(v) the wild-type Lys (K) residue at position 525 is replaced with an Ala (A), Cys (C), Glu (E), Ile (I), Ser (S), Thr (T) or Val (V) residue (K525A, K525C, K525E, K525I, K525S, K525T or K525V);

(vi) the wild-type Gly (G) residue at position 480 is replaced with an Asp (D) residue (G480D);

(vii) the wild-type Ser (S) residue a position 472 is replaced with an Asp (D) residue (S472D);

(viii) the wild-type Asn (N) residue at position 476 is replaced with a Glu (E) or Gly (G) residue (N476E or N476G);

(ix) the wild-type Asn (N) residue at position 527 is replaced with an Asp (D) residue (N527D);

(x) the wild-type Gln (Q) residue at position 531 is replaced with an Arg (R) or Thr (T) residue (Q531R or Q531T);

(xi) the wild-type Arg (R) residue at position 422 is replaced with a His (H) residue (R422H);

(xii) the wild-type Ser (S) residue at position 446 is replaced with an Asp (D) residue (S446D);

(xiii) the wild-type residue at position 448 is replaced with an Ala (A) residue (K448A);

(xiv) the wild-type His (H) residue at position 523 is replaced with a Glu (E) residue (H523E);

(xv) the wild-type Leu (L) residue at position 424 is replaced with a Phe (F) residue (L424F); and

(xvi) the wild-type Asn (N) residue at position 542 is replaced with an Asp (D) residue (N542D);

wherein the amino acid residues are numbered relative to full length *Fok*I wild-type cleavage domain as shown in SEQ ID NO:1, beginning at amino acid residue 384 of the full length *Fok*I protein.

[0013b] In another aspect, described herein is a heterodimer comprising a first engineered cleavage half-domain as described herein and a second cleavage half-domain.

[0013c] In another aspect, described herein is an artificial nuclease comprising an engineered cleavage half-domain as described herein, and a DNA-binding domain.

[0013d] In another aspect, described herein is one or more polynucleotides encoding the engineered cleavage half-domain as described herein, the first and second engineered cleavage domains as described herein or an artificial nuclease as described herein.

[0013e] In another aspect, described herein is an isolated cell comprising the engineered cleavage half-domain as described herein, the heterodimer as described herein, an artificial nuclease as described herein, or the polynucleotide as described herein.

[0013f] In another aspect, described herein is an *in vitro* method for cleaving genomic cellular chromatin in a region of interest, the method comprising: expressing an artificial nuclease comprising an engineered cleavage domain as described herein in a cell, wherein the nuclease site-specifically cleaves to a nucleotide sequence in the region of interest of the genomic cellular chromatin.

[0013g] In another aspect, described herein is an *in vitro* method of cleaving at least two target sites in genomic cellular chromatin, the method comprising: cleaving at least first and second target sites in genomic cellular chromatin, wherein each target site is cleaved using a composition comprising an artificial nuclease as described herein.

[0013h] In another aspect, described herein is the engineered cleavage half-domain as described herein, the heterodimer as described herein, the artificial nuclease as described herein or the isolated cell as described herein for use for use in the treatment or prevention of disease.

[0013i] In another aspect, described herein is a composition comprising a first polynucleotide as described herein encoding a first artificial nuclease and a second polynucleotide as described herein encoding a second artificial nuclease, wherein the ratio of the first and second artificial nucleases is not one to one, optionally a ratio of 1:2, 1:3, 1:4, 1:5, 1:6, 1:8, 1:9, 1:10, 1:20 or 1:30.

[0013j] In another aspect, described herein is an *in vitro* method for increasing engineered nuclease cleavage specificity, the method comprising contacting a cell with an engineered nuclease complex comprising engineered cleavage half-domain partners as described herein, where the ratio of the partners is other than one to one, optionally wherein: (i) the ratio is 1:2, 1:3, 1:4, 1:5, 1:6, 1:8, 1:9, 1:10 or 1:20, or any value therebetween, or is greater than 1:30; and/or (ii) each partner is delivered to the

CLAIMS

What is claimed is:

1. An engineered *Fok*I cleavage half-domain, wherein the engineered cleavage half-domain comprises one or more substitution mutations as follows:

(i) the wild-type Gln (Q) residue at position 481 is replaced with an Cys (C), Asp (D), Glu (E) or Ser (S) residue (Q481C, Q481D, Q481E or Q481S);

(ii) the wild-type Ser (S) residue at position 418 is replaced with a Glu (E) or Asp (D) residue (S418E or S418D);

(iii) the wild-type Ile (I) residue at position 479 is replaced with a Gln (Q) or Thr (T) residue (I479Q or I479T);

(iv) the wild-type Pro (P) residue at position 478 is replaced an Asp (D) residue (P478D);

(v) the wild-type Lys (K) residue at position 525 is replaced with an Ala (A), Cys (C), Glu (E), Ile (I), Ser (S), Thr (T) or Val (V) residue (K525A, K525C, K525E, K525I, K525S, K525T or K525V);

(vi) the wild-type Gly (G) residue at position 480 is replaced with an Asp (D) residue (G480D);

(vii) the wild-type Ser (S) residue a position 472 is replaced with an Asp (D) residue (S472D);

(viii) the wild-type Asn (N) residue at position 476 is replaced with a Glu (E) or Gly (G) residue (N476E or N476G);

(ix) the wild-type Asn (N) residue at position 527 is replaced with an Asp (D) residue (N527D);

(x) the wild-type Gln (Q) residue at position 531 is replaced with an Arg (R) or Thr (T) residue (Q531R or Q531T);

(xi) the wild-type Arg (R) residue at position 422 is replaced with a His (H) residue (R422H);

(xii) the wild-type Ser (S) residue at position 446 is replaced with an Asp (D) residue (S446D);

(xiii) the wild-type residue at position 448 is replaced with an Ala (A) residue (K448A);

(xiv) the wild-type His (H) residue at position 523 is replaced with a Glu (E) residue (H523E);

(xv) the wild-type Leu (L) residue at position 424 is replaced with a Phe (F) residue (L424F); and

(xvi) the wild-type Asn (N) residue at position 542 is replaced with an Asp (D) residue (N542D);

wherein the amino acid residues are numbered relative to full length *Fok*I wild-type cleavage domain as shown in SEQ ID NO:1, beginning at amino acid residue 384 of the full length *Fok*I protein.

2. The engineered cleavage half-domain of claim 1, comprising mutations as follows: the K448A and I479Q mutations; the K448A and Q481A mutations; and/or the K448A mutation and the K525A, K525C, K525E, K525I, K525S, K525T or K525V mutation.

3. The engineered cleavage half-domain of claim 1 or claim 2, further comprising an additional amino acid mutation at one or more of positions 432, 441, 483, 486, 487, 490, 496, 499, 527, 537, 538 and 559.

4. A heterodimer comprising a first engineered cleavage half-domain of any of claims 1 to 3 and a second cleavage half-domain.

5. An artificial nuclease comprising an engineered cleavage half-domain of any of claims 1 to 3, and a DNA-binding domain.

6. The artificial nuclease of claim 5, wherein the DNA-binding domain comprises a zinc finger protein, a TALE-effector domain or a single guide RNA (sgRNA).

7. The artificial nuclease of claim 6, wherein the zinc finger protein comprises 3 to 6 zinc finger domains, wherein each zinc finger DNA-binding domain comprises two beta sheets, an alpha helix, a recognition helix region that binds to a nucleotide sequence, and further wherein one or more of the zinc finger DNA-binding domains comprise one or more

mutations at positions (-5), (-9) and/or (-14), numbered relative to the start of the alpha helix region.

8. One or more polynucleotides encoding the engineered cleavage half-domain of any of claims 1 to 3, the first and second engineered cleavage domains of claim 4 or an artificial nuclease according to any of claims 5 to 7.

9. An isolated cell comprising the engineered cleavage half-domain of any of claims 1 to 3, the heterodimer of claim 4, an artificial nuclease according to any of claims 5 to 7, or the polynucleotide of claim 8.

10. An *in vitro* method for cleaving genomic cellular chromatin in a region of interest, the method comprising:

expressing an artificial nuclease comprising an engineered cleavage domain according to any of claims 5 to 7 in a cell, wherein the nuclease site-specifically cleaves to a nucleotide sequence in the region of interest of the genomic cellular chromatin.

11. The *in vitro* method of claim 10, further comprising contacting the cell with a donor polynucleotide; wherein cleavage of the cellular chromatin facilitates homologous recombination between the donor polypeptide and the cellular chromatin.

12. An *in vitro* method of cleaving at least two target sites in genomic cellular chromatin, the method comprising:

cleaving at least first and second target sites in genomic cellular chromatin, wherein each target site is cleaved using a composition comprising an artificial nuclease according to any of claims 5 to 7.

13. The engineered cleavage half-domain of any of claims 1 to 3, the heterodimer of claim 4, the artificial nuclease according to any of claims 5 to 7 or the isolated cell of claim 9 for use in the treatment or prevention of disease.

14. A composition comprising a first polynucleotide according to claim 8 encoding a first artificial nuclease and a second polynucleotide of claim 8 encoding a second

artificial nuclease, wherein the ratio of the first and second artificial nucleases is not one to one, optionally a ratio of 1:2, 1:3, 1:4, 1:5, 1:6, 1:8, 1:9, 1:10, 1:20 or 1:30.

15. An *in vitro* method for increasing engineered nuclease cleavage specificity, the method comprising contacting a cell with an engineered nuclease complex comprising engineered cleavage half-domain partners according to any one of claims 1 to 3, where the ratio of the partners is other than one to one, optionally wherein:

(i) the ratio is 1:2, 1:3, 1:4, 1:5, 1:6, 1:8, 1:9, 1:10 or 1:20, or any value therebetween, or is greater than 1:30; and/or

(ii) each partner is delivered to the cell as an mRNA or is delivered in a viral or non-viral vector where different quantities of mRNA or vector encoding each partner are delivered.