEKCCAAADPHECYAKVFDEFKPLVEEPQNLIKQNC ELF EQLGEYKFQNA  
LLVRYTKKVPQVSTPTLVEVSRNLGKVGSKCKKHPEAKRMPCAEDYLSVVLNQLCVLH  
EKTPVSDRVT KCCTESLVNRRPCFSALEVDETYVPKEFNAETFTFHADICTLSEKERQ  
IKKQTALVELVKHKPKATKEQLKAVMDDFAAFVEKCKADDKETCFAEEGKKLVAASQ  
AALGLSGGSGGSGGSGGSGSSMRICSFNVR SFGESKQEDKNAMDVIVKVIKRCDIILV

MEIKDSNNRICPILMEKLNRRNSRRGITYNYVISSRLGRNTYKEQYAFLYKEKLVSVKR  
 SYHYHDYQDGDADVFSREPFVWFQSPHTAVKDFVIIPLHTTPETSVKEIDELVEVYT  
 DVKHRWKAENFIEMGDFNAGCSYVPKKAWKNIRLRDPRFVWLIGDQEDTTVKKSTNC  
 AYDRIVLRGQEIVSSVVPKSNVDFDFQKAYKLTEEEALDVSDHFPVEFKLQ

5

**SEQ ID NO: 27**

Albumin – DNASE1L3 Variant – Fusion Protein. (Albumin, **DNASE1L3 Variant**):

DAHKSEVAHRFKDLGEENFKALVLIAFAQYLQCCPFEDHVKLVNEVTEFAKTCVADES  
AENCDKSLHTLFGDKLCTVATLRETYGEMADCCAKQEPERNECFLQHKDDNPPLRLV  
 10 RPEVDVMCTAFHDNEETFLKKYLYEIARRHPYFYAPELLFFAKRYKAAFTECCQAADK  
AACLLPKLDELRLDEGKASSAKQRLKCASLQKFGERAFAKAWAVARLSQRFPAEFAEVS  
KLVTDLTKVHTECCHGDLLECADDRADLAKYICENQDSISSKLKECCEKPLLEKSHCI  
AEVENDEMPADLPSLAADFVESKDVKCNIAEAKDVFLGMFLYFYARRHPDYSVLLLR  
LAKTYETTLEKCCAAADPHECYAKVFDEFKPLVEEPQNLIKQNCLEFEQLGEYKFQNA  
 15 LLVRYTKKVPQVSTPTLVEVSRNLGKVGSKCCKHPEAKRMPCAEDYLSVVLNQLCVLH  
EKTPVSDRVTKCCTESLVNRRPCFSALEVDETYVPKEFNAETFTFHADICTLSEKERQ  
IKKQTALVELVKHKPKATKEQLKAVMDDFAAFVEKCKADDKETCFAEEGKKLVAASQ  
AALGLSGSGSGSGSGSGSGSGSGSGSGSGSGSGSMRICESFNVSFGESEKQEDKNAM  
DVIVKVIKRCDIILVMEIKDSNNRICPILMEKLNRRNSRRGITYNYVISSRLGRNTYKE  
 20 QYAFLYKEKLVSVKRSYHYHDYQDGDADVFSREPFVWFQSPHTAVKDFVIIPLHTTP  
ETSVKEIDELVEVYTDVKHRWKAENFIEMGDFNAGCSYVPKKAWKNIRLRDPRFVWL  
IGDQEDTTVKKSTNCAYDRIVLRGQEIVSSVVPKSNVDFDFQKAYKLTEEEALDVSDH  
FPVEFKLQ

25 **SEQ ID NO: 28**

Albumin – DNASE1L3 Variant – Albumin – Fusion Protein. (Albumin, **DNASE1L3 Variant**):

DAHKSEVAHRFKDLGEENFKALVLIAFAQYLQCCPFEDHVKLVNEVTEFAKTCVADES  
AENCDKSLHTLFGDKLCTVATLRETYGEMADCCAKQEPERNECFLQHKDDNPPLRLV  
 30 RPEVDVMCTAFHDNEETFLKKYLYEIARRHPYFYAPELLFFAKRYKAAFTECCQAADK  
AACLLPKLDELRLDEGKASSAKQRLKCASLQKFGERAFAKAWAVARLSQRFPAEFAEVS  
KLVTDLTKVHTECCHGDLLECADDRADLAKYICENQDSISSKLKECCEKPLLEKSHCI  
AEVENDEMPADLPSLAADFVESKDVKCNIAEAKDVFLGMFLYFYARRHPDYSVLLLR  
LAKTYETTLEKCCAAADPHECYAKVFDEFKPLVEEPQNLIKQNCLEFEQLGEYKFQNA

20

**Albumin – DNASE1L3 Isoform 2 – Fusion Protein. (Albumin, **DNASE1L3 Isoform 2**):**

30

DVIVKVIKRCDIILVMEIKDSNNRICPILMEKLNREQYAFLYKEKLVSVKRSYHYHDY  
 QDGDADVFSREPFVWFQSPHTAVKDFVIIPLHTTPETSVKEIDELVEVYTDVKHRWK  
 AENFIEMGDFNAGCSYVPKKAWKNIRLRTPRFVWLIGDQEDTTVKKSTNCAYDRIVL  
 RGQEIVSSVVPKSNVSFDFQKAYKLTTEEALDVSDHFPVEFKLQSSRAFTNSKKSRTL  
 5 RKKTKSKRS

**SEQ ID NO: 30**

Albumin – DNASE1L3 Isoform 2 Variant – Fusion Protein. (Albumin, **DNASE1L3 Isoform 2**):

10 DAHKSEVAHRFKDLGEENFKALVLI AFAQYLQQCPFEDHVKLVNEVTEFAKTCVADES  
AENCDKSLHTLFGDKLCTVATLRETYGEMADCCAKQEPERNECFLOHKDDNPNLPRLV  
RPEVDVMCTAFHDNEETFLKKYLYEIIARRHPYFYAPELLFFAKRYKAAFTECCQAADK  
AACLLPKLDEL RDEGKASSAKQRLK CASLQKGERAFKAWAVARLSQRFPKAEFAEVS  
KLVTDLTKVHTECCHGDLLECADDRADLAKYICENQDSISSKLKECCEKPLEKSHCI  
 15 AEVENDEMPADLPSLAADFVESKD VCKNYAEAKDVFLGMFLYEYARRHPDYSVVL LRL  
LAKTYETTL EKCCAAADPHECYAKVFDEFKPLVEEPQNLIKQNC ELFQ LGEYKFQNA  
LLVRYTKKVPQVSTPTLVEVSRNLGKVGSKCKKHPEAKRMPCAEDYLSVVLNQLCVLH  
EKTPVSDRVTKCCTESLVNRRPCFSALEVD ETYVPKEFNAETFTFHADICTLSEKERQ  
IKKQTALVELVKHKPKATKEQLKAVMDDFAAFVEKCKADDKETCFAEEGKKLVAASQ  
 20 AALGLSGGSGGSGGSGGSGGSGGSGGSGGSGGSGGSGGSSMRICSFNVR SFGESKQEDKNAM  
 DVIVKVIKRCDIILVMEIKDSNNRICPILMEKLNREQYAFLYKEKLVSVKRSYHYHDY  
 QDGDADVFSREPFVWFQSPHTAVKDFVIIPLHTTPETSVKEIDELVEVYTDVKHRWK  
 AENFIEMGDFNAGCSYVPKKAWKNIRLRTPRFVWLIGDQEDTTVKKSTNCAYDRIVL  
 RGQEIVSSVVPKSNVSFDFQKAYKLTTEEALDVSDHFPVEFKLQ

25

**LINKER SEQUENCES****SEQ ID NO: 31**

GGGGS

30

**SEQ ID NO: 32**

GGGGSGGGSGGGGS

**SEQ ID NO: 33**

APAPAPAPAPAPAP

**SEQ ID NO: 34**

AEAAAKEAAKA

5

**SEQ ID NO: 35**

SGGSGSS

**SEQ ID NO: 36**

10 SGGSGGSGGSGGSGSS

**SEQ ID NO: 37**

SGGSGGSGGSGGSGGSGGSGGSGGSGGSGS

15 **SEQ ID NO: 38**

GGSGGSGGSGGSGGSGGSGGSGGSGGSGS

**OTHER SEQUENCES**

**SEQ ID NO: 39**

20 Human Serum Albumin (Mature Protein):

DAHKSEVAHRFKDLGEENFKALVLI AFAQYLQQCPFEDHVKLVNEVTEFAKTCVADES  
 AENCCKSLHTLFGDKLCTVATLRETYGEMADCCAKQEPERNECFLQHKDDNP NLPRLV  
 RPEVDVMCTAFHDNEETFLKKYLYE IARRHPYFYAPELLFFAKRYKAAFTECCQAADK  
 AACLLPKLDEL RDEGKASSAKQRLK CASLQKFGERAFAKAWAVARLSQRFPKAEFAEVS  
 25 KLVTDLT KVHTECCHGDLLECADDRADLAKY ICENQDSISSKLKECCEKPLLEKSHCI  
 AEVENDEMPADLPSLAADFVESKDVCKNYAEAKDVFLGMFLY EYARRHPDYSVVL LRLR  
 LAKTYET TLEKCCAAADPHECYAKVFDEFKPLVEEPQNLIKQNC ELFELGEYKFQNA  
 LLVRYTKKVPQVSTPTLVEVSRNLGKVGSKCKHPEAKRMPCAEDYLSVVLNQLCVLH  
 EKT PVSDRVTKCTESLVNRRPCFSALEVD ETYVPKEFNAETFTFHADICTLSEKERQ  
 30 IKKQTALVELVKHKPKATKEQLKAVMDDFAAFVEKCKADDKETCFAEEGKKLVAASQ  
 AALGL

**SEQ ID NO: 40**

Human Factor XI:

MIFLYQVVHFILEFTSVSGECVTQLLKDTCFEGGDITTVFTPSAKYCQVVCTYHPRCLL  
 FTFTAESPSEDPTRWFTCVLKDSVTETLPRVNRATAISGYSFKQCSHQISACNKDIYV  
 DLDMKGINYNSSVAKSAQECQERCTDDVHCHFFTYATRQFPSLEHRNICLLKHTQTGT  
 PTRITKLDKVVSGFSLKSCALSNLACIRDIFPNTVFADSNIDSVMAPDAFVCGRICTH  
 5 HPGCLFFTFFSQEWPKESQRNLCLLKTSESGLPSTRIKSKALSGFSLQSCRHSIPVF  
 CHSSFYHDTDFLGEELDIVAAKSHEACQKLCTNAVRCQFFTYTPAQASCNEGKGKCYL  
 KLSSNGSPTKILHGRGGISGYTLRLCKMDNECTTKIKPRIVGGTASVRGEWPWQVTLH  
 TTSPTQRHLCGGSIIIGNQWILTAAHCFYGVESPKILRVYSGILNQSEIKEDTSFFGVQ  
 EIIIIHDQYKMAESGYDIALLKLETTVNYTDSQRPICLPSKGDRNVIYTDWVTGWGYR  
 10 KLRDKIQNTLQAKIPLVTNEECQKRYRGHKITHKMICAGYREGGKDACKGDSGGPLS  
 CKHNEVWHLVGITSWGEGCAQRERPGVYTNVVEYVDWILEKTQAV

**SEQ ID NO: 41**

Human prekallikrein:

15 MILEFKQATYFISLFATVSCGCLTQLYENAFFRGGDVASMYTPNAQYCMRCTFHPRCL  
 LFSFLPASSINDMEKRFGCFKDSVTGTLPKVHRTGAVSGHSLKQCGHQISACHRDIY  
 KGVDMRGVNFNVSKVSSVEECQKRCTNNIRCQFFSYATQTFHKAEYRNNCLLKYSPPG  
 TPTAIKVLSNVESGFSLKPCALSEIGCHMNIFQHAFSDVDVARVLTDAFVCRTICT  
 YHPNCLFFTFYTNVWKIESQRNVCLLKTSESGTPSSSTPQENTISGYSLLTCKRTLPE  
 20 PCHSKIYPGVDFGGEELNVTFVKGVNVCQETCTKMIRCQFFTYSLLPEDCKEEKCKCF  
 LRLSMDGSPTRIAYGTOGSSGYSLRLCNTGDNSVCTTKTSTRIVGGTNSSWGEWPWQV  
 SLQVKLTAQRHLCGGSILGHQWVLTAAHCFDGLPLQDVWRIYSGILNLSDITKDTFVS  
 QIKEIIIIHQNYKVSEGNHDIALIKLQAPLNYTEFQKPICLPSKGDTSTIYTNCWVTGW  
 GFSKEKGEIQNILQKVNIPLVTNEECQKRYQDYKITQRMVCAGYKEGGKDACKGDSGG  
 25 PLVCKHNGMWRLVGITSWGEGCARREQPGVYTKVAEYMDWILEKTQSSDGKAQMOSPA

**ACTIVATABLE LINKER SEQUENCES****SEQ ID NO: 42**

FXIIa-susceptible linker (Factor XI peptide):

30 CTTIKIPRIVGGTASVRGEWPWQVT

**SEQ ID NO: 43**

FXIIa-susceptible linker

GGGGSPRIGGGGS

**SEQ ID NO: 44**

FXIIa-susceptible linker (Prekallikrein peptide):

5 VCTTKTSTRIVGGTNSSWGEWPQVS

**EQ ID NO: 45**

FXIIa-susceptible linker (Prekallikrein peptide):

STRIVGG

10

**SIGNAL PEPTIDES**

**SEQ ID NO: 46**

Alpha mating factor (P01149):

15 MRFPSIFTAVLFAASSALAAPVNTTTEDETAQIPAEAVIGYSDLEGDFDVAVL PFSNS  
TNNGLLFINTTIIASIAAKEEGVS

**SEQ ID NO: 47**

Human Albumin Secretory Signal Peptide + Propeptide (P02768):

20 MKWVTFISLLFLFSSAYS RGVFRR

**SEQ ID NO: 48**

Human DNASE1L3 Signal Peptide (Q13609):

MSRELAPLLLLLLLSIHSALA

25

## WHAT IS CLAIMED IS:

1. A DNase1-like 3 (D1L3) variant, wherein the D1L3 protein variant has one or more of: increased resistance to proteolytic degradation; increased circulation half-life;  
5 higher production levels with in vitro expression systems; and not substantially less, the same, or better chromatin and/or NET-degrading activity in vitro; in each case as compared to wild-type D1L3 protein of SEQ ID NO: 4 and/or SEQ ID NO: 5.
2. The D1L3 variant of claim 1, wherein the D1L3 variant is a fusion protein  
10 comprising an amino acid sequence that is at least 80% identical to the mature enzyme defined by SEQ ID NO:4 or SEQ ID NO:5, an albumin amino acid sequence at the N-terminal side of the mature enzyme, and a linking amino acid sequence between the albumin amino acid sequence and the amino acid sequence of the mature enzyme.
- 15 3. The D1L3 variant of claim 2, wherein the D1L3 variant comprises an amino acid sequence that is at least 90% identical to the mature enzyme defined by SEQ ID NO:4 or SEQ ID NO:5.
4. The D1L3 variant of claim 2, wherein the D1L3 variant has a deletion of at least  
20 5 amino acids of the C-terminal basic domain, the C-terminal basic domain being defined by the 23 C-terminal amino acids of SEQ ID NO:4 or SEQ ID NO:5.
5. The D1L3 variant of claim 4, wherein the D1L3 has a deletion of at least 10 amino  
25 acids of the C-terminal basic domain.
6. The D1L3 variant of claim 4, wherein the D1L3 has a deletion of at least 15 amino acids of the C-terminal basic domain.
7. The D1L3 variant of claim 6, wherein the D1L3 has a deletion of the entire C-  
30 terminal basic domain.
8. The D1L3 variant of any one of claims 1 to 7, wherein the D1L3 has an amino acid substitution at the position corresponding to position 101 of SEQ ID NO:4.

9. The D1L3 variant of claim 8, wherein the D1L3 has an amino acid substitution that is Q101R.
10. The D1L3 variant of claim 2, wherein the linker is flexible, rigid, or comprises a  
5 protease cleavage site.
11. The D1L3 variant of claim 10, wherein the linker is a flexible linker.
12. The D1L3 variant of claim 10 or 11, wherein the linker has at least 10 amino  
10 acids, or at least 15 amino acids.
13. The D1L3 variant of claim 12, wherein the linker has from 15 to 35 amino acids.
14. The D1L3 variant of claim 1, wherein the variant comprises the amino acid  
15 sequence of any one of SEQ ID NOS: 8 to 30, in each case optionally having from one to twenty amino acid modifications independently selected from insertions, deletions, or substitutions.
15. The D1L3 variant of claim 14, wherein the variant has the amino acid sequence  
20 of SEQ ID NO: 19, SEQ ID NO: 22, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, or SEQ ID NO: 30.
16. The D1L3 variant of any one of claims 1 to 14, comprising one or more building  
block substitutions from D1, and/or one or more deletions or substitutions of cysteine  
25 residues as compared to the enzyme of SEQ ID NO: 4.
17. The D1L3 variant of claim 16, wherein the amino acid corresponding to C68 of  
SEQ ID NO: 4 is substituted.
- 30 18. The D1L3 variant of claim 17, wherein the amino acid corresponding to C68 of  
SEQ ID NO: 4 is substituted with an amino acid selected from Ala, Ser, and Gly.
19. The D1L3 variant of claim 16, wherein the variant comprises the substitution  
N64\_I70delinsHLTAVGK, which is optionally modified by one, two, or three amino

acid substitutions, deletions, or insertions, with the proviso that the building block substitution does not modified to include a cysteine residue.

20. The D1L3 variant of claim 1, comprising a PEG conjugation to the amino acid  
5 corresponding to C68 and/or C194.

21. The D1L3 variant of claim 1 or 2, having one or more substitutions of arginine  
and lysine residues that are present in SEQ ID NO: 4 and/or SEQ ID NO: 5, wherein the  
substitution renders the D1L3 variant less sensitive to proteolysis by plasmin, thrombin,  
10 and/or trypsin, as compared to the protein of SEQ ID NO: 4 or the protein of SEQ ID  
NO: 5.

22. The D1L3 variant of claim 21, wherein one or more of the arginine and lysine  
substitutions correspond to the following position(s) of SEQ ID NO: 4: K180, K200,  
15 K259, and R285.

23. The D1L3 variant of claim 22, wherein the arginine or lysine substitution is  
selected from one or more of K180A, K200A, K259A, and R285A with respect to SEQ  
ID NO: 4.  
20

24. The D1L3 variant of claim 22, wherein the variant comprises a substitution  
selected from: K180\_A181delinsGL, P198\_A201delinsRPSQ, and K259A with respect  
to SEQ ID NO: 4.

25. The D1L3 variant of claim 21, wherein the variant comprises one or more  
mutations of a paired basic residue, the paired basic residue corresponding to a position  
selected from K50/R51, R80/R81, K114/R115, K199/K200, K226/K227, K291/K292,  
R297/K298/K299, and K303/R304 of SEQ ID NO:4.

26. The D1L3 variant of claim 25, wherein the one or more mutations of a paired  
basic residue include an amino acid substitution selected from R114T, R114A, R114D,  
R114Q, K227S, and K227E with respect to SEQ ID NO: 4.  
30

27. The D1L3 variant of claim 25, wherein the one or more mutations of a paired basic residue include an amino acid substitution selected from R51K, R81K, R115K, and R304K with respect to SEQ ID NO: 4.
- 5 28. The D1L3 variant of claim 10, wherein the linker is cleavable by a coagulation pathway protease.
29. The D1L3 variant of claim 28, wherein the linker is cleavable by Factor XII or a neutrophil protease.
- 10 30. A DNase 1 (D1) variant comprising an amino acid sequence that is at least 80% identical to the enzyme defined by SEQ ID NO: 1, with one or more deletions or substitutions of cysteine residues.
- 15 31. The D1 variant of claim 30, wherein one or more amino acids corresponding to C123 and C126 of SEQ ID NO:1 are deleted or substituted.
32. The D1 variant of claim 31, wherein the cysteine(s) is/are substituted with an amino acid independently selected from Ala, Ser, and Gly.
- 20 33. The D1 variant of claim 30, wherein the variant comprises the substitution G122\_N128delinsYQGDA.
34. The D1 variant of any one of claims 30 to 33, wherein the D1 variant is a fusion  
25 protein comprising an albumin amino acid sequence and optionally a peptide linker.
35. The D1 variant of claim 34, wherein the albumin domain of the fusion protein is located at the N-terminal side of the D1 domain.
- 30 36. The D1 variant protein of claim 34 or 35, wherein a peptide linker is interposed between the albumin domain and the D1 domain.
37. The D1 variant of claim 36, wherein the peptide linker is a flexible linker, a rigid linker, or a protease-cleavable linker.

38. The D1 variant of claim 37, wherein the linker has at least 10 amino acids, or at least 15 amino acids.
- 5 39. The D1 variant of claim 37, wherein the linker has from 15 to 35 amino acids.
40. The D1 variant of any one of claims 34 to 39, wherein the D1 domain of the fusion protein comprises an amino acid sequence that is at least 80% identical to the mature enzyme defined by SEQ ID NO:4 or SEQ ID NO:5.
- 10 41. The D1 variant of claim 40, wherein the D1 variant has DNA-degrading activity that at least 80% as compared to the wild-type D1 protein of SEQ ID NO: 1.
42. The D1 variant of claim 40 or 41, wherein the D1 variant comprises an amino acid sequence that is at least 85%, or at least 90%, or at least 95%, or at least 98% identical to the enzyme of SEQ ID NO:1.
- 15 43. A D1 variant comprising an amino acid sequence that is at least 80% identical to the enzyme defined by SEQ ID NO: 1, and having a PEG conjugation to the amino acid corresponding to C123 and/or C126.
- 20 44. A DNase1-like 1 (D1L1) variant comprising an amino acid sequence that is at least 80% identical to the enzyme defined by SEQ ID NO: 2, with one or more deleted or substituted cysteine residues.
- 25 45. The D1L1 variant of claim 44, wherein at least one substituted or deleted cysteine residue is the position corresponding to C22 or C50 of SEQ ID NO:2.
46. The D1L1 variant of claim 45, wherein one or more of the substituted cysteine residues is substituted by an amino acid that is Gly, Arg, or Ser.
- 30 47. The D1L1 variant of any one of claims 44 to 46, wherein the D1L1 variant comprises an amino acid sequence that is at least 85%, or at least 90%, or at least 95%, or at least 98% identical to the enzyme of SEQ ID NO: 2.

48. The D1L1 variant of any one of claims 44 to 47, wherein the variant is a fusion protein comprising an albumin amino acid sequence, and optionally a peptide linker between a D1L1 domain and an albumin domain.

5

49. The D1L1 variant of claim 48, wherein the albumin domain is located to the N-terminal side of the D1L1 domain.

50. The D1L1 variant of claim 48 or 49, wherein the fusion protein comprises a peptide linker, wherein the peptide linker is a flexible linker, a rigid linker, or a protease-cleavable linker.

51. A D1L1 variant comprising an amino acid sequence that is at least 80% identical to the enzyme defined by SEQ ID NO: 2, and having a PEG conjugation to the amino acid corresponding to C22 and/or C50.

15

52. A DNase1-like 2 (D1L2) variant comprising an amino acid sequence that is at least 80% identical to the enzyme defined by SEQ ID NO: 3, with one or more substituted or deleted cysteine residues.

20

53. The D1L2 variant of claim 52, wherein the cysteine residue corresponding to C43 of SEQ ID NO: 3 is substituted or deleted.

54. The D1L2 variant of claim 52 or 53, wherein one or more of the substituted cysteine residues is substituted by an amino acid that is Gly, Arg, or Ser.

25

55. The D1L2 variant of any one of claims 52 to 54, wherein the D1L2 variant comprises an amino acid sequence that is at least 85%, or at least 90%, or at least 95%, or at least 98% identical to the enzyme of SEQ ID NO: 2.

30

56. The D1L2 variant of any one of claims 52 to 55, wherein the variant is a fusion protein comprising an albumin amino acid sequence, and optionally a peptide linker between a D1L2 domain and an albumin domain.

57. The D1L2 variant of claim 56, wherein the albumin domain is located to the N-terminal side of the D1L2 domain.
58. The D1L2 variant of claim 56 or 57, wherein a peptide linker is interposed  
5 between the albumin domain and the D1L2 domain.
59. The D1L2 variant of claim 58, wherein the peptide linker is a flexible linker, a rigid linker, or a protease-cleavable linker.
- 10 60. A D1L2 variant comprising an amino acid sequence that is at least 80% identical to the enzyme defined by SEQ ID NO: 3, and having a PEG conjugation to the amino acid corresponding to C43.
61. An isolated polynucleotide encoding the D1, D1L1, D1L2, or D1L3 variant of  
15 any one of claims 1 to 60.
62. The isolated polynucleotide of claim 61, wherein the polynucleotide is an mRNA.
63. The isolated polynucleotide of claim 61, wherein the polynucleotide is DNA.  
20
64. A vector comprising the polynucleotide of any one of claims 61 to 63.
65. A host cell comprising the vector of claim 64.
- 25 66. A host cell modified to express D1, D1L1, D1L2, or D1L3 variant of any one of claim 1 to 60.
67. A pharmaceutical composition comprising the D1, D1L1, D1L2, or D1L3 variant of any one of claims 1 to 60, the polynucleotide according to any one of claims 61 to 63,  
30 the vector according to claim 64, or the host cell according to claim 65 or 66, and a pharmaceutically acceptable carrier.
68. The pharmaceutical composition of claim 67, formulated for topical, parenteral, or pulmonary administration.

69. The pharmaceutical composition of claim 68, formulated for intradermal, intramuscular, intraperitoneal, intraarticular, intravenous, subcutaneous, intraarterial, oral, sublingual, pulmonary, or transdermal administration.

5

70. A method for treating a subject in need of extracellular DNA degradation, extracellular chromatin degradation, extracellular trap (ET) degradation and/or neutrophil extracellular trap (NET) degradation, the method comprising administering a therapeutically effective amount of a composition of any one of claims 67 to 69.

10

71. The method of claim 70, wherein the subject has a loss of function mutation in the D1L3 gene.

72. The method of claim 70 or 71, wherein the subject has a condition selected from chronic neutrophilia, neutrophil aggregation or leukostasis, thrombosis or vascular occlusion, ischemia-reperfusion injury, surgical or traumatic tissue injury, an acute or chronic inflammatory reaction or disease, an autoimmune disease, cardiovascular disease, metabolic disease, systemic inflammation, inflammatory disease of the respiratory tract, renal inflammatory disease, inflammatory disease related to transplanted tissue and cancer.

20

73. The method of claim 70 or 71, wherein the subject has, or is at risk of, NETs occluding ductal systems, wherein the condition is optionally selected from pancreatitis, cholangitis, conjunctivitis, mastitis, dry eye disease, obstructions of vas deferens, and renal disease.

25

74. The method of claim 70 or 71, wherein the subject has, or is at risk of, NETs accumulating on endothelial surfaces.

75. The method of claim any one of claims 70 to 74, wherein the D1L3 variant is a fusion protein comprising an albumin amino acid sequence, and which is parenterally administered to the subject, wherein the administration is approximately weekly.

30

76. A method for producing a recombinant DNase variant of any one of claims 1 to 60, comprising culturing cells expressing a polynucleotide encoding the DNase variant, and recovering the recombinant protein.
- 5 77. The method of claim 76, wherein the cells are *Pichia pastoris*.
78. The method of claim 77, wherein the *Pichia pastoris* cells have a genetic deletion or inactivation, or pharmacological inhibition of, Aspartic proteinase 3 and/or Kexin.
- 10 79. The method of claim 76, wherein the cells are CHO cells.
80. The method of claim 79, wherein the CHO cells have a genetic deletion or inactivation, or pharmacological inhibition of, Furin protease.
- 15 81. The method of any one of claims 76 to 80, wherein the DNase variant is a D1L3 variant comprising one or more mutations of a paired basic residue, the paired basic residue corresponding to a position selected from K50/R51, R80/R81, K114/R115, K199/K200, K226/K227, K291/K292, R297/K298/K299, and K303/R304 of SEQ ID NO:4.
- 20 82. The method of claim 81, wherein the one or more mutations of a paired basic residue include an amino acid substitution selected from R114T, R114A, R114D, R114Q, K227S, and K227E.
- 25 83. The method of claim 81, wherein the one or more mutations of a paired basic residue include an amino acid substitution selected from R51K, R81K, R115K, and R304K.
84. A method for producing a recombinant D1 protein family member of variant thereof, comprising culturing cells expressing a polynucleotide encoding the DNase variant in the presence of a polyanion compound, and recovering the recombinant protein.
- 30

85. The method of claim 84, wherein the recombinant D1 protein family member is a variant of D1, D1L3, D1L1, or D1L2.
86. The method of claim 84 or 85, wherein the polyanion is selected from one or  
5 more of dextran sulfate, heparins, ferric citrate, and EDTA.
87. The method of claim 86, wherein the polyanion is dextran sulfate.
88. The method of any one of claims 84 to 87, wherein the cells are prokaryotic or  
10 eukaryotic.
89. The method of claim 88, wherein the DNase is expressed using a non-mammalian expression system, which is optionally *Pichia pastoris*, *Saccharomyces spp*, or *Escherichia coli*.
- 15 90. The method of claim 89, wherein the expression system is *Pichia pastoris*.
91. The method of claim 90, wherein the *Pichia pastoris* cells have a genetic deletion or inactivation, or pharmacological inhibition of, Aspartic proteinase 3 and/or Kexin.
- 20 92. The method of claim 88, wherein the cells are CHO cells.
93. The method of claim 92, wherein the CHO cells have a genetic deletion or inactivation, or pharmacological inhibition of, Furin protease.
- 25 94. The method of any one of claims 84 to 93, wherein the recombinant D1 family member comprises a fusion of albumin at the N-terminus through a peptide linker.
95. The method of claim 94, wherein the linker is a flexible linker.
- 30 96. The method of claim 95, wherein the linker is a cleavable linker, optionally cleavable by a coagulation pathway protease or a neutrophil protease.
97. The method of claim 96, wherein the coagulation factor protease is Factor XII.

98. The method of claim 97, wherein the peptide linker is selected from all or part of the FXII-cleavage site in Factor XI or prekallikrein.

5 99. The method of any one of claims 84 to 98, wherein the recombinant D1 family member or variant is expressed with a construct that encodes a signal peptide directing secretion of the recombinant protein from the cells.

100. The method of any one of claims 84 to 99, wherein the recombinant D1 family  
10 member or variant is purified from the polyanion compound using an anion exchange resin.

101. The method of claim 100, wherein the recombinant D1 family member or variant is further purified using a cation exchange resin.

15

FIG. 1

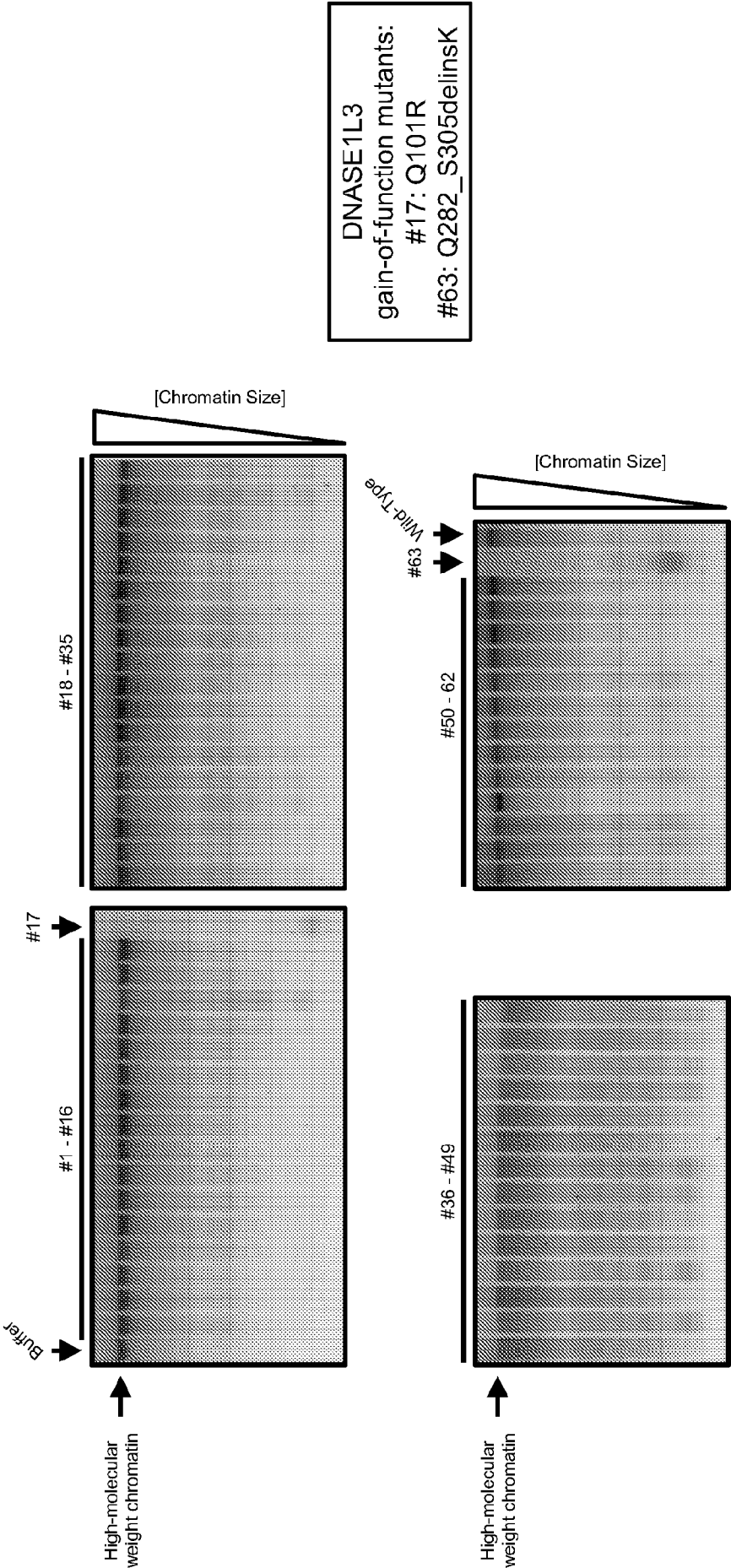


FIG. 2

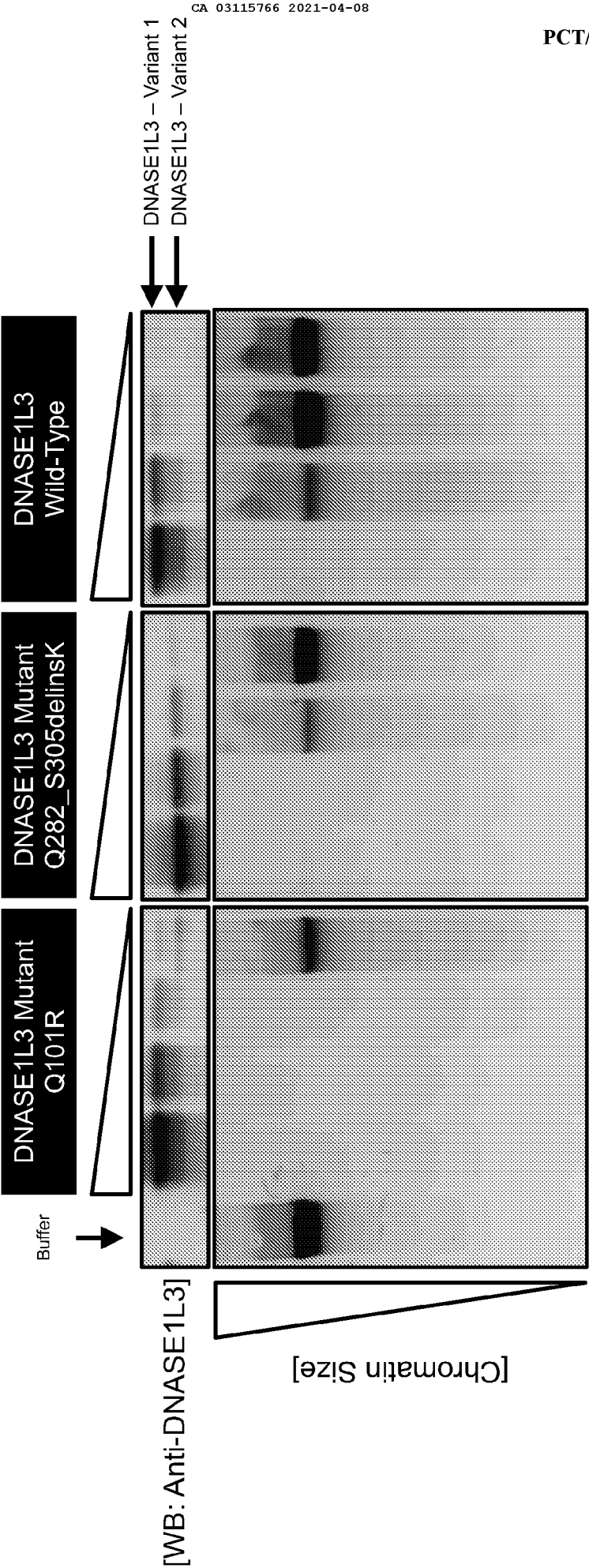


FIG. 3

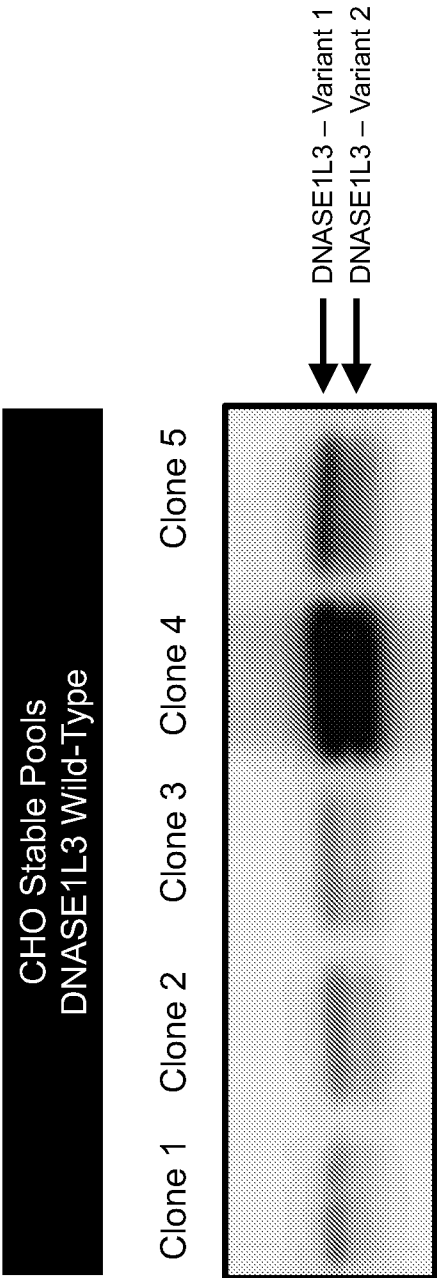
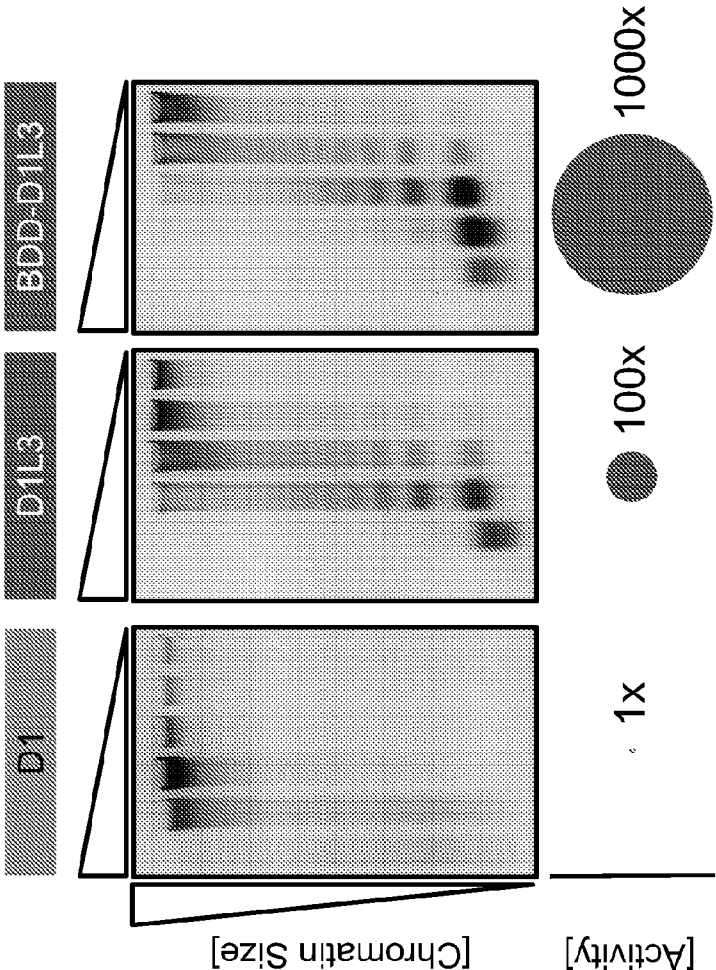
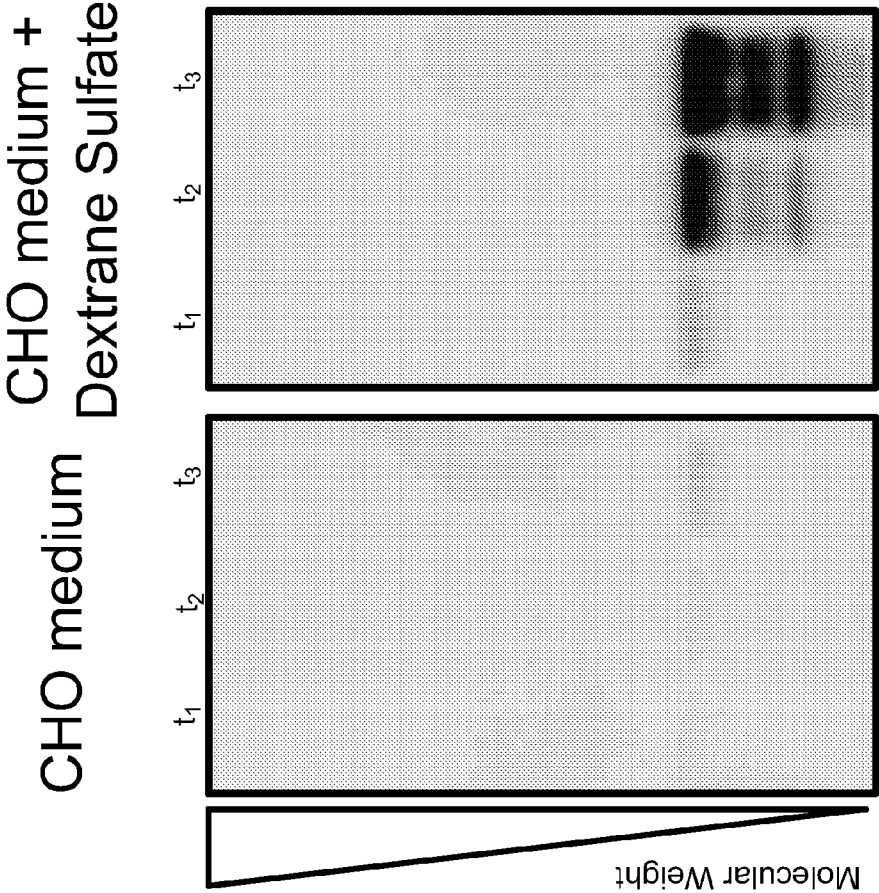


FIG. 4

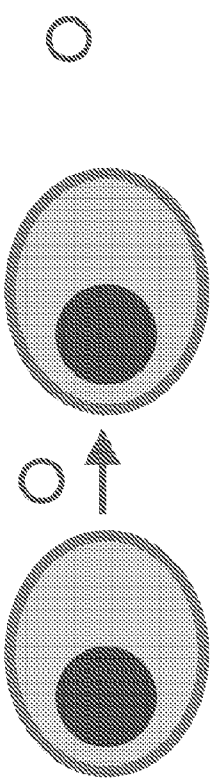


| Input cDNA                    | C-Terminal Amino Acid Sequence | Relative Amount |
|-------------------------------|--------------------------------|-----------------|
|                               | K291_S305del                   | 54%             |
|                               | K292_S305del                   | 17%             |
|                               | S293_S305del                   | 29%             |
| Wild-Type DNASE1L3            |                                |                 |
| Basic Domain Deleted DNASE1L3 | F275Y                          |                 |
|                               | F279_K280delinsVM              |                 |
|                               | Q282_S305delinsK               | 100%            |

FIG. 5



**cGMP-Expression System:**  
*Chinese Hamster Ovary Cells*



**Expression Vector:**

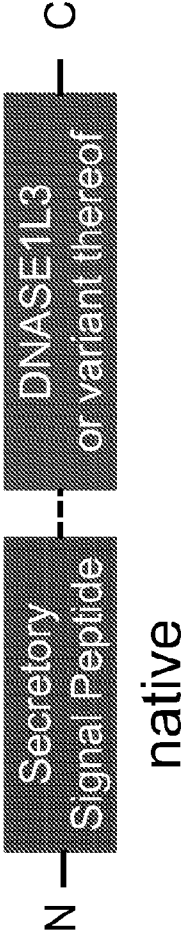
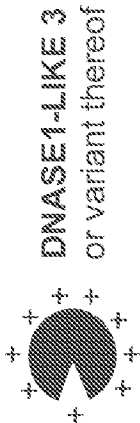
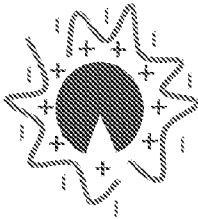


FIG. 6  
Scavenging of DNASE1L3 with polyanions



A

POLYANION  
[e.g. Dextrane Sulfate (DS)]

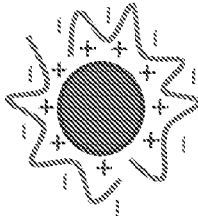


Formation of  
D1L3-DS complex

STEP 1

B

ANION EXCHANGE SURFACE  
(e.g. resin)

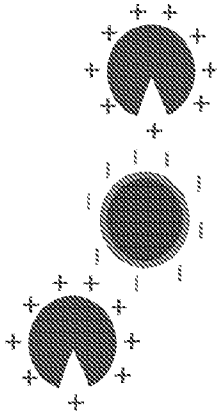


Dissociation of  
D1L3-DS complex

STEP 2

C

CATION EXCHANGE SURFACE  
(e.g. resin)



Affinity purification  
of DS-free D1L3

STEP 3

# FIG. 7

**Mutation of identified trypsin cleavage sites**

| #  | AA in D1L3<br>(SEQ ID NO: 4) | Amino Acid<br>Substitution | D1 (SEQ ID NO: 1)<br>Building Block Mutation |
|----|------------------------------|----------------------------|----------------------------------------------|
| 1  | R22                          | R22A/V/S, R22H/Q/E         | M21_R22delinsLK                              |
| 2  | R29                          | R29A/V/S, R29H/Q/E         | V28_S30delinsIQT                             |
| 3  | R51                          | R51A/V/S, R51H/Q/E         | N/A (conserved residue)                      |
| 4  | R66                          | R66A/V/S, R66H/Q/E         | N64_I70delinsHLTAVGK                         |
| 5  | R80                          | R80A/V/S, R80H/Q/E         | R77_I83delinsQDAPD                           |
| 6  | R81                          | R81A/V/S, R81H/Q/E         | R77_I83delinsQDAPD                           |
| 7  | R95                          | R95A/V/S, R95H/Q/E         | N/A (conserved residue)                      |
| 8  | K99                          | R99A/V/S, R99H/Q/E         | N/A (conserved residue)                      |
| 9  | R115                         | R115A/V/S, R115H/Q/E       | V113_R115delinsAVD                           |
| 10 | K147                         | K147A/V/S, K147H/Q/E       | K147_D148delinsRE                            |
| 11 | K163                         | K163A/V/S, K163H/Q/E       | K163A                                        |
| 12 | K180                         | K180A/V/S, K180H/Q/E       | K180_A181delinsGL                            |
| 11 | R208                         | R208A/V/S, R208H/Q/E       | R208W                                        |
| 12 | R212                         | R212A/V/S, R212H/Q/E       | R212T                                        |
| 13 | R235                         | K235A/V/S, K235H/Q/E       | N/A (conserved residue)                      |
| 14 | R239                         | K239A/V/S, K239H/Q/E       | L238_R239delinsVA                            |
| 15 | K250                         | K250A/V/S, K250H/Q/E       | K250D                                        |
| 16 | K262                         | K262A/V/S, K262H/Q/E       | K262G                                        |

Selection of Amino  
Acid Substitution:

**Grantham's distance**

Arg (R) / His (H): 29

Arg (R) / Glu (E): 54

Arg (R) / Gln (Q): 53

Lys (K) / His (E): 32

Lys (K) / Gln (H): 54

Lys (K): Glu (E): 56

FIG. 8

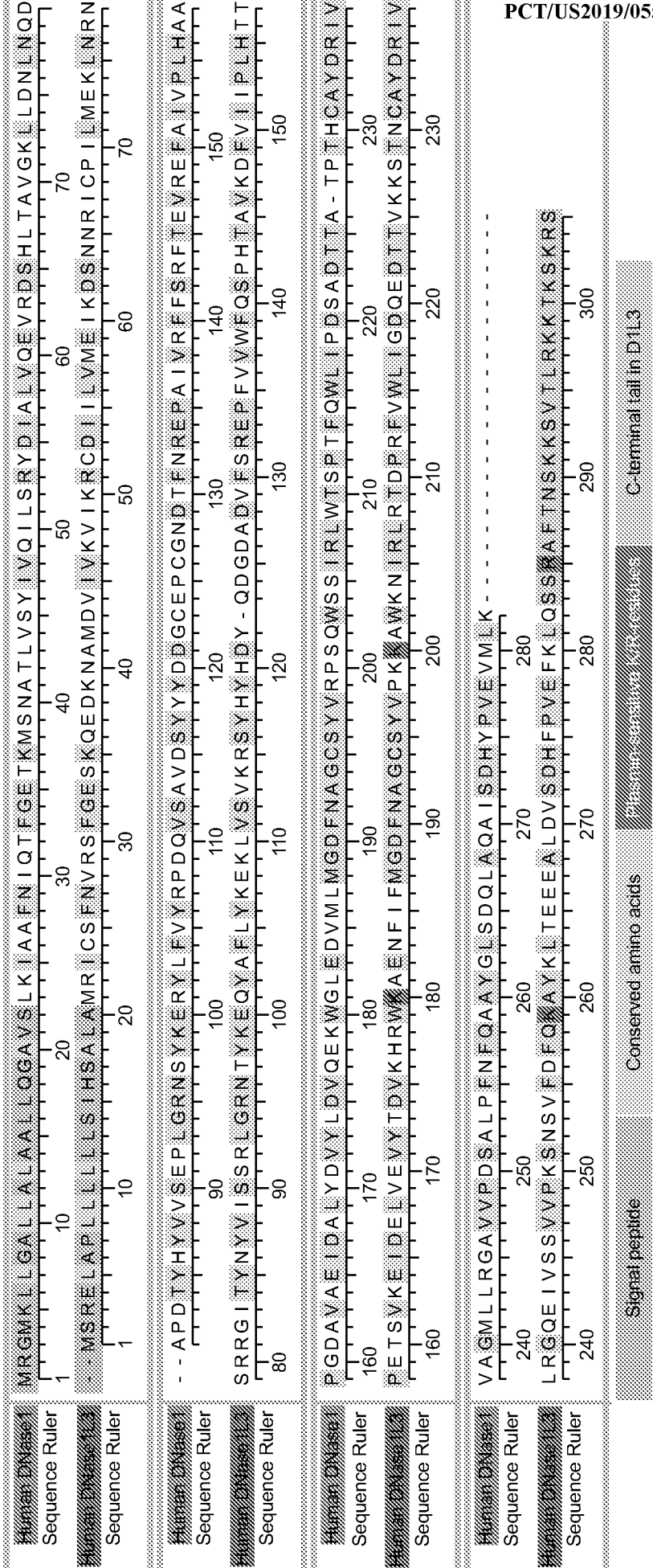
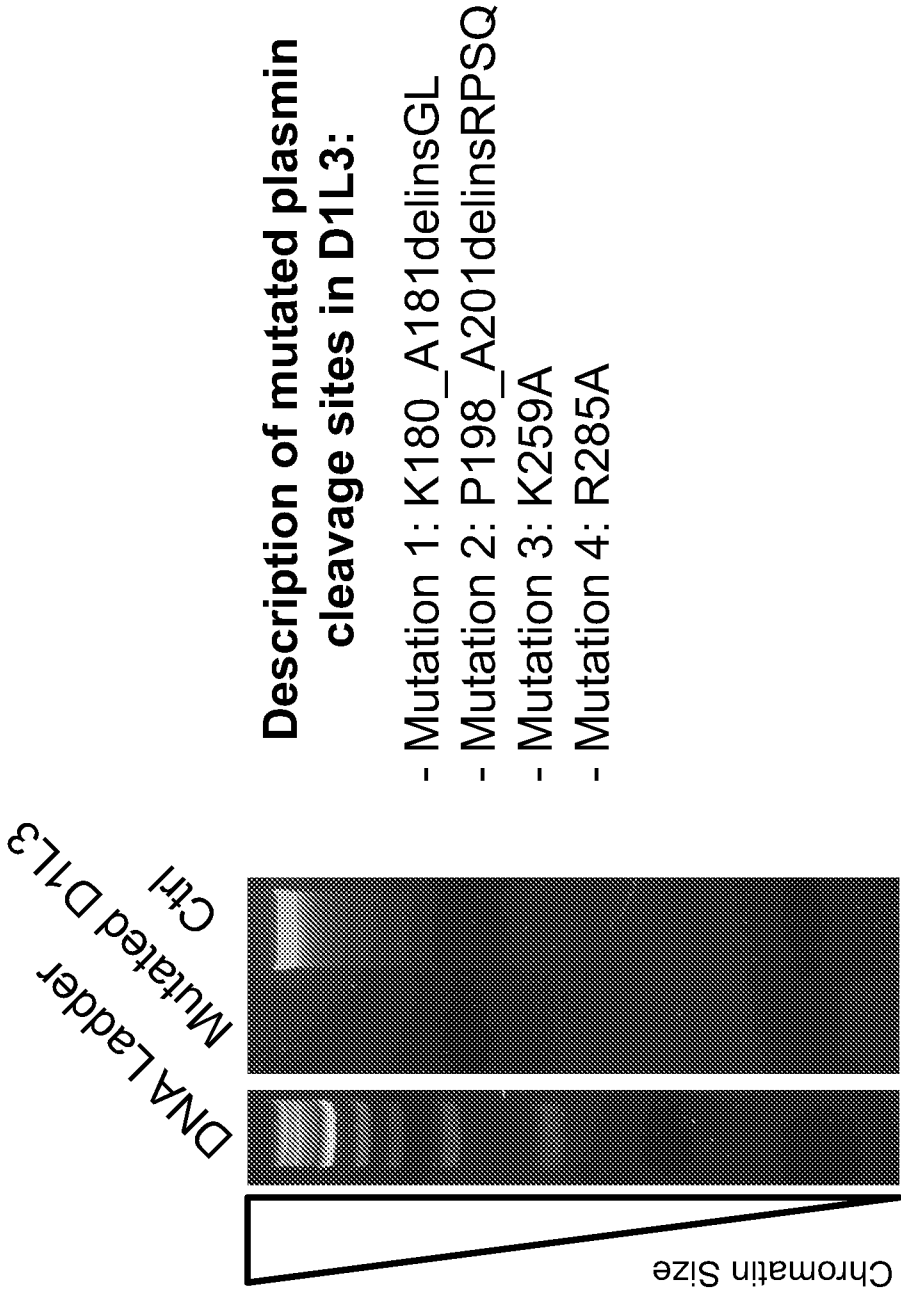


FIG. 9

Mutation of predicted plasmin cleavage sties

| # | AA in D1L3<br>(SEQ ID NO: 4) | Amino Acid<br>Substitution | D1 (SEQ ID NO: 1)<br>Building Block Mutation |
|---|------------------------------|----------------------------|----------------------------------------------|
| 1 | K180                         | K180A, K180H/Q/E           | K180_A181delinsGL                            |
| 2 | K200                         | K200A, K200H/Q/E           | P198_A201delinsRPSQ                          |
| 3 | K259                         | K259A, K259H/Q/E           | A259A                                        |
| 4 | R285                         | R285A, R285H/Q/E           | N/A (absent in D1)                           |

FIG. 10



# FIG. 11

Mutation of identified plasmin cleavage sites

| #  | AA in D1L3<br>(SEQ ID NO: 4) | Amino Acid<br>Substitution | D1 (SEQ ID NO: 1)<br>Building Block Mutation |
|----|------------------------------|----------------------------|----------------------------------------------|
| 1  | R22                          | R22H/Q/E                   | M21_R22delinsLK                              |
| 2  | R29                          | R29H/Q/E                   | V28_S30delinsIQT                             |
| 3  | K45                          | K45H/Q/E                   | N/A (conserved residue)                      |
| 4  | K47                          | K47H/Q/E                   | K47_K50delinsQILS                            |
| 5  | K74                          | K74H/Q/E                   | M72_K74delinsLDN                             |
| 6  | R81                          | R81H/Q/E                   | R77_I83delinsQDAPD                           |
| 7  | R92                          | R92H/Q/E                   | S91_R92delinsEP                              |
| 8  | K107                         | K107H/Q/E                  | K107_L110delinsRPDQ                          |
| 9  | K176                         | K176H/Q/E                  | K176_R178delinsQEK                           |
| 10 | R212                         | R212H/Q/E                  | R212T                                        |
| 11 | K226                         | R226H/Q/E                  | V225_S228delinsATP                           |
| 12 | K227                         | K227H/Q/E                  | V225_S228delinsATP                           |
| 13 | K250                         | K250H/Q/E                  | K250D                                        |
| 14 | K259                         | K259H/Q/E                  | K259A                                        |
| 15 | K262                         | K262H/Q/E                  | K262G                                        |

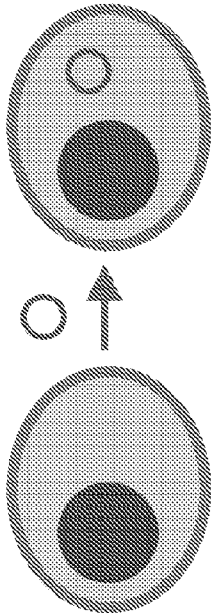
Selection of Amino  
Acid Substitution:  
**Grantham's distance**

Arg (R) / His (H): 29  
Arg (R) / Glu (E): 54  
Arg (R) / Gln (Q): 53

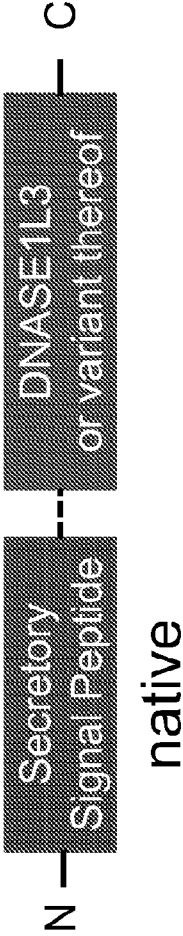
Lys (K) / His (E): 32  
Lys (K) / Gln (H): 54  
Lys (K): Glu (E): 56

FIG. 12

**A** **cGMP-Expression System:**  
*Chinese Hamster Ovary Cells*



**Expression Vector:**



**B**

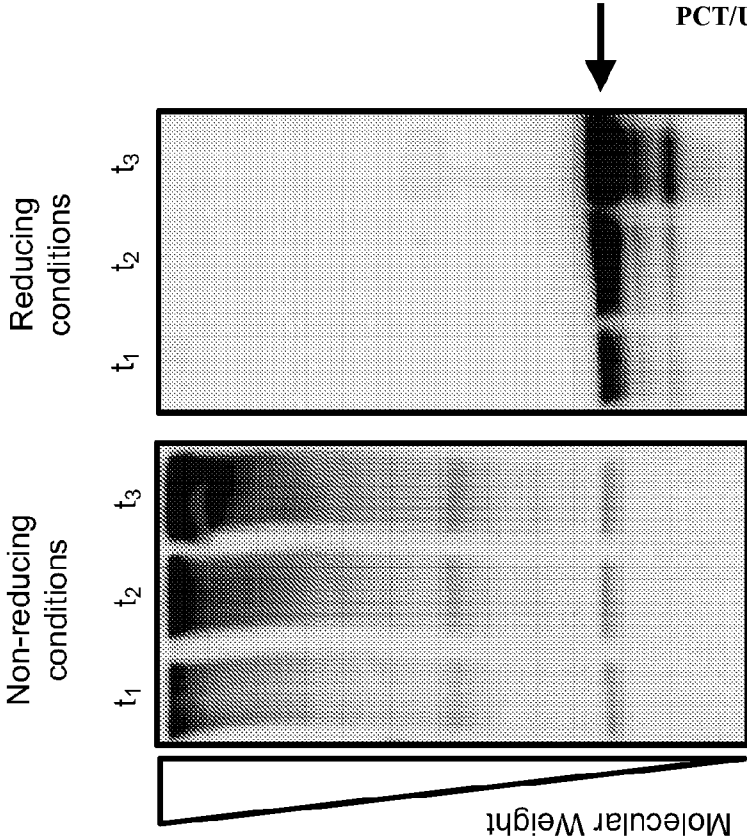


FIG. 13

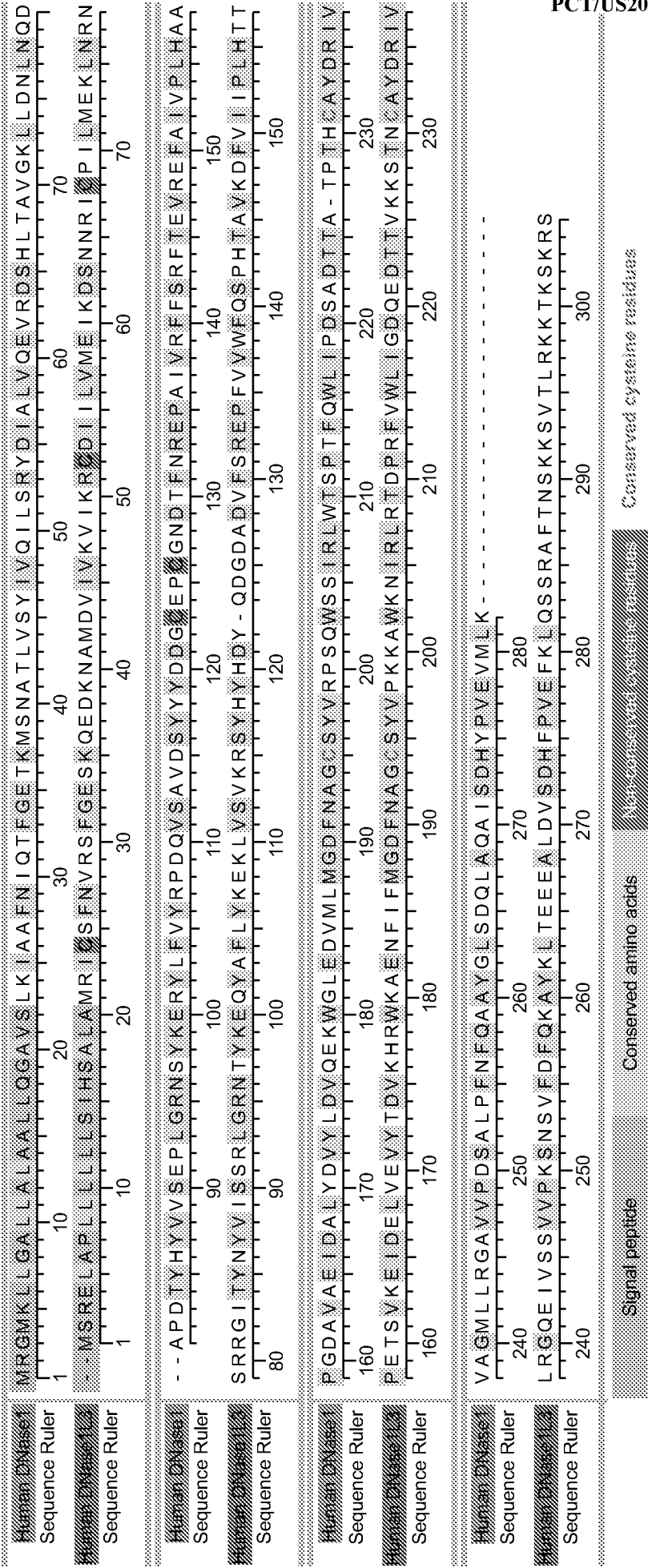


FIG. 14

Mutation of identified plasmin cleavage sites

| # | AA in D1L3<br>(SEQ ID NO: 4) | Amino Acid<br>Substitution | D1 (SEQ ID NO: 1)<br>Building Block Mutation |
|---|------------------------------|----------------------------|----------------------------------------------|
| 1 | C24                          | C24A/S/G                   | C24_S25delinsAA                              |
| 2 | C52                          | C52A/S/G                   | C52Y                                         |
| 3 | C68                          | C68A/S/G                   | N64_I70delinsHLTAVGK                         |
| 4 | C194                         | C194A/S/G                  | N/A (conserved residue)                      |
| 5 | C231                         | C231A/S/G                  | N/A (conserved residue)                      |

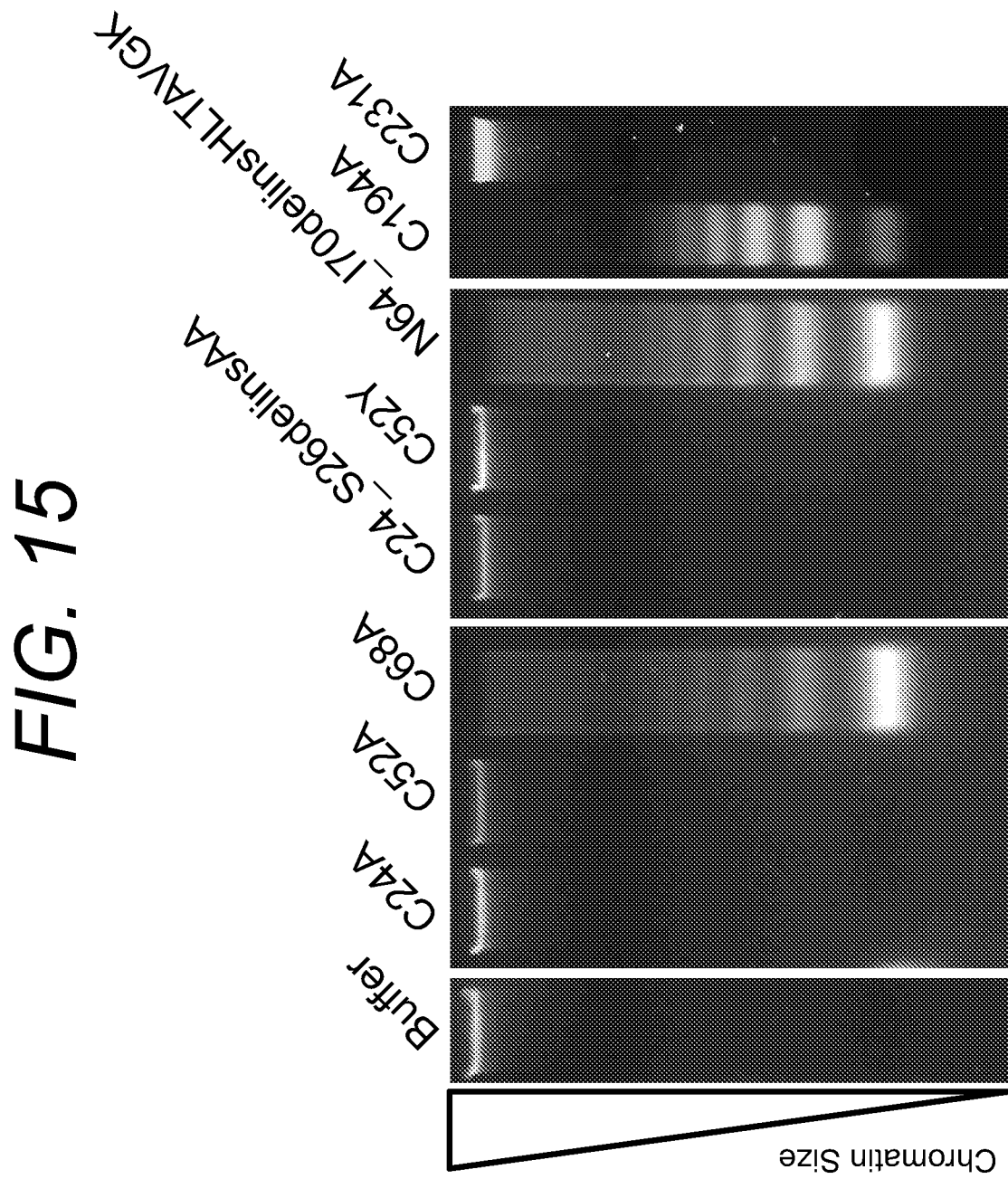


FIG. 16

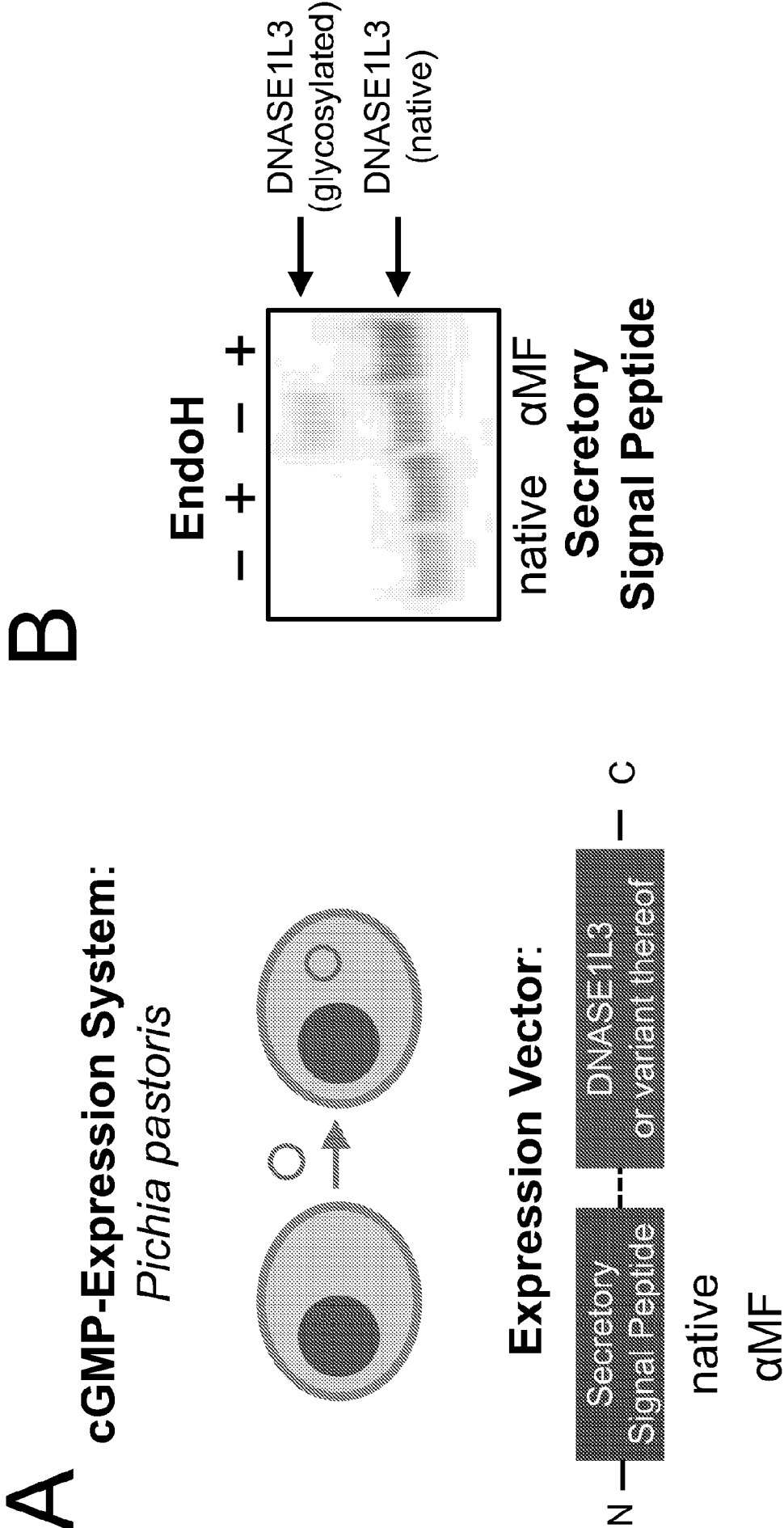


FIG. 17

A  
cGMP-Expression System:  
*Pichia pastoris*

Expression Vector:

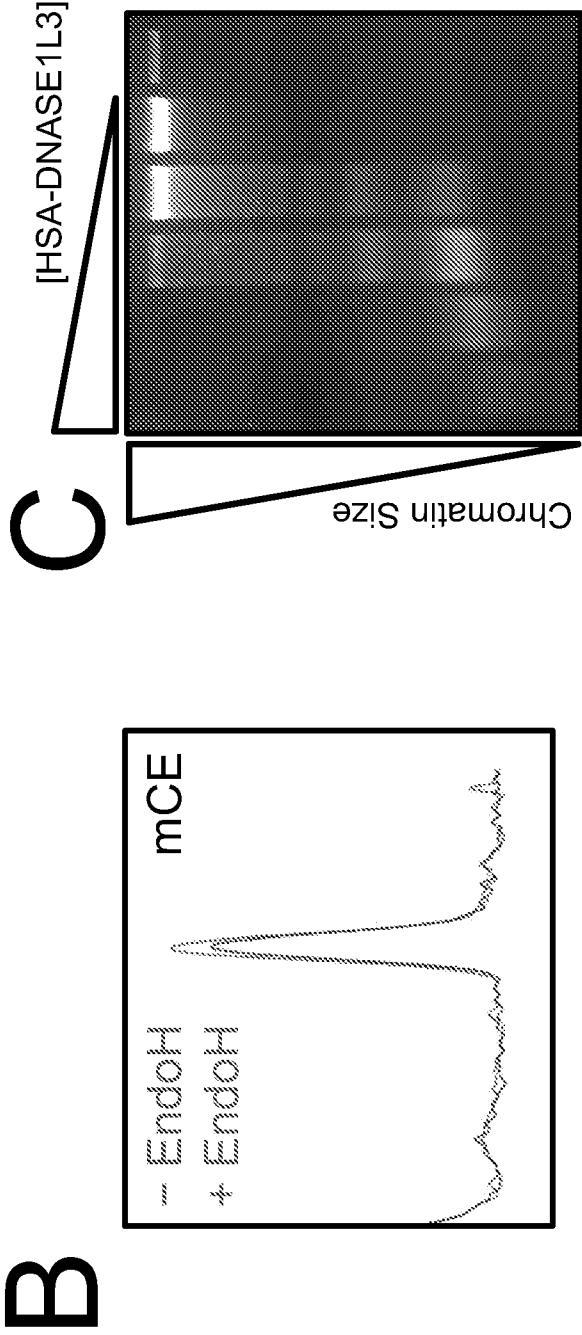
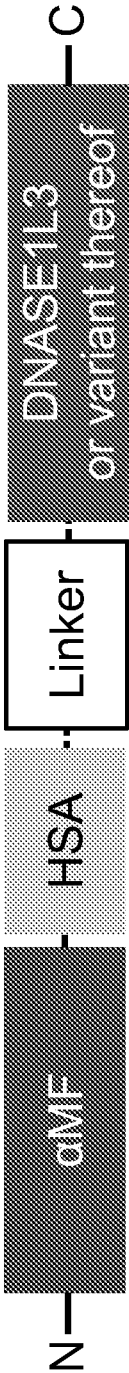


FIG. 18

|               | Expression Vector |          |                | Expression Level (rel. Units) |
|---------------|-------------------|----------|----------------|-------------------------------|
| SEQ ID NO: 14 |                   | BDD-D1L3 |                | 4.3±0.5                       |
| SEQ ID NO: 17 | HSA               | -        | BDD-D1L3       | 4.3±0.4                       |
| SEQ ID NO: 18 | HSA               | L1       | BDD-D1L3       | 12±1.9                        |
| SEQ ID NO: 19 | HSA               | L2       | BDD-D1L3       | 32±3.2                        |
| SEQ ID NO: 20 |                   |          | L1<br>BDD-D1L3 | <1                            |
| SEQ ID NO: 21 |                   |          | L2<br>BDD-D1L3 | 1.8±0.2                       |
| SEQ ID NO: 4  |                   |          | D1L3           | 1.1±0.1                       |
| SEQ ID NO: 22 | HSA               | L2       | D1L3           | 22±1.3                        |

---

|               | Linker | Sequence             | Length |
|---------------|--------|----------------------|--------|
| SEQ ID NO: 31 | L1     | GGGGS                | 5 AA   |
| SEQ ID NO: 32 | L2     | (GGGGS) <sub>3</sub> | 15 AA  |

# FIG. 19

| SEQ ID NO: 22 | Expression Vector |    |      | Expression Level (rel. Units) |
|---------------|-------------------|----|------|-------------------------------|
|               | HSA               | L2 | D1L3 |                               |
|               | HSA               | L3 | D1L3 |                               |
|               | HSA               | L4 | D1L3 |                               |
| SEQ ID NO: 23 |                   |    |      | 17±5.5                        |
| SEQ ID NO: 24 |                   |    |      | 22±6.9                        |
| SEQ ID NO: 24 |                   |    |      | 11±4.9                        |

---

| SEQ ID NO: 32 | Linker | Sequence                | Length | Property |
|---------------|--------|-------------------------|--------|----------|
|               | L2     | (GGGGS) <sub>3</sub>    | 15     | flexible |
|               | L3     | (AP) <sub>7</sub>       | 14     | rigid    |
|               | L4     | A(EAAAK) <sub>2</sub> A | 12     | rigid    |

FIG. 20

| Expression Vector |     |    | Linker   |               | Sequence | Length                  |    |
|-------------------|-----|----|----------|---------------|----------|-------------------------|----|
| SEQ ID NO: 25     | HSA | L5 | BDD-D1L3 | SEQ ID NO: 35 | L5       | SGSGSS                  | 7  |
| SEQ ID NO: 26     | HSA | L6 | BDD-D1L3 | SEQ ID NO: 36 | L6       | S(GGS) <sub>4</sub> GSS | 16 |
| SEQ ID NO: 27     | HSA | L7 | BDD-D1L3 | SEQ ID NO: 37 | L7       | S(GGS) <sub>9</sub> GSS | 31 |

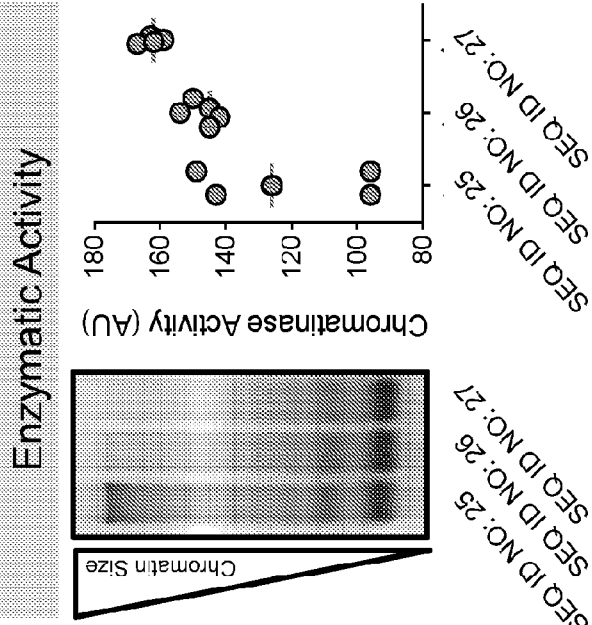
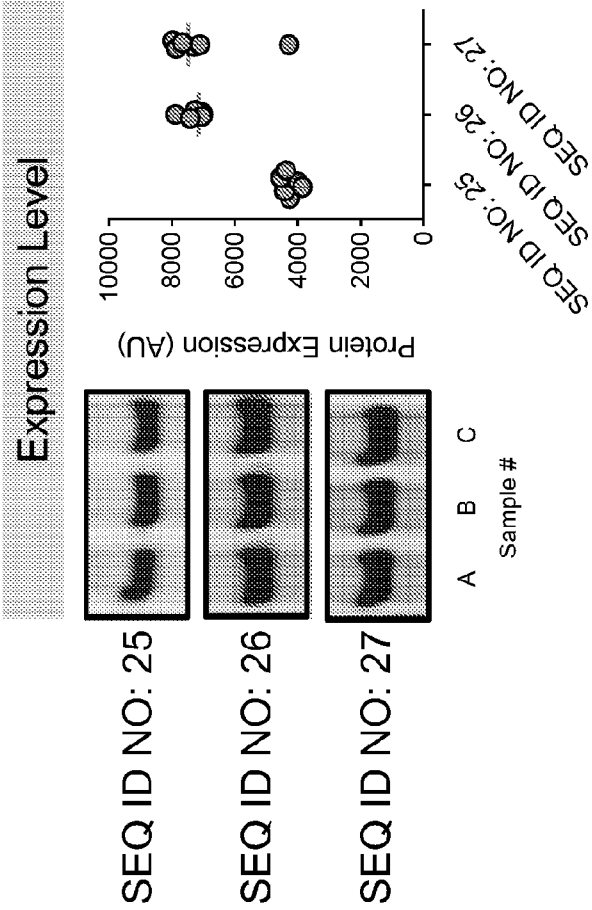
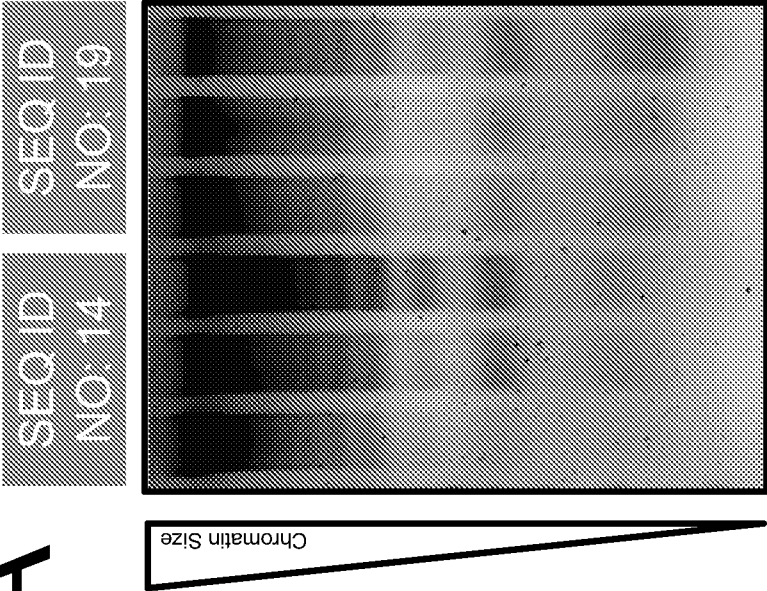
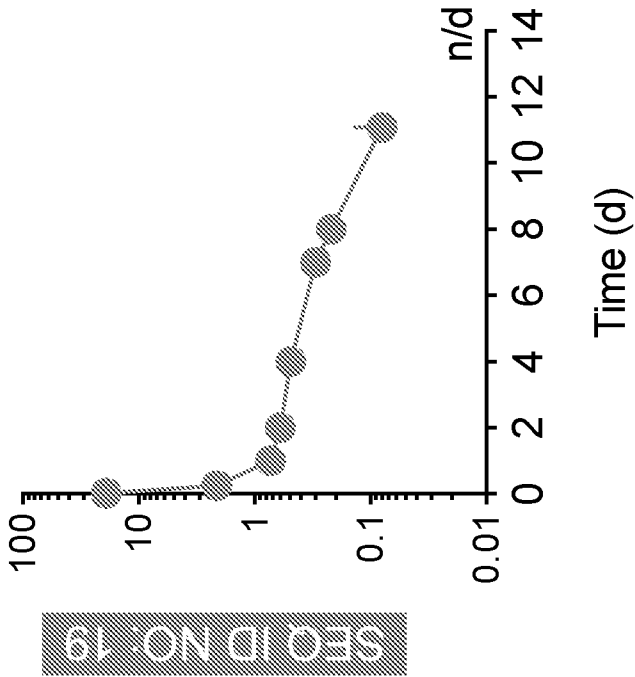


FIG. 21

A



B



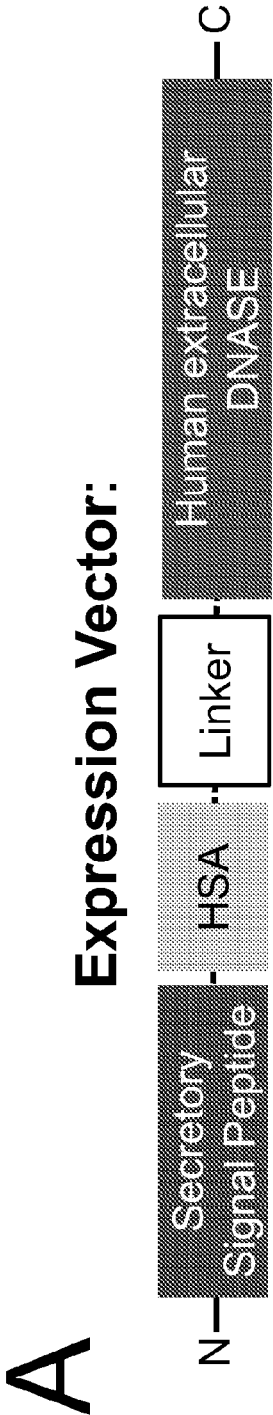
$t_{\max}$  = 5 Minutes  
 $C_{\max}$  = 19  $\mu\text{g/ml}$   
 $t_{1/2}$  = 3.3 Days

FIG. 22

| Expression Vector |     |    |          | Expression Level (rel. Units) |
|-------------------|-----|----|----------|-------------------------------|
| SEQ ID NO: 27     | HSA | L7 | BDD-D1L3 | 32±5.9                        |
| SEQ ID NO: 28     | HSA | L7 | BDD-D1L3 | 30±8.3                        |
|                   |     |    | L8       | HSA                           |

| Linker           | Sequence                | Length |
|------------------|-------------------------|--------|
| SEQ ID NO: 37 L7 | S(GGS) <sub>9</sub> GSS | 31     |
| SEQ ID NO: 38 L8 | (GGS) <sub>9</sub> GS   | 29     |

FIG. 23



**Factor Xlla Cleavable Linker:**

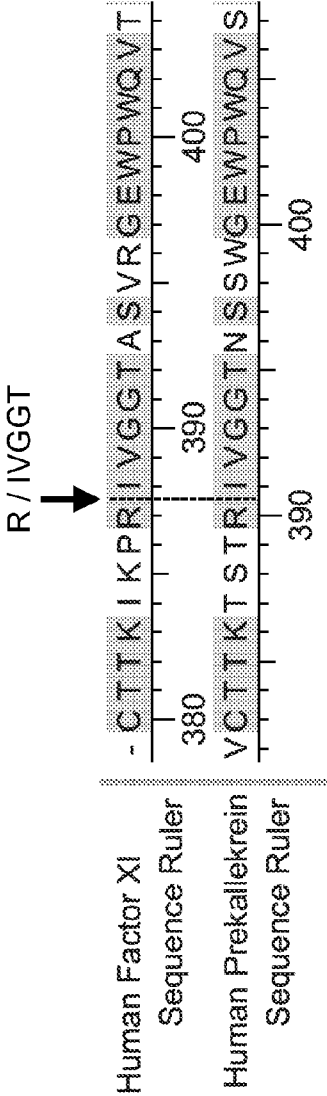
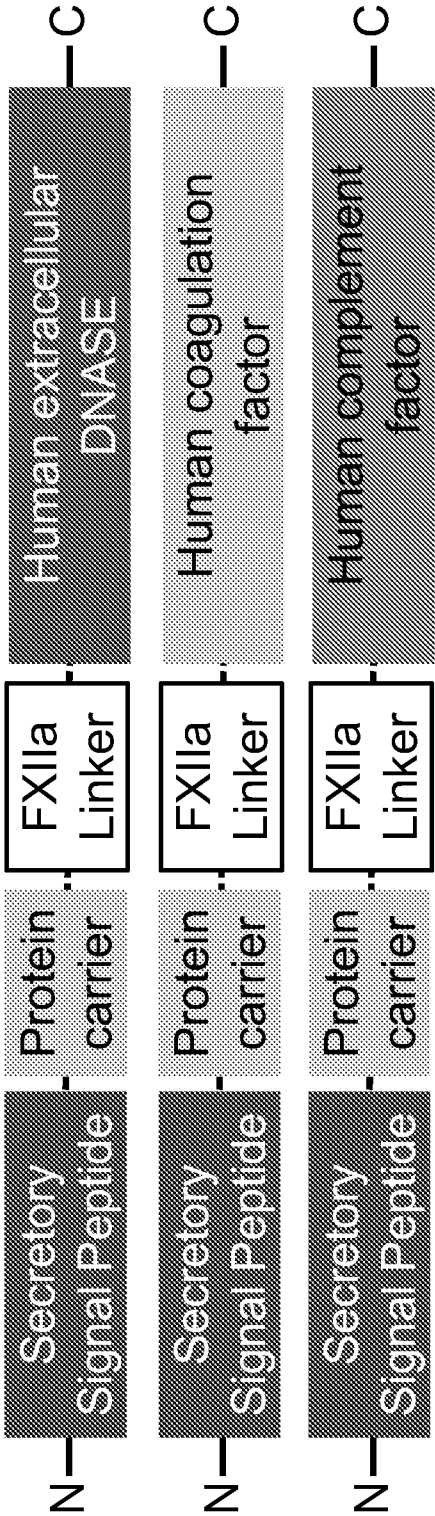
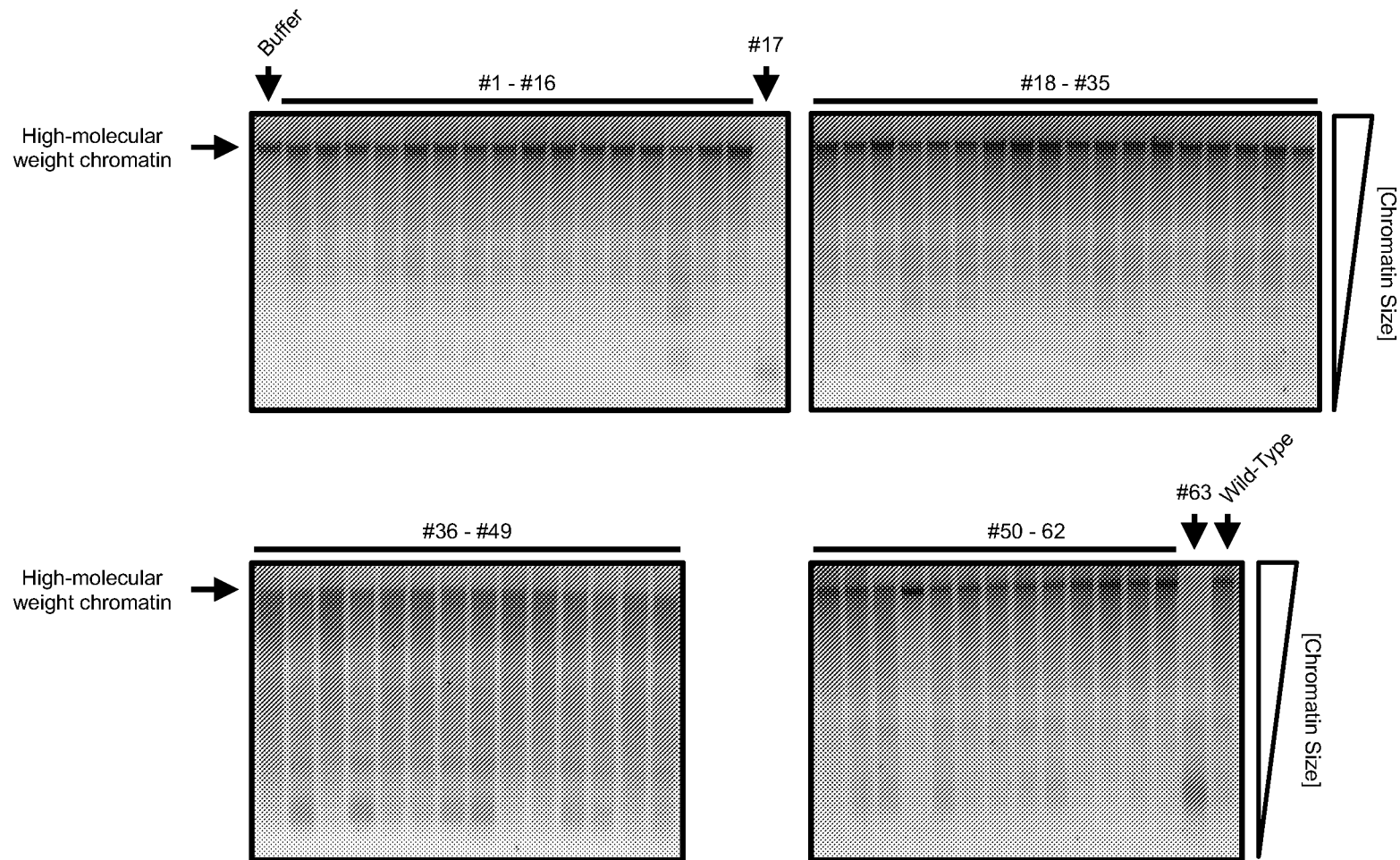


FIG. 24

Expression Vector:



# FIG. 1



DNASE1L3  
gain-of-function mutants:  
#17: Q101R  
#63: Q282\_S305delinsK