

Claims

1. A method for generating obligate DNA recombinases for genome editing, wherein said method comprises the steps of:
 - 5 i. providing a nucleic acid molecule encoding a first recombinase enzyme and a nucleic acid molecule encoding a second recombinase enzyme, wherein said first recombinase enzyme binds to a first half site of an asymmetric recombinase target site and said second recombinase enzyme binds to a second half site of an asymmetric recombinase target site, wherein said first recombinase enzyme and said second recombinase enzyme form a
10 heterodimer, which is capable to induce a site-specific DNA recombination of a sequence of interest at said asymmetric recombinase target site in a DNA sequence, wherein said asymmetric recombinase target site comprises a first half site and a second half site of an upstream target site and/or a downstream target site of a DNA recombinase wherein said first half site and a second half site are not identical and which are not palindromic;
 - 15 ii. mutagenesis to create libraries of nucleic acid molecules encoding mutant first recombinase enzymes and of nucleic acid molecules encoding mutant second recombinase enzymes, wherein mutations are introduced in said first recombinase enzyme and said second recombinase enzyme;
 - iii. creating expression vectors, by cloning the library of the nucleic acid molecules
20 encoding a first mutant recombinase enzyme and the library of nucleic acid molecules encoding a second mutant recombinase enzyme into expression vectors, wherein said expression vectors carry a DNA sequence of interest, which is to be recombined;
 - iv. transfecting a cell with the expression vectors of step iii) and expressing the libraries of said mutant first recombinase enzyme and said mutant second recombinase enzyme
25 in the same cell resulting in the formation of recombinase heterodimers comprising a mutant first recombinase enzyme and a mutant second recombinase enzyme;
 - v. positive selection screen for heterodimers obtained in step iv. that are capable to induce a site-specific DNA recombination of a sequence of interest at an asymmetric recombinase target site in a DNA;
 - 30 vi. negative selection screen for heterodimers obtained in step iv. or v. that are not capable to induce a site-specific DNA recombination of a sequence of interest at an off-target, preferably symmetric recombinase target site in a DNA;

vii. selecting an obligate DNA recombinase which is capable of recombining a DNA sequence of interest at a recombinase target site in a DNA comprising a first half site and a second half site of an upstream target site and/or a downstream target site of a DNA recombinase, wherein said first half site and said second half site are not identical and are not palindromic; and
5 which is not capable of recombining a DNA sequence of interest at an off-target, preferably symmetric recombinase target site in a DNA;

wherein in said obligate DNA recombinase obtained in step vii., said first mutant recombinase enzyme and said second mutant recombinase enzyme each comprise at least one mutation in a catalytic site, which render said first recombinase enzyme and said second
10 recombinase enzyme catalytically inactive when expressed in isolation.

2. The method according to claim 1,

wherein the first recombinase enzyme and the second recombinase enzyme according to steps ii. to vi. are evolved by substrate linked directed evolution (SLiDE) or directed evolution,
15 and/or

wherein the selection according to steps v. and vi. iterates between the selection for obligate heterodimers that are catalytically active on the asymmetric target sites (positive selection), and between heterodimers that are not catalytically active on off-target sites, preferably symmetric target sites.
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3. A genetically engineered DNA recombining enzyme for genome editing, wherein said DNA recombining enzyme comprises an obligate complex of recombinases, wherein said complex comprises at least a first recombinase enzyme and at least a second recombinase enzyme, wherein said first recombinase enzyme and said second recombinase enzyme specifically
25 recognize a first half-site and a second half-site of an upstream target site and/or a downstream target site of a DNA recombinase; wherein said first recombinase enzyme and said second recombinase enzyme each comprises at least one mutation in a catalytic site; wherein said first recombinase enzyme and said second recombinase enzyme carrying said at last one mutation in a catalytic site, when expressed in isolation, do not show the catalytic activity of a DNA
30 recombinase; and wherein the catalytic activity as a DNA recombinase in said obligate complex of recombinases is complemented.

4. The DNA recombining enzyme according to claim 3,

wherein the at least one first recombinase and the at least one second recombinase are of the same type, and/or

wherein the at least one first recombinase and the at least one second recombinase are both Cre-, Dre-, VCre-, SCre-, Vika-, lambda-Int-, Flp-, R-, Kw-, Kd-, B2-, B3-, Nigri- or Panto-
5 recombinases, and/or

wherein the DNA recombining enzyme is a complex of recombinases in form of a heterotetramer, and/or

wherein the at least one mutation is a single amino acid substitution in the catalytic region of the recombinase, preferably wherein the single amino acid substitution is at a position of a
10 conserved amino acid in the catalytic region, and/or

wherein:

(i) the first recombinase enzyme comprises a Cre recombinase and the second recombinase enzyme comprises a Cre recombinase, wherein each Cre recombinase comprises a single amino acid substitution within an amino acid region selected from the group consisting of
15 SEQ ID NO: 109 to SEQ ID NO: 114;

(ii) the first recombinase enzyme comprises a Vika recombinase and the second recombinase enzyme comprises a Vika recombinase, wherein each Vika recombinase comprises a single amino acid substitution within an amino acid region selected from the group consisting of SEQ ID NO: 115 to SEQ ID NO: 120;

20 (iii) the first recombinase enzyme comprises a Dre recombinase and the second recombinase enzyme comprises a Dre recombinase, wherein each Dre recombinase comprises a single amino acid substitution within an amino acid region selected from the group consisting of SEQ ID NO: 121 to SEQ ID NO: 126; or

(iv) the first recombinase enzyme comprises a Panto recombinase and the second
25 recombinase enzyme comprises a Panto recombinase, wherein each Panto recombinase comprises a single amino acid substitution within an amino acid region selected from the group consisting of SEQ ID NO: 127 to SEQ ID NO: 132.

5. The DNA recombining enzyme according to any one of claims 3 to 4, wherein the at least
30 one mutation or the single amino acid substitution in the at least one first and in the at least one second recombinase enzyme is selected from the group consisting of: E129R, Q133H, R173A, R173C, R173D, R173E, R173F, R173G, R173I, R173K, R173L, R173M, R173N, R173P, R173Q, R173S, R173T, R173V, R173W, R173Y, E176H, E176I, E176L, E176M, E176V, E176W, E176Y,

K201A, K201C, K201D, K201F, K201G, K201H, K201I, K201L, K201M, K201N, K201P, K201Q, K201R, K201S, K201T, K201V, K201W, K201Y, H289D, H289E, H289I, H289K, H289R, H289W, R292A, R292C, R292E, R292F, R292G, R292H, R292I, R292L, R292M, R292N, R292P, R292Q, R292S, R292T, R292V, R292W, R292Y, Q311R, W315C, W315E, W315G, W315I, W315K, W315L, W315M, W315N, W315Q, W315R, W315S, W315T, W315V, Y324A, Y324C, Y324E, Y324F, Y324H, Y324I, Y324K, Y324L, Y324M, Y324N, Y324Q, Y324R, Y324S, Y324T, Y324V, and Y324W of SEQ ID NO: 1, or in a corresponding amino acid position of another recombinase, preferably in a corresponding position of SEQ ID NO: 14, SEQ ID NO: 17 or SEQ ID NO: 20, and/or

wherein the at least one mutation or the single amino acid substitution in the at least one first and in the at least one second recombinase enzyme is at a position selected from the group consisting of:

- (i) E129, Q133, R173, E176, K201, H289, R292, Q311, W315, and Y324 of SEQ ID NO: 1;
- (ii) E146, Q151, R191, N194, K219, H308, R311, Q330, W334, and Y343 of SEQ ID NO: 14;
- (iii) E130, Q134, R174, E177, K202, H290, R293, Q312, W316, and Y325 of SEQ ID NO: 17;
- (iv) E131, Q135, R175, E178, K202, H290, R293, Q312, W316, and Y325 of SEQ ID NO: 20;

or

(v) at an amino acid position in another recombinase, wherein said amino acid position in the other recombinase corresponds to position E129, Q133, R173, E176, K201, H289, R292, Q311, W315, or Y324 of SEQ ID NO: 1.

6. The method or the genetically engineered DNA recombining enzyme according to any of the preceding claims, wherein said first recombinase enzyme and said second recombinase enzyme do not comprise monomer-monomer-interface mutations, and/or

wherein said genetically engineered DNA recombining enzyme is a mutant of a naturally occurring site-specific recombinase or a mutant of designer DNA recombinase.

7. The method or genetically engineered DNA recombining enzyme of any one of the preceding claims, wherein

- said first recombinase enzyme comprises the mutation selected from the group consisting of mutation K201R of SEQ ID NO: 1, 2 or 7, K202R of SEQ ID NO: 18 or 21, mutation K219R of SEQ ID NO: 15, and mutation K221R of SEQ ID NO: 24; and

- said second recombinase enzyme comprises the mutation selected from the group consisting of mutation Q311K of SEQ ID NO: 4 or 11, mutation Q311R of SEQ ID NO: 5 or 12, mutation Q312R of SEQ ID NO: 19 or 22, mutation Q330R of SEQ ID NO: 16, and mutation Q336R of SEQ ID NO: 25.

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8. The method or the genetically engineered DNA recombining enzyme according to any one of the preceding claims, wherein said recombinase target site is a target site of a tyrosine site-specific recombinase such as Cre-, Dre-, VCre-, SCre-, Vika-, lambda-Int-, Flp-, R-, Kw-, Kd-, B2-, B3-, Nigri- and Panto-recombinase, and said genetically engineered DNA recombining enzyme is selected from the group consisting of:

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- a genetically engineered DNA recombining enzyme, comprising a first recombinase enzyme and a second recombinase enzyme, wherein the first recombinase enzyme is a polypeptide which has an amino acid sequence with at least 70 %, preferably 80 %, more preferably 90 % sequence identity with a sequence according to SEQ ID NO: 7 and comprises the single mutation K201R in the catalytic site; and wherein the second recombinase enzyme is a polypeptide which has an amino acid sequence with at least 70 %, preferably 80 %, more preferably 90 % sequence identity with a sequence according to SEQ ID NO: 11 and comprises the single mutation Q311R in the catalytic site;

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- a genetically engineered DNA recombining enzyme, comprising a first recombinase enzyme and a second recombinase enzyme, wherein the first recombinase enzyme is a polypeptide which has an amino acid sequence with at least 70 %, preferably 80 %, more preferably 90 % sequence identity with a sequence according to SEQ ID NO: 7 and comprises the single mutation K201R in the catalytic site; and wherein the second recombinase enzyme is a polypeptide which has an amino acid sequence with at least 70 %, preferably 80 %, more preferably 90 % sequence identity with a sequence according to SEQ ID NO: 12 and comprises the single mutation Q311K in the catalytic site;

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- Cre recombinase, comprising a first recombinase enzyme having the single mutation K201R in its catalytic site and having an amino acid sequence which is at least 70 %, preferably 80 %, more preferably 90 % identical with a polypeptide having the sequence according to SEQ ID NO: 2; and comprising a second recombinase enzyme having the single mutation Q311R in its catalytic site and having an amino acid sequence which is at least 70 %, preferably 80 %, more preferably 90 % identical with a polypeptide having the sequence according to SEQ ID NO: 5;

- Cre recombinase, comprising a first recombinase enzyme having the single mutation K201R in its catalytic site and having an amino acid sequence which is at least 70 %, preferably 80 %, more preferably 90 % identical with a polypeptide having the sequence according to SEQ ID NO: 2; and comprising a second recombinase enzyme having the single mutation Q311K in its catalytic site and having an amino acid sequence which is at least 70 %, preferably 80 %, more preferably 90 % identical with a polypeptide having the sequence according to SEQ ID NO: 4;
- Vika recombinase, comprising a first recombinase enzyme having the single mutation K219R in its catalytic site and having an amino acid sequence which is at least 70 %, preferably 80 %, more preferably 90 % identical with a polypeptide having the sequence according to SEQ ID NO: 15; and a second recombinase enzyme having the single mutation Q330R in its catalytic site and having an amino acid sequence which is at least 70 %, preferably 80 %, more preferably 90 % identical with a polypeptide having the sequence according to SEQ ID NO: 16;
- Panto recombinase, comprising a first recombinase enzyme having the single mutation K202R in its catalytic site and having an amino acid sequence which is at least 70 %, preferably 80 %, more preferably 90 % identical with a polypeptide having the sequence according to SEQ ID NO: 18; and a second recombinase enzyme having the single mutation Q312R in its catalytic site and having an amino acid sequence which is at least 70 %, preferably 80 %, more preferably 90 % identical with a polypeptide having the sequence according to SEQ ID NO: 19;
- Dre recombinase, comprising a first recombinase enzyme having the single mutation K202R in its catalytic site and having an amino acid sequence which is at least 70 %, preferably 80 %, more preferably 90 % identical with a polypeptide having the sequence according to SEQ ID NO: 21; and a second recombinase enzyme having the single mutation Q312R in its catalytic site and having an amino acid sequence which is at least 70 %, preferably 80 %, more preferably 90 % identical with a polypeptide having the sequence according to SEQ ID NO: 22; and
- Vcre recombinase, comprising a first recombinase enzyme having the single mutation K221R in its catalytic site and having an amino acid sequence which is at least 70 %, preferably 80 %, more preferably 90 % identical with a polypeptide having the sequence according to SEQ ID NO: 24; and a second recombinase enzyme having the single mutation Q336R in its catalytic site and having an amino acid sequence which is at least 70 %, preferably 80 %, more preferably 90 % identical with a polypeptide having the sequence according to SEQ ID NO: 25.

9. The method or the genetically engineered DNA recombining enzyme according to claim 8, wherein said genetically engineered DNA recombining enzyme specifically recognizes the

upstream recombinase target sequence of the loxF8 target site, which has the nucleic acid sequence

ATAAATCTGTGGAAACGCTGCCACACAATCTTAG (SEQ ID NO: 65) or a reverse complement sequence thereof;

5 and recognizes the downstream recombinase target sequence of the loxF8 target site, which has the nucleic acid sequence

CTAAGATTGTGTGGCAGCGTTCCACAGATTAT (SEQ ID NO: 66) or a reverse complement sequence thereof;

and which catalyzes the recombination of a gene sequence between the upstream recombinase target site sequence of SEQ ID NO: 65 and the downstream recombinase target site sequence of
10 SEQ ID NO: 66 of the loxF8 recombinase target site; and

wherein the capability to catalyze the recombination of a gene sequence between the upstream recombinase target sequence of SEQ ID NO: 65 and the downstream recombinase target sequence of SEQ ID NO: 66 of the loxF8 recombinase target site is tested by a method
15 comprising the steps of:

a) expressing the genetically engineered DNA recombining enzyme comprising a first recombinase enzyme and a second recombinase enzyme in a cell; and

b) analyzing, whether the genetically engineered DNA recombining enzyme expressed in step a) is capable of recombining of a DNA sequence on a human chromosome in
20 said cell.

10. A nucleic acid molecule or a plurality of nucleic acid molecules each comprising or consisting of a nucleic acid sequence encoding a genetically engineered DNA recombining enzyme or a subunit thereof according to any one of claims 3 to 9.

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11. An expression vector comprising the nucleic acid molecule or the plurality of nucleic acid molecules according to claim 10 and expression-controlling elements operably linked with said nucleic acid to drive expression thereof.

30 12. A host cell comprising a nucleic acid molecule or the plurality of nucleic acid molecules according to claim 10 or the expression vector of claim 11.

13. A pharmaceutical composition comprising the genetically engineered DNA recombining enzyme according to any one of claims 3 to 9 or the nucleic acid molecule or the plurality of nucleic acid molecules according to claim 10, or the expression vector according to claim 11, or the cell of claim 12, preferably further comprising one or more therapeutically acceptable diluents or carriers.
14. The genetically engineered DNA recombining enzyme according to any one of claims 3 to 9, or the nucleic acid molecule or the plurality of nucleic acid molecules according to claim 10, or the expression vector according to claim 11, or the pharmaceutical composition according to claim 13, for use in medicine, preferably for use in the treatment of hemophilia A, more preferably for use in the treatment of severe hemophilia A.
15. A method for inversion of a DNA sequence on genomic level in a cell in vitro, comprising a genetically engineered DNA recombining enzyme according to the invention, wherein said method comprises the steps of:
- i. providing a nucleic acid molecule encoding a first recombinase enzyme as claimed in any one of claims 3 to 9, wherein said first recombinase enzyme comprises at least one mutation that inactivates the catalytic activity as a DNA recombinase of said first recombinase enzyme and wherein said first recombinase monomer specifically recognizes a first half-site of a recombinase target site;
 - ii. providing a nucleic acid molecule encoding a second recombinase enzyme as claimed in any one of claims 3 to 9, wherein said second recombinase enzyme comprises at least one mutation that inactivates the catalytic activity as a DNA recombinase of said second recombinase enzyme and wherein said second recombinase monomer specifically recognizes a second half-site of a recombinase target site;
 - iii. creating an expression vector by cloning the nucleic acid molecule encoding the first recombinase enzyme and the nucleic acid molecule encoding the second recombinase enzyme into an expression vector;
 - iv. delivering said expression vector to a cell, which comprises a DNA sequence, which is to be inverted, an expression vector of step iii) or an RNA molecule encoding a genetically engineered DNA recombining enzyme as claimed in any one of claims 3 to 9;
 - v. expressing the genetically engineered DNA recombining enzyme as claimed in any one of claims 3 to 9;

vi. inversion of a DNA sequence, which is to be inverted, on a human chromosome in said cell with said genetically engineered DNA recombining enzyme as claimed in any one of claims 3 to 9 expressed in said cell.