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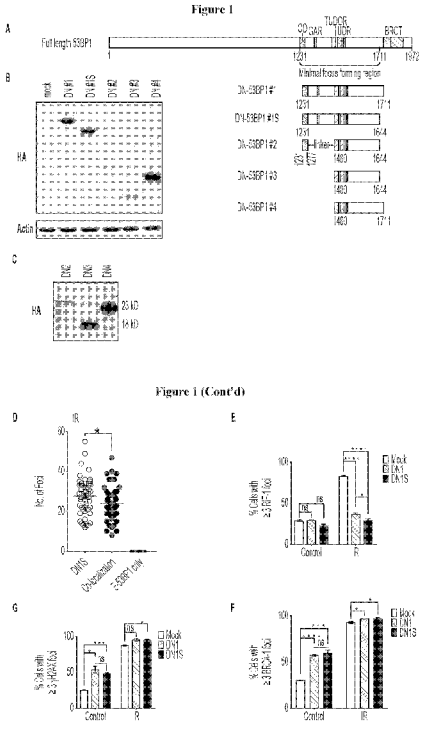

(57) **Abstract:** Provided herein are fusion polypeptides each comprising a gene-editing nuclease enzyme such as CRISPR/Cas9 and a dominant-negative variant of p53 binding protein 1 (53BP1 DN variant). Such a fusion polypeptide can be used in gene editing to inhibit non- homologous end-joining (NHEJ) and enhancing homology-directed DNA repair.

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FUSION PROTEINS FOR USE IN IMPROVING GENE CORRECTION VIA HOMOLOGOUS RECOMBINATION

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit under 35 U.S.C. § 119(e) of U.S. provisional application number 62/578,593, filed October 30, 2017, the contents of which are incorporated by reference herein in its entirety.

BACKGROUND OF THE INVENTION

Genome editing technologies have enabled a new paradigm to manipulate genome of host cells to achieve therapeutic effects, including correction of gene mutations associated with diseases and addition of therapeutic genes to desired sites of the genome. Genome editing technologies involve the use of endonucleases such as CRISPR/Cas9, zinc finger nuclease (ZFN), transcription activator-effector nucleases (TALENs) and meganuclease. Such endonucleases cleave DNAs at specific sites to create double strand breaks (DSBs), which would trigger endogenous cellular DNA repair systems.

There are two major cellular pathways to repair DSBs: homology-directed repair (HDR) and nonhomologous end-joining (NHEJ). The HDR pathway repairs the DSBs using a templated having homologous sequences as the damaged DNA, thereby allowing for precise genome editing. By contrast, the NHEJ pathway repairs the DSBs by ligation of DNA ends after they have been processed, thus resulting in imprecise small insertions and deletions. The NHEJ error prone repair occurs much more rapidly than HDR, and is therefore the far more predominant repair that can result in further mutations that are clinically undesirable.

Most cells, and especially hematopoietic stem cells, primarily utilize NHEJ to repair the genome from naturally occurring DNA breaks, which occur at a relatively high frequency. Hence, small molecules and decoys that have been used to inhibit NHEJ repair pathway in cells (global NHEJ inhibition) can be genotoxic and impair the natural DSB repair.

It is therefore of great interest to develop new strategies to inhibit NHEJ and/or enhance HDR so as to achieve precise genome editing.

SUMMARY OF THE INVENTION

The present disclosure is based, at least in part, on the development of a number of dominant-negative p53 binding protein 1 variants (53BP1 DN variants or mutants), which can be recruited to DNA damage sites but cannot recruit other proteins of the NHEJ machinery. When fused with a gene-editing enzyme such as Cas9, the fusion proteins successfully inhibited NHEJ and increased HDR in gene editing, specifically only at the Cas9 nuclease cut site (site-specific NHEJ inhibition).

Accordingly, one aspect of the present disclosure features a fusion polypeptide, comprising a gene-editing nuclease enzyme and a dominant-negative variant of a p53 binding protein 1 (53BP1). The dominant-negative variant of 53BP1 is a truncated 53BP1, which may comprise the minimum focus forming region. In some embodiments, the dominant-negative variant of 53BP1 comprises (a) deletion in a docking domain, (b) a deletion of a BRCT domain, or (c) both (a) and (b). In some embodiments, the dominant negative variant of 53BP1 comprises (a) a deletion of region 1-1231 of SEQ ID NO:1 or a portion thereof, (b) a deletion of region 1711-1972 of SEQ ID NO:1 or a portion thereof, or (c) both (a) and (b). In some examples, the gene-editing nuclease enzyme is covalently linked directly to the dominant-negative variant of 53BP1 in the fusion polypeptide disclosed herein. Alternatively the gene-editing nuclease enzyme is linked to the dominant-negative variant of 53BP1 via a peptide linker.

The gene-editing nuclease enzyme in any of the fusion proteins disclosed herein may be any site-specific nuclease, such as a Cas9 enzyme (e.g., from a suitable bacterium), a Cas12 enzyme, a zinc finger nuclease (ZFN) or a transcription activator-like effector nuclease (TALEN) or meganuclease such as a homing endonuclease. In some examples, the gene-editing nuclease enzyme is a Cas9 enzyme, which may comprise the amino acid sequence of SEQ ID NO:4 or SEQ ID NO:5 (which are encoded by the 5' portion of the nucleotide sequences (upstream to the linker sequence) shown in SEQ ID NO: 7 and SEQ ID NO: 9, respectively.

In some embodiments, the dominant-negative variant of 53BP1 comprises region 1480-1644 of SEQ ID NO:1. For example, the dominant-negative variant of 53BP1 comprises region 1231-1644 of SEQ ID NO:1, region 1231-1711 of SEQ ID NO:1, or 1480-1711 of SEQ ID NO:1. In some examples, the dominant-negative variant of 53BP1 consists of 1231-1711 of SEQ ID NO:1, 1231-1644 of SEQ ID NO:1, or 1480-1711 of SEQ ID NO:1.

In specific examples, the the fusion polypeptide comprises the amino acid sequence of SEQ ID NO:4 or SEQ ID NO:5, which are encoded by the nucleotide sequences shown in Figures 8 and 9 respectively.

In other aspects, provided herein is a nucleic acid, comprising a nucleotide sequence coding for any of the fusion polypeptides disclosed herein. Also provided herein is a vector that comprises such a nucleic acid. In some instances, the nucleotide sequence coding for the fusion polypeptide in the vector is in operable linkage to a promoter, for example, a mammalian promoter. In some examples, the vector can be a viral vector such as an integration defective viral vector, *e.g.*, a retroviral vector, an adenoviral vector, an adeno-associated viral vector, or a hybrid vector. In specific examples, the viral vector can be a retroviral vector, for example, a lentiviral vector.

In some embodiments, any of the vectors described herein may further comprise a nucleotide sequence coding for a guide RNA, and/or a sequence that serves as a template for homologous recombination, or both.

In yet another aspect, the present disclosure features a method for enhancing homology directed DNA repair (HDR) in gene editing of a cell (*e.g.*, a mammalian cell such as a human cell). The method comprises introducing into a cell any of the fusion polypeptides disclosed herein or a vector that comprises a nucleotide sequence coding for the fusion polypeptide, and optionally a guide RNA targeting a gene of interest. The fusion polypeptide may be introduced into the cell in the form of a protein, an RNA or a DNA that encode the fusion polypeptide. In some instances, the guide RNA can be a single guide RNA. In some embodiments, the fusion polypeptide and the gRNA can be introduced into the cell by delivering a vector that expresses both the fusion polypeptide and the guide RNA into the cell.

In some embodiments, the method may further comprise introducing into the cell a donor template nucleic acid, which comprises homologous arms flanking a cleavage site in the gene of interest directed by the guide RNA. In some examples, the fusion polypeptide can be delivered into the cells in a ribonucleoprotein complex (RNP) form, which may further comprise a guide RNA, modified guide RNA, and/or a donor template nucleic acid as disclosed herein.

The details of one or more embodiments of the invention are set forth in the description below. Other features or advantages of the present invention will be apparent

from the following drawings and detailed description of several embodiments, and also from the appended claims.

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present disclosure, which can be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

Figure 1 includes diagrams showing identification of the homology directed DNA repair (HDR)-enhancer fragment of p53 binding protein 1 (53BP1). **A**: Schematic diagram of 53BP1 protein showing (a) the minimal focus forming region (FFR) including the oligomerization domain (OD), a glycine-arginine rich (GAR) motif, a tandem Tudor domain (TD), and the ubiquitin-dependent recruitment (UDR) motif; (b) the different truncated 53BP1 proteins (DN1, DN1s, DN2, DN3, and DN4) that were tested for their ability to compete with the endogenous 53BP1 and recruit HDR proteins and not NHEJ proteins to the DNA DSB. The amino acid sequence regions are indicated for each 53BP1 truncated fragment. **B**: A Western blot photo showing relative expression levels of the different truncated human 53BP1 proteins (DN1, DN1S, DN2, DN3 or DN4, which was HA-tagged) which were analyzed by western blot using anti-HA antibodies and control anti-actin antibodies in HeLa cells transduced with empty vector (Mock) or lentiviral vectors encoding the HA-tagged truncated 53BP1 proteins. Each arm had equal lentiviral transduction rates in HeLa cells. **C**: A photo showing the relative expression levels of DN2, DN3 and DN4 as shown **B**, at higher exposure to show DN2 levels. **D**: a chart showing quantification of the numbers of the cells with HA+ (DN1S) foci or HA+ endogenous 53BP1+ (Co-localization) foci or endogenous 53BP1+ only foci. **E**: A chart showing quantification of the numbers of the cells with ≥3 RIF-1 foci in control cells or irradiated cells with or without the presence of DN1/DN1S. **F**: A chart showing quantification of the numbers of the cells with ≥3 BRCA-1 foci in control cells or irradiated cells with or without the presence of DN1/DN1S. **G**: A chart showing quantification of the numbers of the cells with ≥3 γH2AX foci in control cells or irradiated cells with or without the presence of DN1/DN1S. The cumulative data in Figure 1 shows that DN1 and DN1S versions of 53BP1 are expressed at high levels and function as a dominant negative (DN) protein: like the endogenous 53BP1 are both recruited to DSBs (at γ-

H2AX foci), where they either co-localize or displace endogenous 53BP1, reduce binding of the downstream NHEJ recruiter protein RIF-1, and increase binding of the HDR protein BRCA-1.

Figure 2 includes diagrams showing Cas9-DN1S fusion protein locally inhibits NHEJ, thereby reducing cellular toxicity that is normally seen with global NHEJ inhibition. **A**: a schematic diagram showing the different truncated 53BP1 proteins (DN1, DN1S, DN2 and DN2L) fused with Cas9. **B**: a photo showing the relative expression levels of the different Cas9 fusion proteins as analyzed by western blot using anti-FLAG antibodies and control anti-actin antibodies in HeLa cells transfected transduced with plasmids encoding an FLAG-tagged Cas9 fusion protein and mCherry fluorophore. Each arm was sorted for mCherry+ cells. The blot shows that DN1, DN1S, DN2 and DN2L fusions with Cas9 result in the expected size stable protein expression. **C**: A representative photo showing representative immunofluorescence (IF) images showing HA tagged DN1S or dCas9-DN1S/gRNA (red fluorescence) and endogenous 53BP1 (green fluorescence) recruitment to the DNA break site. Nuclie are labeled with DAPI (blue). **D.** A chart showing quantification of the numbers of the cells with ≥1 HA+ (DN1S) foci. **E**: A chart showing quantification of the number of the cells with ≥3 53BP1 foci in mock (cells transduced with empty vector), DN1S (cells transduced with lentiviral vectors expressing DN1S fragment), dCas9 (cells transduced with lentiviral vectors expressing dCas9), dCas9-DN1S (cells transduced with lentiviral vectors expressing dCas9-DN1S) and dCas9-DN1S/gRNA (cells transduced with lentiviral vectors expressing dCas9-DN1S/gRNA). **F**: Ionizing radiation (IR) sensitizes cells to apoptosis and death if cellular NHEJ –based repair pathways are compromised. This was tested using a colony forming assay. A chart showing viability of HeLa cells treated with ionizing radiation (IR) at the indicated doses after expressing the fusion protein and gRNA, and global NHEJ inhibition (with shRNA to 53BP1 or NU7441) or their appropriate controls, as indicated. The viable colonies were determined by crystal violet staining. IR treatment resulted in a significant loss of viability with global NHEJ inhibition (using sh53BP1 or NU7441), and a dose-dependent decrease in colony formation when compared with the controls. The data are presented as mean ± SEM of three tests, *P<0.05, **P<0.01. **G**: A chart showing viability of cells treated with IR at the indicated doses and expressing a catalytically inactive form of Cas9 (dCas9) fused to DN1S (dCas9-DN1S with gRNA). The cell viability was determined by crystal violet staining. IR treatment resulted in no significant

dose dependent or dCas9-DN1S/gRNA dependent decrease in colony formation when compared with the controls. dCas9-DN1S/gRNA transfected HeLa cells show that despite continuous presence of the catalytically dead Cas9-DN1S fusion protein (tethered to the Cas9 specific sites by the gRNA), cells were not sensitized to IR. These data show that a dead Cas9 is not dragged by the DN1S to IR induced DSB and cause toxicity.

The cumulative data in Figure 2 shows that fusion of the DN 53BP1 fragment to Cas9 results in stable fusion protein expression. Unlike the DN 53BP1 alone, which localizes to DSBs, the Cas9-DN constructs form very limited foci.

Figure 3 includes diagrams illustrating the Traffic Light Reporter (TLR) system. **A**: a diagram showing construction of the TLR system in exemplary host cells, the 293T cells. **B**: a diagram showing the use of CRISPR/Cas9 to the TLR locus in 293T cells to test the relative efficiency of NHEJ and HDR. Nucleotide sequence of AAVS1-1 (top): SEQ ID NO:10. Nucleotide sequence of AAVS1-2 (bottom): SEQ ID NO:11. **C.** Bar diagram showing the relative frequencies of HDR and NHEJ induced by different Cas9 fusion proteins (DN1, DN1S, DN2, DN2L) as compared to those induced by Cas9 using the TLR system in 293T cells. DN1 or DN1S significantly increase HDR, and the HDR/NHEJ ratio as compared to Cas9 alone**. D.** Relative frequencies of HDR induced by different SpCas9-DNIS fusion proteins with different tags and linkers were compared to those induced by Cas9 using TLR system in 293T cells.The DN1S can be fused to Cas9 using a variety of linkers and tags, with the same effect on increased HDR, compared to the respective Cas9 controls.

Figure 4 includes diagrams showing HDR stimulation by the Cas9-DN1S fusion protein, which takes place at different target genes/loci in multiple cell lines as indicated. A: Cas9 derived from Streptococcus pyogenes (SpCas9) was used as indicated. A chart showing relative frequencies of HDR induced by different SpCas9 fusion proteins as indicated, which were compared to those induced by Cas9. 293T cells were targeted either at the AAVS1 locus (left panel) or the LMO2 locus (middle panel) using the spCas9 plasmid and a GFP donor homology template. Relative frequencies of GFP+ (HDR) cells using either spCas9 or spCas9-DN1S fusion protein and the percentage of GFP+ cells shown. GFP was targeted in frame into the CD45 gene locus of K562 cells (right panel). **B**: Cas9 derived from Staphylococcus aureus (SaCas9), or its fusion with DN1S (SaCas9-DN) shows a highly significant reduction of NHEJ and increase in HDR in EBV transformed primary human B cells (LCL) at the CD45 locus (left panel) or the AAVS1 locus (middle panel). Similar

extremely high efficiency HDR, reaching >90% was observed in the K562 hematopoietic cells targeted with the SaCas9-DN. Red bars denote NHEJ frequency and Green bars denote HDR. For hematopoietic cells, SaCas9 and SaCas9-DN proteins were complexed with gRNA to the indicated loci and transfected as a ribonucleoprotein complex (RNP). **C**: A bar plot showing the gene editing efficiency (NHEJ or HDR) of SaCas9 or SaCas9-DN1S using the CD45 reporter system in K562 cells. When the CD45 locus was targeted in K562 cells using the SaCas9-DN, we did not observe increased HDR over SaCas9 alone. However, there was a very highly significant reduction in NHEJ/error prone repair.

The cumulative data from Figure 4 shows that the fusion of the dominant negative 53BP1 fragment significantly increases HDR and reduces NHEJ at different target genes, in multiple cell lines, using different Cas9 nucleases. Occasionally, if HDR is not increased, a highly significant decrease in NHEJ occurs at the nuclease induced DSB.

Figure 5 includes diagrams showing targeting CD18 at the AAVS1 locus in B lymphocytes derived from a patient with Leukocyte Adhesion Deficiency (which results from defects in the CD18 gene) showed higher levels and quality of HDR. **A:** A diagram showing epresentative flow cytometry plots of EBV immortalized primary B cells from a patient with Leukocyte Adhesion Defect (LAD) transfected with the indicated conditions for targeted integration of CD18 at the AAVS1 locus. LAD results from lack of expression of the CD18 integrin (adhesion molecule). The fractions of unedited cells (black) or HDR+ cells (green at the right gated portion; percentage indicated) are shown with appropriate controls. Here, LAD B cells were transfected either with SaCas9/gRNA RNP, or SaCas9-DN RNP along with a homology donor template (DT) carrying the CD18 gene was embedded in the AAV-6 virus for efficient delivery. No RNP and no DT controls are indicated. The gRNA was designed to target the AAVS1 locus. The flow cytometry plots show that not only was a higher percentage of HDR (GFP+ cells) were seen with SaCas9-DN, but the HDR population showed much brighter GFP fluorescence. **B**: A bar diagram showing the quantification of the HDR efficiency and NHEJ efficiency of the SaCas9 or SaCas9-DN1S by flow cytometry (green) and TIDE assay (red), respectively in LAD B cells with AAVS1 CD18 donor, showing a highly significant increase in HDR and proportionate decrease in NHEJ in EBV immortalized LAD B lymphocytes. **C.** The GFP+ HDR cells could be separated into a GFP^{bright} (bi-allelic HDR) and GFP^{dim} (mono-allelic HDR), as confirmed by sorting this population and subjecting the bright and dim cells to PCR for HDR. The relative frequencies

of mono-allelic and bi-allelic HDR frequency detected by MFI of CD18 staining shows that the SaCas9-DN fusion protein highly significantly improves the HDR on both alleles. A bi-allelic correction would result in perfect correction of the CD18 expression to normal levels.

Figure 6 is a bar plot showing the NHEJ editing efficiency of Cas9 or Cas9-DN1S at the top four off target sites of AAVS1 gRNA in EBV immortalized B cells from a LAD patient. The Cas9-DN1S fusion decreases or does not increase the off target cutting.

DETAILED DESCRIPTION OF THE INVENTION

Double-strand breaks (DSBs) are very common both in quiescent cells and when cells undergo replication. Normally, cells (especially hematopoietic stem cells) use non-homologous end joining (NHEJ) to repair DNA double strand breaks. Hence, global blockade of the NHEJ pathway using small molecule inhibitors to 53BP1, DNA-PK, Ku70/80, ligase 4, etc. are toxic to hematopoietic stem cells (resulting in apoptosis due to unrepaired DSBs) or can potentially result in deleterious, even cancer-causing mutations.

Upon DNA damage, ATM phosphorylates 53BP1. 53BP1 is the first protein to be recruited to DNA damage sites, which then recruits the RIF-1/PTIP protein complex to recruit the other NHEJ proteins to the damage sites to carry out NHEJ repair. We discovered that dominant negative variants of 53BP1, when fused to CRISPR/Cas9 inhibited NHEJ and enhanced HDR in gene editing as observed in multiple cell lines and at various target gene sites. Accordingly, described herein are fusion proteins containing gene editing nucleases such as SpCas9 or SaCas9 (or other site-specific nucleases such as Cas12 (Cpf1) or ZFN) and a dominant negative mutant of the 53BP1 protein, which can be recruited to the DNA DSB sites but unable to recruit other NHEJ proteins. Such fusion proteins can be used to inhibit at sites where the gene editing nuclease creates a DSB, thereby inhibiting NHEJ and increase homology directed repair.

Unexpected, the studies provided herein showed that DN 53BP1 alone, while recruited to DSB, is toxic to cells as it interferes with naturally occurring NHEJ repair of cells. Further, it is reported here that DN 53BP1 alone competes with endogenous 53BP1 and displaces endogenous 53BP1 at a high enough level to result in toxicity. Moreover, it was discovered that fusing DN 53BP1 fragment to Cas9 not only decreases NHEJ only at the Cas9 cut site, but promotes HDR in multiple human cell types and loci.

Fusion Polypeptides Containing Dominant-Negative 53BP1 Variants and Gene-Editing Enzymes

One aspect of the present disclosure relates to fusion polypeptides each comprising a gene-editing nuclease enzyme and a dominant-negative 53BP1 variant. As used herein, a fusion polypeptide refers to a polypeptide comprising at least two fragments derived from different parent proteins, for example, one fragment from a gene-editing nuclease enzyme and one fragment from a 53BP1 protein. In the fusion polypeptides described herein, the gene-editing nuclease enzyme can be linked directly to the 53BP1 DN variant. Alternatively, the gene-editing nuclease enzyme can be linked to the 53BP1 DN variant through a peptide linker, for example, a TGS linker (see below) and an XTEN linker. In some instances, the gene-editing nuclease enzyme is located at the N-terminus of the fusion polypeptide. In other instances, the 53BP1 DN variant is located at the N-terminus of the fusion polypeptide.

(A) 53BP1 Dominant-Negative Variants

Tumor suppressor p53-binding protein 1 (53BP1) is a protein that plays an essential role in DNA damage repair. In humans, 53BP1 is encoded by the *TP53BP1* gene. 53BP1 binds to the DNA-binding domain of p53 and enhances p53-mediated transcriptional activation. 53BP1 plays multiple roles in the DNA damage response, including promoting checkpoint signaling following DNA damage, acting as a scaffold for recruitment of DNA damage response proteins to damaged chromatin, and promoting NHEJ pathways by limiting end resection following a double-strand break.

53BP1 proteins of various species have been well characterized. Information of one exemplary human 53BP1 can be found under UniProtKB-Q12888. Exemplary amino acid sequence is provided below (SEQ ID NO:1).

```
         10          20          30          40          50
MDPTGSQLDS DFSQQDTPCL IIEDSQPESQ VLEDDSGSHF SMLSRHLPNL
         60          70          80          90         100
QTHKENPVLD VVSNPEQTAG EERGDGNSGF NEHLKENKVA DPVDSSNLDT
        110         120         130         140         150
CGSISQVIEQ LPQPNRTSSV LGMSVESAPA VEEEKGEELE QKEKEKEEDT
        160         170         180         190         200
SGNTTHSLGA EDTASSQLGF GVLELSQSQD VEENTVPYEV DKEQLQSVTT
        210         220         230         240         250
NSGYTRLSDV DANTAIKHEE QSNEDIPIAE QSSKDIPVTA QPSKDVHVVK
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        260         270         280         290         300
EQNPPPARSE DMPFSPKASV AAMEAKEQLS AQELMESGLQ IQKSPEPEVL
        310         320         330         340         350
STQEDLFDQS NKTVSSDGCS TPSREEGGCS LASTPATTLH LLQLSGQRSL
        360         370         380         390         400
VQDSLSTNSS DLVAPSPDAF RSTPFIVPSS PTEQEGRQDK PMDTSVLSEE
        410         420         430         440         450
GGEPFQKKLQ SGEPVELENP PLLPESTVSP QASTPISQST PVFPPGSLPI
        460         470         480         490         500
PSQPQFSHDI FIPSPSLEEQ SNDGKKDGDM HSSSLTVECS KTSEIEPKNS
        510         520         530         540         550
PEDLGLSLTG DSCKLMLSTS EYSQSPKMES LSSHRIDEDG ENTQIEDTEP
        560         570         580         590         600
MSPVLNSKFV PAENDSILMN PAQDGEVQLS QNDDKTKGDD TDTRDDISIL
        610         620         630         640         650
ATGCKGREET VAEDVCIDLT CDSGSQAVPS PATRSEALSS VLDQEEAMEI
        660         670         680         690         700
KEHHPEEGSS GSEVEEIPET PCESQGEELK EENMESVPLH LSLTETQSQG
        710         720         730         740         750
LCLQKEMPKK ECSEAMEVET SVISIDSPQK LAILDQELEH KEQEAWEEAT
        760         770         780         790         800
SEDSSVVIVD VKEPSPRVDV SCEPLEGVEK CSDSQSWEDI APEIEPCAEN
        810         820         830         840         850
RLDTKEEKSV EYEGDLKSGT AETEPVEQDS SQPSLPLVRA DDPLRLDQEL
        860         870         880         890         900
QQPQTQEKTS NSLTEDSKMA NAKQLSSDAE AQKLGKPSAH ASQSFCESSS
        910         920         930         940         950
ETPFHFTLPK EGDIIPPLTG ATPPLIGHLK LEPKRHSTPI GISNYPESTI
        960         970         980         990        1000
ATSDVMSESM VETHDPILGS GKGDSGAAPD VDDKLCLRMK LVSPETEASE
       1010        1020        1030        1040        1050
ESLQFNLEKP ATGERKNGST AVAESVASPQ KTMSVLSCIC EARQENEARS
       1060        1070        1080        1090        1100
EDPPTTPIRG NLLHFPSSQG EEEKEKLEGD HTIRQSQQPM KPISPVKDPV
       1110        1120        1130        1140        1150
SPASQKMVIQ GPSSPQGEAM VTDVLEDQKE GRSTNKENPS KALIERPSQN
       1160        1170        1180        1190        1200
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       1210        1220        1230        1240        1250
GSGEKPVSAP GDDTESLHSQ GEEEFDMPQP PHGHVLHRHM RTIREVRTLV
       1260        1270        1280        1290        1300
TRVITDVYYV DGTEVERKVT EETEEPIVEC QECETEVSPS QTGGSSGDLG
       1310        1320        1330        1340        1350
DISSFSSKAS SLHRTSSGTS LSAMHSSGSS GKGAGPLRGK TSGTEPADFA
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RGRPPSRTTG TRETAVPGPL GIEDISPNLS PDDKSFSRVV PRVPDSTRRT
       1460        1470        1480        1490        1500
DVGAGALRRS DSPEIPFQAA AGPSDGLDAS SPGNSFVGLR VVAKWSSNGY
       1510        1520        1530        1540        1550
FYSGKITRDV GAGKYKLLFD DGYECDVLGK DILLCDPIPL DTEVTALSED
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      1560        1570        1580        1590        1600
EYFSAGVVKG HRKESGELYY SIEKEGQRKW YKRMAVILSL EQGNRLREQY
      1610        1620        1630        1640        1650
GLGPYEAVTP LTKAADISLD NLVEGKRKRR SNVSSPATPT ASSSSSTTPT
      1660        1670        1680        1690        1700
RKITESPRAS MGVLSGKRKL ITSEEERSPA KRGRKSATVK PGAVGAGEFV
      1710        1720        1730        1740        1750
SPCESGDNTG EPSALEEQRG PLPLNKTLFL GYAFLLTMAT TSDKLASRSK
      1760        1770        1780        1790        1800
LPDGPTGSSE EEEEFLEIPP FNKQYTESQL RAGAGYILED FNEAQCNTAY
      1810        1820        1830        1840        1850
QCLLIADQHC RTRKYFLCLA SGIPCVSHVW VHDSCHANQL QNYRNYLLPA
      1860        1870        1880        1890        1900
GYSLEEQRIL DWQPRENPFQ NLKVLLVSDQ QQNFLELWSE ILMTGGAASV
      1910        1920        1930        1940        1950
KQHHSSAHNK DIALGVFDVV VTDPSCPASV LKCAEALQLP VVSQEWVIQC
      1960        1970
LIVGERIGFK QHPKYKHDYV SH
```

As illustrated in Figure 1, panel A, an example 53BP1 includes 1972 amino acids. The N-terminal portion (1-1231) contains domains for interacting with other proteins of cellular DNA repair machinery (docking domains). The C-terminal portion (1711-1972) contains two breast cancer susceptibility gene 1 (BRCT) motifs, which are common motifs presented in several proteins involved in DNA repair and/or DNA damage-signaling pathways. Rappold et al., J. Cell Biol. 2001, 153(3):613-620. In between the docking domains and the BRCT domains is the minimal focus forming region (FFR), which may include residues 1231-1711 of SEQ ID NO:1. The FFR region includes an oligomerization domain (OD), a glycine-arginine rich (GAR) motif, a tandem Tudor domain (TD), and an ubiquitin-dependent recruitment (UDR) motif.

The dominant-negative variant of p53BP1 protein as disclosed herein can comprise a fragment of a wild-type 53BP1 from a suitable species (*e.g.*, a mammal such as a human). An exemplary human 53BP1 is provided above. 53BP1 proteins from other species are well known in the art and their sequences can be retrieved from publically available gene database, for example, using SEQ ID NO:1 as a search query. A dominant-negative variant of a 53BP1 protein refers to a mutant of the wild-type 53BP1 protein and adversely affects the normal bioactivity of the wild-type counterpart within the same cells. In some instances, the dominant-negative variant disclosed herein can maintain substantially similar binding activity to a DNA damage site (*e.g.*, a DSB site) but has little or no activity in recruiting other protein components of the NHEJ repair machinery.

Without being bound by theory, such a dominant-negative variant can compete against wild-type 53BP1 from binding to the DSB site but cannot recruit other NHEJ repair proteins, thereby inhibiting the NHEJ repair function of the wild-type 53BP1 counterpart in the same cells. When fused to a site specific nuclease (*e.g.*, Cas9), the dominant-negative variant of a 53BP1 protein may only restrict NHEJ at the specific site where the site-specific nuclease induces a DSB, and would not inhibit normal cellular NHEJ.

The 53BP1 dominant-negative (53BP1 DN) variants described herein may be a truncated version of a wild-type 53BP1, in which one or more of the docketing domains and/or one or more of the BRCT domains are deleted. A docketing domain in a 53BP1 protein is a functional domain, usually located in the N-terminal portion of the protein (*e.g.*, residues 1-1230 of SEQ ID NO:1), that recruits RIF-1, PTIP and the rest of the proteins involved in NHEJ. For example, the 53BP1 DN variant may have a deletion of the fragment corresponding to residues 1-1230 of SEQ ID NO:1 (containing docking domains) or a portion thereof. Alternatively or in addition, the 53BP1 DN variant may have a deletion of the fragment corresponding to residues 1722 to 1972 of SEQ ID NO:1 (containing BRCT domains) or a portion thereof. The 53BP1 DN variants disclosed herein may contain the minimal focus forming region (*e.g.*, residues 1231-1711 of SEQ ID NO:1) or a portion thereof, which binds DSB sites.

In some examples, the 53BP1 DN variant has the complete fragment corresponding to residues 1-1230 of SEQ ID NO:1 deleted or has the complete fragment corresponding to residues 1722 to 1972 of SEQ ID NO:1 deleted. In specific examples, the 53BP1 DN variant has both fragments corresponding to 1-1230 of SEQ ID NO:1 and residues 1722-1972 of SEQ ID NO:1 deleted.

In some embodiments, the 53BP1 DN variant disclosed herein contains one or more functional domains within the minimal focus forming region illustrated in Figure 1, panel A. For example, the 53BP1 DN variant may contain one or more of the OD domain, the GAR) motif, the TD domain, and the UDR) motif. In some examples, the 53BP1 DN variant contains the fragment corresponding to 1231-1711 of SEQ ID NO:1 or a fragment thereof. Specific examples of 53BP1 DN variants are provided in Figure 1, panel A, including DN-53BP1 #1 (consisting of the fragment corresponding to residues 1231-1711 of SEQ ID NO:1), DN-53BP1 #1S (consisting of the fragment corresponding to residues 1231-1644 of SEQ ID NO:1), DN-53BP1 #2 (containing the fragment corresponding to

residues 1231-1644 of SEQ ID NO:1 with a linker replacing the fragment of 1277-1480); DN-53BP1 #3 (consisting of the fragment corresponding to residues 1480-1644 of SEQ ID NO:1), and DN-53BP1 #4 (consisting of the fragment corresponding to residues 1480-1711 of SEQ ID NO:1).

The amino acid sequence of 53BP1 DN1S and its encoding nucleotide sequence, as an example, is provided below:

PHGHVLHRHMRTIREVRTLVTRVITDVYYVDGTEVERKVTEETEEPIVECQECETEVSPSQTGGSSG
DLGDISSFSSKASSLHRTSSGTSLSAMHSSGSSGKGAGPLRGKTSGTEPADFALPSSRGGPGKLSPR
KGVSQTGTPVCEEDGDAGLGIRQGGKAPVTPRGRGRRGRPPSRTTGTRETAVPGPLGIEDISPNLSP
DDKSFSRVVPRVPDSTRRTDVGAGALRRSDSPEIPFQAAAGPSDGLDASSPGNSFVGLRVVAKWSSN
GYFYSGKITRDVGAGKYKLLFDDGYECDVLGKDILLCDPIPLDTEVTALSEDEYFSAGVVKGHRKES
GELYYSIEKEGQRKWYKRMAVILSLEQGNRLREQYGLGPYEAVTPLTKAADISLDNLVEGKRKRRSN
VSSPATPTASSS (SEQ ID NO:2)

CCACATGGCCATGTCTTACATCGTCACATGAGAACAATCCGGGAAGTACGCACACTTGTCACTCGTGT
CATTACAGATGTGTATTATGTGGATGGAACAGAAGTAGAAAGAAAAGTAACTGAGGAGACTGAAGAGC
CAATTGTAGAGTGTCAGGAGTGTGAAACTGAAGTTTCCCCTTCACAGACTGGGGGCTCCTCAGGTGAC
CTGGGGGATATCAGCTCCTTCTCCTCCAAGGCATCCAGCTTACACCGCACATCAAGTGGGACAAGTCT
CTCAGCTATGCACAGCAGTGGAAGCTCAGGGAAAGGAGCCGGACCACTCAGAGGGAAAACCAGCGGGA
CAGAACCCGCAGATTTTGCCTTACCCAGCTCCCGAGGAGGCCCAGGAAAACTGAGTCCTAGAAAAGGG
GTCAGTCAGACAGGGACGCCAGTGTGTGAGGAGGATGGTGATGCAGGCCTTGGCATCAGACAGGGAGG
GAAGGCTCCAGTCACGCCTCGTGGGCGTGGGCGAAGGGGCCGCCCACCTTCTCGGACCACTGGAACCA
GAGAAACAGCTGTGCCTGGCCCCTTGGGCATAGAGGACATTTCACCTAACTTGTCACCAGATGATAAA
TCCTTCAGCCGTGTCGTGCCCCGAGTGCCAGACTCCACCAGACGAACAGATGTGGGTGCTGGTGCTTT
GCGTCGTAGTGACTCTCCAGAAATTCCTTTCCAGGCTGCTGCTGGCCCTTCTGATGGCTTAGATGCCT
CCTCTCCAGGAAATAGCTTTGTAGGGCTCCGTGTTGTAGCCAAGTGGTCATCCAATGGCTACTTTTAC
TCTGGGAAAATCACACGAGATGTCGGAGCTGGGAAGTATAAATTGCTCTTTGATGATGGGTACGAATG
TGATGTGTTGGGCAAAGACATTCTGTTATGTGACCCCATCCCGCTGGACACTGAAGTGACGGCCCTCT
CGGAGGATGAGTATTTCAGTGCAGGAGTGGTGAAAGGACATAGGAAGGAGTCTGGGGAACTGTACTAC
AGCATTGAAAAAGAAGGCCAAAGAAAGTGGTATAAGCGAATGGCTGTCATCCTGTCCTTGGAGCAAGG
AAACAGACTGAGAGAGCAGTATGGGCTTGGCCCCTATGAAGCAGTAACACCTCTTACAAAGGCAGCAG
ATATCAGCTTAGACAATTTGGTGGAAGGGAAGCGGAAACGGAGATCTAACGTCAGCTCCCCAGCCACC
CCTACTGCCTCCTCGAGC (SEQ ID NO:3)

Any of the 53BP1 DN variants disclosed herein may contain fragments from a native 53BP1 protein from any suitable species (*e.g.*, human, monkey, chimpanzee, mouse, rat, pig, etc.). Alternatively, it may contain a functional variant of the fragment from the native counterpart. A functional variant would maintain substantially similar bioactivity of the functional domains contained in the fragment of the native counterpart and share a high amino acid sequence homology with the native counterpart (*e.g.*, at least 85%, at least 90%, at least 95%, at least 97%, at least 98% or above). The "percent identity" of two amino acid sequences is determined using the algorithm of Karlin and Altschul Proc. Natl. Acad. Sci. USA 87:2264-68, 1990, modified as in Karlin and Altschul

Proc. Natl. Acad. Sci. USA 90:5873-77, 1993. Such an algorithm is incorporated into the NBLAST and XBLAST programs (version 2.0) of Altschul, et al. J. Mol. Biol. 215:403-10, 1990. BLAST protein searches can be performed with the XBLAST program, score=50, wordlength=3 to obtain amino acid sequencfes homologous to the protein molecules of interest. Where gaps exist between two sequences, Gapped BLAST can be utilized as described in Altschul et al., Nucleic Acids Res. 25(17):3389-3402, 1997. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (e.g., XBLAST and NBLAST) can be used.

In some instances, a functional variant may contain conservative amino acid residue substitutions relative to the native counterpart. As used herein, a "conservative amino acid substitution" refers to an amino acid substitution that does not alter the relative charge or size characteristics of the protein in which the amino acid substitution is made. Variants can be prepared according to methods for altering polypeptide sequence known to one of ordinary skill in the art such as are found in references which compile such methods, e.g. Molecular Cloning: A Laboratory Manual, J. Sambrook, et al., eds., Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989, or Current Protocols in Molecular Biology, F.M. Ausubel, et al., eds., John Wiley & Sons, Inc., New York. Conservative substitutions of amino acids include substitutions made amongst amino acids within the following groups: (a) M, I, L, V; (b) F, Y, W; (c) K, R, H; (d) A, G; (e) S, T; (f) Q, N; and (g) E, D.

Any of the 53BP1 DN variants disclosed herein may have a length of up to 200-amino acid, up to 300-amino acid, 400-amino acid, up to 450-amino acid, up to 500-amino acid, up to 550-amino acid, up to 600-amino acid, or up to 700-amino acid.

(B) Gene-Editing Nuclease Enzyme

Any of the nucleases used in commonly known gene-editing methods can be used in making the fusion polypeptides disclosed herein. Genome editing methods are generally classified based on the type of endonuclease that is involved in generating double stranded breaks in the target nucleic acid. In some embodiments, the gene-editing nuclease enzyme disclosed herein is an RNA-guided endonuclease, which cleaves DNA at a site specific to a guide RNA. Exemplary gene-editing nuclease enzymes include, but are not limited to, zinc finger nucleases (ZFN), transcription activator-like effector-based

nuclease (TALEN), meganucleases, and Cas9 or variants thereof (*e.g.*, Cas12) for use in the CRISPR/Cas systems.

Zinc-finger nucleases (ZFNs) are artificial restriction enzymes generated by fusing a zinc finger DNA-binding domain to a DNA-cleavage domain. Zinc finger domains can be engineered to target specific desired DNA sequences and this enables zinc-finger nucleases to target unique sequences within complex genomes. By taking advantage of endogenous DNA repair machinery, these reagents can be used to precisely alter the genomes of higher organisms.

Transcription activator-like effector nucleases (TALEN) are restriction enzymes that can be engineered to cut specific sequences of DNA. They are made by fusing a TAL effector DNA-binding domain to a DNA cleavage domain. TALEs can be engineered to bind to practically any desired DNA sequence, so when combined with a nuclease, DNA can be cut at specific locations. The restriction enzymes can be introduced into cells, for use in gene editing. Exemplary TALEN nucleases can be found at GenBank Accession No. AKB90849 or GenBank Accession No. AKB90848.

Cas9 enzymes together with CRISPR sequences form the basis of a technology known as CRISPR/Cas9 that can be used to edit genes within organisms. This type of gene editing process has a wide variety of applications including use as a basic biology research tool, development of biotechnology products, and potentially to treat diseases.

Meganucleases are endodeoxyribonucleases characterized by a large recognition site (double-stranded DNA sequences of 12 to 40 base pairs); as a result this site generally occurs only once in any given genome. Exemplary meganucleases for use in gene editing include homing endonucleases. Meganucleases can be used to replace, eliminate or modify sequences in a highly targeted way. By modifying their recognition sequence through protein engineering, the targeted sequence can be changed.

Cas9 (CRISPR associated protein 9) is an RNA-guided DNA endonuclease enzyme used in the CRISPR technology for gene editing. In some embodiments, the Cas9 enzyme can be from Streptococcus pyogenes. Cas9 proteins have been routinely used as a genome engineering tool to induce site-directed double strand breaks in DNA. These breaks can lead to gene inactivation or the introduction of heterologous genes through non-homologous end joining and homologous recombination respectively in many laboratory model organisms. When fused with the 53BP1 DN variants disclosed herein,

the resultant fusion polypeptides can be used in CRISPR systems to inhibit NHEJ and enhance repair via homologous recombination. Exemplary Cas9 proteins for use in the present disclosure includes those encoded by the nucleotide sequences shown in Figures 8 and 9.

In some embodiments, the Cas endonuclease is a Cas9 enzyme or variant thereof. In some embodiments, the Cas9 endonuclease is derived from *Streptococcus pyogenes*, *Staphylococcus aureus*, *Neisseria meningitidis*, *Streptococcus thermophilus*, or *Treponema denticola*. In some embodiments, the nucleotide sequence encoding the Cas endonuclease may be codon optimized for expression in a host cell. In some embodiments, the endonuclease is a Cas9 homolog or ortholog.

In some embodiments, the nucleotide sequence encoding the Cas9 endonuclease is further modified to alter the activity of the protein. In some embodiments, the Cas9 endonuclease is a catalytically inactive Cas9. For example, dCas9 contains mutations of catalytically active residues (D10 and H840) and does not have nuclease activity. Alternatively or in addition, the Cas9 endonuclease may be fused to another protein or portion thereof. In some embodiments, dCas9 is fused to a repressor domain, such as a KRAB domain. In some embodiments, such dCas9 fusion proteins are used with the constructs described herein for multiplexed gene repression (e.g. CRISPR interference (CRISPRi)). In some embodiments, dCas9 is fused to an activator domain, such as VP64 or VPR. In some embodiments, such dCas9 fusion proteins are used with the constructs described herein for gene activation (*e.g.*, CRISPR activation (CRISPRa)). In some embodiments, dCas9 is fused to an epigenetic modulating domain, such as a histone demethylase domain or a histone acetyltransferase domain. In some embodiments, dCas9 is fused to a LSD1 or p300, or a portion thereof. In some embodiments, the dCas9 fusion is used for CRISPR-based epigenetic modulation. In some embodiments, dCas9 or Cas9 is fused to a Fok1 nuclease domain. In some embodiments, Cas9 or dCas9 fused to a Fok1 nuclease domain is used for genome editing. In some embodiments, Cas9 or dCas9 is fused to a fluorescent protein (*e.g.,* GFP, RFP, mCherry, etc.). In some embodiments, Cas9/dCas9 proteins fused to fluorescent proteins are used for labeling and/or visualization of genomic loci or identifying cells expressing the Cas endonuclease.

Provided below are amino acid sequences of two exemplary Cas9 proteins:

DKKYSIGLDIGTNSVGWAVITDEYKVPSKKFKVLGNTDRHSIKKNLIGALLFDSGETAEATRLKRTAR
RRYTRRKNRICYLQEIFSNEMAKVDDSFFHRLEESFLVEEDKKHERHPIFGNIVDEVAYHEKYPTIYH
LRKKLVDSTDKADLRLIYLALAHMIKFRGHFLIEGDLNPDNSDVDKLFIQLVQTYNQLFEENPINASG
VDAKAILSARLSKSRRLENLIAQLPGEKKNGLFGNLIALSLGLTPNFKSNFDLAEDAKLQLSKDTYDD
DLDNLLAQIGDQYADLFLAAKNLSDAILLSDILRVNTEITKAPLSASMIKRYDEHHQDLTLLKALVRQ
QLPEKYKEIFFDQSKNGYAGYIDGGASQEEFYKFIKPILEKMDGTEELLVKLNREDLLRKQRTFDNGS
IPHQIHLGELHAILRRQEDFYPFLKDNREKIEKILTFRIPYYVGPLARGNSRFAWMTRKSEETITPWN
FEEVVDKGASAQSFIERMTNFDKNLPNEKVLPKHSLLYEYFTVYNELTKVKYVTEGMRKPAFLSGEQK
KAIVDLLFKTNRKVTVKQLKEDYFKKIECFDSVEISGVEDRFNASLGTYHDLLKIIKDKDFLDNEENE
DILEDIVLTLTLFEDREMIEERLKTYAHLFDDKVMKQLKRRRYTGWGRLSRKLINGIRDKQSGKTILD
FLKSDGFANRNFMQLIHDDSLTFKEDIQKAQVSGQGDSLHEHIANLAGSPAIKKGILQTVKVVDELVK
VMGRHKPENIVIEMARENQTTQKGQKNSRERMKRIEEGIKELGSQILKEHPVENTQLQNEKLYLYYLQ
NGRDMYVDQELDINRLSDYDVDHIVPQSFLADDSIDNKVLTRSDKNRGKSDNVPSEEVVKKMKNYWRQ
LLNAKLITQRKFDNLTKAERGGLSELDKAGFIKRQLVETRQITKHVAQILDSRMNTKYDENDKLIREV
KVITLKSKLVSDFRKDFQFYKVREINNYHHAHDAYLNAVVGTALIKKYPALESEFVYGDYKVYDVRKM
IAKSEQEIGKATAKYFFYSNIMNFFKTEITLANGEIRKAPLIETNGETGEIVWDKGRDFATVRKVLSM
PQVNIVKKTEVQTGGFSKESILPKRNSDKLIARKKDWDPKKYGGFDSPTVAYSVLVVAKVEKGKSKKL
KSVKELLGITIMERSSFEKNPIDFLEAKGYKEVKKDLIIKLPKYSLFELENGRKRMLASAGELQKGNE
LALPSKYVNFLYLASHYEKLKGSPEDNEQKQLFVEQHKHYLDEIIEQISEFSKRVILADANLDKVLSA
YNKHRDKPIREQAENIIHLFTLTNLGAPAAFKYFDTTIDRKRYTSTKEVLDATLIHQSITGLYETRID
LSQLGGD (SEQ ID NO:4)

MDKKYSIGLDIGTNSVGWAVITDEYKVPSKKFKVLGNTDRHSIKKNLIGALLFDSGETAEATRLKRTA
RRRYTRRKNRICYLQEIFSNEMAKVDDSFFHRLEESFLVEEDKKHERHPIFGNIVDEVAYHEKYPTIY
HLRKKLVDSTDKADLRLIYLALAHMIKFRGHFLIEGDLNPDNSDVDKLFIQLVQTYNQLFEENPINAS
GVDAKAILSARLSKSRRLENLIAQLPGEKKNGLFGNLIALSLGLTPNFKSNFDLAEDAKLQLSKDTYD
DDLDNLLAQIGDQYADLFLAAKNLSDAILLSDILRVNTEITKAPLSASMIKRYDEHHQDLTLLKALVR
QQLPEKYKEIFFDQSKNGYAGYIDGGASQEEFYKFIKPILEKMDGTEELLVKLNREDLLRKQRTFDNG
SIPHQIHLGELHAILRRQEDFYPFLKDNREKIEKILTFRIPYYVGPLARGNSRFAWMTRKSEETITPW
NFEEVVDKGASAQSFIERMTNFDKNLPNEKVLPKHSLLYEYFTVYNELTKVKYVTEGMRKPAFLSGEQ
KKAIVDLLFKTNRKVTVKQLKEDYFKKIECFDSVEISGVEDRFNASLGTYHDLLKIIKDKDFLDNEEN
EDILEDIVLTLTLFEDREMIEERLKTYAHLFDDKVMKQLKRRRYTGWGRLSRKLINGIRDKQSGKTIL
DFLKSDGFANRNFMQLIHDDSLTFKEDIQKAQVSGQGDSLHEHIANLAGSPAIKKGILQTVKVVDELV
KVMGRHKPENIVIEMARENQTTQKGQKNSRERMKRIEEGIKELGSQILKEHPVENTQLQNEKLYLYYL
QNGRDMYVDQELDINRLSDYDVDHIVPQSFLKDDSIDNKVLTRSDKNRGKSDNVPSEEVVKKMKNYWR
QLLNAKLITQRKFDNLTKAERGGLSELDKAGFIKRQLVETRQITKHVAQILDSRMNTKYDENDKLIRE
VKVITLKSKLVSDFRKDFQFYKVREINNYHHAHDAYLNAVVGTALIKKYPKLESEFVYGDYKVYDVRK
MIAKSEQEIGKATAKYFFYSNIMNFFKTEITLANGEIRKRPLIETNGETGEIVWDKGRDFATVRKVLS
MPQVNIVKKTEVQTGGFSKESILPKRNSDKLIARKKDWDPKKYGGFDSPTVAYSVLVVAKVEKGKSKK
LKSVKELLGITIMERSSFEKNPIDFLEAKGYKEVKKDLIIKLPKYSLFELENGRKRMLASAGELQKGN
ELALPSKYVNFLYLASHYEKLKGSPEDNEQKQLFVEQHKHYLDEIIEQISEFSKRVILADANLDKVLS
AYNKHRDKPIREQAENIIHLFTLTNLGAPAAFKYFDTTIDRKRYTSTKEVLDATLIHQSITGLYETRI
DLSQLGGD (SEQ ID NO:5)

Alternatively or in addition, the Cas endonuclease is a Cpf1 nuclease. In some embodiments, the host cell expresses a Cpf1 nuclease derived from *Provetella spp.* or *Francisella spp.* In some embodiments, the nucleotide sequence encoding the Cpf1 nuclease may be codon optimized for expression in a host cell. Exemplary Cpf1 nucleases can be found under, e.g., GenBank accession no. ASK09413 and GenBank accession no. A0Q7Q2.

(C) Preparation of Fusion Polypeptides

Any of the fusion polypeptides can be prepared via routine recombinant technology. For example, the coding sequences of the 53BP1 DN variant and the gene-editing nuclease enzyme can be fused in-frame via routine technology, either directed or via any linker, and cloned into a suitable vector and the recombinant protein generated. Alternatively, the fusion polypeptide and gene editing nuclease can be synthesized using peptide synthesis technology. In other circumstances, the 53BP1 DN fusion with a gene editing nuclease can be expressed as mRNA. The coding sequence can also be in operable in DNA or RNA viruses, expressed linkage to a suitable promoter (*e.g.*, a mammalian promoter) for expression of the fusion polypeptide in a suitable host cell.

Vectors of the present disclosure can drive the expression of one or more sequences in mammalian cells using a mammalian expression vector. Examples of mammalian expression vectors include pCDM8 (Seed, *Nature* (1987) 329: 840) and pMT2PC (Kaufman, et al., *EMBO J.* (1987) 6: 187). When used in mammalian cells, the expression vector's control functions are typically provided by one or more regulatory elements. For example, commonly used promoters are derived from polyoma, adenovirus 2, cytomegalovirus, simian virus 40, and others disclosed herein and known in the art. For other suitable expression systems for both prokaryotic and eukaryotic cells see, *e.g.*, Chapters 16 and 17 of Sambrook, ct al., MOLECULAR CLONING: A LABORATORY MANUAL. 2nd eds., Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989.

The vectors of the present disclosure are capable of directing expression of the nucleic acid preferentially in a particular cell type (*e.g.*, tissue- specific regulatory elements are used to express the nucleic acid). Such regulatory elements include promoters that may be tissue specific or cell specific. The term “tissue specific” as it applies to a promoter refers to a promoter that is capable of directing selective expression of a nucleotide sequence of interest to a specific type of tissue (*e.g.*, seeds) in the relative absence of expression of the same nucleotide sequence of interest in a different type of tissue. The term “cell type specific” as applied to a promoter refers to a promoter that is capable of directing selective expression of a nucleotide sequence of interest in a specific type of cell in the relative absence of expression of the same nucleotide sequence of interest in a different type of cell within the same tissue. The term “cell type specific” when applied to a promoter also means a promoter capable of promoting selective expression of a nucleotide sequence of interest in a region

within a single tissue. Cell type specificity of a promoter may be assessed using methods well known in the art, *e.g.*, immunohistochemical staining.

Non-limiting examples of viral vectors include, but are not limited to, retroviral vectors (*e.g.*, lentiviral vectors or gammaretroviral vectors), adenoviral vectors, adeno-associated viral vectors (AAV), and hybrid vectors (containing components from different viral genomes). Additional examples of viral vectors are provided in US Patent Patent No. 5,698,443, US Patent No. 5,650,309, and US Patent No, 5,827,703, the relevant disclosures of each of which are herein incorporated by reference for the purpose and subject matter referenced herein.

Any of the nucleic acids encoding the fusion polypeptides disclosed herein, vectors comprising such, and host cells comprising the vectors are within the scope of the present disclosure.

Two exemplary fusion polypeptides, both containing the 53BP1 DN1S variant linked to a Cas9 protein via a TGS linker, are provided below (including both amino acid sequences and nucleotide sequences):

DKKYSIGTDIGTNSVGWAVITDEYKVPSKKFKVTGNTDRHSIKKNTIGATTFDSGETAEATRTKRT
ARRRYTRRKNRICYTQEIFSNEMAKVDDSFFHRTEESFTVEEDKKHERHPIFGNIVDEVAYHEKYP
TIYHTRKKTVDSTDKADTRTIYTATAHMIKFRGHFTIEGDTNPDNSDVDKTFIQTVQTYNQTFEEN
PINASGVDAKAITSARTSKSRRTENTIAQTPGEKKNGTFGNTIATSTGTTPNFKSNFDTAEDAKTQ
TSKDTYDDDTDNTTAQIGDQYADTFTAAKNTSDAITTSDITRVNTEITKAPTSASMIKRYDEHHQD
TTTTKATVRQQTPEKYKEIFFDQSKNGYAGYIDGGASQEEFYKFIKPITEKMDGTEETTVKTNRED
TTRKQRTFDNGSIPHQIHTGETHAITRRQEDFYPFTKDNREKIEKITTFRIPYYVGPTARGNSRFA
WMTRKSEETITPWNFEEVVDKGASAQSFIERMTNFDKNTPNEKVTPKHSTTYEYFTVYNETTKVKY
VTEGMRKPAFTSGEQKKAIVDTTFKTNRKVTVKQTKEDYFKKIECFDSVEISGVEDRFNASTGTYH
DTTKIIKDKDFTDNEENEDITEDIVTTTTTFEDREMIEERTKTYAHTFDDKVMKQTKRRRYTGWGR
TSRKTINGIRDKQSGKTITDFTKSDGFANRNFMQTIHDDSTTFKEDIQKAQVSGQGDSTHEHIANT
AGSPAIKKGITQTVKVVDETVKVMGRHKPENIVIEMARENQTTQKGQKNSRERMKRIEEGIKETGS
QITKEHPVENTQTQNEKTYTYYTQNGRDMYVDQETDINRTSDYDVDHIVPQSFTADDSIDNKVTTR
SDKNRGKSDNVPSEEVVKKMKNYWRQTTNAKTITQRKFDNTTKAERGGTSETDKAGFIKRQTVETR
QITKHVAQITDSRMNTKYDENDKTIREVKVITTKSKTVSDFRKDFQFYKVREINNYHHAHDAYTNA
VVGTATIKKYPATESEFVYGDYKVYDVRKMIAKSEQEIGKATAKYFFYSNIMNFFKTEITTANGEI
RKAPTIETNGETGEIVWDKGRDFATVRKVTSMPQVNIVKKTEVQTGGFSKESITPKRNSDKTIARK
KDWDPKKYGGFDSPTVAYSVTVVAKVEKGKSKKTKSVKETTGITIMERSSFEKNPIDFTEAKGYKE
VKKDTIIKTPKYSTFETENGRKRMTASAGETQKGNETATPSKYVNFTYTASHYEKTKGSPEDNEQK
QTFVEQHKHYTDEIIEQISEFSKRVITADANTDKVTSAYNKHRDKPIREQAENIIHTFTTTNTGAP
AAFKYFDTTIDRKRYTSTKEVTDATTIHQSITGTYETRIDTSQTGGD***TGSTGSTGSTGSMG***PHGHV
LHRHMRTIREVRTTVTRVITDVYYVDGTEVERKVTEETEEPIVECQECETEVSPSQTGGSSGDTGD
ISSFSSKASSLHRTSSGTSTSAMHSSGSSGKGAGPTRGKTSGTEPADFALPSSRGGPGKTSPRKGV
SQTGTPVCEEDGDAGTGIRQGGKAPVTPRGRGRRGRPPSRTTGTRETAVPGPLGMEDISPNLSPDD
KSFSRVVPRVPDSTRRTDVGAGALRRSDSPEIPFQAAAGPSDGLDASSPGNSFVGTRVVAKWSSNG
YFYSGKITRDVGAGKYKLTFDDGYECDVLGKDITLCDPIPTDTEVTATSEDEYFSAGVVKGHRKES

GETYYSIEKEGQRKWYKRMAVITSLEQGNRTREQYGTGPYEAVTPTTKAADISLDNLVEGKRKRRS
NVSSPATPTASSS (SEQ ID NO:6)

GACAAGAAGTACAGCATCGGCCTGGACATCGGCACCAACTCTGTGGGCTGGGCCGTGATCACCGACGAGTACAAG
GTGCCCAGCAAGAAATTCAAGGTGCTGGGCAACACCGACCGGCACAGCATCAAGAAGAACCTGATCGGAGCCCTG
CTGTTCGACAGCGGCGAAACAGCCGAGGCCACCCGGCTGAAGAGAACCGCCAGAAGAAGATACACCAGACGGAAG
AACCGGATCTGCTATCTGCAAGAGATCTTCAGCAACGAGATGGCCAAGGTGGACGACAGCTTCTTCCACAGACTG
GAAGAGTCCTTCCTGGTGGAAGAGGATAAGAAGCACGAGCGGCACCCCATCTTCGGCAACATCGTGGACGAGGTG
GCCTACCACGAGAAGTACCCCACCATCTACCACCTGAGAAAGAAACTGGTGGACAGCACCGACAAGGCCGACCTG
CGGCTGATCTATCTGGCCCTGGCCCACATGATCAAGTTCCGGGGCCACTTCCTGATCGAGGGCGACCTGAACCCC
GACAACAGCGACGTGGACAAGCTGTTCATCCAGCTGGTGCAGACCTACAACCAGCTGTTCGAGGAAAACCCCATC
AACGCCAGCGGCGTGGACGCCAAGGCCATCCTGTCTGCCAGACTGAGCAAGAGCAGACGGCTGGAAAATCTGATC
GCCCAGCTGCCCGGCGAGAAGAAGAATGGCCTGTTCGGAAACCTGATTGCCCTGAGCCTGGGCCTGACCCCCAAC
TTCAAGAGCAACTTCGACCTGGCCGAGGATGCCAAACTGCAGCTGAGCAAGGACACCTACGACGACGACCTGGAC
AACCTGCTGGCCCAGATCGGCGACCAGTACGCCGACCTGTTTCTGGCCGCCAAGAACCTGTCCGACGCCATCCTG
CTGAGCGACATCCTGAGAGTGAACACCGAGATCACCAAGGCCCCCCTGAGCGCCTCTATGATCAAGAGATACGAC
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NO:9)

The sequences above in boldface and italicized refer to the linker amino acid and coding nucleotide sequences. The option at the N-terminal or 5' end of the linker sequences are the Cas9 protein and the option at the C-terminal or 3' end of the linker sequences are the 53BP1 DN1 variant.

Uses of Fusion Polypeptides in Gene Editing

Any of the fusion polypeptides disclosed herein can be used in gene editing, following routine methodology associated with the specific gene-editing nuclease contained in the fusion polypeptide. For example, the fusion polypeptide, or a suitable vector encoding such, can be delivered into host cells where gene-editing is needed. gRNAs specific to the genetic site to be edited and optionally a template nucleic acid guiding homologous recombination can be co-delivered into the host cells via routine methods, *e.g.*, electroporation of nucleic acid or RNP complex, or viral particle infection.

In one example, the fusion polypeptide comprises a Cas protein (a Cas9 protein or a homolog thereof such as Cas12) fused to a 53BP1 DN variant. Such a fusion polypeptide can be used in the CRISPR-Cas system to edit a specific gene of interest. CRISPR-Cas system has been successfully utilized to edit the genomes of various organisms, including, but not limited to bacteria, humans, fruit flies, zebra fish and plants. See, *e.g.*, Jiang et al., *Nature Biotechnology* (2013) 31(3):233; Qi et al, *Cell* (2013) 5:1173; DiCarlo et al., *Nucleic Acids Res.* (2013) 7:4336; Hwang et al., *Nat. Biotechnol* (2013), 3:227); Gratz et al., *Genetics* (2013) 194:1029; Cong et al., *Science* (2013) 6121:819; Mali et al., *Science* (2013) 6121:823; Cho et al. *Nat. Biotechnol* (2013) 3: 230; and Jiang et al., *Nucleic Acids Research* (2013) 41(20):el88.

The method disclosed herein may utilize the CRISPR/Cas9 system that hybridizes with a target sequence in a gene of interest, where the CRISPR/Cas9 system comprises a Cas9/53BP1 DN variant fusion polypeptide and an engineered crRNA/tracrRNA (or a single guide RNA). CRISPR/Cas9 complex can bind to the genetic site to be edited and allow the cleavage of the target site, thereby modifying the gene of interest.

The CRISPR/Cas system of the present disclosure may bind to and/or cleave the gene of interest in a coding or non-coding region, within or adjacent to the gene, such as, for example, a leader sequence, trailer sequence or intron, or within a non-transcribed region, either upstream or downstream of the coding region. The guide RNAs (gRNAs) used in the present disclosure may be designed such that the gRNA directs binding of the Cas9-gRNA complexes to a pre-determined cleavage sites (target site) in a genome. The cleavage sites may be chosen so as to release a fragment that contains a region of unknown sequence, or a region containing a SNP, nucleotide insertion, nucleotide deletion, rearrangement, etc.

Cleavage of a gene region may comprise cleaving one or two strands at the location of the target sequence by the Cas enzyme. In one embodiment, such, cleavage can result in decreased transcription of a target gene. In another embodiment, the cleavage can further comprise repairing the cleaved target polynucleotide by homologous recombination with an exogenous template polynucleotide, wherein the repair results in an insertion, deletion, or substitution of one or more nucleotides of the target polynucleotide. It is expected that the repair efficiency via homologous recombination would be enhanced when a Cas/53BP1 DN variant fusion polypeptide is used.

The terms "gRNA" and "guide RNA" may be used interchangeably throughout and refer to a nucleic acid comprising a sequence that determines the specificity of a Cas DNA binding protein of a CRISPR/Cas system. A gRNA hybridizes to (complementary to, partially or completely) a target nucleic acid sequence in the genome of a host cell. The gRNA or portion thereof that hybridizes to the target nucleic acid may be between 15-25 nucleotides, 18-22 nucleotides, or 19-21 nucleotides in length. In some embodiments, the gRNA sequence that hybridizes to the target nucleic acid is 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 nucleotides in length. In some embodiments, the gRNA sequence that hybridizes to the target nucleic acid is between 10-30, or between 15-25, nucleotides in length.

In addition to a sequence that binds to a target nucleic acid, in some embodiments, the gRNA also comprises a scaffold sequence. Expression of a gRNA encoding both a sequence complementary to a target nucleic acid and scaffold sequence has the dual function of both binding (hybridizing) to the target nucleic acid and recruiting the endonuclease to the target nucleic acid, which may result in site-specific CRISPR activity. In some embodiments, such a chimeric gRNA may be referred to as a single guide RNA (sgRNA).

As used herein, a "scaffold sequence," also referred to as a tracrRNA, refers to a nucleic acid sequence that recruits a Cas endonuclease to a target nucleic acid bound (hybridized) to a complementary gRNA sequence. Any scaffold sequence that comprises at least one stem loop structure and recruits an endonuclease may be used in the genetic elements and vectors described herein. Exemplary scaffold sequences will be evident to one of skill in the art and can be found, for example, in Jinek, *et al. Science* (2012) 337(6096):816-821, Ran, *et al. Nature Protocols* (2013) 8:2281-2308, PCT Application No. WO2014/093694, and PCT Application No. WO2013/176772.

In some embodiments, the gRNA sequence does not comprises a scaffold sequence and a scaffold sequence is expressed as a separate transcript. In such embodiments, the gRNA sequence further comprises an additional sequence that is complementary to a portion of the scaffold sequence and functions to bind (hybridize) the scaffold sequence and recruit the endonuclease to the target nucleic acid.

In some embodiments, the gRNA sequence is at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or at least 100% complementary to a target nucleic acid (see also US Patent 8,697,359, which is incorporated by reference for its teaching of complementarity of a gRNA sequence with a target polynucleotide sequence). It has been demonstrated that mismatches between a CRISPR guide sequence and the target nucleic acid near the 3' end of the target nucleic acid may abolish nuclease cleavage activity (Upadhyay, et al. *Genes Genome Genetics* (2013) 3(12):2233-2238). In some embodiments, the gRNA sequence is at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or at least 100% complementary to the 3' end of the target nucleic acid (*e.g.*, the last 5, 6, 7, 8, 9, or 10 nucleotides of the 3' end of the target nucleic acid).

The target nucleic acid is flanked on the 3' side by a protospacer adjacent motif (PAM) that may interact with the endonuclease and be further involved in targeting the endonuclease activity to the target nucleic acid. It is generally thought that the PAM sequence flanking the target nucleic acid depends on the endonuclease and the source from which the endonuclease is derived. For example, for Cas9 endonucleases that are derived from *Streptococcus pyogenes*, the PAM sequence is NGG. For Cas9 endonucleases derived from *Staphylococcus aureus*, the PAM sequence is NNGRRT. For Cas9 endonucleases that are derived from *Neisseria meningitidis*, the PAM sequence is NNNNGATT. For Cas9 endonucleases derived from *Streptococcus thermophilus*, the PAM sequence is NNAGAA. For Cas9 endonuclease derived from *Treponema denticola*, the PAM sequence is NAAAAC. For a Cpf1 nuclease, the PAM sequence is TTN.

In some embodiments, genetically engineering a cell also comprises introducing a Cas endonuclease into the cell. In some embodiments, the Cas endonuclease and the nucleic acid encoding the gRNA are provided on the same nucleic acid (*e.g.*, a vector). In some embodiments, the Cas endonuclease and the nucleic acid encoding the gRNA are provided on different nucleic acids (*e.g.*, different vectors). Alternatively or in addition, the Cas endonuclease may be provided or introduced into the cell in protein form.

The present disclosure further provides engineered, non-naturally occurring vectors and vector systems, which can encode one or more components of a CRISPR/Cas9 complex, wherein the vector comprises a polynucleotide encoding (i) a (CRISPR)-Cas system guide RNA that hybridizes to the gene of interest and (ii) a Cas9/53BP1 DN variant fusion polypeptide.

Conventional viral and non-viral based gene transfer methods can be used to introduce nucleic acids encoding CRISPR/Cas9 in mammalian cells or target tissues. Such methods can be used to administer nucleic acids encoding components of a CRISPR-Cas system to cells in culture, or in a host organism. Non-viral vector delivery systems include DNA plasmids, RNA (*e.g.*, a transcript of a vector described herein), naked nucleic acid, and nucleic acid complexed with a delivery vehicle. Viral vector delivery systems include DNA and RNA viruses, which have either episomal or integrated genomes after delivery to the cell. For a review of gene therapy procedures.

Viral vectors can be administered directly to patients (*in vivo*) or they can be used to manipulate cells *in vitro* or *ex vivo*, where the modified cells may be administered to patients. In one embodiment, the present disclosure utilizes viral based systems including, but not limited to retroviral, lentivirus, adenoviral, adeno-associated and herpes simplex virus vectors for gene transfer. Furthermore, the present disclosure provides vectors capable of integration in the host genome, such as retrovirus or lentivirus. Preferably, the vector used for the expression of a CRISPR-Cas system of the present disclosure is a lentiviral vector.

In one embodiment, the disclosure provides for introducing one or more vectors encoding CRISPR-Cas into eukaryotic cell. The cell can be a cancer cell. Alternatively, the cell is a hematopoietic cell, such as a hematopoietic stem cell. Examples of stem cells include pluripotent, multipotent and unipotent stem cells. Examples of pluripotent stem cells include embryonic stem cells, embryonic germ cells, embryonic carcinoma cells and induced pluripotent stem cells (iPSCs). In a preferred embodiment, the disclosure provides introducing CRISPR-Cas9 into a hematopoietic stem cell.

The vectors of the present disclosure are delivered to the eukaryotic cell in a subject. Modification of the eukaryotic cells via CRISPR/Cas9 system can takes place in a cell culture, where the method comprises isolating the eukaryotic cell from a subject prior to the modification. In some embodiments, the method further comprises returning said eukaryotic cell and/or cells derived therefrom to the subject.

General techniques

The practice of the present disclosure will employ, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature, such as *Molecular Cloning: A Laboratory Manual,* second edition (Sambrook, et al., 1989) Cold Spring Harbor Press; *Oligonucleotide Synthesis* (M. J. Gait, ed. 1984); *Methods in Molecular Biology*, Humana Press; *Cell Biology: A Laboratory Notebook* (J. E. Cellis, ed., 1989) Academic Press; Animal Cell Culture (R. I. Freshney, ed. 1987); Introuction to Cell and Tissue Culture (J. P. Mather and P. E. Roberts, 1998) Plenum Press; Cell and Tissue Culture: Laboratory Procedures (A. Doyle, J. B. Griffiths, and D. G. Newell, eds. 1993-8) J. Wiley and Sons; Methods in Enzymology (Academic Press, Inc.); Handbook of Experimental Immunology (D. M. Weir and C. C. Blackwell, eds.): Gene Transfer Vectors for Mammalian Cells (J. M. Miller and M. P. Calos, eds., 1987); Current Protocols in Molecular Biology (F. M. Ausubel, et al. eds. 1987); PCR: The Polymerase Chain Reaction, (Mullis, et al., eds. 1994); Current Protocols in Immunology (J. E. Coligan et al., eds., 1991); Short Protocols in Molecular Biology (Wiley and Sons, 1999); Immunobiology (C. A. Janeway and P. Travers, 1997); Antibodies (P. Finch, 1997); Antibodies: a practice approach (D. Catty., ed., IRL Press, 1988-1989); Monoclonal antibodies: a practical approach (P. Shepherd and C. Dean, eds., Oxford University Press, 2000); Using antibodies: a laboratory manual (E. Harlow and D. Lane (Cold Spring Harbor Laboratory Press, 1999); The Antibodies (M. Zanetti and J. D. Capra, eds. Harwood Academic Publishers, 1995); *DNA Cloning: A practical Approach,* Volumes I and II (D.N. Glover ed. 1985); *Nucleic Acid Hybridization* (B.D. Hames & S.J. Higgins eds.(1985»; *Transcription and Translation* (B.D. Hames & S.J. Higgins, eds. (1984»; *Animal Cell Culture* (R.I. Freshney, ed. (1986»; *Immobilized Cells and Enzymes* (lRL Press, (1986»; and B. Perbal, *A practical Guide To Molecular Cloning* (1984); F.M. Ausubel *et al.* (eds.).

Without further elaboration, it is believed that one skilled in the art can, based on the above description, utilize the present invention to its fullest extent. The following specific embodiments are, therefore, to be construed as merely illustrative, and not limitative of the remainder of the disclosure in any way whatsoever. All publications cited herein are

incorporated by reference for the purposes or subject matter referenced herein.

Example 1: **Identification of HDR-Enhancer Fragment of 53BP1**

Several dominant negative (DN) p53 binding protein 1 (53BP1) mutants were designed based on presence of binding to DNA double strand breaks (DSB), but inability to recruit other non-homologous end joining (NHEJ). Five DN 53BP1 mutants were designed as shown in Figure 1, panel A. Nucleic acids encoding these DN mutants (with an HA tag) were cloned into a lentiviral vector, which was delivered into host cells (*e.g.*, HeLa cells) for expression. Expression of the HA-tagged DN mutants were examined by Western blot and the results are shown in Figure 1, panels B and C. The results show that the vectors carrying the DN1, DN1s, DN3, and DN4 mutants expressed the recombinant 53BP1 mutants at the correct size. Figure 1, panel B. Expression of DN2 was observed at higher exposure. Figure 1, panel C. Expression of the 53BP1 DN mutants were also observed by in immunofluorescence (IF) imaging via detection of HA and DAP1. Representative immunofluorescence (IF) images showed HA tagged DN1S and endogenous 53BP1 recruitment to the irradiation-induced DNA break site. Mock has no HA whereas HA co-localizes with or even displaces all the endogenous 53BP1 foci at low or high expression of DN1S, respectively. Lesser number of endogenous 53BP1 was observed in cells expressing DN1 mutant, indicating that the mutant successfully replaced endogenous 53BP1 in DNA damage foci.

Next, recruitment of the 53BP1 DN mutants to DNA damage foci in HeLa cells was examined. HeLa cells expression the 53BP1 DN mutants noted above were stained for DAP1 (damage response protein 1), endogenous 53BP1, and HA tag (indicating 53BP1 DN mutants) and analysed by immunofluorescence (IF). Representative immunofluorescence (IF) images showing HA tagged DN1/DN1S and endogenous 53BP1 recruitment to the irradiation-induced DNA break site. Mock has no HA whereas HA co-localizes with all the endogenous 53BP1 foci in DN1/DN1S arm. See also Figure 1, panel D. Similarly, representative immunofluorescence (IF) images showing HA tagged DN1/DN1S and RIF-1 or γH2AX recruitment to the irradiation-induced DNA break site. Mock has no HA and many RIF-1 foci in each cell whereas DN1/DN1S arm with HA+ cells has reduced/no RIF-1 or γH2AX foci in each cell. See also Figure 1, panel E and panel G.

BRCA1 is a key protein in the homology directed repair. Similar results were observed in connection with BRCA-1 recruitment. It is normally present in S phage foci in untreated cells. Representative immunofluorescence (IF) images showing HA tagged DN1S and BRCA-1 recruitment to the DNA break site. Mock has no HA and few BRCA-1 foci in each cell whereas DN1S arm with HA+ cells has higher BRCA-1 foci in each cell. The percentage of cells with BRCA1 foci was significantly increased in HeLa cells containing 53BP1 DN1, DN1s, DN2, and DN4 mutants with DN1 and DN1s having the highest number of cells with BRCA1. Figure 1, panel F.

The results from this study indicate that the designed 53BP1 DN mutants expressed in host cells and are recruited to DNA damage foci in the host cells expressing such. The results also show that the 53BP1 DN mutants can replace endogenous 53BP1 proteins at the DNA damage sites, indicating that the mutants can block activity of the endogenous 53BP1.

Example 2: Cas9-53BP1 DN Fusion Proteins Inhibited NHEJ and Enhanced Homologous Recombination

Fusion proteins containing a 53BP1 DN mutant and Cas9 were constructed via routine recombinant technology. Figure 2, panel A. Plasmids were generated to express each of the four Cas9-53BP1 DN fusion proteins and transfected into 293T cells carrying the Traffic Light Reporter. Expression of the fusion proteins were detected by Western blot analysis as shown in Figure 2, panel B. Representative immunofluorescence (IF) imaging shows HA tagged DN1S or dCas9-DN1S/gRNA and endogenous 53BP1 recruitment to the DNA break site. Figure 2, panel E. Panels C and D show the level of cells having ≥1 HA foci and ≥ 53BP1 foci, respectively. It was also observed that the 53BP1 DN mutants, either alone or in fusion with Cas9, locally inhibited NHEJ and reduced cellular toxicity. Figure 2 panels F and G. Ionizing radiation (IR) induces DSBs and if cellular NHEJ –based repair pathways are compromised, cell undergo apoptosis. Cas9-DN 53BP1 fusion only inhibits NHEJ at Cas9 cut sites and does not sensitize cells to apoptosis in Figure 2, panels F-G.

The Traffic Light Reporter (TLR) system in host cells such as 293T cells was used to examine the effect of Cas9-53BP1 DN fusion proteins in NHEJ repair and HDR. As illustrated in Figure 3, panel A, a TLR reporter system was introduced into 293T cells. The TLR system includes a venus reporter (green) for detecting homologous recombination and a red fluorescent protein (RFP) for detecting NHEJ. Upon introducing a Cas9/53BP1 DN

fusion protein together with the gRNA shown in Figure 3, panel A, the Cas9 enzyme creates a DSB at the site directed by the gRNA. If repair of the DSB is via NHEJ, expression of the the otherwise 'out of frame' RFP reporter via NHEJ would a third of the time cause a frame shift, leading to expression of the RFP reporter in frame, and making cells fluoresce red. If repair of the DSB is via HDR, the venus reporter targeted would become functional and the RFP reporter would be rendered nonfunctional during HDR, resulting in expression of only the venus reporter, making cells with HDR fluoresce green. Figure 3, panel B.

Results from this study show that, relative to the Cas9 protein, the gene editing efficiency via NHEJ was reduced and the gene editing efficiency via HDR was enhanced, particularly when the Cas9-DN1 or Cas9-DN1S fusion proteins were used. Furthermore, use of different tags or linkers, or fusing the DN at the amino terminus or carboxy terminus of Cas9 all showed increased HDR, Figure 3D. These results were observed in various cell lines and at different target genes using different Cas9 nucleases. At rare loci, where HDR is not improved, NHEJ is still very highly significantly reduced, and this is clinically relevant to not cause inadvertent indels or mutations. Figure 4, panels A-C.

Similar results were observed in patient derived EBV transformed B lymphocyte cells using AAVS1 gRNA and Cas9-DN1S fusion protein to target the normal CD18 gene into the AAVS1 locus. CD18 deficiency leads to leukocyte adhesion defect and therefore inability of leukocytes to adhere and kill invading organisms, resulting in immune deficiency. This disease was chosen for two reasons: CD18 surface expression can be detected by flow cytometry, making the readout possible at a single cell level. Second, CD18 deficiency correction requires high level CD18 expression (low levels of CD18 expression from lentivirus vectors do not correct the defect in dogs with LAD, while high expression corrects the disease). It was observed that Cas9-DN resulted in higher HDR and higher bi-allelic HDR, resulting is very high CD18 expression, Figure 5A-C.

The cumulative data from Figure 5 shows that when the DN1S is fused to different site specific nucleases (either SpCas9 or SaCas9), there is a highly significant reduction in site-specific NHEJ. This results in a highly significant increase in HDR. Even occasional loci that show no increase in HDR show a highly significant reduction in NHEJ (error-creating) repair. Moreover, there is not only a quantitative, but also a qualitative improvement in HDR, as observed with higher biallelic HDR in patient derived B lymphocytes.

Furthermore, while we see improved HDR and reduced NHEJ at Cas9 on-target sites, we observe less off target cutting of Cas9-DN at known off target sites described by this gRNA/Cas9 at this locus, Figure 6, suggesting that the fusion of DN 53BP1 to a gene editing nuclease reduces its genotoxicity and lowers its affinity to off target sites.

Overall, we observe that DN 53BP1 fusion to Cas9 gene editing nucleases a) inhibits NHEJ only at sites where gene editing nucleases are designed to cut, b) increases both the quantity of HDR (% cells with HDR) and the quality of HDR (bi-allelic HDR), and c) reduces the off-target cutting of Cas9.

OTHER EMBODIMENTS

All of the features disclosed in this specification may be combined in any combination. Each feature disclosed in this specification may be replaced by an alternative feature serving the same, equivalent, or similar purpose. Thus, unless expressly stated otherwise, each feature disclosed is only an example of a generic series of equivalent or similar features.

From the above description, one skilled in the art can easily ascertain the essential characteristics of the present invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions. Thus, other embodiments are also within the claims.

EQUIVALENTS

While several inventive embodiments have been described and illustrated herein, those of ordinary skill in the art will readily envision a variety of other means and/or structures for performing the function and/or obtaining the results and/or one or more of the advantages described herein, and each of such variations and/or modifications is deemed to be within the scope of the inventive embodiments described herein. More generally, those skilled in the art will readily appreciate that all parameters, dimensions, materials, and configurations described herein are meant to be exemplary and that the actual parameters, dimensions, materials, and/or configurations will depend upon the specific application or applications for which the inventive teachings is/are used. Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific inventive embodiments described herein. It is, therefore, to be

understood that the foregoing embodiments are presented by way of example only and that, within the scope of the appended claims and equivalents thereto, inventive embodiments may be practiced otherwise than as specifically described and claimed. Inventive embodiments of the present disclosure are directed to each individual feature, system, article, material, kit, and/or method described herein. In addition, any combination of two or more such features, systems, articles, materials, kits, and/or methods, if such features, systems, articles, materials, kits, and/or methods are not mutually inconsistent, is included within the inventive scope of the present disclosure.

All definitions, as defined and used herein, should be understood to control over dictionary definitions, definitions in documents incorporated by reference, and/or ordinary meanings of the defined terms.

All references, patents and patent applications disclosed herein are incorporated by reference with respect to the subject matter for which each is cited, which in some cases may encompass the entirety of the document.

The indefinite articles "a" and "an," as used herein in the specification and in the claims, unless clearly indicated to the contrary, should be understood to mean "at least one."

The phrase "and/or," as used herein in the specification and in the claims, should be understood to mean "either or both" of the elements so conjoined, i.e., elements that are conjunctively present in some cases and disjunctively present in other cases. Multiple elements listed with "and/or" should be construed in the same fashion, i.e., "one or more" of the elements so conjoined. Other elements may optionally be present other than the elements specifically identified by the "and/or" clause, whether related or unrelated to those elements specifically identified. Thus, as a non-limiting example, a reference to "A and/or B", when used in conjunction with open-ended language such as "comprising" can refer, in one embodiment, to A only (optionally including elements other than B); in another embodiment, to B only (optionally including elements other than A); in yet another embodiment, to both A and B (optionally including other elements); etc.

As used herein in the specification and in the claims, "or" should be understood to have the same meaning as "and/or" as defined above. For example, when separating items in a list, "or" or "and/or" shall be interpreted as being inclusive, i.e., the inclusion of at least one, but also including more than one, of a number or list of elements, and, optionally, additional unlisted items. Only terms clearly indicated to the contrary, such as "only one of"

or "exactly one of," or, when used in the claims, "consisting of," will refer to the inclusion of exactly one element of a number or list of elements. In general, the term "or" as used herein shall only be interpreted as indicating exclusive alternatives (i.e. "one or the other but not both") when preceded by terms of exclusivity, such as "either," "one of," "only one of," or "exactly one of." "Consisting essentially of," when used in the claims, shall have its ordinary meaning as used in the field of patent law.

As used herein in the specification and in the claims, the phrase "at least one," in reference to a list of one or more elements, should be understood to mean at least one element selected from any one or more of the elements in the list of elements, but not necessarily including at least one of each and every element specifically listed within the list of elements and not excluding any combinations of elements in the list of elements. This definition also allows that elements may optionally be present other than the elements specifically identified within the list of elements to which the phrase "at least one" refers, whether related or unrelated to those elements specifically identified. Thus, as a non-limiting example, "at least one of A and B" (or, equivalently, "at least one of A or B," or, equivalently "at least one of A and/or B") can refer, in one embodiment, to at least one, optionally including more than one, A, with no B present (and optionally including elements other than B); in another embodiment, to at least one, optionally including more than one, B, with no A present (and optionally including elements other than A); in yet another embodiment, to at least one, optionally including more than one, A, and at least one, optionally including more than one, B (and optionally including other elements); etc.

It should also be understood that, unless clearly indicated to the contrary, in any methods claimed herein that include more than one step or act, the order of the steps or acts of the method is not necessarily limited to the order in which the steps or acts of the method are recited.

CLAIMS

What Is Claimed Is:

1. A fusion polypeptide, comprising a gene-editing nuclease enzyme and a dominant-negative variant of a p53 binding protein 1 (53BP1), wherein the dominant-negative variant of 53BP1 is a truncated 53BP1, which comprises (a) deletion in a docking domain, (b) a deletion of a BRCT domain, or (c) both (a) and (b).

2. The fusion polypeptide of claim 1, wherein the dominant negative variant of 53BP1 comprises (a) a deletion of region 1-1231 of SEQ ID NO:1 or a portion thereof, (b) a deletion of region 1711-1972 of SEQ ID NO:1 or a portion thereof, or (c) both (a) and (b).

3. The fusion polypeptide of claim 1 or claim 2, wherein the gene-editing nuclease enzyme is a Cas9 enzyme, a Cas12 enzyme, a zinc finger nuclease (ZFN) or a transcription activator-like effector nuclease (TALEN) or a meganuclease.

4. The fusion polypeptide of claim 3, wherein the Cas9 enzyme comprises the amino acid sequence of SEQ ID NO:4 or SEQ ID NO:5.

5. The fusion polypeptide of any one of claims 1-4, wherein the dominant-negative variant of 53BP1 comprises region 1480-1644 of SEQ ID NO:1.

6. The fusion polypeptide of claim 5, wherein the dominant-negative variant of 53BP1 comprises region 1231-1644 of SEQ ID NO:1, region 1231-1711 of SEQ ID NO:1, or 1480-1711 of SEQ ID NO:1.

7. The fusion polypeptide of claim 6, wherein the dominant-negative variant of 53BP1 consists of 1231-1711 of SEQ ID NO:1, 1231-1644 of SEQ ID NO:1, or 1480-1711 of SEQ ID NO:1.

8. The fusion polypeptide of any one of claims 1-7, wherein the gene-editing nuclease enzyme is covalently linked directly to the dominant-negative variant of 53BP1.

9. The fusion polypeptide of any one of claims 1-8, wherein the fusion polypeptide comprises the amino acid sequence of SEQ ID NO:6 or SEQ ID NO:8.

10. A nucleic acid, comprising a nucleotide sequence coding for a fusion polypeptide or mRNA of any one of claims 1-9.

11. A vector comprising the nucleic acid of claim 10, wherein the nucleotide sequence coding for the fusion polypeptide is in operable linkage to a promoter.

12. The vector of claim 11, wherein the promoter is a mammalian promoter.

13. The vector of claim 11 or claim 12, which is a viral vector.

14. The vector of claim 13, wherein the viral vector is a retroviral vector, an adenoviral vector, an adeno-associated viral vector, or a hybrid vector.

15. The vector of claim 14, wherein the viral vector is a retroviral vector, which is a lentiviral vector, a foamy virus vector or a gamma retrovirus vector.

16. The vector of any one of claims 11-15, further comprising a nucleotide sequence coding for a guide RNA.

17. A method for enhancing homology directed DNA repair (HDR) in gene editing of a cell, comprising introducing into a cell (a) the fusion polypeptide of any one of claims 1-9 or a vector that comprises a nucleotide sequence coding for the fusion polypeptide.

18. The method of claim 17, further comprising introducing into the cell (b) a guide RNA targeting a gene of interest.

19. The method of claim 18, wherein the guide RNA is a single guide RNA.

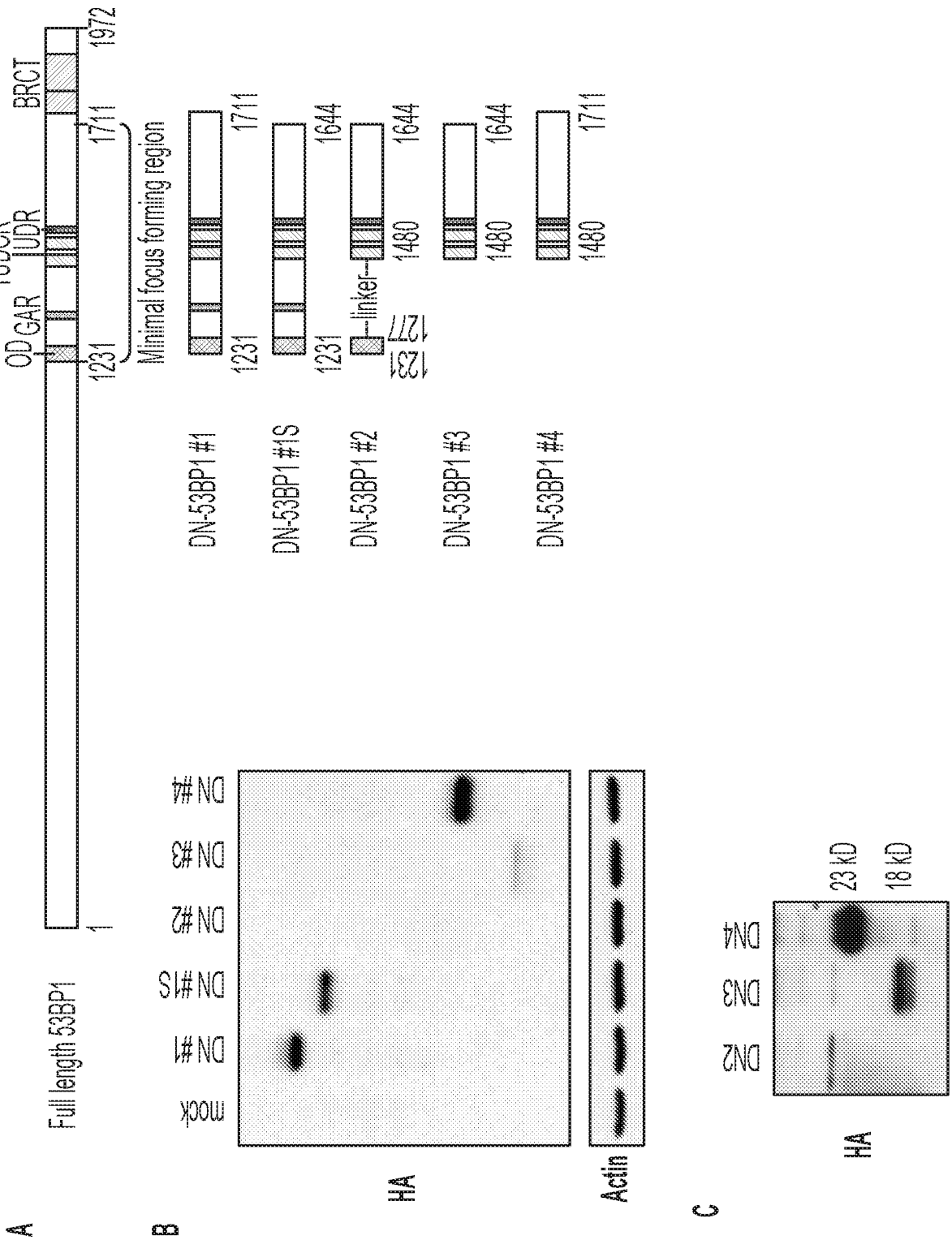
20. The method of any one of claims 17-19, wherein the cell is a mammalian cell.

21. The method of claim 20, wherein the mammalian cell is a human cell.

22. The method of any one of claims 17-21, wherein (a) and (b) are introduced into the cell by delivering a vector that expresses both the fusion polypeptide and the guide RNA into the cell.

23. The method of any one of claims 17-22, wherein the method further comprising introducing into the cell a template nucleic acid, which comprises homologous arms flanking a cleavage site in the gene of interest directed by the guide RNA.

Figure 1


A
Full length 53BP1
1
1231
1711
1972
OD
GAR
TUDOR
UDR
BRCT
Minimal focus forming region
DN-53BP1 #1
1231
1711
DN-53BP1 #1S
1231
1644
DN-53BP1 #2
1231
1277
linker
1480
1644
DN-53BP1 #3
1480
1644
DN-53BP1 #4
1480
1711
B
mock
DN #1
DN #1S
DN #2
DN #3
DN #4
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Actin
C
23 kD
18 kD
DN2
DN3
DN4
HA

Figure 1 (Cont'd)

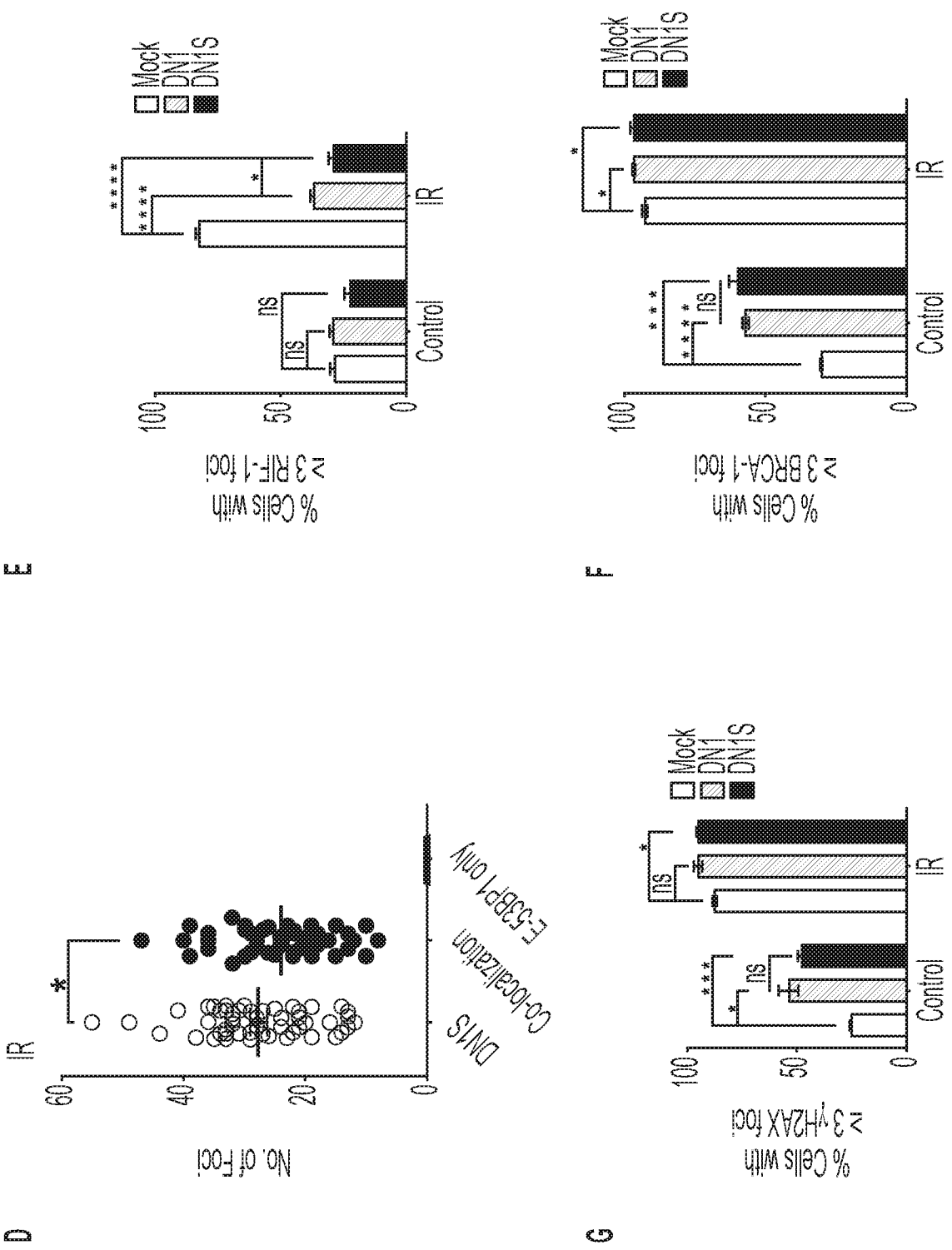
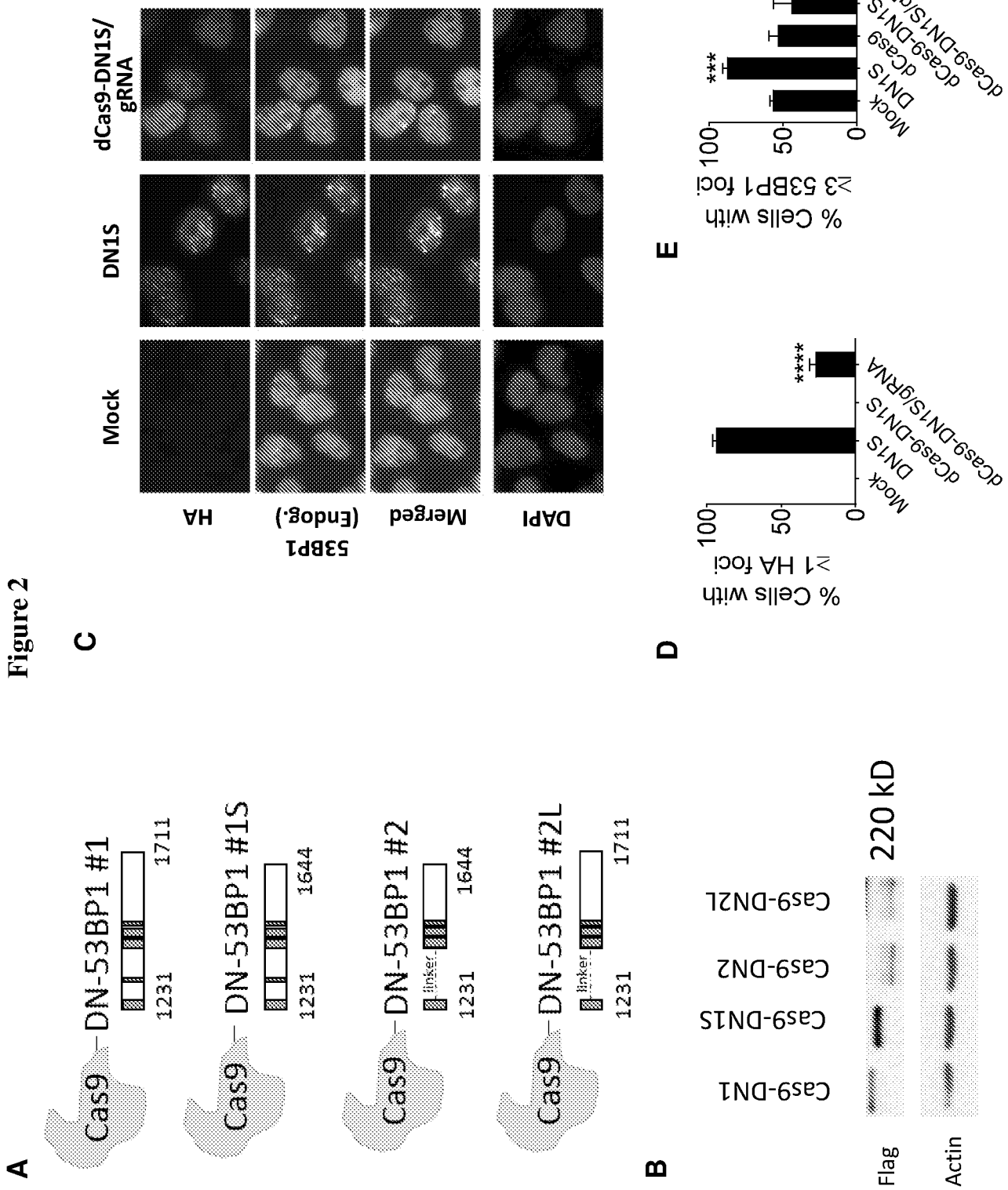


Figure 2


A
Cas9
DN-53BP1 #1
1231
1711
DN-53BP1 #1S
1644
DN-53BP1 #2
linker
DN-53BP1 #2L
B
Cas9-DN1
Cas9-DN1S
Cas9-DN2
Cas9-DN2L
220 kD
Flag
Actin
C
Mock
DN1S
dCas9-DN1S/gRNA
HA
53BP1 (Endog.)
Merged
DAPI
D
% Cells with ≥1 HA foci
100
50
0
****
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DN1S
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dCas9-DN1S/gRNA
E
% Cells with ≥3 53BP1 foci
***
dCas9

Figure 2 (Cont'd)

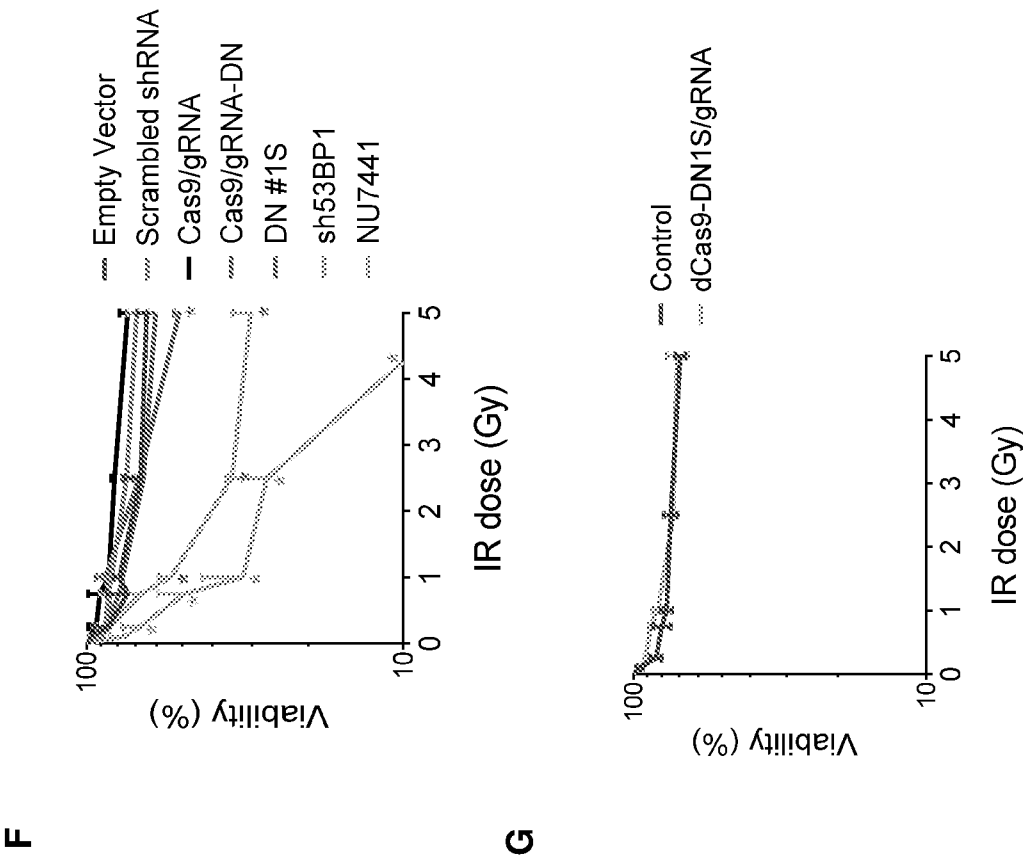
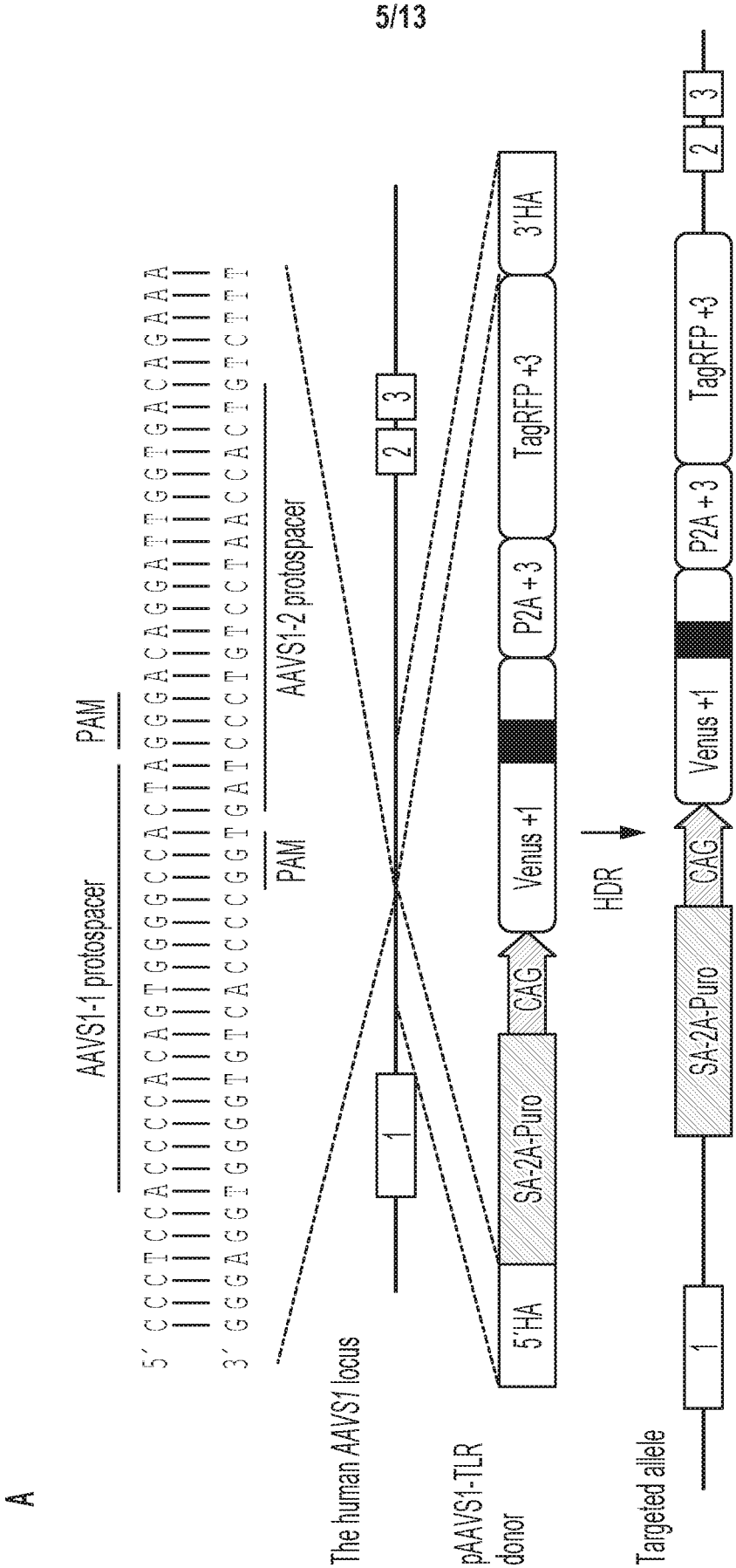


Figure 3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3′ GGGAGGTGGGGTGTCACCCCGGTGATCCCTGTCCTAACCACTGTCTTT
PAM
AAVS1-2 protospacer
The human AAVS1 locus
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2
3
pAAVS1-TLR donor
5'HA
SA-2A-Puro
CAG
Venus +1
P2A + 3
TagRFP +3
3'HA
HDR
Targeted allele
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CAG
Venus +1
P2A + 3
TagRFP +3
2
3

Figure 3 (Cont'd)

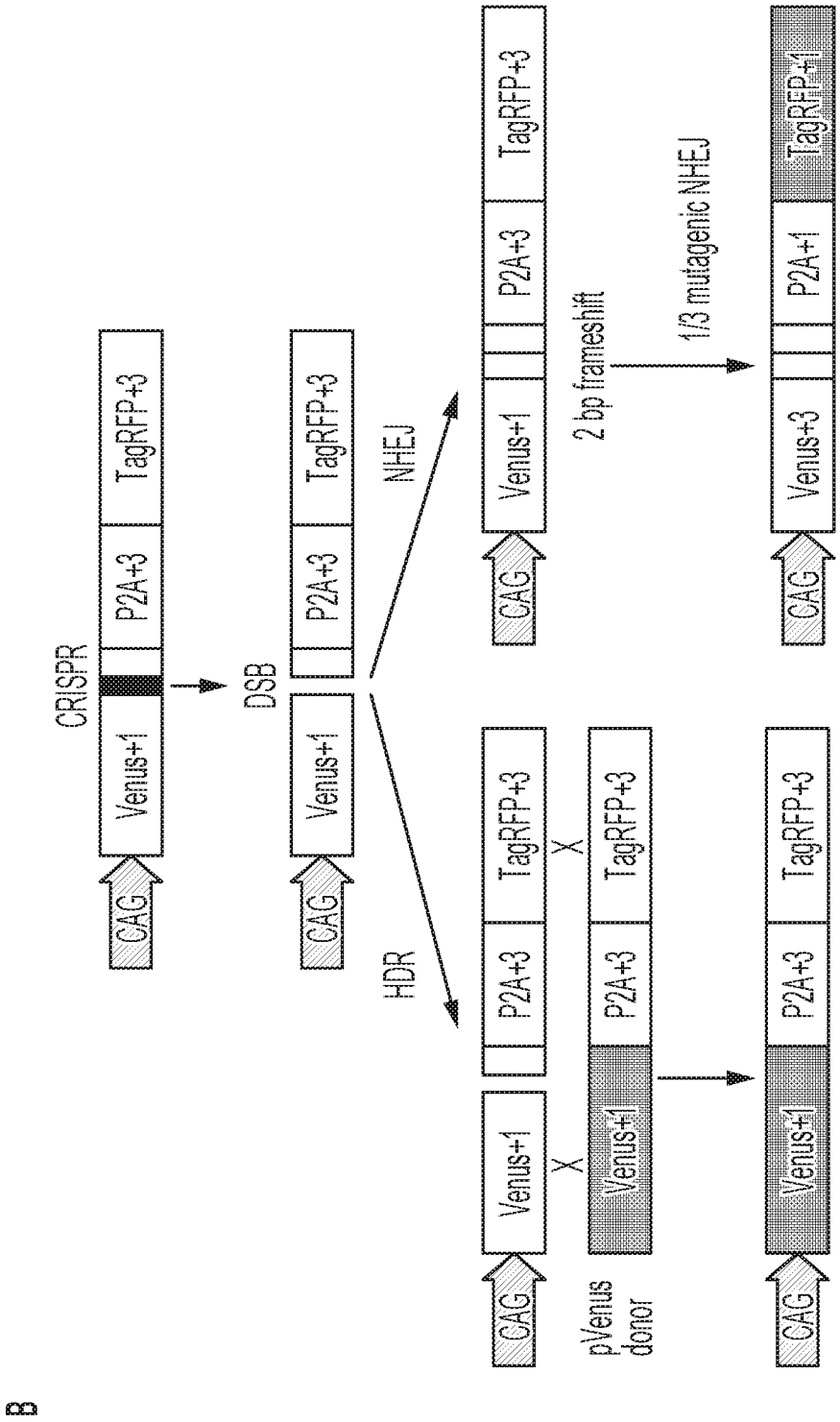


Figure 3 (Cont'd)

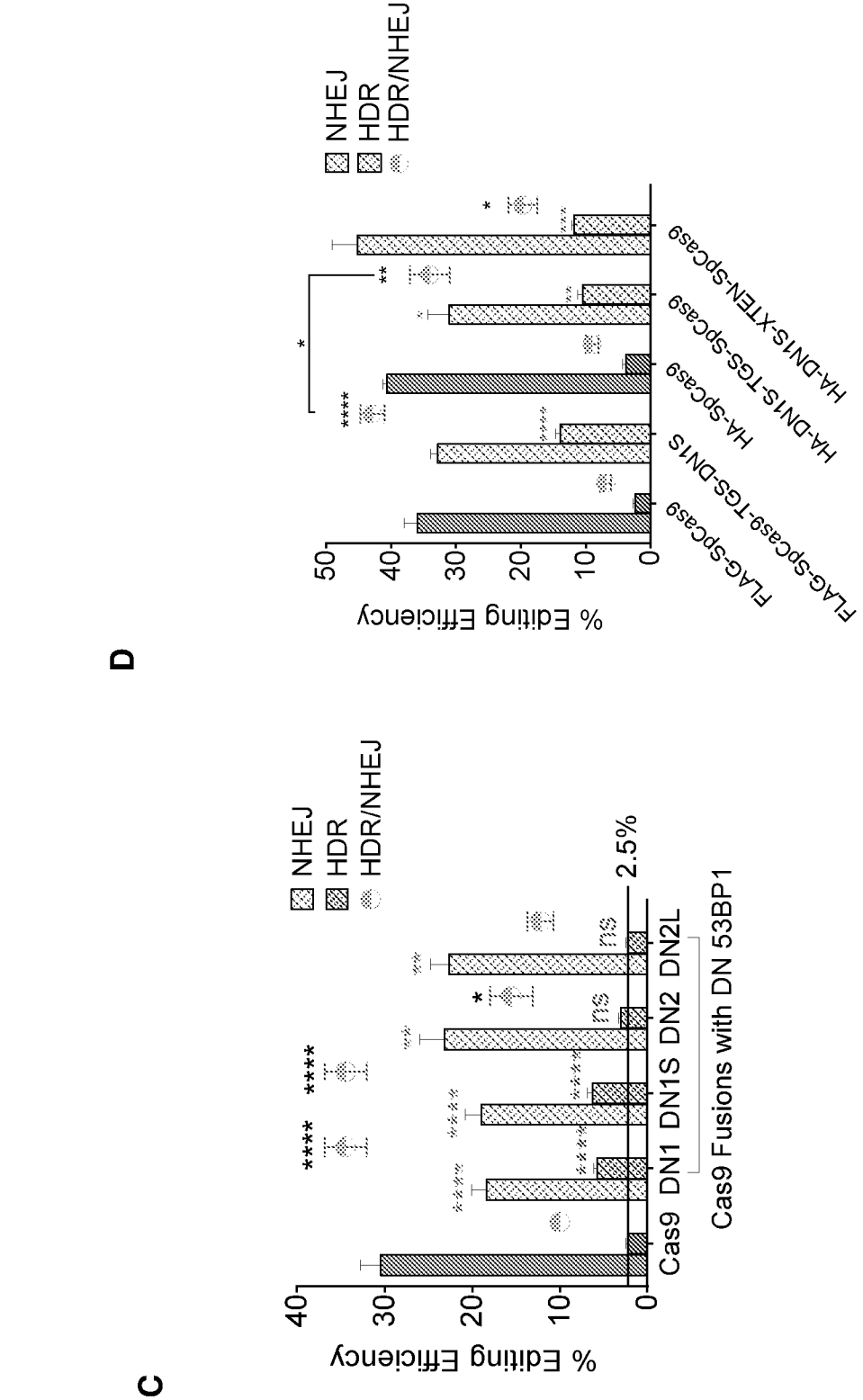


Figure 4

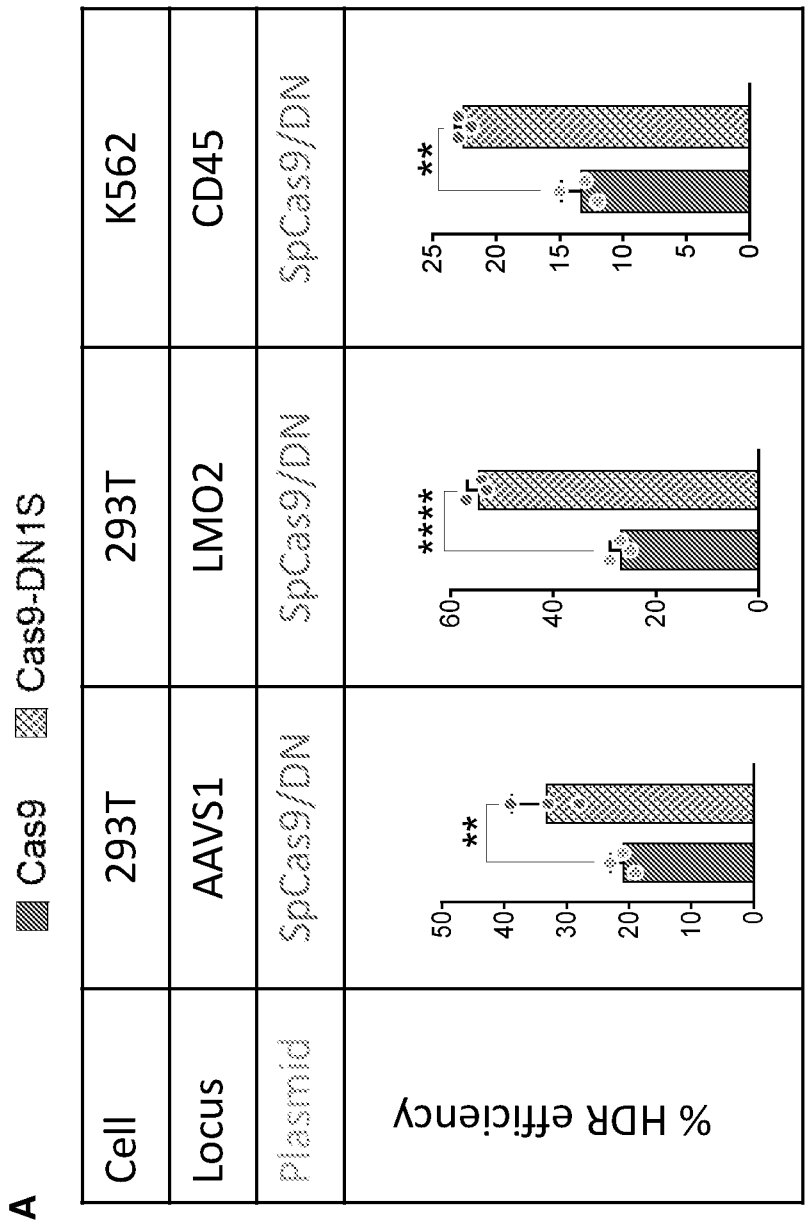

A
Cas9
Cas9-DN1S
Cell
293T
293T
K562
Locus
AAVS1
LMO2
CD45
Plasmid
SpCas9/DN
SpCas9/DN
SpCas9/DN
% HDR efficiency
50
40
30
20
10
0
**
60
40
20
0
****
25
20
15
10
5
0
**

Figure 4 (Cont'd)

B

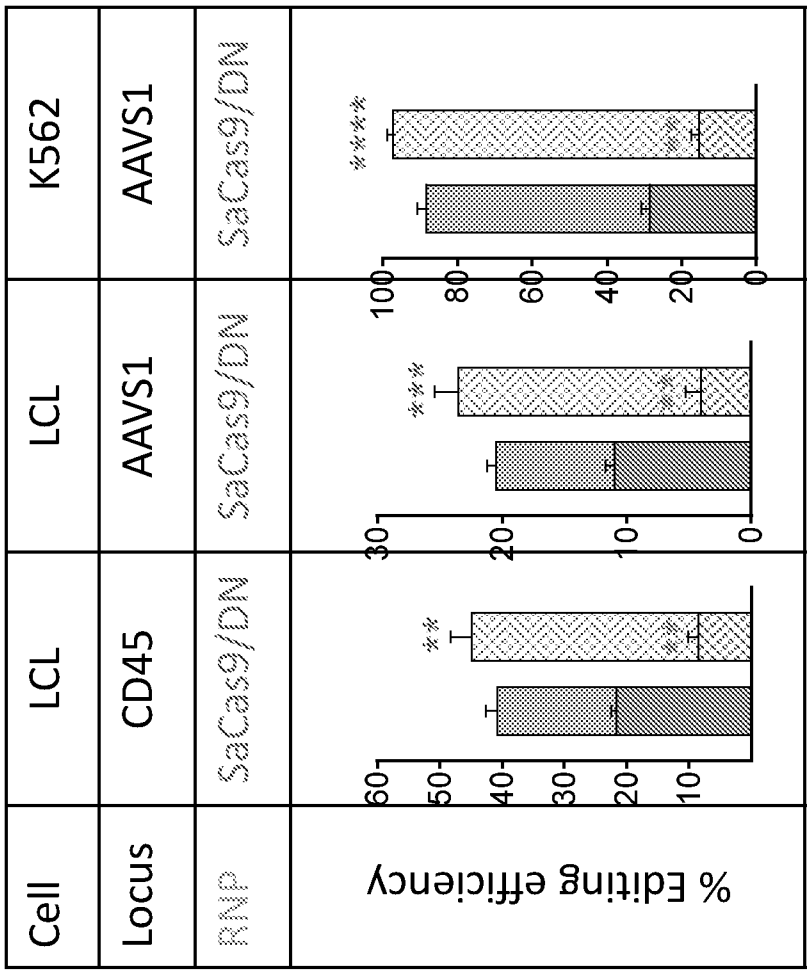


Figure 4 (Cont'd)

C

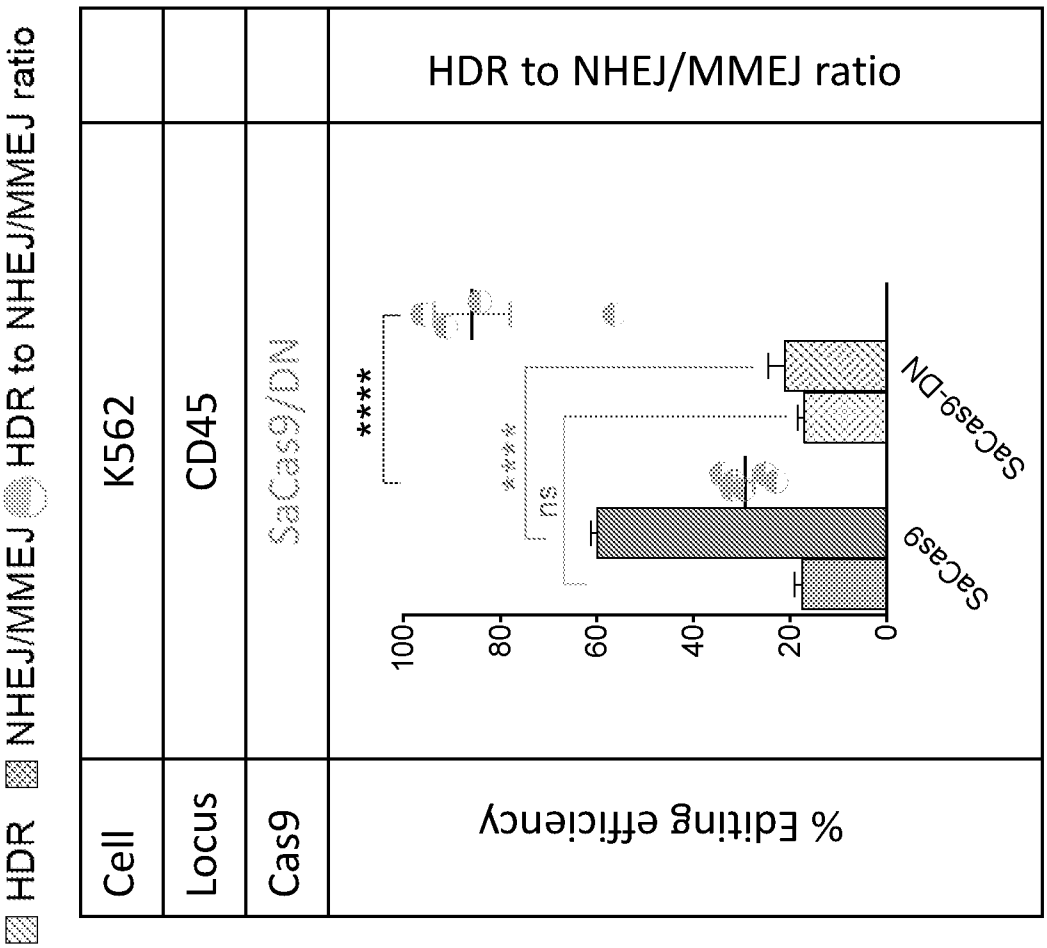


Figure 5

A

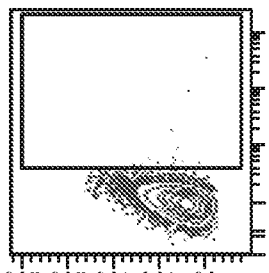
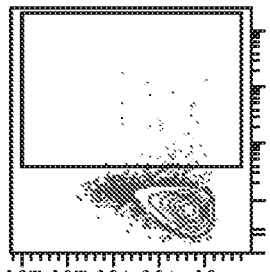
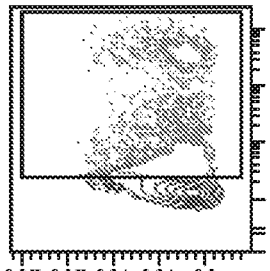
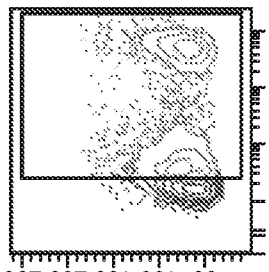
Locus	NA	NA	AAVS1	AAVS1
RNP	-	-	SaCas9	SaCas9-DN
Donor Template (AAV6)	-	+	+	+
	0.1%	0.2%	26%	51%
				

Figure 5 (Cont'd)

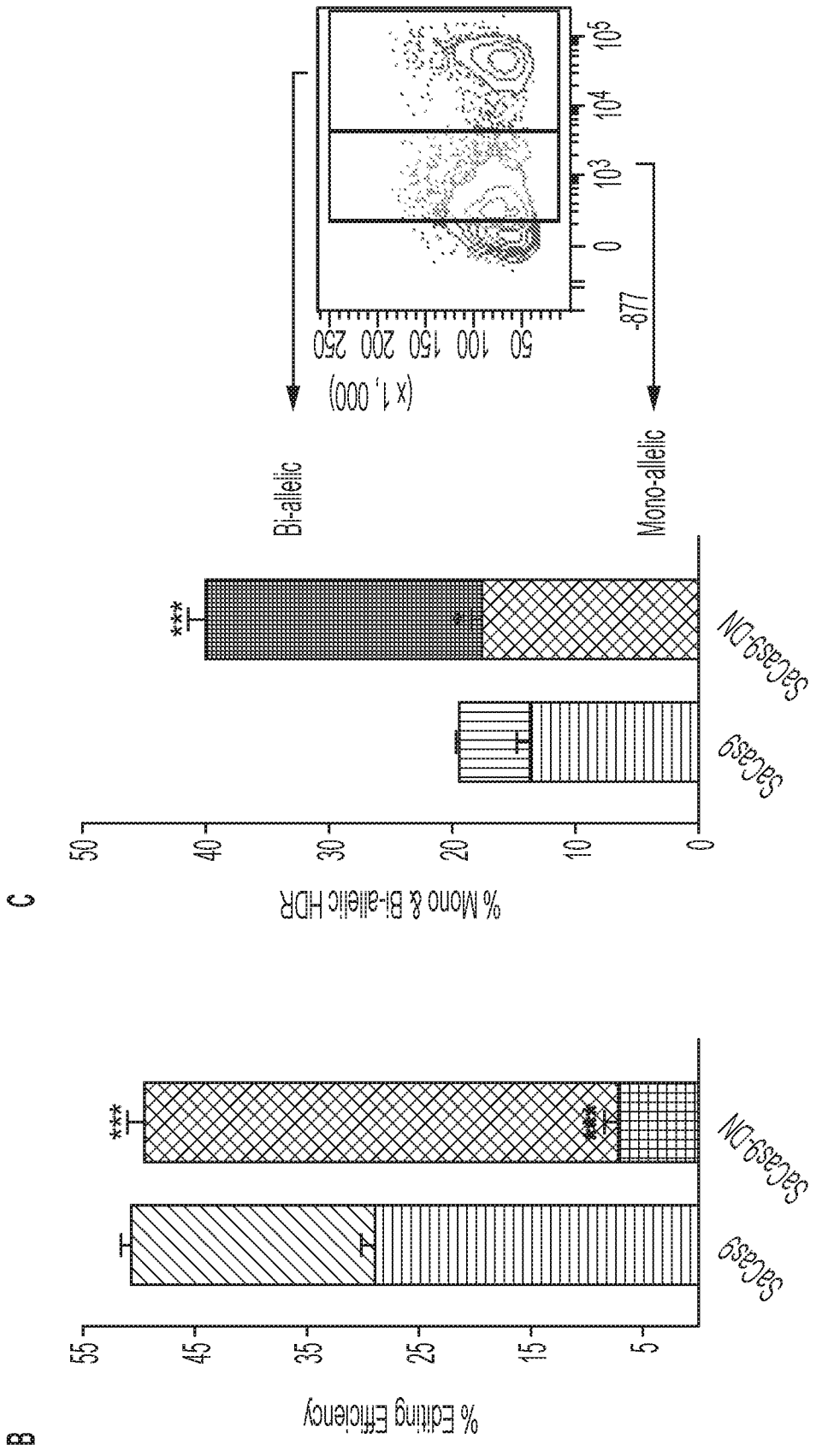
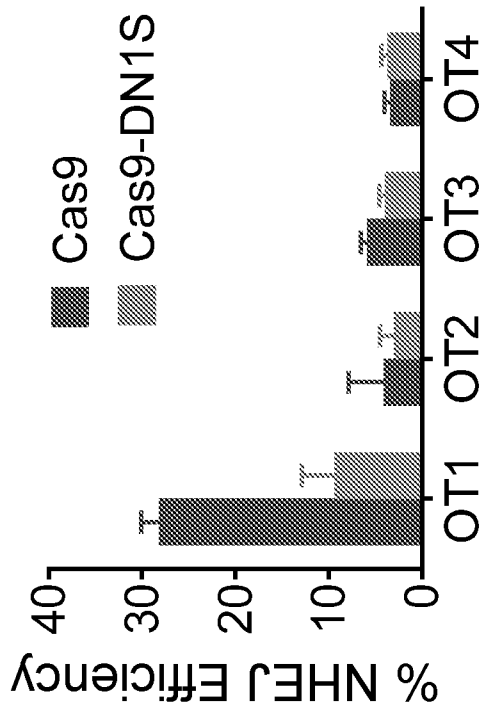


Figure 6



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 18/58254

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C12N 15/00; C12N 15/87; C07H 21/04; A61K 38/00; A61K 48/00 (2019.01)
CPC - C12N 15/63; C12N 15/907; C12N 15/902; C12N 2800/80; C12N 15/86; C12N 15/867; C12N 15/8645; C12N 15/861; C12N 2800/10; C07H 21/00; A61K 47/48246; A61K 48/00

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History Document

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

See Search History Document

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

See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2016/0201089 A1 (DUKE UNIVERSITY) 14 July 2016 (14.07.2016), Abstract, para [0006], [0008], [0026], [0033], [0102], [0104], [0152], [0172], [0268], [0271], and [0306]	1-4
Y	WO 2017/172775 A1 (CHILDREN'S MEDICAL CENTER CORPORATION) 05 October 2017 (05.10.2017), Abstract, para [0007], [0011], [0016], [0024], [0025], [0031], [0032], [0041], [0046], and [0068]	1-4
Y	WO 2017/093969 A1 (NOVARTIS AG et al.) 08 June 2017 (08.06.2017), pg 51, ln 11; and SEQ ID NO: 7830	4
A	GenPept_NP_001135452, TP53-binding protein 1 isoform 1 [Homo sapiens]. GenPept Accession Number: NP_001135452, 04 June 2017 [online]. [Retrieved on 2019.01.21]. Retrieved from the Internet: <URL: https://www.ncbi.nlm.nih.gov/protein/213972636?sat=46&satkey=74344033> Protein; and Region	1-4
A	US 2007/0178044 A1 (HALAZONETIS et al.) 02 August 2007 (02.08.2007), para [0030], [0033], [0088], and SEQ ID NO: 2	1-4
A	ZHANG et al., The p53-binding protein 1-Tudor-interacting repair regulator complex participates in the DNA damage response. J Biol Chem. 2017, Vol. 292(16), p. 6461-6467. Epub 2017 Feb 17. Entire documentation, especially Abstract; pg 6461, col 1, and col 2, top para and middle para; pg 6462, Fig 1; and pg 6463, Fig 2	1-4
A	THANASSOULAS et al., Thermal and chemical denaturation of the BRCT functional module of human 53BP1. Int J Biol Macromol. 2011, Vol. 49(3), p. 297-304. Entire documentation, especially Abstract	1-4

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 January 2019	01 APR 2019
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 18/58254

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 5-23
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Groups I+, Claims 1-4, directed to a fusion polypeptide, comprising a gene-editing nuclease enzyme and a dominant-negative variant of a p53 binding protein 1 (53BP1); wherein the gene-editing nuclease enzyme comprising a Cas9 enzyme. The Cas9 enzyme will be searched to the extent that the Cas9 enzyme encompasses the amino acid sequence of SEQ ID NO: 4. It is believed that claims 1-3, 4 (in part) encompass this first named invention, and thus these claims will be searched without fee to the extent that they encompass wherein the Cas9 enzyme comprises the amino acid sequence of SEQ ID NO: 4. Additional Cas9 enzyme will be searched upon the payment of additional fees. Applicants must specify the claims that encompass any additionally elected Cas9 enzyme. Applicants must further indicate, if applicable, the claims which encompass the first invention, if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched. An exemplary election would be wherein the Cas9 enzyme comprises the amino acid sequence of SEQ ID NO: 5 [claims (1-3), 4 (in part)]. **********Continued in the extra sheet**********

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-3, 4 (in part), limited to SEQ ID NO: 4

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

Continuation of:
Box No III (unity of invention is lacking)

The inventions listed as Groups I+ do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special Technical Feature
Among Groups I+, each SEQ ID NO represents a structurally different protein sequence.

Common Technical Features
The inventions of Groups I+ share the technical features of a fusion polypeptide, comprising a gene-editing nuclease enzyme and a dominant-negative variant of a p53 binding protein 1 (53BP1), wherein the dominantnegative variant of 53BP1 is a truncated 53BP1, which comprises (a) deletion in a docking domain, (b) a deletion of a BRCT domain, or (c) both (a) and (b) (claim 1); and
-- wherein the gene-editing nuclease enzyme is a Cas9 enzyme (claim 3).

However, these shared technical features do not represent a contribution over prior art as being obvious over US 2016/0201089 A1 to DUKE UNIVERSITY (hereinafter 'Duke'), in view of WO 2017/172775 A1 to CHILDREN'S MEDICAL CENTER CORPORATION (hereinafter 'CHILDREN_MED') as follows:

Duke discloses a fusion polypeptide (para [0008] - 'fusion protein comprising two heterologous polypeptide domain'), comprising
-- a gene-editing nuclease enzyme (para [0008] - 'fusion protein comprising two heterologous polypeptide domains. The first polypeptide domain comprises a Clustered Regularly Interspaced Short Palindromic Repeats associated (Cas) protein'; Abstract - 'Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated (Cas) 9-based system ...for altering gene expression'; para [0026] - 'site-specific nuclease may comprise ... CRISPR/Cas9 system') and
-- a second heterologous polypeptide functional domain (para [0008] - 'fusion protein comprising two heterologous polypeptide domains... comprises ...Cas...and the second polypeptide domain has an activity ... transcription activation activity, ...nucleic acid association activity, methylase activity, and demethylase activity').

Duke further discloses wherein the gene-editing nuclease enzyme is a Cas9 enzyme (Abstract; para [0008]; para [0026]; All quotations as above), and wherein the second heterologous polypeptide functional domain may have an additional nucleic acid associated activity for specifically targeted gene-editing (Abstract; para [0008]; para [0006] - 'Engineered nucleases have been used for gene editing in a variety of human stem cells and cell lines ...facilitates successful genome modification'; para [0102] - 'Cas9 fusion protein that combines the DNA sequence targeting function of the CRISPR/Cas9-based system with an additional activity'; para [0172] - 'fusion protein may comprise two heterologous polypeptide domains, wherein the first polypeptide domain comprises a Cas protein and the second polypeptide domain has an activity such as transcription activation activity, ... nucleic acid association activity, ... or demethylase activity'), and further wherein gene-editing can be achieved by non-homologous end joining (NHEJ) or homology-directed repair (HDR) (para [0306] - 'at least one CRISPR/Cas9-based system, ...which specifically targets a gene'; para [0006] - 'Site-specific nucleases can be used to introduce site-specific double strand breaks at targeted genomic loci. ... break will be repaired by non-homologous end joining (NHEJ) ...Engineered nucleases have been used for gene editing in a variety of human stem cells ... facilitates successful genome modification'; para [0033] - 'multiplex gene editing may comprise correcting at least one mutant gene or inserting a transgene. ...Correcting the at least one mutant gene may comprise nuclease-mediated non-homologous end joining or homology-directed repair'; para [0268] - 'genome editing with a site-specific nuclease ...site-specific nucleases may involve using homology-directed repair or nuclease-mediated non-homologous end joining (NHEJ)-based correction approaches,...with an efficient gene editing').

**********Contined in the next extra sheet**********

Continuation of:
The previous extra sheet - Box No III (unity of invention is lacking)

Duke does not specifically teach wherein the second heterologous polypeptide functional domain is a dominant-negative variant of a p53 binding protein 1 (53BP1), wherein the dominant negative variant of 53BP1 is a truncated 53BP1, which comprises (b) a deletion of a BRCT domain, or (c) both (a) deletion in a docking domain and (b). CHILDREN_MED discloses a method of using a dominant-negative variant of a p53 binding protein 1 (53BP1) for increasing targeted gene-editing through non-homologous end joining (NHEJ) or homology-directed repair (HDR) (Abstract - 'improvements in the efficiency and/or accuracy of targeted alterations to a nucleic acid sequence, e.g, gene editing technologies, by creating nick or DSB in a target nucleic in the presence of template molecule, an inhibitor of NHEJ and an agonist of HDR'; para [0068] - 'Nucleases often cleave both strands of dsDNA molecule within several bases of each other, reulting in double-stranded break (DSB)'; para [0007] - 'altering a target sequence of a target nucleic acid molecule... contacting the target nucleic acid molecule with: a) a nuclease; b) at least one inhibitor of non-homologous end joining (NHEJ); c) at least one agonist of homology-directed repair (HDR); ... the inhibitor of NHEJ is selected from ...an inhibitor of 53BP1'; para [0011] - 'inhibitor of 53BP1 ...dominant-negative 53BP1 (dn53BP1) polypeptide. ... target nucleic acid is contacted with the dn53BP1 polypeptide by delivering a polypeptide to the target nucleic acid'), using nuceases including Cas9 nuclease (para [0007]; para [0013] - 'programmable nuclease is selected from the group consisting of: Cas9'; para [0068] - 'Nucleases often cleave both strands of dsDNA molecule within several bases of each other, reulting in double-stranded break (DSB). Exemplary nucleases include,... Cas9'; para [0016] - 'on-target or off-target cutting specificity of Cas9 activity is not altered by inclusion of the at least one inhibitor of NHEJ and/or at least one agonist of HDR'), wherein the dominant negative variant of 53BP1 increases HDR frequency and improves precise genomic modifications at various targeted loci (para [0024] - 'dominant negative 53BP1 (dn53BP1) increases HDR frequency. Fig. 1A ...Factors suppressing NHEJ and promoting HDR are shown'; para [0025] - 'dn53BP1 improve precise genomic modifications at various targeted loci'; para [0031] - 'over-expression of ... dn53BP1 does not alter Cas9 specificity'; para [0032] - 'co-expression of ... dn53BP1 improves HDR frequency using Cas9-Nickase at multiple loci and in human cells'), and further wherein the dominant negative variant of 53BP1 (dn53BP1) is a truncated 53BP1, which comprises (b) a deletion of a BRCT domain, or (c) both (a) deletion in a docking domain and (b) (para [0046] - 'an inhibitor of 53BP1 can be a dominant negative 53BP1 (dn53BP1) polypeptide... "dn53BP1" refers to a variant of 53BP1 which lacks the BRCT domain(s) but does comprise the Tudor domain(s) of wild-type 53BP1. ... dn53BP1 can lack the residues corresponding to about residues 1774-1977 of SEQ ID NO:12. ... dn53BP1 consists essentially of the sequence corresponding to about residues 1493-1537 of SEQ ID NO:12.... dn53BP1 consists essentially of the sequence corresponding to about residues 1218-1715 of SEQ ID NO:12. ... dn53BP1 consists essentially of the sequence corresponding to about residues 1-1715 of SEQ ID NO:12', wherein each of 'dn53BP1 can lack the residues corresponding to about residues 1774-1977 of SEQ ID NO:12' and 'dn53BP1 consists essentially of the sequence corresponding to about residues 1-1715 of SEQ ID NO:12' is 'wherein the dominant negative variant of 53BP1 is a truncated 53BP1, which comprises (b) a deletion of a BRCT domain'; and wherein each of 'dn53BP1 consists essentially of the sequence corresponding to about residues 1493-1537 of SEQ ID NO:12.... residues 1218-1715 of SEQ ID NO:12' is 'wherein the dominant negative variant of 53BP1 is a truncated 53BP1, which comprises (c) both (a) deletion in a docking domain and (b)', see the quotations and explanations that follow; para [0041] - 'human 53BP1 ... polypeptide (e.g., NP 001135452.1 (SEQ ID NO: 12)'; Please see GenPept_NP_001135452: Protein - 'tumor protein 53-binding protein'; Region 1488..1609 /region_name="53-BP1_Tudor"; Region 1774..1841 /region_name="BRCT" /note="Breast Cancer Suppressor Protein (BRCA1); Please also see Specification; Specification: pg 11, ln 21-24 - 'an example 53BP1 includes 1972 amino acids. The N-terminal portion (1-1231) contains domains for interacting with other proteins of cellular DNA repair machinery (docking domains). The C-tenninal portion (1711-1972) contains two breast cancer susceptibility gene 1 (BRCT) motifs').

It would have been obvious to one of ordinary skill in the art at the time the invention was made to combine the teachings of Duke and CHILDREN_MED, to obtain a fusion polypeptide, comprising a gene-editing nuclease enzyme and a second heterologous polypeptide functional domain, based on the teaching of Duke, and further wherein the second heterologous polypeptide functional domain is a dominant-negative variant of a p53 binding protein 1 (53BP1), wherein the dominant negative variant of 53BP1 is a truncated 53BP1, which comprises (b) a deletion of a BRCT domain, or (c) both (a) deletion in a docking domain and (b), based on the combination of CHILDREN_MED and Duke, in order to combine methods, gene-editing nucleases, and protein functional domains available in the art for facilitating obtaining a fusion protein for targeted gene editing with a desired effect with an expected success and without undue experimentation.

The said combination further discloses wherein the gene-editing nuclease enzyme is a Cas9 enzyme (Duke: para [0008] - 'fusion protein comprising ... The first polypeptide domain comprises a Clustered Regularly Interspaced Short Palindromic Repeats associated (Cas) protein'; Abstract - 'Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated (Cas) 9-based system ...for altering gene expression'; para [0026] - 'site-specific nuclease may comprise ... CRISPR/Cas9 system'; CHILDREN_MED: para [0007]; para [0068] - 'Nucleases often cleave both strands of dsDNA molecule within several bases of each other, reulting in double -stranded break (DSB). Exemplary nucleases include,... Cas9'; para [0032] - 'co-expression of ... dn53BP1 improves HDR frequency using Cas9-Nickase at multiple loci and in human cells').

Without a shared special technical feature, the inventions lack unity with one another.

Groups I+ therefore lack unity under PCT Rule 13 because they do not share a same or corresponding special technical feature.

Continuation of item 4: Claims 5-23 are not drafted in accordance with the second and third sentences of Rule 6.4 (a). These claims are improper multiple dependent claims.

Note:

I) Claim 1 contains the first appeared abbreviation 'BRCT' with no full name introduced.