METHOD TO OVERCOME DNA CHEMICAL MODIFICATIONS SENSITIVITY OF ENGINEERED TALE DNA BINDING DOMAINS

Field of the invention

The present invention relates to polypeptides and more particularly to Transcription Activator-Like Effector derived proteins that allow to efficiently target and/or process nucleic acids. Particularly, the present invention reports the characterization of TALE derived proteins that can efficiently target methylated DNA. The present invention more specifically relates to TALE derived proteins that allow activation of methylated promoters responsible for gene silencing. The present invention also concerns methods to use these proteins. The present invention also relates to vectors, compositions and kits in which Repeat Variable Diresidue (RVD) domains and Transcription Activator-Like Effector (TALE) proteins of the present invention are used.

Background of the invention

Transcription activator-like effectors (TALEs), a group of bacterial plant pathogen proteins have recently emerged as new engineerable scaffolds for production of tailored DNA binding domains with chosen specificities (1, 2). TALE DNA binding domain is composed by a variable number of 33-35 amino acid repeat modules. These repeat modules are nearly identical to each other except for two variable amino acids located at positions 12 and 13 (i.e. Repeat Variable Di residues, RVD). The nature of residues 12 and 13 determines base preferences of individual repeat module. Moscou M. J and Bogdanove A. J and Boch et al. described the following code: HD for recognizing C; NG for recognizing T; NI for recognizing A; NN for recognizing G or A; NS for recognizing A or C or G or T; HG for recognizing T; IG for recognizing T; NK for recognizing G; HA for recognizing C; ND for recognizing C; HI for recognizing C; HN for recognizing G; NA for recognizing G; SN for recognizing G or A; and YG for recognizing T (International PCT Applications WO 2011/072246 and 3, 4). This remarkably simple cipher, consisting in a one-repeat-to-one-base pair code, allowed for prediction of TAL effector binding site and more importantly for construction of custom TAL effector

repeat domains that could be tailored to bind DNA sequence of interest. This unprecedented feature unmasked exciting perspectives to develop new molecular tools for targeted genome applications and within the past two years, TALE-derived proteins have been fused to transcription activator/repressor or nuclease domains and successfully used to specifically regulate transcription of chosen genes (5) or to perform targeted gene modifications and insertions (6-9).

Critical to the efficiency of engineered TALE-derived proteins is their ability to access and efficiently bind their chromosomal target sites. Numerous factors may hinder binding, including DNA packaging into chromatin, position of nucleosomal proteins with respect to the target site and chemical DNA modifications such as methylation. In higher eukaryotes, DNA methylation is involved in the regulation of genes expression and predominantly occurs at the C5 position of cytosine found in the dinucleotide sequence CpG (10) and also CpA, CpT and CpC (11). The presence of such additional methyl moiety may hinder recognition of modified cytosine by RVD HD that is commonly used to target cytosine. This feature may represent an important epigenetic drawback for genome engineering applications using TALE-derived proteins.

There remains a need for designing new RVDs, repeat sequences and TALE derived proteins comprising RVDs to overcome chemical DNA modifications and to efficiently detect, target and process nucleic acids comprising these chemical modifications.

Unexpectedly, the inventors have found as part of their laboratory intensive research that shorter TAL repeats including a gap at the level of amino acid positions 12 and/or 13 (which could be regarded as forming "incomplete RVDs") can better accommodate chemically modified nucleic acid bases in particular methylated bases. Based on this finding, they have synthetized TALEs that can efficiently target methylated target nucleic acid sequences, and more generally chemically modified bases, as a way to overcome the above limitations of current TALE-derived proteins.

Brief summary of the invention

In a general aspect, the present invention relates to polypeptides that allow to efficiently detect, target and/or process nucleic acids comprising chemical modifications. More particularly, the present invention reports the characterization of TALE derived protein sensitivity to chemical modifications such as cytosine methylation and presents an efficient method to overcome such sensitivity. This method relies on the utilization of RVDs "star", which means incomplete RVDs including a gap symbolized by "*" to accommodate chemically modified nucleic acid base within a nucleic acid target sequence. This gap is revealed when the TAL repeat is aligned using ClustalW alignment with other standard di-residues. The invention more particularly relies on the inclusion of the RVDs N* and H* or **, in TALE repeat domains to specifically target methylated bases, especially 5-methyl-cytosine. The present invention also concerns methods to use Transcription Activator-Like Effector proteins comprising such RVDs. The present invention also relates to vectors, compositions and kits in which RVDs and Transcription Activator-Like Effector proteins of the present invention are used.

Brief description of the figures and tables

In addition to the preceding features, the invention further comprises other features which will emerge from the description which follows, as well as to the appended drawings. A more complete appreciation of the invention and many of the attendant advantages thereof will be readily obtained as the same becomes better understood by reference to the following Figures in conjunction with the detailed description below.

Figure 1: Close up structure of the eighth RVD HD of PthXoI Tal repeat domain interacting with the eighth deoxycytidine of its cognate target (12). Distances between deoxycytidine C5 and aspartate Cβ and hydrogen bond between deoxycytidine N4 and aspartate O2 are indicated with dashed lines.

Figure 2: Chemical structures of cytosine, 5-methyl-cytosine.

Figure 3: Description of XPCT1_HD and XPCT1_N* TALE-nucleases. **A.** Description of *xpc1* locus target. **B.** sequences of XPCT1L_HD, XPCT1L_N* and XPCT1R TAL repeat arrays used to generate XPCT1_HD and XPCT1_N* TALE-nucleases. "T" as the first nucleotide of the target DNA sequence (5' to 3') is recognized and bound by "RVD0" repeat, named for a postulated 0^{th} repeat (16) at the C-terminus extremity of the N-terminal domain of a natural TALE.

Figure 4: Tal repeats array_HD or N* assembly and subcloning into yeast and mammalian expression plasmids. **A.** Legend of materials used for TAL repeat assembly. **B.** Immobilization of the first biotinylated TAL repeat fragment on a streptavidin coated solid support and ligation to a second TAL repeat harboring SfaNI compatible overhangs (BbvI overhangs displayed in red). **C.** Consecutive ligation/restriction of TAL repeats to generate the complete XPCT1L TAL repeats array. **D.** SfaNI digestion of the XPCT1L TAL repeats array. **E.** BbvI digestion and recovery of the XPCT1L TAL repeats array. Subcloning of XPCT1L TAL repeats array into yeast or mammalian expression plasmids harboring the Nterminal domain of AvrBs3 TAL effector, the eleven first amino acids of its Cterminal domain fused to FokI type IIS restriction endonuclease.

Figure 5: Nuclease activity of XPCT1_HD or XPCT1_N* TALE-nucleases toward the unmethylated extrachromosomal DNA target and toward the methylated endogenous xpc1 locus. **A.** Increasing amounts of DNA coding for both TALE-nucleases were transfected in CHO KI and processed according to the protocol described in Material and Methods section. Nuclease activities of XPCT1 TALE-nucleases toward their extrachromosomal unmethylated targets are displayed. **B.** and **C.** Increasing amounts of DNA coding for both TALE-nucleases were transfected in 293H cells and three days post transfection, genomic DNA was extracted, *xpc1* locus was amplified and amplicons were either analyzed by deep sequencing or used to perform a T7 nuclease assay according to the protocol described by Miller et al (6). **B.** Results obtained from the T7 nuclease assay. **C.** Results obtained from deep sequencing analysis.

Figure 6: Ability of naturally occurring TAL repeats H* and NG to overcome XPCT1 TALE-nuclease sensitivity to 5-methyl-cytosine. **A.** Schematic representation of the XPCT1 TALE-nuclease model used to investigate the influence of TAL repeat H* and NG on TAL DNA

binding domain sensitivity to 5-methyl-cytosine. **B.** Targeted mutagenesis (TM) of endogenous methylated XPC1 target, induced by 5 µg of XPCT1-HD, N*, H* or NG TALE-nucleases encoding plasmids in 293H cells, determined by deep sequencing and **C.** determined by EndoT7 assay. **D.** Toxicity assay results obtained with XPCT1 TALE-nucleases bearing either HD, N*, H* or NG at position +2 of its Left TAL DNA binding domain. Increasing amounts of XPCT1 TALE-nucleases were transfected in CHO KI cells with a constant amount of GFP-encoding plasmid. GFP intensity levels were monitored by flow cytometry 1 and 6 days post-transfection. Cell survival was calculated as a ratio (TALE-nuclease-transfected cells expressing GFP at Day 6/control transfected cells expressing GFP at Day 6) (19).

Figure 7: TAL repeat N*, a universal 5-methyl-cytosine binding module. **A.** Schematic representation of the XPCT1, T2 and T3 TALE-nucleases used to challenge the ability of TAL repeat N* to overcome TAL DNA binding domain sensitivity to 5-methyl-cytosine. XPC1, XPC2 and XPC3 DNA targets are colored in blue and the position of 5-methyl-cytosines (5mC) are indicated by dots. TAL DNA binding domains are colored in grey and N-term, C-term and FokI domains are colored in black. **B.** Targeted mutagenesis (TM) of endogenous methylated XPC1, XPC2 and XPC3 targets induced by their respective TALE-nucleases, determined by EndoT7 assay. TALE-nucleases containing different combinations of TAL repeats HD or N* on their right (R) and left (L) DNA binding domains were assayed. As an example for the sake of clarity, XPCT3 bearing TAL repeat HD and N* on its right and left DNA binding domains respectively, is indicated as XPCT3 R-HD, L-N*. **C.** Toxicity assay results obtained with XPCT2 and XPCT3 TALE-nucleases bearing either HD or N* at different positions of their left and right TAL DNA binding domains (XPCT3-HD stands for XPCT3 bearing TAL repeats HD on its left and right DNA binding domains and XPCT3-N* stands for XPCT3 bearing TAL repeats N* on its left and right DNA binding domains).

Figure 8: Ability engineered TAL repeats T* and Q* to overcome TAL DNA binding domain sensitivity to 5-methylated Cytosine. Frequency of Targeted mutagenesis (TM) of endogenous methylated XPC1 target, induced by 10 µg of XPCT1-HD, NG, HG, N*, H*, Q* and T* TALE-nuclease encoding plasmids in 293H cells, determined by deep sequencing. The results shown in this figure were obtained from a number of experiments ≥ 2.

Detailed description of the invention

Unless specifically defined herein, all technical and scientific terms used have the same meaning as commonly understood by a skilled artisan in the fields of gene therapy, biochemistry, genetics, and molecular biology.

All methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, with suitable methods and materials being described herein. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will prevail. Further, the materials, methods, and examples are illustrative only and are not intended to be limiting, unless otherwise specified.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of cell biology, cell culture, molecular biology, transgenic biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. See, for example, Current Protocols in Molecular Biology (Frederick M. AUSUBEL, 2000, Wiley and son Inc, Library of Congress, USA); Molecular Cloning: A Laboratory Manual, Third Edition, (Sambrook et al, 2001, Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press); Oligonucleotide Synthesis (M. J. Gait ed., 1984); Mullis et al. U.S. Pat. No. 4,683,195; Nucleic Acid Hybridization (B. D. Harries & S. J. Higgins eds. 1984); Transcription And Translation (B. D. Hames & S. J. Higgins eds. 1984); Culture Of Animal Cells (R. I. Freshney, Alan R. Liss, Inc., 1987); Immobilized Cells And Enzymes (IRL Press, 1986); B. Perbal, A Practical Guide To Molecular Cloning (1984); the series, Methods In ENZYMOLOGY (J. Abelson and M. Simon, eds.-in-chief, Academic Press, Inc., New York), specifically, Vols.154 and 155 (Wu et al. eds.) and Vol. 185, "Gene Expression Technology" (D. Goeddel, ed.); Gene Transfer Vectors For Mammalian Cells (J. H. Miller and M. P. Calos eds., 1987, Cold Spring Harbor Laboratory); Immunochemical Methods In Cell And Molecular Biology (Mayer and Walker, eds., Academic Press, London, 1987); Handbook Of Experimental Immunology, Volumes I-IV (D. M. Weir and C. C. Blackwell, eds., 1986); and Manipulating the Mouse Embryo, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986).

Transcription activator like effector derived protein has recently emerged as a new tool for genome engineering. However, relevant chemical modification in the genome such as DNA methylation as non limiting example interferes with TALE gene targeting. In the present

study, the inventors showed that RVD "stars" are capable of targeting chemically modified nucleic acid base.

In a general aspect, the present invention relates to Transcription Activator-Like Effector derived proteins that allow to efficiently target and/or process chemically modified nucleic acids. More particularly the present invention relates to repeat modules or sequences comprising Repeat Variable-Diresidue (RVD) that allow to efficiently detect, target and/or process nucleic acids with chemical modifications such as alkylation as a non-limiting example. The present invention reports the characterization of TALE derived protein sensitivity to chemical nucleic acid base modifications such as cytosine methylation and presents an efficient method to overcome such sensitivity. This method relies on the utilization of RVDs X* or ** as an entity capable of efficient binding of chemically modified base, wherein X represents one amino acid residue selected from the group of A, G, V, L, I, M, S, T, C, P, D, E, F, Y, W, Q, N, H, R and K and * represents a gap in one position of the RVD.

Recently, applicant has discovered a new class of modular base per base nucleic acid binding domains (MBBBD) in the genome of an endosymbiont species *Burkholderia rhizoxinica* displaying some similarities with TALEs from *Xanthomonas.* These new modular proteins and their use for targeting nucleic acid sequences into a genome are the subject-matter of an application filed on July 6, 2012 under US 61/668,721 and US 61/675,160. Although the modules from such proteins are very different and share less than 50 % homology with TALE repeats, while displaying much more inter-variability, their specificity with respect to nucleic acid bases is apparently similarly driven by amino acids in 12^{th} and 13^{th} positions (RVD-like). Position 13^{th} in MBBBDs could determine the specificity of the nucleic acid base by itself. However, it has been observed in these modules that position 13^{th} can be absent and thus be «star» as in the present invention. Given this fact, it is considered the teaching of the present invention is applicable to such new MBBBD domains, as well as other proteins bearing RVD-like structures. The present invention thus extends to the introduction of "*" in RVD-like structures in order to target methylated nucleic acid sequences without being limited to the RVDs found in *Xanthomonas* TALEs.

I. TALE-derived protein capable of binding chemically modified base.

The present invention relates to a Repeat Variable Diresidue (RVD) X* or **, preferably N*, Q*, T* or H* that is capable of efficient binding chemically modified base, wherein X represents one amino acid residue selected from the group of A, G, V, L, I, M, S, T, C, P, D, E, F, Y, W, Q, N, H, R and K and * represents a gap in one position of the RVD.

Repeat Variable Diresidue (RVD) is included in one repeat module or sequence responsible for the binding of a nucleic acid base in a nucleic acid target sequence at the level of variable amino acids located at positions 12 and 13 (i.e. Repeat Variable Di residues, RVD).

In the present invention, said RVD region responsible for the binding of a nucleic acid base comprises any known amino acid residues in positions 12 and 13. In a preferred embodiment, RVDs comprise one amino acid residue from the group consisting of A, G, V, L, I, M, S, T, C, P, D, E, F, Y, W, Q, N, H, R and K in position 12 according to amino acid one-letter code. In another preferred embodiment, RVDs comprise one amino acid residue from the group consisting of A, G, V, L, I, M, S, T, C, P, D, E, F, Y, W, Q, N, H, R and K in position 13 according to amino acid one-letter code. In another embodiment, RVDs comprise a combination of amino acid residues A, G, V, L, I, M, S, T, C, P, D, E, F, Y, W, Q, N, H, R and K according to amino acid one-letter code in positions 12 and 13 for recognizing one nucleic acid base in nucleic acid target sequence. In a preferred embodiment, RVDs responsible for the binding of a modified nucleic acid base comprise a gap in position 12 and/or 13, more particularly RVDs are X* or **, preferably N*, Q*, T* or H* and are capable of efficient binding of chemically modified base, wherein X represents one amino acid residue selected from the group of A, G, V, L, I, M, S, T, C, P, D, E, F, Y, W, Q, N, H, R and K and * represents a gap in one position of the RVD.

Said RVD of the present invention is capable of binding a modified nucleotide comprises a base different from the classical purine and pyrimidine bases, i.e respectively Adenine, Guanine and Cytosine, Uracil and Thymine. In another aspect, said chemically modified nucleic acid base recognized by the RVD of the present invention is a nucleotide comprising one or several additional chemical groups such as alkyl or hydroxyl as non-limiting example. Said additional group may be a methyl group which refers to the transfer of one carbon group on a nucleotide. Alkylation refers to the transfer of a long chain carbon group. In another embodiment, said chemically modified nucleotide comprises a 5-methyl cytosine

base. In another embodiment, said modified nucleic acid base comprises a base selected from the group consisting of 5-hydroxymethylcytosine, 5-formylcytosine and 5-carboxylcytosine. In another embodiment, said RVD of the present invention is capable of binding DNA sequences comprising molecular lesions such as a non-limiting example pyrimidine dimers formed from cytosine or thymine bases via photochemical reactions.

The present invention also relates to a repeat sequence or repeat module of a Transcription Activator-Like Effector (TALE) comprising a RVD responsible for the binding of a modified nucleic acid base in a nucleic acid target sequence. In addition to the different aspects listed above for variable residues in positions 12 and 13, said repeat sequence named TALE like repeat sequence of the invention can comprise one or several additional mutations in one or several of the 30 to 42 amino acids constituting said RVD, more preferably 33 to 35 amino acids, again more preferably 33 or 34 amino acids. By mutations are encompassed substitutions toward any natural amino acids from the group consisting of A, G, V, L, I, M, S, T, C, P, D, E, F, Y, W, Q, N, H, R and K according to amino acid one-letter code, but also insertions and deletions of one or several amino acid residues.

In other words, the scope of the present invention encompasses one repeat module or sequence responsible for the binding of a modified nucleic acid base in a nucleic acid target sequence at the level of variable amino acids located at positions 12 and 13 (i.e. Repeat Variable Di residues, RVD). In particular, the repeat sequence or module of a TALE comprises a RVD selected from the group consisting of X* and **, preferably N*, Q*, T* or H* for binding chemical modified base nucleic acid wherein X represents one amino acid residue selected from the group of A,G, V, L, I, M, S, T, C, P, D, E, F, Y, W, Q, N, H, R and K and * represents a gap in one position of the RVD.

The present invention also relates to a TALE binding domain specific for a nucleic acid target sequence comprising a plurality of TALE repeat sequences comprising each one a Repeat Variable Diresidue region (RVD) which is responsible for the binding of one specific nucleic acid base in said nucleic acid target sequence and wherein said TALE DNA binding domain comprises one or more RVD selected from the group consisting of X* and **, preferably N*, Q*, T* or H* for binding chemically modified nucleic acid base wherein X represents one amino acid residue selected from the group of A,G, V, L, I, M, S, T, C, P, D, E, F, Y, W, Q, N, H,

R and K and * represents a gap in one position of the RVD. In a preferred embodiment, said repeat domain comprises between 8 and 30 repeat sequences derived from a TALE, more preferably between 8 and 20, again more preferably 5, 6,7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 repeat sequences.

II. TALE chimeric protein capable of processing chemically modified base

The present invention also relates to a chimeric protein derived from a TALE corresponding to a fusion between a TALE DNA binding domain as mentioned above and an additional protein domain to process the DNA within or adjacent to the specific nucleic acid target sequence. In other words, said polypeptide of the present invention is a chimeric protein derived from a TALE comprising:

(a) A TALE DNA binding domain specific for a nucleic acid target sequence comprising a plurality of TALE repeat sequences containing each one a Repeat Variable Diresidue region (RVD) which is responsible for the binding of one specific nucleic acid base in said nucleic acid target sequence wherein said TALE DNA binding domain comprises one or more RVD selected from the group consisting of X* and **, preferably N*, Q*, T* or H* for binding chemically modified nucleic acid base wherein X represents one amino acid residue selected from the group of A,G, V, L, I, M, S, T, C, P, D, E, F, Y, W, Q, N, H, R and K and * represents a gap in one position of the RVD,

(b) An additional protein domain to process the DNA within or adjacent to the specific nucleic acid target sequence

In particular embodiment, said chimeric protein according to the present invention can comprise at least one peptidic linker to fuse said TALE DNA binding domain and said additional protein domain processing the DNA. In a preferred embodiment, said peptidic linker is flexible. In another preferred embodiment, said peptidic linker is structured.

In a particular embodiment, the additional protein domain of the chimeric protein may be a transcription activator or repressor (i.e. a transcription regulator), or a protein that interacts with or modifies other proteins implicated in DNA processing. Non-limiting examples of DNA processing activities of said chimeric protein of the present invention include, for example,

creating or modifying epigenetic regulatory elements, making site-specific insertions, deletions, or repairs in DNA, controlling gene expression, and modifying chromatin structure.

The additional protein domain fused to the TALE DNA binding domain may have a catalytical activity selected from the group consisting of nuclease activity, polymerase activity, kinase activity, phosphatase activity, methylase activity, topoisomerase activity, integrase activity, transposase activity, ligase activity, helicase activity, recombinase activity. In a preferred embodiment, said protein domain is an endonuclease; in another preferred embodiment, said protein domain is an exonuclease.

When comprising an endonuclease, said chimeric protein of the present invention derived from a TALE is a TALE-nuclease; in other words, in the scope of the present invention is a TALE-nuclease comprising:

(a) A Transcription Activator-Like Effector (TALE) DNA binding domain specific for a nucleic acid target sequence comprising a plurality of TALE repeat sequences containing each one a Repeat Variable Diresidue region (RVD) which is responsible for the binding of one specific nucleic acid base pair in said nucleic acid target sequence and wherein said TALE DNA binding domain comprises one or more RVD selected from the group consisting of of X* and **, preferably N*, Q*, T* or H* for binding chemically modified nucleic acid base wherein X represents one amino acid residue selected from the group of A,G, V, L, I, M, S, T, C, P, D, E, F, Y, W, Q, N, H, R and K and * represents a gap in one position of the RVD ;

(b) An endonuclease domain to process the DNA within or adjacent to the specific nucleic acid target sequence.

Depending on the endonuclease domain that constitutes said TALE nuclease, cleavage in the nucleic acid target sequence corresponds to either a double-stranded break or a single-stranded break.

As non limiting example, said endonuclease can be a type IIS FokI endonuclease domain or functional variant thereof which functions independently of the DNA binding domain and induces nucleic acid double-stranded cleavage as a dimer (Li, Wu et al. 1992; Kim, Cha et al.

1996). Amino acid sequence of FokI variants can be prepared by mutations in the DNA, which encodes the catalytic domain. Such variants include, for example, deletions from, or insertions or substitutions of, residues within the amino acid sequence. Any combination of deletion, insertion, and substitution may also be made to arrive at the final construct, provided that the final construct possesses the desired activity. Said nuclease domain of FokI variant according to the present invention comprises a fragment of a protein sequence having at least 80%, more preferably 90%, again more preferably 95 % amino acid sequence identity with the protein sequence of FokI. In particular embodiment, a first and a second chimeric proteins can function respectively as monomer to act together as a dimer to process the nucleic acid within or adjacent to a specific nucleic acid target. As a non-limiting example, the two monomers can recognize different adjacent nucleic acid target sequences and the two protein domains constituting each chimeric protein derived from a TALE, function as subdomains that need to interact in order to process the nucleic acid within or adjacent to said specific nucleic acid target sequence.

In another particular embodiment, said chimeric protein is a monomeric TALE-nuclease that does not require dimerization for specific recognition and cleavage. As non limiting example, such monomeric TALE-nuclease comprises a TALE DNA binding domain fused to the catalytic domain of I-TevI or a variant thereof.

In a preferred embodiment, said TALE-nuclease according to the present invention can comprise at least one peptidic linker to fuse said TALE DNA binding domain and said endonuclease domain. In a preferred embodiment, said peptidic linker is flexible or structured.

In a more specific embodiment, the invention relates to a TALE-nuclease comprising amino acid sequence selected from the group consisting of SEQ ID NO: 38 to SEQ ID NO: 49

In a more preferred embodiment, the DNA binding domain of the TALE-nuclease according to the present invention comprises one or more Repeat Variable Diresidue region (RVD) which is responsible for the binding of one chemically modified nucleic acid base in a nucleic acid target sequence. RVDs of said TALE-nuclease can take one or several of the different aspects statements previously listed for RVDs and repeat sequences of a TALE.

It is understood that RVDs, DNA binding domains, TALE-nucleases, chimeric protein according to the present invention can also comprise single or plural additional amino acid substitutions or amino acid insertion or amino acid deletion introduced by mutagenesis process well known in the art. Is also encompassed in the scope of the present invention variants, functional mutants and derivatives from RVDs, DNA binding domains, TALE-nucleases, chimeric protein and polypeptides according to the present invention. Are also encompassed in the scope of the present invention RVDs, DNA binding domains, TALE-nucleases, chimeric proteins and polypeptides which present a sequence with high percentage of identity or high percentage of homology with sequences of RVDs, DNA binding domains, TALE-nucleases, chimeric proteins and polypeptides according to the present invention, at nucleotidic or polypeptidic levels. By high percentage of identity or high percentage of homology it is intended 70%, more preferably 75%, more preferably 80%, more preferably 85%, more preferably 90%, more preferably 95, more preferably 97%, more preferably 99% or any integer comprised between 70% and 99%.

In another aspect of the present invention are polynucleotides encoding for or comprising a coding sequence for the polypeptides, TALE DNA binding domain, chimeric protein derived from a TALE and TALE-nuclease according to the present invention. Are also encompassed vectors comprising such polynucleotides.

Is also encompassed in the scope of the present invention a host cell which comprises a vector and/or a recombinant polynucleotide encoding for or comprising a coding sequence for the polypeptides, TALE DNA binding domain, chimeric protein derived from a TALE and TALE-nuclease according to the present invention.

Is also encompassed in the scope of the present invention a non-human transgenic animal comprising a vector and/or a recombinant polynucleotide encoding for or comprising a coding sequence for the polypeptides, TALE DNA binding domain, chimeric protein derived from a TALE and TALE-nuclease according to the present invention. Is also encompassed in the scope of the present invention a transgenic plant comprising a vector and/or a recombinant polynucleotide encoding for or comprising a coding sequence for the

polypeptides, TALE DNA binding domain, chimeric protein derived from a TALE and TALE-nuclease according to the present invention.

The present invention also relates to a kit comprising at least a polypeptide or a TALE DNA binding domain or a chimeric protein derived from a TALE or a TALE-nuclease according to the present invention or a vector and/or a recombinant polynucleotide encoding for or comprising a coding sequence for such recombinant molecules and instructions for use said kit.

The present invention also relates to a composition comprising at least a polypeptide or a TALE DNA binding domain or a chimeric protein derived from a TALE or a TALE-nuclease according to the present invention or a vector and /or a recombinant polynucleotide encoding for or comprising a coding sequence for such recombinant molecules and a carrier. More preferably, is a pharmaceutical composition comprising such recombinant molecules and a pharmaceutically active carrier. For purposes of therapy, the chimeric protein according to the present invention and a pharmaceutically acceptable excipient are administered in a therapeutically effective amount. Such a combination is said to be administered in a "therapeutically effective amount" if the amount administered is physiologically significant. An agent is physiologically significant if its presence results in a detectable change in the physiology of the recipient. In the present context, an agent is physiologically significant if its presence results in a decrease in the severity of one or more symptoms of the targeted disease and in a genome correction of the lesion or abnormality.

III. Methods

1. *Method for synthesizing a TALE derived protein capable of binding chemically modified nucleic acid base*

In another aspect, the present invention also relates to methods for synthesizing polynucleotides encoding TALE DNA binding domains (also named TALE arrays), TALE derived protein, TALE-nucleases and chimeric proteins according to the present invention for various applications ranging from targeted DNA cleavage to targeted gene regulation.

One aspect of the invention is a method for synthesizing a transcription activator-like effector (TALE) protein to nucleic acid target sequence comprising a chemically modified nucleic acid base. Said method comprises assembling a plurality of TALE-like repeat sequences, each of said sequences comprising a repeat variable-diresidue (RVD) specific to each nucleic acid base of said sequence. RVD(s) that specifically targets the chemically modified nucleic acid base included in the nucleic acid target sequence are selected from X* or ** , wherein X represents one amino acid residue selected from the group of A,G, V, L, I, M, S, T, C, P, D, E, F, Y, W, Q, N, H, R and K and * represents a gap in one position of the RVD, in order to accommodate said chemically modified nucleic acid base.

In a preferred embodiment, said method comprises at least one of the following steps:

(a) determining a nucleic acid target sequence comprising chemically modified nucleic acid base in the genome of a cell;

(b) assembling TALE-like repeat polynucleotide sequences, each repeat being specific to each nucleic acid base of said nucleic acid target sequence by encoding a repeat variable-diresidue (RVD) comprising at least one RVD selected from the group consisting of:

- HD for recognizing C;
- NG for recognizing T;
- NI for recognizing A;
- NN for recognizing G or A;
- NS for recognizing A or C or G or T;
- HG for recognizing T;
- IG for recognizing T;
- NK for recognizing G;
- HA for recognizing C;
- ND for recognizing C;
- HI for recognizing C;
- HN for recognizing G;
- NA for recognizing G;
- SN for recognizing G or A; and
- YG for recognizing T;

wherein the RVD(s) specifically targeting the chemically modified nucleic acid base(s) in the nucleic target sequence are selected from the RVDs X* and **, where

- X represents one amino acid residue selected from the group of A, G, V, L, I, M, S, T, C, P, D, E, F, Y, W, Q, N, H, R and K, and * represents a gap in one position of the RVD,

(c) expressing said polynucleotide sequence assembled in step (b) in said cell.

In a more preferred embodiment, said chemically modified base corresponds to modified nucleic acid base as described above and preferably is a methylated base, in particular a 5-methyl cytosine.

The present invention also relates to a method to synthesize a chimeric protein as described above to process nucleic acid at a locus defined by a nucleic acid target sequence that comprises a chemically modified base, said method comprising:

(a) synthesizing a polynucleotide sequence comprising a fusion of :

(i) a first polynucleotide encoding a transcription activator-like effector (TALE) protein comprising a plurality of TALE-like repeat sequences, each repeat comprising a repeat variable-diresidue (RVD) specific to each nucleic acid base of said nucleic acid target sequence, wherein the RVD(s) that specifically targets the chemically modified nucleic acid base within said nucleic acid target sequence are selected from X* or **, wherein X represents one amino acid residue selected from the group of A,G, V, L, I, M, S, T, C, P, D, E, F, Y, W, Q, N, H, R and K and * represents a gap in one position of the RVD;

(ii) a second polynucleotide encoding an additional protein domain to process nucleic acid within or adjacent to said nucleic acid target sequence that comprises a chemically modified base;

(b) expressing said polynucleotide sequence of step a) into a host cell.

In another preferred embodiment, said RVD specifically targeting the chemically modified base(s) are preferentially selected from RVD N*, T*, Q* and H*. In another particular embodiment, said RVD specifically targeting the chemically modified base(s) are preferentially selected from RVD NG and HG.

In a preferred embodiment, said additional protein domain has a catalytical activity selected from the group consisting of nuclease activity, polymerase activity, kinase activity, phosphatase activity, methylase activity, topoisomerase activity, integrase activity, transposase activity, ligase activity, helicase activity, recombinase activity. In another preferred embodiment, the protein domain of the chimeric protein can be a transcription activator that can potentially allows site specific activation of methylated promoters responsible for gene silencing. In a more preferred embodiment, said additional protein domain is an endonuclease and thus the chimeric protein is a TALE-nuclease.

As non limiting example, each TALE-like repeat can be assembled together using a solid support method composed of consecutive restriction, ligation, washing step as shown in figure 4 then can be further in a vector. Other methods such as Golden Gate cloning methods and variants or FLASH assembly method may be used as non limiting example (5, 21, 23, 24).

As used herein, the term "expressed" refers to generation of a polynucleotide (transcript) or a polypeptide product. The methods of the invention involve introducing polynucleotide into a cell. The TALE derived protein or chimeric protein may be synthesized *in situ* in the cell as a result of the introduction of polynucleotide encoding polypeptide into the cell. Alternatively, the TALE derived protein or chimeric protein could be produced outside the cell and then introduced thereto. Methods for introducing a polynucleotide construct into bacteria, plants, fungi and animals are known in the art and including as non limiting examples stable transformation methods wherein the polynucleotide construct is integrated into the genome of the cell, transient transformation methods wherein the polynucleotide construct is not integrated into the genome of the cell and virus mediated methods. Said polynucleotides may be introduced into a cell by for example, recombinant viral vectors (e.g. retroviruses, adenoviruses), liposomes and the like. For example, transient transformation methods include for example microinjection, electroporation or particle bombardment. Said polynucleotides may be included in vectors, more particulary plasmids or virus, in view of being expressed in prokaryotic or eukaryotic cells. Alternatively, polynucleotide transcript may be introduced into the cell.

More particularly, the present invention relates to a method to generate a nucleic acid encoding a TALE DNA binding domain insensitive to cytosine methylation comprising the steps of:

(a) determining a DNA target sequence in the genome of a cell,

(b) synthesizing a nucleic acid encoding a TALE DNA binding domain specific for said DNA target sequence comprising a plurality of TALE repeat sequences containing each one a Repeat Variable Diresidue region (RVD) which is responsible for the binding of one specific nucleotide in said DNA target sequence wherein said TALE DNA binding domain comprises one or more RVD selected from the group consisting of X* and ** for binding chemically modified nucleic acid base wherein X represents one amino acid residue selected from the group of A,G, V, L, I, M, S, T, C, P, D, E, F, Y, W, Q, N, H, R and K and * represents a gap in one position of the RVD;

(c) introducing said nucleic acid into said cell,

thereby obtaining a nucleic acid encoding a TALE DNA binding domain which binds said DNA target sequence independently of its cytosine methylation status when expressed in appropriate conditions.

In a particular embodiment, said TALE DNA binding domain which binds the DNA target sequence promotes transcription activation around said DNA target sequence independently of chemically modification, when expressed in appropriate conditions.

In another embodiment, the present invention relates to a method to generate a nucleic acid encoding a TALE-nuclease insensitive to cytosine methylation comprising the steps of:

(a) determining a DNA target sequence in the genome of a cell,

(b) synthesizing a nucleic acid encoding (i) a TALE DNA binding domain specific for said DNA target sequence comprising a plurality of TALE repeat sequences containing each one a Repeat Variable Diresidue region (RVD) which is responsible for the binding of one specific nucleotide in said DNA target sequence wherein said TALE DNA binding domain comprises one or more RVD selected from the group consisting of X* and ** for binding chemically modified nucleic acid base wherein X represents one amino acid residue

selected from the group of A,G, V, L, I, M, S, T, C, P, D, E, F, Y, W, Q, N, H, R and K and * represents a gap in one position of the RVD, (ii) an endonuclease domain to process the DNA within or adjacent to the specific DNA target sequence,

(c) introducing said nucleic acid into said cell,

thereby obtaining a nucleic acid encoding a TALE-nuclease wherein said TALE-nuclease process the DNA within or adjacent to the specific DNA target sequence independently of its cytosine methylation status, when expressed in appropriate conditions.

In a preferred embodiment, said TALE-nuclease according to the present invention can comprise at least one peptide linker to fuse said TALE DNA binding domain and said endonuclease domain. In a preferred embodiment, said peptidic linker is flexible. In another preferred embodiment, said peptidic linker is structured.

More particularly, the present invention encompasses a chimeric protein obtainable by a method comprising at least the steps of:

(a) Determining a DNA target sequence of interest;

(b) Synthesizing a repeat sequence domain specific for said DNA target sequence comprising a plurality of TALE repeat sequences containing each one a Repeat Variable Diresidue region (RVD) which is responsible for the binding of one specific nucleotide pair in said DNA target sequence and wherein said TALE DNA binding domain comprises one or more RVD selected from the group consisting of X* and ** for binding chemically modified nucleic acid base wherein X represents one amino acid residue selected from the group of A,G, V, L, I, M, S, T, C, P, D, E, F, Y, W, Q, N, H, R and K and * represents a gap in one position of the RVD;

(c) Providing a protein domain to process the DNA within or adjacent to the specific DNA target sequence;

(d) Optionally designing a peptidic linker to link polypeptides obtained in b) and c);

(e) Assembling said chimeric protein;

(f) Testing the activity of said chimeric protein.

In a further embodiment, synthesis step b) can be done using a solid support method composed of consecutive restriction/ligation/washing steps as shown in figure 4 and examples section; step c) can be done by cloning said protein domain of interest into a plasmidic vector; in the case where said chimeric protein according to the invention is a TALE-nuclease, as non-limiting example, said protein domain can be cloned together in a same vector with chosen peptidic linker and eventual additional N and C terminal backbones for a RVD. Assembling step e) can be done by cloning repeat sequence domain of step b) in the vector resulting from step e). Testing step f) can be done, in the case where said chimeric protein is a TALE-nuclease as a non-limiting example, in yeast by using a yeast target reporter plasmid containing the DNA target sequence as previously described (International PCT Applications WO 2004/067736 and in [Epinat, Arnould et al. 2003 (13); Chames, Epinat et al. 2005 (17); Arnould, Chames et al. 2006 (14); Smith, Grizot et al. 2006 (18)]. The activity of said TALE-nuclease can be tested at 30°C and 37°C in a yeast SSA assay previously described (International PCT Applications WO 2004/067736 and in [Epinat, Arnould et al. 2003 (13); Chames, Epinat et al. 2005 (17); Arnould, Chames et al. 2006 (14); Smith, Grizot et al. 2006 (18)*]*.

2. *Method for processing target nucleic acid sequence comprising chemically modified nucleic acid base*

In another aspect, the present invention also relates to methods for use of protein comprising TALE domain according to the present invention for various applications ranging from targeted nucleic acid cleavage to targeted gene regulation.

In a particular embodiment, the present invention relates to a method for binding a nucleic acid target sequence comprising at least one chemically modified nucleic acid base, said method comprising contacting: (i) a nucleic acid target sequence comprising chemically modified nucleic acid base and (ii) a TALE protein comprising a repeat variable-diresidue (RVD) specific to each nucleotide base of said nucleic acid target sequence, wherein the RVD(s) that specifically targets the chemically modified nucleic acid base within said nucleic acid target sequence are selected from X* or **, preferably N*, Q*, T* or H* , wherein X

represents one amino acid residue selected from the group of A,G, V, L, I, M, S, T, C, P, D, E, F, Y, W, Q, N, H, R and K and * represents a gap in one position of the RVD.

More particularly, the present invention relates to a method to bind a nucleic acid target sequence comprising at least one chemically modified nucleic acid base, said method comprising:

(a) providing a cell containing a nucleic acid target sequence that comprises a chemically modified base,

(b) synthesizing within said cell a TALE protein directed to said nucleic acid target sequence as described above and,

(c) testing the binding affinity of said TALE protein with said nucleic acid target sequence that comprises said chemically modified base.

In a preferred embodiment, said specific DNA sequence comprising at least one chemically modified dinucleotide selected from the group consisting of CpG, CpA, CpT, CpC.

In another aspect, the present invention relates to a method to process a nucleic acid target sequence comprising at least one chemically modified nucleic acid base by using a chimeric protein as previously defined. Said method preferably comprises the following steps of:

(a) providing a cell containing a nucleic acid target sequence that comprises a chemically modified nucleic acid base;

(b) synthesizing within said cell a chimeric protein directed to said nucleic acid target sequence, so that said chimeric protein process the nucleic acid within or adjacent to said nucleic acid target sequence independently of chemical modification and,

(c) testing the nucleic acid processing at the locus of said nucleic acid target sequence.

In general, the chimeric protein of the present invention can have a catalytical activity selected from the group consisting of nuclease activity, polymerase activity, kinase activity, phosphatase activity, methylase activity, topoisomerase activity, integrase activity, transposase activity, ligase activity, helicase activity, recombinase activity. In another preferred embodiment, the protein domain of the chimeric protein can be a transcription

activator that can potentially allows site specific activation of methylated promoters responsible for gene silencing. In another preferred embodiment, the protein domain can also be a transcription repressor.

Any nucleic acid target sequence can be processed by the present methods. For example, the nucleic acid target sequence can be chromosomal, organelle sequences such as mitochondrial or choloroplast sequences, or the nucleic acid target sequence can be a plasmid or viral sequence. The term "processing" as used herein means that the sequence is considered modified simply by the binding of the polypeptide. The term "processing" as used herein means for example promoting transcription activation around said nucleic acid target sequence. For example, said chimeric protein can comprise a TALE domain according to the present invention fused to a transcription activator such as VP16. Said method is particularly well-suited to reactivate genes in cells wherein their promoters have been silenced by methylation. In other words, the present invention relates to a method to activate transcription of genes in cells where their transcription is normally silenced by methylation. In a preferred embodiment, said cells are eukaryotic cells or primary cells, stem cells, induced Pluripotent Stem (iPS) cells or cells lines derived from any previous types of cells.

As non limiting example, the binding affinity can be tested by detecting signal of reporter proteins such as fluorescent proteins fused to said TALE proteins, or by detecting the presence of the TALE protein with for example antibodies. In a preferred embodiment, the binding affinity, particularly the nucleic acid processing may be tested by a nuclease activity or transcriptional activity. For example, in the case where said chimeric protein is a TALE-nuclease, nucleic acid processing can be tested in yeast by using a yeast target reporter plasmid containing the nucleic acid target sequence as previously described (International PCT Applications WO 2004/067736 and in [Epinat, Arnould et al. 2003 (13); Chames, Epinat et al. 2005 (17); Arnould, Chames et al. 2006 (14); Smith, Grizot et al. 2006 (18)]. The activity of said TALE-nuclease can be tested at 30°C and 37°C in a yeast SSA assay previously described (International PCT Applications WO 2004/067736 and in [Epinat, Arnould et al. 2003 (13); Chames, Epinat et al. 2005 (17); Arnould, Chames et al. 2006 (14); Smith, Grizot et al. 2006 (18)

In a particular embodiment, said additional protein domain is a catalytic domain which has nuclease activity, more preferably, endonuclease activity and the present invention more particularly relates to a method for modifying the genetic material of a cell within or adjacent to a nucleic acid target sequence.

The double strand breaks caused by endonucleases are commonly repaired through non-homologous end joining (NHEJ). NHEJ comprises at least two different processes. Mechanisms involve rejoining of what remains of the two DNA ends through direct re-ligation (Critchlow and Jackson 1998) or via the so-called microhomology-mediated end joining (Ma, Kim et al. 2003). Repair via non-homologous end joining (NHEJ) often results in small insertions or deletions and can be used for the creation of specific gene knockouts. The present invention relates to a method for processing the genetic material in a cell within or adjacent to a nucleic acid target sequence by using chimeric protein, preferably A TALE-nuclease according to the present invention that allows nucleic acid cleavage that will lead to the loss of genetic information and any NHEJ pathway will produce targeted mutagenesis. In a preferred embodiment, the present invention related to a method for modifying the genetic material of a cell within or adjacent to a nucleic acid target sequence by generating at least one nucleic acid cleavage and a loss of genetic information around said nucleic acid target sequence thus preventing any scarless re-ligation by NHEJ. Said modification may be a deletion of the genetic material, insertion of nucleotides in the genetic material or a combination of both deletion and insertion of nucleotides.

The present invention also relates to a method for modifying nucleic acid target sequence further comprising the step of expressing an additional catalytic domain into a host cell. In a more preferred embodiment, the present invention relates to a method to increase mutagenesis wherein said additional catalytic domain is a DNA end-processing enzyme. Non limiting examples of DNA end-processing enzymes include 5-3' exonucleases, 3-5' exonucleases, 5-3' alkaline exonucleases, 5' flap endonucleases, helicases, hosphatase, hydrolases and template-independent DNA polymerases. Non limiting examples of such catalytic domain comprise of a protein domain or catalytically active derivate of the protein domain seleced from the group consisting of hExoI (EXO1_HUMAN), Yeast ExoI (EXO1_YEAST), E.coli ExoI, Human TREX2, Mouse TREX1, Human TREX1, Bovine TREX1, Rat TREX1, TdT (terminal deoxynucleotidyl transferase) Human DNA2, Yeast DNA2

(DNA2_YEAST). In a preferred embodiment, said additional catalytic domain has a 3'-5'-exonuclease activity, and in a more preferred embodiment, said additional catalytic domain has TREX exonuclease activity, more preferably TREX2 activity. In another preferred embodiment, said catalytic domain is encoded by a single chain TREX polypeptide. Said additional catalytic domain may be fused to the chimeric protein according to the invention optionally by a peptide linker.

Endonucleolytic breaks are known to stimulate the rate of homologous recombination. Therefore, in another preferred embodiment, when a chimeric protein with nuclease activity, such as a TALE-nuclease, is used the present invention relates to a method for inducing homologous gene targeting in the nucleic acid target sequence further comprising providing to the cell an exogeneous nucleic acid comprising at least a sequence homologous to a portion of the nucleic acid target sequence, such that homologous recombination occurs between the nucleic acid target sequence and the exogeneous nucleic acid. Following cleavage of the nucleic acid target sequence, a homologous recombination event is stimulated between the genome containing the nucleic acid target sequence and the exogenous nucleic acid. Preferably, homologous sequences of at least 50 bp, preferably more than 100 bp and more preferably more than 200 bp are used within said exogenous nucleic acid. Therefore, the exogenous nucleic acid is preferably from 200 bp to 6000 bp, more preferably from 1000 bp to 2000 bp. Indeed, shared nucleic acid homologies are located in regions flanking upstream and downstream the site of the cleavageand the nucleic acid sequence to be introduced should be located between the two arms.

In another embodiment, said exogenous nucleic acid comprises two sequences homologous to portions or adjacent portions of said nucleic acid target sequence flanking a sequence to introduce in the nucleic acid target sequence. Particularly, said exogenous nucleic acid comprises first and second portions which are homologous to region 5' and 3' of the nucleic acid target, respectively. Said exogenous nucleic acid in these embodiments can also comprise a third portion positioned between the first and the second portion which comprises no homology with the regions 5' and 3' of the nucleic acid target sequence. In this case, said exogenous sequence allows introducing new genetic material into a cell. Said new genetic material introduced into a cell can confer a selective or a commercial advantage to said cell. In another embodiment, said exogenous sequence allows to replace genetic

material into a cell. In another embodiment, said exogenous sequence allows to repair genetic material into a cell.

In particular embodiments, said exogenous nucleic acid can comprise a positive selection marker between the two homology arms and eventually a negative selection marker upstream of the first homology arm or downstream of the second homology arm. The marker(s) allow(s) the selection of the cells having inserted the sequence of interest by homologous recombination at the target site. Depending on the location of the targeted genome sequence wherein break event has occurred, such exogenous nucleic acid can be used to knock-out a gene, e.g. when exogenous nucleic acid is located within the open reading frame of said gene, or to introduce new sequences or genes of interest. Sequence insertions by using such exogenous nucleic acid can be used to modify a targeted existing gene, by correction or replacement of said gene (allele swap as a non-limiting example), or to up- or down-regulate the expression of the targeted gene (promoter swap as non-limiting example), said targeted gene correction or replacement.

Cells in which a homologous recombination event has occurred can be selected by methods well-known in the art. As a non-limiting example, PCR analysis using one oligonucleotide matching within the exogenous nucleic acid sequence and one oligonucleotide matching the genomic nucleic acid of cells outside said exogenous nucleic acid but close to the targeted locus can be performed. Therefore, cells in which methods of the invention allowed a mutagenesis event or a homologous recombination event to occur, can be selected.

In another embodiment, said exogenous sequence to be introduced into a cell can be optimized in order to be not cleavable by the protein used to generate the initial double-stranded break. In other words, in the case where a nucleic acid target sequence has to be corrected by replacement consecutively to a double-stranded break generated by a protein or a chimeric protein according to the present invention, exogenous replacement sequence can be modified in order to be not cleavable again by the original protein or chimeric protein. Said modifications include as non-limiting example silent mutations when targeted sequence is in a coding sequence of a gene or mutations when targeted sequence is in a non-coding sequence of a gene.

In other word, the present invention relates to a method to overcome nucleotide chemical modification sensitivity of a TALE array for binding a DNA target sequence comprising the steps of:

(a) determining a DNA target sequence in the genome of a cell, wherein said DNA target sequence comprises at least one chemically modified nucleic acid base,

(b) synthesizing a nucleic acid encoding a TALE DNA binding domain specific for said DNA target sequence comprising a plurality of TALE repeat sequences containing each one a Repeat Variable Diresidue region (RVD) which is responsible for the binding of one specific nucleotide in said DNA target sequence wherein said TALE DNA binding domain comprises one or more RVD selected from the group consisting of X* and ** for binding chemically modified nucleic acid base wherein X represents one amino acid residue selected from the group of A,G, V, L, I, M, S, T, C, P, D, E, F, Y, W, Q, N, H, R and K and * represents a gap in one position of the RVD,

(c) introducing said nucleic acid into said cell,

thereby obtaining a nucleic acid encoding a TALE DNA binding domain which binds said DNA target sequence independently of its cytosine methylation status, when expressed in appropriate conditions.

More particularly, the present invention relates to a method for targeting a genetic material in a cell comprising:

(a) Providing a cell containing a target DNA sequence, wherein said DNA target sequence comprises at least one CpG sequence,

(b) Introducing a protein comprising at least one (i) Transcription Activator-Like Effector (TALE) domain wherein said TALE domain comprises a plurality of TALE repeat sequences containing each one a Repeat Variable Diresidue region (RVD) which is responsible for the binding of one specific nucleotide pair in the target DNA sequence wherein said TALE DNA binding domain comprises one or more

RVD selected from the group consisting of X* and ** for binding cytosine or 5-methyl-cytosine wherein X represents one amino acid residue selected from the group of A,G, V, L, I, M, S, T, C, P, D, E, F, Y, W, Q, N, H, R and K and * represents a gap in one position of the RVD, (ii) an additional protein domain to process the DNA within or adjacent to the specific DNA target sequence,

such that the TALE domain binds said target DNA sequence independently of its cytosine methylation status, when expressed in appropriate conditions.

As non-limiting example, said protein or chimeric protein can be introduced as a transgene encoded by a plasmidic vector; said plasmidic vector may contain a selection marker which allows to identify and/or select cells which received said vector by method well-known in the art. Said protein expression can be induced in selected cells and said TALE domain of the protein binds target DNA sequence in selected cells, thereby obtaining cells in which TALE domain binds a specific target DNA sequence. In another embodiment, said protein or chimeric protein comprising TALE domain can be directly introduced in cells as a protein by well-known method of the art.

In a preferred embodiment, the present invention relates to a method for modifying the genetic material of a cell comprising:

(a) Providing a cell containing a target DNA sequence, wherein said DNA target sequence comprises at least one CpG sequence,

(b) Introducing a protein comprising at least:

(i) A Transcription Activator-Like Effector (TALE)DNA binding domain specific for a DNA target sequence comprising a plurality of TALE repeat sequences containing each one a Repeat Variable Diresidue region (RVD) which is responsible for the binding of one specific nucleotide pair in said DNA target sequence wherein said TALE DNA binding domain comprises one or more RVD selected from the group consisting of X* and ** for binding cytosine or 5-methyl-cytosine wherein X represents one amino acid residue selected from the group of A,G, V, L, I, M, S, T, C, P, D, E, F, Y, W, Q, N, H, R and K and * represents a gap in one position of the RVD,

(ii) An endonuclease,

such that the TALE DNA binding domain binds said target DNA sequence and the endonuclease generates a double-stranded break within or adjacent to the specific DNA target sequence independently of its cytosine methylation status, when expressed in appropriate conditions.

In a preferred embodiment, the present invention relates to a method for modifying the genetic material of a cell comprising:

(a) Providing a cell containing a target DNA sequence, , wherein said DNA target sequence comprises at least one CpG sequence,

(b) Introducing a protein comprising at least:

(i) A Transcription Activator-Like Effector (TALE)DNA binding domain specific for a DNA target sequence comprising a plurality of TALE repeat sequences containing each one a Repeat Variable Diresidue region (RVD) which is responsible for the binding of one specific nucleotide pair in said DNA target sequence wherein said TALE DNA binding domain comprises one or more RVD selected from the group consisting of X*and ** for binding cytosine or 5-methyl-cytosine wherein X represents one amino acid residue selected from the group of A,G, V, L, I, M, S, T, C, P, D, E, F, Y, W, Q, N, H, R and K and * represents a gap in one position of the RVD,

(ii) An endonuclease,

(c) Inducing the expression of the protein of (b);

(d) Selecting the cells in which a double-stranded break within or adjacent to the specific DNA target sequence has occurred.

As a non-limiting example, said protein comprising at least a TALE DNA binding domain fused to an endonuclease can be introduced as a transgene encoded by a plasmidic vector in said provided cell containing a DNA target sequence; said plasmidic vector contains a selection marker which allows to identify and/or select cells which received said vector. Said protein expression can be induced in selected cells and said TALE domain of the protein can bind

target DNA sequence in selected cells and fused endonuclease can generate a double-stranded break within or adjacent to the specific DNA target sequence; thereby obtaining cells in which protein comprising at least a TALE DNA binding domain fused to an endonuclease has generated a targeted double-stranded break. Cells in which said protein has been introduced is selected by a selection method well-known in the art.

Cells in which a cleavage-induced mutagenesis event, i.e a mutagenesis event consecutive to an NHEJ event, has occurred can be identified and/or selected by well-known method in the art. As a non-limiting example, deep-sequencing analysis can be generated from the targeted cell genome around the targeted locus. Insertion/deletion events (mutagenesis events) can be therefore detected. As another non-limiting example, assays based on T7 endonuclease that recognizes non-perfectly matched DNA can be used, to quantify from a locus specific PCR on genomic DNA from provided cells, mismatches between reannealed DNA strands coming from cleaved/non-cleaved DNA molecules.

3. *Method to detect chemically modified base(s)*

In another embodiment, the present invention relates to methods to detect the presence of chemically modified nucleic acid base in a nucleic acid target sequence in the genome of a cell.

According to a further aspect, the present invention relates to a method to detect at least one chemically modified nucleic acid base in a nucleic acid target sequence comprising:

(a) binding said nucleic acid target sequence with a transcription activator-like effector (TALE) protein comprising a plurality of TALE-like repeat sequences, each of said sequences comprising a repeat variable-diresidue (RVD) specific to each nucleic acid base of said nucleic acid target sequence wherein at least one RVD is selected from the group consisting of:

- HD for recognizing C;
- NG for recognizing T;
- NI for recognizing A;
- NN for recognizing G or A;

- NS for recognizing A or C or G or T;
- HG for recognizing T;
- IG for recognizing T;
- NK for recognizing G;
- HA for recognizing C;
- ND for recognizing C;
- HI for recognizing C;
- HN for recognizing G;
- NA for recognizing G;
- SN for recognizing G or A; and
- YG for recognizing T;

(b) binding the same nucleic acid target sequence with another transcription activator-like effector (TALE) protein comprising a plurality of TALE-like repeat sequences, similar to that used in step a), wherein at least one RVD has been replaced by a RVD consisting of X* or **, preferably H*, T*, Q* or N*, wherein

- X represents one amino acid residue selected from the group of A,G, V, L, I, M, S, T, C, P, D, E, F, Y, W, Q, N, H, R and K,

- and * represents a gap in one position of the RVD,

(c) determining the binding affinity with said nucleic acid sequence under a) and b),

(d) calculating the ratio of binding activities determined under c) , wherein said ratio, when close to 0, indicates the presence of chemically modified nucleic acid base(s) in said nucleic acid target sequence and, when close to 1, the absence of chemically modified nucleic acid base(s) in said nucleic acid target sequence.

In another embodiment, the invention relates to said method wherein the binding affinity is measured (or tested) by a nuclease activity or transcriptional activity. In a preferred embodiment, the invention relates to said method wherein binding affinity is measured by detecting signal of reporter proteins such as fluorescent proteins, fused to said TALE proteins (a) and (b). Said reporter proteins can be luciferase, β-galactosidase, and β-lactamase as non-

limiting examples or other reporter proteins which are usable in systems such as split systems known in the art.

More particularly, the present invention also relates to a method to detect the presence of 5-methyl-cytosine in a DNA target sequence in the genome of a cell comprising at least one of the steps of:

(a) determining a first DNA target sequence in the genome of a cell, wherein said first DNA target sequence comprises at least one CpG sequence,

(b) synthesizing a first nucleic acid encoding (i) a TALE array specific for said first DNA target sequence comprising a plurality of TALE repeat sequences containing each one a Repeat Variable Diresidue region (RVD) which is responsible for the binding of one specific nucleotide in said first DNA target sequence wherein said TALE DNA binding domain comprises one or more RVD selected from the group consisting of X* and ** for binding cytosine or 5-methyl-cytosine wherein X represents one amino acid residue selected from the group of A,G, V, L, I, M, S, T, C, P, D, E, F, Y, W, Q, N, H, R and K and * represents a gap in one position of the RVD, (ii) a first subdomain of two of a reporter protein wherein said reporter protein is only active when said first and second subdomains interact,

(c) synthesizing a second nucleic acid encoding (i) a TALE array specific for said first DNA target sequence comprising a plurality of TALE repeat sequences containing each one a Repeat Variable Diresidue region (RVD) which is responsible for the binding of one specific nucleotide in said first DNA target sequence wherein said TALE DNA binding domain comprises one or more RVD HD for binding cytosine, (ii) a first subdomain of two of a reporter protein wherein said reporter protein is only active when said first and second subdomains interact,

(d) synthesizing a third nucleic acid encoding (i) a TALE array specific for a second DNA target sequence adjacent to said first DNA target sequence comprising a plurality of TALE repeat sequences containing each one a Repeat Variable Diresidue region (RVD) which is responsible for the binding of one specific nucleotide in said DNA target sequence, (ii) a second subdomain of two of a reporter protein wherein said reporter protein is only active when said first and second subdomains interact,

(e) introducing said first and third nucleic acids into said cell, thereby obtaining a first and a third nucleic acids encoding TALE arrays which bind said first and second DNA target sequences when expressed in appropriate conditions and transmits a reporter protein signal independently of the cytosine methylation status of said first DNA target,

(f) introducing said second and third nucleic acids into said cell, thereby obtaining a second and a third nucleic acids encoding TALE arrays which bind said first and second DNA target sequences when expressed in appropriate conditions and transmits a reporter protein signal when 5-methyl-cytosine is absent of said first DNA target,

(g) determining a ratio : reporter protein signal of (f)/ reporter protein signal of (e),

wherein said ratio, when close to 0, indicates the presence of 5-methyl cytosine in said first DNA target sequence and wherein said ratio, when close to 1, indicates the absence of 5-methyl cytosine in said first DNA target sequence,

thereby obtaining the methylation status of the at least one CpG sequence comprised in said first DNA target sequence.

In another embodiment, when two CpGs are present in said first and second DNA target sequences, respectively, in the genome of a cell, the present invention relates to a method to detect the methylation status of each CpGs comprising at least one of the steps of:

(a) synthesizing a first nucleic acid encoding (i) a TALE array specific for said first DNA target sequence comprising a plurality of TALE repeat sequences containing each one a Repeat Variable Diresidue region (RVD) which is responsible for the binding of one specific nucleotide in said first DNA target sequence wherein said TALE DNA binding domain comprises one or more RVD selected from the group consisting of X*and ** for binding cytosine or 5-methyl-cytosine wherein X represents one amino acid residue selected from the group of A,G, V, L, I, M, S, T, C, P, D, E, F, Y, W, Q, N, H, R and K and * represents a gap in one position of the RVD, (ii) a first subdomain of two of a reporter protein wherein said reporter protein is only active when said first and second subdomains interact,

(b) synthesizing a second nucleic acid encoding (i) a TALE array specific for said first DNA target sequence comprising a plurality of TALE repeat sequences containing each one a Repeat Variable Diresidue region (RVD) which is responsible for the binding of one specific nucleotide in said first DNA target sequence wherein said TALE DNA binding

domain comprises one or more RVD HD for binding cytosine, (ii) a first subdomain of two of a reporter protein wherein said reporter protein is only active when said first and second subdomains interact,

(c) synthesizing a third nucleic acid encoding (i) a TALE array specific for said second DNA target sequence comprising a plurality of TALE repeat sequences containing each one a Repeat Variable Diresidue region (RVD) which is responsible for the binding of one specific nucleotide in said second DNA target sequence wherein said TALE DNA binding domain comprises one or more RVD selected from the group consisting of X* and ** for binding cytosine or 5-methyl-cytosine wherein X represents one amino acid residue selected from the group of A,G, V, L, I, M, S, T, C, P, D, E, F, Y, W, Q, N, H, R and K and * represents a gap in one position of the RVD, (ii) a second subdomain of two of a reporter protein wherein said reporter protein is only active when said first and second subdomains interact,

(d) synthesizing a fourth nucleic acid encoding (i) a TALE array specific for said second DNA target sequence comprising a plurality of TALE repeat sequences containing each one a Repeat Variable Diresidue region (RVD) which is responsible for the binding of one specific nucleotide in said second DNA target sequence wherein said TALE DNA binding domain comprises one or more RVD HD for binding cytosine, (ii) a second subdomain of two of a reporter protein wherein said reporter protein is only active when said first and second subdomains interact,

(e) introducing said first and third nucleic acids into said cell, thereby obtaining a first and a third nucleic acids encoding TALE arrays which bind said first and second DNA target sequences when expressed in appropriate conditions and transmits a reporter protein signal independently of the cytosine methylation status of said first DNA target,

(f) introducing said second and third nucleic acids into said cell, thereby obtaining a second and a third nucleic acids encoding TALE arrays which bind said first and second DNA target sequences when expressed in appropriate conditions and transmits a reporter protein signal when 5-methyl-cytosine is absent of said first DNA target,

(g) introducing said first and fourth nucleic acids into said cell, thereby obtaining a first and a fourth nucleic acids encoding TALE arrays which bind said first and second DNA target sequences when expressed in appropriate conditions and transmits a reporter protein signal when 5-methyl-cytosine is absent of said second DNA target,

(h) determining a ratio : reporter protein signal of (f)/ reporter protein signal of (e), wherein said ratio, when close to 0, indicates the presence of 5-methyl cytosine in said first DNA target sequence and wherein said ratio, when close to 1, indicates the absence of 5-methyl cytosine in said first DNA target sequence.

(i) determining a ratio : reporter protein signal of (g)/ reporter protein signal of (e), wherein said ratio, when close to 0, indicates the presence of 5-methyl cytosine in said second DNA target sequence and wherein said ratio, when close to 1, indicates the absence of 5-methyl cytosine in said second DNA target sequence,

thereby obtaining the methylation status of the two CpG sequences comprised in said first and second DNA target sequences, respectively.

In another embodiment, said first and second subdomains of a reporter protein according to the present invention can be subdomains of fluorescent proteins, luciferase, β-galactosidase, and β-lactamase as non-limiting examples or other reporter proteins which are usable in systems such as split systems known in the art.

In another embodiment, the cell targeted or modified by the methods of the present invention is a eukaryotic cell preferably a mammalian cell or a plant cell. In another embodiment, the cell targeted or modified by the methods of the present invention is an algae cell.

In another embodiment, the DNA sequence targeted or modified by the methods of the present invention is a chromosomal sequence or an episomal sequence. In another embodiment, said sequence is an organelle sequence.

In another embodiment, said methods of the present invention can be used to generate animals or plants wherein a targeted double-stranded break occurred.

Other definitions

- Amino acid residues in a polypeptide sequence are designated herein according to the one-letter code, in which, for example, Q means Gln or Glutamine residue, R means Arg or Arginine residue and D means Asp or Aspartic acid residue.

- Amino acid substitution means the replacement of one amino acid residue with another, for instance the replacement of an Arginine residue with a Glutamine residue in a peptide sequence is an amino acid substitution.

- DNA or nucleic acid processing activity refers to a particular / given enzymatic activity of a protein domain comprised in a chimeric protein or a polypeptide according to the invention such as in the expression "a protein domain to process the nucleic acid within or adjacent to the nucleic acid target sequence". Said DNA or nucleic acid processing activity can refer to a cleavage activity, either a cleavase activity either a nickase activity, more broadly a nuclease activity but also a polymerase activity, a kinase activity, a phosphatase activity, a methylase activity, a topoisomerase activity, an integrase activity, a transposase activity, a ligase, a helicase or recombinase activity as non-limiting examples.

- Nucleotides or nucleic acid base are designated as follows: one-letter code is used for designating the base of a nucleoside: a is adenine, t is thymine, c is cytosine, and g is guanine. For the degenerated nucleotides, r represents g or a (purine nucleotides), k represents g or t, s represents g or c, w represents a or t, m represents a or c, y represents t or c (pyrimidine nucleotides), d represents g, a or t, v represents g, a or c, b represents g, t or c, h represents a, t or c, and n represents g, a, t or c.

- by "peptide linker" or "peptidic linker" it is intended to mean a peptide sequence which allows the connection of different monomers or different parts comprised in a fusion protein such as between a TALE DNA binding domain and a protein domain in a chimeric protein or a polypeptide according to the present invention and which allows the adoption of a correct conformation for said chimeric protein activity and/or specificity. Peptide linkers can be of various sizes, from 3 amino acids to 50 amino acids as a non limiting indicative range. Peptide linkers can also be qualified as structured or unstructured. Peptide linkers can be qualified as active linkers when they comprise active domains that are able to change their structural conformation under appropriate stimulation.

- by "subdomain" it is intended a protein subdomain or a protein part that interacts with another protein subdomain or protein part to form an active entity and / or a catalytic active entity bearing nucleic acid or DNA processing activity of said chimeric protein or polypeptide according to the invention.

- by "DNA target", "DNA target sequence", "target DNA sequence", "nucleic acid target sequence", "target sequence" , or "processing site" is intended a polynucleotide sequence that can be bound and/or processed by a TALE derived protein or chimeric protein according to the present invention. These terms refer to a specific nucleic acid location, preferably a genomic location in a cell, but also a portion of genetic material that can exist independently to the main body of genetic material such as plasmids, episomes, virus, transposons or in organelles such as mitochondria or chloroplasts as non-limiting examples. The nucleic acid target sequence is defined by the 5' to 3' sequence of one strand of said target.

- Adjacent is used to qualify the second nucleic acid sequence recognized and bound by a set of specific RVDs comprised in the TALE DNA binding domain of a polypeptide or a chimeric protein according to the present invention, compared to a first nucleic acid sequence recognized and bound by another set of specific RVDs comprised in the TALE DNA binding domain of a polypeptide or a chimeric protein according to the present invention, both sequences possibly surrounds a spacer sequence wherein a protein domain of a chimeric protein according to the present invention, process the targeted DNA spacer. Said nucleic acid sequences can be adjacent and located on a different DNA strand.

- By " delivery vector" or " delivery vectors" is intended any delivery vector which can be used in the present invention to put into cell contact (i.e "contacting") or deliver inside cells or subcellular compartments agents/chemicals and molecules (proteins or nucleic acids) needed in the present invention. It includes, but is not limited to liposomal delivery vectors, viral delivery vectors, drug delivery vectors, chemical carriers, polymeric carriers, lipoplexes, polyplexes, dendrimers, microbubbles (ultrasound contrast agents), nanoparticles, emulsions or other appropriate transfer vectors. These delivery vectors allow delivery of molecules, chemicals, macromolecules (genes, proteins), or other vectors such as plasmids, peptides developed by Diatos. In these cases, delivery vectors are molecule carriers. By "delivery vector" or "delivery vectors" is also intended delivery methods to perform transfection.

- The terms "vector" or "vectors" refer to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. A "vector" in the present invention includes, but is not limited to, a viral vector, a plasmid, a RNA vector or a linear or circular DNA or RNA molecule which may consists of a chromosomal, non chromosomal, semi-synthetic or synthetic nucleic acids. Preferred vectors are those capable of autonomous

replication (episomal vector) and/or expression of nucleic acids to which they are linked (expression vectors). Large numbers of suitable vectors are known to those of skill in the art and commercially available.

Viral vectors include retrovirus, adenovirus, parvovirus (e. g. adenoassociated viruses), coronavirus, negative strand RNA viruses such as orthomyxovirus (e. g., influenza virus), rhabdovirus (e. g., rabies and vesicular stomatitis virus), paramyxovirus (e. g. measles and Sendai), positive strand RNA viruses such as picornavirus and alphavirus, and double-stranded DNA viruses including adenovirus, herpesvirus (e. g., Herpes Simplex virus types 1 and 2, Epstein-Barr virus, cytomegalovirus), and poxvirus (e. g., vaccinia, fowlpox and canarypox). Other viruses include Norwalk virus, togavirus, flavivirus, reoviruses, papovavirus, hepadnavirus, and hepatitis virus, for example. Examples of retroviruses include: avian leukosis-sarcoma, mammalian C-type, B-type viruses, D type viruses, HTLV-BLV group, lentivirus, spumavirus (Coffin, J. M., Retroviridae: The viruses and their replication, In Fundamental Virology, Third Edition, B. N. Fields, et al., Eds., Lippincott-Raven Publishers, Philadelphia, 1996).

- By "lentiviral vector" is meant HIV-Based lentiviral vectors that are very promising for gene delivery because of their relatively large packaging capacity, reduced immunogenicity and their ability to stably transduce with high efficiency a large range of different cell types. Lentiviral vectors are usually generated following transient transfection of three (packaging, envelope and transfer) or more plasmids into producer cells. Like HIV, lentiviral vectors enter the target cell through the interaction of viral surface glycoproteins with receptors on the cell surface. On entry, the viral RNA undergoes reverse transcription, which is mediated by the viral reverse transcriptase complex. The product of reverse transcription is a double-stranded linear viral DNA, which is the substrate for viral integration in the DNA of infected cells.

- By "integrative lentiviral vectors (or LV)", is meant such vectors as non limiting example, that are able to integrate the genome of a target cell.

- At the opposite by "non integrative lentiviral vectors (or NILV)" is meant efficient gene delivery vectors that do not integrate the genome of a target cell through the action of the virus integrase.

One type of preferred vector is an episome, i.e., a nucleic acid capable of extra-chromosomal replication. Preferred vectors are those capable of autonomous replication and/or expression of nucleic acids to which they are linked. Vectors capable of directing the expression of genes to which they are operatively linked are referred to herein as "expression vectors. A vector according to the present invention comprises, but is not limited to, a YAC (yeast artificial chromosome), a BAC (bacterial artificial), a baculovirus vector, a phage, a phagemid, a cosmid, a viral vector, a plasmid, a RNA vector or a linear or circular DNA or RNA molecule which may consist of chromosomal, non chromosomal, semi-synthetic or synthetic DNA. In general, expression vectors of utility in recombinant DNA techniques are often in the form of "plasmids" which refer generally to circular double stranded DNA loops which, in their vector form are not bound to the chromosome. Large numbers of suitable vectors are known to those of skill in the art. Vectors can comprise selectable markers, for example: neomycin phosphotransferase, histidinol dehydrogenase, dihydrofolate reductase, hygromycin phosphotransferase, herpes simplex virus thymidine kinase, adenosine deaminase, glutamine synthetase, and hypoxanthine-guanine phosphoribosyl transferase for eukaryotic cell culture; TRP1 for *S. cerevisiae*; tetracyclin, rifampicin or ampicillin resistance in *E. coli*. Preferably said vectors are expression vectors, wherein a sequence encoding a polypeptide of interest is placed under control of appropriate transcriptional and translational control elements to permit production or synthesis of said polypeptide. Therefore, said polynucleotide is comprised in an expression cassette. More particularly, the vector comprises a replication origin, a promoter operatively linked to said encoding polynucleotide, a ribosome binding site, a RNA-splicing site (when genomic DNA is used), a polyadenylation site and a transcription termination site. It also can comprise an enhancer or silencer elements. Selection of the promoter will depend upon the cell in which the polypeptide is expressed. Suitable promoters include tissue specific and/or inducible promoters. Examples of inducible promoters are: eukaryotic metallothionine promoter which is induced by increased levels of heavy metals, prokaryotic lacZ promoter which is induced in response to isopropyl-β-D-thiogalacto-pyranoside (IPTG) and eukaryotic heat shock promoter which is induced by increased temperature. Examples of tissue specific promoters are skeletal muscle creatine kinase, prostate-specific antigen (PSA), α-antitrypsin protease, human surfactant (SP) A and B proteins, β-casein and acidic whey protein genes.

- Inducible promoters may be induced by pathogens or stress, more preferably by stress like cold, heat, UV light, or high ionic concentrations (reviewed in Potenza C et al. 2004, In vitro Cell Dev Biol 40:1-22). Inducible promoter may be induced by chemicals (reviewed in (Moore, Samalova et al. 2006); (Padidam 2003); (Wang, Zhou et al. 2003); (Zuo and Chua 2000).

Delivery vectors and vectors can be associated or combined with any cellular permeabilization techniques such as sonoporation or electroporation or derivatives of these techniques.

- By cell or cells is intended any prokaryotic or eukaryotic living cells, cell lines derived from these organisms for *in vitro* cultures, primary cells from animal or plant origin.

- By "primary cell" or "primary cells" are intended cells taken directly from living tissue (i.e. biopsy material) and established for growth in vitro, that have undergone very few population doublings and are therefore more representative of the main functional components and characteristics of tissues from which they are derived from, in comparison to continuous tumorigenic or artificially immortalized cell lines. These cells thus represent a more valuable model to the in vivo state they refer to.

- In the frame of the present invention, "eukaryotic cells" refer to a fungal, plant or animal cell or a cell line derived from the organisms listed below and established for in vitro culture. More preferably, the fungus is of the genus Aspergillus, Penicillium, Acremonium, Trichoderma, Chrysoporium, Mortierella, Kluyveromyces or Pichia; More preferably, the fungus is of the species Aspergillus niger, Aspergillus nidulans, Aspergillus oryzae, Aspergillus terreus, Penicillium chrysogenum, Penicillium citrinum, Acremonium Chrysogenum, Trichoderma reesei, Mortierella alpine, Chrysosporium lucknowense, Kluyveromyces lactis, Pichia pastoris or Pichia ciferrii.

More preferably the plant is of the genus Arabidospis, Nicotiana, Solanum, lactuca, Brassica, Oryza, Asparagus, Pisum, Medicago, Zea, Hordeum, Secale, Triticum, Capsicum, Cucumis, Cucurbita, Citrullis, Citrus, Sorghum; More preferably, the plant is of the species Arabidospis thaliana, Nicotiana tabaccum, Solanum lycopersicum, Solanum tuberosum, Solanum melongena, Solanum esculentum, Lactuca saliva, Brassica napus, Brassica oleracea, Brassica rapa, Oryza glaberrima, Oryza sativa, Asparagus officinalis, Pisum sativum, Medicago sativa, zea mays, Hordeum vulgare, Secale cereal, Triticum aestivum, Triticum durum, Capsicum

sativus, Cucurbita pepo, Citrullus lanatus, Cucumis melo, Citrus aurantifolia, Citrus maxima, Citrus medica, Citrus reticulata.

More preferably the animal cell is of the genus Homo, Rattus, Mus, Sus, Bos, Danio, Canis, Felis, Equus, Salmo, Oncorhynchus, Gallus, Meleagris, Drosophila, Caenorhabditis; more preferably, the animal cell is of the species Homo sapiens, Rattus norvegicus, Mus musculus, Sus scrofa, Bos taurus, Danio rerio, Canis lupus, Felis catus, Equus caballus, Salmo salar, Oncorhynchus mykiss, Gallus gallus, Meleagris gallopavo, Drosophila melanogaster, Caenorhabditis elegans.

In the present invention, the cell can be a plant cell, a mammalian cell, a fish cell, an insect cell or cell lines derived from these organisms for *in vitro* cultures or primary cells taken directly from living tissue and established for *in vitro* culture. As non limiting examples cell lines can be selected from the group consisting of CHO-K1 cells; HEK293 cells; Caco2 cells; U2-OS cells; NIH 3T3 cells; NSO cells; SP2 cells; CHO-S cells; DG44 cells; K-562 cells, U-937 cells; MRC5 cells; IMR90 cells; Jurkat cells; HepG2 cells; HeLa cells; HT-1080 cells; HCT-116 cells; Hu-h7 cells; Huvec cells; Molt 4 cells. Are also encompassed in the scope of the present invention stem cells and induced Pluripotent Stem cells (iPS).

All these cell lines can be modified by the method of the present invention to provide cell line models to produce, express, quantify, detect, study a gene or a protein of interest; these models can also be used to screen biologically active molecules of interest in research and production and various fields such as chemical, biofuels, therapeutics and agronomy as non-limiting examples.

- by "mutation" is intended the substitution, deletion, insertion of one or more nucleotides/amino acids in a polynucleotide (cDNA, gene) or a polypeptide sequence. Said mutation can affect the coding sequence of a gene or its regulatory sequence. It may also affect the structure of the genomic sequence or the structure/stability of the encoded mRNA.

- In the frame of the present invention, the expression "double-strand break-induced mutagenesis" (DSB-induced mutagenesis) refers to a mutagenesis event consecutive to an

NHEJ event following an endonuclease-induced DSB, leading to insertion/deletion at the cleavage site of an endonuclease.

- By "gene" is meant the basic unit of heredity, consisting of a segment of DNA arranged in a linear manner along a chromosome, which codes for a specific protein or segment of protein. A gene typically includes a promoter, a 5' untranslated region, one or more coding sequences (exons), optionally introns, a 3' untranslated region. The gene may further comprise a terminator, enhancers and/or silencers.

- As used herein, the term "locus" is the specific physical location of a DNA sequence (e.g. of a gene) on a chromosome. The term "locus" usually refers to the specific physical location of a polypeptide or chimeric protein's nucleic target sequence on a chromosome. Such a locus can comprise a target sequence that is recognized and/or cleaved by a polypeptide or a chimeric protein according to the invention. It is understood that the locus of interest of the present invention can not only qualify a nucleic acid sequence that exists in the main body of genetic material (i.e. in a chromosome) of a cell but also a portion of genetic material that can exist independently to said main body of genetic material such as plasmids, episomes, virus, transposons or in organelles such as mitochondria or chloroplasts as non-limiting examples.

- By "fusion protein" is intended the result of a well-known process in the art consisting in the joining of two or more genes which originally encode for separate proteins or part of them, the translation of said "fusion gene" resulting in a single polypeptide with functional properties derived from each of the original proteins.

- By "chimeric protein" according to the present invention is meant any fusion protein comprising at least one RVD to bind a nucleic acid sequence and one protein domain to process a nucleic acid target sequence within or adjacent to said bound nucleic acid sequence.

- By "protein domain" is meant the nucleic acid target sequence processing part of said chimeric protein according to the present invention. Said protein domain can provide any catalytical activity as classified and named according to the reaction they catalyze [Enzyme Commission number (EC number) at

http://www.chem.qmul.ac.uk/iubmb/enzyme/)]. Said protein domain can be a catalytically active entity by itself. Said protein domain can be a protein subdomain that needs to interact with another protein subdomain to form a dimeric protein domain active entity.

- By a "TALE-nuclease" (TALEN) is intended a fusion protein consisting of a DNA-binding domain derived from a Transcription Activator Like Effector (TALE) and one nuclease catalytic domain to cleave a nucleic acid target sequence. Said TALE-nuclease is a subclass of chimeric protein according to the present invention.

- by "variant(s)", it is intended a RVD variant, a chimeric protein variant, a DNA binding variant, a TALE-nuclease variant, a polypeptide variant obtained by replacement of at least one residue in the amino acid sequence of the parent molecule.

- by "functional mutant" is intended a catalytically active mutant of a protein or a protein domain; such mutant can have the same activity compared to its parent protein or protein domain or additional properties. This definition applies to chimeric proteins or protein domains that constitute chimeric proteins according to the present invention. Are also encompassed in the scope of this definition "derivatives" of these proteins or protein domains that comprise the entirety or part of these proteins or protein domains fused to other proteic or chemical parts such as tags, antibodies, polyethylene glycol as non-limiting examples.

- "identity" refers to sequence identity between two nucleic acid molecules or polypeptides. Identity can be determined by comparing a position in each sequence which may be aligned for purposes of comparison. When a position in the compared sequence is occupied by the same base, then the molecules are identical at that position. A degree of similarity or identity between nucleic acid or amino acid sequences is a function of the number of identical or matching nucleotides at positions shared by the nucleic acid sequences. Various alignment algorithms and/or programs may be used to calculate the identity between two sequences, including FASTA, or BLAST which are available as a part of the GCG sequence analysis package (University of Wisconsin, Madison, Wis.), and can be used with, e.g., default setting.

The above written description of the invention provides a manner and process of making and using it such that any person skilled in this art is enabled to make and use the same, this enablement being provided in particular for the subject matter of the appended claims, which make up a part of the original description.

As used above, the phrases "selected from the group consisting of," "chosen from," and the like include mixtures of the specified materials.

Where a numerical limit or range is stated herein, the endpoints are included. Also, all values and subranges within a numerical limit or range are specifically included as if explicitly written out.

The above description is presented to enable a person skilled in the art to make and use the invention, and is provided in the context of a particular application and its requirements. Various modifications to the preferred embodiments will be readily apparent to those skilled in the art, and the generic principles defined herein may be applied to other embodiments and applications without departing from the spirit and scope of the invention. Thus, this invention is not intended to be limited to the embodiments shown, but is to be accorded the widest scope consistent with the principles and features disclosed herein.

Having generally described this invention, a further understanding can be obtained by reference to certain specific examples, which are provided herein for purposes of illustration only, and are not intended to be limiting unless otherwise specified.

Examples

Example 1

To investigate the sensitivity of TAL repeats domain to CpG methylation an engineered TAL nuclease model named XPCT1 (or XPC4T3) was specifically designed to bind and cleave *xpc1* locus (also named xpc4) (SEQ ID NO: 1) containing one methylated CpG. XPCT1 TALE-nuclease was composed of two independent entities XPCT1L (XPCT4T3.3) and XPCT1R (XPC4T3.4), each containing a TALE-derived DNA binding domain fused to the catalytic domain of the FokI restriction enzyme. XPCT1L and XPCT1R were engineered to bind to two DNA target sequences (Left and Right targets respectively) separated by a 11 bp spacer sequence (*xpc1* locus, figures 3A and B). Binding of XPCT1L and XPCT1 R to *xpc1* locus was expected to allow FokI to dimerize and create a double-strand break within the spacer.

The abilities of RVD HD and N* to bind to 5-methyl-cytosine located at position +2 of the Left target (figure 3A in red) were compared by engineering two variants of XPCT1L containing either RVD HD or RVD N* in position +2 of the TALE repeat stretch (figure 3B). Each of these two variants were coupled with their counterpart XPCT1R and the nuclease activity of the resulting TALE-nucleases named XPCT1_HD (XPC4T3_HD) or XPCT1_N* (XPC4T3_N*) was determined according to four different protocols (see Material and Methods section for details).

Briefly, the first and second protocols consisted in determining the nuclease activities of XPCT1_HD and XPCT1_N* in yeast and mammalian cells according to the protocol described respectively in Epinat et al. 2003 and Arnould et al. 2006, using an extrachromosomal target containing the unmethylated *xpc1* locus whereas, the third and fourth protocols consisted in determining and comparing their nuclease activities toward the methylated endogenous *xpc1* locus in mammalian cells. Nuclease activities were assessed by T7 nuclease assay (6) or by deep sequencing.

Material and methods

-Tal repeats array assembly and subcloning into yeast and mammalian expression plasmids

The TAL repeats arrays XPCT1L_HD, XPCT1L_N* and XPCT1R (SEQ ID NO: 2, SEQ ID NO: 3 and SEQ ID NO: 4, respectively, encoding SEQ ID NO: 14, SEQ ID NO: 15 and SEQ ID NO: 16) were

synthesized using a solid support method consisting in a sequential assembly of TAL repeats through consecutive restriction/ligation/washing steps as shown in figure 4. Briefly, as an example, to assemble XPCT1L_HD repeats array, the first TAL repeat (SEQ ID NO: 5 encoding SEQ ID NO: 17) was immobilized on a solid support through biotin/streptavidin interaction, digested by SfaNI type IIS restriction endonuclease and then ligated to a second TAL repeat (SEQ ID NO: 5 encoding SEQ ID NO: 17) harboring SfaNI compatible overhangs at its 5' end (figure 4B). The resulting TAL repeats array (i.e containing TAL repeats 1 and 2) was then used as template for subsequent additions of the appropriate TAL repeats (SEQ ID NO: 6-9, encoding SEQ ID NO: 18-21 for NI, NN, respectively targeting nucleotides A, G and HD, N* respectively targeting nucleotides C) to generate the complete TAL repeats arrays XPCT1L_HD or N* according to the same protocol (figure 4C). The complete TAL repeats array was finally digested by SfaNI to generate SfaNI overhangs at its 3' end (figure 4D) and then striped of the solid support using BbvI type IIS restriction endonuclease (figure 4E). The digested TAL repeats array was recovered and subcloned into yeast or mammalian expression plasmids harboring the Nterminal domain of AvrBs3 TAL effector and the eleven first amino acids of its Cterminal domain fused to FokI type IIS restriction endonuclease (pCLS 7802 and pCLS 11170, i.e. SEQ ID NO: 10 and SEQ ID NO: 11 respectively encoding SEQ ID NO: 22 and SEQ ID NO: 23, figure 4F). pCLS7802 was derived from pCLS0542 (SEQ ID NO: 24) using NcoI and XhoI restriction sites and pCLS11170 was derived from pCLS8391(SEQ ID NO: 25) using NcoI and EagI restriction sites.

-Cells culture and transfections

Human 293H cells (Life Technologies, Carlsbad, CA) and hamster CHO-KI cells (ATCC) were cultured at 37°C with 5 % CO2 in complete medium DMEM or F12-K respectively, supplemented with 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B (Fongizone, Life Technologies,) and 10 % FBS. Concerning the extrachromosomal assays, CHO-KI cells were plated at 2500 cells per well in 96 wells plate. The next day, cells were transfected with an increasing amount of DNA (from 0.04 to 50 ng total) using Polyfect transfection reagent (Qiagen) according to the manufacturer's protocol. Concerning the mutagenesis assays, 293H cells were plated at a density of 1.2×10^6 cells per 10 cm dish. The next day, cells were transfected with 2, 5 or 10 µg of DNA using

Lipofectamine 2000 transfection reagent (Life Technologies) according to the manufacturer's protocol.

Monitoring TALE-nuclease extrachromosomal SSA activity

CHO-KI cells were plated at 2500 cells per well in 96 wells plate. The next day, cells were cotransfected by increasing amounts of DNA encoding XPC TALE-nuclease (from 0 to 25 ng each) and a constant amount of XPC extrachromosomal unmethylated target (75ng) using polyfect transfection reagent (Qiagen) according to the manufacturer's protocol. TALE-nucleases single strand annealing (SSA) activities were determined according to the protocol described in (19,20).

-Monitoring of targeted modification induced by XPCT1 TALE-nucleases via deep sequencing or T7 nuclease assay

To evaluate the ability of different XPC TALE-nucleases to induce Targeted Mutagenesis (TM) at their endogenous loci, 293H cells were first plated at a density of 1.2 x 10^6 cells per 10 cm dish. The next day, cells were transfected with a total amount of 2, 5 or 10 µg of TALE-nuclease expressing vector or empty vector using Lipofectamine 2000 transfection reagent (Life Technologies) according to the manufacturer's protocol. Two or three days post-transfection, genomic DNA was extracted and the loci of interest were amplified with locus specific primers (respectively XPCMID1_F, SEQ ID NO: 12 and XPC_R, SEQ ID NO: 13) linked to adaptor sequences needed for deep sequencing method. Amplicons were analyzed either by EndoT7 assay according to the protocol described in (21) or by deep sequencing using the 454 system (Life Sciences, an average of 5000 sequences per sample were analyzed).

Results

Our results showed that XPCT1_HD or XPCT1_N* TALE-nucleases displayed similar nuclease activities toward an XPC1 unmethylated extrachromosomal DNA target in yeast and mammalian cells with a slight advantage for XPCT1_HD TALE-nuclease (data not shown and figure 5A). In stark contrast, when the two TALE-nucleases were assayed at the endogenous methylated locus, XPCT1_N* was the only one showing detectable nuclease activity as seen by the presence of T7 nuclease digestion band (figure 5B, red stars). Accordingly, the frequency of targeted modification (TM) induced by XPCT1_N* was much higher than the

one induced by XPCT1_HD TALE-nuclease which was almost undetectable under our best experimental conditions (17.2 % and 0.8 % respectively, figure 5C). Differences of nuclease activity observed between the two TALE-nucleases were not due to variation of transfection efficiency from one TALE-nuclease to another (data not shown). Taken together, our results showed that TAL DNA binding domain using RVD HD to target cytosine are sensitive to cytosine methylation and that such sensitivity can be overcome by substituting RVD HD by RVD N*.

Example 2 : Ability of naturally occurring TAL repeats H* and NG to overcome TAL DNA binding domain sensitivity to 5-methyl-cytosine.

We hypothesized that naturally occuring TAL repeats, other than TAL repeat N*, either lacking the glycine 13 or harboring small side chain residues at the same position, could efficiently bind 5-methyl-cytosine. To confirm this, we assessed the ability of TAL repeats H* and NG to substitute HD in position +2 of XPCT1 TAL DNA binding domain (figure 6A) and rescue its activity toward its endogenous methylated locus in 293H cells (SEQ ID NO: 1).

Material and Methods

-Materials

TALE-nucleases XPCT1L-HD, XPCT1L-N*, XPCT1L-NG, XPCT1L-H* and XPCT1R (SEQ ID NO: 26-30 respectively encoding SEQ ID NO: 38-42) were obtained according to the method described in earlier examples. Active TALE-nucleases were formed by a combination of one "TALE-nuclease L" (XPCT1L-HD, XPCT1L-N*, XPCT1L-NG or XPCT1L-H*) and one "TALE-nuclease R" (XPCT1 R).

See example 1 for monitoring TALE-nuclease extrachromosomal SSA activity and monitoring of TALE-nuclease-induced Targeted Mutagenesis methods

-Toxicity assay

The CHO-KI cell line was transfected in 96 wells plate as described above, with increasing amounts of TALE-nuclease expression vectors and a constant amount of GFP-encoding plasmid. GFP levels were monitored by flow cytometry (Guava EasyCyte, Guava

Technologies) 1 and 6 days post-transfection. Cell survival was calculated as a ratio (TALE-nuclease-transfected cells expressing GFP at Day 6/control transfected cells expressing GFP at Day 6). Ratios were corrected for the transfection efficiency determined at Day 1 and plotted as a function of final concentration of DNA transfected. Toxic and non-toxic controls were used in each experiment (19).

Results

To first control whether substitution of HD to H* and NG affected the intrinsic nuclease activity of XPCT1, we performed a single strand annealing (SSA) assay in Chinese Hamster Ovary (CHO) cells (19), using an unmethylated extrachromosomal XPC1 target (SEQ ID NO: 1) and XPCT1-HD and N* as controls (figure 6B). Our results showed that the XPCT1-N* and H* TALE-nucleases (SEQ ID NO: 39 with SEQ ID NO: 42 and SEQ ID NO: 41 with SEQ ID NO: 42) displayed similar SSA activities and was slightly less active than XPCT1-HD (SEQ ID NO: 38 with SEQ ID NO: 42, figure 6B). On another hand, XPCT1-NG (SEQ ID NO: 40 with SEQ ID NO: 42) displayed a marked decrease of activity with respect to XPCT1-HD, consistent with the poor ability of NG to recognize cytosine (3,4). We then assessed the ability of these TALE-nucleases to disrupt the endogenous methylated XPC1 target in 293H cells by targeted mutagenesis (TM). TALE-nuclease-induced TM, consisting of small insertion or deletion of nucleotide generated via imprecise non-homologous end joining, was determined by an endoT7 assay and by deep sequencing as described previously (21,22). Our results showed that both TAL repeats H* and NG could rescue XPCT1 activity, with a clear advantage for H*, which was almost as efficient as N* (figure 6C). We thus conclude that although small amino acids in position 13 can accommodate 5-methyl-cytosine, complete absence of such amino acids, the hallmark of the TAL repeat "*", leads to more proficient 5-methyl-cytosine recognition.

We verified that HD to N*, HD to H* or HD to NG substitutions within TAL DNA binding domains of XPCT1L, did not increase TALE-nuclease-induced toxicity in CHO cells using the protocol described by Grizot *& al.* (19). For all TALE-nucleases tested, we found that the presence of TAL repeats N*, H* or NG in position 2 of the TAL DNA binding domain of XPCT1L, did not influence its toxicity as seen by similar cell survival patterns obtained between HD, N*, H* and NG variants (figure 6D)

Example 3: TAL repeat N*, a universal 5-methyl-cytosine binding module.

To evaluate the ability of TAL repeat N* to overcome TAL DNA binding domain sensitivity to 5-methyl-cytosine in different contexts (i.e at other endogenous methylated targets), we engineered two other TALE-nucleases, XPCT2 and XPCT3, specifically designed to process the methylated endogenous XPC targets called XPC2 and XPC3 (SEQ ID NO: 50 and SEQ ID NO: 51). These targets contained respectively one and two 5-methyl-cytosine located at different positions (figure 7A), making it possible to evaluate the influence of the number and position of N* repeats in a TALE DNA binding domain.

Material and Methods

See examples 1 and 2 for methods

-Materials

TALE-nucleases XPCT2L-HD, XPCT2L-N*, XPCT2R, XPCT3L-HD, XPCT3L-N*, XPCT3R-HD and XPCT3R-N* (SEQ ID NO 31-37 respectively encoding SEQ ID NO: 43-49) were obtained according to the method described in earlier examples. Active TALE-nucleases were formed by a combination of one "TALE-nuclease L" and one "TALE-nuclease R" as described in example 1.

Results

TALE-nuclease activities of XPCT2-N* and XPCT3-N* (figure 7) were determined in 293H cells according to the protocol described in example 1, and then compared to their HD counterparts (figure 7B). XPCT1-HD and N* (see example 2) was used as a control in the experiment described below. Our EndoT7 assays showed that N* variants were always the most active, indicating that TAL repeat N* is able to successfully bind 5-methyl-cytosine in different contexts. Interestingly, the basal activities of TALE-nucleases-HD and the fold induction achieved by HD/N* substitution, were different form one TALE-nuclease to another, suggesting that the binding penalty induced by 5-methyl-cytosine depends on its position within TAL DNA binding site.

We verified that HD/N* substitution within TAL DNA binding domains, did not increase TALE-nuclease-induced toxicity in CHO cells using the protocol described by Grizot *& al* (19). For all TALE-nucleases tested, we found that the presence of single or multiple TAL N* repeats did not influence TALE-nuclease-induced toxicity as seen by similar cell survival patterns obtained between HD and N* variants of XPCT2 and T3 (figure 7C and 7D respectively). In full agreement with their lack of toxicity, TALE-nucleases-N* displayed similar TM frequencies in 293H cells, 3 or 7 days post transfection (data not shown). Consistent with this absence of toxicity, naturally occurring TAL effectors were reported to bear up to 20% of TAL repeat N*50 within their DNA binding domain while retaining high specificity. Therefore, taken together, our results showed that the TAL repeat N* could be used as a universal 5-methyl-cytosine binding module without affecting toxicity of engineered TAL DNA binding domains.

In summary, our work unraveled the hidden cipher governing 5-methyl-cytosine recognition by TAL repeats N*, H* and NG. Based on this finding, we present a simple, efficient and universal method to overcome TALE DNA binding domain sensitivity to cytosine methylation. Such method presents three major advantages. First, it allows one to bypass the need for chemical demethylation of endogenous targets which is unsuitable for cell engineering and therapeutic applications. Second, it is readily applicable to all TAL derived proteins, and in particular, to engineered transcription activators, thus potentially enabling site specific activation of methylated promoters responsible for genes silencing. Third, it is transposable to the broad range of cellular systems including ES, iPS mammalian cells and plant cells that have already been shown to be engineerable with TALE-nuclease technology.

Example 4: Ability of engineered TAL repeats T*, Q* and natural TAL repeat HG to overcome TAL DNA binding domain sensitivity to 5-mC.

We hypothesized that engineered TAL repeats "*", namely T* and Q* and natural TAL repeat HG could efficiently bind 5mC. To confirm this, we assessed the ability of TAL repeats T*, Q* and HG to substitute HD in position +2 of XPCT1 TAL DNA binding domain and to rescue its activity toward its endogenous methylated locus in 293H cells.

Material and Methods

See examples 1 to 3 for methods

TALE-nucleases XPCT1L-T*, XPCT1L-Q*, XPCT1L-HG and XPCT1R (SEQ ID NO: 52, 53, 54, and 30 respectively encoding SEQ ID NO: 55, 56, 57 and 42) were obtained according to the method described in earlier examples or else, by de *novo gene* synthesis. Active TALE-nucleases were formed by a combination of one "TALE-nuclease L" (XPCT1L-T*, XPCT1L-Q* or XPCT1L-HG) and one "TALE-nuclease R" (XPCT1 R). The nuclease activity of TALE-nucleases XPCT1L-HD, N*, NG, and H*(SEQ ID NO: 26-29 respectively encoding SEQ ID NO: 38-41) were also determined and used here as control experiments

See example 1 for a comprehensive description of the monitoring of TALE-nuclease-induced Targeted Mutagenesis.

Results

TALE-nuclease activities of XPCT1L-T*, XPCT1L-Q* and XPCT1L-HG were determined in 293H cells according to the protocol described in example 1, and then compared to their HD counterparts (figure 8). XPCT1L-HD, N*, NG, and H*(SEQ ID NO: 26-29 respectively encoding SEQ ID NO: 38-41, see example 2) were used as controls in the experiment described below. Our Deep sequencing results showed that T*, and Q* variants were more active than the HD variant, indicating that TAL repeat T*, Q* and HG can bind 5-methyl-Cytosine more efficiently than does HD. Thus, TAL repeat T*, Q* and HG could be potentially used to design TALE-nuclease targeting methylated endogenous loci.

List of cited references

1. Baker, M. Gene-editing nucleases. Nat Methods 9, 23-6 (2012)

2. Bogdanove, A.J. & Voytas, D.F. TAL effectors: customizable proteins for DNA targeting. Science 333, 1843-6 (2011).

3. Boch, J. et al. Breaking the code of DNA binding specificity of TAL-type III effectors. Science 326, 1509-12 (2009).

4. Moscou, M.J. & Bogdanove, A.J. A simple cipher governs DNA recognition by TAL effectors. Science 326, 1501 (2009).

5. Zhang, F. et al. Efficient construction of sequence-specific TAL effectors for modulating mammalian transcription. Nat Biotechnol 29, 149-53 (2011).

6. Miller, J.C. et al. A TALE nuclease architecture for efficient genome editing. Nat Biotechnol 29, 143-8 (2011).

7. Huang, P. et al. Heritable gene targeting in zebrafish using customized TALENs. Nat Biotechnol 29, 699-700 (2011).

8. Sander, J.D. et al. Targeted gene disruption in somatic zebrafish cells using engineered TALENs. Nat Biotechnol 29, 697-8 (2011).

9. Li, T. et al. Modularly assembled designer TAL effector nucleases for targeted gene knockout and gene replacement in eukaryotes. Nucleic Acids Res 39, 6315-25 (2011).

10. Jaenisch, R. & Bird, A. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. Nat Genet 33 Suppl, 245-54 (2003).

11. Ziller, M.J. et al. Genomic distribution and inter-sample variation of non-CpG methylation across human cell types. Plos Genet 7(12):e1002389 (2011)

12. Mak, A.N., Bradley, P., Cernadas, R.A., Bogdanove, A.J. & Stoddard, B.L. The crystal structure of TAL effector PthXo1 bound to its DNA target. Science 335, 716-9 (2012).

13. Epinat, J. C. et al. A novel engineered meganuclease induces homologous recombination in yeast and mammalian cells. Nucleic Acids Res 31(11): 2952-62 (2003).

14. Arnould, S. et al. Engineering of large numbers of highly specific homing endonucleases that induce recombination on novel DNA targets. J Mol Biol 355(3): 443-58 (2006).

15. Grizot, S. et al. Generation of redesigned homing endonucleases comprising DNA-binding domains derived from two different scaffolds. Nucleic Acids Res 38(6): 2006-18 (2009).

16. Bogdanove, A. J., S. Schornack, et al. TAL effectors: finding plant genes for disease and defense. Curr Opin Plant Biol 13(4): 394-401 (2010).

17. Chames, P., J. C. Epinat, et al. In vivo selection of engineered homing endonucleases using double-strand break induced homologous recombination. Nucleic Acids Res 33(20): e178 (2005).

18. Smith, J., S. Grizot, et al. A combinatorial approach to create artificial homing endonucleases cleaving chosen sequences. Nucleic Acids Res (2006).

19. Grizot, S., Smith, J. et al. Efficient targeting of a SCID gene by an engineered single-chain homing endonuclease. Nucleic Acids Res 37(16): 5405-19 (2009)

20. Daboussi, F., Zaslavskiy, M. et al. Chromosomal context and epigenetic mechanisms control the efficacy of genome editing by rare-cutting designer endonucleases. Nucleic Acids Res, Epub ahead of print (2012)

21. Reyon, D., Tsai, S. Q. et al. FLASH assembly of TALENs for high-throughput genome editing. Nat Biotechnol 30(5): 460-5 (2012)

22. Mussolino, C., Morbitzer, R. et al. A novel TALE nuclease scaffold enables high genome editing activity in combination with low toxicity. Nucleic Acids Res 39(21): 9283-93 (2011)

23. Weber, E., Gruetzner, R. et al. Assembly of designer TAL effectors by Golden Gate cloning. Plos One 6, e19722 (2011).

24. Morbitzer, R. Elsaesser, J. et al. Assembly of custom ALE-type DNA binding domains by modular cloning. Nucleic Acids Res 39, 5790-5799 (2011)

CLAIMS

1. A method for synthesizing a transcription activator-like effector (TALE) protein to target nucleic acid sequence comprising a 5-methyl-cytosine , said method comprising assembling a plurality of TALE-like repeat sequences, each of said sequences comprising a repeat variable-diresidue (RVD) specific to each nucleic acid base of said sequence, wherein the RVD(s) that specifically targets the 5-methyl-cytosine within said nucleic acid target sequence are selected from X*, where X represents one amino acid residue selected from the group of A,G, V, L, I, M, S, T, C, P, D, E, F, Y, W, Q, N, H, R and K and * represents a gap in one position of the RVD.

2. The method according to claim 1 wherein said method comprises:

(a) determining a nucleic acid target sequence comprising 5-methyl-cytosine in the genome of a cell;

(b) assembling TALE-like repeat polynucleotide sequences, each repeat being specific to each nucleic acid base of said nucleic acid target sequence by encoding a repeat variable-diresidue (RVD) comprising at least one RVD selected from the group consisting of:

- HD for recognizing C;
- NG for recognizing T;
- NI for recognizing A;
- NN for recognizing G or A;
- NS for recognizing A or C or G or T;
- HG for recognizing T;
- IG for recognizing T;
- NK for recognizing G;
- HA for recognizing C;
- ND for recognizing C;
- HI for recognizing C;
- HN for recognizing G;
- NA for recognizing G;
- SN for recognizing G or A; and
- YG for recognizing T;

wherein said RVD(s) specifically targeting the chemically modified nucleic acid base(s) in the nucleic acid target sequence are selected from the RVDs X* and **, where

- X represents one amino acid residue selected from the group of A, G, V, L, I, M, S, T, C, P, D, E, F, Y, W, Q, N, H, R and K, and

- * represents a gap in one position of the RVD,

(c) expressing said polynucleotide sequence assembled in step b) in said cell.

3. The method according to claim 1 or 2 wherein said RVD specifically targeting the 5-methyl-cytosine(s) are preferentially selected from RVD N*, T*, Q* and H*.

4. A method to synthesize a chimeric protein to process nucleic acid at a locus defined by a nucleic acid target sequence that comprises a 5-methyl-cytosine , said method comprising:

(a) synthesizing a polynucleotide sequence comprising a fusion of:

(i) a first polynucleotide encoding a transcription activator-like effector (TALE) protein comprising a plurality of TALE-like repeat sequences, each repeat comprising a repeat variable-diresidue (RVD) specific to each nucleic acid base of said nucleic acid target sequence, wherein the RVD(s) that specifically targets the 5-methyl-cytosine within said nucleic acid target sequence are selected from X*, preferably N*, Q*, T* or H* where X represents one amino acid residue selected from the group of A,G, V, L, I, M, S, T, C, P, D, E, F, Y, W, Q, N, H, R and K and * represents a gap in one position of the RVD;

(ii) a second polynucleotide encoding an additional protein domain to process nucleic acid within or adjacent to said nucleic acid target sequence that comprises a 5-methyl-cytosine ;

(c) expressing said polynucleotide sequence of step a) into a host cell.

5. The method of claim 4 wherein said additional protein domain is an endonuclease.

6. A method to bind a nucleic acid target sequence comprising at least one 5-methyl-cytosine , said method comprising contacting:

(i) a nucleic acid target sequence comprising 5-methyl-cytosine and

(ii) a TALE protein comprising a repeat variable-diresidue (RVD) specific to each nucleic acid base of said nucleic acid target sequence, wherein the RVD(s) that specifically targets the 5-methyl-cytosine within said nucleic acid target sequence are selected from X*, preferably N*, Q*, T* or H* where X represents one amino acid residue selected from the group of A,G, V, L, I, M, S, T, C, P, D, E, F, Y, W, Q, N, H, R and K and * represents a gap in one position of the RVD.

7. A method to bind a nucleic acid target sequence comprising at least one 5-methyl-cytosine , said method comprising:

(a) providing a cell containing a nucleic acid target sequence that comprises a 5-methyl-cytosine ,

(b) synthesizing within said cell a TALE protein according to claims 1 to 4 directed to said nucleic acid target sequence and,

(c) testing the binding affinity of said TALE protein with said nucleic acid target sequence that comprises said 5-methyl-cytosine.

8. The method according to claim 6 or 7 wherein the said nucleic acid target sequence comprising at least one methylated dinucleotide selected from the group consisting of CpG, CpA, CpT, CpC.

9. A method to process a nucleic acid target sequence comprising at least one 5-methyl-cytosine comprising:

(a) providing a cell containing a nucleic acid target sequence that comprises a 5-methyl-cytosine;

(b) synthesizing within said cell a chimeric protein according to claims 4 to 5 directed to said nucleic acid sequence, so that said chimeric protein processes the nucleic acid within or adjacent to said nucleic acid target sequence independently of methylation and,

(c) testing the nucleic acid processing at the locus of said nucleic acid target sequence.

10. The method according to claim 9 wherein said protein domain has a catalytical activity selected from the group consisting of nuclease activity, polymerase activity, kinase activity, phosphatase activity, methylase activity, topoisomerase activity, integrase activity, transposase activity, ligase activity, helicase activity, recombinase activity.

11. The method according to claim 9 wherein said additional protein domain is a transcription activator.

12. The method according to claim 9 wherein said nucleic acid target sequence is a methylated promoter sequence.

13. The method according to claim 9 wherein said additional protein domain is an endonuclease.

14. The method according to claim 13 further comprising providing to the cell an exogenous nucleic acid comprising a sequence homologous to at least a portion of the nucleic acid target sequence, such that homologous recombination occurs between the nucleic acid target sequence and the exogenous nucleic acid.

15. A method to detect at least one 5-methyl-cytosine in a nucleic acid target sequence comprising:

(a) binding said nucleic acid target sequence with a transcription activator-like effector (TALE) protein comprising a plurality of TALE-like repeat sequences, each of said sequences comprising a repeat variable-diresidue (RVD) specific to each nucleic acid base of said nucleic acid target sequence wherein at least one RVD selected from the group consisting of:
    - HD for recognizing C;
    - NG for recognizing T;
    - NI for recognizing A;
    - NN for recognizing G or A;
    - NS for recognizing A or C or G or T;
    - HG for recognizing T;
    - IG for recognizing T;
    - NK for recognizing G;

- HA for recognizing C;
- ND for recognizing C;
- HI for recognizing C;
- HN for recognizing G;
- NA for recognizing G;
- SN for recognizing G or A; and
- YG for recognizing T;

(b) binding the same nucleic acid target sequence with another transcription activator-like effector (TALE) protein comprising a plurality of TALE-like repeat sequences, similar to that used in step a), wherein at least one RVD has been replaced by a RVD consisting of X*, preferably H*, T*, Q* or N*, wherein

- X represents one amino acid residue selected from the group of A,G, V, L, I, M, S, T, C, P, D, E, F, Y, W, Q, N, H, R and K,
- and * represents a gap in one position of the RVD,

(c) determining the binding affinity with said nucleic acid sequence under a) and b),

(d) calculating the ratio of binding activities determined under c), wherein said ratio, when close to 0, indicates the presence of methylated nucleic acid in said nucleic acid target sequence and, when close to 1, the absence of methylated nucleic acid in said nucleic acid target sequence.

16. The method according to claim 15 wherein binding affinity is measured by a nuclease activity.

17. The method according to claim 15 wherein binding affinity is measured by a transcriptional activity.

18. The method according to claim 15 wherein binding affinity is measured by detecting signal of fluorescent proteins fused to said TALE proteins of (a) and (b).

19. A composition for processing a nucleic acid target sequence comprising at least one 5-methyl-cytosine, wherein said composition comprises a TALE-nuclease comprising a plurality of TALE-like repeat sequences, each of said sequences comprising a repeat variable-

diresidue (RVD) specific to each nucleic acid base of said target sequence, wherein the RVD(s) that specifically target(s) the 5-methyl-cytosine within said nucleic acid target sequence are selected from X*, where X represents one amino acid residue selected from the group of A,G, V, L, I, M, S, T, C, P, D, E, F, Y, W, Q, N, H, R and K and * represents a gap in one position of the RVD.

20. The composition of claim 19, wherein said TALE-nuclease comprises or consist of a sequence selected from the group consisting of SEQ ID Nos: 39, 41, 44, 47, 49-51, 55 and 56.

21. A TALE-nuclease comprising at least one TALE-like repeat sequences with a repeat variable-diresidue (RVD) selected from the group consisting of T* and Q*, where * represents a gap in one position of the RVD.

22. The TALE-nuclease of claim 21, wherein said TALE-nuclease comprises or consist of a sequence selected from the group consisting of SEQ ID Nos: 55 and 56.