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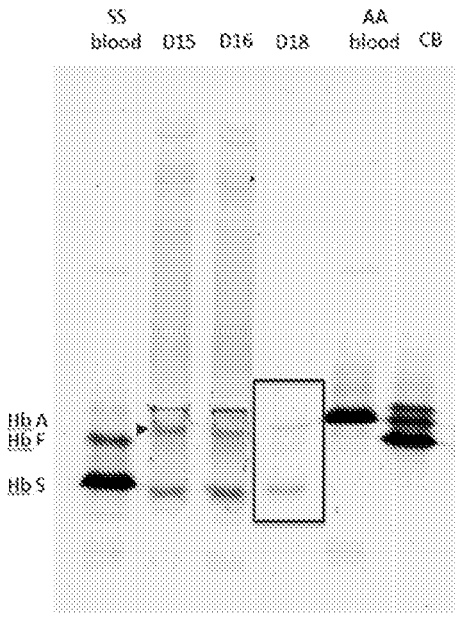
[Continued on next page]

(54) Title: CRISPR/CAS9 COMPLEX FOR GENOMIC EDITING

(57) Abstract: Provided herein are CRISPR/Cas9 complexes and method of using same.

FIGURE 10



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CRISPR/CAS9 COMPLEX FOR GENOMIC EDITING

This application claims the benefit of U.S. Provisional Application No. 62/181,138, filed June 17, 2015, and U.S. Provisional Application No. 62/266,316, filed December 11, 2015, both of which are hereby incorporated herein in their entireties by this reference.

BACKGROUND

Clustered regularly interspaced short palindromic repeats (CRISPR)-associated (Cas) systems (CRISPR-Cas9 systems) are used for gene editing at desired genomic sites in mammalian cells. In CRISPR-Cas9 systems, a Cas9 nuclease is targeted to a genomic site by complexing with a guide RNA that hybridizes to a target site in the genome. This results in a double-strand break that initiates either non-homologous end-joining (NHEJ) or homology-directed repair (HDR) of genomic DNA via a double-strand or single-strand DNA repair template. However, repair of a genomic site via HDR is inefficient.

SUMMARY

Provided herein is a complex for correcting a mutation in the genome of a cell or populations of cells. The complex comprises a guide RNA (gRNA) comprising a first nucleotide sequence that hybridizes to a target DNA in the genome of the cell, wherein the target DNA comprises a mutation, and a second nucleotide sequence that interacts with a site-directed nuclease. The complex further comprises a recombinant site-directed nuclease operably linked to a supercharged protein, wherein the site-directed nuclease comprises an RNA-binding portion that interacts with the second nucleotide sequence of the guide RNA and wherein the site-directed nuclease specifically binds and cleaves the target DNA to create a double stranded break. The complex also comprises a single-stranded donor oligonucleotide (ssODN) that hybridizes to a genomic sequence flanking the double stranded break in the target DNA and integrates into the target DNA to correct a mutation in the target DNA.

Methods of site-specific modification of a target DNA in a cell or a population of cells are also provided. The methods comprise introducing a complex for correcting a mutation in the genome of the cell, wherein the complex is introduced into the cells under conditions that allow homology-directed repair (HDR) and integration of the ssODN into the target DNA. The method further provides for a high rate of cell survival in corrected cells.

Further provided is a method of treating a disease associated with a mutation in the genomic sequence encoding hemoglobin in a subject. The method comprises introducing into a population of cells obtained from the subject a complex for correcting a mutation in the genomic sequence encoding hemoglobin under conditions that allow homology-directed repair (HDR) to correct the mutation in the genomic sequence encoding hemoglobin and transplanting the corrected cells into the subject.

DESCRIPTION OF THE FIGURES

Figures 1A-1C show that *in vitro* differentiation of JAK3 C1837T patient induced pluripotent stem cells (iPSCs) recapitulates SCID phenotypes. Figures 1A and 1B show flow cytometry of iPSC-derived T cells. JAK3 WT iPSCs (Control) and JAK3- deficient iPSCs (JAK3 C1837T) were differentiated into CD34+ cells on OP9 stromal cells and, subsequently, into T cells on OP9-DL4 monolayers. T-cell differentiation from JAK3-deficient iPSCs was absent compared to controls; no CD3+ T cells or CD3-CD16+CD56+ NK cells were observed (Figure 1A), and no CD4+CD8+ double positive (DP), CD4+ single positive (SP), or CD8+ single positive (SP) T cells were detected (Figure 1B). Figure 1C shows the results of RT-qPCR assays for transcripts of key genes that regulate early events during specification of the T cell lineage. RNA levels are shown relative to GAPDH expression.

Figures 2A-2C show that BCL2 partially rescues T cell developmental defects in JAK3–deficient, *in-vitro* derived cells. Figure 2A shows apoptosis of JAK3-deficient, iPSC-derived T cells compared to JAK3 WT controls. Annexin V-positive cells were analyzed at T cell induction day 10 (TD10) and 17 (TD17). Four independent experiments were performed with control JAK3 WT cells (Control) and 5 independent experiments were performed with JAK3-deficient cells (JAK3 C1837T). *$P < 0.005$. Figure 2B shows the results of RT-qPCR assays for anti-apoptotic BCL2 and proapoptotic BAX expression in two lines (1 and 2) from JAK3 WT (Control) and JAK3-deficient cells (JAK3 C1837T). ND, not determined (due to insignificant JAK3 qPCR signal). RNA levels are shown relative to GAPDH expression. Figure 2C shows flow cytometry of JAK3–deficient iPSCderived T cells transduced with BCL2-2A-GFP lentivirus to assess effects on NK (CD16+56+) and T cell (CD3+) development and DP (CD4+CD8+) to SP (CD4+ or CD8+) T cell maturation.

Figures 3A-3D show that CRISPR/Cas9 enhanced correction of the JAK3 C1837T mutation in patient-specific iPSCs. Figure 3A depicts the strategy for genome modification

using CRISPR/Cas9 to induce double-strand breaks in the JAK3 locus and a template for homology directed repair. Top line, structure of the JAK3 gene. Open boxes, exons. Asterisk, C1837T mutation. Arrows, guide RNAs. Figure 3B, top, shows PCR analysis demonstrating homologous recombination; primers for 5' and 3' analysis are indicated. (Lower Left) RT-PCR analysis demonstrating JAK3 mRNA expression in JAK3 WT (Control), JAK3-deficient (JAK3 C1837T), and corrected (JAK3 Corrected) T cells. (Lower Right) Western Blot analysis demonstrating JAK3 protein expression in JAK3 WT (Control), JAK3-deficient (JAK3 C1837T), and corrected (JAK3 Corrected) T cells. Figure 3C provides a summary of targeting efficiencies of guide RNAs. (Figure 3D) Sanger sequencing of the PCR amplicons from parental JAK3 iPSCs (Left), heterozygous corrected (Middle) and homozygous corrected iPSCs (Right). The two heterozygous clones were corrected with gRNA2 + wild type Cas9, and the homozygous clone was corrected with gRNA1 + gRNA2 + nickase Cas9 (D10A).

Figures 4A-4C show *in vitro* differentiation of JAK3 corrected patient iPSCs produces T cells with phenotypic and functional characteristics of mature T cells. Figure 4A shows the expression of T cell developmental markers in JAK3 WT (Control, n=3), JAK3-deficient (JAK3 C1837T, n=5) and JAK3- corrected (JAK3 Corrected, n=6) T cells. Cells were stained with indicated antibodies and analyzed by flow cytometry at T cell induction Day 14, 21, 28 and 35 (TD 14, 21, 28 and 35). Figure 4B shows T cell receptor (TCR) Vβ analysis of JAK3-corrected T cells. A highly diverse repertoire of TCR Vβ is represented in T cells derived from corrected SCID patient iPSCs. Figure 4C shows flow cytometry demonstrating T cell activation in JAK3-corrected T cells. T cells derived from JAK3 WT (Control) and JAK3-corrected iPSCs were stimulated with anti-CD3/28 beads for 3 days before analysis of activation markers CD25 and CD69. The data were gated on CD3+ populations.

Figures 5A-5C show *in vitro* generation of CD34+ HSCs from hiPSCs by co-culture with human bone marrow stromal cells (hMSC). Human iPSCs were cultured on hMSCs for 18 days before analysis for hematopoietic markers, CD34 and CD43 (Figure A). CD34+ cells were purified on beads and differentiated into T cells (Figure B), erythroid and myeloid cells (Figure C). To generate T cells, purified CD34+ cells were plated on OP9-DL4 cells for 3 to 4 weeks. For the CFC assay to generate myeloid and erythroid cells, purified CD34+ cells were plated in MethoCult H4434 Classic medium according to the manufacturer's protocol. These data demonstrate that hiPSC can be efficiently differentiated into multipotent HSC after co-culture on hMSC.

Figure 6A-6C show *in vitro* generation of T cells by culturing hiPSC derived CD34+ cells with hMSC-DL4. To generate CD7+ T progenitor cells, hiPSC derived CD34+ cells were co-cultured on hMSC-DL4 for 3 to 4 weeks (Figure 6A). When CD7+ cells from Figure 6A were purified on magnetic beads and co-cultured on OP9-DL4, fully mature CD4+/CD8+/CD3+/TCR-αβ+ cells were produced in 10 days or less (Figures B and C). These data demonstrate that hiPSC can be efficiently differentiated into CD7+ lymphoid progenitors after co-culture on hMSC-DL4.

Figure 7 shows *in vitro* generation of γδ T cells from hiPSC. Human iPSC were transduced with a lentiviral vector carrying a pre-rearranged human V γδ1 cDNA linked with a 2A-GFP cDNA fragment. After co-culture with OP9 for 18 days, hiPSC derived CD34+ cells were purified on magnetic beads. These cells were subsequently plated on OP9-DL4 cells for T cell differentiation. Cells were harvested at Day 32 and T cell surface markers were analyzed by FACS. The GFP+ population represents Vδ1-2A-GFP lentiviral transduced cells. A high percentage of these GFP positive cells expressed Vδ1 (66%). A low percentage of GFP negative cells expressed Vδ1 (1%). These results demonstrate that Vδ T cells expressing recombinant T Cell Receptors (TCR) can be efficiently produced from genetically modified iPSC. Production of Vδ T cells expressing recombinant T Cell Receptors (TCR) specific for tumor antigens provides a powerful cellular therapy for many types of cancer.

Figure 8 shows that a correction complex including guide RNA, a modified Cas9 and a single stranded oligonucleotide donor sequence (ssODN) can correct a sickle cell mutation. The complex was introduced into sickle iPSC by nucleoporation, and 2 days later genomic DNA was analyzed by digital PCR (ddPCR) and sequenced. Over 65% of the cells contained at least one corrected gene. The results were confirmed as follows. Two days after introduction of the correction complex, the cells were plated in culture dishes, and 43 individual iPSC colonies were isolated. Genomic DNA was isolated from these colonies and the beta-globin gene was sequenced. Sixty-five percent of the colonies contained at least one corrected beta-globin gene (S corrected to A).

Figure 9 shows that introduction of a sickle cell correction complex (gRNA-modified recombinant Cas9-ssODN) into patient primary bone marrow CD34+ cells can correct a sickle cell mutation. After twelve days of *in vitro* differentiation, DNA was analyzed by digital PCR (ddPCR) and sequenced. Approximately equal amounts of betaA and betaS mRNA were observed.

Figure 10 is an isoelectric focusing (IEF) gel of *in vitro* differentiated red blood cells from the corrected sickle patient CD34+ cells of Figure 9, showing an HbA (normal

hemoglobin) to HbS (hemoglobin with sickle cell mutation) ratio of about 1:3, which is sufficient to inhibit sickling and treat sickle cell anemia.

Figure 11 shows that engineered positively charged Cas9 RNPs/ssODN (EpcCas9 RNPs/ssODN) efficiently correct the sickle mutation in human patient iPSCs. Wild type Cas9 (Cas9WT) RNP and eight engineered positively charged (EpcCas9) RNPs were co-nucleoporated with correction ssODN into human sickle iPSCs. Sickle correction efficiencies in the pooled cells were determined by Sanger sequencing at two days post nucleofection. The arrow indicates the position of sickle correction (T->A) and the scissors indicate the Cas9WT-36GFP RNP cutting site on the sickle HBB DNA.

Figure 12 shows the results of deep sequencing of on-target modifications in human sickle iPSC populations. On-target deep sequencing analysis of human sickle iPSCs nucleoporated with Cas9WT RNP/ssODN, Cas9WT-EGFP, or four EpcCas9 RNPs/ssODNs is shown. Black bars indicate the corrected base and the space below the black bars indicates the sickle cell mutation. The negative control and the ssODN alone both show only the sickle cell mutation. All iPSC samples also contain a SNP near the sickle mutation (column on right hand side).

Figure 13 shows that TAT-CAs9WT-EGFP RNP suppresses on-target indels. Human sickle iPSCs were nucleoporated with Cas9WT and TAT-Cas9WT-EGFP RNPs with (+ssODN) or without correction ssODN (-ssODN). Indel and correction efficiencies were analyzed by Sanger sequencing at two days post nucleoporation. The arrows indicate the position of sickle correction (T->A) and the scissors indicate the Cas9WT-36GFP RNP cutting site on the sickle HBB DNA.

Figure 14 shows that EpcCas9 RNPs suppress on-target indels in human sickle iPSCs. Human sickle iPSCs were nucleoporated with Cas9WT and five EpcCas9 RNPs, with or without correction ssODN. Indel and correction efficiencies were analyzed by Sanger sequencing at two days post nucleoporation. The arrows indicate the position of sickle correction (T->A) and the scissors indicate the Cas9WT-36GFP RNP cutting site on the sickle HBB DNA.

Figure 15 shows that EpcCas9 RNPs enhance cell survival after nucleoporation in human sickle iPSCs. Human sickle iPSCs were nucleoporated with Cas9WT RNP and seven EpcCas9 RNPs with or without correction ssODN. Cell survival was assessed by light microscopy at two days post nucleofection.

Figures 16A and 16B show ssODN:Cas9 RNP ratios for sickle correction in human iPSCs. Correction ssODN and Cas9WT-36GFP/T2 RNP were nucleoporated into sickle

patient iPSC at molar ratios of 0, 0.2, 0.5, 1.0, 1.15, 1.35, 1.5 and 2.0. (A Cas9WT-36GFP:T2 gRNA molar ratio of 1:1.35 was fixed for these experiments. For example, the r=0.5 value in the graph below is 0.5 ssODN:1.0 Cas9WT-36GFP:1.35 T2 gRNA.) Forty-eight hours after nucleoporation of the ssODN:Cas9WT-36GFP RNPs, sickle corrections were quantitated by digital droplet PCR (ddPCR) (Fig. 16A) and Sanger sequencing (Fig. 16B). The percent correction (betaA/betaS alleles x 100) was plotted verses r (ssODN:Cas9WT-36GFP RNP). A dashed sigmoidal curve was fitted with the data points. (B) An arrow indicates the position of sickle correction (T->A) and scissors indicate the Cas9WT-36GFP RNP cutting site on sickle HBB DNA.

Figure 17 shows Cas9:sgRNA ratios for sickle correction in human iPSCs. Cas9-36GFP:sgRNA molar ratios of 1:1.15, 1:1.35 and 1:1.50 with ssODN molar ratios of 1.15 or 1.35 were tested to determine optimal correction efficiency of the sickle mutation in patient iPSC. The mixtures were nucleoporated into human sickle iPS cells and the Sanger sequencing results for the pooled cells were analyzed at two days post nucleofection. Arrows indicate the position of sickle correction (T->A) and scissors indicate the Cas9WT-36GFP RNP cutting site on sickle HBB DNA.

Figure 18 shows correction of human sickle iPSCs by EpcCas9 RNP/ssODN. Sanger sequencing analysis of pooled human sickle iPS cells nucleofected with TAT-Cas9WT-36GFP-INF7 RNP/ ssODN was performed. The arrow indicates the position of sickle correction (T->A) and the scissors indicate the position of EpcCas9 RNP induced DSB on the sickle HBB DNA.

Figures 19A and 19B show correction of human iPSCs with EpcCas9 RNP and wobble ssODNs. Human sickle iPSC were nucleoporated with TAT-Cas9-36GFP-INF7 RNP and ssODNs containing wobble bases near the gRNA cleavage sites. (A) Sanger sequencing of iPSC populations nucleoporated with T1 gRNA and T1-wb ssODN was performed. The arrow on the left hand side of Figure 19A indicates the position of sickle mutation and the 3 arrows located downstream of the sickle mutation indicate positions of wobble bases. Scissors point to the T1 cleavage site. (B) Sanger sequencing of iPSC populations nucleoporated with T2 gRNA and T2-wb ssODN was performed. The arrow on the left hand side indicates the position of the sickle mutation and the 2 arrows downstream of the sickle mutation indicate the positions of wobble bases. Scissors point to the T2 cleavage site.

Figures 20A and 20B show the results of whole genome sequencing (WGS) analysis of 4 iPSC clones corrected with TAT-Cas9WT-36GFP-INF7 RNP/ssODNs. (A) On-target

sequence analysis demonstrates sickle correction and wobble-base substitution. (B) WGS off-target analysis of genomic loci with homology to T1 and T2 sgRNA is shown.

Figures 21A-D show gene correction of sickle patient bone marrow CD34+ HSPCs. (A) Human sickle bone marrow CD34+ cells were nucleoporated by Cas9WT, Cas9WT-36GFP and TAT-Cas9WT-3xTAT RNPs/ssODN. Gene correction efficiency for pooled populations cells was analyzed six days after nucleofection. The arrow indicates the position of sickle correction (T->A) and the scissor indicate the Cas9WT-36GFP RNP cutting site on the sickle HBB DNA. (B) mRNA correction by RT-PCR and Sanger sequencing in Cas9WT-36GFP nucleoporated sickle CD34+ cells that were harvested after 10-day culturing in erythroid differentiation media. (C) IEF Gel analysis of *in vitro* differentiated RBCs from Cas9WT-36GFP RNP/ssODN nucleofected sickle CD34+ cells. Human sickle child patient blood lysate (SS) and human normal adult blood lysate (AA) that represent HbF, HbS and HBA proteins were also loaded as controls. (D) Mass spectrometry analysis of *in vitro* differentiated RBCs derived from sickle CD34+ cells nucleofected with Cas9WT-36GFP RNP/ssODN. The peaks demonstrate signals from uncorrected HbS protein and corrected HbA protein.

Figures 22A-C show correction of colonies derived from single CD34+ progenitors. (A) BFU-E and CFU-GEMM colonies derived from nucleoporated human sickle CD34+ cells. (B) Representative Sanger Sequencing results of colonies obtained from human sickle CD34+ cells after nucleoporation with TAT-Cas9WT-36GFP-INF7 RNP/ssODN. (C) Colony survival after nucleoporation with Cas9WT, Cas9WT-36GFP, and TAT-Cas9WT-3xTAT RNPs plus ssODNs.

Figure 23 is a graphical summary of deep sequencing data from Table 6.

Figure 24 shows non-specific modifications near Cas9WT RNP targeting site. BFU-E colonies from Cas9WT RNP/ssODN nucleoporated sickle CD34+ cells contain indels that do not appear to be initiated at the cutting site. The top sequence labeled 'Upstream' is representative of non-specific modifications upstream of the expected cleavage site. The bottom sequence labeled 'Downstream' represents non-specific modifications observed downstream of the expected cleavage site. Arrows indicate the position of the sickle mutation and scissors indicate the expected cleavage site of Cas9WT RNP.

Figure 25 shows isoelectric focusing (IEF) gel analysis of blood six weeks after primary transplantation of sickle mouse fetal liver c-kit+ cells nucleoporated with Cas9 RNP/ssODN into irradiated C57BI/6 mice to correct a sickle cell mutation. Mouse fetal liver c-kit+ cells are equivalent to human cord-blood Cd34+ cells.

Figure 26 shows ddPCR analysis of FACS purified bone marrow cells at twelve weeks post-transplantation into irradiated C57BI6 mice. Twelve weeks after nucleoporation and transplantation, approximately 50% of erythroid cells (Ter119+) and myeloid cells (CD11b+ and CD11b+/GR1+) are corrected. Erythroid and myeloid cells are relatively short lived; therefore, these cells are derived from transplanted HSCs. Correction levels in B and T cells can rise to approximately 50% after secondary transplantation at twelve weeks (twenty-four weeks total). After twenty-four weeks, most if not all hematopoietic cells will be derived from long-term HSCs.

Figure 27 shows IEF gel analysis of blood from mice twelve weeks after primary transplantation and six weeks after secondary transplantation of cells nucleoporated with Cas9 RNP/ssODN to correct a sickle cell mutation. Human HbA is produced in mice after transplantation of HSCs nucleoporated with Cas9 RNP/ssODN to correct a sickle cell mutation.

DETAILED DESCRIPTION

Provided herein are CRISPR/Cas9 complexes for genomic modification of cells. Methods of using the complexes provided herein result in increased efficiency of modification, an increased cell survival ratio and/or an increased ratio of HDR to NHEJ in the cells. These complexes and methods can be used for therapeutic purposes, for example, to correct a mutation in cells, wherein the mutation is associated with a disease or disorder.

Provided herein is a complex for correcting a mutation in the genome of a cell comprising (a) a guide RNA (gRNA) comprising a first nucleotide sequence that hybridizes to a target DNA in the genome of a cell, wherein the target DNA comprises a mutation, and a second nucleotide sequence that interacts with a site-directed nuclease; (b) a recombinant site-directed nuclease operably linked to a supercharged protein, wherein the site-directed nuclease comprises an RNA-binding portion that interacts with the second nucleotide sequence of the guide RNA and wherein the site-directed nuclease specifically binds and cleaves the target DNA to create a double stranded break; and (c) a single-stranded donor oligonucleotide (ssODN) that hybridizes to a genomic sequence flanking the double stranded break in the target DNA and integrates into the target DNA to correct a mutation in the target DNA.

It is understood that the complex comprising a guide RNA (gRNA), a recombinant site-directed nuclease and a donor nucleotide described herein does not occur in nature. The complex, however, provides the elements necessary with the required configuration and stoichiometry to efficiently and effectively modify cells. The gRNA molecule binds to the

site-directed nuclease and targets the nuclease to a specific location within the target DNA. A gRNA comprises a first nucleotide sequence that hybridizes to a target DNA in the genome of a cell, wherein the target DNA comprises a mutation, and a second nucleotide sequence that interacts with a site-directed nuclease. The complexes described herein can comprise one or two separate gRNAs. Therefore, the term guide RNA includes both a single guide RNA and a double guide RNA. An example of a guide sequence that can be used to correct a mutation associated with sickle cell anemia is set forth herein as TAACGGCAGACTTCTCCAC (SEQ ID NO: 1). An example of a guide sequence comprising a stem loop for Cas9 binding is provided herein as GTAACGGCAGACTTCTCCACGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGG CTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTT (SEQ ID NO: 2). It is noted that the 5'G of SEQ ID NO: 2 was added by T7 during *in vitro* transcription.

In the complexes described herein, the recombinant site-directed nuclease can be an RNA-guided site-directed nuclease, for example, a Cas protein from any bacterial species or a functional fragment thereof. For example, the Cas protein can be a Cas9 protein or a functional fragment thereof. As used herein, the term "Cas9" means a Cas9 protein or a fragment thereof present in any bacterial species that encodes a Type II CRISPR/Cas9 system. See, for example, Makarova et al. *Nature Reviews*, Microbiology, 9: 467-477 (2011), including supplemental information, hereby incorporated by reference in its entirety. For example, the Cas9 protein or a fragment thereof can be from *Streptococcus pyogenes*. Full-length Cas9 is an endonuclease that includes a recognition domain and two nuclease domains (HNH and RuvC, respectively). In the amino acid sequence, HNH is linearly continuous, whereas RuvC is separated into three regions, one left of the recognition domain, and the other two right of the recognition domain flanking the HNH domain. Cas9 from *Streptococcus pyogenes* is targeted to a genomic site in a cell by interacting with a guide RNA that hybridizes to a 20-nucleotide DNA sequence that immediately precedes an NGG motif recognized by Cas9. This results in a double-strand break that is repaired via HDR by a donor nucleotide, for example, a ssODN or a double stranded DNA construct that hybridizes to a genomic sequence flanking the double stranded break in the target DNA and integrates into the target DNA to correct a mutation in the target DNA.

In the complexes provided herein, the molar ratio of gRNA to site-directed nuclease operably linked to a supercharged protein to ssODN can be from about 1:1:0.2 to about 1.5:1:2.0. For example, the molar ratio of gRNA to site-directed nuclease operably linked to a

supercharged protein to ssODN can be about 1:1:1, 1.1:1:1, 1:1:1.15, 1:1:1.25, 1:1:1.30; 1:1:1.35; 1:1:1.40; 1:1:1.50, 1.2:1:1, 1.3:1:1. 1.4:1:1, 1.5:1:1, 1.5:1:1.15, 1.5:1:1.25, 1.5:1:1.35; 1.5:1:1.40, 1.5:1:1.45; 1.5:1:1.50; 1.5:1:1.55; 1.5:1:1.60; 1.5:1:1.65; 1.5:1:1.70; 1.5:1:1.75; 1.5:1:1.80; 1.5:1:1.85; 1.5:1:1.90; 1.5:1:1.95; 1.5:1: 2.0 or any ratio in between these ratios. Complexes having these molar ratios can be used in any of the methods described herein. Methods for preparing a complex prior to introducing the complex into a cell or a population of cells are set forth in the Examples.

As used herein, a supercharged protein can be a superpositively charged protein that has an overall positive charge that is greater than its corresponding unmodified protein. For example, the superpositively charged protein can be a superpositively charged green fluorescent protein (GFP) that has an overall positive charge from about +5 to about +40. For example, the overall positive charge can be about +5, +6, +7, +8, +9, +10, +11, +12, +13, +14, +15, +16, +17, +18, +19, +20, +21, +22, +23, +24, +25, +26, +27, +28, +29, +30, +31, +32, +33, +34, +35, +36, +37, +38, +39 or +40.

The supercharged protein can be operably linked to the amino-terminus or the carboxy-terminus of the nuclease. It is also contemplated that the supercharged protein can be associated with the nuclease, without necessarily being covalently linked to the nuclease. An example of a supercharged protein is a superpositively charged GFP, for example, +36 GFP. +36 GFP can be operably linked to the amino or carboxy- terminus of Cas9 or a functional fragment thereof. See, for example, McNaughton et al., "Mammalian cell penetration, siRNA transfection, and DNA transfection by supercharged proteins," *PNAS* 106(15): 6111-6116. An example of a polypeptide comprising +36 GFP operably linked to the carboxy-terminus of Cas9 is provided herein as SEQ ID NO: 3.

The nuclease can also be operably linked to a supercharged protein and one or more positively charged peptides, for example, one or more transactivating transcriptional activator (TAT) peptide can be operably linked to the amino-terminus or the carboxy-terminus of the nuclease. For example,and not to be limiting, a superpositively charged protein can be operably linked to the carboxy-terminus of the nuclease and one or more TAT peptides (for example, 1X TAT, 2X TAT, 3X TAT, 4X TAT, etc.) can be operably linked to the amino-terminus of the nuclease. An example of polypeptide comprising a TAT peptide operably linked to the amino-terminus of the nuclease and a superpositively charged GFP operably linked to the carboxy-terminus of the nuclease is provided herein as SEQ ID NO: 4. Polypeptide sequences that are at least about 75% identical to SEQ ID NO: 3 or SEQ ID NO:

4 are also provided. For example, polypeptide sequences that are at least about 75%, 80%, 85%, 90%, 95%, 99% or any percentage in between are also provided.

The nuclease can also be operably linked to a supercharged protein and one or more negatively charged peptides, for example, a negatively charged peptide of about 10 to about 25 amino acids in length, for example, SEQ ID NO: 50, can be operably linked to the carboxy-terminus of the site-directed nuclease. For example,and not to be limiting, a superpositively charged protein can be operably linked to the carboxy-terminus of the nuclease and a negatively charged peptide can be operably linked to the carboxy-terminus of the superpositively charged protein.

As used throughout, recombination is a process of exchange of genetic information between two polynucleotides. Homology-directed repair (HDR) refers to DNA repair that takes place, for example, during repair of double-strand breaks in cells. This process requires nucleotide sequence homology and uses a donor molecule, for example, a single stranded or a double stranded nucleotide sequence as a template for repair of a target genomic sequence, i.e., the genomic sequence with the double-strand break, and leads to the transfer of genetic information from the donor to the target genomic sequence. Homology-directed repair can result in a modification of the sequence of the target genomic sequence. For example, HDR can result in an insertion, a deletion or a mutation in the target genomic sequence. Part or all of the sequence of the donor polynucleotide can be incorporated into the target DNA. It is also contemplated that the donor polynucleotide, a portion of the donor polynucleotide, a copy of the donor polynucleotide, or a portion of a copy of the donor polynucleotide integrates into the target DNA.

As used throughout, by non-homologous end joining (NHEJ) is meant the repair of double-strand breaks in DNA by direct ligation of the break ends to one another without the need for a homologous template (in contrast to homology-directed repair, which requires a homologous sequence to guide repair).

The complexes and methods provided herein can be used to correct any mutation in a target DNA by HDR. For example, and not to be limiting, the complexes can be used to replace an incorrect nucleotide sequence with a correct nucleotide sequence (e.g., to restore function to a target polynucleotide sequence that is impaired due to a loss of function mutation, i.e., a SNP) at a specific site in the genome. These mutations can be associated with an autoimmune disorder, a genetic disease, a blood disorder, a T cell disorder, a monogenic disorder, cancer, a neurodegenerative disease, a cardiovascular disease or an infectious disease, to name a few. For example, and not to be limiting, the complexes and

methods provided herein can be used to correct a mutation associated with sickle cell disease (i.e., a mutation in a hemoglobin gene, for example, a GAG to GTG mutation at codon 6 of the beta-globin gene that results in a glutamic acid to valine substitution), severe combined immunodeficiency (SCID) (for example, a mutation in JAK3), beta thalassemia or Wiskott-Aldrich Syndrome.

Correction of single mutations or multiple mutations can be performed with one or more complexes. The complexes and methods provided herein can also be used to insert sequences into a specific site in the genome to correct a deletion, as opposed to making a correction or a substitution. The complexes and methods provided herein can also be used to insert a nucleotide sequence that encodes an a functional polypeptide into a specific site in the genome of the cell, in order to express the functional polypeptide in the cell. The functional polypeptide can be a polypeptide that is endogenous (i.e., normally expressed by the cell) or exogenous to the cell (i.e. not normally expressed by the cell). For example, chimeric antigen receptor (CAR) sequences can be inserted into the genome of a T cell precursor in order to generate cancer specific T cells for the treatment of cancer. In another example, the complexes and methods provided herein can be used to inhibit the activity of a gene at a specific site in the genome of the cell. For example, the complexes and methods provided herein can be used to insert sequences into the CXCR4 or CCR5 receptor to treat or prevent HIV infection.

The complexes provided herein can modify or alter target DNA with surprisingly high efficiency as compared to conventional CRISPR/Cas systems. The efficiency of alteration in a population of cells can be at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75% or 80% or higher or any percentage in between these percentages. The efficiency of alteration can also be greater than or equal to about 80%. Therefore, also provided herein are populations of cells, wherein at least 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75% or 80% or higher or any percentage in between are altered. For example, a mutation associated with sickle cell disease or another disorder has been corrected. If a population of cells comprising a mutation associated with sickle cell disease is contacted with a CRISPR/Cas complex described herein and the mutation is corrected in about 5% of the cells, the efficiency of modification or alteration is about 5%. Optionally, a population of cells wherein the mutation associated with sickle cell disease is corrected in about 30% of the cells, including, for example, 27%, 28% and 29% is sufficient to treat sickle cell disease, upon transplantation in a subject with sickle cell disease. Optionally, a mutation associated with sickle cell disease is corrected in about

40%, 50%, 60%, 70%, 80%, 90% or higher or any percentage in between, of the cells in the population.

In addition to altering the target DNA with high efficiency, the complexes provided herein can also increase the ratio of HDR to NHEJ in a population of cells contacted with the complex. The HDR/NHEJ ratio can be from about 10 to about 0.5. For example, the HDR/NHEJ ratio can be about 10, 9, 8, 7, 6, 5, 4, 3, 2, 1, 0.5 or less or any ratio in between these ratios. In addition to high efficiency of correction and high rate of HDR to NHEJ, the cell survival rate for corrected cells can be at least about 50%, 60%, 70%, 80%, 90% or higher and any percentage in between.

Any cell(s) can be modified or derived using the complexes described herein. Introduction of the complex into the cells can be cell cycle dependent or cell cycle independent. Methods of synchronizing cells to increase the proportion of cells in a particular phase, for example, the S-phase , are known in the art. See, for example, Takahashi et al. "Efficient introduction of a gene into hematopoietic cells in S-phase by electroporation," *Exp. Hematol.* 19(5):343-346 (1991). Depending on the type of cell to be modified, one of skill in the art can readily determine if cell cycle synchronization is necessary.

The cell(s) can be a eukaryotic cell, for example, a mammalian cell. The cell can also be prokaryotic or a plant cell. The cell can be a human cell. The cell can be a germ cell, a somatic cell, a stem cell, a precursor cell or a progenitor cell. The precursor cell can be, for example, a pluripotent stem cell or a multipotent stem cell, like a hematopoietic stem cell. As used throughout, pluripotent cells include induced pluripotent stem cells. Methods of making induced pluripotent stem cells and known in the art and described in the Examples. The cell can also be CD34+ cell, optionally derived from an induced pluripotent stem cell. The CD34+ cell can be selected from the group consisting of a primary CD34+ hematopoietic progenitor cell, a CD34+ peripheral blood cell, a CD34+ cord blood cell and a CD34+ bone marrow cell. The cell can also be a primary cell, for example, a primary CD34+ hematopoietic progenitor cell. The cells are cells that are not cancer cells, cells that are not tumor cells or cells that are not transformed cells. Cells can be screened before or after correction for evidence of undesirable genetic characteristics. Further provided is a cell comprising any of the complexes described herein. The cell can be *in vitro*, *ex vivo* or *in vivo*.

Further provided is a method of site-specific modification of a target DNA in a population of cells comprising introducing into the cells any of the complexes described herein, wherein the complex is introduced into the cells under conditions that allow

homology-directed repair (HDR) and integration of a donor nucleotide, for example, a ssODN or double stranded nucleotide sequence into the target DNA. The complex can be introduced into the cell via nucleoporation. Methods for nucleoporation are known in the art. See, for example, Maasho et al. "Efficient gene transfer into the human natural killer cell line, NKL, using the amaxa nucleofection system," *Journal of Immunological Methods* 284(1-2): 133-140 (2004); and Aluigi et al. "Nucleofection is an efficient non-viral transduction technique for human bone marrow derived mesenchymal stem cells," *Stem Cells* 24(2): 454-461 (2006)), both of which are incorporated herein in their entireties by this reference.

In some of the methods provided herein, the donor nucleotide, for example, a ssODN or a double stranded nucleotide sequence integrates into a target DNA and corrects a mutation in the target DNA. In the methods provided herein the ratio of HDR to NHEJ in a population of cells is increased relative to other CRISPR-Cas9 delivery methods. The HDR/NHEJ ratio can be from about 10 to about 0.5. For example, the HDR/NHEJ ratio can be about 10, 9, 8, 7, 6, 5, 4, 3, 2, 1, 0.5 or less or any ratio in between these ratios. In the methods provided herein, the efficiency of alteration by HDR can be at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80% or greater or any percentage in between these percentages. The efficiency of alteration by HDR can also be greater than or equal to about 80%. For example, if a population of cells comprising a mutation associated with sickle cell anemia is contacted with a CRISPR/Cas complex described herein and the mutation is corrected in about 5% of the cells, the efficiency of alteration by HDR is about 5%. The population of cells can be obtained from the subject having a disorder such that at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75% or 80% or greater or any percentage in between these percentages, of the cells undergo HDR to correct a mutation associated with the disorder. In some cases greater than 80% of the cells from the subject will undergo HDR to correct a mutation associated with the disorder. In the methods described herein, between about 50% and 99% of the cells survive after introduction of the complex. For example, great than about 50%, 60%, 70%, 80%, 90%, 95%, 99% or any percentage in between these percentages, of corrected cells survive after introduction of the complex.

Further provided is a method of treating a disease associated with a mutation in the genomic sequence encoding hemoglobin in a subject comprising: (a) introducing into a population of cells obtained from the subject a complex comprising (1) a guide RNA (gRNA) comprising a first nucleotide sequence that hybridizes to a target DNA in the genome of a cell, wherein the target DNA is a hemoglobin gene that comprises a mutation, and a second

nucleotide sequence that interacts with a site-directed nuclease; (2) a recombinant site-directed nuclease operably linked to a supercharged protein, wherein the site-directed nuclease comprises an RNA-binding portion that interacts with the second nucleotide sequence of the guide RNA and wherein the site-directed nuclease specifically binds and cleaves the target DNA to create a double stranded break; and (3) a single-stranded donor oligonucleotide (ssODN) that hybridizes to a genomic sequence flanking the double stranded break in the target DNA and integrates into the target DNA to correct the mutation in hemoglobin gene; and (b) transplanting the corrected cells into the subject.

In the methods for treating a disease associated with a mutation in the genomic sequence encoding hemoglobin in a subject, for example, sickle cell anemia, the subject with sickle cell anemia can optionally be a transfusion dependent subject or a subject with at least one silent infarction. The subject can also be less than about twelve months, eleven months, ten months, nine months, eight months, seven months, six months, five months, four months, three months, two months, or one month in age. As infants are routinely screen for sickle cell disease, infants can be treated before symptoms of the disease manifest. The methods provided herein can further comprise diagnosing a subject with a disorder, for example, sickle cell disease.

As set forth above, cells can be obtained from the subject with the disease or from a related donor. For example, bone marrow cells can be obtained or harvested from the subject. Bone marrow harvesting involves collecting stem cells with a needle placed into the soft center of the bone, the marrow. Bone marrow can be harvested for example, from the hip bones or sternum of the subject. From about 500 ml to about 1 liter of bone marrow can be obtained from the subject.

In any of the methods provided herein the cell(s) can be a eukaryotic cell, for example, a human cell. The cell can be a germ cell, a stem cell, a precursor cell. The precursor cell can be, for example, a pluripotent stem cell or a hematopoietic stem cell. As used throughout, pluripotent cells include induced pluripotent stem cells. Methods of making induced pluripotent stem cells and known in the art and described in the Examples. The cell can also be CD34+ cell. The CD34+ cell can be selected from the group consisting of a primary CD34+ hematopoietic progenitor cell, a CD34+ peripheral blood cell, a CD34+ cord blood cell and a CD34+ bone marrow cell. The cell can also be a primary cell, for example, a primary CD34+ hematopoietic progenitor cell. The cells are that are not cancer cells, cells that are not tumor cells or cells that are not transformed cells. The cell can be *in vitro* or *ex vivo*. The cells can also be in a pharmaceutically acceptable composition.

The methods provided herein can further comprise culturing the cells corrected with HDR. For example, the cells can be cultured under conditions for expansion or under conditions that promote differentiation of the corrected cells into T-cells. For example, and not to be limiting, using the methods provided herein, after a mutation has been corrected in induced pluripotent stem cells via HDR, the corrected cells can be co-cultured with human bone marrow stromal cells to generate CD34+ cells. The CD34+ cells can then be cultured under conditions that differentiate the CD34+ cells into T cells.

The methods provided herein can further comprise screening the corrected cells for the proper correction, other mutations, or NEJ prior to transplantation. Optionally cells can be screened to detect cells with one or more corrections.

In the methods provided herein, the cells can be transplanted into the subject after modification, for example, after correction of a mutation by HDR. The cells can be transplanted into the subject with or without differentiation. For example, modified hematopoietic stem cells (HSCs) can be administered in a bone marrow transplant, wherein the HSCs are allowed to differentiate and mature *in vivo* in a subject Alternatively, the modified cells can be differentiated into a desired population of cells prior to transplantation.

As used herein, transplanting, introducing or administering cells to a subject refers to the placement of cells into a subject. For example, the cells described herein comprising a target DNA sequence corrected or modified according to the methods described herein can be transplanted into a subject, by an appropriate route which results in at least partial localization of the transplanted cells at a desired site. The cells can be implanted directly to the desired site, or alternatively can be administered by any appropriate route which results in delivery to a desired location in the subject where at least a portion of the implanted cells remain viable. For example, the cells can be administered systemically, via intravenous infusion. The period of viability of the cells after administration to a subject can be as short as a few hours, e. g. twenty-four hours, to a few days, to as long as several years.

For *ex vivo* methods, cells can be autologous cells, i.e., a cell or cells taken from a subject who is in need of modification of a target DNA in the cell or cells (i.e., the donor and recipient are the same individual). As described herein, the modification can be, for example correction of a mutation, insertion of a sequence that inhibits activity of a protein or insertion of a sequence that increases expression of a protein, for example, insertion of a sequence encoding a chimeric antigen receptor that can be used to target cancer cells. Autologous cells can be used to avoid immunological reactions that can result in rejection of the cells. In other words, when using autologous cells, the donor and recipient are the same subject.

Alternatively, the cells can be heterologous, e.g., taken from a donor, preferably a related donor. The second subject can be of the same or different species. Typically, when the cells come from a donor, they will be from a donor who is sufficiently immunologically compatible with the recipient to reduce the chances of transplant rejection, and/or to reduce the need for immunosuppressive therapy. The cells can also be obtained from a xenogeneic source, i.e., a non-human mammal that has been genetically engineered to be sufficiently immunologically compatible with the recipient, or the recipient's species. Any of the methods of treating a disorder described herein can further comprise administering one or more immunosuppressants to the subject.

In the methods involving transplantation, a subject optionally undergoes myeloablative therapy prior to transplantation of any of the cells described herein. The myeloablative therapy can include administering one or more doses of chemotherapy, radiation therapy, or both, that results in severe or complete depletion of healthy bone marrow cells. In another example, the subject can undergo submyeloablative therapy that includes administering one or more doses of chemotherapy, radiation therapy, or both, that depletes a portion of the healthy bone marrow cells. The cells can also be transplanted into subjects that have undergone nonablative chemotherapy. For example, the cells can be transplanted into a subject that has been treated with Busulfan, Fludarabine and/or Treosulfan.

In the methods involving transplantation, an effective dose or amount of corrected cells is administered to the subject. The terms effective amount and effective dosage are used interchangeably. The term effective amount is defined as any amount necessary to produce a desired physiologic response. In some methods, about 1 X 10^6 to about 7 X 10^6 corrected cells/kg can be administered, but this amount can vary depending on the associated disorder. The percentage of corrected cells that Effective amounts and schedules for administering the cells may be determined empirically, and making such determinations is within the skill in the art. The dosage ranges for administration are those large enough to produce the desired effect (e.g., treatment of a disease, for example, sickle cell anemia). The dosage should not be so large as to cause substantial adverse side effects, such as unwanted cross-reactions, anaphylactic reactions, and the like. Generally, the dosage will vary with the age, condition, sex, type of disease, the extent of the disease or disorder, route of administration, or whether other drugs are included in the regimen, and can be determined by one of skill in the art. The dosage can be adjusted by the individual physician in the event of any contraindications.

Dosages can vary, and the agent can be administered in one or more dose administrations daily, for one or multiple days as needed.

As used throughout, a subject can be a vertebrate, more specifically a mammal (e.g., a human, horse, cat, dog, cow, pig, sheep, goat, mouse, rabbit, rat, and guinea pig). The term does not denote a particular age or sex. Thus, adult and newborn subjects, whether male or female, are intended to be covered. As used herein, patient or subject may be used interchangeably and can refer to a subject with or at risk of developing a disorder. The term patient or subject includes human and veterinary subjects.

As used herein the terms treatment, treat, or treating refers to a method of reducing one or more of the effects of the disorder or one or more symptoms of the disorder, for example, sickle cell disease, by eliciting an immune response in the subject. Thus in the disclosed method, treatment can refer to a 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100% reduction in the severity of sickle cell disease and other disorders. For example, a method for treating sickle cell disease is considered to be a treatment if there is a 10% reduction in one or more symptoms of the infection in a subject as compared to a control. Thus the reduction can be a 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, or any percent reduction in between 10% and 100% as compared to native or control levels. It is understood that treatment does not necessarily refer to a cure or complete ablation of the disorder or symptoms of the disorder.

Also provided is a method of correcting a mutation associated with a T-cell disorder comprising introducing into a population of cells obtained from a subject with the T-cell disorder a complex comprising: (a) a guide RNA (gRNA) comprising a first nucleotide sequence that hybridizes to a target DNA in the genome of a cell, wherein the target DNA comprises the mutation associated with the T-cell disorder, and a second nucleotide sequence that interacts with a site-directed nuclease; (b) a recombinant site-directed nuclease operably linked to a supercharged protein, wherein the site-directed nuclease comprises an RNA-binding portion that interacts with the second nucleotide sequence of the gRNA and wherein the site-directed nuclease specifically binds and cleaves the target DNA that comprises the mutation associated with the T-cell disorder to create a double stranded break in the target DNA; and (c) a single stranded donor oligonucleotide (ssODN) comprising a third nucleotide sequence that hybridizes to a genomic sequence flanking the double stranded break in the target DNA and that integrates into the target DNA to correct the mutation associated with the T-cell disorder, wherein the complex is introduced into the cell under conditions that

allow homology-directed repair (HDR) to correct the mutation associated with the T-cell disorder.

In the methods provided herein, the target DNA comprising a mutation associated with a T-cell disorder can be a target DNA that encodes a protein associated with T-lymphocyte development. For example, the target DNA can encode JAK3. Such corrected cells can be used, for example, in the treatment of SCID.

In addition to correcting mutations in the genome of a cell, the complexes and methods provided herein can also be used to insert functional polypeptides at specific sites in the genome of a cell, such that the polypeptide is expressed by the cell. The polypeptide can be expressed in the cell or on the cell surface.

Also provided is a method of making tumor-specific T-cell precursor cells comprising introducing into a population of T-cell precursor cells a complex comprising: (a) a guide (gRNA) comprising a first nucleotide sequence that hybridizes to a target DNA in the genome of the T cell precursor cells and a second nucleotide sequence that interacts with a site-directed nuclease; (b) a recombinant site-directed nuclease operably linked to a supercharged protein, wherein the site-directed nuclease comprises an RNA-binding portion that interacts with the second nucleotide sequence of the gRNA and wherein the site-directed nuclease specifically binds and cleaves the target DNA to create a double stranded break; and (c) donor nucleotide sequence comprising a third nucleotide sequence that encodes a chimeric antigen receptor (CAR) and a fourth nucleotide sequence that hybridizes to a genomic sequence flanking the double stranded break in the target DNA, wherein the complex is introduced into the T-cell precursor cells under conditions that allow homology-directed repair (HDR) and integration of the third nucleotide sequence into the target DNA to form modified T-cell precursor cells that express the CAR.

The T cell precursor cells can be obtained from a subject with cancer. As set forth above, the HDR/NHEJ ratio can be from about 10 to about 0.5. For example, the HDR/NHEJ ratio can be about 10, 9, 8, 7, 6, 5, 4, 3, 2, 1, 0.5 or any ratio in between these ratios. In the methods provided herein, the efficiency of alteration by HDR can be at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80% or any percentage in between these percentages. The efficiency of alteration by HDR can also be greater than or equal to about 80%. For example, when using the methods described herein, if a nucleotide sequence encoding an functional polypeptide, for example, a nucleotide sequence that encodes a CAR, is inserted in about 5% of the cells, the efficiency of alteration by HDR is about 5%. The population of cells can be obtained from the subject that has

cancer such that at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75% or 80% or any percentage in between these percentages, of the cells undergo HDR to insert a nucleotide sequence that encodes a chimeric antigen receptor (CAR) and form cells that express the CAR. In some cases greater than 80% of the cells from the subject will undergo HDR to correct a mutation associated with the disorder.

The modified T-cell precursor cells that express the CAR can be transplanted into a subject with cancer. As used herein, cancer is a disease characterized by the rapid and uncontrolled growth of aberrant cells. Cancer cells can spread locally or through the bloodstream and lymphatic system to other parts of the body. Examples of cancers include but are not limited to, breast cancer, prostate cancer, ovarian cancer, cervical cancer, skin cancer, pancreatic cancer, colorectal cancer, renal cancer, liver cancer, brain cancer, lymphoma, leukemia, lung cancer and the like. The modified T-cell precursor cells that express the CAR exhibit anti-tumor immunity when the antigen binding domain binds to its corresponding antigen.

Disclosed are materials, compositions, and components that can be used for, can be used in conjunction with, can be used in preparation for, or are products of the disclosed methods and compositions. These and other materials are disclosed herein, and it is understood that when combinations, subsets, interactions, groups, etc. of these materials are disclosed that while specific reference of each various individual and collective combinations and permutations of these compounds may not be explicitly disclosed, each is specifically contemplated and described herein. For example, if a method is disclosed and discussed and a number of modifications that can be made to a number of molecules including the method are discussed, each and every combination and permutation of the method, and the modifications that are possible are specifically contemplated unless specifically indicated to the contrary. Likewise, any subset or combination of these is also specifically contemplated and disclosed. This concept applies to all aspects of this disclosure including, but not limited to, steps in methods using the disclosed compositions. Thus, if there are a variety of additional steps that can be performed, it is understood that each of these additional steps can be performed with any specific method steps or combination of method steps of the disclosed methods, and that each such combination or subset of combinations is specifically contemplated and should be considered disclosed.

Publications cited herein and the material for which they are cited are hereby specifically incorporated by reference in their entireties.

EXAMPLES

Example 1

Correction of SCID by CRISPR/Cas9 enhanced gene replacement

Mutations of the Janus family kinase JAK3 gene cause severe combined immunodeficiency (SCID). JAK3 deficiency in humans is characterized by the absence of circulating T cells and natural killer (NK) cells with normal numbers of poorly functioning B cells (T–B+NK–). As shown herein, using SCID patient-specific induced pluripotent stem cells (iPSCs) and a T cell *in vitro* differentiation system, a complete block in early T cell development of JAK3-deficient cells was demonstrated. Correction of the novel JAK3 mutation by CRISPR/Cas9 enhanced gene replacement restores normal T cell development, including the production of mature T-cell populations with a broad T Cell Receptor (TCR) repertoire. Whole genome sequencing of corrected cells demonstrated no CRISPR/Cas9 off-target modifications. Thus, provided herein is a novel approach for the study of human lymphopoiesis and a method for gene replacement therapy in humans with immunodeficiencies.

Allogeneic hematopoietic stem cell (HSC) transplantation is currently the only established therapy for SCID; however, delayed immune recovery and risk of graft-vs-host disease present significant risks. Treatment by retroviral-based gene therapy has been successfully demonstrated for X-linked SCID. However, severe adverse effects of insertional mutagenesis have been observed with retroviral gene therapy. Self-inactivating lentiviral vectors have been used effectively in recent clinical trials, but long-term follow-up is needed to thoroughly address safety concerns.

Provided herein is an alternative therapeutic strategy in which patient-specific induced pluripotent stem cells (iPSCs) are derived, and disease-causing mutations are corrected by gene replacement using a CRISPR-Cas9 complex. These corrected iPSCs could optionally be differentiated into hematopoietic progenitors for transplantation into patients to treat the disease (Hanna et al., "Treatment of sickle cell anemia mouse model with iPS cells generated from autologous skin," *Science* 318: 1920-1923 (2007)). As shown herein, differentiation of JAK3-deficient human T cells is blocked at an early developmental stage. Also demonstrated is that correction of the human JAK3 mutation by CRISPR/Cas9 enhanced gene replacement restores the differentiation potential of early T cell progenitors. These corrected progenitors are capable of producing NK cells and mature T cell populations expressing a broad

repertoire of T-cell antigen receptors (TCR). These studies establish a powerful system for determining the mechanism of immunodeficiency in human SCID patients and for testing pharmacological and genetic therapies for the disorder.

Patient Information

The male patient was enrolled in an Institutional Review Board-approved study in accordance with the Declaration of Helsinki. The family history was negative for immune deficiencies. For the first 8 months of age he had poor weight gain, diarrhea, and recurrent bronchiolitis requiring frequent hospitalization. He was admitted to the hospital at 8 months of age with severe respiratory distress and oral thrush. Bronchoscopy with bronchial alveolar lavage demonstrated bacterial (pseudomonas, H flu, S. pneumonia) and viral organisms (respiratory syncytial virus). Immunologic evaluations demonstrated severe hypogammaglobulinemia, with an IgE<3, IgA<4, IgG=29, IgM=26. Immune phenotyping of peripheral blood demonstrated complete absence of CD3+ T cells and NK cells, though B cells were present (absolute B cell count=875). Mitogen studies demonstrated a complete lack of response to concanavalin A, poke weed mitogen and phytohemagglutinin A. The diagnosis of SCID was confirmed by genetic testing, with a homozygous C>T nucleotide substitution in exon 14 of the JAK3 gene, resulting in the replacement of an arginine codon (CGA) with a stop codon (TGA) at amino acid position 613. This is the first report linking this JAK3 variant (rs149316157) to a clinical case of SCID. The patient underwent a reduced intensity conditioning matched unrelated bone marrow transplant, and is doing well now two years off therapy with complete immune reconstitution.

Human iPSC reprogramming and characterization

For iPSC induction, $5x10^4$ primary keratinocytes were seeded into one well of a 6-well plate. On the following day, keratinocytes were transduced with 1 mL of virus supernatant and 1 mL of human keratinocyte medium containing polybrene at a final concentration of 4 μg/mL. The keratinocytes were spinfected at 800 x g for 45 minutes (day 1). The transduction procedure was repeated again the next day. On day 3, cells were changed to fresh human keratinocyte medium and cultured for two more days. On day 5, the keratinocytes were trypsinized and transferred to a 10 cm dish pre-seeded with mitomycin C-treated murine embryonic fibroblasts (MEFs) and cultured in human keratinocyte medium. On day 7, cells were changed to human ES medium and continuously cultured in the same dish for 3-4 weeks. ES medium was changed daily. Potential iPSC colonies were visible after 2-3 weeks. These colonies were individually picked and expanded on MEFs for analysis. To

remove the integrated lentiviral and polycistronic sequences, iPSCs were infected with a Cre-expressing adenovirus (rAd-Cre-IE). Individual colonies were picked and Cre-mediated removal of floxed sequences was verified by PCR using the primers gctaattcactcccaaagaagacaag (SEQ ID NO: 5) and cttcagcaagccgagtcctg (SEQ ID NO: 6).

Generation of CD34+ cells and T cells with OP9 co-culture

The procedure was described previously (Chang et al., "Broad T-cell receptor repertoire in T-lymphocytes derived from human induced pluripotent stem cells," PloS one 9, e97335 (2014)). This method was used with the following modifications. Cultures of hiPSCs in one well of a 6 well plate were treated as described by Ohnuki et al (Ohnuki M, "Generation and characterization of human induced pluripotent stem cells. *Curr Protoc Stem Cell Biol* Chapter 4: Unit 4A *2* (2009)) with CTK solution to make small cell clumps. Cell clumps were then transferred to a 10 cm plate that was pre-seeded with 2-day old OP9 cells in α-MEM-based medium containing 10% FBS, 1X penicillin/streptomycin and 100 μM mono-thioglycerol. The medium was changed every other day, and cells were cultured for 18 days without splitting. After 18 days of co-culture, cells were harvested by treating with dissociation solution (0.15% collagenase IV and 0.015% hyaluronidase in α-MEM medium) for about 30 minutes and followed by 0.25% trypsin for another 30 minutes. CD34+ cells were then purified on anti- CD34+ magnetic beads (MicroBead Kit; Miltenyi Biotec, Bergisch Gladbach, Germany). For T cell differentiation, these CD34+ cells were plated onto OP9-DL4 cells and cultured with α-MEM medium containing 20% FBS, 5 ng/mL hFlt3-L, 5 ng/mL hIL-7, and 10 ng/mL hSCF. The medium was changed every other day, and cells were transferred to new OP9-DL4 plates every 4 days.

T cell stimulation

In vitro derived T cells from hiPSCs were stimulated by incubation with CD3/28 beads (Invitrogen, Carlsbad, CA) according to the manufacturers' protocol for 3 days prior to analysis by flow cytometry, as previously described (Chang et al., 2014).

Flow Cytometry

Cells were harvested and washed before analysis with an LSRFortessa cell analyzer (BD Bioscience, San Jose, CA). For cell surface staining, propidium iodide (PI, Sigma-Aldrich, St. Louis, MO) was used to exclude dead cells. For apoptosis assay, harvested cells were first stained with cell surface antibodies for 30 min. After washing once with 1X PBS, the cells were resuspended in 100 μL of Annexin Binding Buffer (Invitrogen, Carlsbad, CA) containing Annexin V-647 (Invitrogen, Carlsbad, CA) and PI and incubated for 15 min before adding 400 μL of Annexin Binding Buffer with PI. Antibodies were obtained from BD

Biosciences unless otherwise indicated: CD3 (Percp-Cy5-5, clone UCHT1), CD4 (PE-Cy7, clone SK3), CD7 (APC, BV510, clone M-T701), CD8 (APC-Cy7, clone SK1), CD16 (PE, clone B73.1), CD25 (FITC, clone 2A3), CD34 (PE-Cy7, clone WM59), CD43 (PE, clone 1G10), CD56- PE (clone MY31), CD69 (FITC, clone L78), NKG2D-PE (clone 1D11), TCR-αβ (FITC, PE, clone T10B9.1A-31), TCR-Vδ1-FITC (Fisher Scientific, Pittsburgh, PA, Clone TS8.2), TCR-Vδ2-PE (clone B6), TCRVγ9- FITC (clone B3), TNF-α-PE-Cy7 (clone MAB11), Beta Mark TCR Repertoire Kit (Beckman Coulter, Atlanta, GA).

Vector Construction

The polycistronic OSKM vector was previously described (Chang et al.,"Polycistronic lentiviral vector for "hit and run" reprogramming of adult skin fibroblasts to induced pluripotent stem cells," *Stem cells* 27: 1042-1049 (2009)). The Lenti-hDL4-mCherry plasmid was constructed by cloning a PCR-amplified human DL4 cDNA (Open Biosystems, LaFayette, CO), an IRES fragment (Open Biosystems) and mCherry cDNA into a lentiviral vector (pDL171) which contains the EF1α promoter. PCR reactions were performed using PrimeStar polymerase (Takara, Mountain View).

To construct CRISPR plasmids, gRNA oligos were designed and introduced into pX330 and pX335 plasmids following the Zhang lab protocol (Addgene, Cambridge, MA). To construct the JAK3 repair plasmid, wild type human genomic DNA was PCR amplified using JAK3 primer sets (5' arm: gtcgacgtcgacgctcagtgaagctgaagtattccttctgcttcacagggcgaccactac (SEQ ID NO: 7) and atttaaatcctcccctcgaacccttaccaaactcctatgcatactacag (SEQ ID NO:8); 3' arm: ttaattaattaattagcattttaggttcaggttgtgagaacactagaagagaacaagtca (SEQ ID NO: 9) and gtatacgtatacgcatacctggagaggggacaaggtcttgagatgcgagggt (SEQ ID NO: 10). After digesting with enzymes (5' arm: SalI and SwaI; 3' arm: PacI and BstZ17I), the PCR products were cloned into a plasmid containing a LoxP-PGK-Neo-LoxP fragment. All of the oligos used in this study were synthesized by Integrated DNA Technologies (IDT, Coralville, IA). To construct the BCL2 lentiviral plasmid, a primer set (forward: agccaccttaattaagccaccatggcgcacgctgggagaacggggtacgata (SEQ ID NO: 11) and reverse: taacagagagaagttcgtggctccggatcccttgtggcccagataggcacccagggtgat (SEQ ID NO: 12)) was used to amplify the human BCL2 cDNA (Open Biosystems) fragment. The product was linked with GFP through a 2A sequence by PCR and cloned into the pDL171 vector. gRNA-F1 caccGTG AGA TAC AGA TAC AGA CA (SEQ ID NO: 13) gRNA-R1 aaacTGT CTG TAT CTG TAT CTC AC (SEQ ID NO: 14) gRNA-F2 caccgAAT GAT TTG CCT GGA ATG CC (SEQ ID NO: 14) gRNA-R2 aaacGGC ATT CCA GGC AAA TCA TTc (SEQ ID NO: 15)

gRNA-F3 caccgCAG CCT AGG CAA AGG CCT GC (SEQ ID NO: 16) gRNA-R3 aaacGCA GGC CTT TGC CTA GGC TGc (SEQ ID NO: 17) gRNA-F4 caccgTGC CAA CAG AAC TGC CTG AT (SEQ ID NO: 18) gRNA-R4 aaacATC AGG CAG TTC TGT TGG Cac (SEQ ID NO: 19) gRNA-F5 caccGAC CAG GGT GCA AGT GTG GA (SEQ ID NO: 20) gRNA-R5 aaacTCC ACA CTT GCA CCC TGG TC (SEQ ID NO: 21) gRNA-F6 caccGCT CCT CAG CCT GGC ATT CA (SEQ ID NO: 22) gRNA-R6 aaacTGA ATG CCA GGC TGA GGA GC (SEQ ID NO: 23)

Cell culture

IPSCs were cultured on mitomycin C-treated MEFs derived from E14.5 CF-1 embryos in ES cell media consisting of DMEM F-12 supplemented with 1X non-essential amino acids, 1X penicillin-streptomycin, 1X L-glutamine (all from Mediatech, Corning, NY), 20% KnockOut Serum Replacement (Invitrogen), 2-βME (Sigma) and 5-10 ng/mL bFGF (Invitrogen). Human primary keratinocytes were cultured in DermaLife K Medium Complete Kit (LifeLine Cell Technology, Frederick, MD). OP9 cells were purchased from ATCC and grown in α-MEM medium with 20% FBS and penicillin-streptomycin. OP9-DL4 cells were established by transducing OP9 cells with a lentivirus containing hDL4 and mCherry.

Virus Production

For preparation of lentivirus, 10 μg of the lentiviral vector, 2.5 μg of the envelope plasmid (pMDG), and 7.5 μg of the packaging plasmid (pCMBVdR8.9.1) were co-transfected into 5x106 293T cells by Fugene 6 (Roche, Nutley, NJ or Promega, Madison, WI). Virus-containing supernatant was collected 2 days after transfection and passed through a 0.45 μm filter.

Gene targeting

IPSCs were treated with 0.25% trypsin for 5 minutes to generate single cell suspensions. After washing twice with 1X PBS, 1 to 2 million cells were mixed with 5 μg of JAK3 repair plasmid and 5 μg of pX330-JAK3 or pX335-JAK3 plasmids for Nucleofection (Human Stem Cell Nucleofector Kit, program A-023, Lonza, Alpharetta, GA) and plating onto MEFs. Two to four days later, hES medium containing 30 μg/mL of G418 was added to the plates to select for drug resistant colonies. The colonies were picked 3 to 4 weeks later and expanded for genomic DNA extraction. For PCR genotyping, a 5' primer set (tgctaaagcgcatgctccagact (SEQ ID NO: 24) and gtcttcatctcagggtcggct (SEQ ID NO: 25) and a 3' primer set (cctctctgtgcattatggcag (SEQ ID NO: 26) and gccttctatcgccttcttg (SEQ ID NO: 27)) were used. To remove the Neo selection marker, hiPSCs were infected with a Cre-expressing adenovirus (rAd-Cre-IE).

RT-PCR

Total RNA was isolated from *in-vitro* derived cells with Trizol reagent (Invitrogen, Carlsbad, CA). cDNA was synthesized with 0.5 to 2 μg of total RNA using Superscript First-strand Synthesis System (Invitrogen) according to the manufacturer's instructions. SYBR Green PCR Master Mix (Life Technologies, Carlsbad, CA) was used for qPCR according to the manufacturer's instructions. Primer sets used for qPCR are GAPDH (F: actcctccacctttgacgct (SEQ ID NO: 28), R: tcccctcttcaagggtctacatg (SEQ ID NO: 29)); PU.1 (F: gtgcaaaatggaagggtttc (SEQ ID NO: 30), R: ggagctccgtgaagttgttc (SEQ ID NO: 31)); GATA3 (F: tgtttcctttcactggccaca (SEQ ID NO: 32), R: aacggcaactggtgaacggta (SEQ ID NO: 33)); BCL11B (F: ggcgatgccagaatagatgccg (SEQ ID NO: 34), R: ccaggccacttggctcctctatctccaga (SEQ ID NO: 35)); RAG1 (F: ccttactgttgagactgcaatatcc (SEQ ID NO: 36), R: ctgaagtcccagtatatacttcacac (SEQ ID NO: 37)); RAG2 (F: cccagaagcagtaataatcatcgag (SEQ ID NO: 38), R: atgtgggatgtagtagatcttgc (SEQ ID NO: 39)); pTa (F: gggtcttacctcagcagttac (SEQ ID NO: 40), R: cctcacacagtgtgacgcag (SEQ ID NO: 41)); BCL2 (F: gactgagtacctgaaccggc (SEQ ID NO: 42), R: gggccaaactgagcagagtc (SEQ ID NO: 43)); BAX (F: aagaccagggtggttgggac (SEQ ID NO: 44), R: gtaagaaaaatgcccacgtc (SEQ ID NO: 45)); and JAK3 (F: agtcagacgtctggagcttc (SEQ ID NO: 46), R: gtgagcagtgaaggcatgagtc (SEQ ID NO: 47)). All values were normalized relative to GAPDH expression.

Whole Genome Sequencing and Analysis

DNA from iPSCs was sheared using a Covaris S2 Focused-ultrasonicator: 130 μL samples in microTUBEs were subjected to two 40-second cycles of 10% Duty Cycle, Intensity of 4, and 200 Cycles per Burst in Frequency Sweeping Mode. DNA Chip (DNA 1000 Kit; Agilent Technologies, Santa Clara, CA) analysis using an Agilent 2100 Bioanalyzer indicated an average fragment size of 400 bp. Library preparation was performed using an NEBNext Ultra DNA Library Prep Kit for Illumina (NEB #E7370), and the final library concentration was determined by qPCR using a KAPA Illumina Library Quantification Kit (KK4835; KAPA Biosystems, Wilmington, MA) and an Applied Biosystems ViiA 7 Real-Time PCR System (Life Technologies). Sequencing clusters were produced on the flow cell using an Illumina TruSeq PE Cluster Kit v3 – cBot – HS (PE-401-3001) and an Illumina cBot. WGS was performed using an Illumina TruSeq SBS Kit v3 – HS – 200 cycles (FC-401-3001) and an Illumina HiSeq 2500 upgrade to generate 2x100 single-index paired-end reads for bioinformatic analysis. Probable off-target sites were identified by aligning the CRISPR/Cas9 guide sequences to the hg19 reference genome using EMBOSS fuzznuc software (v6.6.0.0) (Rice et al., "EMBOSS: the European Molecular Biology Open

Software Suite," *Trends in Genetics* : TIG 16: 276-277 (2000)) and allowing for a maximum of three mismatches; 1193 sites were predicted for the first guide sequence (GTGAGATACAGATACAGACA) (SEQ ID NO: 48) and 257 sites for the second guide sequence (AATGATTTGCCTGGAATGCC) (SEQ ID NO: 49). All of the reads from the WGS for each sample were mapped to the hg19 reference genome using the BWA (v0.7.5a) mem algorithm (Li and Durbin, "Fast and accurate long-read alignment with Burrows-Wheeler transform," *Bioinformatics* 26: 589-595 (2010)) and duplicate reads were removed using Picard-tools (v1.100) (http://picard.sourceforge.net). Local realignment and base quality re-calibration were performed using GATK (v2.7-2) (McKenna et al., "The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data," *Genome research* 20: 1297-1303 (2010)). Both SNVs and indels were called using the GATK HaplotypeCaller. Additionally, SNVs and indels were separately re-calibrated as described in GATK Best Practices and quality filters were applied. The variants from the reference genome that were common to all four iPSC samples were excluded from CRISPR/Cas9 off-target analysis. The non-excluded variants were screened using Bedtools (v2.17.0) (Quinlan and Hall, "BEDTools: a flexible suite of utilities for comparing genomic features," Bioinformatics 26: 841-842 (2010)) to determine if they fell within the probable off-target sites. The analysis shows that none of these variants reside in the off-target sites and suggests these mutations were randomly accumulated. All of the functional variants (excluded and non-excluded) with a low allele frequency (< 1%, dbSNP 138) were then annotated using the ANNOVAR software package and screened for known associations with diseases in HGMD and ClinVar (v20140902); additionally, all of the hits with a high CADD score (CADD >= 20) were also screened for associations with complex diseases using the GWAS Catalog and COSMIC (v70). No validated disease-associated variants were identified in the databases queried. Of particular interest, the JAK3 C1837T (p.R613X) mutation was also not validated to associate with a disease, though the SNP (rs149316157) is predicted to be significantly deleterius, with a GERP score of 3.85 and a CADD score (CADD phred-like score) of 38. Therefore, the JAK3 C1837T variant was associated for the first time with a clinical case of SCID.

Accession codes

The WGS data can be accessed at the NCBI SRA database with the accession number SRP056149.

JAK3-deficient human T cells express low levels of BCL2 and die at an early developmental stage

IPSCs were generated from skin keratinocytes (Chang et al., 2009) of a SCID patient homozygous for a C>T nucleotide substitution in exon 14 of the JAK3 gene. This mutation replaces a CGA codon (arginine at 613) with a TGA stop codon (p.R613X). As described above, the four-month-old patient presented with a T-B+NK- clinical phenotype. To determine whether this SCID phenotype can be recapitulated *in vitro*, differentiation of patient-specific iPSCs to T lymphocytes using a two-step OP9 and OP9-DL4 system (Chang et al., 2014) was attempted. JAK3-deficient iPSCs grew at a rate comparable to control iPSCs derived from healthy donors, and these iPSCs efficiently differentiated into CD34+ hematopoietic progenitors (HPs) on OP9 stromal cell monolayers. However, when the JAK3-deficient, iPSC-derived CD34+ HPs were plated on OP9-DL4 stromal monolayers, T-cell differentiation was absent compared to controls (Fig. 1). No CD3+ T cells or CD3-CD16+CD56+ NK cells were observed (Fig.1A), and no CD4+CD8+ double positive (DP), CD4+ single positive (SP), or CD8+ single positive (SP) T cells were detected (Fig.1B). Jak3 knockout (KO) mice have a small thymus due to a block in thymocyte differentiation at the CD4-CD8- double negative (DN) stage prior to productive TCR rearrangement. To further understand the developmental defects resulting from a JAK3 mutation in humans, T lineage commitment and maturation of JAK3-deficient cells compared to normal JAK3 WT controls was assayed. IPSC-derived CD34+ cells were plated on OP9-DL4 monolayers, and cells were harvested and analyzed for lymphocyte markers at T-cell induction day (TD) 14, 21, 28 and 35 (Fig. 4A). In normal controls, 1.2 X 107 CD7+ cells (84% of cells counted in the lymphoid gate) were generated at TD14 from 1-2 X 106 CD34+ cells. T cell markers CD4, CD8, CD3 and TCR αβ were sequentially detected upon T cell maturation. At TD35, more than 50% of the population was CD8 SP cells. In JAK3-deficient cells, only 4.5 X 104 CD7+ cells (38.9% of cells counted in lymphoid gate) were generated at TD14 from 1-2 X 10^6 CD34+ cells. The number of CD7+ cells decreased during extended culture and T cell markers CD3, CD4, CD8 and TCR αβ were not significantly expressed. During the transition through early T cell progenitors (ETPs), the CD4-CD8- (DN) to CD4+CD8+ (DP) stages are directed by precise activation and repression of specific transcription factors. In control cells, the silencing of PU.1 and induction of GATA3 and BCL11B (Fig. 1C) suggest that these cells proceed to the onset of T lineage commitment (DN2 to DN3) followed by TCR rearrangement. In contrast, in JAK3-deficient cells PU.1 accumulates and GATA3 and BCL11B levels are reduced (Fig. 1C). These data suggest that human JAK3-deficient cells

arrest before or at the DN2 stage, which is similar to the stage at which T cells die in Jak3 KO mice. Interestingly, human JAK3-deficient cells may express sufficient RAG1, RAG2 and PTCRA (Fig. 1C) to perform TCR rearrangement, but the cells do not survive long enough to proceed to this important developmental stage. These profound defects in lymphocyte development of JAK3-deficient cells can be explained by the absence of IL-7 signaling which plays an important role in lymphoid progenitor survival and differentiation. IL-7/JAK3 signaling maintains thymocyte homeostasis by regulating the BCL2 family of apoptotic regulators. Thymocytes and peripheral T cells from Jak3 KO mice have a high apoptotic index in part through selectively elevating BAX, a pro-apoptotic factor, and by reducing expression of BCL2, an anti-apoptotic factor. Similarly, in these studies, an increase in apoptosis of *in vitro*-derived human JAK3-deficient cells compared to controls at TD10 (9% to 2.2%) and TD17 (7% to 1.9%) (Fig. 2A). Consistent with this phenotype, BAX levels were increased and BCL2 levels were reduced in JAK3-deficient cells compared to controls (Fig. 2B). Forced expression of Bcl2 rescues T, but not B or NK cell development in γc–deficient mice (Kondo et al., *Immunity* 7: 155-162 (1997)). Transplantation of Jak3 KO mice with Bcl2-expressing Jak3 KO bone marrow cells also improves peripheral T cell numbers (Wen et al., *Molecular and cellular biology* 21: 678-689 (2001)). To determine whether overexpression of BCL2 will rescue T cell developmental defects of human JAK3-deficient cells, in vitro-derived, JAK3-deficient CD34+ cells were transduced with a lentivirus containing a BCL2- 2A-GFP polycistron driven by EF1a promoter. After transduction, CD34+ cells were plated on OP9-DL4 monolayers and assayed for NK and T cell markers at TD 28. No CD3-CD16+CD56+ NK cells were found in GFP- (JAK3-; BCL2 low) or GFP+ cells (JAK3-; BCL2+) (Fig 2C). These findings suggest that BCL2 released the blockage at the DN stage in JAK3-deficient cells. Interestingly, a second developmental arrest was evident at the DP stage; no further differentiation of CD8+CD4+ DP positive cells was observed in GFP+ cells (Fig 2C). In summary, the studies described above demonstrate that human SCID phenotypes can be recapitulated *in vitro* with patient-derived iPSCs. JAK3 deficiency results in proliferative defects in DN thymocytes. Forced expression of BCL2 enhances survival of DN cells, which further differentiate into DP thymocytes. Nevertheless, DP thymocytes fail to mature to SP T cells, and this defect may result from the absence of IL7/JAK3 signaling.

Correction of the JAK3 deficiency in SCID hiPSCs by CRISPR/Cas9 enhanced gene replacement

To determine whether normal T cell development can be restored in JAK3-deficient SCID patient cells, the JAK3 mutation was corrected in iPSCs by CRISPR/Cas9 enhanced gene replacement. Six guide RNAs within introns upstream and downstream of exon 14 were designed to target wtCas9 or nCas9 near the C1837T mutation, and a correction template was used for gene replacement (Fig. 3A). IPSCs were nucleofected with two plasmids expressing the D10A Cas9 nickase and paired guide RNAs or a single plasmid expressing wild-type Cas9 and a single guide RNA. Cells were grown in medium containing G418 for 2 weeks post nucleofection. Individual colonies were picked, expanded, and genotyped by PCR (Fig. 3B Top). The efficiency of CRISPR/Cas9–mediated JAK3 gene correction is shown in Fig. 3C. Three clones from WT Cas9 + gRNA #1, 3 clones from WT Cas9 + gRNA #2 and 6 clones from Cas9 nickase + paired gRNAs #1 and #2 were further verified by Sanger sequencing. In 12 sequenced clones, 2 homozygous corrected clones (1 clone from Cas9 nickase + paired gRNA #1 and #2, and 1 clone from WT Cas9 + gRNA #1) and 10 heterozygous corrected clones were identified (Fig. 3D). Restoration of JAK3 gene expression was demonstrated by RT-PCR (JAK3 mRNA) (Fig. 3B; lower left panel) and western blot (JAK3 protein) (Fig. 3B; lower right).

Specificity of CRISPR/Cas9 directed JAK3 correction

The potential for off-target, CRISPR/Cas9 directed genome modifications raises some concerns about the use of this approach for therapy in humans. In cancer cell lines, relatively high levels of off-target mutagenesis by Cas9-gRNAs have been described. To determine the specificity of CRISPR/Cas9 directed JAK3 correction in human SCID iPSCs, Whole genome sequencing was performed before and after gene replacement. The genomes of two heterozygous and one homozygous corrected clones were sequenced. The two heterozygous clones were corrected with gRNA #2 + wild type Cas9, and the homozygous clone was corrected with gRNA #1 + gRNA #2 + nickase Cas9 (D10A). The 20-base CRISPR guide sequences were mapped to the human reference genome, allowing up to 3 mismatches in order to identify possible off-target sites. These sites were then analyzed for variations in the iPSC samples following CRISPR/Cas9 directed gene replacement. WGS analysis of one homozygous and two heterozygous corrected iPSC lines demonstrated that no mutations (SNVs nor indels) were introduced into the predicted off-target sites, suggesting a strong specificity for the CRISPR/Cas9 directed gene replacement.

Restoration of T cell development after CRISPR/Cas9 directed JAK3 correction

To determine whether T cell development is restored after JAK3 gene correction, T cell lineage commitment and maturation were assayed. T cell differentiation sequentially passes through intermediates observed *in vivo*: CD34+CD7+ T/NK committed stage; CD7+CD4+CD8− immature, SP stage; CD4+CD8+ DP stage; and finally, CD3+CD8+ TCRαβ mature stage. Mature T cells are polyclonal, proliferate, and secrete cytokines in response to mitogens. Therefore, JAK3 corrected hiPSCs were differentiated into hematopoietic progenitors on OP9 monolayers, and CD34+ cells were positively selected on anti-CD34 magnetic beads. These cells were plated on OP9-DL4 monolayers, and nonadherent cells were analyzed for lymphocyte markers at TD14, 21, 28 and 35 (Fig. 4). Similar to control cells, 1-2 X 106 CD34+ JAK3 corrected cells differentiated into 4.7 X 106 CD7+ cells (91% of cells counted in lymphoid gate) at TD14. After further differentiation to TD21, TD28 and TD35, T cell maturation markers CD3, CD4, CD8 and TCR αβ were abundantly observed (Fig. 4A). To determine whether TCR rearrangement is reestablished in JAK3-corrected T cells, TCR Vβ typing was performed by flow cytometry and summarized in Figure 4B. JAK3-corrected T cells expressed all the Vβ segments that we tested (19 of 25); therefore, a broad TCR repertoire was restored. Finally, the integrity of the TCR signaling pathway, a surrogate of T cell function, in JAK3-corrected T cells, was examined by measuring cell surface activation markers following anti-CD3/CD28 stimulation. On Day 3 post-stimulation, the percentage of CD3+CD25+CD69+ T cells increased from 0.68% to 59.7% in JAK3-corrected T cells similar to the increase observed in control cells (0.01% to 37.6%) (Fig. 4C). These data and results described above demonstrate that correction of the JAK3 C1837T (p.R613X) mutation by CRISPR/Cas9 enhanced gene replacement in an *in vitro* iPSC model system restores normal T cell development with the capacity to produce functional, mature T cell populations with a broad TCR repertoire.

In humans, the phenotype of lymphocytes in the peripheral blood of SCID patients has been well described, but studies on critical steps of lymphoid commitment and thymocyte development have been difficult to perform. Access to bone marrow and thymocyte samples from untreated patients with SCID is challenging since these conditions are rare and infants typically present with life-threatening infections requiring urgent HSC transplantation to survive. The strategy described herein for studying human SCID bypasses these restrictions; large numbers of hematopoietic progenitors can be produced from patient specific iPSCs *in vitro*, and the mechanisms responsible for immunodeficiency can be precisely determined. Demonstrated herein is that T cell development in human JAK3-

deficient SCID is completely blocked before or at the CD4-CD8- (DN2) stage. Interestingly, forced expression of BCL2 enhances survival of DN cells, which further differentiate into DP thymocytes. However, DP thymocytes fail to mature to SP T cells, and this defect may result from the absence of IL7/JAK3 signaling. It is also demonstrated that correction of the human JAK3 mutation by CRISPR/Cas9 enhanced gene replacement restores the differentiation potential of early T cell progenitors. Corrected progenitors are capable of producing NK cells and mature T cell populations expressing a broad TCR repertoire. Whole-genome sequencing analysis of one homozygous and two heterozygous corrected iPSC lines demonstrates that no mutations (SNVs nor indels) are introduced into the predicted off-target sites, suggesting a strong specificity for the CRISPR/Cas9 directed gene replacement.

In the methods described herein, CD34+ HSCs can be generated from hiPSCs by co-culturing with human bone marrow stromal stem (hMSC) cells (See Figure 5). The HSCs produced by this method from patient-specific iPSC after gene correction/modification could be transplanted back into the patient to treat diseases such as sickle cell disease (SCD), SCID or cancer. In the methods described herein, T cells can be generated by culturing hiPSC derived CD34+ cells by co-culturing the hiPSC derived CD34+ cells with hMSC-DL4 (See Figure 6). HSCs produced by this method from patient-specific iPSC after correction/modification could be transplanted back into the patient to treat diseases. The T cells can comprise γδ T cells. As shown in Figure 7, γδ T cells expressing recombinant T cell receptor (TCR) can be efficiently produced from genetically modified iPSC. Production of γδ T cells expressing TCR specific for tumor antigens provide a cellular therapy for cancer.

Example 2

Correction of a mutation associated with Sickle Cell Anemia by CRISPR/Cas9 enhanced gene replacement

Vector Construction

The human codon optimized *S. pyogenes* Cas9 with both N-terminal and C-terminal nuclear localization sequences (nls-Cas9-nls) were PCR cloned from px330 vector (Addgene ID: 42230) into a modified pET-28b (EMD Biosciences) vector with a His_6-SUMO tag at the N-terminus. A gene block cassette containing a short linker peptide followed by a supercharged GFP with a net charge of +36 and a 23 amino acid influenza virus hemagglutinin HA-2 variant peptide INF7 (GLFEAIEGFIENGWEGMIDGWYG)(SEQ ID NO: 50) was codon optimized for *E. coli* and synthesized (IDT DNA) and cloned to fuse with

the C-terminus of the nls-Cas9-nls. An HIV-TAT peptide (YGRKKRRQRRRPPQ))(SEQ ID NO: 51) coding sequence was also synthesized (IDT DNA) and cloned to fuse with the N-terminus of the nls-Cas9-nls.

Protein Overexpression and Purification

The pET-SUMO-scCas9 plasmid was transformed into *E. coli* strain Rosetta™ 2(DE3) cells (EMD Millipore, Billerica, MA) in LB medium. The cells were grown at 37°C until the optical density reached 0.6 at 600nm. Induction of protein overexpression was achieved by adding 0.5 mM isopropyl-1-thio-ß-D-galactopyranoside (IPTG) and culturing overnight at 18°C in a shaker. The harvested cells were re-suspended in Ni-binding buffer (20mM Tris-HCl pH 8.0, 1.5 M NaCl, 25 mM imidazole and 0.2 mM TCEP) and lysed by Emulsiflex C3 high pressure homogenizer (Avestin). Polyethyleneimine (PEI) with final concentration of 0.4% was added into the cleared lysate to precipitate the nucleic acids. The proteins in the supernatant after centrifugation was then precipitated by ammonium sulfate to remove the PEI and re-dissolved in the Ni-binding buffer. The proteins were first purified by a HisTrap nickel affinity column (GE Healthcare) followed by overnight digestion with SUMO protease Ulp1 at 4°C. The cleaved His-SUMO tag was then removed via a second HisTrap column. The flow though containing the scCas9 protein was diluted to reach the final NaCl concentration of 0.5 M and purified on a HiTrap Heparin column (GE Healthcare) by gradient elution with buffer containing 20mM Tris-HCl pH 8.0, 2.0 M NaCl, and 0.2 mM TCEP. The eluted scCas9 protein was further purified by a size exclusion column Superdex 200 16/600 (GE Healthcare) in gel filtration buffer (20mM Tris-HCl pH 8.0, 0.5 M NaCl, and 0.2 mM TCEP), sterilized by passing through a 0.22 μm filter and concentrated by an Amicon Centrifugal Unit (EMD Millipore) with 100 kDa cutoff. The concentrated protein was quantified by UV spectrophotometer and flash frozen in liquid nitrogen.

Guide RNA Preparation

Template DNA for sgRNA transcription was generated by PCR with primer set adding a T7 promoter and a polyA sequences. sgRNA was *in vitro* transcribed by T7 RNA polymerase using T7 Ribomax Express System (Promega, Madison, WI) according to the manufacturer's manual. The transcribed RNA was purified by phenol: chloroform extraction, ethanol precipitation and followed by column purification with MEGAclear™ Transcription Clean-Up Kit (Ambion, Austin, TX). The purified gRNA was quantified by UV spectrophotometer and stored in -80°C freezer.

Single-stranded DNA Donors

Single-stranded DNA (ssODN) donors were synthesized by IDT DNA.

Single-stranded Donor DNAs for HBB sickle correction	
HBB-T2-ssODN	ATCCACGTTCACCTTGCCCCACAGGGCAGTAACGGCAGACTTCT CCtCAGGAGTCAGGTGCACCATGGTGTCTGTTTGAGGTTGCTAGT GA (SEQ ID NO: 52)
HBB-T2-ssODN-wobble	CTTCATCCACGTTCACCTTGCCCCACAGGGCAGTAACGGCAGAtT TtTCCtCAGGAGTCAGGTGCACCATGGTGTCTGTTTGAGGTTGCT AGTGA (SEQ ID NO: 53)

Cell Culture

Human sickle patient iPSC were derived from skin fibroblasts and were maintained on Matrigel (BD) in mTeSR™1 medium (Stem Cell Technologies, Vancouver, CA) with penicillin/streptomycin.

scCas9-sgRNA-ssODN complex preparation and nucleofection

1/10 volume of 10x PBS was added into sgRNA to reach 1x final concentration. The sgRNA was annealed on PCR thermo cycler with slow decreasing of temperature from 95°C to 4°C. After annealing, scCas9 protein was added into the sgRNA with a 1:1.5 protein-to-RNA molar ratio and mixed quickly by tapping the tube until all the transient precipitation was gone. The mixture was incubated in room temperature for 10 minutes in dark. Then, 1 molar ratio amount of ssODN was added into the mixture and incubated for additional 10 minutes in dark to form the scCas9-sgRNA-ssODN complex.

One day before nucleofection, cells were detached by Accutase (Stem Cell Technologies) and $1x10^6$ cells /well cells were seeded on a 6-well plate with 10 μM Rock inhibitor (Y-27632) (EMD Millipore). For each experiment, $5x10^5$ hsIPSCs were resuspended as single cells in 100μl supplemented Human Stem Cell Nucleofector Solution 1(Lonza) and scCas9-sgRNA-ssODN complex was then mixed with the cell solution. The cells were nucleofected with program A-023 using a Nucleofector II device (Lonza, Basel, Switzerland). The efficiency of HBB genome correction was analyzed by ddPCR two days post nucleofection.

Detection of sickle correction by ddPCR

The cells nucleofected with the scCas9-sgRNA-ssODN complex were lysed by prepGEM Tissue DNA extraction reagent (ZyGEM, Hamilton, NZ) following manufacturer's manual and 1:3 diluted with water. In a 22 μl ddPCR reaction, 11 μl 2 x ddPCR mix (Bio-rad) was mixed with 1ul each of 5μM allele-specific FAM or VIC Taqman probes set forth

below, 0.2 µl each of a 100 µM forward and reverse primer, and 8.6 µl diluted genomic DNA. Droplets were generated by QX200 Droplet Generator (Bio-rad, Hercules, CA) according to the manufacturer's manual. The reaction mix was then transferred into a 96-well PCR plate and the PCR was performed on a standard thermal cycler (Bio-rad). The program for PCR was : Step 1: 95°C 10 min; Step 2: 95°C 30s; Step 3: 55°C 1 min; repeat steps 2-3 for 39 times; Step 4: 98°C 10 min; Step 5: 8°C hold. After PCR was done, the plate was then analyzed by QX200 Droplet Reader (Bio-rad).

T7-sgRNA transcription template primers	
T7-T2-F	TAATACGACTCACTATAGGGTAACGGCAGACTTCTCCAC (SEQ ID NO: 54)
T7-polyA-R	AAAAAGCACCGACTCGGTGCC (SEQ ID NO: 55)

Taqman Probes:

HBB-wb-FAM-TM	FAM-TCCTGaGGAaAAaT-MGB (SEQ ID NO: 56)
HBB-wt-FAM-TM	FAM-TGACTCCTGAGGAGAA-MGB (SEQ ID NO: 57)
HBB-sk-VIC-TM	VIC-ACTCCTGTGGAGAAG-MGB (SEQ ID NO: 58)

ddPCR Primers:

R196	HBB-TaqM-f2	CAGAGCCATCTATTGCTTACATTTG (SEQ ID NO: 59)
R197	HBB-TaqM-r1	GGCCTCACCACCAACTTCAT (SEQ ID NO: 60)

As set forth above, a complex that includes a guide RNA (gRNA), modified recombinant Cas9 protein (mrCas9) and a single-stranded oligodeoxyribonucleotide (ssODN) can be introduced into human stem cells or derivatives thereof to correct a single base mutation that causes disease. Table 1 and Figure 8 illustrate results from the introduction of a sickle cell correction complex (gRNA-mrCas9-ssODN) into induced Pluripotent Stem Cells (iPSC) derived from skin cells of a sickle cell patient. IPSCs were derived as described in Example 1. The correction complex was introduced into sickle iPSC by nucleoporation and 2 days later genomic DNA was analyzed by digital PCR, using the primers set forth above, and sequenced. Over 65% of the cells contained at least one

corrected gene. One corrected gene is sufficient to cure the disease. The results were confirmed as follows. Two days after introduction of the correction complex, the cells were plated in culture dishes, and 43 individual iPSC colonies were isolated. Genomic DNA was isolated from these colonies and the beta-globin gene was sequenced. Sixty-five percent of the colonies contained at least one corrected beta-globin gene (S corrected to A).

Table 1

	gRNA-mrCas9-ssODN	
Pooled ddPCR result (2-day)	68.6%	
Total colonies picked after 2 weeks	48	
Mixed colonies	5	
Total single colonies	43	
A/A	14	32.6%
A/S	4	9.3%
S/S	3	7.0%
A/indel	10	23.3%
S/indel	6	14.0%
Indel/indel	6	14.0%
Clones with at least 1 allele corrected	28	65.1%
Clones with indels	22	51.2%
Clones with genome modification	40	93.0%
Total number of alleles	86	
Total "A" alleles (corrected)	42	48.8%
Total "S" alleles (uncorrected)	16	18.6%
Total "indel" alleles	28	32.6%
A (A+S) *comparable to ddPCR result	42/58 = 72.4%	
HR:NHEJ (A:indel) ratio	1.50	

Similar studies were performed with patient primary bone marrow CD34+ cells. The protocol was as follows. Bone marrow was obtained from a sickle patient by an IRB approved protocol. CD34+ cells were purified on a Miltenyi anti-CD34+ beads (Miltenyi, Bergisch Gladbach, Germany). The cells were nucleoporated with the complex prepared as described above. After nucleoporation, the cells plated in methycult and BFU-E, CFU-E and CFU-GEMM colonies were picked after two weeks and analyzed for corrected alleles. Table 2 and Figure 9 illustrate results from the introduction of a sickle cell correction complex (gRNA-mrCas9-ssODN) into patient primary bone marrow CD34+ cells. After twelve days of *in vitro* differentiation, DNA was analyzed by digital PCR (ddPCR) and sequenced.

Approximately equal amounts of betaA and betaS mRNA were observed (See Figure 9). Immediately after nucleoporation, some of the cells were culture in erythroid differentiation medium for up to eightenn days and enucleated red blood cells were analyzed for HbA. An isoelectric focusing (IEF) gel of *in vitro* differentiated red blood cells from the corrected sickle patient CD34+ cells showed an HbA (normal hemoglobin) to HbS (hemoglobin with sickle cell mutation) ratio of about 1:3, which is sufficient to inhibit sickling and treat the disease (See Figure 10).

Table 2

Complex for nucleofection	Cas9wt-36GFP-T2-ssODN	
Nucleofection Program	P4 DN-100	
BFU-E/CFU-E/GEMM colonies picked on D10 and D15	21/23/7	
Total colonies*	**51**	
A/A	2	4%
A/S	4	8%
S/S	19	37%
A/indel	5	10%
S/indel	15	29%
Indel/indel	6	12%
Clones with at least 1 allele corrected	11	22%
Clones with indels	24	47%
Clones with genome modification	29	57%
Total number of alleles	**102**	
Total "A" alleles (corrected)	13	13%
Total "S" alleles (uncorrected)	57	56%
Total "indel" alleles	32	31%
A:(A+S) *comparable to ddPCR result	13/70 = 18.6%	
HR:NHEJ (A: indel) ratio	0.41	

Example 3

Correction of a mutation associated with Sickle Cell Anemia by CRISPR/Cas9 enhanced gene replacement

iPSCs have the potential to generate all cell types including HSPCs (human stem/progenitor cells); therefore, iPSC based gene therapy could provide a curative therapy for sickle cell disease. Correction of sickle iPSCs can provide an unlimited number of cells from which to generate corrected HSPCs, and these corrected HSPCs can be used for autologous transplantation. Importantly, corrected iPSCs and the HSPCs derived from them can be fully characterized and evaluated for safety before transplantation. Described below is CRISPR/Cas9 enhanced gene correction of iPSCs derived from fibroblasts of a sickle patient.

Cell Culture

Human sickle iPSCs

Human sickle iPSCs were derived from fibroblasts of a skin biopsy obtained from a consented sickle patient at the UAB Kirklin Clinic. The cells were maintained on Matrigel (BD) in mTeSR™1 medium (Stem Cell Technologies) with penicillin/streptomycin. Human sickle iPSCs were passaged every 3-4 days by incubating colonies with Accutase (Stem Cell Technologies), and single cells were seeded on Matrigel coated plates with 10 μM Rock inhibitor (Y-27632) (EMD Millipore). After one day, the media was changed with no rock inhibitor.

Human sickle bone marrow CD34+ cells

Bone marrow from a consented sickle patient was aspirated in the adult sickle clinic at UAB. The CD34+ cells were purified on anti-Cd34+ beads, aliquoted and stored in liquid nitrogen.

Cas9 Expression Plasmids for *E. coli* overexpression

Cas9WT

The *S. pyogenes* Cas9WT coding sequence with both N-terminal and C-terminal fused nuclear localization sequences (nls-Cas9WT-nls) were PCR cloned from the px330 vector (Addgene ID: 42230) into a modified pET-28b (EMD Biosciences) vector with a His_6-SUMO tag at the N-terminus, resulting in a pSUMO-Cas9WT plasmid.

TAT-Cas9WT-EGFP

Synthesized genes block (IDT DNA) containing a short linker peptide and the coding region of EGFP were ligated to the C-terminus of the nls-Cas9WT-nls and cloned. Coding sequence for a HIV-TAT peptide (YGRKKRRQRRRPPQ)(SEQ ID NO: 51) was also synthesized, ligated to the N-terminus of the nls-Cas9WT-nls and cloned, resulting in the pSUMO-TAT-Cas9WT-EGFP plasmid.

Cas9WT-36GFP

A synthesized gene block (IDT DNA) containing the *E. coli* codon optimized coding sequence of supercharged GFP with a net positive charge of +36 (Lawrence et al. "Supercharging Proteins Can Impart Unusual Resilience," *J. Am. Chem. Soc.* 129(33): 10110 (2007))) and short linker peptide was ligated with the C-terminus of the nls-Cas9WT-nls and cloned, resulting in a pSUMO-Cas9WT-36GFP plasmid.

TAT-Cas9WT-36GFP

The coding sequence of a HIV-TAT peptide (YGRKKRRQRRRPPQ)(SEQ ID NO: 51) was synthesized, ligated with the C-terminus of Cas9WT-36GFP and cloned, resulting in the pSUMO-TAT-Cas9WT-36GFP vector.

TAT-Cas9WT-36GFP-INF7

A synthesized gene block (IDT DNA) containing a short linker peptide followed by a supercharged GFP with a net charge of +36 (Lawrence, 2007) and a 23 amino acid influenza virus hemagglutinin HA-2 variant peptide INF7 (GLFEAIEGFIENGWEGMIDGWYG)(SEQ ID NO: 50) (Plank, 1994) was codon optimized for *E. coli,* ligated with the C-terminus of the nls-Cas9WT-nls and cloned. An HIV-TAT peptide (YGRKKRRQRRRPPQ)(SEQ ID NO: 51) coding sequence was also synthesized, ligated with the N-terminus of nls-Cas9-nls and cloned, resulting in the pSUMO-TAT-Cas9WT-36GFP-INF7 plasmid.

Cas9WT-3xTAT

The coding sequence of 3 tandem repeats of the coding region for HIV-TAT peptide separated with short linkers (YGRKKRRQRRRPPQAGGGSGGSYGRKKRRQRRRPPQAGGGSGGSYGRKKRRQRRRPPQAG) (SEQ ID NO: 61) was codon optimized for E.coli, synthesized, ligated with the C-terminus of nls-Cas9WT-nls and cloned, resulting in the pSUMO-Cas9WT-3xTAT plasmid.

TAT-Cas9WT-3xTAT

The coding sequence of a HIV-TAT peptide was (YGRKKRRQRRRPPQ)(SEQ ID NO: 51) synthesized, ligated with the N-terminus of nls-Cas9WT-3xTAT and cloned, resulting in a pSUMO-TAT-Cas9WT-3xTAT plasmid.

Protein Overexpression and Purification

The Cas9WT or Engineered positively charged Cas9 (EpcCas9) expression plasmid was transformed into the *E. coli* strain Rosetta™ 2(DE3) cells (EMD Millipore) in LB medium. The cells were grown at 37°C until the optical density reached 0.6 at 600nm. Induction of protein overexpression was achieved by adding 0.5 mM isopropyl-1-thio-ß-D-galactopyranoside (IPTG) and culturing overnight at 18°C in a shaker incubator. The harvested cells were re-suspended in Ni-binding buffer (20mM Tris-HCl pH 8.0, 1.5 M NaCl, 25 mM imidazole and 0.2 mM TCEP) and lysed with a Emulsiflex C3 high pressure homogenizer (Avestin). Polyethyleneimine (PEI) was added to the cleared lysate supernatant to a final concentration of 0.4% to precipitate nucleic acids. The supernatant after centrifugation was then precipitated by ammonium sulfate to remove the PEI and the protein pellet was re-dissolved in the Ni-binding buffer. The protein solution was first purified by a HisTrap nickel affinity column (GE Healthcare, Atlanta, GA) followed by overnight digestion with SUMO protease Ulp1 at 4°C. The cleaved His-SUMO tag was then removed by passing through a second HisTrap column. The flow through containing the Cas9 protein was diluted to reach a final NaCl concentration of 0.5 M and purified on a HiTrap Heparin column (GE Healthcare) by gradient elution with buffer containing 20mM Tris-HCl pH 8.0, 2.0 M NaCl, and 0.2 mM TCEP. The eluted Cas9 protein was further purified by a size exclusion column Superdex 200 16/600 (GE Healthcare) in gel filtration buffer (20mM Tris-HCl pH 8.0, 0.5 M NaCl, and 0.2 mM TCEP), sterilized by passing through a 0.22 μm filter and concentrated by an Amicon Centrifugal Unit (EMD Millipore) with a 100 kDa cutoff. The concentrated protein was quantified by UV spectrophotometer, flash frozen in liquid nitrogen and stored at -80°C.

Single Guide RNA Preparation

The DNA template for sgRNA *in vitro* transcription was generated by PCR with primers adding a T7 promoter at 5' end and a polyA sequence at the 3' end. The sgRNAs was *in vitro* transcribed by T7 RNA polymerase using a T7 Ribomax Express Kit (Promega) according to the manufacturer's manual. The transcribed RNA was then isolated by phenol: chloroform extraction, ethanol precipitation and column purification with the MEGAclear™ Transcription Clean-Up Kit (Ambion). The sgRNA was eluted in nuclease free water, and the concentration was measured by UV spectrophotometer. The stock sgRNA was then aliquoted and stored in a -80°C freezer.

Cas9 RNP/ssODN Assembly

Before complexing with Cas9 protein, 10x PBS was added into the stock sgRNA solution to reach 1x PBS final salt concentration. The sgRNA was annealed on a thermo-cycler by slowly decreasing the temperature from 95°C to 4°C. To form Cas9 RNP, stock Cas9 protein was added to the annealed sgRNA at a 1:1.5 protein:RNA molar ratio and mixed thoroughly by quickly tapping the tube until all the transient precipitation was gone. The mixture was incubated at room temperature for 10 minutes in the dark. Subsequently, ssODN was added at a 1:1 molar ratio with Cas9 RNP for nucleoporation.

Nucleoporation of human sickle iPSCs with Cas9 RNP/ssODN

One day before nucleoporation, human sickle iPSCs were detached by accutase (Stem Cell Technologies) and incubated to obtain a single cell suspension in mTesR1 media supplemented with 10 μM Rock inhibitor (Stem Cell Technologies). This single cell suspension was seeded into 6-well plate at density of $5x10^5$ cells/well. On the day of nucleoporation, $5x10^5$ human sickle iPSC cells were prepared with Accutase as described above and resuspended in 100 μl of Human Stem Cell Nucleofector Solution 1 (Lonza) and 7.5 μM of Cas9RNP/ssODN was mixed with the cell suspension in the nucleoporation cuvette. The cells were nucleoporated with program A-023 using a Nucleofector II (Lonza) and transferred into pre-warmed media immediately. The correction efficiencies for the cell population were assayed 2 days after nucleoporation.

Detection of sickle correction by ddPCR

Two to five days after nucleoporation, Cas9 RNP/ssODN nucleoporated cells were lysed by prepGEM Tissue DNA extraction reagent (ZyGEM) following the manufacturer's manual and the cell lysate was diluted 1:3 with water. In a 22 μl ddPCR reaction, 11 μl 2 x ddPCR mix (Bio-Rad) was mixed with 1ul each of 5μM allele-specific FAM or VIC Taqman probes, 0.2 μl each of a 100 μM forward and reverse primer, and 8.6 μl diluted cell lysate. Droplets were generated by a QX200 Droplet Generator (Bio-Rad) according to the manufacturer's instructions. The reaction mix was then transferred into a 96-well PCR plate, and PCR was performed on a standard thermal cycler (Bio-Rad). The program for PCR was: Step 1: 95°C 10 min; Step 2: 95°C 30s; Step 3: 55°C 1min; repeat steps 2-3 for 39 times; Step 4: 98°C 10min; Step 5: 8°C hold. After PCR was completed, the plate was analyzed on a QX200 Droplet Reader (Bio-Rad).

Generation of single iPSC clone after Cas9 RNP/ssODN nucleoporation

To generate single iPSC clones, Cas9 RNP/ssODN nucleoporated sickle iPSCs were seeded in BD matrix gel coated 96-well plates after serial dilution to a density of 20, 10 and 5

cells/well. Fresh mTesR1 media with 10 μM rock inhibitor was changed every 2 days during the first 6 days of culture. mTesR1 media without rock inhibitor was changed every day after day 6. Ten to twelve days after seeding, single iPSC colonies were picked, and the cell lysates were analyzed by Sanger sequencing for genome modification.

Activation and Nucleoporation of human patient bone marrow sickle CD34+ cells

To activate the cell cycle, frozen human sickle bone marrow CD34+ cells were thawed and resuspended into pre-warmed Stemspan media supplemented with CC110 cytokine cocktail (STEMCELL Technology). The cells were cultured in a 37°C incubator with 5% CO_2 and fresh media was partially changed every day for 2 days before nucleoporation. On the day of nucleoporation, $5x10^5$ live CD34+ cells were rinsed with 1xPBS and harvested by centrifugation at 150g for 15 mins. The cell pellet was resuspended in 100 μl P4 primary cell nucleofection solution (Lonza) and 15 μM of Cas9 RNP/ssODN complex was mixed with the cell suspension in the nucleoporation cuvette. The cells were nucleoporated with program DN-100 using a 4D-Nucleofector (Lonza) and transferred into pre-warmed media immediately. The efficiency of gene correction was analyzed 6 days after nucleoporation.

Erythroid Colony forming Unit (CFU) assay for Cas9 RNP nucleoporated CD34+ cells

After nucleoporation with Cas9 RNP/ssODN complex, CD34+ cells were seeded into Methocult media (Stem Cell Technologies) at a density of 500-1000 cells/mL in 35mm tissue culture plates. Cells were grown in a 37°C incubator with 5% CO_2 for 12-15 days until the colonies were large enough to pick individually for analysis.

***In vitro* erythroid differentiation of CD34+ HSPCs into RBCs**

One day after the nucleoporation of CD34+ cells with Cas9 RNP/ssODN complex, the media was changed to Erythroid expansion media (Stemspan SFEM (STEMCELL Technologies) supplemented with 1u/mL erythropoietin (EPO), 2 nM dexamethasone (DEX), 1nM β-Estradiol, 20ng /mL human SCF, and 5ng /mL human IL-3.) The media was changed every 2 days. After the first 7 days of expansion and differentiation, the media were supplemented with a higher concentration of EPO (2 u/mL) until differentiated RBCs are harvested at day 15-18.

Mass spectrometry analysis of corrected hemoglobin beta protein in RBCs

Hemolysates of RBCs differentiated from human sickle bone marrow CD34+ HSPCs were separated by PAGE. The globin band was cut out of the gel and trypsinized. Peptides were separated and analyzed by LC-MS/MS.

Sequences

***In vitro* transcribed sgRNA sequences:**

T1 sgRNA:

GGGUCUGCCGUUACUGCCCUGGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUA AGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUUUUU (SEQ ID NO: 62)

T2 sgRNA:

GGGUAACGGCAGACUUCUCCACGUUUUAGAGCUAGAAAUAGCAAGUUAAAAU AAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUUUU U (SEQ ID NO: 63)

91-nt correction ssODN:

ATCCACGTTCACCTTGCCCCACAGGGCAGTAACGGCAGACTTCTCCtCAGGAGTC AGGTGCACCATGGTGTCTGTTTGAGGTTGCTAGTGA (SEQ ID NO: 52)

95-nt T2 wobble ssODN:

CTTCATCCACGTTCACCTTGCCCCACAGGGCAGTAACGGCAGAtTTtTCCtCAGGAG TCAGGTGCACCATGGTGTCTGTTTGAGGTTGCTAGTGA (SEQ ID NO: 53)

90-nt T1 wobble ssODN:

ACCTCAAACAGACACCATGGTGCACCTGACTCCTGAGGAGAAGTCTGCCGTCAC AGCTCTGTGGGGCAAGGTGAACGTGGATGAAGTTGG (SEQ ID NO: 64)

PCR primers for T2 sgRNA *in vitro* transcription template

T7-T2F: TAATACGACTCACTATAGGGTAACGGCAGACTTCTCCAC (SEQ ID NO: 54)

T7-R: AAAAAGCACCGACTCGGTGCC (SEQ ID NO: 55)

PCR primers for T1 sgRNA *in vitro* transcription template

T7-T1F: TAATACGACTCACTATAGGGTCTGCCGTTACTGCCCTG (SEQ ID NO: 65)

T7-R: AAAAAGCACCGACTCGGTGCC (SEQ ID NO: 55)

PCR primer for on-target Sanger sequencing

R157: TCCACATGCCCAGTTTCTAT (SEQ ID NO: 66)

R158: AGTAGCAATTTGTACTGATGGTATG (SEQ ID NO: 67)

Engineered positively charged Cas9 RNPs/ssODN (EpcCas9 RNPs/ssODN) efficiently correct the sickle mutation in human patient iPSC (induced Pluripotent Stem Cells)

To correct the sickle HBB gene, human sickle patient derived iPSCs were nucleoporated with Cas9WT/T2 RNP, Cas9WT-EGFP/T2 or 8 different EpcCas9/T2 RNPs (Engineered positively-charged Cas9/T2 RNPs) together with a 91-nt ssODN correction

template (SEQ ID NO: 51). Cas9/T2 RNPs induce a double strand break near (2 bp downstream) the sickle mutation. The proximity of the cut site to the mutation enhances HDR of the sickle mutation (T->A) using the 91-nt ssODN correction template. On-target Sanger sequencing data for the population of iPSCs demonstrate correction of the sickle mutation at high efficiency in Cas9WT RNP/ssODN nucleoporated cells (Fig 11). The addition of an EGFP (Enhanced Green Fluorescent Protein) domain at the C-terminus of Cas9WT did not affect the level of correction.

Correction efficiencies vary in cells nucleoporated with the 8 different EpcCas9s RNPs. The addition of a positively charged HIV TAT peptide at the N-terminus of the Cas9WT-EGFP (TAT-Cas9WT-EGFP) results in a small decrease in correction efficiency compared to the Cas9WT and Cas9WT-EGFP and a small decrease in indels. Addition of 3x tandem repeats of TAT at the N-term of the Cas9WT-EGFP (3xTAT-Cas9WT-EGFP) almost completely abolishes correction and indel levels, indicating loss of Cas9 enzymatic activity from this modification. This result suggests that a relatively high number of positive charges linked to the N-terminus of Cas9 severely inhibits enzymatic activity. Interestingly, addition of positive charges at the C-terminus of Cas9 (Cas9WT-3xTAT or Cas9WT-36GFP) results in a high level of correction and a relatively low level of indels. These results suggest that positive charges linked to the C-terminus of Cas9 significantly inhibit exonuclease digestion of cleaved ends and stimulate religation of ends without formation of indels. Similar levels of correction and indels were observed from EpcCas9 with a C-terminal addition of 3x tandem repeats of TAT peptides or a positively charged +36GFP.

EpcCas9s with both N-terminal and C-terminal positively charged modifications (TAT-Cas9WT-3xTAT and TAT-Cas9WT-36GFP) produce significantly less indels. Interestingly, further addition of a negatively charged INF7 peptide to the C-terminus of TAT-Cas9WT-36GFP (TAT-Cas9WT-36GFP –INF7) significantly enhances the correction efficiency compared to TAT-Cas9WT-36GFP. Sanger sequencing results were verified by deep sequencing analysis of on-target correction and indels for iPSC populations after nucleoporation with Cas9WT and selected EpcCas9 RNPs (Fig. 12).

EpcCas9 RNPs suppress on-target indels in human sickle iPSC

To study further the effects of positively charged modifications on the efficiency of HDR based gene corrections and NHEJ based indels, human sickle iPSC were nucleoporated with Cas9 RNPs plus or minus a 91-nt ssODN correction template. On-target Sanger sequencing analysis demonstrated that addition of ssODN (+ssODN) to both Cas9WT RNP and TAT-Cas9WT-EGFP corrects the sickle mutation with a similar high efficiency (Fig 13).

However, in the absence of ssODN (-ssODN), indel formation is dramatically lower with TAT-Cas9WT-EGFP compared to Cas9WT. Since HDR requires DSBs (Double Strand Breaks), the enzymatic activity of Cas9 is apparently not lowered by the addition of 1XTAT. Therefore, the large discrepancy in indel formation is not due to lower transduction efficiency or lower enzymatic activity of TAT-Cas9WT-EGFP.

To confirm these observations, the correction and indel efficiencies in 5 other EpcCas9 RNPs with (+) or without (-) ssODN (Fig. 14) was evaluated. Sanger sequencing analyses confirmed that all EpcCas9 RNPs result in significantly fewer on-target indels in the absence of ssODN (-ssODN). Although the correction efficiencies of EpcCas9 RNPs/+ssODN vary with different positively charged modifications (Fig. 14), indel formation is suppressed by all positively charged Cas9 modifications.

EpcCas9 RNPs enhance cell survival after nucleoporation in human sickle iPSC

To determine whether positively charged modifications affect cell survival, sickle iPSC were nucleoporated with Cas9WT RNP or 7 different EpcCas9s with (+) or without (-) a correction ssODN. Immediately after nucleoporation, cells were plated in culture dishes and growth was examined after 48 hours. Cell survival was poor with Cas9WT and increased dramatically with higher positively charged modifications (Fig. 15). Excellent cell survival was achieved with Cas9WT-36GFP and EpcCas9s containing both N-terminal and C-terminal positively charged modifications (Fig 15). Considering cell survival and correction/indel efficiency, the optimum balance of high correction, low indel formation and excellent cell survival is achieved with Cas9WT-36GFP and TAT-Cas9-36GFP-INF7 RNPs in human sickle iPSCs.

ssODN: Cas9 RNP ratios for sickle correction in human iPSC

The ratio of ssODN correction template to Cas9 RNP (ssODN:Cas9 RNP) is ~~critical~~ important for HDR and cell survival. Single stranded ODN is toxic to cells; therefore, high ssODN:Cas9 RNP ratios may result in poor cell survival after nucleoporation. However, low ssODN:Cas9 RNP ratios may result in inefficient HDR. To achieve high correction efficiencies with high cell survival, ssODN:Cas9 RNP ratios were optimized. The efficiency of sickle mutation correction with increasing doses of ssODN in sickle patient iPSC was determined. A Cas9WT-36GFP:T2 sgRNA molar ratio of 1:1.35 was fixed for these experiments, and the molar ratios of ssODN:Cas9WT-36GFP RNP ranged from 0 to 2.0 (r= 0, 0.2, 0.5, 1.0, 1.15, 1.35, 1.5 and 2.0). For example, the r=0.5 value in Fig. 6 is 0.5 ssODN:1.0 Cas9WT-36GFP:1.35 T2 sgRNA. Forty-eight hours after nucleoporation of the ssODN:Cas9WT-36GFP RNPs, sickle corrections were quantitated by digital droplet PCR

(ddPCR) (Fig. 16A) and Sanger sequencing (Fig. 16B). The percent correction (betaA/betaS alleles x 100) was plotted verses r (ssODN:Cas9WT-36GFP RNP). Correction efficiencies increased with r=0.2 to r=1.0 and reached a plateau at 1.15 (65.7%). Increasing r above 1.15 did not significantly increase correction efficiency and dramatically inhibited cell survival.

Cas9:sgRNA ratios for sickle correction in human iPSC

Theoretically, the optimal Cas9:sgRNA molar ratio is 1:1. Saturation of the Cas9 protein with sgRNA ensures maximal Cas9 enzymatic activity and reduces the possibility of free Cas9 interactions with other small RNAs that may produce unpredictable off-target genome modifications. Small RNAs are sensitive to nucleases; therefore, molar ratios of Cas9:sgRNA greater than 1:1 may be necessary to saturate Cas9. Cas9-36GFP:sgRNA molar ratios of 1:1.15, 1:1.35 and 1:1.5 were tested with ssODN molar ratios of 1.15 or 1.35 to determine optimal correction efficiency of the sickle mutation in patient iPSC. Sanger sequencing results and cell survival analyses demonstrated that optimal correction efficiencies and cell survivals were achieved with a Cas9-36GFP: sgRNA: ssODN molar ratio of 1:1:35:1.15 (Fig. 17).

Colony analysis for sickle correction in human iPSC

Human sickle iPSC were nucleoporated with TAT-Cas9WT-36GFP-INF7:T2 sgRNA:ssODN at a molar ratio of 1.0:1.35:1.0 to investigate the correction efficiency in cell populations (Fig. 18) and, subsequently, at a single cell level (Table 3). For single cell analysis, nucleoporated iPSCs were plated in a 96-well plate after serial dilution. Two weeks later, single iPSC colonies were picked, genomic DNA isolated, and Sanger sequencing performed. Forty-three single iPSC colonies were analyzed for on-target modifications. Table 3 summarizes the Sanger sequencing results for these iPSC clones. Twenty-eight of the 43 colonies contained at least one corrected allele (A/A, A/S or A/indel); therefore, 65.1% of the clones contained at least one corrected allele. iPSC containing at least one corrected allele will produce red blood cells that do not sickle.

Table 3. Summary for Sanger sequencing results of iPSC colonies corrected by EpcCas9 RNP/ssODN

Total single colonies	43	
A/A	14	32.6%
A/S	4	9.3%
S/S	3	7.0%
A/indel	10	23.3%
S/indel	6	14.0%
Indel/indel	6	14.0%
Colonies with at least 1 allele corrected	28	65.1%
Colonies with indels	22	51.2%
Colonies with genome modification	40	93.0%
Total number of alleles	86	
Total "A" alleles (corrected)	42	48.8%
Total "S" alleles (uncorrected)	16	18.6%
Total "indel" alleles	28	32.6%

Genome-editing events were also assessed at the allele level for these iPSC clones. Forty-two of 86 alleles (48.8%) were corrected, 28 of 86 alleles (32.6%) contained indels and 16 of 86 alleles (18.6%) were unmodified. This high rate of genome modification (81.4% of alleles and 93% of cells) demonstrates highly efficient gene targeting with the biochemical complex is possible.

Correction of human iPSC with EpcCas9 RNPs and wobble ssODNs

Retargeting of corrected DNA is a potential pitfall for the CRISPR/Cas system in HDR based gene correction. Compared to plasmid or viral delivery, the risk of retargeting for Cas9 RNP is low due to the RNPs short half-life; however, retargeting is difficult to avoid completely. In this example, the sickle mutation is located within the T2 sgRNA targeting sequence and is only 2 base pairs from the PAM. After correction with the ssODN, the corrected DNA contains a 1 base mismatch with the sgRNA target sequence. This difference reduces but does not eliminate retargeting. One strategy to prevent retargeting is to introduce wobble base changes into the correction template. These base changes do not alter the translated protein sequence but alter the DNA sequences at or near the PAM sequence so that the corrected DNA will no longer be a target for the Cas9 RNP. Based on this strategy, sickle iPSC were nucleoporated with TAT-Cas9WT-36GFP-INF7/T1sgRNA/T1wb-ssODN and TAT-Cas9WT-36GFP-INF7/T2sgRNA/T2wb-ssODN to determine whether EpcCas9 RNP could correct the sickle mutation at high efficiencies with wobble ssODNs.

Sanger sequencing results for the nucleoporated cell populations verified correction of the sickle mutation in both populations of nucleoporated cells (Fig. 19). The sickle correction

efficiency with T2wb-ssODN (Fig. 19B) was similar to the correction efficiency of the ssODN without wobble bases (Fig 18). However, the sickle correction efficiency with T1 sgRNA and T1wb ssODN is lower than T2wb-ssODN, probably due to differences in sgRNA targeting efficiencies, distance from the sickle mutation to the sgRNA cleavage sites and the number of wobble bases. Therefore, T2wb-ssODN is the preferred ssODN.

Whole Genome Sequencing analysis of EpcCas9 corrected iPSC colonies

To determine the specificity of EpcCas9 RNP directed correction of human sickle patient iPSCs, Whole Genome Sequencing (WGS) was performed on uncorrected sickle iPSC and 4 homozygous corrected clones wereproduced with TAT-Cas9WT-36GFP-INF7 RNP. Within the 4 corrected iPSC clones, 2 (T2-cl1 and T2-cl2) were corrected with T2 sgRNA and the 91-nt ssODN without wobble bases; 1 clone (T1w) was corrected with T2 sgRNA and a 95-nt T2wb ssODN and 1 clone (T1w) was corrected with T1 sgRNA and a 90-nt T1wb ssODN (Table 4). These WGS data confirmed homozygous correction of the sickle mutation and the absence of on-target indels in the 4 homozygous corrected iPSC clones (Fig 20A). Analysis of 4720 potential off-target sites with homology to the T1 sgRNA and 1476 potential off-target sites with homology to the T2 sgRNA (1-5 mismatches) demonstrated no off-target modifications (Fig 20B). Furthermore, analysis of the whole genome sequence data as described in Chang et al. (*Cell Reports* 12(10): 1668-77 (2015), demonstrated no disease-causing variants in sequences with or without homology to the sgRNAs. Four homozygous corrected clones were produced with TAT-Cas9WT-36GFP-INF7 RNP.

Table 4. Whole Genome Sequencing analysis of EpcCas9 corrected iPSC colonies

Clone ID	Cas9 protein	sgRNA	sgRNA sequence	Wobble donor ssODN
T1w	TAT-Cas9WT-36GFP-INF7	T1	GGTCTGCCGTTACTGCCCTG SEQ ID NO: 68	T1 wobble
T2w		T2	GTAACGGCAGACTTCTCCAC SEQ ID NO: 69	T2 wobble
T2-cl1		T2	GTAACGGCAGACTTCTCCAC	No wobble
T2-cl2		T2	GTAACGGCAGACTTCTCCAC	No wobble

Gene correction of sickle patient bone marrow CD34+ HSPCs

Correction of primary CD34+ HSPCs from a sickle patient followed by autologous transplant is a powerful and simple approach for SCD gene therapy. To determine whether

EpcCas9 RNP can also correct the sickle mutation in bone marrow progenitors, obtained CD34+ HSPCs were obtained from bone marrow of a consenting sickle cell patient. Sickle CD34+ cells were purified on anti-CD34 beads, and the cell cycle was activated by culture for 2 days in media with specific cytokines (SCF, TPO and FLT-3). Subsequently, the cells were nucleoporated with Cas9WT, Cas9-36GFP or TAT-Cas9-3xTAT plus T2 sgRNA and ssODN. The efficiency of sickle correction was determined 6 days after nucleoporation by the Sanger sequencing (Fig 21A). The highest correction efficiency was obtained with Cas9WT; however, indel frequency was high. Although the correction efficiency with the 2 EpcCas9 RNPs was lower than with Cas9WT, the frequency of indels was dramatically lower.

Correction of the sickle mutation with one EpcCas9 (Cas9-36GFP) was verified at the mRNA and protein levels (Fig 21B-D). After expansion of the nucleoporated cells in human erythroid expansion media for 10 days, RT-PCR and Sanger sequencing were performed (Fig 21B). Approximately equal amounts of betaA and betaS mRNA were observed (peaks are essentially superimposed). Cells were also cultured in human erythroid differentiation media containing Erythropoietin (Epo) for 15-18 days. The red blood cells (RBCs) derived from this culture were lysed, and hemoglobins were resolved on an IEF gel (Fig 21C). Approximately 35% of total hemoglobin was HbA (Fig. 21C), and this result was confirmed by mass spectrometry (Fig 21D). *In vivo*, RBCs containing HbA survive 5-10 times longer than rbcs containing only HbS. Therefore, if about 30% of cells are corrected in the bone marrow after transplantation, HbA levels of 60-70% will be achieved in peripheral blood.

EpcCas9 RNPs enhance the correction/indel ratio in sickle patient bone marrow CD34+ HSPCs

In addition to examining correction of the sickle mutation in populations of patient bone marrow CD34+ cells, we analyzed colonies derived from single CD34+ progenitors. After nucleoporation with TAT-Cas9WT-36GFP-INF7, CD34+ cells were mixed with semi-solid MethoCult media and plated into dishes. Two weeks after plating, colonies derived from single cells were isolated, DNA was extracted and Sanger sequence performed. The colonies that we examined were BFU-E (Burst Forming Units-Erythroid), CFU-E (Colony Forming Units-Erythroid) and CFU-GEMM (Colony Forming Units-Granulocyte, Erythrocyte, Monocyte, Megakaryocyte). Figure 11 illustrates typical BFU-E and CFU-GEMM colonies (A) and representative Sanger Sequencing results of the six genotypes that were obtained (B). Table 5 summarizes of the Sanger sequencing results from 95, 96, and 96 colonies (BFU-E, CFU-E and CFU-GEMM) obtained after nucleoporation of Cas9WT,

Cas9WT-36GFP, and TAT-Cas9WT-3xTAT RNPs and ssODNs, respectively. The highest correction efficiency was obtained with Cas9WT (51.6%); however, indel/indel frequency in cells treated with Cas9WT was also very high (40.0%). This level of indel/indel may result in beta-thalassemia because these HSCs will compete effectively for a limited number of bone marrow niches and red blood cells derived from these HSCs cannot synthesize HbA. Although the correction efficiency obtained with Cas9WT-36GFP RNP was lower (28.1%), this level of correction is sufficient to cure the disease as discussed above, and the frequency of indels (8.3%) is much safer. For TAT-Cas9WT-3xTAT RNP, the correction efficiency (32.3%) and indel frequency (14.6%) were intermediate. The correction/indel ratios after nucleoporation of Cas9WT, Cas9WT-36GFP, and TAT-Cas9WT-3xTAT RNPs plus ssODNs are 1.29 (51.6/40.0), 3.39 (28.1/8.3) and 2.21 (32.3/14.6), respectively. Therefore, Cas9WT-36GFP that has a correction/indel ratio of 3.39 is our preferred EpcCas9.

Table 5. Summary of the Sanger sequencing results from 95, 96, and 96 colonies (BFU-E, CFU-E and CFU-GEMM) obtained after nucleoporation of human sickle patient bone marrow CD34+ HSPC with Cas9WT, Cas9WT-36GFP, and TAT-Cas9WT-3xTAT RNPs and ssODNs, respectively.

	Cas9WT RNP + ssODN		Cas9WT-36GFP RNP + ssODN		TAT-Cas9WT-3xTAT RNP + ssODN	
Total colonies	95		96		96	
GEMM /BFU-E /CFU-E colonies	7/81/7		16/80/0		10/86/0	
A/A	18	18.9%	3	3.1%	10	10.4%
A/S	2	2.1%	14	14.6%	4	4.2%
S/S	5	5.3%	46	47.9%	27	28.1%
A/indel	29	30.5%	10	10.4%	17	17.7%
S/indel	3	3.2%	15	15.6%	24	25.0%
Indel/indel	**38**	**40.0%**	**8**	**8.3%**	**14**	**14.6%**
Colonies with at least 1 allele corrected	**49**	**51.6%**	**27**	**28.1%**	**31**	**32.3%**
Colonies with indels	70	73.7%	41	42.7%	55	57.3%
Colonies with genome modification	90	94.7%	50	52.1%	69	71.9%
GEMM correction	**5**	**71.4%**	**4**	**25.0%**	**1**	**10.0%**
Total number of alleles	190		192		192	
Total "A" alleles (corrected)	67	35.3%	30	15.6%	41	21.4%
Total "S" alleles (uncorrected)	15	7.9%	121	63.7%	82	43.2%
Total "indel" alleles	108	56.8%	41	21.6%	69	36.3%

As discussed above, the sickle correction efficiency of the Cas9WT-36GFP RNP/ssODN complex (28.1% of total CFU; 25% of CFU-GEMM) is high enough to cure the disease. This level of correction in the bone marrow after transplantation would result in 60-70% corrected RBC in peripheral blood. In addition, only 8.3% of colonies are homozygous indels (indel/indel); therefore, thalassemia is unlikely to result after transplantation.

EpcCas9 RNPs enhance cell survival after nucleoporation in sickle patient bone marrow CD34+ HSPCs

The data in Fig. 22C demonstrate that EpcCas9 RNPs enhance cell survival after nucleoporation in sickle patient bone marrow CD34+ HSPCs. The number of erythroid colonies (BFU-E and CFU-E) obtained after nucleoporation of sickle patient bone marrow CD34+ HSPCs was compared with Cas9WT, Cas9WT-36GFP, and TAT-Cas9WT-3xTAT RNPs plus ssODNs. The number of colonies obtained with Cas9WT RPN/ssODN was normalized to 1. The number of colonies obtained with Cas9WT-36GFP RNP/ssODN was 2.5-fold higher than the Cas9WT control and TAT-Cas9WT-3xTAT RNP/ssODN was 1.6-fold higher. It was concluded that Epc (Engineered positive charge) protects human bone marrow progenitors/stem cells from the toxic effects of single stranded oligodeoxynucleotides (ssODNs).

These results are significant because the dose of CD34+ HPSCs is critical for bone marrow reconstitution after transplantation. In general, two million CD34+ cells/kg are transplanted into human recipients. Cell doses below this level result in poor long-term reconstitution. A 75kg patient requires a dose of approximately 150 million cells. One liter of bone marrow can be harvested from a 75kg patient under anesthesia and approximately 200 million CD34+ cells can be isolated for transplantation. As indicated above, 2.5-fold fewer cells are obtained after nucleoporation of CD34+ cells with Cas9WT RNP/ssODN compared to Cas9-36GFP RNP/ssODN. Therefore, our preferred complex for correction is Cas9WT-36GFP RNP/ssODN.

EpcCas9 results in higher genome editing specificity

To evaluate the specificity of genome editing by EpcCas9 RNPs in nucleoporated CD34+ cells, deep sequencing analysis was conducted at five potential off-target genomic loci. The five potential off-target sites were the top 5 sites predicted by the Zhang MIT server (http://crispr.mit.edu) based on sequence homology to the sgRNA. In Cas9 RNP/ssODN nucleoporated sickle patient CD34+ cells, deep sequencing measured approximately 0.1% off-target indels at OT5 site (Table 6). In contrast, in Cas9WT-36GFP or TAT-Cas9WT-3xTAT RNP/ssODN nucleoporated cells, no off-target modifications were observed (Fig. 23).

Table 6 Deep sequencing analysis of 5 potential off-target genomic loci to evaluate editing specificity of EpcCas9 RNPs in nucleoporated CD34+ cells

		OT1 chr3:37684838 3MMs [1:5:7]	OT2 chr12:112746615 3MMs [2:4:11]	OT3 chr11:132762118 3MMs [2:5:19]	OT4 chr14:101366447 4MMs [1:2:5:7]	OT5 chr10:95158973 4MMs [1:2:3:7]
Neg ctrl	Indel reads	13	34	28	4	8
	Non-indel reads	209990	900262	700844	449423	226882
	Total reads	210003	900296	700872	449427	226890
	Indel percentage	0.0062%	0.0038%	0.0040%	0.0009%	0.0035%
Cas9WT	Indel reads	6	33	37	7	240
	Non-indel reads	199453	862095	754410	425039	226916
	Total reads	199459	862128	754447	425046	227156
	Indel percentage	0.0030%	0.0038%	0.0049%	0.0016%	0.1057%
Cas9WT-36GFP	Indel reads	4	37	23	4	8
	Non-indel reads	189683	777630	615613	482843	207613
	Total reads	189687	777667	615636	482847	207621
	Indel percentage	0.0021%	0.0048%	0.0037%	0.0008%	0.0039%
TAT-Cas9WT-3xTAT	Indel reads	9	24	32	3	9
	Non-indel reads	193690	843834	685044	458625	199515
	Total reads	193699	843858	685076	458628	199524
	Indel percentage	0.0046%	0.0028%	0.0047%	0.0007%	0.0045%

In addition, in erythroid colonies derived from Cas9WT RNP/ssODN nucleoporated sickle CD34+ cells, 5 out of 95 colonies containing non-specific modifications near (upstream or downstream) the targeting site were observed (Fig. 24). These non-specific modifications are random gene replacements or indels that do not appear to be initiated at the expected Cas9 RNP cutting site. In contrast, 0 out of 96 colonies derived from EpcCas9 RNPs nucleoporated cells contain non-specific modifications.

Example 4

Correction of Sickle Cell Mutation in mice

Figure 25 shows an isoelectric focusing (IEF) gel analysis of blood six weeks after primary transplantation of Sickle Mouse Fetal Liver c-Kit+ cells nucleoporated with Cas9 RNP/ssODN to correct a sickle cell mutation. Mouse fetal liver c-kit+ cells are equivalent to human cord-blood Cd34+ cells. Figure 26 shows ddPCR analysis of FACS purified bone marrow cells at twelve weeks post-transplantation into irradiated C57Bl6 mice. Twelve weeks after nucleoporation and transplantation, approximately 50% of erythroid cells (Ter119+) and myeloid cells (CD11b+ and CD11b+/GR1+) are corrected. Erythroid and myeloid cells are relatively short lived; therefore, these cells are derived from transplanted HSCs. Correction levels in B and T cells should rise to approximately 50% after secondary transplantation at twelve weeks (twenty-four weeks total). After twenty-four weeks, most if

not all hematopoietic cells will be derived from long-term HSCs. Figure 27 shows IEF gel analysis of the blood in mice twelve weeks after primary transplantation and six weeks after secondary transplantation of cells nucleoporated with Cas9 RNP/ssODN to correct a sickle cell mutation. Human HbA is produced in mice after transplantation of HSCs nucleoporated with Cas9 RNP/ssODN to correct a sickle cell mutation. The mouse hemoglobin band will disappear in six more weeks.

Sequences

SEQ ID NO: 1

TAACGGCAGACTTCTCCAC

SEQ ID NO: 2

GTAACGGCAGACTTCTCCACGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGG
CTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTT

SEQ ID NO:3
Cas9-supercharged GFP construct

mdykdhdgdykdhdidykddddkmapkkkrkvgihgvpaadkkysigldigtnsvgwavitdeykvpskkfkvlgntdrh
sikknligallfdsgetaeatrlkrtarrytrrknricylqeifsnemakvddsffhrleesflveedkkherhpifgnivdevayhek
yptiyhlrkklvdstdkadlrliylalahmikfrghfliegdlnpdnsdvdklfiqlvqtynqlfeenpinasgvdakailsarlsksrrl
enliaqlpgekknglfgnlialslgltpnfksnfdlaedaklqlskdtydddldnllaqigdqyadlflaaknlsdaillsdilrvnteitk
aplsasmikrydehhqdltllkalvrqqlpekykeiffdqskngyagyidggasqeefykfikpilekmdgteellvklnredllrk
qrtfdngsiphqihlgelhailrrqedfypflkdnrekiekiltfripyyvgplargnsrfawmtrkseetitpwnfeevvdkgasaqs
fiermtnfdknlpnekvlpkhsllyeyftvyneltkvkyvtegmrkpaflsgeqkkaivdllfktnrkvtvkqlkedyfkkiecfds
veisgvedrfnaslgtyhdllkiikdkdfldneenediledivltltlfedremieerlktyahlfddkvmkqlkrrrytgwgrlsrkli
ngirdkqsgktildflksdgfanrnfmqlihddsltfkediqkaqvsgqgdslhehianlagspaikkgilqtvkvvdelvkvmgr
hkpeniviemarenqttqkgqknsrermkrieegikelgsqilkehpventqlqneklylyylqngrdmyvdqeldinrlsdydv
dhivpqsflkddsidnkvltrsdknrgksdnvpseevvkkmknywrqllnaklitqrkfdnltkaergglseldkagfikrqlvetr
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dykvydvrkmiakseqeigkatakyffysnimnffkteitlangeirkrplietngetgeivwdkgrdfatvrkvlsmpqvnivkk
tevqtggfskesilpkrnsdkliarkkdwdpkkyggfdsptvaysvlvvakvekgkskklksvkellgitimerssfeknpidfle
akgykevkkdliiklpkyslfelengrkrmlasagelqkgnelalpskyvnflylashyeklkgspedneqkqlfveqhkhyldei
ieqisefskrviladanldkvlsaynkhrdkpireqaeniihlftltnlgapaafkyfdttidrkrytstkevldatlihqsitglyetridls
qlggdkrpaatkkagqakkkkgsgsngssgsaskgerlfrgkvpilvelkgdvnghkfsvrgkgkgdatrgkltlkficttgklpv
pwptlvttltygvqcfsrypkhmkrhdffksampkgyvqertisfkkdgkyktraevkfegrtlvnriklkgrdfkekgnilghkl
rynfnshkvyitadkrkngikakfkirhnvkdgsvqladhyqqntpigrgpvllprnhylstrsklskdpkekrdhmvllefvtaa
gikhgrderyk

SEQ ID NO: 4
TAT-Cas9-supercharged GFP construct

ygrkkrrqrrrppqaggsmdykdhdgdykdhdidykddddkmapkkkrkvgihgvpaadkkysigldigtnsvgwavitd
eykvpskkfkvlgntdrhsikknligallfdsgetaeatrlkrtarrytrrknricylqeifsnemakvddsffhrleesflveedkkh
erhpifgnivdevayhekyptiyhlrkklvdstdkadlrliylalahmikfrghfliegdlnpdnsdvdklfiqlvqtynqlfeenpin
asgvdakailsarlsksrrlenliaqlpgekknglfgnlialslgltpnfksnfdlaedaklqlskdtydddldnllaqigdqyadlflaa
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itpwnfeevvdkgasaqsfiermtnfdknlpnekvlpkhslllyeyftvyneltkvkyvtegmrkpaflsgeqkkaivdllfktnrk
vtvkqlkedyfkkiecfdsveisgvedrfnaslgtyhdllkiikdkdfldneenediledivltltlfedremieerlktyahlfddkv
mkqlkrrrytgwgrlsrklingirdkqsgktildflksdgfanrnfmqlihddsltfkediqkaqvsgqgdslhehianlagspaikk
gilqtvkvvdelvkvmgrhkpeniviemarenqttqkgqknsrermkrieegikelgsqilkehpventqlqneklylyylqngr
dmyvdqeldinrlsdydvdhivpqsflkddsidnkvltrsdknrgksdnvpseevvkkmknywrqllnaklitqrkfdnltkaer
gglseldkagfikrqlvetrqitkhvaqildsrmntkydendklirevkvitlksklvsdfrkdfqfykvreinnyhhahdaylnavv
gtalikkypklesefvygdykvydvrkmiakseqeigkatakyffysnimnffkteitlangeirkrplietngetgeivwdkgrdf
atvrkvlsmpqvnivkktevqtggfskesilpkrnsdkliarkkdwdpkkyggfdsptvaysvlvvakvekgkskklksvkell
gitimerssfeknpidfleakgykevkkdliiklpkyslfelengrkrmlasagelqkgnelalpskyvnflylashyeklkgsped
neqkqlfveqhkhyldeiieqisefskrviladanldkvlsaynkhrdkpireqaeniihlftltnlgapaafkyfdttidrkrytstke
vldatlihqsitglyetridlsqlggdkrpaatkkagqakkkkgsgsngsssgsaskgerlfrgkvpilvelkgdvnghkfsvrgkgk
gdatrgkltlkficttgklpvpwptlvttltygvqcfsrypkhmkrhdffksampkgyvqertisfkkdgkyktraevkfegrtlvnr
iklkgrdfkekgnilghklrynfnshkvyitadkrkngikakfkirhnvkdgsvqladhyqqntpigrgpvllprnhylstrsklsk
dpkekrdhmvllefvtaagikhgrderykggsggsvdglfeaiegfiengwegmidgwyg

What is claimed is:

1. A complex for correcting a mutation in the genome of a cell comprising
    a. a guide RNA (gRNA) comprising a first nucleotide sequence that hybridizes to a target DNA in the genome of a cell, wherein the target DNA comprises a mutation, and a second nucleotide sequence that interacts with a site-directed nuclease;
    b. a recombinant site-directed nuclease operably linked to a supercharged protein, wherein the site-directed nuclease comprises an RNA-binding portion that interacts with the second nucleotide sequence of the guide RNA and wherein the site-directed nuclease specifically binds and cleaves the target DNA to create a double stranded break; and
    c. a single-stranded donor oligonucleotide (ssODN) that hybridizes to a genomic sequence flanking the double stranded break in the target DNA and integrates into the target DNA to correct a mutation in the target DNA.

2. The complex of claim 1, wherein the supercharged protein is operably linked to the amino-terminus or the carboxy-terminus of the nuclease.

3. The complex of any of claims 1 or 2, wherein the complex further comprises a trans-activating transcriptional activator (TAT) peptide that is operably linked to the amino-terminus of the site-directed nuclease.

4. The complex of any of claims 1-3, wherein the complex further comprises a trans-activating transcriptional activator (TAT) peptide that is operably linked to the carboxy-terminus of the site-directed nuclease.

5. The complex of any of claims 1-4, wherein the complex further comprises a negatively charged peptide of about 10 to about 25 amino acids in length that is operably linked to the carboxy-terminus of the site-directed nuclease.

6. The complex of claim 5, wherein the negatively charged peptide is an INF7 peptide comprising SEQ ID NO: 50.

7. The complex of any of claims 1-6, wherein the supercharged protein has an overall positive charge that is greater than its corresponding unmodified protein.

8. The complex of claim 7, wherein the overall positive charge is from about +5 to about +40.

9. The complex of claim 8, wherein the supercharged protein is superpositively charged green fluorescent protein (GFP).

10. The complex of claim 9, wherein the supercharged protein is a superpositively charged +36 GFP.

11. The complex of any of claims 1-10, wherein the ssODN that hybridizes to the genomic sequence flanking the double stranded break in the target DNA is a template for homology directed repair of a mutation in the target DNA.

12. The complex of claim 11, wherein the ssODN hybridizes to the genomic sequence encoding hemoglobin.

13. The complex of any of claims 1-12, wherein the nuclease is Cas9.

14. The complex of any of claims 1-13, wherein the molar ratio of gRNA to site-directed nuclease operably linked to a supercharged protein to ssODN is from about 1:1:0.2 to about 1.5:1:2.0.

15. The complex of any of claims 1-14, wherein the molar ratio of gRNA to site-directed nuclease operably linked to a supercharged protein to ssODN is from about 1:1:1 to about 1.5:1:1:15.

16. A cell comprising the complex of any of claims 1-15.

17. The cell of claim 16, wherein the cell is a eukaryotic cell.

18. The cell of claim 17, wherein the eukaryotic cell is a human cell.

19. The cell of any of claims 16-18, wherein the cell is a germ cell, a stem cell, or a precursor cell.

20. The cell of claim 19, wherein the stem cell is an induced pluripotent stem cell.

21. The cell of claim 19, wherein the precursor cell is a hematopoietic stem cell.

22. The cell of any of claims 16-21, wherein the cell is *in vitro*, *ex vivo* or *in vivo*.

23. A method of site-specific modification of a target DNA in a population of cells comprising introducing into the cells the complex of any of claims 1-15, wherein the complex is introduced into the cells under conditions that allow homology-directed repair (HDR) and integration of the ssODN into the target DNA.

24. The method of claim 23, wherein the complex is introduced into the cells by nucleoporation.

25. The method of claim 23 or 24, wherein the ssODN integrated into the target DNA corrects a mutation in the target DNA.

26. The method of any of claims 23-25, wherein the ratio of homology-directed repair to nonhomologous end joining (NHEJ) in the population of cells is from about 10 to about 0.5.

27. The method of any of claims 23-26, wherein the mutation is corrected in at least 5% of the cells.

28. The method of claim 27, wherein the the cell survival rate for corrected cells is at least about 50%.

29. The method of any of claims 23-28, wherein the molar ratio of gRNA to site-directed nuclease operably linked to a supercharged protein to ssODN is from about 1:1:0.2 to about 1.5:1:2.0.

30. The method of any of claims 23-29, wherein the target DNA encodes hemoglobin.

31. The method of claim 30, wherein the site-specific modification corrects a hemoglobin mutation associated with sickle cell anemia.

32. The method of claim 30, wherein the site-specific modification corrects a mutation associated with β-thalassemia.

33. A method of treating a disease associated with a mutation in the genomic sequence encoding hemoglobin in a subject comprising:
    a. introducing into a population of cells obtained from the subject the complex of claim 11 under conditions that allow homology-directed repair (HDR) to correct the mutation in the genomic sequence encoding hemoglobin and
    b. transplanting the cells of step (a) into the subject.

34. The method of claim 33, wherein the disease associated with a mutation in the genomic sequence encoding hemoglobin is sickle cell disease or β-thalassemia.

35. The method of claim 33 or 34, wherein the cell is a hematopoietic stem cell or an induced pluripotent stem cell.

36. The method of claim any of claims 33-35, wherein at least 5% of the transplanted cells include a corrected mutation.

37. The method of any of claims 33-36, wherein the ratio of homology-directed repair to nonhomologous end joining in the population of cells is at least about 0.5.

38. The method of any of claims 33-36, wherein the complex is introduced into the cell by nucleoporation.

39. A method of correcting a mutation associated with a T-cell disorder comprising introducing into a population of cells obtained from a subject with the T-cell disorder a complex comprising:
    a. a guide RNA (gRNA) comprising a first nucleotide sequence that hybridizes to a target DNA in the genome of a cell, wherein the target DNA comprises the mutation associated with the T-cell disorder, and a second nucleotide sequence that interacts with a site-directed nuclease;
    b. a recombinant site-directed nuclease operably linked to a supercharged protein, wherein the site-directed nuclease comprises an RNA-binding portion that interacts with the second nucleotide sequence of the gRNA and wherein the site-directed nuclease specifically binds and cleaves the target DNA that comprises the mutation associated with the T-cell disorder to create a double stranded break in the target DNA; and
    c. a single stranded donor oligonucleotide (ssODN) comprising a third nucleotide sequence that hybridizes to a genomic sequence flanking the double stranded break in the target DNA and that integrates into the target DNA to correct the mutation associated with the T-cell disorder,

    wherein the complex is introduced into the cell under conditions that allow homology-directed repair (HDR) to correct the mutation associated with the T-cell disorder.

40. The method of claim 39, wherein the target DNA encodes a protein associated with T-lymphocyte development.

41. The method of claim 39 or 40, wherein the cells are selected from the group consisting of hematopoietic stem cells or pluripotent stem cells.

42. The method of claim 41, wherein the pluripotent stem cells are induced pluripotent stem cells.

43. The method of any of claims 39-42, wherein the supercharged protein is operably linked to the amino-terminus or the carboxy-terminus of the nuclease.

44. The method of any of claims 39-43, wherein the recombinant site-directed nuclease operably linked to a supercharged protein further comprises a trans-activating

transcriptional activator (TAT) peptide operably linked to the amino-terminus of the site-directed nuclease.

45. The method of any of claims 39-44, wherein the complex further comprises a trans-activating transcriptional activator (TAT) peptide that is operably linked to the carboxy-terminus of the site-directed nuclease.

46. The method of any of claims 39-45 wherein the complex further comprises a negatively charged peptide of about 10 to about 25 amino acids in length that is operably linked to the carboxy-terminus of the site-directed nuclease.

47. The method of claim 46, wherein the negatively charged peptide is an INF7 peptide comprising SEQ ID NO: 50.

48. The method of any of claims 39-47, wherein the supercharged protein has an overall positive charge that is greater than its corresponding unmodified protein.

49. The method of claim 48, wherein the overall positive charge is from about +5 to about about +40.

50. The method of any of claims 39-49, wherein the supercharged protein is superpositively charged green fluorescent protein (GFP).

51. The method of claim 50, wherein the supercharged protein is a superpositively charged +36 GFP.

52. The method of any of claims 39-51, wherein the nuclease is Cas9.

53. The method of any of claims 39-52, wherein the molar ratio of gRNA to site-directed nuclease operably linked to a supercharged protein to ssODN is from about 1:1:0.2 to about 1.5:1:2.0.

54. The method of any of claims 39-53, wherein the ratio of homology-directed repair to nonhomologous end joining in the population of cells obtained from a subject with the T-cell disorder is at least about 0.5.

55. The method of any of claims 39-54, wherein the complex is introduced into the cells by nucleoporation.

56. The method of any of claims 39-55, wherein at least 5% of the population of cells obtained from a subject with the T-cell disorder undergo HDR to correct the mutation associated with the T-cell disorder.

57. The method of any of claims 39-56, further comprising isolating the cells corrected with HDR.

58. The method of claim 57, further comprising culturing the cells corrected with HDR.

59. The method of claim 58, wherein the cells are cultured under conditions for expansion.

60. The method of claim 58 or 59, wherein the cells are cultured under conditions that promote differentiation of the cells into T cells.

61. A method of treating a T cell disorder associated with a genetic mutation in a subject comprising transplanting into the subject cells obtained by the method of any one of claims 39-60.

62. The method of claim 61, wherein the transplantation is autologous.

63. The method of any of claims 39-62, wherein the T cell disorder is severe combined immune deficiency.

64. A complex for correcting a mutation associated with a T-cell disorder in the genome of a cell comprising:

a. a guide RNA (gRNA) comprising a first nucleotide sequence that hybridizes to a target DNA in the genome of a cell, wherein the target DNA comprises the mutation associated with the T-cell disorder, and a second nucleotide sequence that interacts with a site-directed nuclease;
b. a recombinant site-directed nuclease operably linked to a supercharged protein, wherein the site-directed nuclease comprises an RNA-binding portion that interacts with the second nucleotide sequence of the gRNA and wherein the site-directed nuclease specifically binds and cleaves the target DNA that comprises the mutation associated with the T-cell disorder to create a double stranded break in the target DNA; and
c. a single stranded donor oligonucleotide (ssODN) comprising a third nucleotide sequence that hybridizes to a genomic sequence flanking the double stranded break in the target DNA and that integrates into the target DNA to correct the mutation associated with the T-cell disorder,

65. The complex of claim 64 wherein the supercharged protein is operably linked to the amino-terminus or the carboxy-terminus of the nuclease.

66. The complex of any of claims 64 or 65 wherein the recombinant site-directed nuclease operably linked to a supercharged protein further comprises a trans-activating transcriptional activator (TAT) peptide operably linked to the amino-terminus of the site-directed nuclease.

67. The complex of any of claims 64-66, wherein the supercharged protein has an overall positive charge that is greater than its corresponding unmodified protein.

68. The complex of claim 67, wherein the overall positive charge is from about +5 to about about +40.

69. The complex of any of claims 64-68, wherein the supercharged protein is superpositively charged green fluorescent protein (GFP).

70. The complex of claim 69, wherein the supercharged protein is a superpositively charged +36 GFP.

71. The complex of any of claims 64-70, wherein the nuclease is Cas9.

72. The complex of any of claims 64-71, wherein the molar ratio of gRNA to site-directed nuclease operably linked to a supercharged protein to ssODN is from about 1:1:0.2 to about 1.5:1:2.0.

73. The complex of any of claims 1-15, wherein the recombinant site-directed nuclease operably linked to a supercharged protein is recombinant Cas9 operably linked to superpositively charged +36 GFP.

74. The complex of any of claims 1-15, wherein the recombinant site-directed nuclease operably linked to a supercharged protein is recombinant Cas9 operably linked to superpositively charged +36 GFP.

75. The complex of claim 74, wherein superpositively charged +36 GFP is operably linked to the carboxy-terminus of Cas9.

76. The complex of claim 74 or 75, wherein the ssODN hybridizes to the genomic sequence encoding hemoglobin and comprises SEQ ID NO: 52.

FIGURE 1A

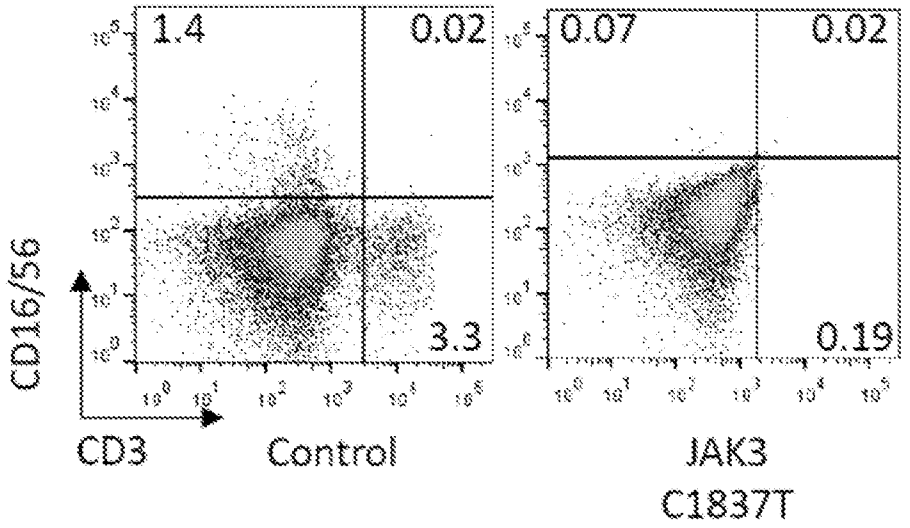


FIGURE 1B

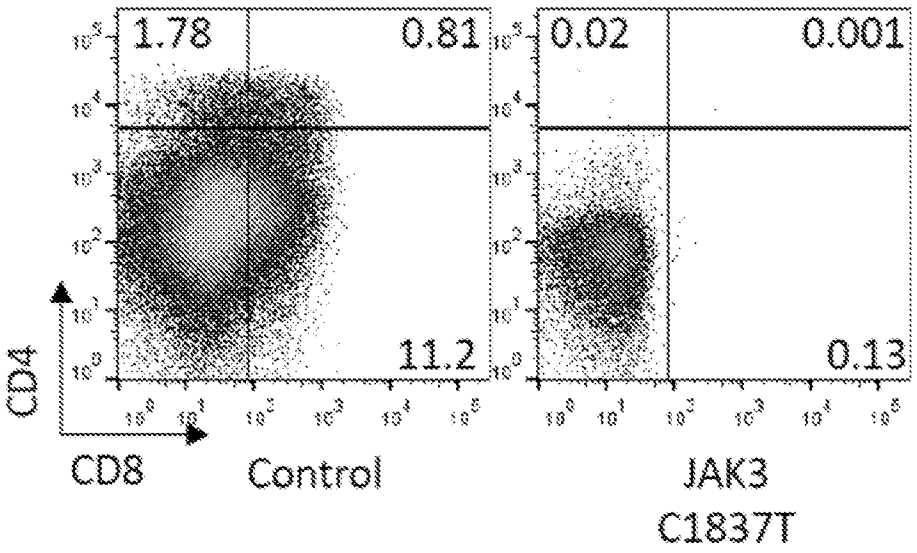


FIGURE 1C

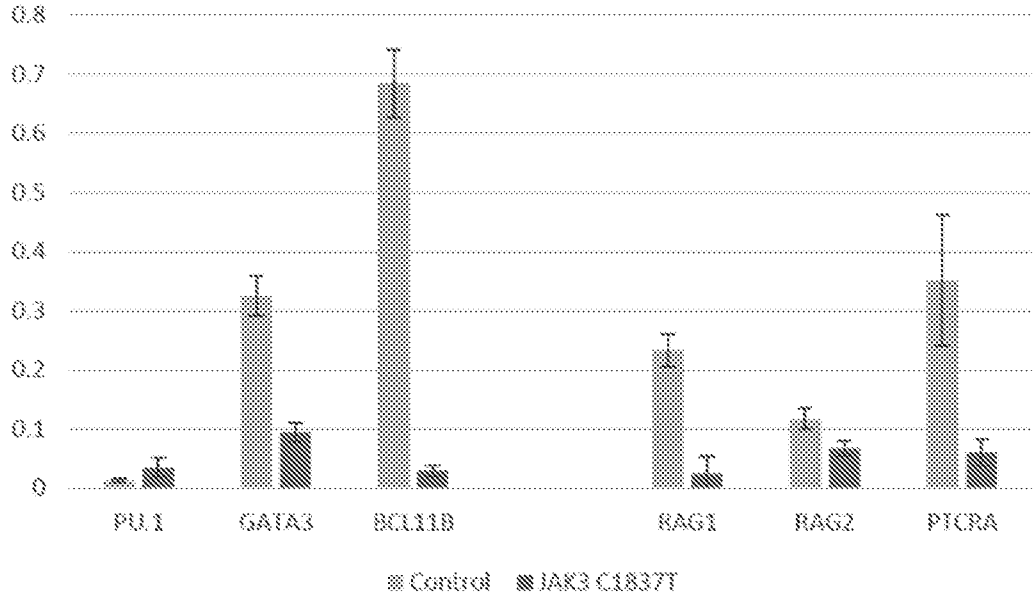

0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
PU.1
GATA3
BCL11B
RAG1
RAG2
PTCRA
Control
JAK3 C1837T

FIGURE 2A

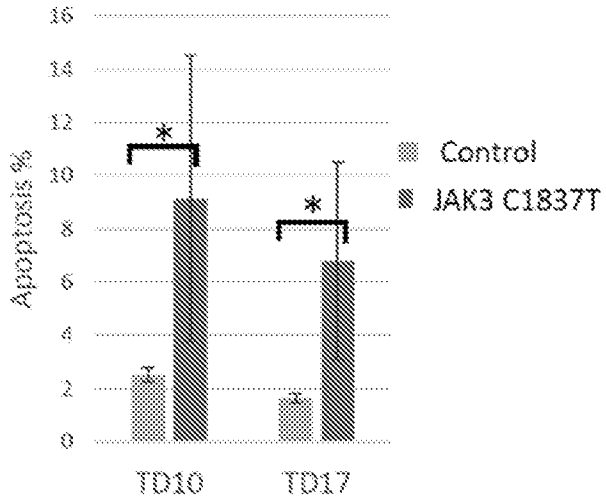


FIGURE 2B

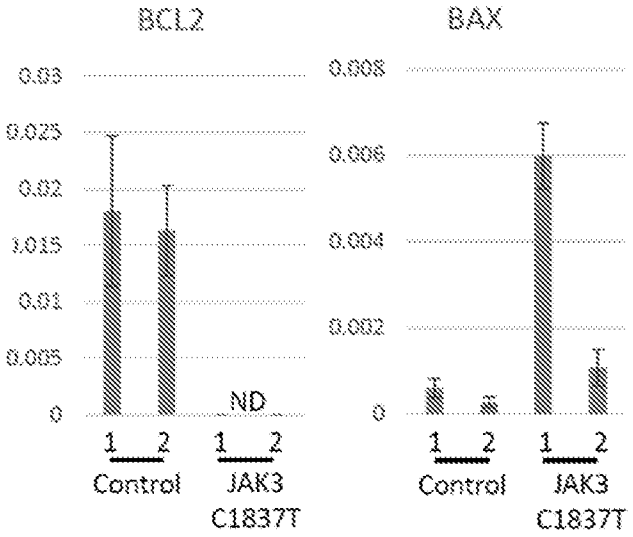


FIGURE 2C

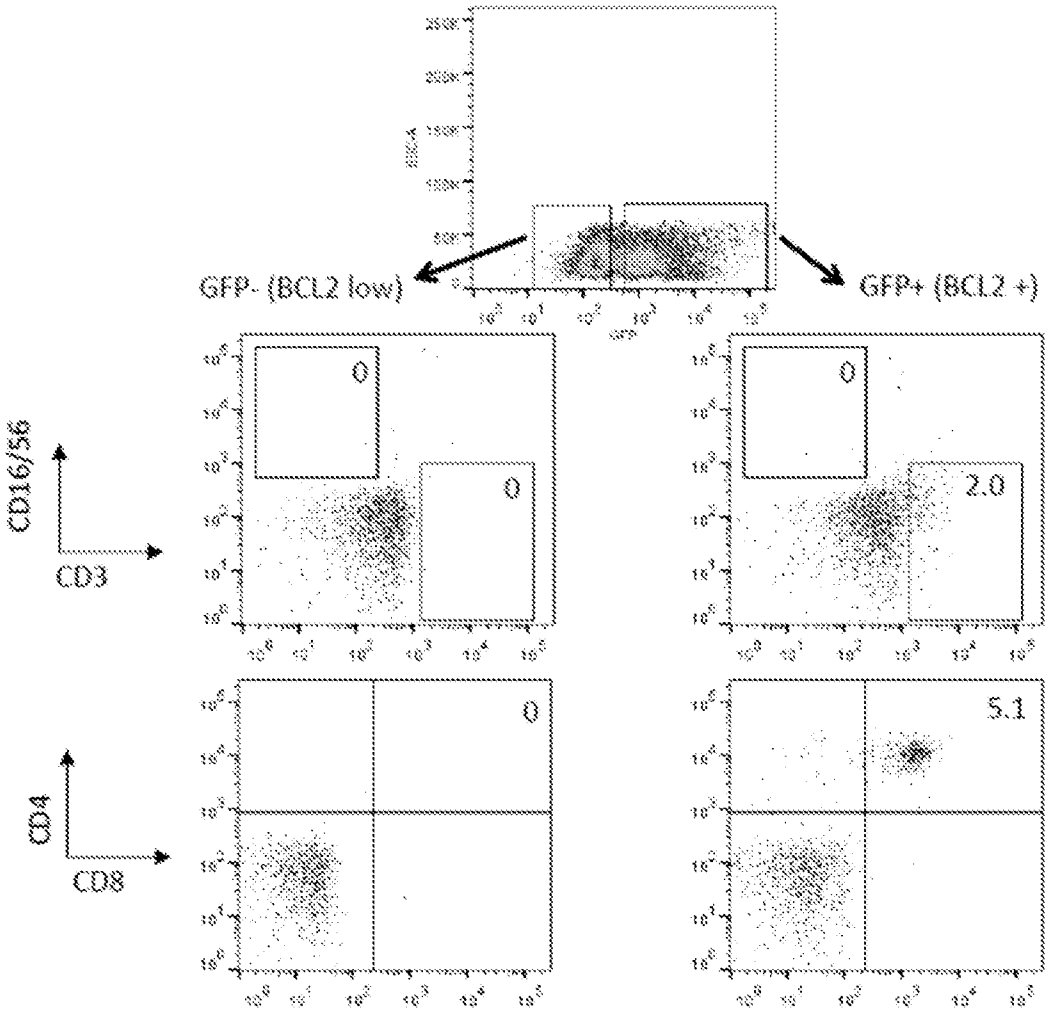


FIGURE 3A

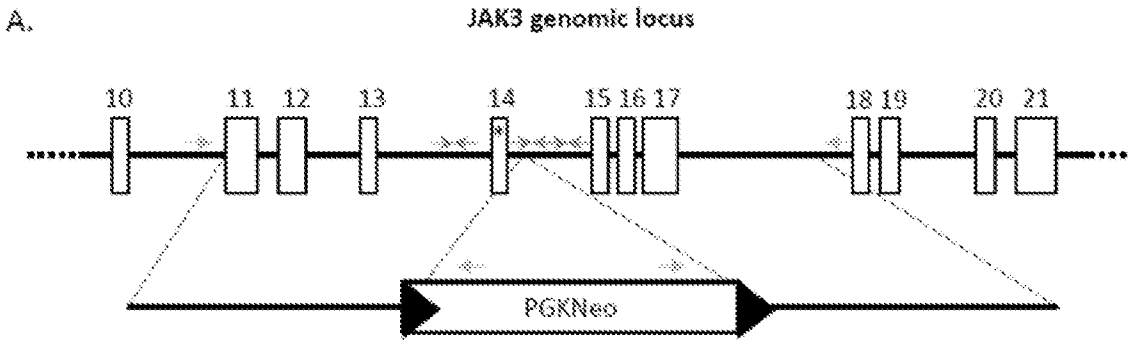


FIGURE 3B

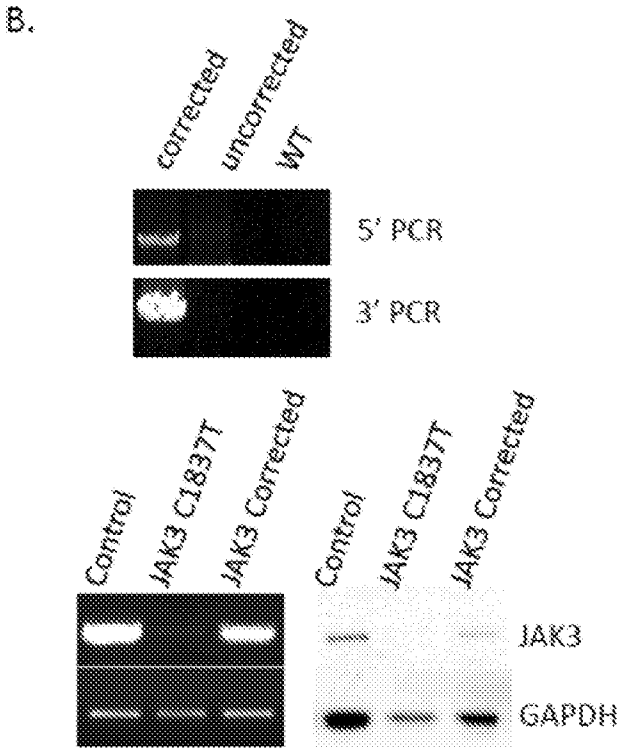


FIGURE 3C

	Colonies examined	PCR positive colonies	%
gRNA #1	39	9	23
gRNA #2	45	33	73.3
gRNA #3	16	1	6.25
gRNA #4	9	3	33.3
gRNA #5	3	0	0
gRNA #6	7	0	0
gRNA #1 + #2	14	14	100
gRNA #3 + #4	3	0	0
gRNA #5 + #6	4	0	0

FIGURE 3D

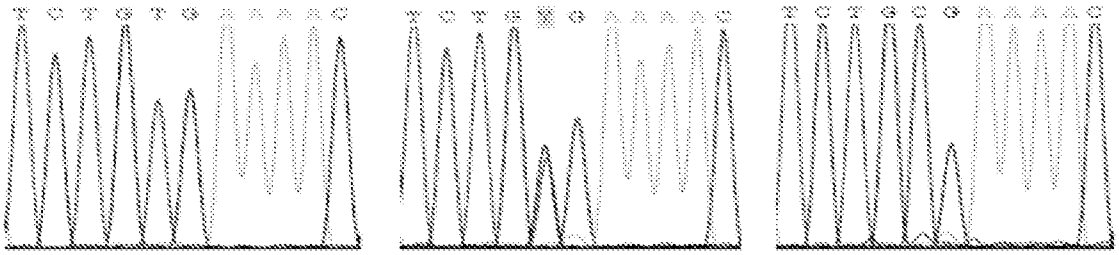


FIGURE 4A

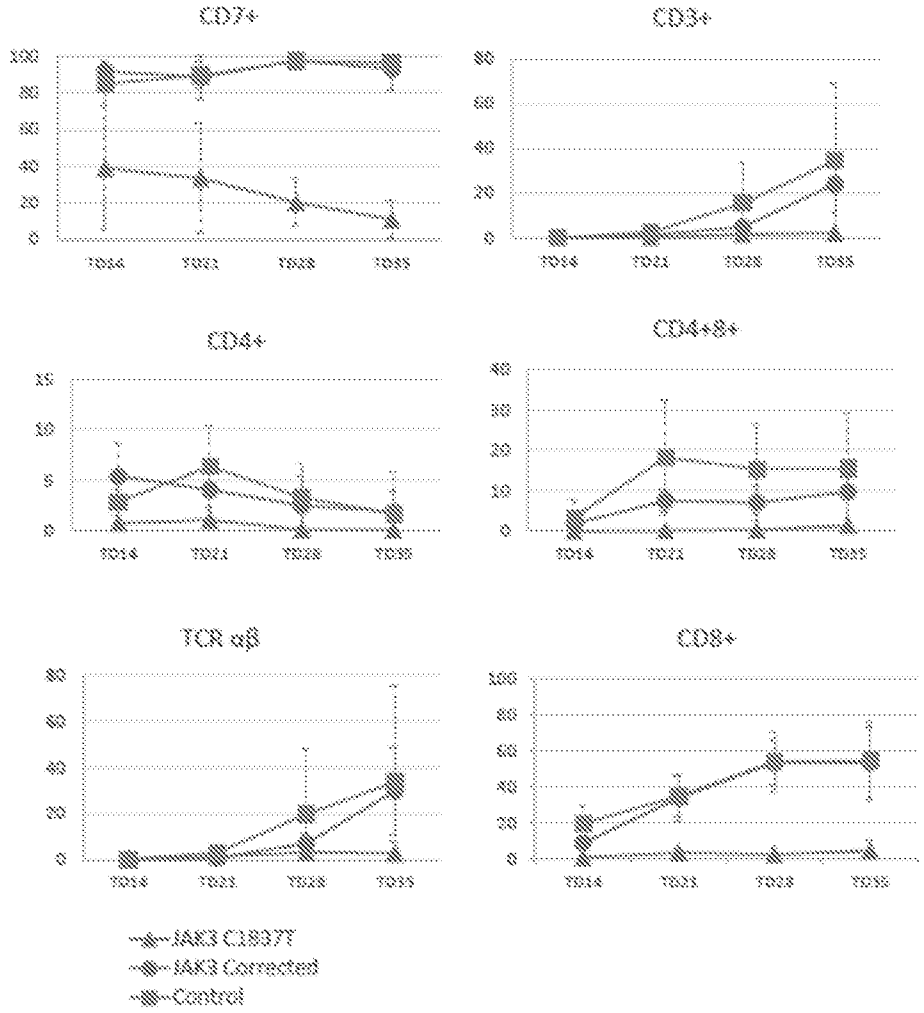

CD7+
CD3+
CD4+
CD4+8+
TCR αβ
CD8+
IAK3 C1837T
IAK3 Corrected
Control

FIGURE 4B

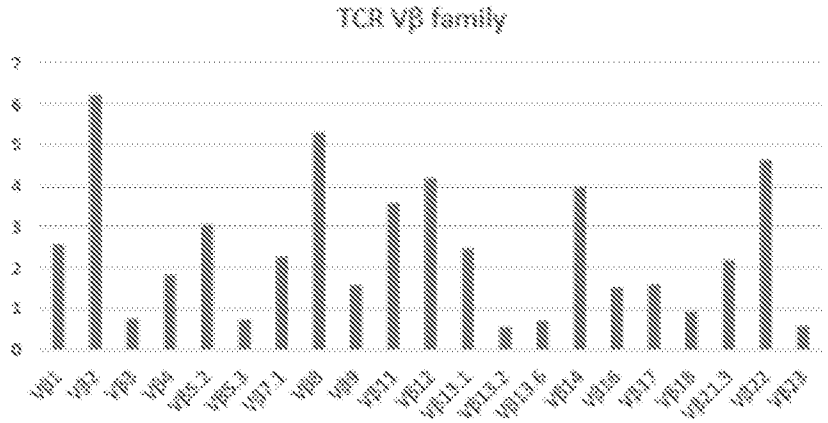


FIGURE 4C

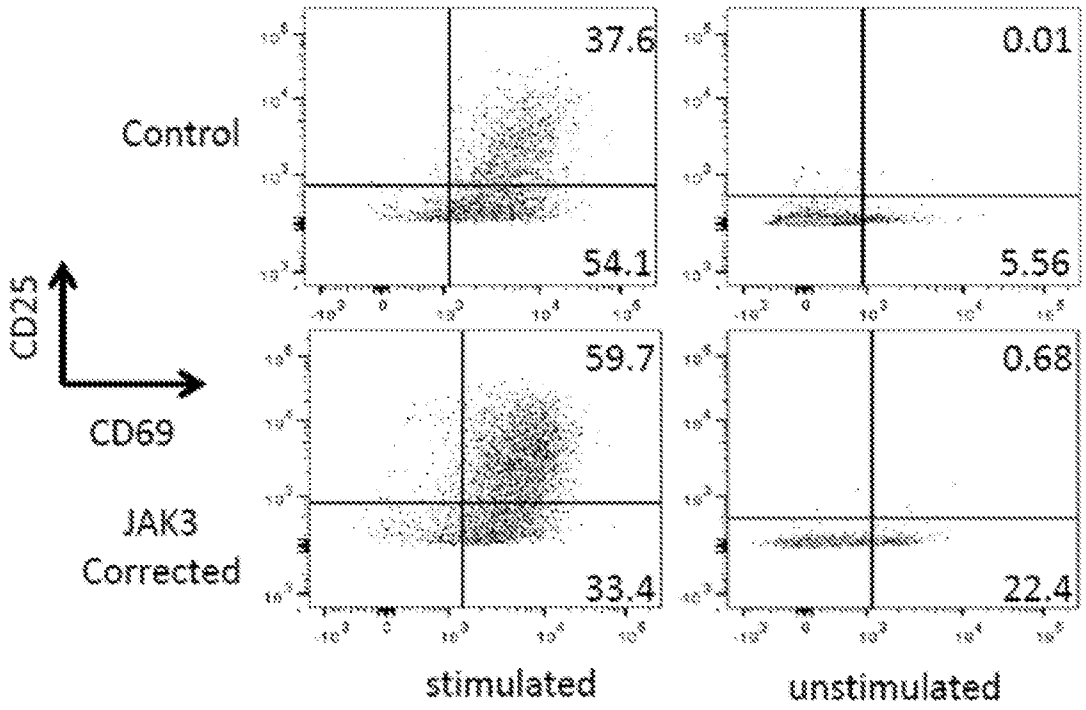


FIGURE 5

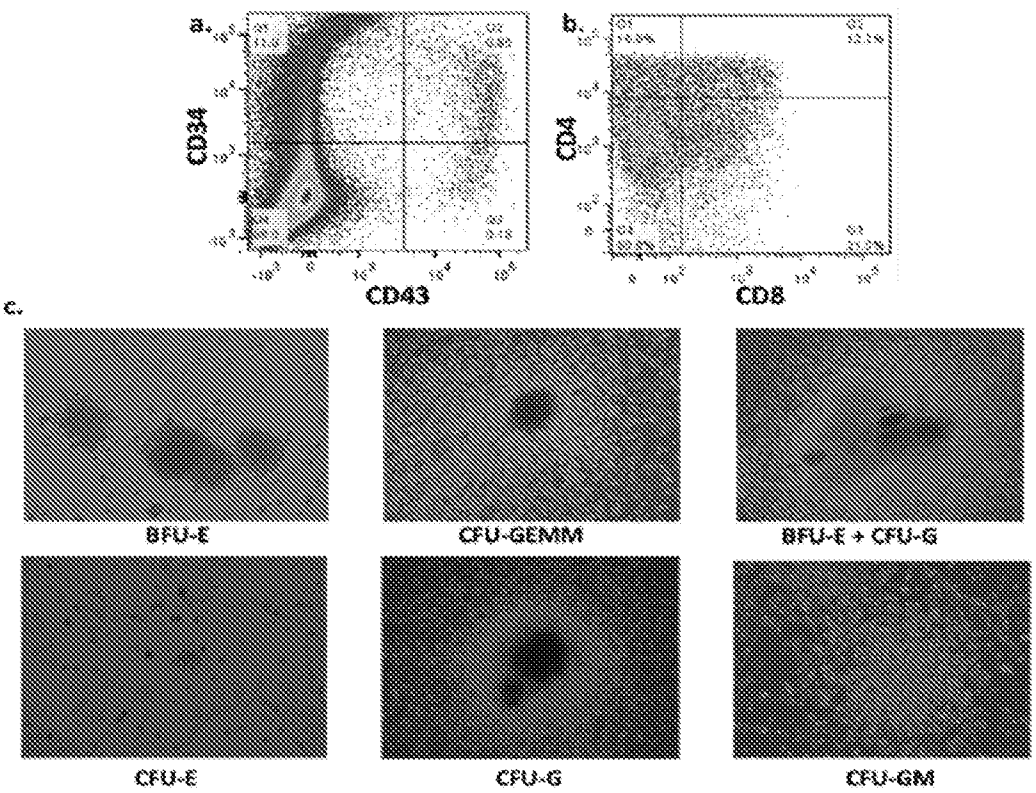


FIGURE 6

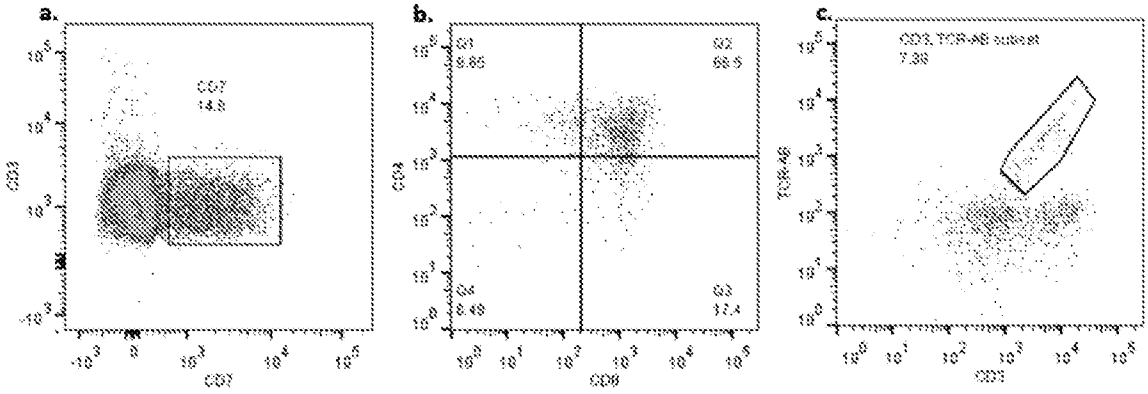


FIGURE 7

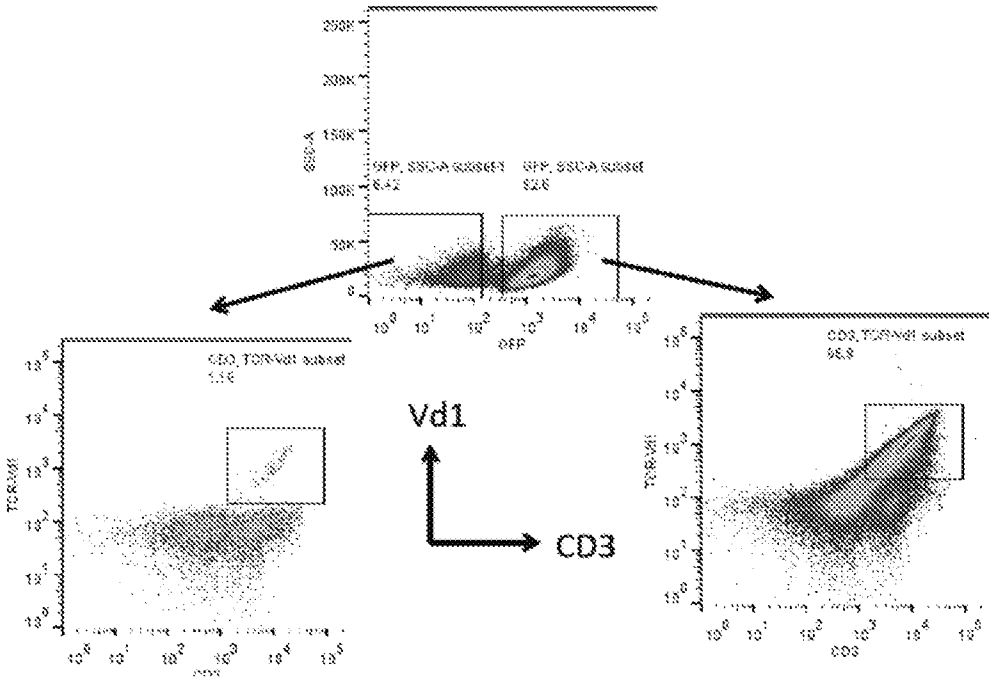


FIGURE 8

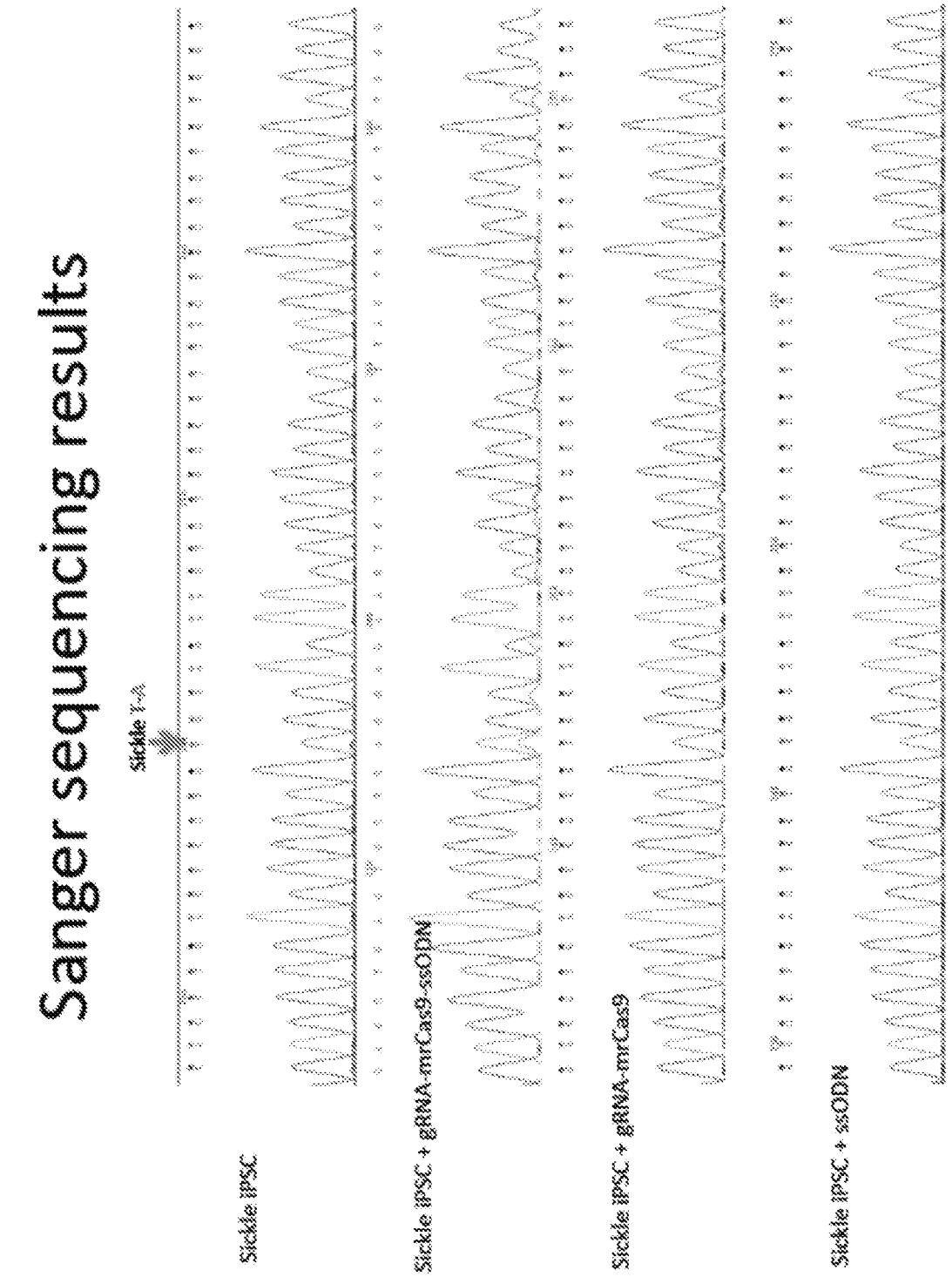

Sanger sequencing results
Sickle T->A
Sickle iPSC
Sickle iPSC + gRNA-mrCas9-ssODN
Sickle iPSC + gRNA-mrCas9
Sickle iPSC + ssODN

FIGURE 9

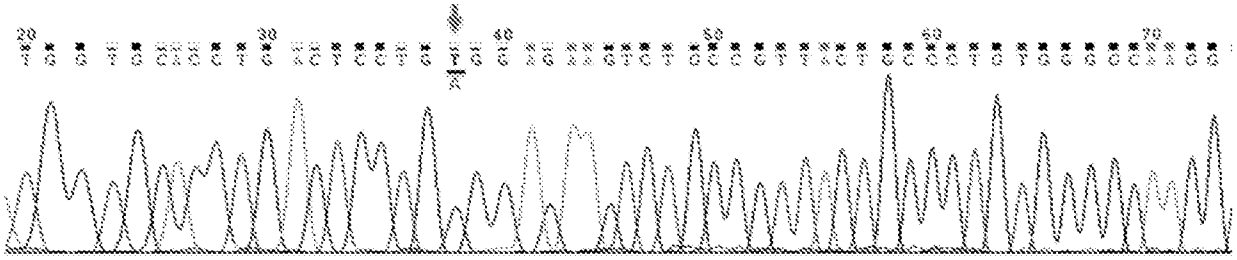


FIGURE 10

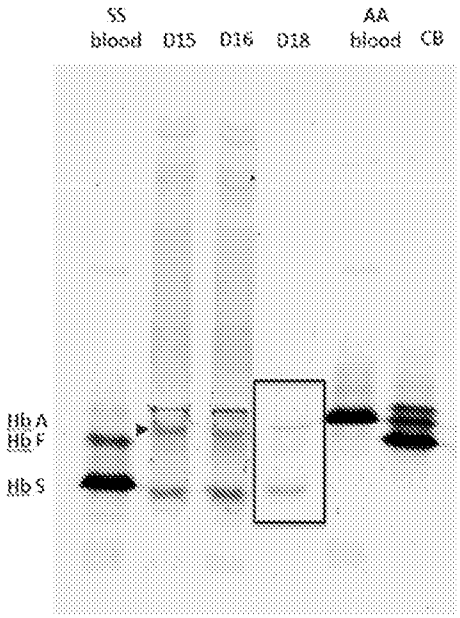


FIGURE 11

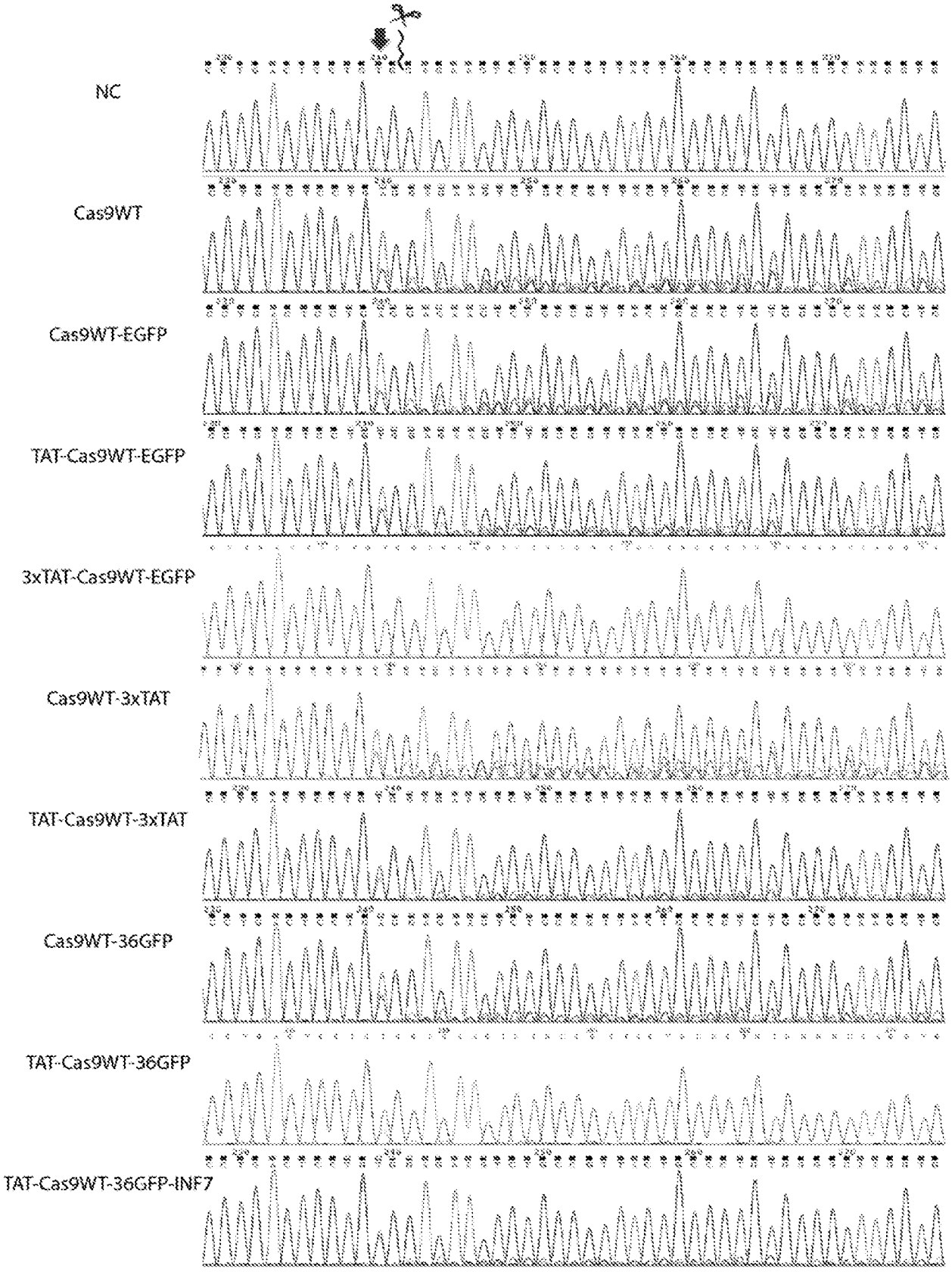

NC
Cas9WT
Cas9WT-EGFP
TAT-Cas9WT-EGFP
3xTAT-Cas9WT-EGFP
Cas9WT-3xTAT
TAT-Cas9WT-3xTAT
Cas9WT-36GFP
TAT-Cas9WT-36GFP
TAT-Cas9WT-36GFP-INF7

FIGURE 12

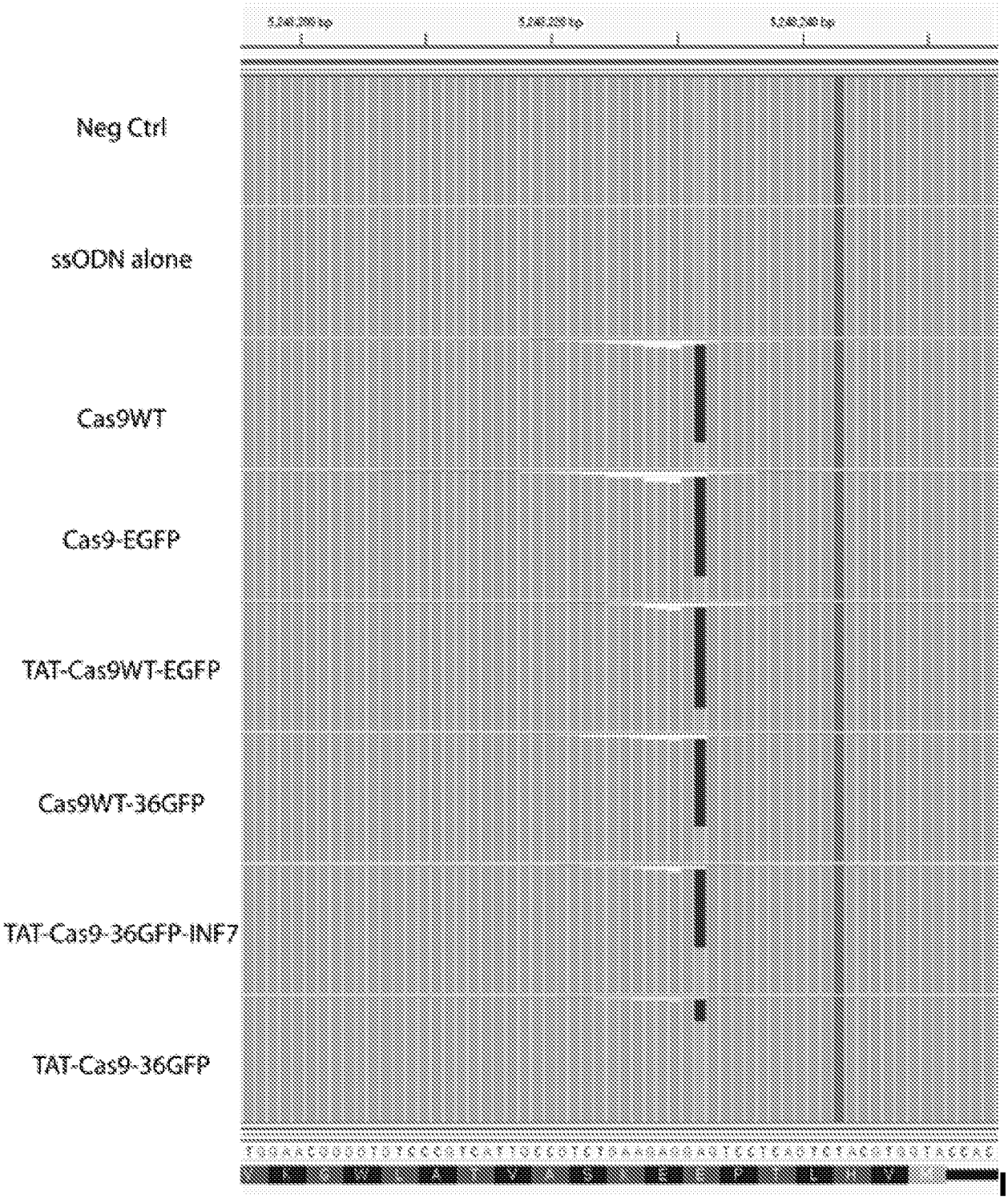

Neg Ctrl
ssODN alone
Cas9WT
Cas9-EGFP
TAT-Cas9WT-EGFP
Cas9WT-36GFP
TAT-Cas9-36GFP-INF7
TAT-Cas9-36GFP

FIGURE 13

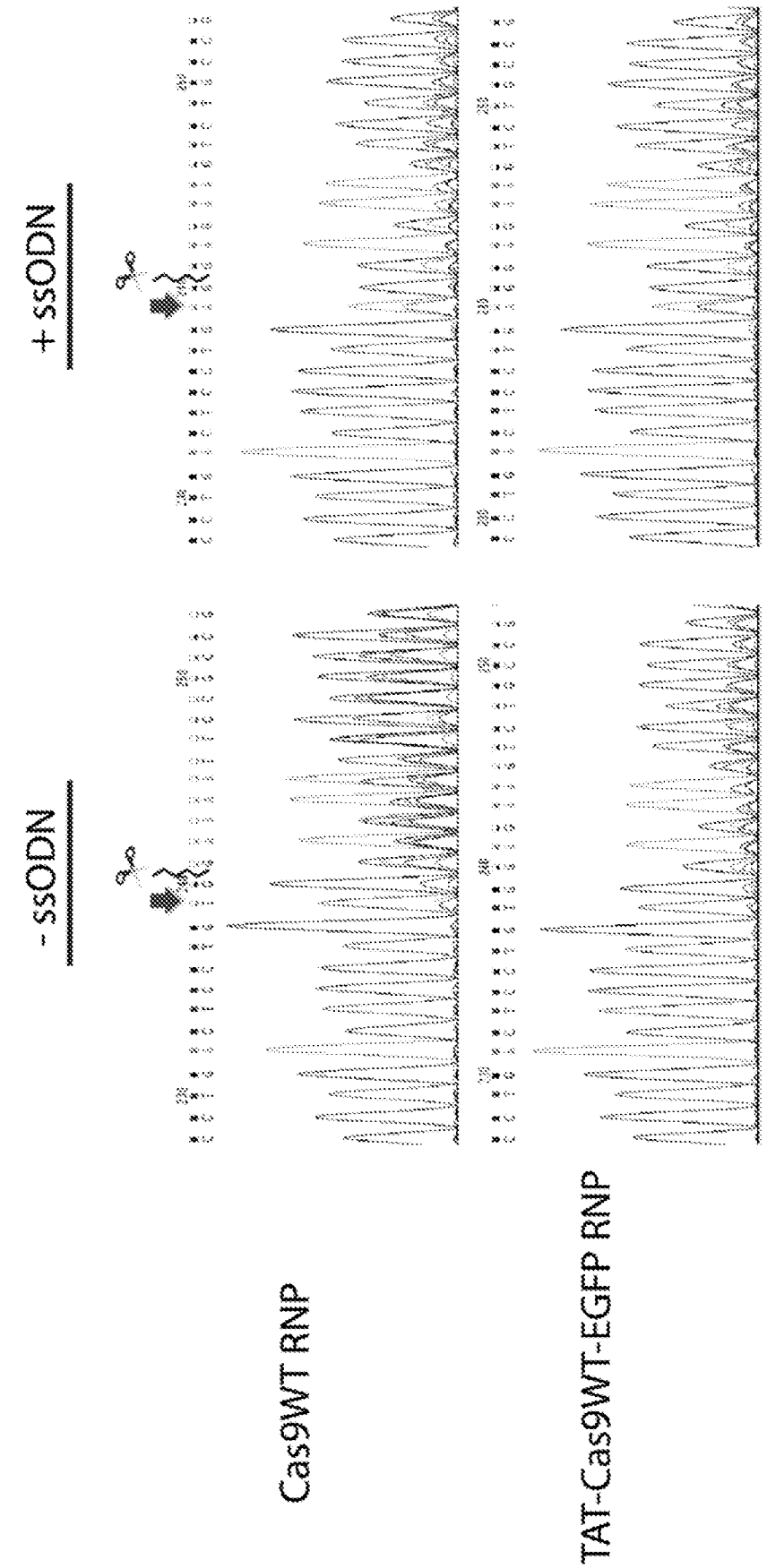


FIGURE 14

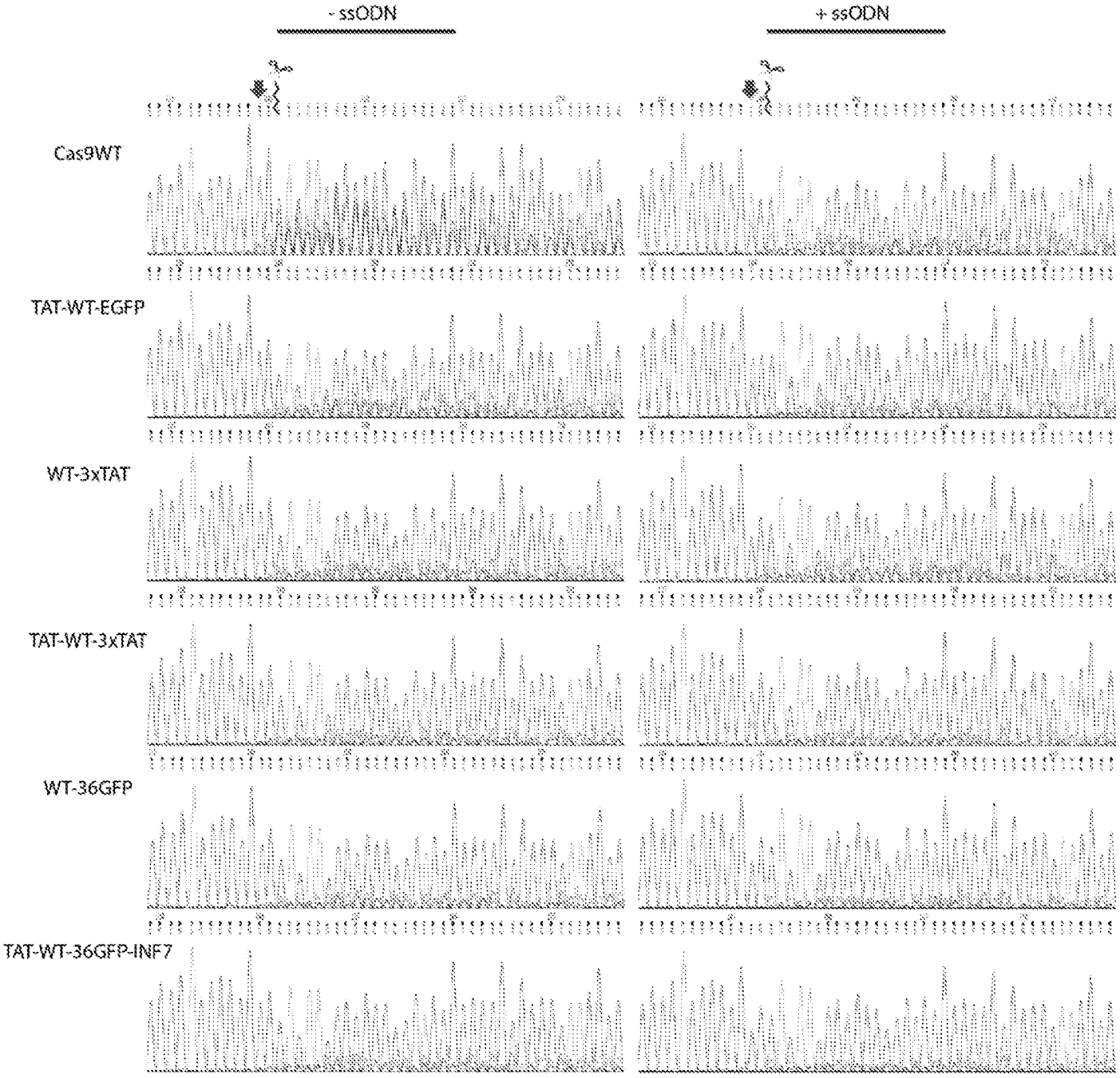

- ssODN
+ ssODN
Cas9WT
TAT-WT-EGFP
WT-3xTAT
TAT-WT-3xTAT
WT-36GFP
TAT-WT-36GFP-INF7

FIGURE 15

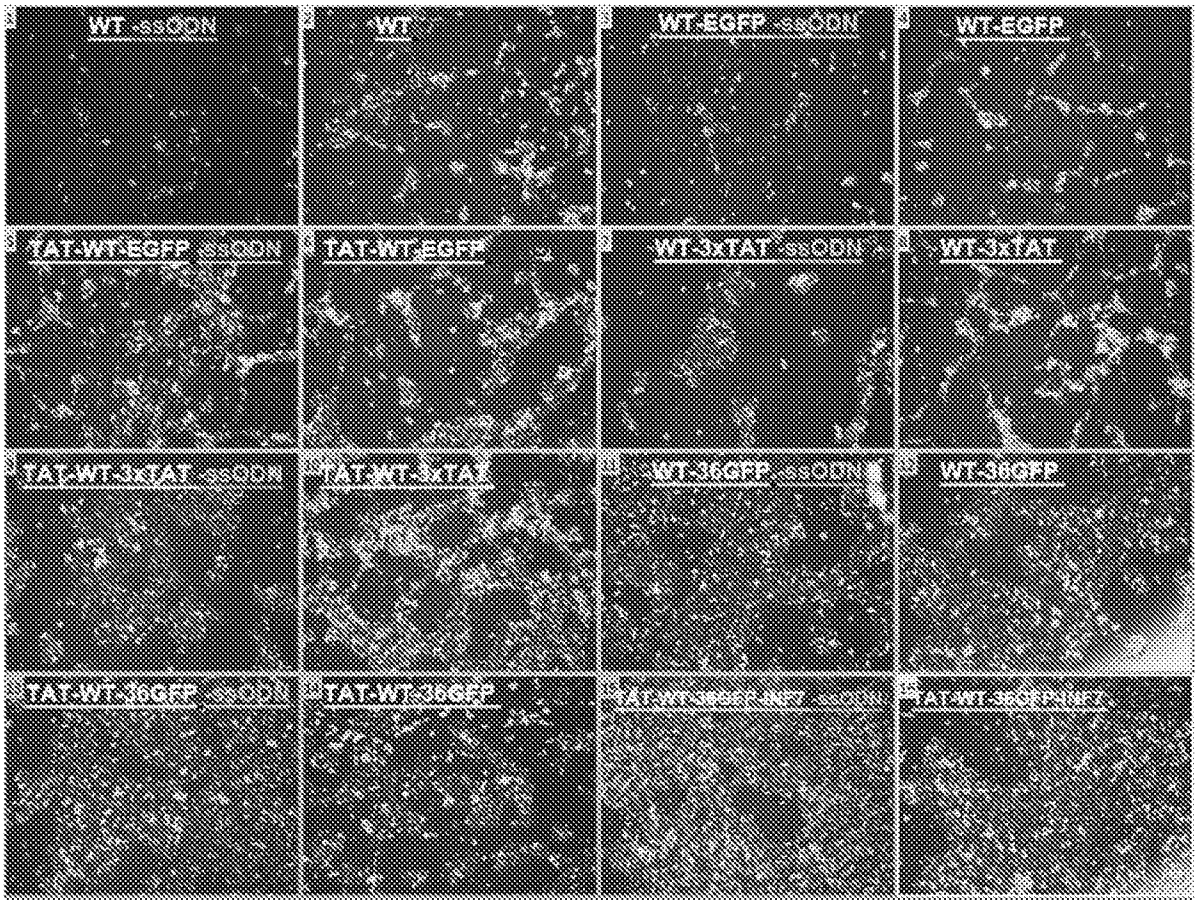

WT ssODN
WT
WT-EGFP ssODN
WT-EGFP
TAT-WT-EGFP ssODN
TAT-WT-EGFP
WT-3xTAT ssODN
WT-3xTAT
TAT-WT-3xTAT ssODN
TAT-WT-3xTAT
WT-3GFP ssODN
WT-3GFP
TAT-WT-3GFP ssODN
TAT-WT-3GFP

FIGURE 16

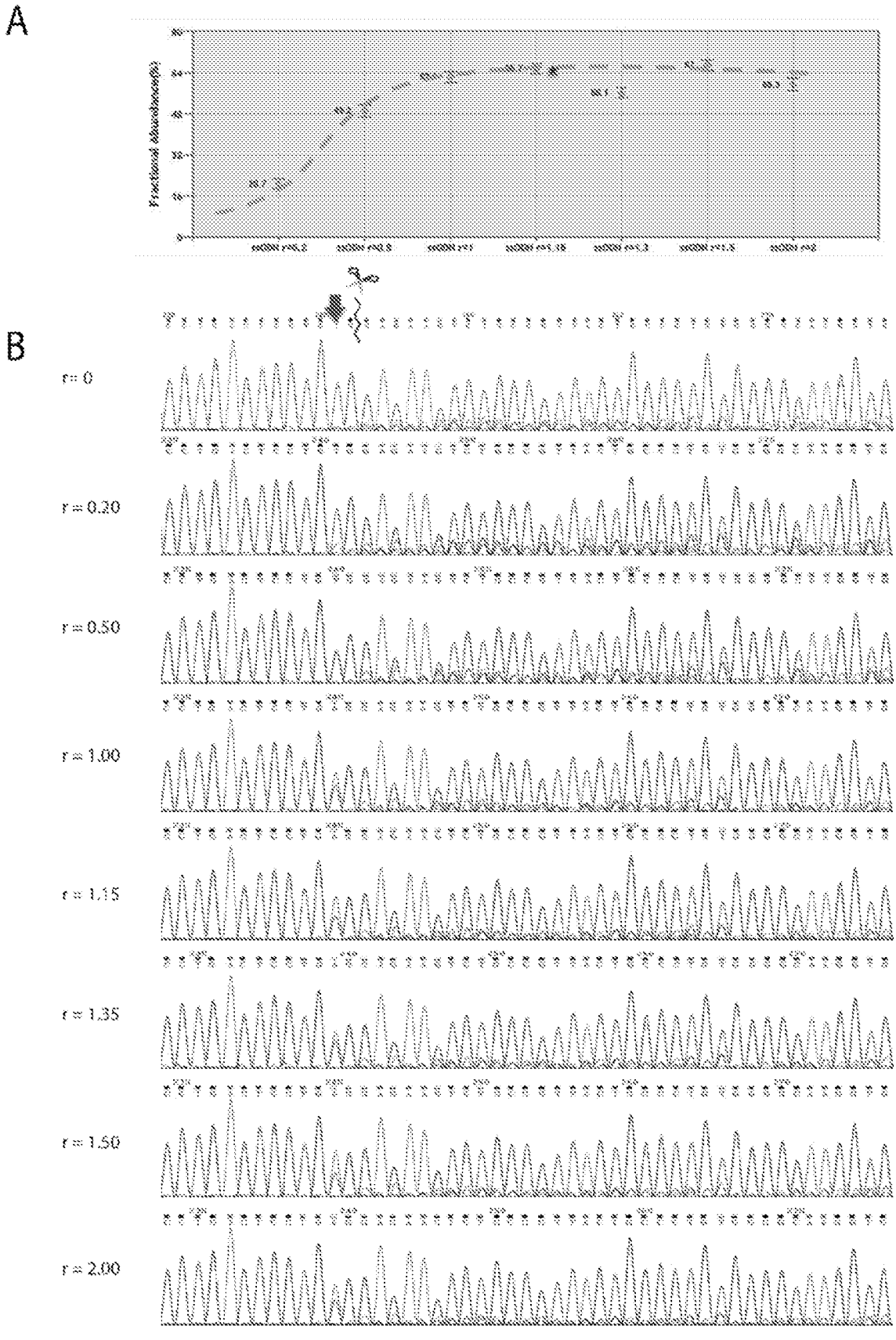


FIGURE 17

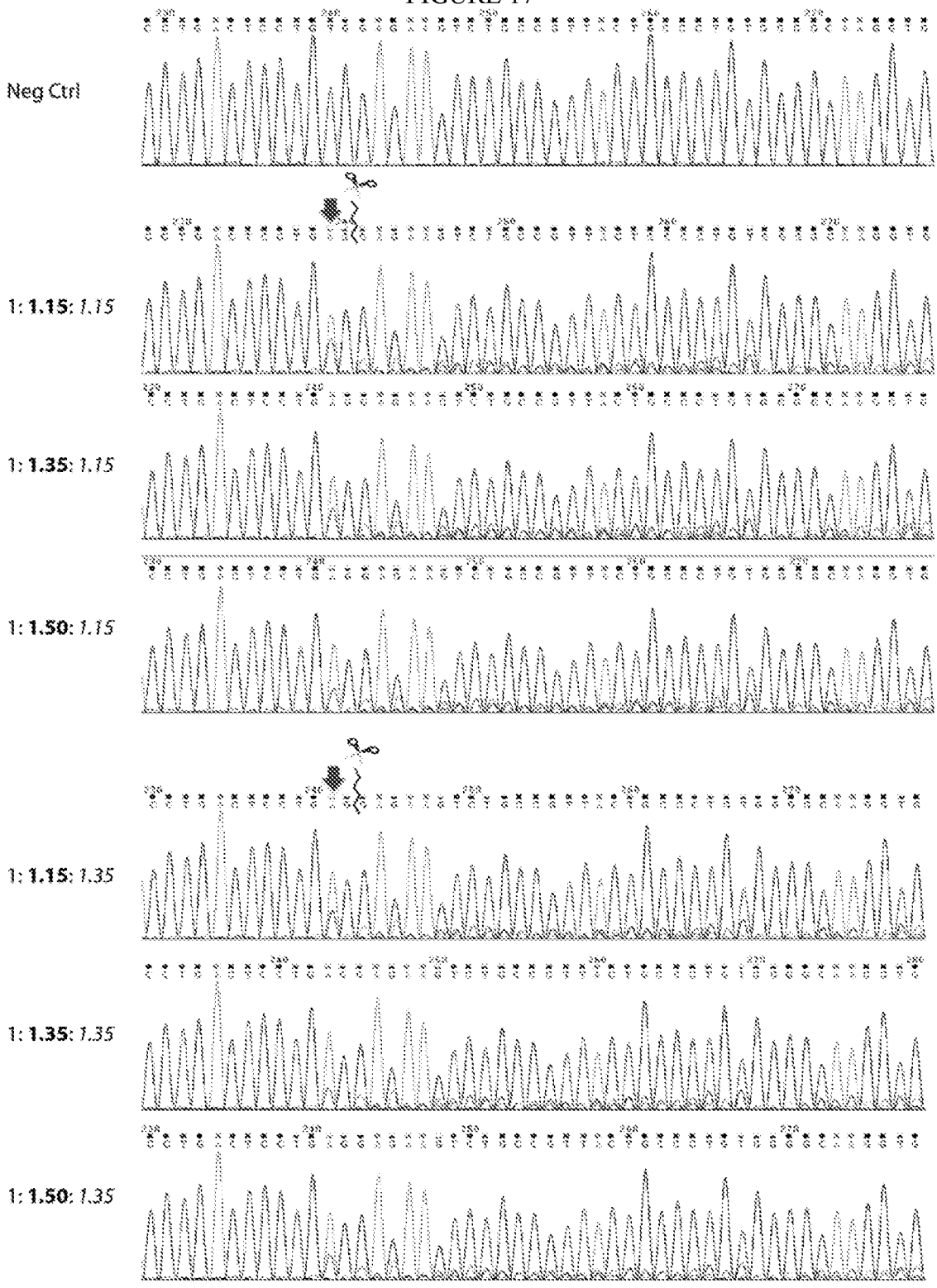


FIGURE 18

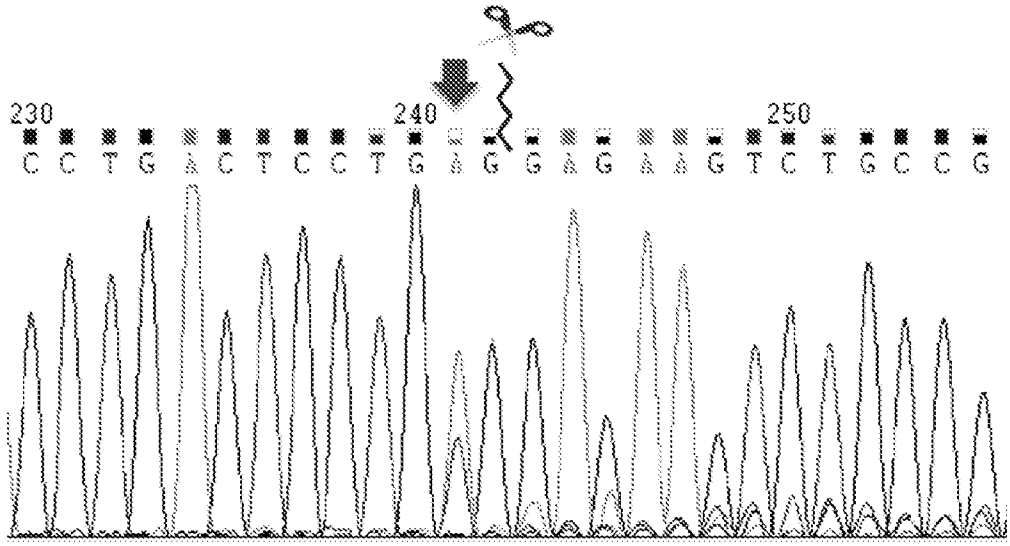


FIGURE 19

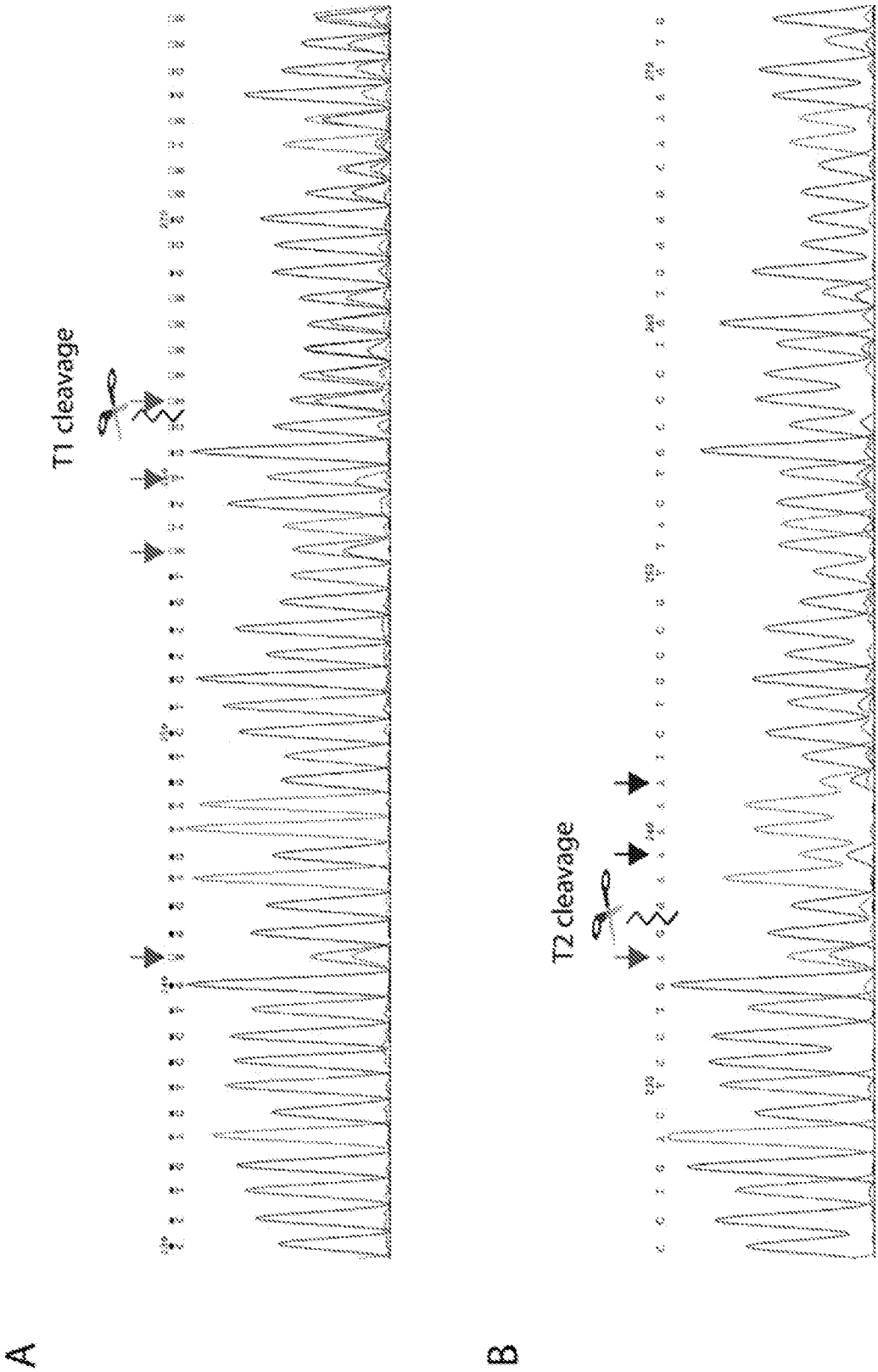
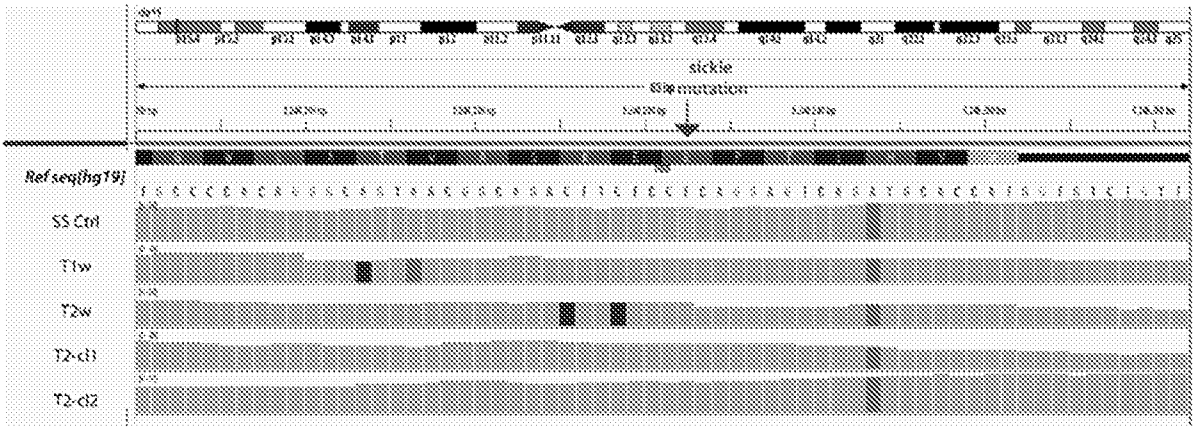


FIGURE 20

A


sickle
SS mutation
Ref seq(hg19)
SS Ctrl
T1w
T2w
T2-cl1
T2-cl2

B

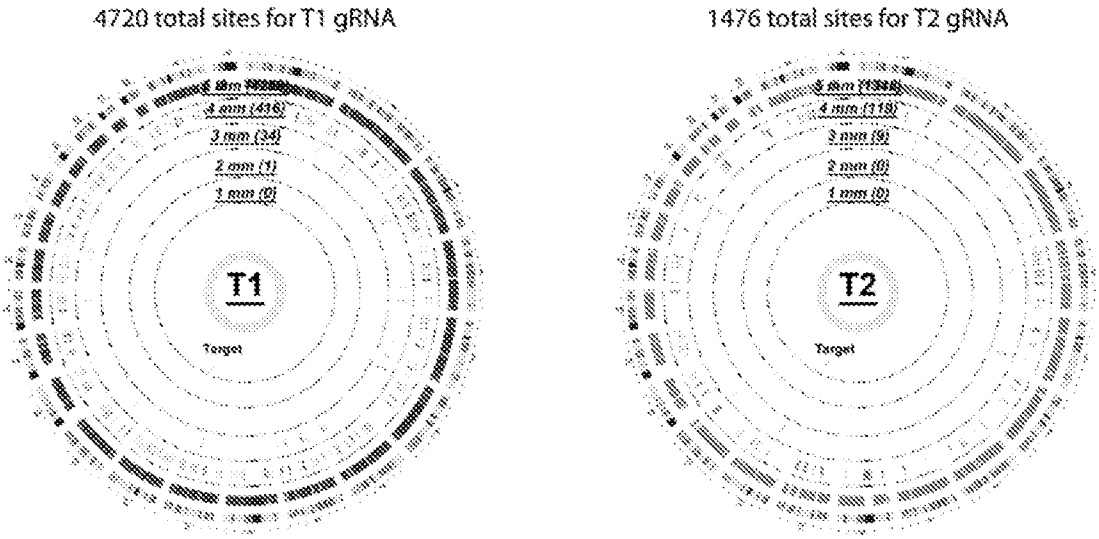

4720 total sites for T1 gRNA
4 mm (416)
3 mm (34)
2 mm (1)
1 mm (0)
T1
Target
1476 total sites for T2 gRNA
4 mm (118)
3 mm (9)
2 mm (0)
1 mm (0)
T2
Target

FIGURE 21

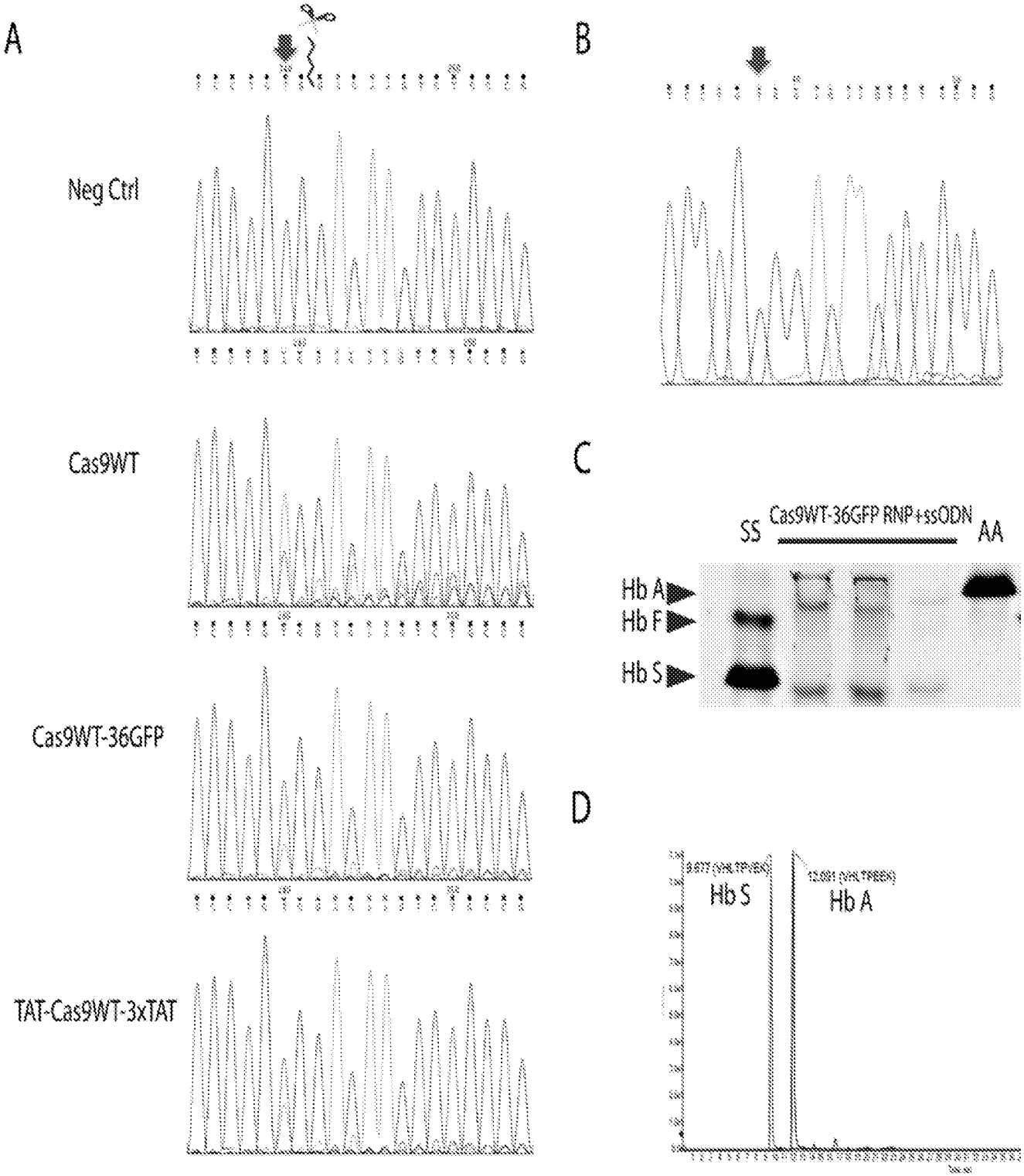

A
Neg Ctrl
Cas9WT
Cas9WT-36GFP
TAT-Cas9WT-3xTAT
B
C
SS
Cas9WT-36GFP RNP+ssODN
AA
Hb A
Hb F
Hb S
D
Hb S
Hb A

FIGURE 22

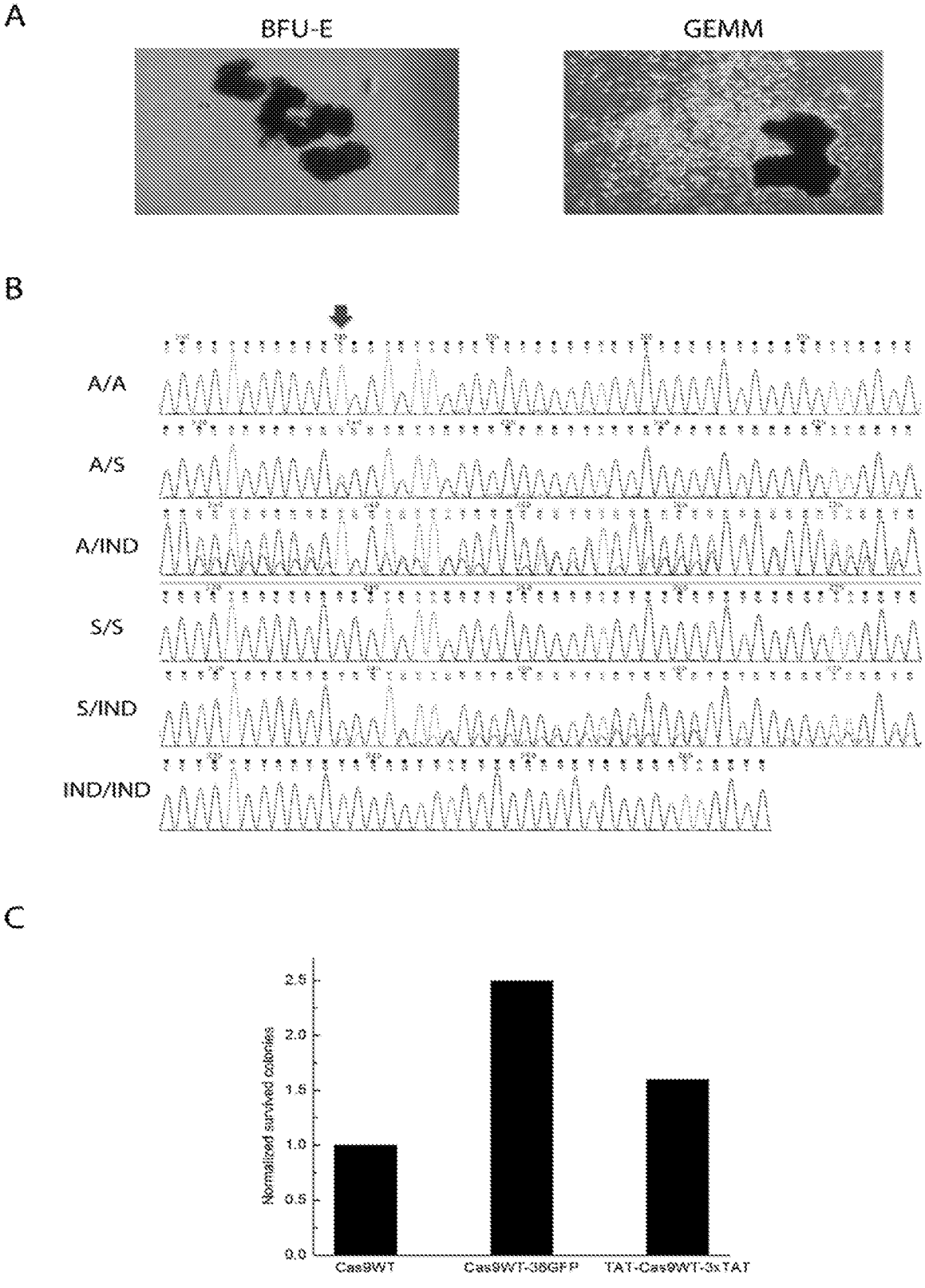

A
BFU-E
GEMM
B
A/A
A/S
A/IND
S/S
S/IND
IND/IND
C
Normalized survived colonies
2.5
2.0
1.5
1.0
0.5
0.0
Cas9WT
Cas9WT-3BGFP
TAT-Cas9WT-3xTAT

FIGURE 23

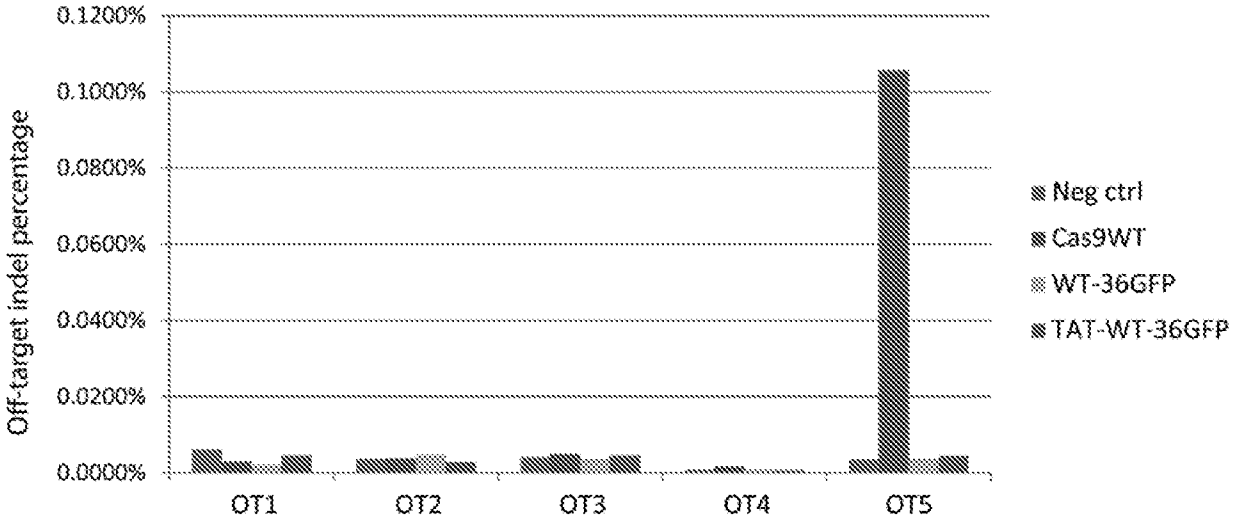

0.1200%
0.1000%
0.0800%
0.0600%
0.0400%
0.0200%
0.0000%
Off-target indel percentage
OT1
OT2
OT3
OT4
OT5
Neg ctrl
Cas9WT
WT-36GFP
TAT-WT-36GFP

FIGURE 24

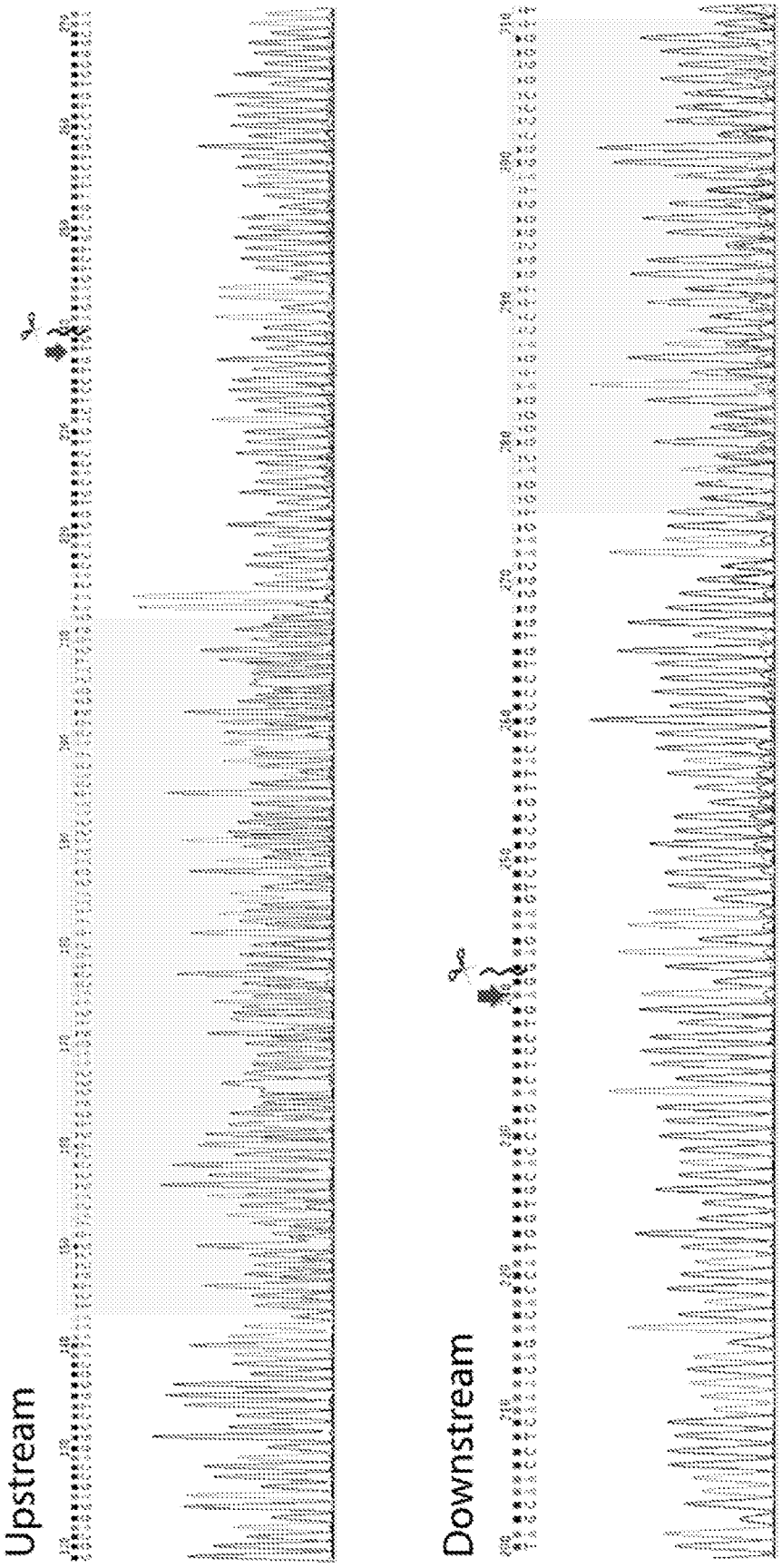
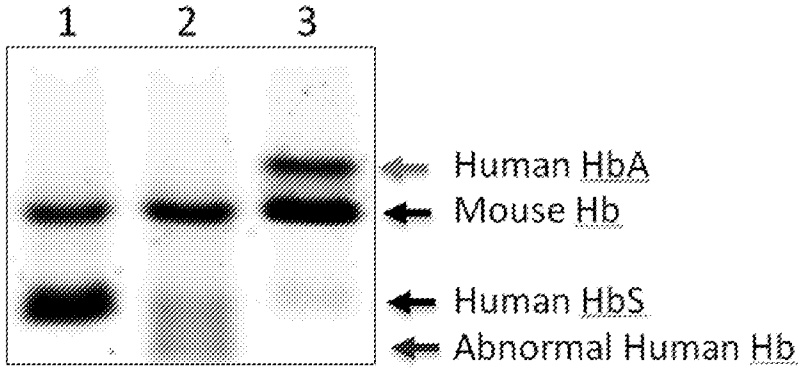


FIGURE 25

Sickle mouse HSCs transplanted into irradiated C57Bl/6 mice



1. Non-nucleoporation control followed by transplant
2. Cas9WT RNP +WB-ssODN nucleoporation followed by transplant
3. mrS-Cas9 RNP + WB-ssODN nucleoporation followed by transplant

FIGURE 26

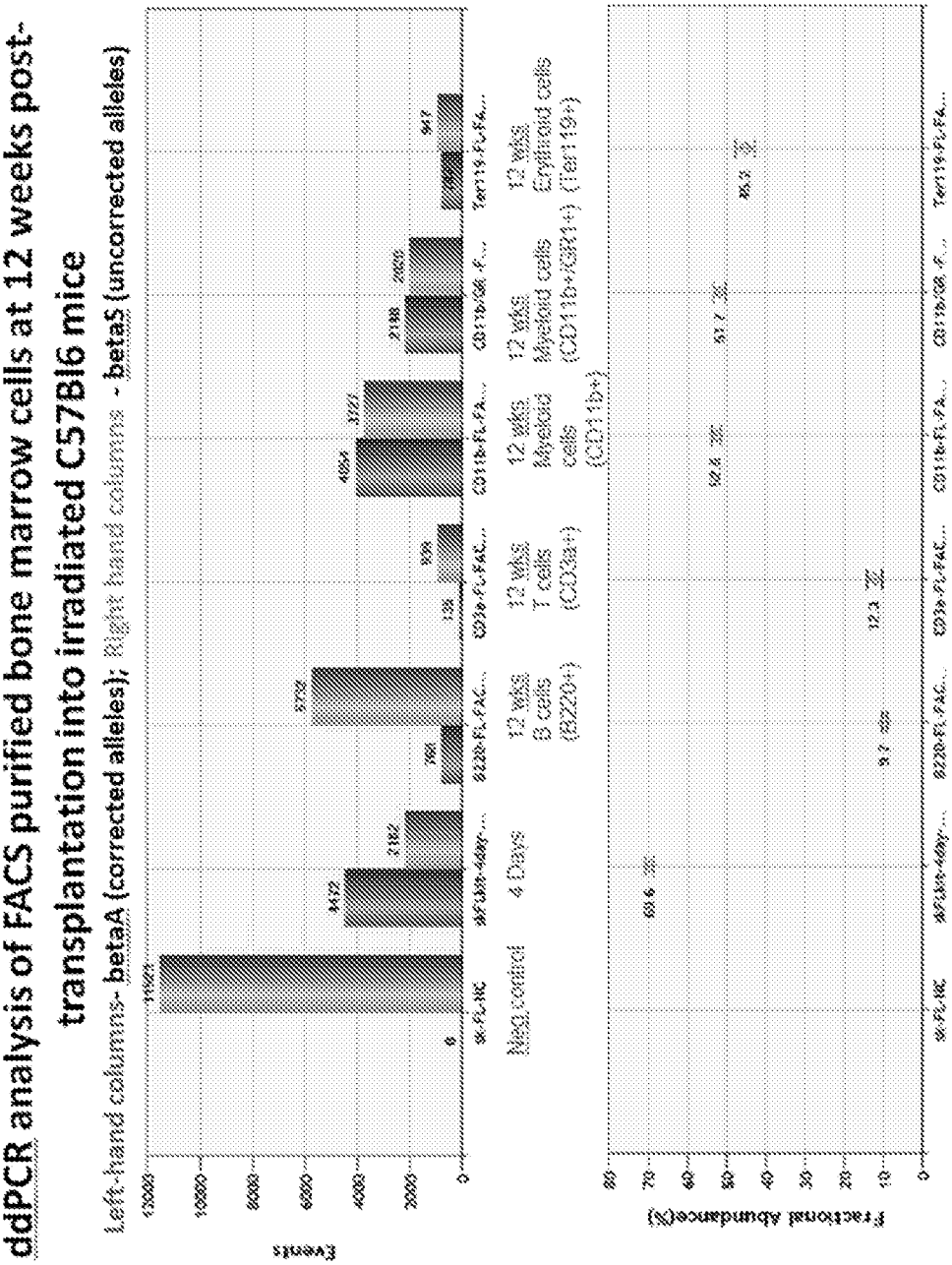
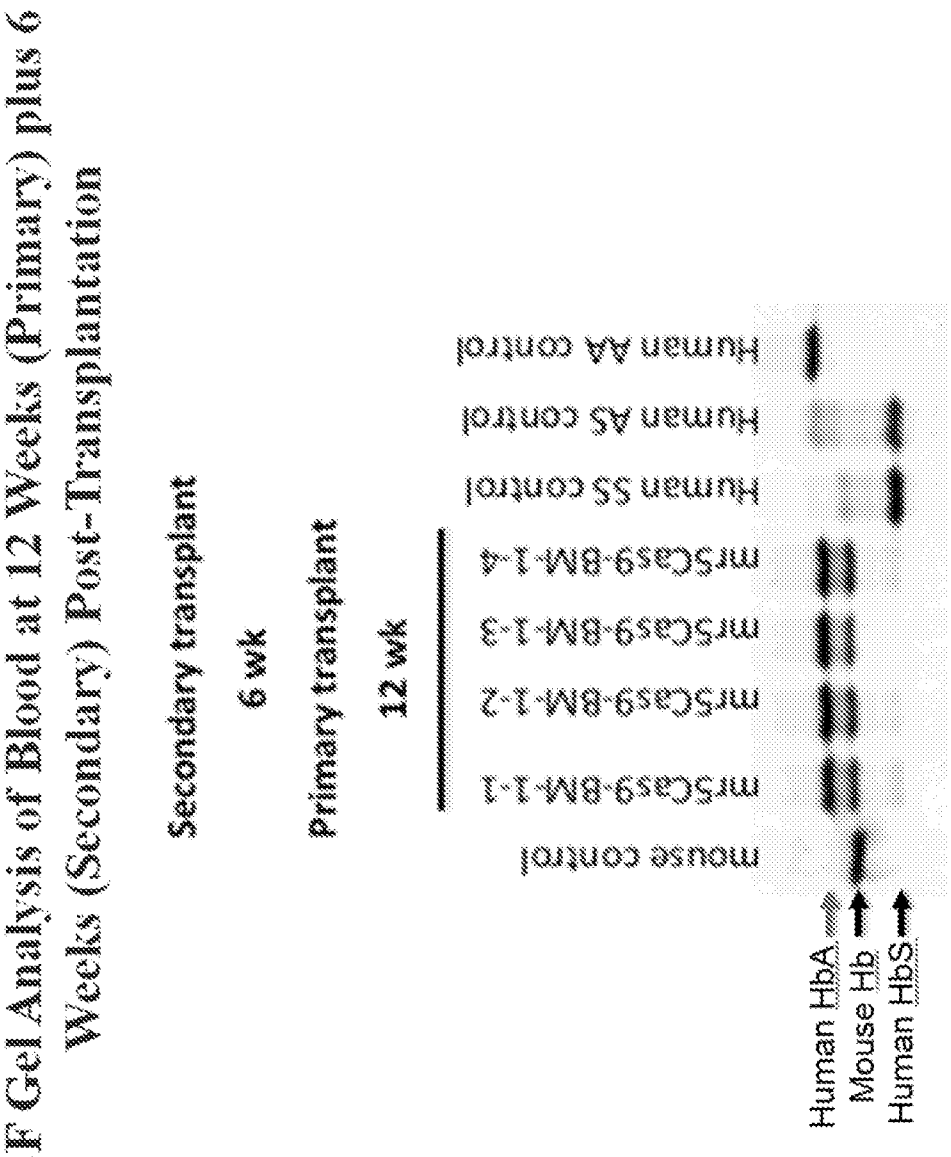

ddPCR analysis of FACS purified bone marrow cells at 12 weeks post-transplantation into irradiated C57Bl6 mice
Left-hand columns- betaA (corrected alleles); Right hand columns - betaS (uncorrected alleles)
Events
Fractional Abundance(%)
Neg control
4 Days
12 wks B cells (B220+)
12 wks T cells (CD3e+)
12 wks Myeloid cells (CD11b+)
12 wks Myeloid cells (CD11b+/GR1+)
12 wks Erythroid cells (Ter119+)

FIGURE 27


IEF Gel Analysis of Blood at 12 Weeks (Primary) plus 6 Weeks (Secondary) Post-Transplantation
Secondary transplant
6 wk
Primary transplant
12 wk
mouse control
mr5Cas9-8M-1-1
mr5Cas9-8M-1-2
mr5Cas9-8M-1-3
mr5Cas9-8M-1-4
human SS control
human AS control
human AA control
Human HbA
Mouse Hb
Human HbS

SEQUENCE LISTING

<110> UAB Research Foundation
Townes, Tim
Ding, Lei
Chang, Chia-Wei

<120> CRISPR/CAS9 COMPLEX FOR GENOMIC EDITING

<130> 035979-1014678 (177WO1)

<150> US 62/181,138
<151> 2015-06-17

<150> US 62/266,316
<151> 2015-12-11

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<170> PatentIn version 3.5

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<212> DNA
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<220>
<223> Synthetic construct

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taacggcaga cttctccac 19

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<220>
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cgttatcaac ttgaaaaagt ggcaccgagt cggtgctttt ttt 103

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Met Asp Tyr Lys Asp His Asp Gly Asp Tyr Lys Asp His Asp Ile Asp
1 5 10 15

Tyr Lys Asp Asp Asp Asp Lys Met Ala Pro Lys Lys Lys Arg Lys Val
20 25 30

```
Gly Ile His Gly Val Pro Ala Ala Asp Lys Lys Tyr Ser Ile Gly Leu
        35                  40                  45

Asp Ile Gly Thr Asn Ser Val Gly Trp Ala Val Ile Thr Asp Glu Tyr
    50                  55                  60

Lys Val Pro Ser Lys Lys Phe Lys Val Leu Gly Asn Thr Asp Arg His
65                  70                  75                  80

Ser Ile Lys Lys Asn Leu Ile Gly Ala Leu Leu Phe Asp Ser Gly Glu
                85                  90                  95

Thr Ala Glu Ala Thr Arg Leu Lys Arg Thr Ala Arg Arg Arg Tyr Thr
            100                 105                 110

Arg Arg Lys Asn Arg Ile Cys Tyr Leu Gln Glu Ile Phe Ser Asn Glu
        115                 120                 125

Met Ala Lys Val Asp Asp Ser Phe Phe His Arg Leu Glu Glu Ser Phe
    130                 135                 140

Leu Val Glu Glu Asp Lys Lys His Glu Arg His Pro Ile Phe Gly Asn
145                 150                 155                 160

Ile Val Asp Glu Val Ala Tyr His Glu Lys Tyr Pro Thr Ile Tyr His
                165                 170                 175

Leu Arg Lys Lys Leu Val Asp Ser Thr Asp Lys Ala Asp Leu Arg Leu
            180                 185                 190

Ile Tyr Leu Ala Leu Ala His Met Ile Lys Phe Arg Gly His Phe Leu
        195                 200                 205

Ile Glu Gly Asp Leu Asn Pro Asp Asn Ser Asp Val Asp Lys Leu Phe
    210                 215                 220

Ile Gln Leu Val Gln Thr Tyr Asn Gln Leu Phe Glu Glu Asn Pro Ile
225                 230                 235                 240

Asn Ala Ser Gly Val Asp Ala Lys Ala Ile Leu Ser Ala Arg Leu Ser
                245                 250                 255

Lys Ser Arg Arg Leu Glu Asn Leu Ile Ala Gln Leu Pro Gly Glu Lys
            260                 265                 270

Lys Asn Gly Leu Phe Gly Asn Leu Ile Ala Leu Ser Leu Gly Leu Thr
```

```
        275                 280                 285

Pro Asn Phe Lys Ser Asn Phe Asp Leu Ala Glu Asp Ala Lys Leu Gln
    290                 295                 300

Leu Ser Lys Asp Thr Tyr Asp Asp Asp Leu Asp Asn Leu Leu Ala Gln
305                 310                 315                 320

Ile Gly Asp Gln Tyr Ala Asp Leu Phe Leu Ala Ala Lys Asn Leu Ser
                325                 330                 335

Asp Ala Ile Leu Leu Ser Asp Ile Leu Arg Val Asn Thr Glu Ile Thr
            340                 345                 350

Lys Ala Pro Leu Ser Ala Ser Met Ile Lys Arg Tyr Asp Glu His His
        355                 360                 365

Gln Asp Leu Thr Leu Leu Lys Ala Leu Val Arg Gln Gln Leu Pro Glu
    370                 375                 380

Lys Tyr Lys Glu Ile Phe Phe Asp Gln Ser Lys Asn Gly Tyr Ala Gly
385                 390                 395                 400

Tyr Ile Asp Gly Gly Ala Ser Gln Glu Glu Phe Tyr Lys Phe Ile Lys
                405                 410                 415

Pro Ile Leu Glu Lys Met Asp Gly Thr Glu Glu Leu Leu Val Lys Leu
            420                 425                 430

Asn Arg Glu Asp Leu Leu Arg Lys Gln Arg Thr Phe Asp Asn Gly Ser
        435                 440                 445

Ile Pro His Gln Ile His Leu Gly Glu Leu His Ala Ile Leu Arg Arg
    450                 455                 460

Gln Glu Asp Phe Tyr Pro Phe Leu Lys Asp Asn Arg Glu Lys Ile Glu
465                 470                 475                 480

Lys Ile Leu Thr Phe Arg Ile Pro Tyr Tyr Val Gly Pro Leu Ala Arg
                485                 490                 495

Gly Asn Ser Arg Phe Ala Trp Met Thr Arg Lys Ser Glu Glu Thr Ile
            500                 505                 510

Thr Pro Trp Asn Phe Glu Glu Val Val Asp Lys Gly Ala Ser Ala Gln
        515                 520                 525
```

```
Ser Phe Ile Glu Arg Met Thr Asn Phe Asp Lys Asn Leu Pro Asn Glu
    530                 535                 540

Lys Val Leu Pro Lys His Ser Leu Leu Tyr Glu Tyr Phe Thr Val Tyr
545                 550                 555                 560

Asn Glu Leu Thr Lys Val Lys Tyr Val Thr Glu Gly Met Arg Lys Pro
                565                 570                 575

Ala Phe Leu Ser Gly Glu Gln Lys Lys Ala Ile Val Asp Leu Leu Phe
            580                 585                 590

Lys Thr Asn Arg Lys Val Thr Val Lys Gln Leu Lys Glu Asp Tyr Phe
        595                 600                 605

Lys Lys Ile Glu Cys Phe Asp Ser Val Glu Ile Ser Gly Val Glu Asp
    610                 615                 620

Arg Phe Asn Ala Ser Leu Gly Thr Tyr His Asp Leu Leu Lys Ile Ile
625                 630                 635                 640

Lys Asp Lys Asp Phe Leu Asp Asn Glu Glu Asn Glu Asp Ile Leu Glu
                645                 650                 655

Asp Ile Val Leu Thr Leu Thr Leu Phe Glu Asp Arg Glu Met Ile Glu
            660                 665                 670

Glu Arg Leu Lys Thr Tyr Ala His Leu Phe Asp Asp Lys Val Met Lys
        675                 680                 685

Gln Leu Lys Arg Arg Arg Tyr Thr Gly Trp Gly Arg Leu Ser Arg Lys
    690                 695                 700

Leu Ile Asn Gly Ile Arg Asp Lys Gln Ser Gly Lys Thr Ile Leu Asp
705                 710                 715                 720

Phe Leu Lys Ser Asp Gly Phe Ala Asn Arg Asn Phe Met Gln Leu Ile
                725                 730                 735

His Asp Asp Ser Leu Thr Phe Lys Glu Asp Ile Gln Lys Ala Gln Val
            740                 745                 750

Ser Gly Gln Gly Asp Ser Leu His Glu His Ile Ala Asn Leu Ala Gly
        755                 760                 765

Ser Pro Ala Ile Lys Lys Gly Ile Leu Gln Thr Val Lys Val Val Asp
    770                 775                 780
```

```
Glu Leu Val Lys Val Met Gly Arg His Lys Pro Glu Asn Ile Val Ile
785                 790                 795                 800

Glu Met Ala Arg Glu Asn Gln Thr Thr Gln Lys Gly Gln Lys Asn Ser
                805                 810                 815

Arg Glu Arg Met Lys Arg Ile Glu Glu Gly Ile Lys Glu Leu Gly Ser
            820                 825                 830

Gln Ile Leu Lys Glu His Pro Val Glu Asn Thr Gln Leu Gln Asn Glu
        835                 840                 845

Lys Leu Tyr Leu Tyr Tyr Leu Gln Asn Gly Arg Asp Met Tyr Val Asp
    850                 855                 860

Gln Glu Leu Asp Ile Asn Arg Leu Ser Asp Tyr Asp Val Asp His Ile
865                 870                 875                 880

Val Pro Gln Ser Phe Leu Lys Asp Asp Ser Ile Asp Asn Lys Val Leu
                885                 890                 895

Thr Arg Ser Asp Lys Asn Arg Gly Lys Ser Asp Asn Val Pro Ser Glu
            900                 905                 910

Glu Val Val Lys Lys Met Lys Asn Tyr Trp Arg Gln Leu Leu Asn Ala
        915                 920                 925

Lys Leu Ile Thr Gln Arg Lys Phe Asp Asn Leu Thr Lys Ala Glu Arg
    930                 935                 940

Gly Gly Leu Ser Glu Leu Asp Lys Ala Gly Phe Ile Lys Arg Gln Leu
945                 950                 955                 960

Val Glu Thr Arg Gln Ile Thr Lys His Val Ala Gln Ile Leu Asp Ser
                965                 970                 975

Arg Met Asn Thr Lys Tyr Asp Glu Asn Asp Lys Leu Ile Arg Glu Val
            980                 985                 990

Lys Val Ile Thr Leu Lys Ser Lys  Leu Val Ser Asp Phe  Arg Lys Asp
        995                 1000                1005

Phe Gln  Phe Tyr Lys Val Arg  Glu Ile Asn Asn Tyr  His His Ala
    1010                1015                1020

His Asp  Ala Tyr Leu Asn Ala  Val Val Gly Thr Ala  Leu Ile Lys
    1025                1030                1035
```

```
Lys Tyr  Pro Lys Leu Glu Ser  Glu Phe Val Tyr Gly  Asp Tyr Lys
    1040                1045                1050

Val Tyr  Asp Val Arg Lys Met  Ile Ala Lys Ser Glu  Gln Glu Ile
    1055                1060                1065

Gly Lys  Ala Thr Ala Lys Tyr  Phe Phe Tyr Ser Asn  Ile Met Asn
    1070                1075                1080

Phe Phe  Lys Thr Glu Ile Thr  Leu Ala Asn Gly Glu  Ile Arg Lys
    1085                1090                1095

Arg Pro  Leu Ile Glu Thr Asn  Gly Glu Thr Gly Glu  Ile Val Trp
    1100                1105                1110

Asp Lys  Gly Arg Asp Phe Ala  Thr Val Arg Lys Val  Leu Ser Met
    1115                1120                1125

Pro Gln  Val Asn Ile Val Lys  Lys Thr Glu Val Gln  Thr Gly Gly
    1130                1135                1140

Phe Ser  Lys Glu Ser Ile Leu  Pro Lys Arg Asn Ser  Asp Lys Leu
    1145                1150                1155

Ile Ala  Arg Lys Lys Asp Trp  Asp Pro Lys Lys Tyr  Gly Gly Phe
    1160                1165                1170

Asp Ser  Pro Thr Val Ala Tyr  Ser Val Leu Val Val  Ala Lys Val
    1175                1180                1185

Glu Lys  Gly Lys Ser Lys Lys  Leu Lys Ser Val Lys  Glu Leu Leu
    1190                1195                1200

Gly Ile  Thr Ile Met Glu Arg  Ser Ser Phe Glu Lys  Asn Pro Ile
    1205                1210                1215

Asp Phe  Leu Glu Ala Lys Gly  Tyr Lys Glu Val Lys  Lys Asp Leu
    1220                1225                1230

Ile Ile  Lys Leu Pro Lys Tyr  Ser Leu Phe Glu Leu  Glu Asn Gly
    1235                1240                1245

Arg Lys  Arg Met Leu Ala Ser  Ala Gly Glu Leu Gln  Lys Gly Asn
    1250                1255                1260

Glu Leu  Ala Leu Pro Ser Lys  Tyr Val Asn Phe Leu  Tyr Leu Ala
```

```
    1265                1270                1275

Ser His  Tyr Glu Lys Leu Lys  Gly Ser Pro Glu Asp  Asn Glu Gln
    1280                1285                1290

Lys Gln  Leu Phe Val Glu Gln  His Lys His Tyr Leu  Asp Glu Ile
    1295                1300                1305

Ile Glu  Gln Ile Ser Glu Phe  Ser Lys Arg Val Ile  Leu Ala Asp
    1310                1315                1320

Ala Asn  Leu Asp Lys Val Leu  Ser Ala Tyr Asn Lys  His Arg Asp
    1325                1330                1335

Lys Pro  Ile Arg Glu Gln Ala  Glu Asn Ile Ile His  Leu Phe Thr
    1340                1345                1350

Leu Thr  Asn Leu Gly Ala Pro  Ala Ala Phe Lys Tyr  Phe Asp Thr
    1355                1360                1365

Thr Ile  Asp Arg Lys Arg Tyr  Thr Ser Thr Lys Glu  Val Leu Asp
    1370                1375                1380

Ala Thr  Leu Ile His Gln Ser  Ile Thr Gly Leu Tyr  Glu Thr Arg
    1385                1390                1395

Ile Asp  Leu Ser Gln Leu Gly  Gly Asp Lys Arg Pro  Ala Ala Thr
    1400                1405                1410

Lys Lys  Ala Gly Gln Ala Lys  Lys Lys Lys Gly Ser  Gly Ser Asn
    1415                1420                1425

Gly Ser  Ser Gly Ser Ala Ser  Lys Gly Glu Arg Leu  Phe Arg Gly
    1430                1435                1440

Lys Val  Pro Ile Leu Val Glu  Leu Lys Gly Asp Val  Asn Gly His
    1445                1450                1455

Lys Phe  Ser Val Arg Gly Lys  Gly Lys Gly Asp Ala  Thr Arg Gly
    1460                1465                1470

Lys Leu  Thr Leu Lys Phe Ile  Cys Thr Thr Gly Lys  Leu Pro Val
    1475                1480                1485

Pro Trp  Pro Thr Leu Val Thr  Thr Leu Thr Tyr Gly  Val Gln Cys
    1490                1495                1500
```

```
Phe Ser  Arg Tyr Pro Lys His  Met Lys Arg His Asp  Phe Phe Lys
    1505                1510                1515

Ser Ala  Met Pro Lys Gly Tyr  Val Gln Glu Arg Thr  Ile Ser Phe
    1520                1525                1530

Lys Lys  Asp Gly Lys Tyr Lys  Thr Arg Ala Glu Val  Lys Phe Glu
    1535                1540                1545

Gly Arg  Thr Leu Val Asn Arg  Ile Lys Leu Lys Gly  Arg Asp Phe
    1550                1555                1560

Lys Glu  Lys Gly Asn Ile Leu  Gly His Lys Leu Arg  Tyr Asn Phe
    1565                1570                1575

Asn Ser  His Lys Val Tyr Ile  Thr Ala Asp Lys Arg  Lys Asn Gly
    1580                1585                1590

Ile Lys  Ala Lys Phe Lys Ile  Arg His Asn Val Lys  Asp Gly Ser
    1595                1600                1605

Val Gln  Leu Ala Asp His Tyr  Gln Gln Asn Thr Pro  Ile Gly Arg
    1610                1615                1620

Gly Pro  Val Leu Leu Pro Arg  Asn His Tyr Leu Ser  Thr Arg Ser
    1625                1630                1635

Lys Leu  Ser Lys Asp Pro Lys  Glu Lys Arg Asp His  Met Val Leu
    1640                1645                1650

Leu Glu  Phe Val Thr Ala Ala  Gly Ile Lys His Gly  Arg Asp Glu
    1655                1660                1665

Arg Tyr  Lys
    1670
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<211> 1720
<212> PRT
<213> Artificial sequence

<220>
<223> Synthetic construct

<400> 4

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Tyr Gly Arg Lys Lys Arg Arg Gln Arg Arg Arg Pro Pro Gln Ala Gly
1               5                  10                  15

Gly Ser Met Asp Tyr Lys Asp His Asp Gly Asp Tyr Lys Asp His Asp
```

20 25 30

Ile Asp Tyr Lys Asp Asp Asp Asp Lys Met Ala Pro Lys Lys Lys Arg
35 40 45

Lys Val Gly Ile His Gly Val Pro Ala Ala Asp Lys Lys Tyr Ser Ile
50 55 60

Gly Leu Asp Ile Gly Thr Asn Ser Val Gly Trp Ala Val Ile Thr Asp
65 70 75 80

Glu Tyr Lys Val Pro Ser Lys Lys Phe Lys Val Leu Gly Asn Thr Asp
85 90 95

Arg His Ser Ile Lys Lys Asn Leu Ile Gly Ala Leu Leu Phe Asp Ser
100 105 110

Gly Glu Thr Ala Glu Ala Thr Arg Leu Lys Arg Thr Ala Arg Arg Arg
115 120 125

Tyr Thr Arg Arg Lys Asn Arg Ile Cys Tyr Leu Gln Glu Ile Phe Ser
130 135 140

Asn Glu Met Ala Lys Val Asp Asp Ser Phe Phe His Arg Leu Glu Glu
145 150 155 160

Ser Phe Leu Val Glu Glu Asp Lys Lys His Glu Arg His Pro Ile Phe
165 170 175

Gly Asn Ile Val Asp Glu Val Ala Tyr His Glu Lys Tyr Pro Thr Ile
180 185 190

Tyr His Leu Arg Lys Lys Leu Val Asp Ser Thr Asp Lys Ala Asp Leu
195 200 205

Arg Leu Ile Tyr Leu Ala Leu Ala His Met Ile Lys Phe Arg Gly His
210 215 220

Phe Leu Ile Glu Gly Asp Leu Asn Pro Asp Asn Ser Asp Val Asp Lys
225 230 235 240

Leu Phe Ile Gln Leu Val Gln Thr Tyr Asn Gln Leu Phe Glu Glu Asn
245 250 255

Pro Ile Asn Ala Ser Gly Val Asp Ala Lys Ala Ile Leu Ser Ala Arg
260 265 270

```
Leu Ser Lys Ser Arg Arg Leu Glu Asn Leu Ile Ala Gln Leu Pro Gly
        275                 280                 285

Glu Lys Lys Asn Gly Leu Phe Gly Asn Leu Ile Ala Leu Ser Leu Gly
    290                 295                 300

Leu Thr Pro Asn Phe Lys Ser Asn Phe Asp Leu Ala Glu Asp Ala Lys
305                 310                 315                 320

Leu Gln Leu Ser Lys Asp Thr Tyr Asp Asp Asp Leu Asp Asn Leu Leu
                325                 330                 335

Ala Gln Ile Gly Asp Gln Tyr Ala Asp Leu Phe Leu Ala Ala Lys Asn
            340                 345                 350

Leu Ser Asp Ala Ile Leu Leu Ser Asp Ile Leu Arg Val Asn Thr Glu
        355                 360                 365

Ile Thr Lys Ala Pro Leu Ser Ala Ser Met Ile Lys Arg Tyr Asp Glu
    370                 375                 380

His His Gln Asp Leu Thr Leu Leu Lys Ala Leu Val Arg Gln Gln Leu
385                 390                 395                 400

Pro Glu Lys Tyr Lys Glu Ile Phe Phe Asp Gln Ser Lys Asn Gly Tyr
                405                 410                 415

Ala Gly Tyr Ile Asp Gly Gly Ala Ser Gln Glu Glu Phe Tyr Lys Phe
            420                 425                 430

Ile Lys Pro Ile Leu Glu Lys Met Asp Gly Thr Glu Glu Leu Leu Val
        435                 440                 445

Lys Leu Asn Arg Glu Asp Leu Leu Arg Lys Gln Arg Thr Phe Asp Asn
    450                 455                 460

Gly Ser Ile Pro His Gln Ile His Leu Gly Glu Leu His Ala Ile Leu
465                 470                 475                 480

Arg Arg Gln Glu Asp Phe Tyr Pro Phe Leu Lys Asp Asn Arg Glu Lys
                485                 490                 495

Ile Glu Lys Ile Leu Thr Phe Arg Ile Pro Tyr Tyr Val Gly Pro Leu
            500                 505                 510

Ala Arg Gly Asn Ser Arg Phe Ala Trp Met Thr Arg Lys Ser Glu Glu
        515                 520                 525
```

```
Thr Ile Thr Pro Trp Asn Phe Glu Glu Val Val Asp Lys Gly Ala Ser
    530                 535                 540

Ala Gln Ser Phe Ile Glu Arg Met Thr Asn Phe Asp Lys Asn Leu Pro
545                 550                 555                 560

Asn Glu Lys Val Leu Pro Lys His Ser Leu Leu Tyr Glu Tyr Phe Thr
                565                 570                 575

Val Tyr Asn Glu Leu Thr Lys Val Lys Tyr Val Thr Glu Gly Met Arg
            580                 585                 590

Lys Pro Ala Phe Leu Ser Gly Glu Gln Lys Lys Ala Ile Val Asp Leu
        595                 600                 605

Leu Phe Lys Thr Asn Arg Lys Val Thr Val Lys Gln Leu Lys Glu Asp
    610                 615                 620

Tyr Phe Lys Lys Ile Glu Cys Phe Asp Ser Val Glu Ile Ser Gly Val
625                 630                 635                 640

Glu Asp Arg Phe Asn Ala Ser Leu Gly Thr Tyr His Asp Leu Leu Lys
                645                 650                 655

Ile Ile Lys Asp Lys Asp Phe Leu Asp Asn Glu Glu Asn Glu Asp Ile
            660                 665                 670

Leu Glu Asp Ile Val Leu Thr Leu Thr Leu Phe Glu Asp Arg Glu Met
        675                 680                 685

Ile Glu Glu Arg Leu Lys Thr Tyr Ala His Leu Phe Asp Asp Lys Val
    690                 695                 700

Met Lys Gln Leu Lys Arg Arg Arg Tyr Thr Gly Trp Gly Arg Leu Ser
705                 710                 715                 720

Arg Lys Leu Ile Asn Gly Ile Arg Asp Lys Gln Ser Gly Lys Thr Ile
                725                 730                 735

Leu Asp Phe Leu Lys Ser Asp Gly Phe Ala Asn Arg Asn Phe Met Gln
            740                 745                 750

Leu Ile His Asp Asp Ser Leu Thr Phe Lys Glu Asp Ile Gln Lys Ala
        755                 760                 765

Gln Val Ser Gly Gln Gly Asp Ser Leu His Glu His Ile Ala Asn Leu
    770                 775                 780
```

```
Ala Gly Ser Pro Ala Ile Lys Lys Gly Ile Leu Gln Thr Val Lys Val
785                 790                 795                 800

Val Asp Glu Leu Val Lys Val Met Gly Arg His Lys Pro Glu Asn Ile
                805                 810                 815

Val Ile Glu Met Ala Arg Glu Asn Gln Thr Thr Gln Lys Gly Gln Lys
            820                 825                 830

Asn Ser Arg Glu Arg Met Lys Arg Ile Glu Glu Gly Ile Lys Glu Leu
        835                 840                 845

Gly Ser Gln Ile Leu Lys Glu His Pro Val Glu Asn Thr Gln Leu Gln
    850                 855                 860

Asn Glu Lys Leu Tyr Leu Tyr Tyr Leu Gln Asn Gly Arg Asp Met Tyr
865                 870                 875                 880

Val Asp Gln Glu Leu Asp Ile Asn Arg Leu Ser Asp Tyr Asp Val Asp
                885                 890                 895

His Ile Val Pro Gln Ser Phe Leu Lys Asp Asp Ser Ile Asp Asn Lys
            900                 905                 910

Val Leu Thr Arg Ser Asp Lys Asn Arg Gly Lys Ser Asp Asn Val Pro
        915                 920                 925

Ser Glu Glu Val Val Lys Lys Met Lys Asn Tyr Trp Arg Gln Leu Leu
    930                 935                 940

Asn Ala Lys Leu Ile Thr Gln Arg Lys Phe Asp Asn Leu Thr Lys Ala
945                 950                 955                 960

Glu Arg Gly Gly Leu Ser Glu Leu Asp Lys Ala Gly Phe Ile Lys Arg
                965                 970                 975

Gln Leu Val Glu Thr Arg Gln Ile Thr Lys His Val Ala Gln Ile Leu
            980                 985                 990

Asp Ser Arg Met Asn Thr Lys Tyr  Asp Glu Asn Asp Lys  Leu Ile Arg
        995                 1000                1005

Glu Val  Lys Val Ile Thr Leu  Lys Ser Lys Leu Val  Ser Asp Phe
    1010                1015                1020

Arg Lys  Asp Phe Gln Phe Tyr  Lys Val Arg Glu Ile  Asn Asn Tyr
```

1025 1030 1035

His His Ala His Asp Ala Tyr Leu Asn Ala Val Val Gly Thr Ala
1040 1045 1050

Leu Ile Lys Lys Tyr Pro Lys Leu Glu Ser Glu Phe Val Tyr Gly
1055 1060 1065

Asp Tyr Lys Val Tyr Asp Val Arg Lys Met Ile Ala Lys Ser Glu
1070 1075 1080

Gln Glu Ile Gly Lys Ala Thr Ala Lys Tyr Phe Phe Tyr Ser Asn
1085 1090 1095

Ile Met Asn Phe Phe Lys Thr Glu Ile Thr Leu Ala Asn Gly Glu
1100 1105 1110

Ile Arg Lys Arg Pro Leu Ile Glu Thr Asn Gly Glu Thr Gly Glu
1115 1120 1125

Ile Val Trp Asp Lys Gly Arg Asp Phe Ala Thr Val Arg Lys Val
1130 1135 1140

Leu Ser Met Pro Gln Val Asn Ile Val Lys Lys Thr Glu Val Gln
1145 1150 1155

Thr Gly Gly Phe Ser Lys Glu Ser Ile Leu Pro Lys Arg Asn Ser
1160 1165 1170

Asp Lys Leu Ile Ala Arg Lys Lys Asp Trp Asp Pro Lys Lys Tyr
1175 1180 1185

Gly Gly Phe Asp Ser Pro Thr Val Ala Tyr Ser Val Leu Val Val
1190 1195 1200

Ala Lys Val Glu Lys Gly Lys Ser Lys Lys Leu Lys Ser Val Lys
1205 1210 1215

Glu Leu Leu Gly Ile Thr Ile Met Glu Arg Ser Ser Phe Glu Lys
1220 1225 1230

Asn Pro Ile Asp Phe Leu Glu Ala Lys Gly Tyr Lys Glu Val Lys
1235 1240 1245

Lys Asp Leu Ile Ile Lys Leu Pro Lys Tyr Ser Leu Phe Glu Leu
1250 1255 1260

```
Glu Asn  Gly Arg Lys Arg Met  Leu Ala Ser Ala Gly  Glu Leu Gln
    1265                1270                1275

Lys Gly  Asn Glu Leu Ala Leu  Pro Ser Lys Tyr Val  Asn Phe Leu
    1280                1285                1290

Tyr Leu  Ala Ser His Tyr Glu  Lys Leu Lys Gly Ser  Pro Glu Asp
    1295                1300                1305

Asn Glu  Gln Lys Gln Leu Phe  Val Glu Gln His Lys  His Tyr Leu
    1310                1315                1320

Asp Glu  Ile Ile Glu Gln Ile  Ser Glu Phe Ser Lys  Arg Val Ile
    1325                1330                1335

Leu Ala  Asp Ala Asn Leu Asp  Lys Val Leu Ser Ala  Tyr Asn Lys
    1340                1345                1350

His Arg  Asp Lys Pro Ile Arg  Glu Gln Ala Glu Asn  Ile Ile His
    1355                1360                1365

Leu Phe  Thr Leu Thr Asn Leu  Gly Ala Pro Ala Ala  Phe Lys Tyr
    1370                1375                1380

Phe Asp  Thr Thr Ile Asp Arg  Lys Arg Tyr Thr Ser  Thr Lys Glu
    1385                1390                1395

Val Leu  Asp Ala Thr Leu Ile  His Gln Ser Ile Thr  Gly Leu Tyr
    1400                1405                1410

Glu Thr  Arg Ile Asp Leu Ser  Gln Leu Gly Gly Asp  Lys Arg Pro
    1415                1420                1425

Ala Ala  Thr Lys Lys Ala Gly  Gln Ala Lys Lys Lys  Lys Gly Ser
    1430                1435                1440

Gly Ser  Asn Gly Ser Ser Gly  Ser Ala Ser Lys Gly  Glu Arg Leu
    1445                1450                1455

Phe Arg  Gly Lys Val Pro Ile  Leu Val Glu Leu Lys  Gly Asp Val
    1460                1465                1470

Asn Gly  His Lys Phe Ser Val  Arg Gly Lys Gly Lys  Gly Asp Ala
    1475                1480                1485

Thr Arg  Gly Lys Leu Thr Leu  Lys Phe Ile Cys Thr  Thr Gly Lys
    1490                1495                1500
```

```
Leu Pro  Val Pro Trp Pro Thr  Leu Val Thr Thr Leu  Thr Tyr Gly
    1505                1510                1515

Val Gln  Cys Phe Ser Arg Tyr  Pro Lys His Met Lys  Arg His Asp
    1520                1525                1530

Phe Phe  Lys Ser Ala Met Pro  Lys Gly Tyr Val Gln  Glu Arg Thr
    1535                1540                1545

Ile Ser  Phe Lys Lys Asp Gly  Lys Tyr Lys Thr Arg  Ala Glu Val
    1550                1555                1560

Lys Phe  Glu Gly Arg Thr Leu  Val Asn Arg Ile Lys  Leu Lys Gly
    1565                1570                1575

Arg Asp  Phe Lys Glu Lys Gly  Asn Ile Leu Gly His  Lys Leu Arg
    1580                1585                1590

Tyr Asn  Phe Asn Ser His Lys  Val Tyr Ile Thr Ala  Asp Lys Arg
    1595                1600                1605

Lys Asn  Gly Ile Lys Ala Lys  Phe Lys Ile Arg His  Asn Val Lys
    1610                1615                1620

Asp Gly  Ser Val Gln Leu Ala  Asp His Tyr Gln Gln  Asn Thr Pro
    1625                1630                1635

Ile Gly  Arg Gly Pro Val Leu  Leu Pro Arg Asn His  Tyr Leu Ser
    1640                1645                1650

Thr Arg  Ser Lys Leu Ser Lys  Asp Pro Lys Glu Lys  Arg Asp His
    1655                1660                1665

Met Val  Leu Leu Glu Phe Val  Thr Ala Ala Gly Ile  Lys His Gly
    1670                1675                1680

Arg Asp  Glu Arg Tyr Lys Gly  Gly Ser Gly Gly Ser  Val Asp Gly
    1685                1690                1695

Leu Phe  Glu Ala Ile Glu Gly  Phe Ile Glu Asn Gly  Trp Glu Gly
    1700                1705                1710

Met Ile  Asp Gly Trp Tyr Gly
    1715                1720
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<210> 6
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<400> 6
cttcagcaag ccgagtcctg 20

<210> 7
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<220>
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<211> 49
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<211> 60
<212> DNA
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<220>
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ttaattaatt aattagcatt ttaggttcag gttgtgagaa cactagaaga gaacaagtca 60

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gtatacgtat acgcatacct ggagagggga caaggtcttg agatgcgagg gt 52

<210> 11
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<220>
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<400> 11
agccacctta attaagccac catggcgcac gctgggagaa cggggtacga ta 52

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taacagagag aagttcgtgg ctccggatcc cttgtggccc agataggcac ccagggtgat 60

<210> 13
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<400> 13
caccgtgaga tacagataca gaca 24

<210> 14
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<220>
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<400> 14
aaactgtctg tatctgtatc tcac 24

<210> 15
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<212> DNA
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<220>
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<400> 15
aaacggcatt ccaggcaaat cattc 25

<210> 16
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<212> DNA
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caccgcagcc taggcaaagg cctgc 25

<210> 17
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<220>
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<400> 17
aaacgcaggc ctttgcctag gctgc 25

<210> 18
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caccgtgcca acagaactgc ctgat 25

<210> 19
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<400> 19
aaacatcagg cagttctgtt ggcac 25

<210> 20
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<220>
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caccgaccag ggtgcaagtg tgga 24

<210> 21
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<212> DNA
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<220>
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<400> 21
aaactccaca cttgcaccct ggtc 24

<210> 22
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<400> 22
caccgctcct cagcctggca ttca 24

<210> 23
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<210> 24
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<400> 24
tgctaaagcg catgctccag act 23

<210> 25
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<210> 26
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<400> 26
cctctctgtg cattatggca g 21

<210> 27
<211> 19
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<400> 27
gccttctatc gccttcttg 19

<210> 28
<211> 20
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<220>
<223> Synthetic construct

<400> 28
actcctccac ctttgacgct 20

<210> 29
<211> 23
<212> DNA
<213> Artificial sequence

<220>
<223> Synthetic construct

<400> 29
tcccctcttc aagggtctac atg 23

<210> 30
<211> 20
<212> DNA
<213> Artificial sequence

<220>
<223> Synthetic construct

<400> 30
gtgcaaaatg gaagggtttc 20

<210> 31
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tgtttccttt cactggccac a 21

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<400> 33
aacggcaact ggtgaacggt a 21

<210> 34
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ggcgatgcca gaatagatgc cg 22

<210> 35
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ccaggccact tggctcctct atctccaga 29

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ccttactgtt gagactgcaa tatcc 25

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ctgaagtccc agtatatact tcacac 26

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<400> 38
cccagaagca gtaataatca tcgag 25

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atgtgggatg tagtagatct tgc 23

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gactgagtac ctgaaccggc 20

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gggccaaact gagcagagtc 20

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aagaccaggg tggttgggac 20

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gtaagaaaaa tgcccacgtc 20

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agtcagacgt ctggagcttc 20

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<400> 47
gtgagcagtg aaggcatgag tc 22

<210> 48
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<400> 48
gtgagataca gatacagaca 20

<210> 49
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<400> 49
aatgatttgc ctggaatgcc 20

<210> 50
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<220>
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<400> 50

Gly Leu Phe Glu Ala Ile Glu Gly Phe Ile Glu Asn Gly Trp Glu Gly
1 5 10 15

Met Ile Asp Gly Trp Tyr Gly
20

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<211> 14
<212> PRT
<213> Artificial sequence

<220>
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<400> 51

Tyr Gly Arg Lys Lys Arg Arg Gln Arg Arg Arg Pro Pro Gln
1 5 10

<210> 52
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<212> DNA
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<220>

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atccacgttc accttgcccc acagggcagt aacggcagac ttctcctcag gagtcaggtg 60

caccatggtg tctgtttgag gttgctagtg a 91

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ggtgcaccat ggtgtctgtt tgaggttgct agtga 95

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<220>
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<400> 54
taatacgact cactataggg taacggcaga cttctccac 39

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<400> 55
aaaaagcacc gactcggtgc c 21

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<400> 56
tcctgaggaa aaat 14

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<400> 57
tgactcctga ggagaa 16

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<220>
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<400> 58
actcctgtgg agaag 15

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cagagccatc tattgcttac atttg 25

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<400> 60
ggcctcacca ccaacttcat 20

<210> 61
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<213> Artificial sequence

<220>
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<400> 61

Tyr Gly Arg Lys Lys Arg Arg Gln Arg Arg Arg Pro Pro Gln Ala Gly
1 5 10 15

Gly Gly Ser Gly Gly Ser Tyr Gly Arg Lys Lys Arg Arg Gln Arg Arg
20 25 30

Arg Pro Pro Gln Ala Gly Gly Gly Ser Gly Gly Ser Tyr Gly Arg Lys
35 40 45

Lys Arg Arg Gln Arg Arg Arg Pro Pro Gln Ala Gly
50 55 60

<210> 62
<211> 104
<212> RNA
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<220>
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<400> 62
gggucugccg uuacugcccu gguuuuagag cuagaaauag caaguuaaaa uaaggcuagu 60

ccguuaucaa cuugaaaaag uggcaccgag ucggugcuuu uuuu 104

<210> 63
<211> 105
<212> RNA
<213> Artificial sequence

<220>
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<400> 63
ggguaacggc agacuucucc acguuuuaga gcuagaaaua gcaaguuaaa auaaggcuag 60

uccguuauca acuugaaaaa guggcaccga gucggugcuu uuuuu 105

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gtggggcaag gtgaacgtgg atgaagttgg 90

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<400> 65
taatacgact cactataggg tctgccgtta ctgccctg 38

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<400> 66
tccacatgcc cagtttctat 20

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<400> 67
agtagcaatt tgtactgatg gtatg 25

<210> 68
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<212> DNA
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<400> 68
ggtctgccgt tactgccctg 20

<210> 69
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<400> 69
gtaacggcag acttctccac 20