monomer can in turn lead to increased off-target activity without providing any beneficial increase in cleavage at the intended target, potentially leading to a decreased specificity overall for any given nuclease pair.

[0009] To decrease off-target cleavage events, engineered obligate heterodimeric cleavage half-domains have been developed. *See, e.g.,* U.S. Patent Nos. 7,914,796; 8,034,598; 8,961,281 and 8,623,618; U.S. Patent Publication Nos. 20080131962 and 20120040398. These obligate heterodimers dimerize and cleave their targets only when the differing engineered cleavage domains are positioned at the appropriate target site by the ZFPs, thereby reducing and/or eliminating monomeric off-target cleavage.

[0010] However, there remains a need for additional methods and compositions to engineered nuclease cleavage systems to decrease off-target cleavage activity.

SUMMARY

[0010a] In one aspect, the present invention refers to an engineered FokI cleavage half-domain, wherein the engineered cleavage half-domain comprises one or more mutations in residues 416, 421, 422, 424, 472, 478, 480, 525 or 542 or in which the wild-type residue at position 418 is replaced with a Glu (E) or Asp (D) residue, the wild-type residue at position 446 is replaced with an Asp (D) residue, the wild-type residue at position 448 is replaced with an Ala (A) residue (K448A), the wild-type residue at position 479 is replaced with a Gln (Q) or Thr (T) residue (I479Q or I479T), the wild type residue at position 481 is replaced with an Ala (A), Cys (C), Asp (D), Ser (S), or Glu (E) residue (Q481A, Q481C, Q481D, Q481S, Q481E), or the wild-type residue at position 476 is replaced with a Glu (E) residue or a Glu (G) residue (N476E, N476G), wherein the amino acid residues are numbered relative to full length FokI wild-type cleavage domain as shown in SEQ ID NO:1. In another aspect, the present invention refers to one or more polynucleotides encoding the engineered cleavage half-domain, the first and second engineered cleavage domains, or an artificial nuclease of the present invention. In another aspect, the present invention refers to an isolated cell comprising the polynucleotide of the present invention. In another aspect of the invention, there is provided a method for cleaving genomic cellular chromatin in a region of interest, the method comprising: expressing an artificial nuclease comprising an engineered cleavage domain of the present

invention in a cell, wherein the nuclease site-specifically cleaves to a nucleotide sequence in the region of interest of the genomic cellular chromatin. In yet another aspect, the present invention refers to a 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 of the present invention. Another aspect of the present invention also refers to an isolated cell or cell line comprising at least one site-specific genomic modification made by an artificial nuclease of the present invention. In another aspect, the present invention refers to a composition comprising first and second polynucleotides of the present invention wherein the artificial nuclease comprises a nuclease comprising first and second DNA-binding domains, wherein the first polynucleotide encodes the first nuclease and a second polynucleotide encodes the second nuclease and the ratio of the first and second 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 ratio. In yet another aspect, the present invention refers to a zinc finger protein comprising at least three zinc finger DNA-binding 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 mutations in amino acid residues (-5), (-9) and/or (-14), numbered relative to the start of the alpha helix region. In still another aspect of the present invention, there is provided a zinc finger nuclease comprising the zinc finger protein of the present invention and a cleavage domain. In another aspect of the invention, there is also provided a zinc finger nuclease dimer comprising first and second dimerizing zinc finger nucleases of the present invention. In another aspect of the invention, there is also provided an isolated cell comprising the one or more polynucleotides of the present invention. In another aspect, the present invention refers to a method for cleaving genomic cellular chromatin in a region of interest, the method comprising: expressing the zinc finger nuclease of the present invention or the one or more polynucleotides of the present invention in a cell, wherein the zinc finger nuclease is expressed in the cell and site-specifically cleaves a nucleotide sequence in the region of interest of the genomic cellular chromatin. In another aspect, the present invention refers to an isolated cell or cell line comprising at least one site-specific genomic modification made by a zinc finger nuclease of the present invention. In another aspect, there is also provided a composition comprising

first and second polynucleotides of the present invention, or the method of the present invention, wherein the zinc finger nuclease comprises first and second zinc finger nucleases, wherein the first polynucleotide encodes the first zinc finger nuclease and a second polynucleotide encodes the second zinc finger nuclease and the ratio of the first and second zinc finger 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 ratio. In another aspect, the present invention refers to a zinc finger nuclease that cleaves a T cell receptor constant region gene (TRAC) gene, the zinc finger nuclease comprising a first zinc finger nuclease comprising an ELD engineered FokI cleavage domain and a first zinc finger DNA-binding domain that binds to a target site in the TRAC gene and a second zinc finger nuclease comprising a KKR FokI cleavage domain and a second zinc finger DNA-binding domain, the first and second zinc finger DNA-binding domains comprising wherein (i) at least one of the ELD or KKR FokI cleavage domains further comprises a mutation in the FokI cleavage domain selected from the group consisting of: K393S, K394S, R398S, K400S, K402S, R416S, R422S, K427S, K434S, R439S, K441S, R447S, K448S, K469S, R487S, R495S, K497S, K506S, K516S, K525S, K529S, R534S, K559S, R569S, and R570S, numbered relative to wild-type FokI; and/or (ii) at least one of the first and second zinc finger DNA-binding domains comprises one or more mutations in amino acid residues (-5), (-9) and/or (-14), numbered relative to the start of the alpha helix region. In another aspect, the present invention refers to one or more polynucleotides encoding the zinc finger nuclease of the present invention. In yet another aspect, the present invention refers to an isolated cell comprising the one or more polynucleotides of the present invention. In another aspect, the present invention refers an isolated cell comprising at least one site-specific genomic modification in a TRAC gene made by a zinc finger nuclease. In another aspect, the present invention provides a composition comprising first and second polynucleotides or the method of the present invention, wherein the zinc finger nuclease comprises first and second zinc finger nucleases, wherein the first polynucleotide encodes the first zinc finger nuclease and a second polynucleotide encodes the second zinc finger nuclease and the ratio of the first and second zinc finger 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 ratio.

[0011] The present disclosure provides methods and compositions to increase the specificity of a nuclease (*e.g.,* nuclease pair) for its intended target relative to other unintended cleavage sites, also known as off-target sites. Thus, described herein are artificial nucleases (*e.g.,* zinc finger nucleases (ZFNs), TALENs, CRISPR/Cas nucleases) comprising mutations in one or more of the DNA binding domain regions (*e.g.,* the backbone of a zinc finger protein or TALE) and/or one or more mutations in a *FokI* nuclease cleavage domain or cleavage half domain. Further, described herein are methods to increase specificity of cleavage activity by using these novel nucleases (*e.g.,* ZFNs, TALENs, etc.) and/or through independent titration of the engineered cleavage half-domain partners of a nuclease complex. When used individually or in combination, the methods and compositions of the invention provide surprising and unexpected increases in targeting specificity via reductions in off-target cleavage activity. The disclosure also provides methods of using these compositions for targeted cleavage of cellular chromatin in a region of interest and/or integration of a transgene via targeted integration at a predetermined region of interest in cells.

[0012] Thus, described herein is an engineered nuclease cleavage half domain comprising one or more mutations as compared to a parental (*e.g.,* wild-type) cleavage domain from which these mutants are derived. In certain examples, the one or more mutations are one or more of the mutations shown in any of the appended Tables and Figures, including any combination of these mutants with each other and with other mutants (such as dimerization and/or catalytic domain mutants as well as nickase mutations). Mutations as described herein, include but are not limited to, mutations that change the charge of the cleavage domain, for example mutations of positively charged residues to non-positively charged residues (*e.g.,* mutations of K and R residues (*e.g.,* mutated to S); N residues (*e.g.,* to D), and Q residues (*e.g.,* to E); mutations to residues that are predicted to be close to the DNA backbone based on molecular modeling and that show variation in *Fok*I homologs (Figures 1 and 17); and/or mutations at other residues (*e.g.,* U.S. Patent No. 8,623,618 and Guo *et al,* (2010) *J. Mol. Biol.* 400(1):96-107).

[0013] The most promising mutations were found using the second criteria. The initial promising mutations were positively charged residues predicted to be close to the DNA backbone when *Fok*I is bound to DNA. The cleavage domains described herein may include one, two, three, four, five or more of the mutations described herein and may further include additional known mutations. Therefore, mutations of

the present disclosure do not include specific mutations disclosed in U.S. Patent No. 8,623,618 (*e.g.,* N527D, S418P, K448M, Q531R, etc.) when used alone; however, provided here are novel mutants that can be used in combination with the mutants of U.S. Patent No. 8,623,618. Nickase mutants wherein one of the catalytic nuclease domains in a dimer pair comprises one or more mutations rendering it catalytically inactive (see U.S. Patent Nos 8,703,489; 9,200,266; and 9,631,186) may also be used in combination with any of the mutants described herein. Nickases can be ZFN nickases, TALEN nickases and CRISPR/dCas systems.

[0014] In certain examples, the engineered cleavage half domains are derived from *Fok*I or *Fok*I homologues and comprise a mutation in one or more of amino acid residues 416, 422, 447, 448, and/or 525, numbered relative to the wild-type full length *Fok*I as shown in SEQ ID NO:1 or corresponding residues in *Fok*I homologues (see, Figure 17). In other examples, the cleavage half domains derived from *FokI* comprises a mutation in one or more of amino acid residues 414-426, 443-450, 467-488, 501-502, and/or 521-531, including one or more of 387, 393, 394, 398, 400, 416, 418, 422, 427, 434, 439, 441, 442, 444, 446, 448, 472, 473, 476, 478, 479, 480, 481, 487, 495, 497, 506, 516, 523, 525, 527, 529, 534, 559, 569, 570, and/or 571. The mutations may include mutations to residues found in natural restriction enzymes homologous to *Fok*I at the corresponding positions (Figure 17). In certain examples, the mutations are substitutions, for example substitution of the wild-type residue with any different amino acid, for example alanine (A), cysteine (C), aspartic acid (D), glutamic acid (E), histidine (H), phenylalanine (F), glycine (G), asparagine (N), serine (S) or threonine (T). Any combination of mutants is contemplated, including but not limited to those shown in the appended Tables and Figures. In certain examples, the *Fok*I nuclease domain comprises a mutation at one or more of 416, 422, 447, 479 and/or 525 (numbered relative to wild-type, SEQ ID NO:1). The nuclease domains may also comprise one or more mutations at positions 418, 432, 441, 448, 476, 481, 483, 486, 487, 490, 496, 499, 523, 527, 537, 538 and 559, including but not limited to ELD, KKR, ELE, KKS. *See, e.g.,* U.S. Patent No. 8,623,618. In still further examples, the cleavage domain includes mutations at one or more of the residues shown in Table 15 (*e.g.,* 419, 420, 425, 446, 447, 470, 471, 472, 475, 478, 480, 492, 500, 502, 521, 523, 526, 530, 536, 540, 545, 573 and/or 574). In certain examples, the variant cleavage domains described herein include mutations to the residues involved in nuclease dimerization (dimerization domain mutations), and one or more

additional mutations; for example to phosphate contact residues: *e.g.* dimerization mutants (such as ELD, KKR, ELE, KKS, etc.) in combination with one, two, three, four, five, six or more mutations at amino acid positions outside of the dimerization domain, for example in amino acid residues that may participate in phosphate contact. In a preferred example, the mutation at positions 416, 422, 447, 448 and/or 525 comprise replacement of a positively charged amino acid with an uncharged or a negatively charged amino acid. In other examples, mutations at positions 446, 472 and/or 478 (and optionally additional residues for example in the dimerization or catalytic domains) are made.

[0015] In other examples, the engineered cleavage half domain comprises mutations in the dimerization domain, for example, amino acid residues 490, 537, 538, 499, 496 and 486 in addition to the mutations described herein. In a preferred example, there is provided fusion proteins wherein the engineered cleavage half-domain comprises a polypeptide in which the wild-type Gln (Q) residue at position 486 is replaced with a Glu (E) residue, the wild-type Ile (I) residue at position 499 is replaced with a Leu (L) residue and the wild-type Asn (N) residue at position 496 is replaced with an Asp (D) or a Glu (E) residue ("ELD" or "ELE") in addition to one or more mutations described herein. In another example, the engineered cleavage half domains are derived from a wild-type *Fok*I or *FokI* homologue cleavage half domain and comprise mutations in the amino acid residues 490, 538 and 537, numbered relative to wild-type *Fok*I (SEQ ID NO:1) in addition to the one or more mutations at amino acid residues 416, 422, 447, 448, or 525. In a preferred example, described herein is a fusion protein, wherein the engineered cleavage half-domain comprises a polypeptide in which the wild-type Glu (E) residue at position 490 is replaced with a Lys (K) residue, the wild-type Ile (I) residue at position 538 is replaced with a Lys (K) residue, and the wild-type His (H) residue at position 537 is replaced with a Lys (K) residue or an Arg (R) residue ("KKK" or "KKR") (see U.S. Patent 8,962,281, incorporated by reference herein) in addition to one or more mutations described herein.

[0016] In another example, the engineered cleavage half domains are derived from a wild-type *Fok*I cleavage half domain or homologues thereof and comprise mutations in the amino acid residues 490, and 538, numbered relative to wild-type *Fok*I in addition to the one or more mutations at amino acid residues 416, 422, 447, 448, or 525. In a preferred example, described herein is a fusion protein, wherein the

engineered cleavage half-domain comprises a polypeptide in which the wild-type Glu (E) residue at position 490 is replaced with a Lys (K) residue, and the wild-type Ile (I) residue at position 538 is replaced with a Lys (K) residue ("KK") in addition to one or more mutations at positions 416, 422, 447, 448, or 525. In a preferred example, described herein is a fusion protein, wherein the engineered cleavage half-domain comprises a polypeptide in which the wild-type Gln (Q) residue at position 486 is replaced with an Glu (E) residue, and the wild-type Ile (I) residue at position 499 is replaced with a Leu (L) residue ("EL") (See U.S. Patent 8,034,598, incorporated by reference herein) in addition to one or more mutations at positions 416, 422, 447, 448, or 525.

[0017] In some examples, also described herein are fusion molecules wherein the engineered cleavage half-domain comprises a polypeptide in which the wild-type amino acid residue at one or more of positions 387, 393, 394, 398, 400, 402, 416, 422, 427, 434, 439, 441, 446, 447, 448, 469, 472, 478, 487, 495, 497, 506, 516, 525, 529, 534, 559, 569, 570, 571 in the *Fok*I catalytic domain are mutated. In some examples, the one or more mutations alter the wild type amino acid from a positively charged residue to a neutral residue or a negatively charged residue. In any of these examples, the mutants described may also be made in a *Fok*I domain comprising one or more additional mutations. In preferred examples, these additional mutations are in the dimerization domain, *e.g.* at positions 499, 496, 486, 490, 538 and 537. Mutations include substitutions, insertions and/or deletions of one or more amino acid residues.

[0018] In yet another aspect, any of the engineered cleavage half domains described above may be incorporated into artificial nucleases, for example by associating them with a DNA-binding domain, including but not limited to zinc finger nucleases, TALENs, CRISPR/Cas nucleases, and the like. The zinc finger proteins of the zinc finger nucleases may comprise non-canonical zinc-coordinating residues (*e.g.* CCHC rather than the canonical C2H2 configuration, see United States Patent 9,234,187).

[0019] In another aspect, fusion molecules comprising a DNA binding domain and an engineered FokI or homologue thereof cleavage half-domain as described herein that produce an artificial nuclease are provided. In certain examples, the DNA-binding domain of the fusion molecule is a zinc finger binding domain (*e.g.,* an engineered zinc finger binding domain). In other examples, the DNA-binding domain is a TALE DNA-binding domain. In still further examples, the DNA binding domain

comprises a DNA binding molecule (*e.g.* guide RNA) and a catalytically inactive Cas9 or Cfp1 protein (dCas9 or dCfp1). In some examples, the engineered fusion molecules form a nuclease complex with a catalytically inactive engineered cleavage half-domain such that the dimeric nuclease is only capable of cleaving only one strand of a double-stranded DNA molecule, forming a nickase (*see* U.S. Patent 9,200,266).

[0020] The methods and compositions of the present disclosure also include mutations to one or more amino acids within the DNA binding domain outside the residues that recognize the nucleotides of the target sequence (*e.g.,* one or more mutations to the 'ZFP backbone' (outside the DNA recognition helix region) or to the 'TALE backbone' (outside of the RVDs)) that can interact non-specifically with phosphates on the DNA backbone. Thus, in certain examples, described herein are mutations of cationic amino acid residues in the ZFP backbone that are not required for nucleotide target specificity. In some examples, these mutations in the ZFP backbone comprise mutating a cationic amino acid residue to a neutral or anionic amino acid residue. In some examples, these mutations in the ZFP backbone comprise mutating a polar amino acid residue to a neutral or non-polar amino acid residue. In preferred examples, mutations at made at position (-5), (-9) and/or position (-14) relative to the DNA binding helix. In some examples, a zinc finger may comprise one or more mutations at (-5), (-9) and/or (-14). In further examples, one or more zinc fingers in a multi-finger zinc finger protein may comprise mutations in (-5), (-9) and/or (-14). In some examples, the amino acids at (-5), (-9) and/or (-14) (*e.g.* an arginine (R) or lysine (K)) are mutated to an alanine (A), leucine (L), Ser (S), Asp (N), Glu (E), Tyr (Y) and/or glutamine (Q).

[0021] In another aspect, polynucleotides encoding any of the engineered cleavage half-domains or fusion proteins as described herein are provided.

[0022] In yet another aspect, cells comprising any of the nucleases, polypeptides (*e.g.,* fusion molecules or fusion polypeptides) and/or polynucleotides as described herein are also provided. In one example, the cells comprise a pair of fusion polypeptides, one fusion polypeptide comprising, in addition to one or more mutations in amino acid residues 393, 394, 398, 416, 421, 422, 442, 444, 447, 448, 473, 480, 530 and/or 525, an ELD or ELE cleavage half-domain and one fusion polypeptide comprising, in addition to one or more mutations at residues 393, 394, 398, 416, 421, 422, 442, 444, 446, 447, 448, 472, 473, 478, 480, 530 and/or 525, a KKK or KKR cleavage half-domain (see U.S. Patent 8,962,281).

[0023] In any of these fusion polypeptides described herein, the ZFP partners may further comprise mutations in the zinc finger DNA binding domain in the (-5), (-9) and/or (-14) positions. In some examples, the Arg (R) at position -5 is changed to a Tyr (Y), Asp (N), Glu (E), Leu (L), Gln (Q), or Ala (A). In other examples, the Arg (R) at position (-9) is replaced with Ser (S), Asp (N), or Glu (E). In further examples, the Arg (R) at position (-14) is replaced with Ser (S) or Gln (Q). In other examples, the fusion polypeptides can comprise mutations in the zinc finger DNA binding domain where the amino acids at the (-5), (-9) and/or (-14) positions are changed to any of the above listed amino acids in any combination.

[0024] Also provided herein are cells that have been modified by the polypeptides and/or polynucleotides of the present disclosure. In some examples, the cells comprise a nuclease-mediated insertion of a transgene, or a nuclease-mediated knock out of a gene. The modified cells, and any cells derived from the modified cells do not necessarily comprise the nucleases of the present disclosure more than transiently, but the genomic modifications mediated by such nucleases remain.

[0025] In yet another aspect, methods for targeted cleavage of cellular chromatin in a region of interest; methods of causing homologous recombination to occur in a cell; methods of treating infection; and/or methods of treating disease are provided. These methods maybe practiced *in vitro, ex vivo* or *in vivo* or a combination thereof. The methods involve cleaving cellular chromatin at a predetermined region of interest in cells by expressing a pair of fusion polypeptides as described herein (*i.e.,* a pair of fusion polypeptides in which one or both fusion polypeptide(s) comprises the engineered cleavage half-domains as described herein). In certain examples, the targeted cleavage of the on-target site is increased by at least 50 to 200% (or any value therebetween) or more, including 50%-60% (or any value therebetween), 60%-70% (or any value therebetween), 70%-80% (or any value therebetween), 80%-90% (or any value therebetween, 90% to 200% (or any value therebetween), as compared to cleavage domains without the mutations as described herein. Similarly, using the methods and compositions as described herein, off-target site cleavage is reduced by 1-100 or more-fold, including but not limited to 1-50-fold (or any value therebetween).

[0026] The engineered cleavage half domains described herein can be used in methods for targeted cleavage of cellular chromatin in a region of interest and/or homologous recombination at a predetermined region of interest in cells. Cells

include cultured cells, cell lines, cells in an organism, cells that have been removed from an organism for treatment in cases where the cells and/or their descendants will be returned to the organism after treatment, and cells removed from an organism, modified using the fusion molecules of the disclosure, and then returned to the organism in a method of treatment (cell therapy). A region of interest in cellular chromatin can be, for example, a genomic sequence or portion thereof. Compositions include fusion molecules or polynucleotides encoding fusion molecules that comprise a DNA binding molecule (*e.g.,* an engineered zinc finger or TALE binding domain or an engineered CRISPR guide RNA) and a cleavage half domain as described.

[0027] A fusion molecule can be expressed in a cell, *e.g.*, by delivering the fusion molecule to the cell as a polypeptide, or by delivering a polynucleotide encoding the fusion molecule to a cell, wherein the polynucleotide, if DNA, is transcribed and is translated, to generate the fusion molecule. Further, if the polynucleotide is an mRNA encoding the fusion molecule, following delivery of the mRNA to the cell, the mRNA is translated, thus generating the fusion molecule.

[0028] In some examples, also described herein are methods and compositions for increasing engineered nuclease specificity. In one aspect, methods are provided for increasing overall on-target cleavage specificity by decreasing off-target cleavage activity. In some examples, the engineered cleavage half-domain partners of an engineered nuclease complex are used to contact a cell, where each partner of the complex is given in a ratio to the other partner other than one to one. In some examples, the ratio of the two partners (half cleavage domains) is given at a 1:2, 1:3, 1:4, 1:5, 1:6, 1:8, 1:9, 1:10 or 1:20 ratio, or any value therebetween. In other examples, the ratio of the two partners is greater than 1:30. In other examples, the two partners are deployed at a ratio that is chosen to be different from 1:1. In some aspects, 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. In further examples, each partner of the nuclease complex may be comprised on a single viral or non-viral vector, but is deliberately expressed such that one partner is expressed at a higher or lower value that the other, ultimately delivering the cell a ratio of cleavage half domains that is other than one to one. In some examples, each cleavage half domain is expressed using different promoters with different expression efficiencies. In other examples, the two cleavage domains are delivered to the cell using a viral or non-viral vector where both are expressed from

the same open reading frame, but the genes encoding the two partners are separated by a sequence (*e.g.* self-cleaving 2A sequence or IRES) that results in the 3' partner being expressed at a lower rate, such that the ratios of the two partners are 1:2, 1:3, 1:4, 1:5, 1:6, 1:8, 1:9, 1:10 or 1:20 ratio, or any value therebetween. In other examples, the two partners are deployed at a ratio that is chosen to be different from 1:1.

[0029] Also provided are methods to decrease off-target nuclease activity when two or more nuclease complexes are used. For example, the disclosure provides methods for varying the ratio of DNA binding molecules when two or more nuclease complexes are used. In some examples, the DNA binding molecules are polypeptide DNA binding domains (*e.g.*, ZFNs, TALENs, dCas-Fok, megaTALs, meganucleases), while in others, the DNA binding molecules are guide RNAs for use with RNA-guided nucleases. In preferred examples, the ratio of the two or more DNA binding molecules is 1:2, 1:3, 1:4, 1:5, 1:6, 1:8, 1:9, 1:10 or 1:20 ratio, or any value therebetween. In other examples, the two DNA binding molecules are deployed at a ratio that is chosen to be different from 1:1. In some aspects, the non-1:1 ratio is achieved by altering the ratio of guide RNAs used to transfect a cell. In other aspects, the ratio is altered by changing the ratio of each Cas9 protein-guide RNA complex used to treat the cells of interest. In a still further aspect, the altered ratio is achieved by using differing ratios of DNAs encoding the guide RNAs (viral or non-viral) for treatment
of the cells, or by using promoters with different expression strengths to differentially express the DNA binding molecules inside the cells. Off-target events can be reduced by 2 to 1000-fold (or any amount therebetween) or more, including but not limited to, reduction by at least 10, 50, 60, 70, 80, 100, 150, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, 1000-fold (of any value therebetween) or more.

[0030] Accordingly, in another aspect, a method for cleaving cellular chromatin in a region of interest can comprise (a) selecting a first sequence in the region of interest; (b) engineering a first DNA-binding molecule to specifically bind to the first sequence; (c) expressing a first fusion molecule in the cell, the first fusion molecule comprising the first DNA-binding molecule (*e.g.*, zinc finger, TALE, sgRNA), and a cleavage domain (or half-domain); and (d) expressing a second fusion protein in the cell, the second fusion molecule comprising a second DNA-binding domain, and a second cleavage domain (or half-domain), wherein at least one of the

fusion molecules comprises a linker as described herein, and further wherein the first fusion molecule binds to the first sequence, and the second fusion molecule binds to a second sequence located between 2 and 50 nucleotides from the first sequence, such that an active nuclease complex can form and cellular chromatin is cleaved in the region of interest. In certain examples, both fusion molecules comprise a linker as described herein between the DNA binding domain and the catalytic nuclease domain.

[0031] Also provided are methods of altering a region of cellular chromatin, for example to introduce targeted mutations. In certain examples, methods of altering cellular chromatin comprise introducing into the cell one or more targeted nucleases to create a double-stranded break in cellular chromatin at a predetermined site, and a donor polynucleotide, having homology to the nucleotide sequence of the cellular chromatin in the region of the break. Cellular DNA repair processes are activated by the presence of the double-stranded break and the donor polynucleotide is used as a template for repair of the break, resulting in the introduction of all or part of the nucleotide sequence of the donor into the cellular chromatin. Thus, a sequence in cellular chromatin can be altered and, in certain examples, can be converted into a sequence present in a donor polynucleotide.

[0032] Targeted alterations include, but are not limited to, point mutations (*i.e.*, conversion of a single base pair to a different base pair), substitutions (*i.e.*, conversion of a plurality of base pairs to a different sequence of identical length), insertions or one or more base pairs, deletions of one or more base pairs and any combination of the aforementioned sequence alterations. Alterations can also include conversion of base pairs that are part of a coding sequence such that the encoded amino acid is altered.

[0033] The donor polynucleotide can be DNA or RNA, can be linear or circular, and can be single-stranded or double-stranded. It can be delivered to the cell as naked nucleic acid, as a complex with one or more delivery agents (*e.g.*, liposomes, nanoparticles, poloxamers) or contained in a viral delivery vehicle, such as, for example, an adenovirus, lentivirus or an Adeno-Associated Virus (AAV). Donor sequences can range in length from 10 to 1,000 nucleotides (or any integral value of nucleotides therebetween) or longer. In some examples, the donor comprises a full-length gene flanked by regions of homology with the targeted cleavage site. In some examples, the donor lacks homologous regions and is integrated into a target locus through homology independent mechanism (*i.e.* NHEJ). In other examples, the donor

comprises a smaller piece of nucleic acid flanked by homologous regions for use in the cell *(i.e.* for gene correction). In some examples, the donor comprises a gene encoding a functional or structural component such as a shRNA, RNAi, miRNA or the like. In other examples, the donor comprises sequences encoding a regulatory element that binds to and/or modulates expression of a gene of interest. In other examples, the donor is a regulatory protein of interest (*e.g.* ZFP TFs, TALE TFs or a CRISPR/Cas TF) that binds to and/or modulates expression of a gene of interest.

[0034] For any of the aforementioned methods, the cellular chromatin can be in a chromosome, episome or organellar genome. Cellular chromatin can be present in any type of cell including, but not limited to, prokaryotic and eukaryotic cells, fungal cells, plant cells, animal cells, mammalian cells, primate cells and human cells.

[0035] In yet another aspect, cells comprising any of the polypeptides (*e.g.*, fusion molecules) and/or polynucleotides as described herein are also provided. In one example, the cells comprise a pair of fusion molecules, each comprising a cleavage domain as disclosed herein. Cells include cultured cells, cells in an organism and cells that have been removed from an organism for treatment in cases where the cells and/or their descendants will be returned to the organism after treatment. A region of interest in cellular chromatin can be, for example, a genomic sequence or portion thereof.

[0036] In some examples, also described herein is a kit comprising a fusion protein as described herein or a polynucleotide encoding one or more zinc finger proteins, cleavage domains and/or fusion proteins as described herein; ancillary reagents; and optionally instructions and suitable containers. The kit may also include one or more nucleases or polynucleotides encoding such nucleases.

[0037] These and other aspects will be readily apparent to the skilled artisan in light of disclosure as a whole.

BRIEF DESCRIPTION OF THE DRAWINGS

[0038] **Figure 1** depicts the amino acid sequence (SEQ ID NO:1) and nucleotide sequence (SEQ ID NO:2) of a portion of a wild-type *Fok*I nuclease. The sequence shows the *Fok*I catalytic nuclease domain, and numbering is with respect to the wild type *Fok*I protein used to generate the crystal structures 1FOK.pdb and 2FOK.pdb (Wah *ibid*) (amino acid Q of the nuclease domain begins at 384). Boxed positions indicate possible mutation sites.

[0039] **Figures 2A through 2C** are schematics showing models of the *Fok*I domain interacting with a DNA molecule. Figure 2A indicates the locations of amino acids R422, R416 and K525. Figure 2B indicates the locations of amino acids R447, K448 and R422. Figure 2C is an illustration showing a subset of the different types of ZFNs that can be made incorporating 1, 2 or 3 (1x, 2x, or 3x, respectively) mutations (either R-> Q or R -> L) in the zinc finger backbone. Black arrows indicate the positions of the mutations.

[0040] **Figures 3A and 3B** show activity of BCL11A-specific ZFNs bearing the novel *Fok*I mutations described herein. Figure 3A shows targeted modification in CD34+ cells for BCL11A-specific ZFNs SBS#51857-ELD/SBS#51949-KKR against the BCL11A cognate target (indicated with the unique 'license plate' identifier PRJIYLFN, SEQ ID NO:13) and two off-target sites also identified by their 'license plate' identifiers NIFMAEVG (SEQ ID NO:14) and PEVYOHIU (SEQ ID NO:20). ZFPs are described in PCT/US2016/032049. All experiments were done with 2μg of

CLAIMS

1. An engineered *Fok*I cleavage half-domain, wherein the engineered cleavage half-domain comprises one or more mutations in residues 416, 421, 422, 424, 472, 478, 480, 525 or 542 or in which the wild-type residue at position 418 is replaced with a Glu (E) or Asp (D) residue, the wild-type residue at position 446 is replaced with an Asp (D) residue, the wild-type residue at position 448 is replaced with an Ala (A) residue (K448A), the wild-type residue at position 479 is replaced with a Gln (Q) or Thr (T) residue (I479Q or I479T), the wild type residue at position 481 is replaced with an Ala (A), Cys (C), Asp (D), Ser (S), or Glu (E) residue (Q481A, Q481C, Q481D, Q481S, Q481E), or the wild-type residue at position 476 is replaced with a Glu (E) residue or a Glu (G) residue (N476E, N476G), wherein the amino acid residues are numbered relative to full length *Fok*I wild-type cleavage domain as shown in SEQ ID NO:1.

2. The engineered cleavage half-domain of claim 1, comprising mutations as follows: mutations at residues 416 and 422; a mutation at position 416 and the K448A mutation; the K448A and I479Q mutations; the K448A and Q481A mutations; and/or the K448A mutation and a mutation at position 525.

3. The engineered cleavage half-domain of claim 1 or claim 2, wherein:

the wild-residue at position 416 is replaced with a Glu (E), Asp (D), His (H) or Asn (N) residue (R416E, R416D, R416H, R416N);

the wild-type residue at position 421 is replaced with a Ser (S) residue (D421S);

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

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

the wild-type residue at position 472 is replaced with an Asp (D) residue (S472D);

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

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

the wild-type 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, K525V); and/or

the wild-type residue at position 542 is replaced with an Asp (D) residue (N542D).

4. The engineered cleavage half-domain of any of claims 1 to 3, 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.

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

6. An artificial nuclease comprising an engineered cleavage half-domain of any of claims 1 to 4, and a DNA-binding domain.

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

8. The artificial nuclease of claim 7, wherein the zinc finger protein comprises one or more mutations at positions -14, -9, and -5.

9. One or more polynucleotides encoding the engineered cleavage half-domain of any of claims 1 to 4, the first and second engineered cleavage domains of claim 5 or an artificial nuclease according to any of claims 6 to 8.

10. An isolated cell comprising the polynucleotide of claim 9.

11. A 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 6 to 8 in a cell,

wherein the nuclease site-specifically cleaves ~~to~~ a nucleotide sequence in the region of interest of the genomic cellular chromatin.

12. The method of claim 11, 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.

13. A 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 6 to 8.

14. An isolated cell or cell line comprising at least one site-specific genomic modification made by an artificial nuclease according to any of claims 6 to 8.

15. A composition comprising first and second polynucleotides of claim 9 or the method of claim 11 wherein the artificial nuclease comprises a nuclease comprising first and second DNA-binding domains, wherein the first polynucleotide encodes the first nuclease and a second polynucleotide encodes the second nuclease and the ratio of the first and second 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 ratio.

16. A zinc finger protein comprising at least three zinc finger DNA-binding 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 mutations in amino acid residues (-5), (-9) and/or (-14), numbered relative to the start of the alpha helix region.

17. The zinc finger protein of claim 16, wherein the amino acids at (-5), (-9) and/or (-14) are mutated to an alanine (A), a leucine (L), a serine (S), an aspartic acid (N), glutamine (E), tyrosine (Y) and/or glutamine (Q) reside.

18. The zinc finger protein of claim 16 or claim 17, wherein the Arg (R) at position -5 is changed to a Tyr (Y), Asp (N), Glu (E), Leu (L), Gln (Q) or Ala (A) residue; the Arg (R) at position (-9) is replaced with Ser (S), Asp (N), or Glu (E); and/or the Arg (R) at position (-14) is replaced with Ser (S) or Gln (Q) residue.

19. A zinc finger nuclease comprising the zinc finger protein of any of claims 16 to 18 and a cleavage domain.

20. The zinc finger nuclease according to claim 19, wherein the cleavage domain comprises an engineered *Fok*I cleavage domain.

21. The zinc finger nuclease according to claim 19 or claim 20, wherein the engineered cleavage half-domain comprises one or more mutations in residues 416, 421, 422, 424, 472, 478, 480, 525 or 542 or in which the wild-type residue at position 418 is replaced with a Glu (E) or Asp (D) residue, the wild-type residue at position 446 is replaced with an Asp (D) residue, the wild-type residue at position 448 is replaced with an Ala (A) residue (K448A), the wild-type residue at position 479 is replaced with a Gln (Q) or Thr (T) residue (I479Q or I479T), the wild type residue at position 481 is replaced with an Ala (A), Cys (C), Asp (D), Ser (S), or Glu (E) residue (Q481A, Q481C, Q481D, Q481S, Q481E), or the wild-type residue at position 476 is replaced with a Glu (E) residue or a Glu (G) residue (N476E, N476G), wherein the amino acid residues are numbered relative to full length *Fok*I wild-type cleavage domain as shown in SEQ ID NO:1.

22. The zinc finger nuclease of claim 21, comprising mutations as follows: mutations at residues 416 and 422; a mutation at position 416 and the K448A mutation; the K448A and I479Q mutations; the K448A and Q481A mutations; and/or the K448A mutation and a mutation at position 525.

23. The zinc finger nuclease of claim 22, wherein:

the wild-residue at position 416 is replaced with a Glu (E), Asp (D), His (H) or Asn (N) residue (R416E, R416D, R416H, R416N);

the wild-type residue at position 421 is replaced with a Ser (S) residue (D421S);

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

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

the wild-type residue at position 472 is replaced with an Asp (D) residue (S472D);

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

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

the wild-type 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, K525V); and/or

the wild-type residue at position 542 is replaced with an Asp (D) residue (N542D).

24. The zinc finger nuclease of any of claims 19 to 23, wherein the cleavage domain is *FokI* cleavage half-domain 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.

25. A zinc finger nuclease dimer comprising first and second dimerizing zinc finger nucleases according to any of claims 19 to 22.

26. One or more polynucleotides encoding the zinc finger protein of any of claims 16 to 18 or the zinc finger nuclease according to any of claims 19 to 25.

27. An isolated cell comprising the one or more polynucleotides of claim 26.

28. A method for cleaving genomic cellular chromatin in a region of interest, the method comprising:

expressing the zinc finger nuclease according to any of claims 25 or the one or more polynucleotides according to claim 26 in a cell,

wherein the zinc finger nuclease is expressed in the cell and site-specifically cleaves a nucleotide sequence in the region of interest of the genomic cellular chromatin.

29. The method of claim 28, 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.

30. An isolated cell or cell line comprising at least one site-specific genomic modification made by a zinc finger nuclease according to any of claims 19 to 25.

31. A composition comprising first and second polynucleotides of claim 26 or the method of claim 28 or claim 29 wherein the zinc finger nuclease comprises first and second zinc finger nucleases, wherein the first polynucleotide encodes the first zinc finger nuclease and a second polynucleotide encodes the second zinc finger nuclease and the ratio of the first and second zinc finger 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 ratio.

32. A zinc finger nuclease that cleaves a T cell receptor constant region gene (TRAC) gene, the zinc finger nuclease comprising a first zinc finger nuclease comprising an ELD engineered *FokI* cleavage domain and a first zinc finger DNA-binding domain that binds to a target site in the TRAC gene and a second zinc finger nuclease comprising a KKR *Fok*I cleavage domain and a second zinc finger DNA-binding domain, the first and second zinc finger DNA-binding domains comprising wherein

(i) at least one of the ELD or KKR *FokI* cleavage domains further comprises a mutation in the *Fok*I cleavage domain selected from the group consisting of: K393S, K394S, R398S, K400S, K402S, R416S, R422S, K427S, K434S, R439S, K441S,

R447S, K448S, K469S, R487S, R495S, K497S, K506S, K516S, K525S, K529S, R534S, K559S, R569S, and R570S, numbered relative to wild-type FokI; and/or

(ii) at least one of the first and second zinc finger DNA-binding domains comprises one or more mutations in amino acid residues (-5), (-9) and/or (-14), numbered relative to the start of the alpha helix region.

33. The zinc finger nuclease of claim 32, wherein the mutation in the ELD and/or KKR *Fok*I cleavage domain is a R416S, R422S, R447S, K448S or K525S mutation.

34. The zinc finger nuclease of claim 32 or claim 33, wherein the amino acid at residue at position -5 is mutated in one, two or three fingers.

35. One or more polynucleotides encoding the zinc finger nuclease according to any of claims 32 to 34.

36. An isolated cell comprising the one or more polynucleotides of claim 35.

37. A method for cleaving a TCR alpha (TRAC) gene in a mammalian cell, the method comprising:

expressing the zinc finger nuclease according to any of claims 32 to 34 or the one or more polynucleotides according to claim 35 in a cell,

wherein the zinc finger nuclease is expressed in the cell and site-specifically cleaves to a nucleotide sequence in the TRAC gene.

38. The method of claim 37, 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.

39. An isolated cell comprising at least one site-specific genomic modification in a TRAC gene made by a zinc finger nuclease according to any of claims 32 to 34.

40. A composition comprising first and second polynucleotides of claim 36 or the method of claim 37 or 38 wherein the zinc finger nuclease comprises first and second zinc finger nucleases, wherein the first polynucleotide encodes the first zinc finger nuclease and a second polynucleotide encodes the second zinc finger nuclease and the ratio of the first and second zinc finger 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 ratio.