SUMMARY

[0006] Disclosed herein are methods, systems, eukaryotic cells (*e.g.*, plant cells or mammalian cells), and compositions (*e.g.*, cell culture compositions, nucleic acids, vectors, kits, or cells) that can provide for increased frequencies of modification of a target editing site of the eukaryotic cell genome with a donor template polynucleotide by Homology-Directed Repair (HDR) in comparison to a control. Features of such methods, systems, eukaryotic cells (*e.g.*, plant cells or mammalian cells), and compositions (*e.g.*, cell culture compositions, nucleic acids, vectors, kits, or cells) that can provide for such increased frequencies of HDR include provision of HDR promoting agents comprising a single-stranded DNA annealing protein (SSAP), an exonuclease which can at least partially convert a double stranded DNA substrate to a single stranded DNA product, and a single stranded DNA binding protein (SSB) in combination with genome editing molecules comprising at least one sequence-specific endonuclease which cleaves a target editing site in a eukaryotic cell genome and a donor template DNA molecule having homology to the target editing site. In certain examples, the donor template DNA molecule is flanked by copies of an endonuclease recognition sequence.

[0007] Methods provided herein include methods for increasing Homology Directed Repair (HDR)-mediated genome modification of a target editing site of a eukaryotic cell genome, comprising: providing genome-editing molecules and HDR promoting agents to a eukaryotic cell, wherein the genome editing molecules comprise: (i) at least one sequence-specific endonuclease which cleaves a DNA sequence in the target editing site or at least one polynucleotide encoding the sequence-specific endonuclease; and (ii) a donor template DNA molecule having homology to the target editing site; and wherein the HDR promoting agents comprise a single-stranded DNA annealing protein (SSAP), an exonuclease which can at least partially convert a double stranded DNA substrate to a single stranded DNA product, and a single stranded DNA binding protein (SSB); whereby the genome editing molecules and HDR promoting agents provide for modification of the target editing site of the eukaryotic cell genome with the donor template polynucleotide by HDR at a frequency that is increased in comparison to a control. In one aspect, the present disclosure refers to a method for increasing Homology Directed Repair (HDR)-mediated genome modification of a target editing site of a eukaryotic cell genome, comprising: providing genome-editing molecules and HDR promoting agents to a eukaryotic cell, wherein the genome editing molecules comprise: (i) at least one sequence-specific endonuclease which cleaves a DNA sequence in the target editing site or at least one polynucleotide encoding the sequence-specific endonuclease; and (ii) a donor template DNA molecule having homology to the target editing site; and wherein the HDR promoting agents comprise a single-stranded DNA annealing protein (SSAP), an exonuclease which can at least partially convert a double stranded DNA substrate to a single stranded DNA product, and a single stranded DNA binding protein (SSB); whereby the genome editing molecules and HDR promoting agents provide for modification of the target editing site of the eukaryotic cell genome with the donor template polynucleotide by HDR at a frequency that is increased in comparison to a control.

[0008] Methods provided herein also include methods for making a eukaryotic cell having a genomic modification, comprising: providing genome editing molecules and Homology Directed Repair (HDR) promoting agents to a eukaryotic cell, wherein the genome editing molecules comprise: (i) at least one sequence-specific endonuclease which

cleaves a DNA sequence in the target editing site or at least one polynucleotide encoding the sequence-specific endonuclease and a donor template DNA molecule having homology to the target editing site; and wherein the HDR promoting agents comprise a single-stranded DNA annealing protein (SSAP), an exonuclease which can at least partially convert a double stranded DNA substrate to a single stranded DNA product, and a single stranded DNA binding protein (SSB);whereby the genome editing molecules and HDR promoting agents provide for modification of the target editing site of the eukaryotic cell genome with the donor template polynucleotide by HDR at a frequency that is increased in comparison to a control; and isolating or propagating a eukaryotic cell comprising the genome modification. In one aspect, the present disclosure refers to a method for making a eukaryotic cell having a genomic modification, comprising: (a) providing genome editing molecules and Homology Directed Repair (HDR) promoting agents to a eukaryotic cell, wherein the genome editing molecules comprise: (i) at least one sequence-specific endonuclease which cleaves a DNA sequence in the target editing site or at least one polynucleotide encoding the sequence-specific endonuclease and a donor template DNA molecule having homology to the target editing site; and wherein the HDR promoting agents comprise a single-stranded DNA annealing protein (SSAP), an exonuclease which can at least partially convert a double stranded DNA substrate to a single stranded DNA product, and a single stranded DNA binding protein (SSB);whereby the genome editing molecules and HDR promoting agents provide for modification of the target editing site of the eukaryotic cell genome with the donor template polynucleotide by HDR at a frequency that is increased in comparison to a control; and (b) isolating or propagating a eukaryotic cell comprising the genome modification, thereby making the eukaryotic cell having a genomic modification.

[0009] Systems provided herein include systems for increasing Homology Directed Repair (HDR)-mediated genome modification of a target editing site of a eukaryotic cell genome, comprising:

(a) a eukaryotic cell;

(b) HDR promoting agents comprising a single-stranded DNA annealing protein (SSAP), an exonuclease which can at least partially convert a double stranded DNA substrate to a single stranded DNA product, and a single stranded DNA binding protein (SSB); and

(c) genome editing molecule(s) comprising at least one sequence-specific endonuclease which cleaves a DNA sequence in the target editing site or at least one polynucleotide encoding the sequence-specific endonuclease and a donor template DNA molecule having homology to the target editing site; wherein the eukaryotic cell is associated with, contacts, and/or contains and effective amount of the HDR promoting agents and the genome editing molecule(s).

[0010] Methods provided herein also include a method of genetic engineering of a eukaryotic cell comprising providing to the eukaryotic cell: i) at least one sequence-specific endonuclease, ii) a donor template DNA molecule having homology to a target editing site in the eukaryotic cell, iii) a single-stranded DNA annealing protein (SSAP), iv) an exonuclease which can at least partially convert a double stranded DNA substrate to a single stranded DNA product, and v) a single stranded DNA binding protein (SSB), wherein the target editing site of the cell is modified by the donor template DNA molecule. In one aspect, the present disclosure refers to a method of genetic engineering of a eukaryotic cell comprising providing to the eukaryotic cell: i) at least one sequence-specific endonuclease, ii) a donor template DNA molecule having homology to a target editing site in the eukaryotic cell, iii) a single-stranded DNA annealing protein (SSAP), iv) an exonuclease which can at least partially convert a double stranded DNA substrate to a single stranded DNA product, and v) a single stranded DNA binding protein (SSB), wherein the target editing site of the cell is modified by the donor template DNA molecule.

[0011] Methods provided herein also include a method for producing a eukaryotic cell with a genetically modified target editing site comprising: (a) providing at least one sequence-specific endonuclease which cleaves a DNA sequence at least one endonuclease recognition sequence in said target editing site or at least one polynucleotide encoding said at

least one sequence-specific endonuclease, and (b) providing at least one donor molecule comprising at least one double-stranded DNA sequence, wherein (i) said DNA sequence has a homology of at least 90% over a length of at least 50 nucleotides to sequences flanking the target editing site and (ii) wherein said donor sequence comprises at least one modification in comparison to said target editing site; and (c) providing at least one Homology Directed Repair (HDR) promoting agent comprising (i) at least one single-stranded DNA annealing protein (SSAP), and (ii) at least one exonuclease which can at least partially convert a double stranded DNA substrate to a single stranded DNA product, and (iii) at least one single stranded DNA binding protein (SSB); and whereby the at least one sequence-specific endonucleases, the at least one donor molecule, and the at least one HDR promoting agent introduce said modification into said target editing site of said eukaryotic cell; and (d) isolating a eukaryotic cell comprising a modification in said target editing site.

[0012] Compositions provided herein include a composition comprising nucleic acids encoding one or more of i) at least one sequence-specific endonuclease, ii) a donor template DNA molecule having homology to a target editing site in the eukaryotic cell, iii) a single-stranded DNA annealing protein (SSAP), iv) an exonuclease which can at least partially convert a double stranded DNA substrate to a single stranded DNA product, and v) a single stranded DNA binding protein (SSB). In one aspect, the present disclosure refers to a composition comprising nucleic acids encoding one or more of i) at least one sequence-specific endonuclease, ii) a donor template DNA molecule having homology to a target editing site in the eukaryotic cell, iii) a single-stranded DNA annealing protein (SSAP), iv) an exonuclease which can at least partially convert a double stranded DNA substrate to a single stranded DNA product, and v) a single stranded DNA binding protein (SSB).

[0013] Vectors provided herein include a vector comprising nucleic acids encoding one or more of i) at least one sequence-specific endonuclease, ii) a donor template DNA molecule having homology to a target editing site in the eukaryotic cell, iii) a single-stranded DNA annealing protein (SSAP), iv) an exonuclease which can at least partially convert a double stranded DNA substrate to a single stranded DNA product, and v) a single stranded DNA binding protein (SSB). In one aspect, the present disclosure refers to a vector comprising nucleic acids encoding one or more of i) at least one sequence-specific endonuclease, ii) a donor template DNA molecule having homology to a target editing site in the eukaryotic cell, iii) a single-stranded DNA annealing protein (SSAP), iv) an exonuclease which can at least partially convert a double stranded DNA substrate to a single stranded DNA product, and v) a single stranded DNA binding protein (SSB).

[0014] Kits provided herein include a kit comprising nucleic acids encoding i) at least one sequence-specific endonuclease, ii) a donor template DNA molecule having homology to a target editing site in the eukaryotic cell, iii) a single-stranded DNA annealing protein (SSAP), iv) an exonuclease which can at least partially convert a double stranded DNA substrate to a single stranded DNA product, and v) a single stranded DNA binding protein (SSB) and instructions for use for genetically engineering a eukaryotic cell. In one aspect, the present disclosure refers to a kit comprising nucleic acids encoding i) at least one sequence-specific endonuclease, ii) a donor template DNA molecule having homology to a target editing site in the eukaryotic cell, iii) a single-stranded DNA annealing protein (SSAP), iv) an exonuclease which can at least partially convert a double stranded DNA substrate to a single stranded DNA product, and v) a single stranded DNA binding protein (SSB) and instructions for use for genetically engineering a eukaryotic cell.

[0015] Cells provided herein include a cell comprising i) at least one sequence-specific endonuclease, ii) a donor template DNA molecule having homology to a target editing site in the eukaryotic cell, iii) a single-stranded DNA annealing protein (SSAP), iv) an exonuclease

which can at least partially convert a double stranded DNA substrate to a single stranded DNA product, and v) a single stranded DNA binding protein (SSB). In one aspect, the present disclosure refers to a cell comprising i) at least one sequence-specific endonuclease, ii) a donor template DNA molecule having homology to a target editing site in the eukaryotic cell, iii) a single-stranded DNA annealing protein (SSAP), iv) an exonuclease which can at least partially convert a double stranded DNA substrate to a single stranded DNA product, and v) a single stranded DNA binding protein (SSB). In one aspect, the present disclosure refers to a cell comprising nucleic acids encoding i) at least one sequence-specific endonuclease, ii) a donor template DNA molecule having homology to a target editing site in the eukaryotic cell, iii) a single-stranded DNA annealing protein (SSAP), iv) an exonuclease which can at least partially convert a double stranded DNA substrate to a single stranded DNA product, and v) a single stranded DNA binding protein (SSB). In one aspect, the present disclosure refers to a genetically engineered cell produced by the method as disclosed herein.

[0016] Cells provided herein also include a progenitor eukaryotic cell or organism for genetic engineering at a target editing site, comprising a subset of i) at least one sequence-specific endonuclease, ii) a donor template molecule having homology to a target editing site in the eukaryotic cell, iii) a single-stranded DNA annealing protein (SSAP), iv) an exonuclease which can at least partially convert a double stranded DNA substrate to a single stranded DNA product, and v) a single stranded DNA binding protein (SSB), wherein the cell does not comprises at least one of i)- v), wherein providing the cell or organism with the at least one of i)- v) that is not comprised in the progenitor cell or organism results in modification of the target editing site by the donor template molecule.

BRIEF DESCRIPTION OF THE DRAWINGS

[0017] **FIG. 1** shows a schematic diagram of the vector pRS08t Length in base pairs is indicated by the labels outside of the vector. Beginning at base pair 1, the vector includes a high copy number origin of replication (High Copy Ori), Cas expression cassette (tomato SlUBI10 promoter, Cas nuclease coding sequence (Cas nuclease CDS), and HSP terminator), guide RNA expression cassette (*A. thaliana* U6 promoter (AtU6), sequence encoding a guide RNA, and 35S promoter), mGFP6 sequence, pea rbcS E9 terminator, ANT1 donor template, and spectinomycin resistance marker (SpnR).

[0018] **FIG. 2** shows a schematic diagram of the vector pRS045. Length in base pairs is indicated by the labels outside of the vector. Beginning at base pair 1, the vector includes an ampicillin resistance marker (AmpR), HDR promoting agents expression cassette (PcUbi promoter, c2 nuclear localization sequence (NLS) fused to an *E. coli* SSB coding sequence (*E. coli* SSB CDS), pea 3A terminator, tomato SlUBI10 promoter, c2 NLS fused to a SSAP coding sequence (Red Beta CDS), HSP terminator, 2x 35S promoter, c2 NLS fused to an exonuclease coding sequence (Red Exo CDS), and 35S terminator), and pUC origin of replication (pUC ori).

[0019] **FIG. 3** shows a schematic diagram of the vector pAP046. Length in base pairs is indicated by the labels outside of the vector. Beginning at base pair 1, the vector includes a high copy number origin of replication (High Copy Ori), Cas expression cassette (tomato SlUBI10 promoter, Cas nuclease coding sequence (Cas nuclease CDS), and HSP terminator), guide RNA and ribozyme expression cassette (35S promoter, sequence encoding a