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(54) **Title:** T-CELL SPECIFIC GENOME EDITING

(57) **Abstract:** The present invention is concerned with the provision of a modified CRISPR/Cas9 system that can be used to directly edit genes in T cells. The modified CRISPR/Cas9 system of the present invention allows single-step direct analysis of gene function within T cells lineage and circumvents the tedious gene targeting and crossing necessary for Cre/loxP-mediated conditional gene ablation.



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T cell specific genome editing

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Field of the invention

The present invention is concerned with the field of genome editing. The invention provides CRISPR/Cas9 vectors and vector systems, wherein the Cas9 protein is under the control of a T cell specific promoter. The invention further provides the use of such vectors for targeted genome editing of T cells.

Background

A functioning immune system is central to the body's capability to fight off diseases caused by pathogens, but also to fight cancer. If the immune system in a body is weakened, diseases can easily develop. On the other hand, if the immune system is not working in a regulated way, it can attack its own cells, causing autoimmune diseases. The immune system comprises the innate immune system and the adaptive immune system, which each comprise both humoral and cell-mediated components. The humoral components comprise B cells, which produce soluble antibodies. The cell-mediated immunity comprises macrophages, natural killer cells and T cells.

Cell-mediated immunity protects the body by activating antigen-specific cytotoxic T-lymphocytes that are able to induce apoptosis in body cells displaying epitopes of foreign antigen on their surface, such as virus-infected cells, cells with intracellular bacteria, and cancer cells displaying tumor antigens. Furthermore, macrophages and natural killer cells are activated, enabling them to destroy pathogens; cells involved in the immune system are also stimulated to secrete a variety of cytokines that influence the function of other cells involved in adaptive immune responses and innate immune responses.

Cell-mediated immunity is directed primarily at microbes that survive in phagocytes and microbes that infect non-phagocytic cells. It is most effective in removing virus-infected cells, but also participates in defending against fungi, protozoans, cancers, and intracellular bacteria. It also plays a major role in transplant rejection.

Very important components of the cell-mediated immunity are the T cells. Among the T cells are the CD8⁺ cytotoxic T cells, and the CD4⁺ T helper cells. T helper cells assist other white blood cells in immunologic processes, including maturation of B cells into plasma cells and memory B cells, and activation of cytotoxic T cells and macrophages. These cells are also known as CD4⁺ T cells because they express the CD4 glycoprotein on their surfaces.

Cytotoxic T cells (CTLs) destroy virus-infected cells and tumor cells, and are also implicated in transplant rejection. These cells are also known as CD8⁺ T cells since they express the CD8 glycoprotein at their surfaces. These cells recognize their targets by binding to antigen associated with MHC class I molecules, which are present on the surface of all nucleated cells. Through IL-10, adenosine, and other molecules secreted by regulatory T cells, the CD8⁺ cells can be inactivated to an anergic state, which prevents autoimmune diseases.

T cells are therefore essential components of the immune system whose malfunction or absence is central to multiple pathologies, including inborn and acquired immune deficiencies, autoimmunity and cancer.

Their important role in widespread diseases such as cancer, HIV, rheumatoid arthritis, and multiple sclerosis makes it necessary to intensely study T cells and their functions in *in vivo* models.

One approach to study the function of cells *in vivo* involves genome modification, wherein the effects of silencing or activating a gene can be observed in a model organism. Since 1987 targeted mutagenesis in the mouse was performed by manipulating embryonic stem (ES) cells *in vitro*. These were used to generate chimeric mice and through participation in the germline the cells could transmit their genetic information, thus founding a new, manipulated mouse line. As extension to this technology recombinases such as Cre/loxP or similar systems are frequently used to overcome lethality, to make inducible and cell-type specific knockouts or transgenes, or to remove antibiotic resistance genes. While widening the scope of the technology recombinase-mediated conditional gene targeting still relies on ES cells for introduction of the recombinase target sites (loxP sites). Further, mouse lines carrying a loxP-containing allele need to be combined with a Cre transgene to address target gene function. Altogether the minimal time for generation of such conditional mutants is beyond a year. Furthermore, there is the possibility that Cre itself has an effect on the cells, like it was shown in mast cells.

As the Cre/loxP system is very time consuming, and also requires many test animals to obtain a valid *in vivo* model organisms to study gene functions, and has undesirable effect on

the model organism, in which the gene functions are to be studied, there is still a need for a system and method that allow single-step direct analysis of gene function within a certain cell lineage circumventing the tedious gene targeting and crossing necessary for Cre/loxP-mediated conditional gene ablation.

5 It was therefore the problem to be solved by the present invention to provide a system for time-efficient and reliable editing of the genome of T cells to obtain a model for studying conditions and diseases associated with T cells. It was a further problem to be solved by the present invention to provide an animal model for studying conditions and diseases associated with T cells, wherein the number of animals required to obtain the model is
10 reduced when compared to prior art methods.

Description of drawings

The invention is also explained with reference to the following figures.

Fig. 1 is concerned with conditional gene editing.

15 In a) a scheme of conditional gene editing is shown. In the Cas9 driver the nuclease is placed under control of a cell type or lineage specific promoter. The gRNA construct is driven by the ubiquitous U6 promoter. Both transgenes are co-injected into oocytes, and double-transgenic animals are analyzed for cell-type specific gene deletion and phenotype

In b) a PCR analysis of tail biopsies for presence of the CD4dsCas9 transgene (835 bp fragment) is shown.

20 In c) a PCR analysis of tail biopsies for the presence of the U6gRNA(CD2) transgene by PCR (407 bp fragment) is shown.

Fig. 2 shows the abrogation of CD2 expression on a small population of T cells in an analysis of PBMC of the indicated mouse strains by flow cytometry. Shown are live cells within a lymphocyte gate. CD4 and CD8 cells are gated on TCR β . The percentage of cells
25 found within the marked gates of the dot plot analysis is shown.

Fig. 3 shows single cell PCR analysis of the target region within the CD2 locus. Peripheral blood lymphocytes were surface stained for CD2, CD4, CD8 and CD19 and single cell sorted by flow cytometry. A 253 bp long region including the gRNA target was amplified by two rounds nested PCR. Obtained products were cloned in pGEM-T and pGEM-Teasy
30 and sequenced

In a) an agarose gel showing PCR products of single cell amplicons of the target region within the CD2 gene locus from sorted peripheral blood single cells is shown.

In b) a table showing the number of amplicons obtained from the indicated sorted cell types and the frequency of obtained mutations is shown.

In c) an alignment of the obtained sequences from the CD2 gene amplicon is shown. Indicated is the cell type (right side) and frequency (left side) of the respective sequence.

5 Fig. 4 shows the analysis of cell death in Cas9 transgenic animals in PBMC of the indicated mouse strains by flow cytometry. Presented are cells lying in a lymphocyte gate. The percentage of cells found within the marked gates of the dot plot analysis are shown. Aqua live dead staining was used for cell death analysis.

Fig. 5 shows a vector map of the vector CD4-DEPE-Cas9 as defined by SEQ ID NO: 1.

10 Fig. 6 shows a vector map of the vector px330gRNA(CD2) as defined by SEQ ID NO: 2.

Description of the present invention

The present invention is concerned with the provision of a modified CRISPR/Cas9 system that can be used to directly edit genes in specific cell lineages, similar to conditional mutagenesis by the Cre/loxP system. The modified CRISPR/Cas9 system of the present invention allows single-step direct analysis of gene function within a certain cell lineage and circumvents the tedious gene targeting and crossing necessary for Cre/loxP-mediated conditional gene ablation. The modified CRISPR/Cas9 system of the present invention allows rapid assessment of cell-type specific phenotypic changes in a time frame incomparable to classical conditional mutagenesis. Compared with modern analysis tools for single cells such an approach is extremely useful.

To be able to study the gene functions of T cells *in vivo*, or *in vitro*, the present invention provides a modified CRISPR/Cas9 system, which allows a T cell specific gene modification.

25 It has been surprisingly found that by using the modified CRISPR/Cas9 system of the present invention, wherein Cas9 is under the control of a T cell specific promoter such as a CD4 promoter, and the gRNA targeting the gene to be modified is under the control of promoter, which shows consistent strong expression across all mammalian cells, a transgenic model organism could be generated, wherein the T cells of the transgenic model organism comprised the gene modified by the CRISPR/Cas9 system.

30 With the modified CRISPR/Cas9 system of the present invention, several advantages to the methods of the prior art are provided. For example, the time to generate the transgenic model organism is reduced tremendously as well as the numbers of model organisms to be modified, because one can directly choose the background strain from the beginning and no intercrossing of strains to obtain homozygosity is necessary.

Furthermore, it has surprisingly been shown that *in vivo* application of the modified CRISPR/Cas9 system of the present invention for conditional gene editing was not leading to increased apoptosis, as Cas9 itself does not lead to apoptosis, which leads to a more reliable model organism. The modified CRISPR/Cas9 system of the present invention can therefore
5 be used to abrogate gene function *in vivo* and can be a useful research tool enabling rapid analysis of gene function in T cells.

The modified CRISPR/Cas9 system of the present invention is a vector system comprising one or more vectors comprising

a first regulatory element operably linked to one or more nucleotide sequences encoding
10 CRISPR-Cas system polynucleotide sequences comprising one or more guide RNAs (gRNA), wherein the gRNA comprises a guide sequence, a trans-activating cr (tracr) RNA, and a tracr mate sequence, wherein the one or more gRNAs hybridize with one or more target sequences in polynucleotide loci in a mammalian T cell,

a second regulatory element operably linked to a nucleotide sequence encoding a Type
15 II Cas9 protein,

wherein components (a) and (b) are located on same or different vectors of the system; wherein the CRISPR-Cas9 system comprises two or more nuclear localization signals (NLSs),

wherein the nucleotide sequence encoding a Type II Cas9 protein is under the control of
20 a T cell specific promoter, such as a mammalian CD4 promoter; and

wherein the one or more gRNAs target the one or more polynucleotide loci in the mammalian T cell and the Cas9 protein cleaves the one or more polynucleotide loci, whereby the sequence of the one or more polynucleotide loci is modified.

The essential components of a classical CRISPR/Cas9 system are known to the person
25 of skill in the art.

CRISPR/Cas consists of two key components: a "guide" RNA (gRNA) and a non-specific CRISPR-associated endonuclease (Cas). The original CRISPR/Cas system from *Streptococcus pyogenes* has been modified for application in molecular biology and now relies on one protein, the endonuclease Cas9, combined with a 102 bp long guide RNA
30 (gRNA) of which 20 bp determine the target sequence. The modifications to the Cas9 enzyme have extended the application of CRISPR/Cas to selectively activate or repress target genes, purify specific regions of DNA, and even image DNA in live cells using fluorescence microscopy. The Cas9 protein can also be codon optimized for expression in a

human cell, for example the protein termed hSpCas9 (humanized Cas9 derived from *S. pyogenes*). The skilled person knows how to obtain a codon optimized protein for expression in a human cell.

5 The gRNA is a short synthetic RNA composed of a “scaffold” sequence necessary for Cas9-binding and a user-defined ~20 nucleotide “spacer” or “targeting” sequence which defines the genomic target to be modified. Thus, the skilled person can change the genomic target of Cas9 by simply changing the targeting sequence present in the gRNA. The gRNA can be preferably formed by one RNA molecule.

10 The two or more nuclear localization signals (NLSs) can be expressed with the nucleotide sequence encoding the Cas9 protein; or can be encoded on the vector comprising the nucleotide sequences encoding the gRNA. In a preferred embodiment the NLSs are comprised in the vector comprising the nucleotide sequences encoding the gRNA.

15 The genomic target to be edited by the CRISPR/Cas9 system can be any ~20 nucleotide DNA sequence, provided it meets two conditions. Firstly, the sequence has to be unique compared to the rest of the genome. Secondly, the target has to be present immediately upstream of a Protospacer Adjacent Motif (PAM). Online tools have been developed for designing functionally competent gRNAs (see e.g. crispr.mit.edu).

20 The PAM sequence is absolutely necessary for target binding and the exact sequence is dependent upon the species of Cas9. The PAM sequence for *Streptococcus pyogenes* Cas9 is 5'-NGG-3'. Once expressed, the Cas9 protein and the gRNA form a riboprotein complex through interactions between the gRNA “scaffold” domain and surface-exposed positively-charged grooves on Cas9. Cas9 undergoes a conformational change upon gRNA binding that shifts the molecule from an inactive, non-DNA binding conformation, into an active DNA-binding conformation. Importantly, the “spacer” sequence of the gRNA remains free to
25 interact with target DNA. The Cas9-gRNA complex will bind any genomic sequence with a PAM, but the extent to which the gRNA spacer matches the target DNA determines whether Cas9 will cut. Once the Cas9-gRNA complex binds a putative DNA target, a “seed” sequence at the 3' end of the gRNA targeting sequence begins to anneal to the target DNA. If the seed and target DNA sequences match, the gRNA will continue to anneal to the target DNA in a 3'
30 to 5' direction. Cas9 will only cleave the target if sufficient homology exists between the gRNA spacer

The T cells to be modified can be comprised in a model organism. The model organism to be modified by the CRISPR/Cas9 system of the present invention can be a mammal. For example the model organism to be modified by the CRISPR/Cas9 system can be a mouse, a

human, a rat, a cow, a pig, a horse, a cat, or a dog. Preferably, the model organism is a mouse. The model organism can also be a chimeric organism. For example, the model organism can be a mouse, which comprises human T cells. In this embodiment, the genes to be modified are part of the human DNA comprised in the T cells, which are in turn comprised in the chimeric mouse. Therefore the mammalian T cell to be modified by the CRISPR/Cas9 system of the present invention can be a murine, human, bovine, equine, porcine, canine, feline, or rat cell, preferably a murine or human T cell.

The modified CRISPR/Cas9 system of the present invention can also be used to edit the genome of a T cell comprised in a tissue *in vitro*. In this embodiment, the model organism is the tissue, and comprises the T cell to be modified.

The T cell specific promoter is preferably a mammalian CD4 promoter. The mammalian CD4 promoter of the present invention can be found in any kind of mammal, because the CD4 system is phylogenetically older than mammals. In a preferred embodiment, the CD4 promoter comprises a nucleotide sequence as defined by nucleotides at positions 4054 to 4470 of SEQ ID NO:1. In a more preferred embodiment, the T cell specific promoter of the present invention is a modified CD4 promoter, which is specific for CD8⁺ T cells and CD4⁺ T cells, because it lacks the silencer sequence, and can, be defined by nucleotides at positions 2262 to 4470 of SEQ ID NO:1, which also includes enhancer sequences.

The gRNA of the present invention, which targets the polynucleotide locus or gene in the T cell to be modified is under control of a promoter. This promoter can show consistent strong expression across all mammalian cells. In a preferred embodiment this promoter is the human U6 promoter. The human U6 promoter is defined by nucleotides at position 1 to 251 of SEQ ID NO:2. The promoter controlling the transcription of the gRNA can also be a cell or tissue specific promoter. This can further enhance the specificity of the cell or tissue specific gene editing obtainable with the system of the present invention.

A "polynucleotide locus" (plural loci) is the specific location or position of a gene on a chromosome. The guided RNAs (gRNAs) of the present invention can hybridize to a polynucleotide sequence in a certain polynucleotide locus. This polynucleotide sequence is termed the "target sequence". The vector system of the present invention can comprise several gRNAs, which hybridize with several different target sequences within the same polynucleotide locus. This results in a more efficient modification of the polynucleotide locus. For example, if it is intended to disrupt (knock-down) the function of the gene at the polynucleotide locus, the same result can be achieved by editing different target sequences in the locus.

In one embodiment of the present invention the polynucleotide locus to be modified is the CD2 gene. In a preferred embodiment, exon 2 of CD2 comprises the targeted the polynucleotide locus. CD2 is an easily detectable surface marker found on all T cells and whose deficiency was reported to not introduce survival biases. Therefore, the disruption of CD2 expression is a good example to observe the efficiency of the cell specific gene modification of the present invention. It is however important to note, that the modified CRISPR/Cas9 system of the present invention is suitable to genetically modify any kind of polynucleotide locus or gene in a mammalian T cell. The target polynucleotide locus or gene can be chosen by the person of skill in the art trying to study the effects of the genetic editing in a T cell.

In one embodiment of the present invention, the one or more gRNAs hybridize with one or more target sequences in the same polynucleotide locus. This allows an increase of the effect of the CRISPR/Cas9 system mediated gene editing.

The vectors comprised in the system of the present invention are preferably bacterial or viral vectors. The origin of the vector can however be chosen freely by the skilled person as long as the vector is applicable to the CRISPR/Cas9 system.

The viral vectors can be retroviral, lentiviral, adenoviral, adeno-associated or herpes simplex viral vectors, or other viral vectors known in the art.

The present invention is also concerned with the use of the modified CRISPR/Cas9 system of the present invention for editing the genome of a mammalian T cell, wherein genome editing does not comprise a method of modifying the germline of a human being, and wherein genome editing does not comprise a method of treatment of the human or animal body. Methods for introducing the vectors of the system of the present invention into a model organism are known to the skilled person. For example, the vector constructs can be co-injected into the pronuclei of oocytes mice and the offspring can be screened for transgenic founders by PCR (see Example 1). The vectors can also be fused to be one vector.

In another embodiment the modified CRISPR/Cas9 system of the present invention can be used in a method of treatment of the human or animal body, or in a diagnostic method.

The mammalian T cell to be edited can be a murine, human, bovine, equine, porcine, canine, feline, or rat, or other mammalian cell, preferably a murine or human T cell.

The use of the modified CRISPR/Cas9 system of the present invention for editing the genome of a mammalian T cell can comprise modifying the sequence of a target

polynucleotide in a mammalian T cell, modifying expression of a polynucleotide in a mammalian T cell, generating a model mammalian T cell comprising a mutated disease gene, knocking out a gene, amplifying a gene, or repairing a mutation associated with DNA repeat instability.

5 The use of the modified CRISPR/Cas9 system of the present invention for editing the genome of a mammalian T cell can further comprise editing or repairing the cleaved target polynucleotide by inserting an exogenous template polynucleotide, wherein the editing or repairing results in a mutation comprising an insertion, deletion, or substitution of one or more nucleotides of the target polynucleotide.

10 The modified CRISPR/Cas9 system of the present invention for editing the genome of a mammalian T cell can also be used in the production of a non-human transgenic animal.

In this regard, one further embodiment of the present invention is a non-human transgenic animal obtained by the use of the modified CRISPR/Cas9 system of the present invention for editing the genome of a mammalian T cell in the production of a non-human
15 transgenic animal.

Examples

Preferred embodiments of the invention are outlined in the following examples which should not be interpreted as restricting the scope or spirit of the invention.

Example 1 – Modified CRISPR/Cas9 system

20 Mice transgenic for Cas9 under control of the CD4 promoter and for U6 promoter-driven gRNA targeting of the CD2 locus were generated. In peripheral blood we found a small population of CD4+ and CD8+ T cells lacking expression of CD2 was found. Sequencing of the CD2 locus in single cells from this population revealed insertion and deletion mutations, which led to abrogation of expression.

25 Cas9 was placed under control of a CD4 promoter, thus directing expression towards T cells (see Fig. 1B). In a second construct a gRNA expression cassette was placed under control of a U6 promoter and a target sequence directed towards exon 2 of CD2 (see Fig. 1B) was introduced. CD2 is an easily detectable surface marker found on all T cells and whose deficiency was reported to not introduce survival biases. Therefore, the disruption of
30 CD2 expression is a good example to observe the efficiency of the cell specific gene modification of the present invention. The two constructs were co-injected into the pronuclei of oocytes of FVB/N mice and offspring screened for transgenic founders by PCR. One transgenic founder carrying both constructs was identified (see Fig. 1C). One founder

carrying solely the Cas9 construct and two founders carrying only the gRNA construct were also obtained. In the double-transgenic founder an analysis of peripheral blood by flow cytometry revealed populations of CD4⁺ and CD8⁺ cells lacking expression of CD2 (see Fig. 2). No such populations were found in wildtype controls as well as Cas9 and gRNA-single transgenic founders (see Fig. 2).

To confirm that the CD2 locus was edited by CRISPR/Cas9 the one founder presenting with T lymphocytes lacking CD2 single cells was sorted and a fragment around the target site after PCR amplification and cloning was sequenced (see Fig. 3A). It was observed that of nine CD2⁻CD4⁺ T cells eight carried a single mutation in the amplicon (see Fig. 3B). Of the three amplicons from CD2⁻CD8⁺ T cells two were wildtype and one mutated (see Fig. 3B). In all amplicons, only a single sequence was found, either mutated or wildtype. This may be either the result of a technical bias leading to amplification of only one allele or alternatively the outcome of repair of the second allele by homologous recombination using an already mutated donor allele. In all 17 amplicons from CD2⁺ T cells as well as nine amplicons from CD19⁺ B cells solely unmutated sequences were obtained (see Fig. 3B), thus indicating the specificity of the system of the present invention. Sequence analysis of the mutations found within the amplicons from CD2⁻ T cells revealed deletions spanning up to 19 bp and insertions of one to four base pairs (see Fig. 3C).

Even though CD2⁻ T cells were observed in a Cas9/gRNA double-transgenic situation, the number of such cells was surprisingly low. This may have been a specific result of this particular founder due to expression variegation. To rule out that the low number of transgenic cells was due to a general problem of the system of the present invention, the T cell compartments were further analyzed in the different transgenic mice. The ratio of CD4⁺TCRβ⁺ and CD8⁺TCRβ⁺ T to CD19⁺ B cells was found to be slightly increased in double-transgenic versus Cas9 single and gRNA transgenic animals (4.9% and 4.8% versus 8% and 1.8% and 1.4% versus 2.2%). Since expression of nucleases such as Cre recombinase were reported to lead to apoptosis in situations of high proliferative activity, CD4⁺ and CD8⁺ T cells were analyzed for cell death by Live/Dead Fixable Aqua Dead Cell Stain Kit. No evidence was found for an increased number of dead cells in the Cas9/gRNA double-transgenic situation compared to either Cas9 or gRNA single transgenic mice (see Fig. 4). Thus, there was no evidence for induction of apoptosis by continuous presence of gRNA and Cas9 in T cells. For rapid investigation of oncogenes and tumor suppressor genes the observed low editing frequency may even prove to be advantageous.

Example 2 - Animal Models

Cas9 was PCR-amplified and placed into the second exon of CD4 in a construct (CD4-DEPE) consisting of the CD4 promoter, the distal and the proximal enhancer, exon 1 and parts of exon 2 but lacking the intronic silencer. The resulting vector was termed CD4-DEPE-Cas9 (see Fig. 5) and is defined by the nucleotide sequence of SEQ ID NO:1. The CD4 promoter is specific for CD8⁺ T cells and CD4⁺ T cells and is defined by nucleotides at positions 4054 to 4470 of SEQ ID NO:1. Subsequently, the plasmid was digested with NotI.

The protospacer specific for exon 2 of CD2 (5'-GACTAGGCTGGAGAAGGACC-3'(SEQ ID NO:3)) was cloned with BbsI into a modified px330 vector (px330ccdBChR), thus replacing a ccdB/chloramphenicol cassette. The resulting vector was termed px330gRNA(CD2) (see Fig. 6) and is defined by the nucleotide sequence of SEQ ID NO:2. The human U6 promoter is defined by nucleotides at position 1 to 251 of SEQ ID NO:2. The transgene was cut out by BciVI and XbaI. Both constructs were injected into FVB/N oocytes. The founders were screened by PCR using following primers: CD4 Cas9 typ fwd: 5'-TGC TCA CAA CCC TTT AGT TT-3' (SEQ ID NO:4), CD4 Cas9 typ rev: 5'-CTT TTT ATC CTC CTC CAC C-3' (SEQ ID NO:5) (product length: 835 bp); U6 fwd: 5'-GAG GGC CTA TTT CCC ATG ATT CC-3' (SEQ ID NO:6), T7 gRNA rev: 5'-GCA CGC GCT AAA AAC GGA-3' (SEQ ID NO:7) (product length: 407 bp). Animals were kept in barrier-SPF level animal facilities at Technische Universität München according to German law.

Example 3 – Flow cytometry

Blood samples were treated with red blood cell lysis buffer (REF) and washed with PBS. They were stained in FACS buffer (REF) with following antibodies: anti-CD2 FITC, anti-TCR β PE, anti-CD8 PerCP, anti-CD19 APC and anti-CD4 PB (all Biolegend). Live/Dead discrimination was performed by propidium iodide or Live Dead Fixable Aqua Dead Cell Stain Kit (life technologies). Single cells were sorted (BD, MoFlow) onto AmpliGrid slides and processed immediately. Cells were acquired at BD Canto II analysed with FlowJo Version 9.4.

Example 4 – Molecular Biology

Single cell PCR was performed as nested PCR. The outer PCR was performed with Advantage2 (Clontech) directly on the AmpliGrid slide. For the inner PCR Herculase II Fusion Enzyme with dNTP combos (Agilent) was used. PCRs were performed with following primers: out fwd: 5'-ATC ACC CTG AAC ATC CCC AAC-3' (SEQ ID NO:8), out rev: 5'-ACT GGA GTC TTC TTG TGG GC-3' (SEQ ID NO:9) (product length: 382 bp); in fwd: 5'-CTG

GTC GCA GAG TTT AAA AGG-3' (SEQ ID NO:10), in rev: 5'-GCT GCT CCC CAA CTT TCT AC-3' (SEQ ID NO:11) (product length 253 bp).

Claims

1. Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-CRISPR associated (Cas) (CRISPR-Cas) vector system comprising one or more vectors comprising
 - a) a first regulatory element operably linked to one or more nucleotide sequences encoding CRISPR-Cas system polynucleotide sequences comprising one or more guide RNAs (gRNA), wherein the gRNA comprises a guide sequence, a trans-activating cr (tracr) RNA, and a tracr mate sequence, wherein the one or more gRNAs hybridize with one or more target sequences in polynucleotide loci in a mammalian T cell,
 - b) a second regulatory element operably linked to a nucleotide sequence encoding a Type II Cas9 protein,wherein components (a) and (b) are located on the same or different vectors of the system; wherein the CRISPR-Cas system comprises two or more nuclear localization signals;
wherein the nucleotide sequence encoding a Type II Cas9 protein is under the control of a mammalian CD4 promoter; and
wherein the one or more gRNAs target the one or more polynucleotide loci in the mammalian T cell and the Cas9 protein cleaves the one or more polynucleotide loci, whereby the sequence of the one or more polynucleotide loci is modified.
2. The system of claim 1, wherein the mammalian CD4 promoter is defined by nucleotides at positions 4054 to 4470 of SEQ ID NO:1, or preferably by nucleotides at positions 2262 to 4470 of SEQ ID NO:1.
3. The system of claim 1 or 2, wherein the mammalian T cell is a murine, human, bovine, equine, porcine, canine, feline, or rat cell, preferably a murine or human T cell.
4. The system of any one of claims 1 to 3, wherein the one or more gRNAs hybridize with one or more target sequences in the same polynucleotide locus.
5. The system of any one of the preceding claims, wherein the polynucleotide locus is the CD2 gene in the mammalian T cell, wherein optionally the one or more guide RNA hybridize with exon 2 of the CD2 gene.
6. The system of any one of the preceding claims, wherein the vectors are bacterial or viral vectors.

7. The system of any one of the preceding claims, wherein the viral vectors are retroviral, lentiviral, adenoviral, adeno-associated or herpes simplex viral vectors.
8. The system of any one of the preceding claims, wherein the Cas9 protein is codon optimized for expression in a human or mouse or other mammalian cell.
9. Use of the system of any of claims 1 to 8 for editing the genome of a mammalian T cell, wherein genome editing does not comprise a method of modifying the germline of a human being, and wherein genome editing does not comprise a method of treatment of the human or animal body.
10. The use of claim 9, wherein the mammalian T cell is a murine, human, bovine, equine, porcine, canine, feline, or rat cell, preferably a murine or human T cell.
11. The use of claim 9 or 10, wherein the genome editing comprises modifying the sequence of a target polynucleotide in a mammalian T cell, modifying expression of a polynucleotide in a mammalian T cell, generating a model mammalian T cell comprising a mutated disease gene, knocking out a gene, amplifying a gene, or repairing a mutation associated with DNA repeat instability.
12. The use of claim 9, wherein the use further comprises editing or repairing said cleaved target polynucleotide by inserting an exogenous template polynucleotide, wherein said editing or repairing results in a mutation comprising an insertion, deletion, or substitution of one or more nucleotides of said target polynucleotide.
13. Use of the system of any one of claims 1 to 8 in the production of a non-human transgenic animal.
14. Non-human transgenic animal obtained by the use of any one of claims 11 to 12.
15. In vitro method for editing the genome function of a mammalian T cell using the system of any one of claims 1 to 8, wherein optionally the T cell is comprised in a cell tissue.

Fig. 1

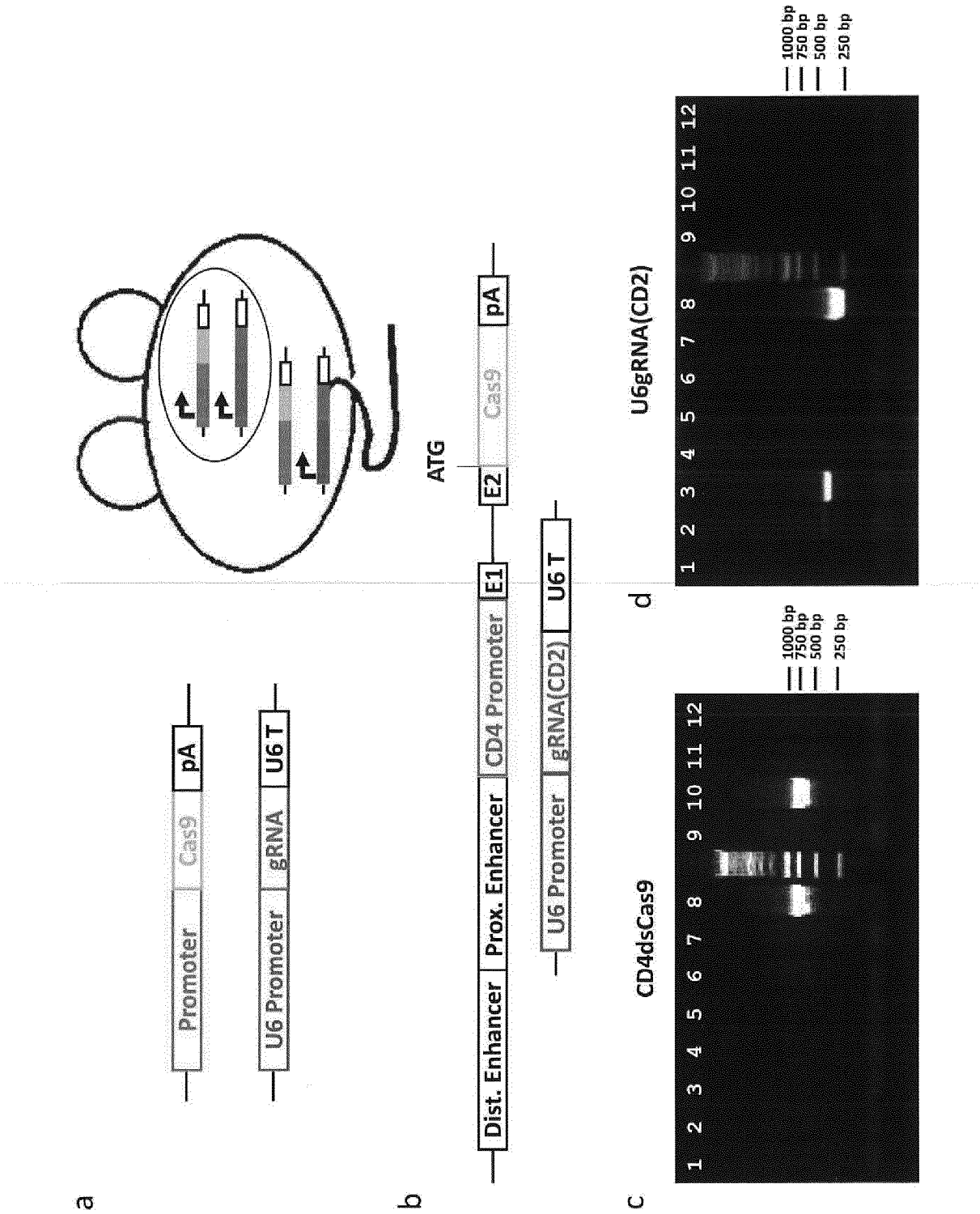


Fig. 2

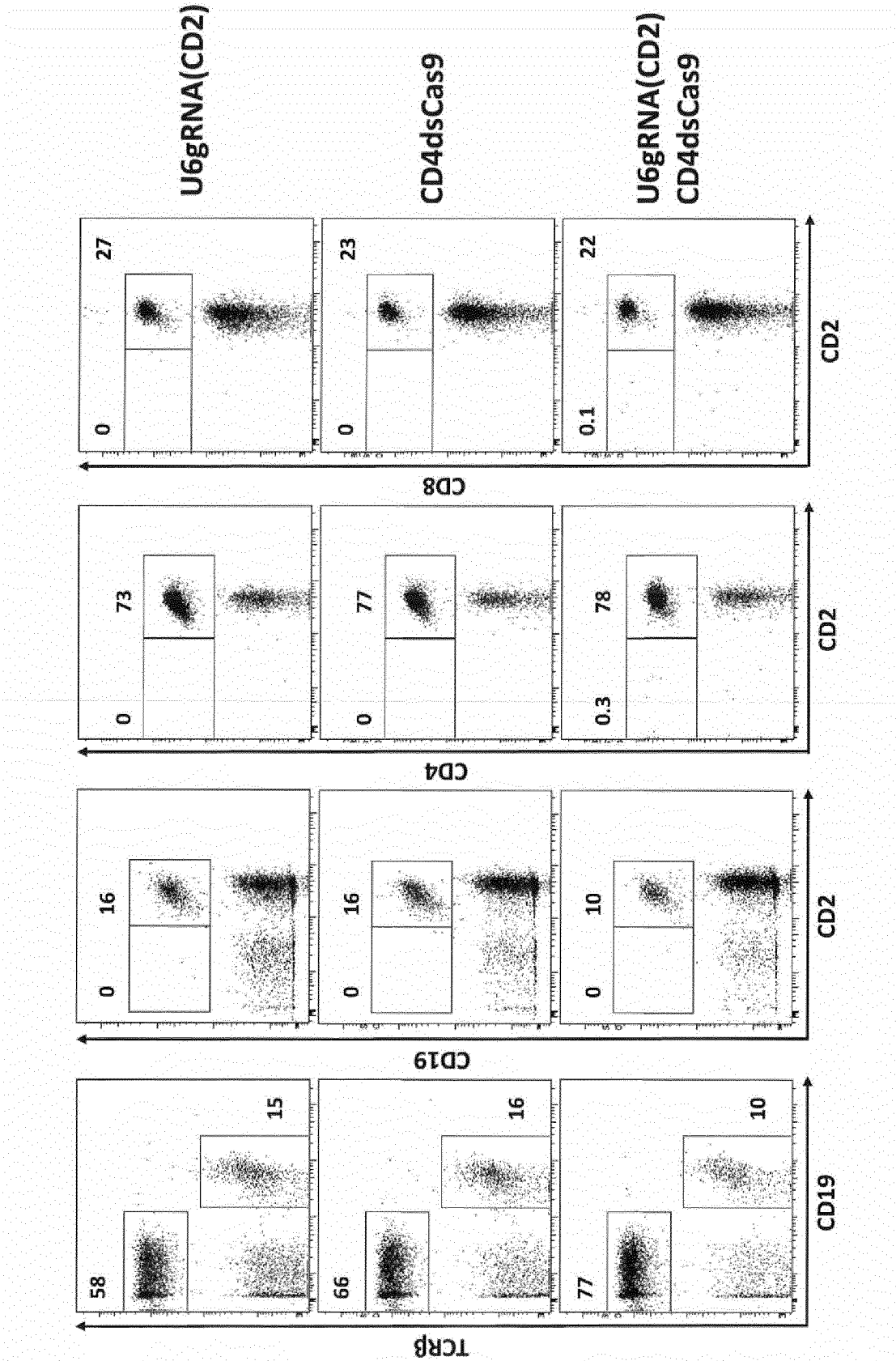


Fig. 3

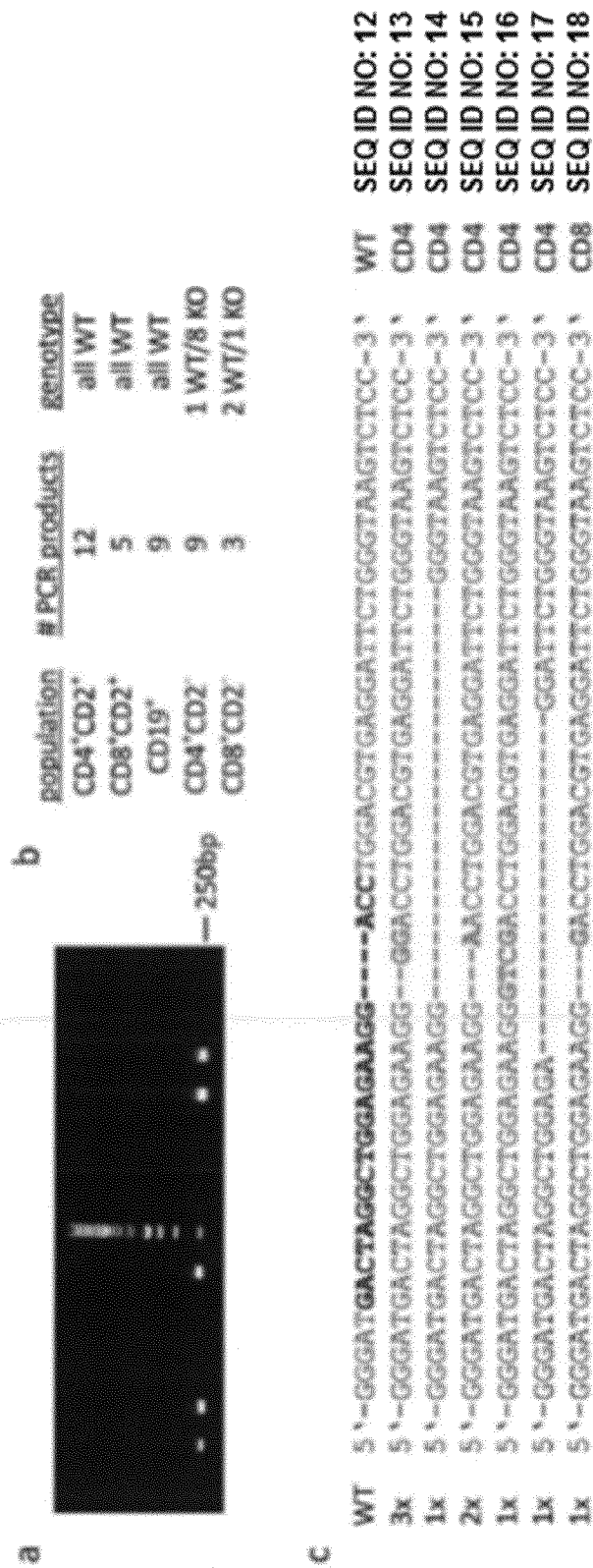


Fig. 4

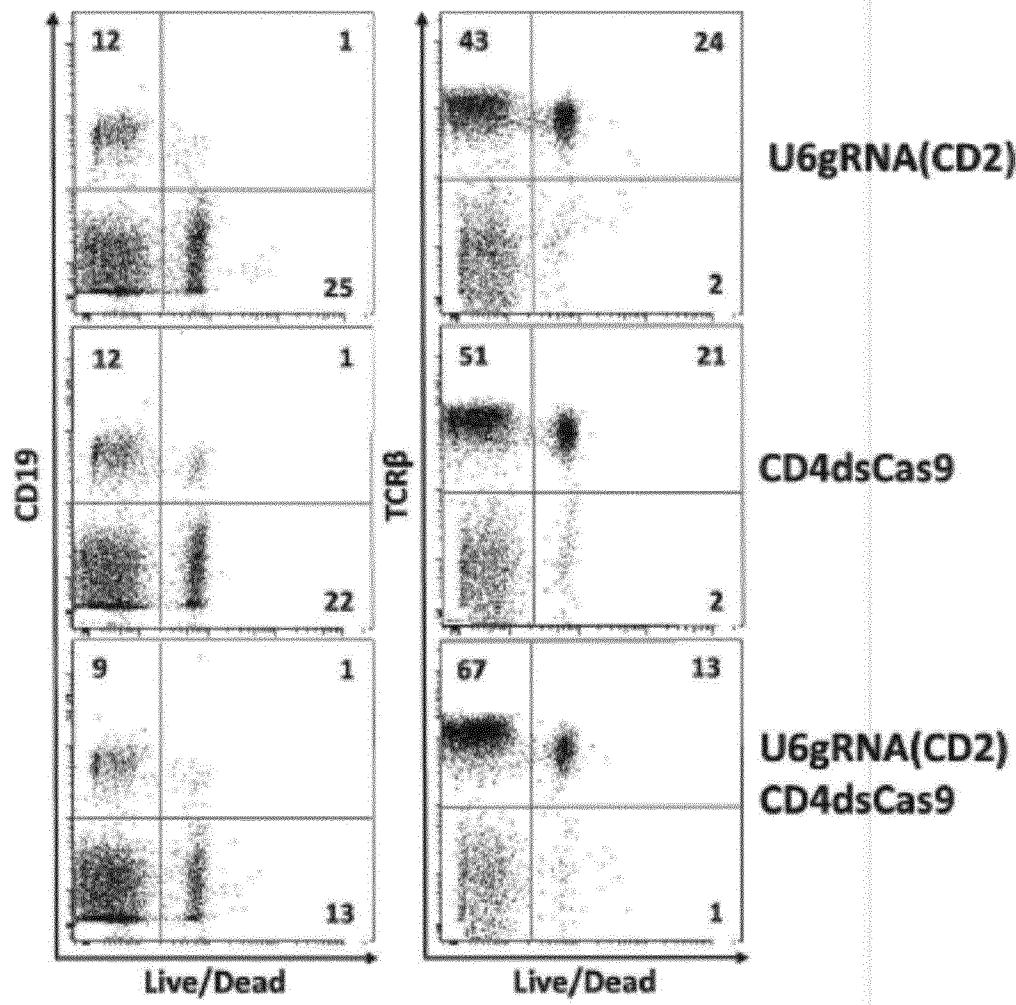


Fig. 5

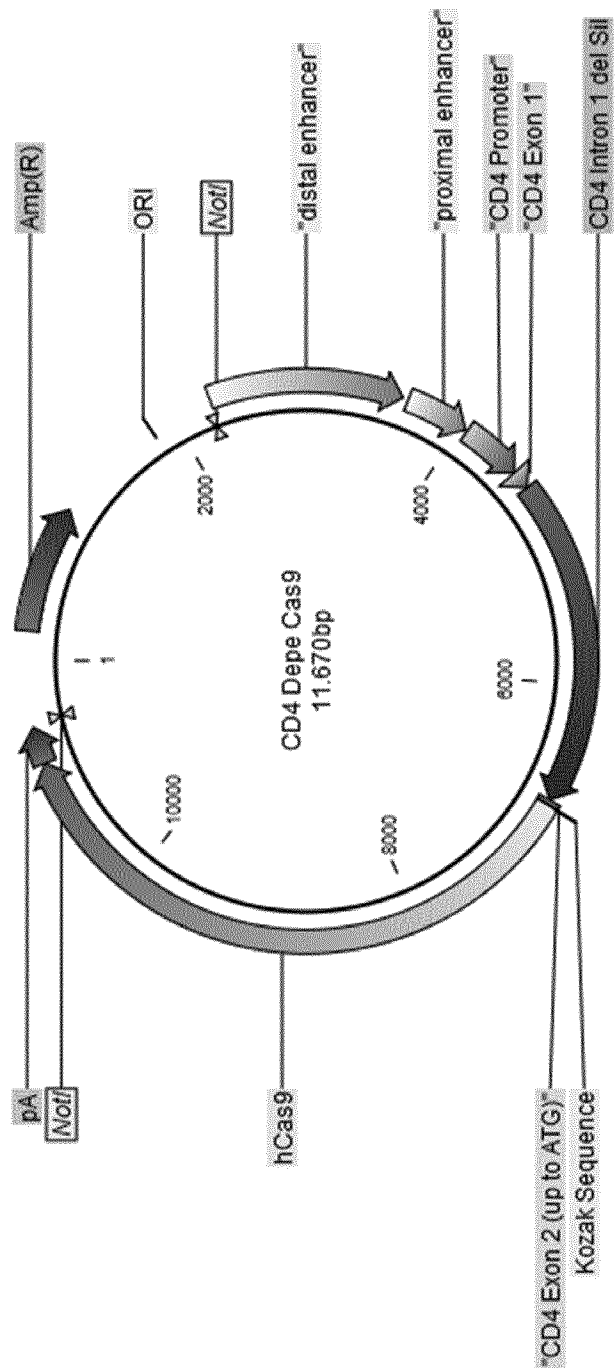
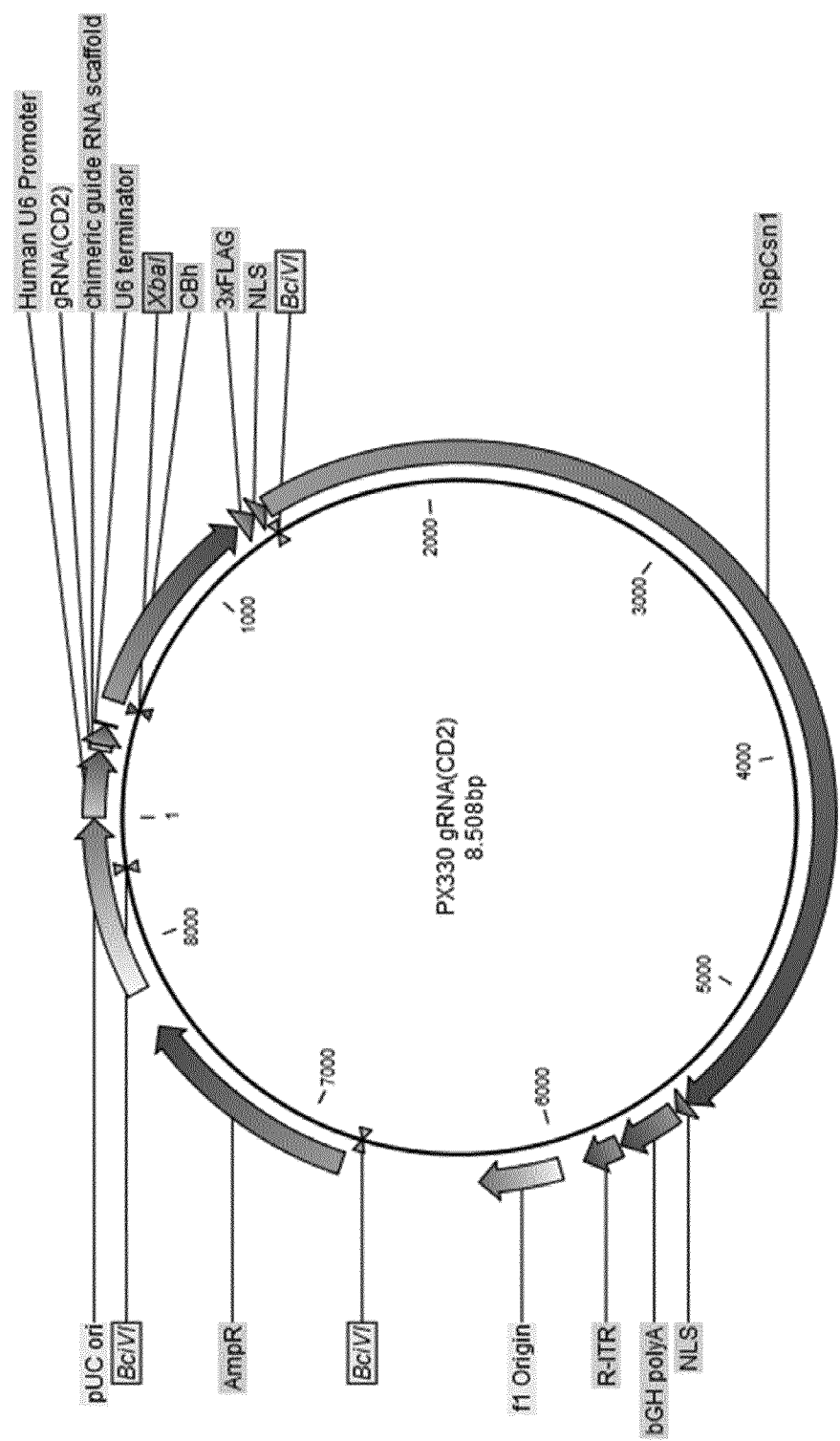


Fig. 6



INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2016/079370

A. CLASSIFICATION OF SUBJECT MATTER
 INV. C12N15/85 A01K67/027
 ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12N A01K

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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X	----- ELENA E PEREZ ET AL: "Establishment of HIV-1 resistance in CD4+ T cells by genome editing using zinc-finger nucleases", NATURE BIOTECHNOLOGY, vol. 26, no. 7, 29 June 2008 (2008-06-29), pages 808-816, XP055024363, ISSN: 1087-0156, DOI: 10.1038/nbt1410 page 813, left-hand column, paragraph 2; figure 4a ----- -/-	14



Further documents are listed in the continuation of Box C.



See patent family annex.

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Date of the actual completion of the international search

31 January 2017

Date of mailing of the international search report

07/02/2017

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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/079370

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
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A	PANPAN HOU ET AL: "Genome editing of CXCR4 by CRISPR/cas9 confers cells resistant to HIV-1 infection", SCIENTIFIC REPORTS, vol. 5, 20 October 2015 (2015-10-20), page 15577, XP055267475, DOI: 10.1038/srep15577 the whole document -----	1-15
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A	WO 2014/093635 A1 (BROAD INST INC [US]; MASSACHUSETTS INST TECHNOLOGY [US]; ZHANG FENG [U] 19 June 2014 (2014-06-19) paragraph [0138] -----	1-15

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Information on patent family members

International application No

PCT/EP2016/079370

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