Supplementary information

Development of an in vivo cleavable donor plasmid for targeted transgene integration by CRISPR-Cas9 and CRISPR-Cas12a

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Supplementary Figure S1 to S10
Supplementary Table S1
**Supplementary Figure S1. Generation of Split-mCherry (SpmCherry) reconstitution reporter cells.**

(A) Design of CMV-SpmCherry-P2A-Puro-pA reporter sequence. The mCherry coding sequence (1–300) was divided by the mouse ActB intron III sequence. (B) Genotyping PCR in 23 SpmCherry reporter HEK293T cell lines. The gel electrophoresis images show the PCR products of the SpmCherry reporter (2,422 bp) using the SpmCherry_F/R primers shown in (A) and an internal control (AAVS1 locus; 55,115,576–55,116,174: 599 bp).
Supplementary Figure S2. *In vitro* pCriMGET_9-12a cleavage using Cas9 or Cas12a nuclease.
pCriMGET_9-12a_SpmCherry_c was incubated with ribonuclease complex of syn-crRNA-TS-sgRNA and 
Cas9 nuclease, or syn-crRNA-TS-crRNA and Cas12a nuclease. Samples were separated by 1.0% agarose 
gel electrophoresis. Black arrowhead shows a single cleavage band and red arrowhead shows double 
cleavage bands. EcoRV digested product was used as a single cleavage marker.
Supplementary Figure S3. Effect of the length of the homology arms on knock-in frequency.

(A) Gating strategy for fluorescence-activated cell sorting (FACS) analysis. Single cells were gated by FSC-H vs FSC-W. Live cells were gated by the DAPI- population. EGFP\textsuperscript{high} cells were gated and the percentages of mCherry\textsuperscript{+} cells were calculated in the EGFP\textsuperscript{high} cell population. (B) FACS analyses of mCherry expression in the Split-mCherry reporter cells transfected with the indicated length of donor sequence. (upper, pCriMGET_9-12a/CRISPR-Cas9 system; lower, pCriMGET_9-12a/CRISPR-Cas12a system). Average percentages of mCherry\textsuperscript{+} cells in each sample are shown in the bar graph (mean ± s.d. from three experiments).
Supplementary Figure S4. Generation of Hipp11^{CAG-tdTomato} knock-in mice by the pCriMGET and pCriMGET_9-12a systems. Genotyping PCR analyses of Hipp11^{CAG-tdTomato} knock-in blastocysts generated by pCriMGET (A) and pCriMGET_9-12a (B). KI and WT bands for 5' and 3' junctions are indicated by red and black arrowheads, respectively.
Supplementary Figure S5. tdTomato expression in tissues of Hipp11\textsuperscript{CAG-tdTomato} Kl/+ mice.

Images of tdTomato expression in brain, heart, lung, liver, kidney, spleen, intestine, and skeletal muscle. Tissues on the left and right are from WT and Hipp11\textsuperscript{CAG-tdTomato} Kl/+ mice, respectively. Images were taken under 540-nm excitation light using a single-lens reflex camera with a 600-nm LP filter. qPCR analyses for expression of \textit{tdTomato} mRNA are shown at the bottom. For qPCR, values were normalized to expression of \textit{G3pdh}. 
Supplementary Figure S6. Generation of Rosa26CAG-LSL-NuM-mCherry knock-in mice by pCriMGET_9-12a and CRISPR-Cas9 with or without syn-crRNA-TS-crRNA. Genotyping PCR analyses of Rosa26CAG-LSL-NuM-mCherry knock-in blastocysts generated by pCriMGET_9-12a and CRISPR-Cas9 in the absence (A) or presence (B) of syn-crRNA-TS-crRNA. KI and WT bands for 5’ and 3’ junctions are indicated by red and black arrowheads, respectively. The table shows the knock-in frequency in the blastocysts.
Supplementary Figure S7. Generation of $\text{Rosa26}^{\text{CAG-LSL-NuM-mCherry}}$ knock-in mice by pCriMGET_9-12a and CRISPR-Cas12a with or without syn-crRNA-TS-crRNA. Genotyping PCR analysis of $\text{Rosa26}^{\text{CAG-LSL-NuM-mCherry}}$ knock-in blastocysts generated by pCriMGET_9-12a and CRISPR-Cas12a in the absence (A) or presence (B) of syn-crRNA-TS-crRNA. KI and WT bands for 5’ and 3’ junctions are indicated by red and black arrowheads, respectively. The table shows the knock-in frequency in the blastocysts.
Supplementary Figure S8. Germline transmission of the donor gene in \textit{Rosa26}^{CAG-LSL-NuM-mCherry} knock-in mice. Genotyping PCR of 4-week-old F\textsubscript{1} pups derived from a \textit{Rosa26}^{CAG-LSL-NuM-mCherry} hemizygous knock-in male mouse and \textit{Tbx3}^{CreERT2} homozygous knock-in female mice. Upper panel shows \textit{Rosa26}^{CAG-LSL-NuM-mCherry} knock-in (KI) (934 bp) and wild-type (WT) (677 bp) bands; lower panel shows \textit{Tbx3}^{CreERT2} KI (657 bp) and WT (335 bp) bands.
Supplementary Figure S9. Donor transgene expression in plantar skin of Rosa26<sup>CAG-LSL-NuM-mCherry</sup> knock-in mice. Representative immunofluorescence images of plantar skin tissue in the Rosa26<sup>CAG-LSL-NuM-mCherry</sup>, Tbx3<sup>CreERT2</sup> mice administered control corn oil and tamoxifen. Tbx3 (green), NuM-mCherry (red), CD49f (white), and DAPI (blue). Scale bar, 20μm.
| Reference genome                  | mismatches |
|----------------------------------|------------|
|                                  | 0 | 1 | 2 | 3 |
| mouse genome (GCRm38/mm10)       | 0 | 0 | 0 | 5 |
| human genome (GCRh38/hg 38)      | 0 | 0 | 0 | 0 |
| mouse genome (GCRm38/mm10)       | 0 | 0 | 0 | 0 |
| human genome (GCRh38/hg 38)      | 0 | 0 | 0 | 0 |

The number of syn-crRNA-TS_9-12a mismatches (0–3 nucleotides) for CRISPR-Cas9 and -Cas12a, and the mismatched bases are shown.
Related to figure 2

Cas9: 1-9, Reporter, DW

Cas12a: 1-9, Reporter, DW

Related to figure 3

5' junction: WT, KI, DW

3' junction: WT, KI, DW

Related to figure 4

5' junction: 1-10

5' junction: 11-15, WT, DW

3' junction: 1-10

3' junction: 11-15, WT, DW
Related to supplementary figure S1

EcoRV, Cas9, Cas12a

Related to supplementary figure S2

1-18

19-23, WT, DW
Related to supplementary figure S4 (A)

- 5' junction: 1-21
- 5' junction: 22-42
- 5' junction: 43-47, WT, DW
- 3' junction: 1-21
- 3' junction: 22-42
- 3' junction: 43-47, WT, DW
Related to supplementary figure S3 (B)

5' junction: 1-21

5' junction: 43-53, WT, DW

5' junction: 22-42

3' junction: 1-21

3' junction: 22-42

3' junction: 43-53, WT, DW
Related to supplementary figure S5 (A) (B)

(A) 5’ junction : 1-21

(A) 5’ junction : 22-42

(A) 5’ junction : 43-52, WT, DW

(B) 5’ junction : 9-29

(B) 5’ junction : 30-41, WT, DW

(A) 3’ junction : 1-21

(A) 3’ junction : 22-42

(A) 3’ junction : 43-52, WT, DW

(B) 3’ junction : 1-8

(B) 3’ junction : 9-29

(B) 3’ junction : 30-41, WT, DW
Supplementary Figure S10. Full-length, unprocessed Scan for PCR gel.