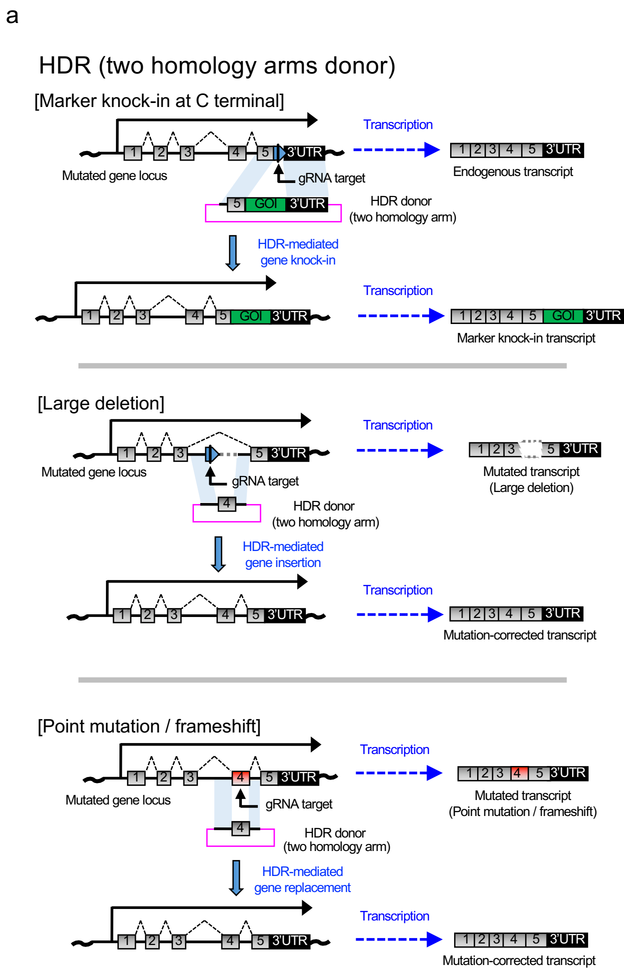
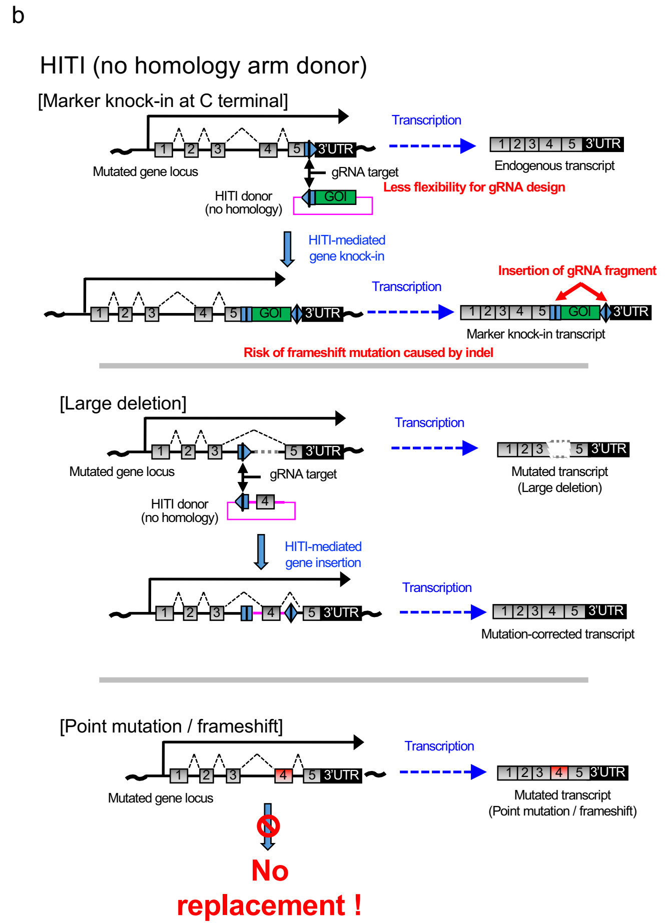
a



b



c

SpCas9/gRNA

N1N2N3N4N5N6N7N8N9N10N11N12N13N14N15N16N17N18N19N20NGG

Chromosomal DNA

Gene

GOI

Donor DNA

Forward (correct) direction

Reverse (undesirable) direction

NHEJ repair

Recut

SpCas9/sgRNA

Homology-Independent Targeted Integration (HITI)

Supplementary Figure S1 | Schematic representation of HDR- and HITI-mediated knock-in methods. a Schematic representation of the HDR-mediated gene-knock-in method. The donor DNA includes two-homology arms where is identical to target genome. HDR can replace the existing mutations, but not active in non-dividing cells. The application for *in vivo* is limited to the tissues that possess dividing capacity. **b** Schematic representation of the HITI-mediated gene-knock-in method. The donor DNA includes Cas9-mediated DSB induction site and no homology for target genome. DSBs are created simultaneously in both genomic target sequences and donor DNA, allowing for donor integration into the genomic DSB site. HITI cannot replace the existing mutations, but active in non-dividing cells. **c** Unidirectional gene knock-in by HITI. The SpCas9 and sgRNA complex introduces double-strand break (DSB) into chromosomal DNA three base pairs upstream of the PAM sequence, resulting in two blunt ends. The same sgRNA target sequence is loaded onto the donor DNA in the reverse direction. Both targeted chromosomal DNA and donor DNA are cleaved by SpCas9/sgRNA complex in the cells. When the blunt ends of targeted chromosomal DNA and the linearized donor DNA are ligated via the cellular non-homologous end joining (NHEJ) repair machinery, the donor DNAs are integrated into target sites. If the donor DNA is integrated in the correct orientation (left), junction sequences are protected from further cleavage by SpCas9. If the donor DNA integrates in the reverse orientation (right), SpCas9 will excise the integrated donor DNA due to the presence of intact sgRNA target sites. We named this integration system as Homology-Independent Targeted Integration (HITI). Blue pentagon, sgRNA target sequence. Black line within blue pentagon, SpCas9 cleavage site. GOI, gene of interest.