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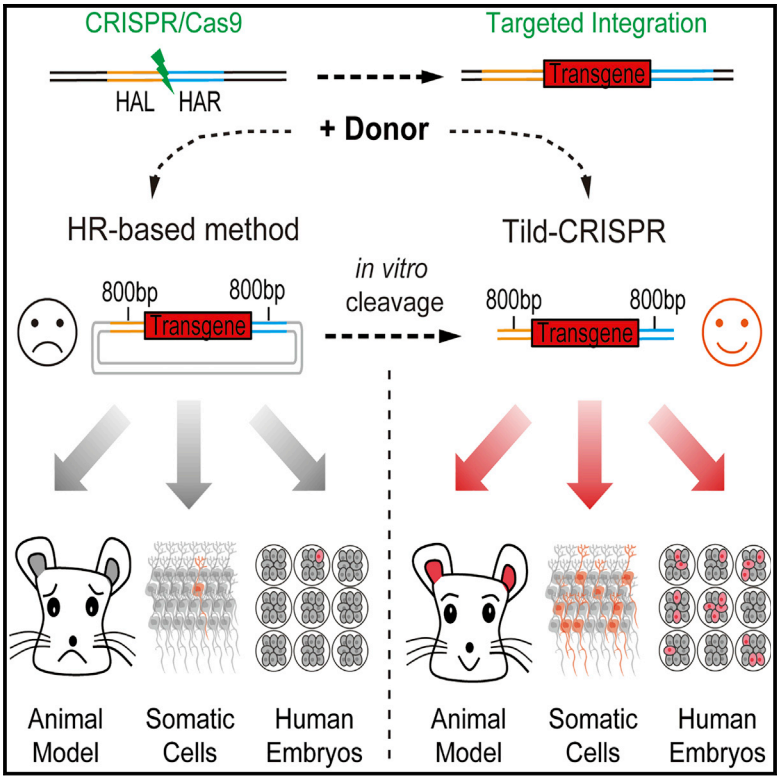
# Tild-CRISPR Allows for Efficient and Precise Gene Knockin in Mouse and Human Cells

## Graphical Abstract




## Highlights

- Tild-CRISPR uses *in vitro* linearized transgene donor with 800-bp HA
- Tild-CRISPR is a simple and efficient method for creating gene-modified mice
- Tild-CRISPR shows robust DNA knockin efficiency in mouse brain and human embryos

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## In Brief

Yao et al. describe Tild-CRISPR, a targeting strategy using PCR-amplified or precisely enzyme-cut transgene donor sequence. Tild-CRISPR yields robust knockin efficiency in mouse and human embryos, as well as mouse brain *in vivo*, suitable for studying gene functions *in vivo* and developing potential gene therapies.

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# Tild-CRISPR Allows for Efficient and Precise Gene Knockin in Mouse and Human Cells

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## SUMMARY

**The targeting efficiency of knockin sequences via homologous recombination (HR) is generally low. Here we describe a method we call Tild-CRISPR (targeted integration with linearized dsDNA-CRISPR), a targeting strategy in which a PCR-amplified or precisely enzyme-cut transgene donor with 800-bp homology arms is injected with Cas9 mRNA and single guide RNA into mouse zygotes. Compared with existing targeting strategies, this method achieved much higher knockin efficiency in mouse embryos, as well as brain tissue. Importantly, the Tild-CRISPR method also yielded up to 12-fold higher knockin efficiency than HR-based methods in human embryos, making it suitable for studying gene functions *in vivo* and developing potential gene therapies.**

## INTRODUCTION

CRISPR/Cas9-mediated genome editing has greatly facilitated the correction of genetically mutated cells and tissues *in situ* (Komor et al., 2017; Yin et al., 2014). However, the precise knockin efficiency via homologous recombination (HR) is generally low (Skarnes et al., 2011; Yang et al., 2013). Recently, non-homologous end-joining (NHEJ)- or microhomology-mediated end-joining (MMEJ)-based methods have reported that *in vivo* cleavage of transgene donors without homology arm (HA) or with short HA (5–25 bp) promotes CRISPR-mediated targeted integration in zebrafish and mice (Auer et al., 2014; Cristea et al., 2013; Hisano et al., 2015; Li et al., 2015; Maresca et al., 2013; Nakade et al., 2014; Suzuki et al., 2016; Yao et al., 2017b). Moreover, two groups have reported a homology-mediated end-joining (HMEJ)-based strategy for *in vivo* cleavage of transgene donors with ~800 bp HA achieving a robust DNA knockin (Yao et al., 2017a; Zhang et al., 2017). However, mice bearing conditional alleles—the most useful genetically engineered models—and human embryos modified by DNA knockin have not been generated by these new approaches. A targeting strategy, Easi-CRISPR, using long single-stranded DNA (ssDNA) donors has been reported to generate mice with conditional alleles efficiently (Quadros et al., 2017). However, using ssDNA as a donor by Easi-CRISPR is costly and there is a size limitation for insertion (usually <1 kb).

Here we report a Tild-CRISPR (targeted integration with linearized dsDNA-CRISPR)-based strategy, using *in vitro* linearized DNA by PCR-amplified or precisely enzyme-cut transgene donor with 800 bp HA, which efficiently achieved DNA-targeted integration in mouse and human embryos, as well as mouse brain *in vivo*.

## RESULTS

### Genome Editing in Mouse Embryos Using Tild-CRISPR

Previous studies have showed that *in vivo* cleavage of transgene donor could promote CRISPR-mediated targeted integration in generating animal models (Auer et al., 2014; Cristea et al., 2013; Hisano et al., 2015; Li et al., 2015; Maresca et al., 2013; Nakade et al., 2014; Suzuki et al., 2016; Yao et al., 2017a, 2017b). We thus examined whether the knockin efficiency could be further improved by prior *in vitro* cleavage of the transgene donor by two restriction enzymes that results in a linearized donor with long HAs (termed Tild donor). We compared the efficiency of Tild donor with two commonly used other types of donors: an HMEJ donor (single guide RNA [sgRNA] target sites plus 800-bp HAs)

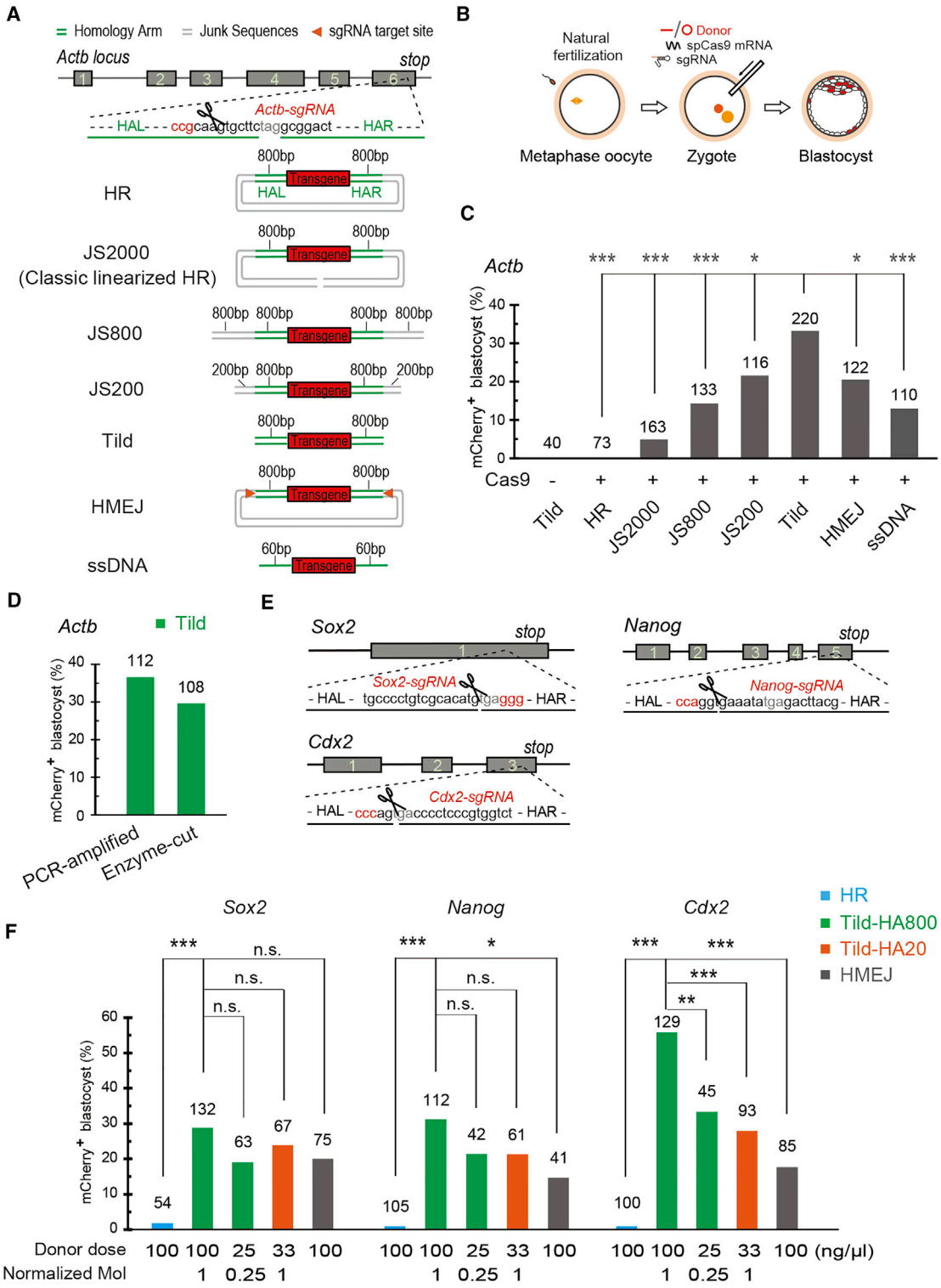



**Figure 1. Tild-CRISPR-Mediated Targeted Integration in Mouse Embryos**

(A) Schematic overview of different gene-targeting donors using CRISPR/Cas9 at the *Actb* locus. Green parallel lines, left/right homology arm; gray parallel lines, junk sequence; triangles, sgRNA target sites; HR, a homologous recombination donor; HMEJ, a homology-mediated end-joining donor; ssDNA, a single-stranded DNA donor; Tild, a linearized donor with 800-bp HAs; JS200 to JS2000, a linearized donor with 200- to 2,000-bp junk sequences adjacent to HAs.

(B) Experimental design. Cas9 mRNA, sgRNA, and different donor vectors were injected into mouse zygotes, and the injected zygotes were cultured into blastocyst stage for fluorescence observation and genotyping analysis.

*(legend continued on next page)*

and an HR donor (only 800-bp HAs) (Figure 1A). We fused a p2A-mCherry reporter gene to the last codon of the *Actb* gene (Figure 1A). Each type of the transgene donor was injected together with Cas9 mRNA and sgRNA targeting the *Actb* gene into mouse zygotes. The injected zygotes were cultured into blastocysts (Figure 1B). The knockin efficiencies were evaluated by mCherry fluorescence signals in blastocysts (Figures 1B and S1A). Interestingly, we observed a much higher rate of mCherry$^+$ blastocysts with the Tild donor (33.2%) than those with the HMEJ donor (20.5%) or HR donor (0%) (Figures 1C and S1A).

Compared with a classic linearized HR donor (JS2000), the Tild donor did not contain a non-homologous sequence adjacent to HAs. We hypothesized that the non-homologous sequence (known as the junk sequence [JS]) of the donor may have reduced the efficiency of transgene integration. To test this idea, we added JSs of different lengths (200, 800, and 2,000 bp) to both ends of the Tild donor (Figure 1A). Interestingly, increased JS length resulted in decreased knockin efficiency from 33.2% (no JS) to 4.9% (2000-bp JS) (Figure 1C). In addition, PCR-amplified transgene donor with long HAs exhibited knockin efficiencies at the *Actb* locus similar to that with the enzyme-cut Tild donor (Figure 1D). We also examined the donor with a long single-strand DNA (ssDNA) that was used in an efficient targeting strategy, Easi-CRISPR (Quadros et al., 2017), and observed a relatively low rate of mCherry$^+$ blastocysts (12.7%) (Figure 1C). We next examined Tild-CRISPR-mediated knockin efficiencies at additional loci, including *Nanog* (pluripotency marker), *Sox2* (pluripotency marker), and *Cdx2* (trophectoderm marker), by fusing p2A-mCherry reporter with the last codon of targeted genes (Figure 1E). We found that the Tild-based method exhibited higher knockin efficiencies than HR- or HMEJ-based methods at all of the three tested loci (Figure 1F). Notably, we found that the knockin efficiency of 800-bp HA was generally higher than that of 20-bp HA (Figure 1F). Genotyping analysis of individual mCherry$^+$ blastocyst by the Tild-CRISPR method showed that almost all examined integration events had precise in-frame integration at 5′ and 3′ junctions (Figure S1).

Next, we tested whether Tild-CRISPR could be used for generating knockin mice. After transplantation of Tild-CRISPR-treated embryos into pseudo-pregnant mice, we successfully obtained gene-edited knockin mice at six different loci, including mice with mCherry integration at the *Dbh* locus (6/29) and the *Cdx2* locus (31/57), Cre integration at the *Sp8* locus (10/35), CAG-LSL-ChR2-Tdtomato (6.0 kb) integration at the *Rosa26* locus (2/29), and conditional floxed alleles at the *Nr3c2* locus (3/16) and *Lhx6* locus (4/12) (Figures 2 and 3; Table 1). Precise integration in these gene-edited mice was confirmed by DNA-sequencing analysis (Figures 2B, 3C, and 3D), and all the knockin mice examined were able to go through germline transmission (Table 1 and Figure S2).

Considering that linearized double-strand DNA (dsDNA) as templates in Tild-CRISPR may result in more random insertions than using equal doses of circular DNAs in HR- or HMEJ-based methods, we performed Southern blot analysis of the samples from the mice bearing mCherry knockin at *Cdx2* locus by the HMEJ-based method and Tild-CRISPR. By using mCherry internal probe, additional randomly integrated transgenes were detected in 2 out of 7 mCherry knockin fetuses by the HMEJ-based method and in 2 out of 8 mCherry knockin mice by Tild-CRISPR (Figure S3). Compared with HMEJ-based and HR-based methods (Yang et al., 2013; Yao et al., 2017a), the random insertion rate of Tild-CRISPR was not significantly increased.

Together, these results indicate that the Tild-CRISPR method yielded robust DNA integration in the mouse embryo.

### *In Vivo* Genome Editing Using Tild-CRISPR

For *in vivo* applications, we set out to discover whether Tild-CRISPR could be applied for *in vivo* DNA integration. We aimed to insert a p2A-mCherry to the *Actb* gene. Tild constructs were delivered to the embryonic day 14.5 mouse brain using *in utero* electroporation (Figures 4A and 4B). Seven days after electroporation, brain sections were stained and counted. We observed that about 16% of electroporated cells with Tild-HA800 constructs (mCherry$^+$/GFP$^+$, relative efficiency) showed mCherry expression (Figures 4C and 4D). By contrast, only about 5%, 9.5% ± 1.7%, and 0.8% ± 0.2% of electroporated cells were mCherry$^+$ using Tild-HA200, HMEJ, and HR donors, respectively (Figures 4C and 4D). Our results indicated that Tild-CRISPR showed high DNA-integration efficiency *in vivo*.

### Genome Editing in Human Embryos Using Tild-CRISPR

CRISPR/Cas9-mediated genome editing is capable of introducing precise genetic modifications in early human embryos by direct injection of Cas9 mRNA, sgRNA, and an HR donor into single-cell zygotes. However, the precise knockin efficiency is generally low (Kang et al., 2016; Liang et al., 2015; Tang et al., 2017). In the next set of experiments, we examined whether Tild-CRISPR could achieve efficient DNA integration in human embryos. We first targeted *OCT4* (the inner cell mass and epiblast marker) with a fluorescent reporter (Figure 5A) by inserting *OCT4*-intron 4-exon 5-2A-GFP-polyA into intron 4 of the *OCT4* locus (Figure 5A). Three sgRNAs for *OCT4* were designed and the cleavage efficiency of sgRNAs was examined by injecting Cas9 mRNA and each sgRNA into human tripronuclear zygotes (Figure 5A). DNA-sequencing analysis showed that sgRNA #3 for *OCT4* yielded highest cleavage activities (Figure 5B). We thus co-injected *OCT4*-sgRNA (50 ng/μL), Cas9 mRNA (100 ng/μL), and Tild donors of *OCT4* (60 ng/μL) into human tripronuclear zygotes (Figure 5C). In the control group, HR donors of *OCT4*

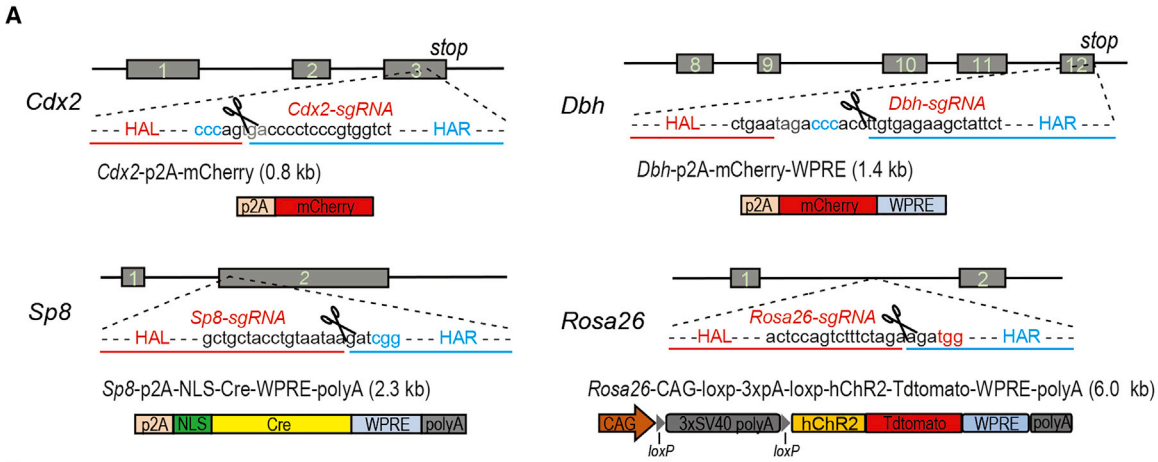
(C) Knockin efficiencies indicated by percentage of mCherry$^+$ blastocysts. Number above each bar denotes total blastocysts counted. *$p < 0.05$, ***$p < 0.001$, $\chi^2$ test.

(D) Knockin efficiencies of PCR-amplified or precisely enzyme-cut Tild donor indicated by percentage of mCherry$^+$ blastocysts. Number above each bar denoted total blastocysts counted.

(E) Schematic overview of Tild-mediated gene-targeting strategy at the *Sox2*, *Nanog*, and *Cdx2* loci in mouse embryos.

(F) Efficiencies of different gene-targeting donors for p2A-mCherry precise integration at the *Sox2*, *Nanog*, and *Cdx2* loci. Number above each bar denotes total blastocysts counted. n.s., not significant; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, $\chi^2$ test.

See also Figure S1.




B

*Cdx2*-p2A-mCherry mice genotyping

5' junction

| | *Cdx2* | HAL | PAM | p2A |
|---|---|---|---|---|
| | 5'-gtcacagtgtcggctaactcTGATTGCAGGGGACACCCAG- - - - // - - - - TAAACTCCACTGTCACCCAGgccactaacttctccctgtt-3' | | | |
| 4/4 (100%) | 5'-gtcacagtgtcggctaactcTGATTGCAGGGGACACCCAG- - - - // - - - - TAAACTCCACTGTCACCCAGgccactaacttctccctgtt-3' | | | |

3' junction

| | mCherry | HAR | 3'-UTR |
|---|---|---|---|
| | 5'-tggacgagctgtacaagtaaGACCCCTCCCGTGGTCTGAAG- - - - // - - - - TCGAGTCACTGATCTGTGTAacgagccaaacaagcctttt-3' | | |
| 4/4 (100%) | 5'-tggacgagctgtacaagtaaGACCCCTCCCGTGGTCTGAAG- - - - // - - - - TCGAGTCACTGATCTGTGTAacgagccaaacaagcctttt-3' | | |

*Dbh*-p2A-mCherry-WRPE mice genotyping

5' junction

| | *Dbh* | HAL | p2A |
|---|---|---|---|
| | 5'-gtgtcagcaccctgctcagaGAATAAAAATAGAGCTATGG- - - - - // - - - - - ACAACAGAGGCAGATGCTGAAgccactaacttctccctgtt-3' | | |
| 3/3 (100%) | 5'-gtgtcagcaccctgctcagaGAATAAAAATAGAGCTATGG- - - - - // - - - - - ACAACAGAGGCAGATGCTGAAgccactaacttctccctgtt-3' | | |

3' junction

| | WPRE | HAR | 3'-UTR |
|---|---|---|---|
| | 5'-ttgggccgcctcccgccacTTGTGAGAAGCTATTCTTCA- - - - - // - - - - - TGTCCAGGATTCCTGCCCTGtgctgagcctttctgggctc-3' | | |
| 3/3 (100%) | 5'-ttgggccgcctcccgccacTTGTGAGAAGCTATTCTTCA- - - - - // - - - - - TGTCCAGGATTCCTGCCCTGtgctgagcctttctgggctc-3' | | |

*Sp8*-p2A-NLS-Cre-WPRE-ploy A mice genotyping

5' junction

| | *Sp8* | HAL | p2A |
|---|---|---|---|
| | 5'-gcatagcccctaatacactcgacGATTTCGAAGGGGAACGCGAGTA- - - - - // - - - - -TGCTTGCTGCTACCTGTAATAAGgccactaacttctccctgttgaa-3' | | |
| 3/3 (100%) | 5'-gcatagcccctaatacactcgacGATTTCGAAGGGGAACGCGAGTA- - - - - // - - - - -TGCTTGCTGCTACCTGTAATAAGgccactaacttctccctgttgaa-3' | | |

3' junction

| | hGH-ployA | PAM | HAR | *Sp8* |
|---|---|---|---|---|
| | 5'-accactgctcccttccctgtcttGATCGGCAGCCCCAGCCCGTCTCC- - - - // - - - - AGGACAGCACCTCATGGACGGTTTcaagccggtattgcccggctctta-3' | | | |
| 3/3 (100%) | 5'-accactgctcccttccctgtcttGATCGGCAGCCCCAGCCCGTCTCC- - - - // - - - - AGGACAGCACCTCATGGACGGTTTcaagccggtattgcccggctctta-3' | | | |

*Rosa26*-CAG-loxp-3xpA-loxp-hChR2-Tdtomato-WPRE-polyA mice genotyping

5' junction

| | *Rosa26* | HAL | linker-CAG |
|---|---|---|---|
| | 5'-gttttggttggcgtaaggcgCCTGTCAGTTAACGGCAGCC- - - - // - - - - GCAACTCCAGTCTTTCTAGAatcgatggcgcgccggattcgacattgatt-3' | | |
| 2/2 (100%) | 5'-gttttggttggcgtaaggcgCCTGTCAGTTAACGGCAGCC- - - - // - - - - GCAACTCCAGTCTTTCTAGAatcgatggcgcgccggattcgacattgatt-3' | | |

3' junction

| | hGH-ployA-linker | PAM | HAR | *Rosa26* |
|---|---|---|---|---|
| | 5'-cggtgggctctatggcggcggccgcAGATGGGCGGGAGTCTTCTG- - - - // - - - - TGAATGCCTCTCTCCTTTTTCTCCAtttataaactgagctattaa-3' | | | |
| 2/2 (100%) | 5'-cggtgggctctatggcggcggccgcAGATGGGCGGGAGTCTTCTG- - - - // - - - - TGAATGCCTCTCTCCTTTTTCTCCAtttataaactgagctattaa-3' | | | |

*(legend on next page)*

**Table 1. Knockin Mice Generated by Tild-Mediated Targeted Integration**

| Gene | Insert Size (kb) | Method | Transferred Embryos (Recipients) | Newborns (Birth Rate %) | 5′ and 3′ Junction (%) | Precise Integration (%) | Germline Transmission (%) |
|---|---|---|---|---|---|---|---|
| *Cdx2*-2A-mCherry | 0.8 | HMEJ | 118 (6) | 43 (36.4)[a] | 7/43 (16.3) | 7/7 (100) | ND |
| | | Tild | 216 (11) | 57 (26.4) | 31/57 (54.4) | 4/4 (100) | 4/4 (100) |
| *Dbh*-2A-mCherry-WPRE | 1.4 | Tild | 84 (4) | 29 (34.5) | 6/29 (20.7) | 3/3 (100) | 3/3 (100) |
| *Sp8*-2A-Cre-WPRE | 2.3 | HMEJ | 125 (5) | 33 (26.4) | 4/33 (12.1) | 4/4 (100) | 3/3 (100) |
| | | Tild | 148 (7) | 35 (23.6) | 10/35 (28.6) | 3/3 (100) | 2/2 (100) |
| *Rosa26-CAG-lsl*-hChR2-Tdtomato-WPRE | 6.0 | Tild | 57 (3) | 29 (50.9) | 2/29 (6.9) | 2/2 (100) | 1/1 (100) |
| *Nr3c2*-exon 5 floxed | 0.6 | Tild | 63 (3) | 16 (25.4) | 3/16 (18.8) | 3/3 (100) | 2/2 (100) |
| *Lhx6*-exon 6–8 floxed | 1.0 | Tild | 66 (3) | 12 (18.2) | 4/12 (33.3) | 4/4 (100) | 2/2 (100) |

Cas9 mRNA (100 ng/μL), sgRNAs (50 ng/μL), and donor vectors (50 ng/μL) were injected into fertilized eggs. Two-cell embryos derived from the injected embryos were transferred into recipients, and newborn pups were obtained and genotyped. ND, not determined.
[a]The fetuses were isolated before parturition and analyzed.

(60 ng/μL), together with sgRNA and Cas9, were injected. The injected embryos were cultured *in vitro* for 2 days, and the single blastomeres isolated from injected human embryos at the 4- to 16-cell stage were used for genotyping (Figure 5C). We found that Tild-CRISPR showed much higher knockin efficiency (21/101 at single-blastomere level, 10/14 at whole-embryo level) than the HR-based method (1/60 at single-blastomere level, 1/9 at whole-embryo level) (Figures 5D–5F and S4). Precise integration was confirmed by Sanger sequencing (Figure S4). We also detected NHEJ-induced mutations or point mutations at the *OCT4* locus in the blastomere with knockin allele (Figure S5). Furthermore, the injected embryos were cultured into blastocysts, and immunostaining showed co-localization of GFP and endogenous OCT4, indicating correct integration of GFP at the *OCT4* locus (Figure 5G).

To test whether double knockins could be achieved by Tild-CRISPR, we co-injected two selected sgRNAs of *OCT4* and *GATA6* (50 ng/μL for each), Cas9 mRNA (100 ng/μL), and Tild donors of *OCT4* and *GATA6* (30 ng/μL for each) into human tripronuclear zygotes (Figure S6). The whole embryos at 4- to 8-cell stage were genotyped, and 11 out of 14 embryos were double-positive at the 5′ and 3′ junctions of *OCT4* and *GATA6* loci (Figure S6). To exclude the possibility that embryos could have been mosaics of either *OCT4* or *GATA6* single targeted cells, we performed single-cell analysis on the blastomeres from these 4- to 16-cell embryos and found that 4 out of 137 blastomeres were $OCT4^{+}/GATA6^{+}$ double-positive (Figure S7).

Taken together, Tild-CRISPR is an efficient method for generating knockin human embryos (Figure S7).

### Mechanism of Tild-CRISPR

Finally, we explored whether the Tild-CRISPR depends on the NHEJ and HR pathways. Mouse embryonic stem cells (ESCs) and N2a cells were transfected with Tild donors for p2A-mCherry knockin at the *Actb* locus and treated with Scr7 (NHEJ inhibitor) and caffeine (HR inhibitor) during the transfection procedure. Consistent with the HMEJ-based method (Yao et al., 2017a), we found that caffeine significantly decreased knockin efficiency in mouse ESCs and N2a cells (Figure 6A). By contrast, Scr7 promoted Tild-CRISPR-mediated knockin in these cells (Figure 6A). We also treated embryos with Scr7, Nu7026 (the other NHEJ inhibitor), or caffeine, and found that Scr7 or Nu7026 repressed Tild-CRISPR-mediated knockin whereas caffeine had no obvious effect (Figure 6B). Overall, transgene integration using Tild-CRISPR is possibly mediated by the HR pathway as well as the NHEJ pathway, and this may account for the high knockin efficiency achieved by Tild-CRISPR.

## DISCUSSION

### Tild-CRISPR Is a Simple and Efficient Method for Creating Gene-Modified Mice

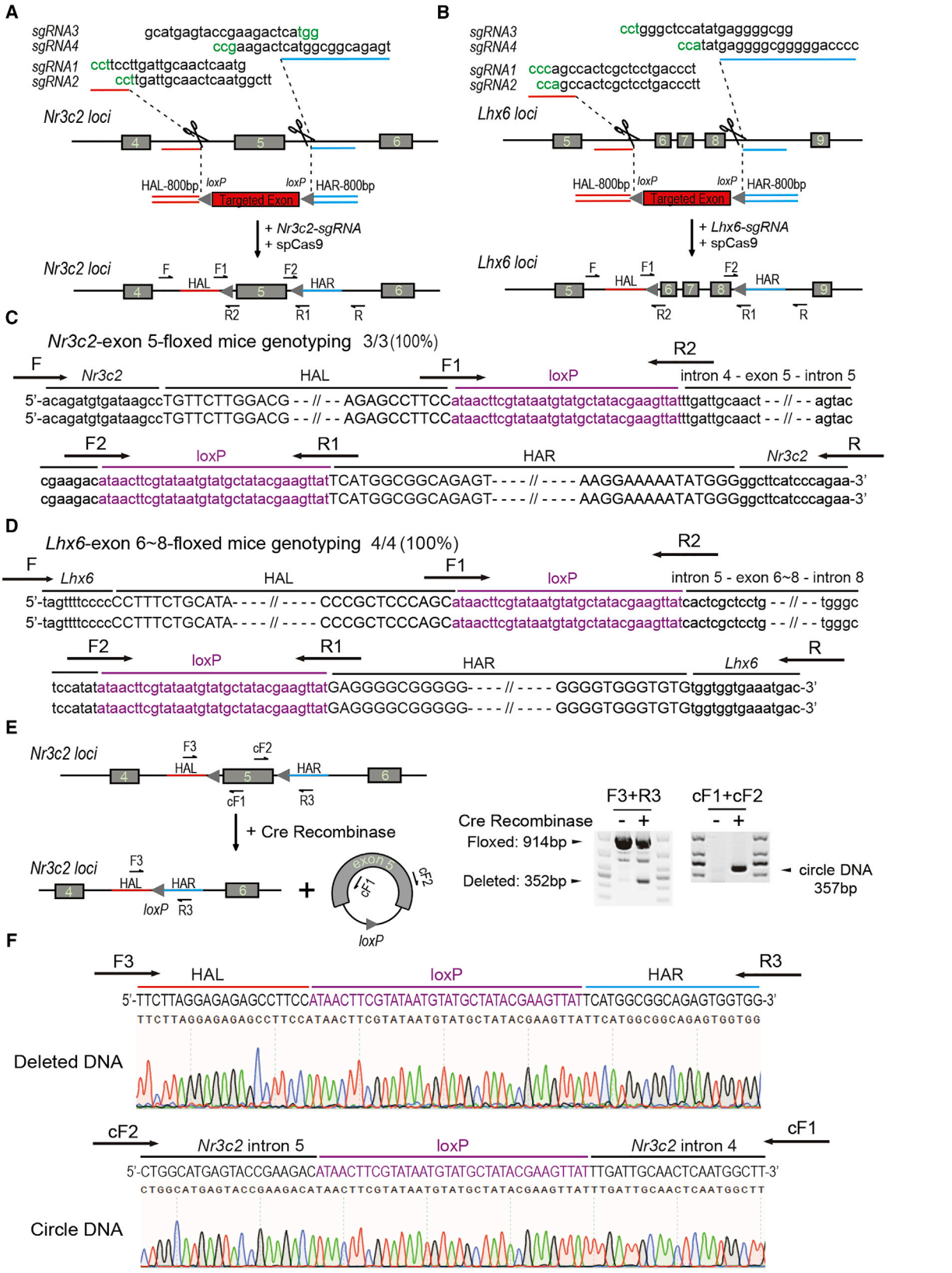
In this study we devised a Tild-CRISPR targeting strategy, whereby PCR-amplified or precisely enzyme-cut transgene donor with 800-bp HA exhibited the highest knockin efficiencies compared with all other targeting strategies with various types of transgene donor. This method is highly generalizable because it has worked for nine loci, including four loci (*Actb*, *Nanog*, *Sox2*, *Cdx2*) in targeting mouse embryos and six loci (*Cdx2*, *Dbh*, *Sp8*, *Nr3c2*, *Lhx6*, *Rosa26*) in generating

**Figure 2. Generation of Knockin Mice Using Tild-CRISPR**

(A) Schematic overview of generating knockin mice at different loci using Tild-CRISPR. Different insertion fragments at different loci: p2A-mCherry at the *Cdx2*, p2A-mCherry-WPRE at the *Dbh*, p2A-NLS-Cre-WPRE-polyA at the *Sp8*, and CAG-LSL-hChR2-tdtomato-WPRE-polyA at the *Rosa26*. hChR2, humanized channelrhodopsin-2.

(B) Sequence analysis of gene-edited mice by Tild-CRISPR. DNA of mouse tails of *Cdx2*-p2A-mCherry, *Dbh*-p2A-mCherry-WPRE, *Sp8*-p2A-Cre-WPRE-polyA, and *Rosa26*-CAG-LSL-hChR2-tdtomato-WPRE-polyA were isolated. PCR products amplified from 5′ and 3′ junction sites were sequenced. Uppercase, homology arm; purple, p2A; gray, polyA; red, mCherry; brown, WPRE; orange, CAG; blue, PAM sequence; HAR or HAL, right or left homology arm. Dashed lines mark the region omitted for clarity.

See also Figures S2 and S3.

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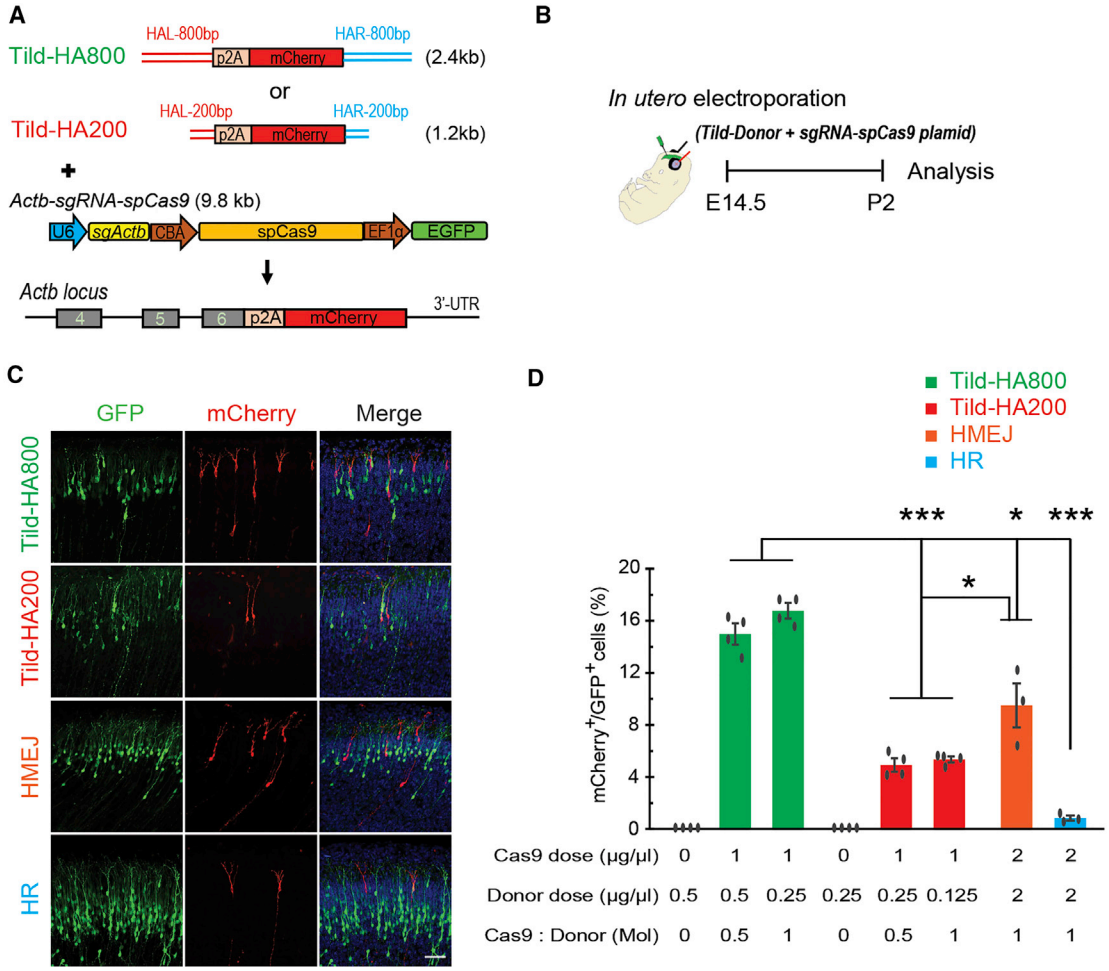



**Figure 4. *In Vivo* Genome Editing via Tild-CRISPR-Mediated Targeted Integration**

(A) Schematic of Tild vectors with different length of HAs for knockin p2A-mCherry to the last codon of *Actb* gene.

(B) Experimental scheme for targeted *Actb*-2A-mCherry knockin in fetal brain via *in utero* electroporation.

(C) Representative immunofluorescence images of neurons showing correct mCherry knockin at the *Actb* locus with Tild-HA800, Tild-HA200, HMEJ, and HR donors. Plasmid expressing *Actb*-sgRNA-spCas9-GFP at a final concentration of 1 μg/μL were mixed with a linearized donor (500 ng/μL or 250 ng/μL for Tild-HA800, 250 ng/μL or 125 ng/μL for Tild-HA200), compared with 2 μg/μL of EFs-spCas9-NLS-SV40polyA (Addgene #97307) mixed with the donor vector for HR (Addgene, #97309) or HMEJ (Addgene, #97308). GFP, transfected cells. Scale bar, 100 μm.

(D) Relative knockin efficiency measured by the percentage of $mCherry^+$ cells among $GFP^+$ cells. As a control, a linearized donor was mixed with plasmid expressing *Actb*-sgRNA-GFP. Results were obtained from at least three mice and presented as mean ± SD. The input data points are shown as black dots. *p < 0.05, ***p < 0.001, unpaired Student's t test.

knockin mice. Of note, when HR donor contains a very long HA (usually 2–4 kb), it occasionally achieves comparable knockin efficiency with Tild-CRISPR in mouse embryos, especially in well-studied loci, as reported by our own and other groups' previous studies (Chu et al., 2016; Menoret et al., 2015; Yang et al., 2013). However, the plasmid construction and targeted

**Figure 3. Generation of Conditional Knockout Mice at the *Nr3c2* and *Lhx6* Loci Using Tild-CRISPR**

(A) Schematic overview of generating a floxed *Nr3c2* allele using Tild-CRISPR in mouse embryos. The wild-type allele (upper), the Tild-HA800 donor designed to flox exon 5 of *Nr3c2* (middle), and the final targeted allele (lower) are shown. Genotyping PCR was performed by the primer combinations (5′*loxP*, F + R2 & F1 + R primers; 3′*loxP*, F + R1 & F2 + R primers). Red parallel lines, left homology arm; blue parallel lines, right homology arm; gray triangles, *loxP*.

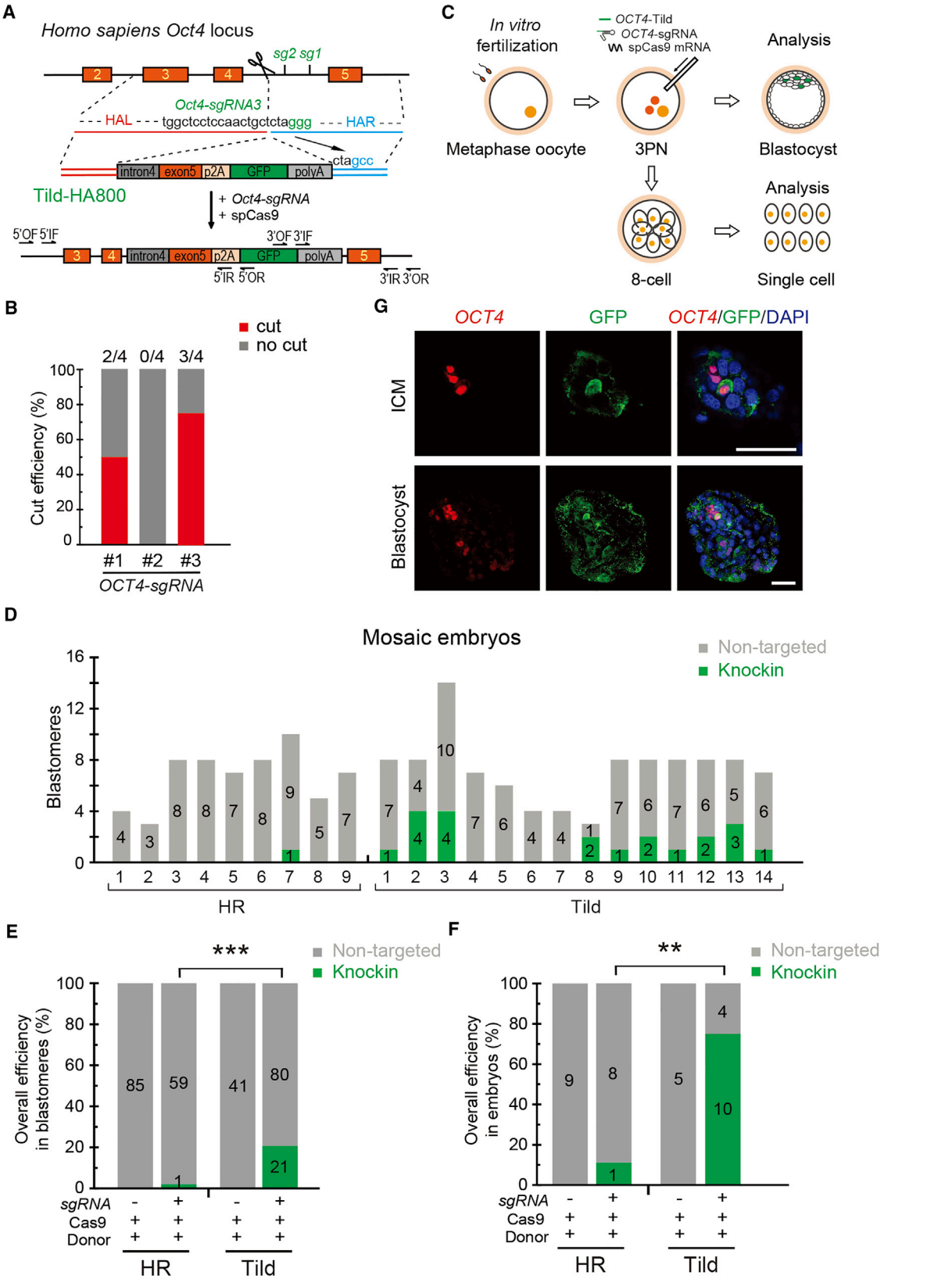
(B) Schematic overview of generating a floxed *Lhx6* allele using Tild-CRISPR in mouse embryos.

(C and D) Sequence analysis of *Nr3c2*-exon 5 floxed (C) and *Lhx6*-exon 6–8 floxed (D) mice using Tild-CRISPR method. PCR products amplified by F + R1 and F1+R were sequenced. Uppercase, homology arm; purple, *loxP*; HAR or HAL, right or left homology arm. Dashed lines mark the region omitted for clarity.

(E) *In vitro* Cre-mediated recombination of the floxed *Nr3c2* allele. The genomic DNA of targeted mouse #14 was incubated with Cre recombinase and used as PCR template. Primers F3 and R3 flanking the floxed allele produce shorter products upon Cre-dependent excision. Primers cF1 and cF2 detect the circular molecule, which only form upon Cre-loxP recombination. The position of each primer is shown in the schematic overview.

(F) Sequence analysis of a deletion allele and circular PCR products showing Cre-mediated recombination. Purple, *loxP*.

See also Figure S2.




*(legend on next page)*

allele identification are laborious. Compared with Easi-CRISPR using ssDNA as a donor, the Tild-CRISPR method has two obvious advantages. First, Tild-CRISPR exhibits higher DNA knockin efficiency than Easi-CRISPR, as shown in Figure 1. Second, there is no size limitation for insertions by Tild-CRISPR. Various insertions, from 0.8 to 6.0 kb, could be precisely integrated into different loci.

## Mechanism of Tild-CRISPR

Our results indicate that the Tild donor, a linearized dsDNA donor consisting of a transgene fragment flanked by 800-bp HAs on each side, is the key factor in achieving high knockin efficiency in CRISPR genome editing. Compared with MMEJ- or HMEJ-based methods, the Tild donor skips the step of *in vivo* cleavage of transgene donor, thus leading to high knockin efficiency. As summarized in Figure 6, we speculate that the MMEJ or HMEJ pathway may be involved in Tild-CRISPR, as the Tild donor is very similar to the fragment derived from *in vivo* CRISPR-mediated cleavage of MMEJ and HMEJ donors. The classic HR donor is a cycle plasmid or a linearized donor with substantial JSs using HR to mediate transgene knockin. Our results indicate that MMEJ- or HMEJ-mediated targeted integration is superior to HR-mediated targeted integration in mouse zygotes, as HR inhibitor had no effect on Tild-CRISPR-mediated transgene integration. Therefore, removal of JSs by *in vivo* CRISPR-mediated cleavage of the HR donor or by *in vitro* enzyme-mediated cleavage of the HR donor promotes transgene targeted integration. For Easi-CRISPR using ssDNA as a donor, single-strand annealing factors may be involved in ssDNA donor-mediated repair. Elucidation of the precise molecular mechanisms underlying this Tild-CRISPR method undoubtedly requires further studies.

## Mosaicism

The majority of gene-edited animals and human embryos generated by CRISPR/Cas9 showed mosaicism (Izpisua Belmonte et al., 2015; Yen et al., 2014). Many previous studies have attempted to produce gene-modified animals without mosaicism in a single step, including injection of Cas9 protein (Suzuki et al., 2014), or injection of a cocktail of sgRNAs (Zuo et al., 2017). However, these approaches achieved gene knockout rather than gene knockin. Recently, Ma et al. (2017) reported the correction of heterozygous mutations without mosaicism in human embryos using CRISPR/Cas9, but this approach relies on the homologous wild-type copy other than the synthetic DNA template, and thus is not suitable for the correction of homozygous recessive mutations and transgene integration. Although Tild-CRISPR showed much higher knockin efficiency (up to 12-fold) than other knockin strategies, it is still critical to eliminate mosaicism in gene-edited embryos for large animals and clinical applications. It would be interesting to combine our Tild-CRISPR strategy with Cas9 protein in early pronuclear zygotes or metaphase oocytes to generate embryos carrying the same genetic modification in future studies.

## Other Potential Applications of Tild-CRISPR

Our Tild-CRISPR showed robust DNA knockin using *in utero* electroporation. With higher editing efficiency compared with HR- or HMEJ-based methods, Tild-CRISPR holds great promise for applications in other systems, such as eyes and livers, to study biological functions of genes. Gene editing has recently been used for studying human embryo development and correcting pathologic gene mutation (Fogarty et al., 2017; Ma et al., 2017). Tild-CRISPR may greatly facilitate targeted gene editing in human embryos.

## STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- CONTACT FOR REAGENT AND RESOURCE SHARING
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  - *In Vitro* Cre Recombination
  - *In Utero* Electroporation
  - Mouse Immunostaining

**Figure 5. Tild-CRISPR-Mediated Targeted Integration in Human Embryos**

(A) Schematic overview of Tild-CRISPR-mediated gene-targeting strategy at the *Homo sapiens OCT4* locus. *OCT4*-Intron 4-Exon 5-p2A-GFP-polyA was designed to be inserted into the intron 4 of *OCT4* locus, leading to GFP expression under the control of *OCT4* promoter. HAL/HAR, left/right homology arm; green, PAM sequence; OF/OR, outer forward/reverse primer; IF/IR, inner forward/reverse primer.

(B) Cleavage efficiencies of each sgRNA targeting *OCT4* in human embryos. Number above the bars denote total embryos analyzed. Each indicated sgRNA, together with Cas9 mRNA, was injected into human tripronuclear zygotes and cultured into 8-cell embryos for genotyping.

(C) Experimental design. *In vitro* fertilization (IVF) was performed on human oocyte and tripronuclear embryos were injected with a mixture of *OCT4*-Tild, *OCT4*-sgRNA, and spCas9 mRNA 24 hr post IVF. The injected embryos were cultured into 8-cell stage for single-cell analysis or blastocyst stage for fluorescence observation.

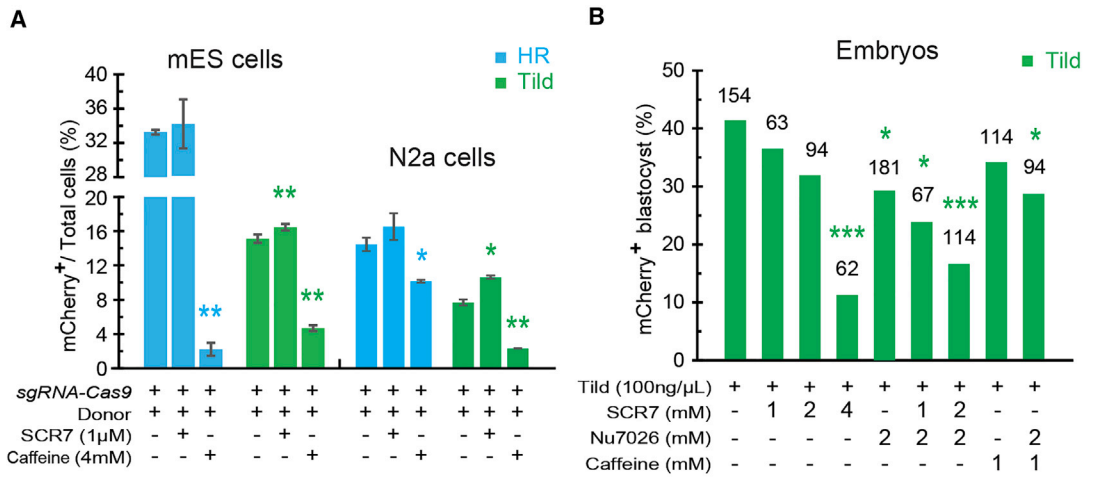
(D) Blastomere genotyping outcomes in mosaic embryos generated by HR-based method and Tild-CRISPR. PCR products of 5′ junction were amplified with 5′OF/O-5′OR and 5′IF/O-5′IR shown in Figure S5A, and used for Sanger sequencing.

(E) Overall efficiency in blastomeres using HR-based method and Tild-CRISPR. ***$p < 0.001$, $\chi^2$ test.

(F) Overall efficiency in embryos using HR-based method and Tild-CRISPR. **$p < 0.01$, $\chi^2$ test.

(G) Representative immunofluorescence images of a gene-edited human embryo at blastocyst stage. Upper panel: a single-layer image for inner cell mass (ICM) of the blastocyst. Bottom panel: a merged image for the whole blastocyst. Red, *OCT4*; green, GFP; blue, DAPI. Scale bars, 50 μm.

See also Figures S4–S7.




C

| Strategy | Map | Mechanism | Efficiency | Limitation | Ref. |
|---|---|---|---|---|---|
| Tild | 800bp Transgene 800bp → *in vitro* cleavage → 800bp Transgene 800bp | HMEJ | + + + + | AAV-mediated *in vivo* delivery | This study |
| HR | 800bp Transgene 800bp (HAL HAR) or 800bp Transgene 800bp (JS-2000bp JS-2000bp) | HR | + | Inefficient *in vivo* | Yang et al, 2013 |
| HMEJ | 800bp Transgene 800bp → *in vivo* cleavage → 800bp Transgene 800bp | HMEJ | + + + | Complex constructions | Yao et al, 2017; Zhang et al, 2017 |
| NHEJ | Transgene → *in vivo* cleavage → Transgene | NHEJ | + | Random insertion, various indels | Auer et al, 2013; Maresca et al, 2013 |
| MMEJ | 20bp Transgene 20bp → *in vivo* cleavage → 20bp Transgene 20bp | MMEJ | + + | Inefficient in mouse embryos | Nakade et al, 2014 |
| ssDNA | ~60bp Transgene ~60bp | SSA | + + + | Costly, insertion size < 2kb | Quadros et al, 2017 |

**Figure 6. Schematic Overview of Tild-CRISPR-Mediated Gene Knockin**

(A and B) Knockin efficiencies of mCherry knockin at *Actb* locus by HR-based and Tild-CRISPR. Knockin efficiencies in culture cell lines (mouse ESC, N2a cells; A) and embryos (B) were measured by FACS and fluorescence observation, respectively, and compared with the group treated with NHEJ inhibitor (Scr7 or NU7026), HR inhibitor (caffeine), or both. Results are presented as mean ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, unpaired Student's t test (A) and $\chi^2$ test (B). (C) Comparison of different targeting strategies for transgene targeted integration.

- Human Egg Retrieval and Sperm Preparation
- *In Vitro* Fertilization (IVF) and Human Embryo Transfer
- Human Embryo Injection and Culture
- Single-Cell PCR
- Human Embryo Immunostaining
- Southern Blot Analysis
- Cell Culture and Transfection

● QUANTIFICATION AND STATISTICAL ANALYSIS

- Quantification of Relative Knock-in Efficiency in Neuron Cells
- Quantification of Knock-in Efficiency in Culture Cells
- Statistical Analysis

## SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and one table and can be found with this article online at https://doi.org/10.1016/j.devcel.2018.04.021.

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## AUTHOR CONTRIBUTIONS

X.Y. and X.W. designed and performed experiments. X.H. performed *in utero* electroporation. W.Y., X.H., and L.S. performed mouse experiments. P.D. and F.M. performed Southern blotting. M.Z., Y.S., N.Y., W.Z., Y.L., and K.W. performed experiments on human embryos. H.Y., Z.-J.C., and W.L. supervised the project. H.Y. and X.Y. wrote the paper.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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## STAR★METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Antibodies | | |
| Goat anti-GFP | GeneTex | Cat# GTX26673; RRID: SCR_000069 |
| Rabbit anti-mCherry | GeneTex | Cat# GTX128508; RRID: SCR_000069 |
| chicken anti-GFP | GeneTex | Cat# GTX13970; RRID: SCR_000069 |
| rabbit anti-OCT4 | GeneTex | Cat# GTX100468; RRID: SCR_000069 |
| FITC-AffiniPure donkey Anti-goat IgG | Jackson Immunoresearch | Cat# 711-095-152; RRID: SCR_010488 |
| Cy3-AffiniPure donkey Anti-Rabbit IgG | Jackson Immunoresearch | Cat# 711-165-152; RRID: SCR_010488 |
| 488-AffiniPure goat Anti-chicken IgG | Jackson Immunoresearch | Cat# 103-545-155; RRID: SCR_010488 |
| Cy3-AffiniPure goat Anti-Rabbit IgG | Jackson Immunoresearch | Cat# 111-165-003; RRID: SCR_010488 |
| Chemicals, Peptides, and Recombinant Proteins | | |
| *Actb*-HR-donor vector | Addgene | 97317 |
| pX330 | Addgene | 42230 |
| pX260 | Addgene | 42229 |
| Lipofectamine® 3000 Transfection Reagent | Life Technology | L3000015 |
| Nuclease-Free Water | Life Technology | AM9930 |
| Gel Extraction Kit | Omega | D2500-02 |
| MMESSAGE MMACHINE T7 ULTRA | Life Technologies | AM1345 |
| MEGACLEAR KIT 20 RXNS | Life Technologies | AM1908 |
| MEGASHORTSCRIPT T7 KIT 25 RXNS | Life Technologies | AM1354 |
| PMSG | Ningbo Sansheng Medicine | S141004 |
| HCG | Ningbo Sansheng Medicine | B141002 |
| Cytochalasin B | Sigma | CAT#C6762 |
| KSOM+AA with D-Glucose and Phenol Red | Millipore | MR-106-D |
| M2 Medium with Phenol Red | Millipore | MR-015-D |
| Mineral oil | Sigma | M8410 |
| G-IVF | Vitrolife, Sweden | 10136 |
| Zeta-Probe GT Blotting Membranes | Bio-Rad | 162-0196 |
| Reverse transcriptase | Takara | 2641A |
| RNaseH | NEB | M0297 |
| Nu7026 | Selleck | S141004 |
| SCR7 | Selleck | B141002 |
| TIANamp Genomic DNA Kit | TIANGEN | DP304-03 |
| Cre recombinase | NEB | M0298 |
| Caffeine | Sigma Aldrich | MR-015-D |
| DPBS, no calcium, no magnesium | Life Technology | 14190144 |
| TRYPSIN 0.05% EDTA | Life Technology | 25300054 |
| DMEM | Gibco | 11965092 |
| FBS | Gibco | 10099141 |
| PEN STREP 100X | Gibco | 15070063 |
| L-Glutamine 100X | Gibco | 25030081 |
| NEAA | Gibco | 11140050 |
| PD0325901 | Selleck | S1036 |
| CHIR99021 | Selleck | S1263 |
| Mouse lif | Millipore | ESG1107 |
| Beta-mercaptoethanol | Sigma | M3148 |

(*Continued on next page*)

| Continued | | |
|---|---|---|
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
| Experimental Models: Organisms/Strains | | |
| Mouse: B6D2F1 | SHANGHAI SLAC | N/A |
| Mouse: ICR | SHANGHAI SLAC | N/A |
| Software and Algorithms | | |
| ImageJ | Schneider et al., 2012 | https://imagej.net/NIH_Image |
| Flowjo | BD FACS Aria II | N/A |
| ZEN | Nikon NiE-A1 plus | N/A |

## CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Hui Yang (huiyang@ion.ac.cn).

## EXPERIMENTAL MODEL AND SUBJECT DETAILS

### Mice

B6D2F1 (C57BL/6 X DBA2J) mice (7-8 weeks old) were used for zygotes collection. ICR females were used for recipients. E14.5 pregnant ICR mice were used for *in utero* electroporation. The use and care of animals complied with the guideline of the Biomedical Research Ethics Committee of Shanghai Institutes for Biological Science, Chinese Academy of Sciences.

### Source of Human Zygotes

This study used discarded human embryos provided by Ren Ji Hospital, approved by ART Ethics Committee of Ren Ji Hospital, Shanghai Jiao Tong University (Approval Reference Number: 20140422), and informed consent was obtained from all the participants during September 2016 to July 2017.

## METHOD DETAILS

### Construction of Linearized Donors

To construct Tild donor (transgene DNA sandwiched by different length of homology arm) for *Actb* gene, an *Actb*-HR-donor vector (Addgene catalog no. 97317) containing 800bpHAL-p2A-mCherry-800bpHAR was linearized with two restriction enzymes Hind III and Xho I or PCR-amplified, and gel purified with Gel Extraction Kit (Omega, D2500-02).

To construct JS-2000 donor (800bpHAL-transgene DNA-800bpHAR sandwiched by 2000 bp of junk sequence) for *Actb* gene, an *Actb*-HR-donor vector (Addgene catalog no. 97317) containing 800bpHAL-p2A-mCherry-800bpHAR was linearized with restriction enzyme digestion and gel purified. To construct JS-800 and JS-200 donors, the *Actb*-HR-donor vectors were PCR-amplified with different length of junk sequences.

To construct Tild donor for human *OCT4/GATA6* gene, donor vector (800bpHAL-intron 4-exon 5-p2A-GFP-polyA-800bpHAR for *Oct4*, 800bpHAL-p2A-mCherry-800bpHAR for *GATA6*) was linearized with PCR-amplified and gel purified as described above.

### Construction of ssDNA Donor

The ssDNA donor for *Actb* gene was prepared from *Actb*-HR-donor vector using the IvTRT (*in vitro* Transcription and Reverse Transcription) method. In brief, T7 promoter was added to donor by PCR amplification of *Actb*-HR-donor vector using *Actb*-IvTRT_F and *Actb*-IvTRT_R (Table S1). PCR products was purified and used as template for *in vitro* transcription (IVT) using mMESSAGE mMACHINE T7 ULTRA kit (Life Technologies), and then purified using MEGA clear kit (Life Technologies). The cDNA (ssDNA) was synthesized by reverse transcriptase (Takara, 2641A) and the RNA template was degraded by RNaseH (NEB, M0297).

### Production of Cas9 mRNA and sgRNA

T7 promoter was added to Cas9 coding region by PCR amplification of px260, using primer Cas9 IVT_F and IVT_R (Table S1). T7-Cas9 PCR product was purified and used as the template for *in vitro* transcription (IVT) using mMESSAGE mMACHINE T7 ULTRA kit (Life Technologies). T7 promoter was added to sgRNA template by PCR amplification of px330, using primers listed in (Table S1). The T7-sgRNA PCR product was purified and used as the template for IVT using MEGA shortscript T7 kit (Life Technologies). Both the Cas9 mRNA and the sgRNAs were purified using MEGA clear kit (Life Technologies) and eluted in RNase-free water.

### Zygote Injection, Embryo Culturing, and Embryo Transplantation

For mice gene editing, super ovulated female B6D2F1 (C57BL/6 X DBA2J) mice (7-8 weeks old) by injecting 5 IU of pregnant mare serum gonadotropin (PMSG), followed by 5 IU of human chorionic gonadotropin (hCG) 48h later were mated to B6D2F1 males, and fertilized embryos were collected from oviducts 20h post hCG injection.

For zygote injection, Cas9 mRNA (100 ng/μl), sgRNA (50 ng/μl) and linearized donor with a series of dose (100 or 25 ng/μl for Tild-HA800; 33 ng/μl for Tild-HA20) were mixed and injected into the cytoplasm of fertilized eggs with well recognized pronuclei in a droplet of HEPES-CZB medium containing 5 μg/ml cytochalasin B (CB) using a FemtoJet microinjector (Eppendorf) with constant flow settings. The injected zygotes were cultured in KSOM medium with amino acids at 37°C under 5% $CO_2$ in air to blastocysts for fluorescence observation and harvested for genotyping analysis.

For Nu7026 (Selleck) and SCR7 (Selleck) treatment, 2 mM Nu7026 or a different dose (1, 2, 4 mM) of SCR7 mixed with Tild-CRISPR components were co-injected into the cytoplasm of fertilized eggs. For caffeine (Sigma Aldrich) treatment, the injected zygotes were cultured in KSOM medium with 1 mM caffeine for 1 day, and then transferred to fresh KSOM medium.

For knock-in mice generation, the injected zygotes were cultured into 2-cell stage and 25-30 2-cell embryos were transferred into oviducts of pseudopregnant ICR females at 0.5 dpc.

### Embryo and Mouse Genotyping Analysis

For picking up and transferring single embryo, a glass capillary was used under a dissection microscope. Single embryos were picked up based on fluorescence, and transferred directly into PCR tubes. 3 μl lysis buffer (0.1% tween 20, 0.1% Triton X-100 and 4 μg/ml proteinase K) were added to each tube. The samples were incubated at 56°C for 30 min and heat inactivate proteinase K at 95°C for 10 min. PCR amplification was performed using nest primer sets (Table S1). ExTaq was activated at 95°C for 3 min, and primary PCR was performed for 30 cycles at 95°C for 30 sec, 60°C for 30 sec, and 72°C for 1min, with a final extension at 72°C for 10 min. Secondary PCR was performed using 1μl primary PCR product and nested inner primer. PCR was carried out in the same reaction mixture. PCR product was gel purified and sequenced.

For mouse genotyping analysis, mouse genomic DNA was extracted from toe or tail samples using the TIANamp Genomic DNA Kit (TIANGEN, DP304-03). PCR amplification was performed using primers designed to amplify the correctly targeted junctions (Table S1). ExTaq was activated at 95°C for 3 min, and PCR was performed for 38 cycles at 95°C for 30 sec, 60°C for 30 sec, and 72°C for 1min, with a final extension at 72°C for 10 min. PCR product was gel purified and sequenced.

### *In Vitro* Cre Recombination

A 10 μl reaction containing 300 ng of genomic DNA and 2 units of recombinant Cre recombinase (NEB, M0298) in 1× buffer was incubated at 37°C for 1 hr. 1 μl of the Cre reaction mix was used as template for PCR reactions with gene-specific primers. For *Nr3c2* target, primers F3 and R3 were used for detecting the deletion products, and primers cF1 and cF2 were used to detect the circular product.

### *In Utero* Electroporation

E14.5 pregnant ICR mice were anaesthetized with Pentobarbital sodium (50 mg/Kg, Sigma) for *in utero* electroporation. Plasmids expressing *Actb*-sgRNA-spCas9-GFP at a final concentration of 1 μg/μl were mixed with linearized donor (500 ng/μl or 250 ng/μl for Tild-HA800, 250 ng/μl or 125 ng/μl for Tild -HA200). As a control, a linearized donor was mixed with plasmid expressing *Actb*-sgRNA-GFP. Mixtures were injected into the embryos' lateral ventricles with 0.005% fast green solution (Sigma). For electroporation, 5 pulses with a 50-ms duration separated by 950-ms were applied at 35 V using ECM 830 (BTX). The uterine horns then were placed back into the abdominal cavity and allowed to develop in utero for the indicated time.

### Mouse Immunostaining

Mice were transcardially perfused with 0.9% saline followed by 4% paraformaldehyde using a peristaltic pump (Gilson) and fixed overnight at 4°C. Then the tissue was dehydrated using 30% sucrose until sinking to the bottom of tube. Tissue sections were performed on a microtome cryostat (Leica, CM1950) at a thickness of 40 μm for brain. Sections were rinsed three times in 0.1M phosphate-buffered (PB) and incubated with primary antibody: goat anti-GFP (1:500, GeneTex) and rabbit anti-mCherry (1:3 000, GeneTex), which was diluted in diluent with 5% NGS overnight at 4°C. The following day, sections were washed three times in PB and then incubated with secondary antibody: FITC-AffiniPure donkey Anti-goat IgG (1:500, Jackson Immunoresearch) and Cy3-AffiniPure donkey Anti-Rabbit IgG (1:500, Jackson Immunoresearch) for 2 hours at room temperature on an orbital shaker. Finally, the sections were counterstained with DAPI for 20 min and mounted with SlowFade Diamond Antifade Mountant (Life) on glass slides.

### Human Egg Retrieval and Sperm Preparation

Cumulus-corona oocyte complexes (COCs) were isolated from the follicle fluid promptly and precisely, and cultured in G-IVF (Vitrolife, Sweden) for three hours. Semen samples were collected on day of oocyte retrieval by masturbation after 3–5 days of abstinence. Semen was kept for 30 minutes at 37°C for liquefaction, followed by a conventional density-gradient separation method. After the second centrifugation, the pellet was usually overlaid with 0.5 ml of G-IVF and incubated for swim-up for 30 min. The supernatant was used for insemination. Patients had signed the informed consent of remaining gamete donation for research before taking eggs.

### *In Vitro* Fertilization (IVF) and Human Embryo Transfer

The COCs were inseminated in 4-well plates with approximately 100 000 motile spermatozoa for each oocyte and tri-pronuclear (3PN) embryos were chosen under microscope for experiment. Sequential culture media from Vitrolife (G-IVF, G1, and G2; Scandinavicn IVF Science, Sweden) were applied in all steps.

### Human Embryo Injection and Culture

For human embryo injection, Cas9 mRNA (100 ng/μl), *OCT4/GATA6*-sgRNA (50 ng/μl) and *OCT4/GATA6*-Tild (30 ng/μl) were mixed and injected into the cytoplasm of tri-pronuclear (3PN) embryos 24 hours post IVF in a droplet of G1 medium using a FemtoJet microinjector (Eppendorf) with constant flow settings. The injected embryos were cultured in G2 medium with amino acids at 37°C under 5% $CO_2$ in air to blastocysts for fluorescence observation and harvested for genotyping analysis.

### Single-Cell PCR

4∼16 cell stage embryos were digested with acid Tyrode solution to remove the zona pellucida, and then transferred into 0.25% trypsin and gently pipetted to separate the individual blastomeres. Finally, the blastomeres were washed in CZB medium for 8 to 10 times and transferred into a PCR tube individually. 2 μl lysis buffer (0.1% tween 20, 0.1% Triton X-100 and 4 μg/ml proteinase K) were added to each tube. The samples were incubated at 56°C for 30 min and heat inactivate proteinase K at 95°C for 10 min. The products of the lysis program were used as templates in a nest PCR analysis with primer sets (Table S1). PCR products were gel purified and sequenced.

### Human Embryo Immunostaining

Human embryo were incubated with 0.9% saline followed by 4% paraformaldehyde for 15 min at room temperature. Then the embryos were blocked in PBST with 5% FBS for 1 hour and then incubated with primary antibody: chicken anti-GFP (1:500, GeneTex) and rabbit anti-OCT4 (1:3 000, GeneTex), which was diluted in PBST with 5% FBS overnight at 4°C. The following day, embryos were washed three times in PB and then incubated with secondary antibody: 488-AffiniPure goat Anti-chicken IgG (1:500, Jackson Immunoresearch) and Cy3-AffiniPure goat Anti-Rabbit IgG (1:500, Jackson Immunoresearch) for 2 hours at room temperature on an orbital shaker. Finally, the embryos were counterstained with DAPI for 15 min and set up for fluorescence observation.

### Southern Blot Analysis

The 10 μg of genomic DNAs from *Cdx2*-p2A-mCherry mice were digested with EcoRI. The digested genomic DNA was then separated on a 0.8% agarose gel and transferred to a Zeta-Probe GT Blotting Membranes (Bio-Rad, 162-0196). Southern blot analysis was performed using the 32 phosphorus radioisotope system. Membranes were hybridized with internal mCherry probe (5'atggtgag caagggcgaggaggataacatggccatcatcaaggagttcatgcgcttcaaggtgcacatggagggctccgtgaacggccacgagttcgagatcgagggcgagggcgaggg ccgcccctacgagggcacccagaccgccaagctgaaggtgaccaagggtggcccccctgcccttcgcctgggacatcctgtcccctcagttcatgtacggctccaaggcctac gtgaagcaccccgccgacatccccgactacttgaagctgtccttccccgagggcttcaagtgggagcgcgtgatgaacttcgaggacggcggcgtggtgaccgtgacccagg actcctccctgcaggacggcgagttcatctacaaggtgaagctgcgcggcaccaacttcccctccgacggccccgtaatgcagaagaagaccatgggctgggaggcctcctc cgagcggatgtaccccgaggacggcgccctgaagggcgagatcaagcagaggctgaagctgaaggacggcggccactacgacgctgaggtcaagaccacctacaaggc caagaagcccgtgcagctgcccggcgcctacaacgtcaacatcaagttggacatcacctcccacaacgaggactacaccatcgtggaacagtacgaacgcgccgagggcc gccactccaccggcggcatggacgagctgtacaagtaa3'). Internal mCherry probe expected fragment size: WT = N/A, Targeted = 4.2 kb.

### Cell Culture and Transfection

Mouse ESCs (129/Sv x C57BL/6 ES cell) were cultured with 2i medium, consists of Dulbecco's modified Eagle's Medium (DMEM) (Gibco,11965-092) plus 15% fetal bovine serum (FBS) (Gibco), 1,000 U/mL mouse Lif, 2 mM glutamine (Gibco), 1% penicillin/streptomycin (Thermo Fisher Scientific), 0.1 mM β-mercaptoethanol (Sigma),0.1 mM non-essential amino acids(Gibco),1 ìM PD0325901 and 3 ìM CHIR99021. N2a cells were cultured with DMEM (Gibco) plus 10% FBS (Gibco). All cells were cultured at 37°C with 5% $CO_2$ incubation. Mouse ESCs and N2a cells were transfected using Lipofectamine 3000 Reagent (Invitrogen) according to the manufacturer's instructions. For each well of a 6-well plate, a total of 5 ìg plasmids (Cas9: donor = 1:1) was used. After 48 hours, transfection-positive ES cells and N2a cells were sorted into 6-well plates using BD FACS Aria II for further culture and analysis.

Treatment with 1 μM SCR7 (Selleck) or 4 mM caffeine (Sigma Aldrich) was started 1 day before transfection and was continued for 2 days after transfection, both in mouse ES cells and N2a cells.

## QUANTIFICATION AND STATISTICAL ANALYSIS

### Quantification of Relative Knock-in Efficiency in Neuron Cells

The relative knock-in efficiency were measured by the percentage of $mCherry^+$ cells among GFP+ transfected positive cells with random view. As a control, a linearized donor was mixed with plasmid expressing *Actb*-sgRNA-GFP. At least 3 mice and 3 brain sections of each animal were counted. Results were presented as mean ± s.d. The input data points were shown as black dots. * $P < 0.05$, *** $P < 0.001$, unpaired Student's t-test.

### Quantification of Knock-in Efficiency in Culture Cells

knock-in efficiencies of mCherry were measured by the percentage of $mCherry^{+}$ cells among total cells using FACS analysis. Results were presented as mean ± s.d. *P < 0.05, **P < 0.01, ***P < 0.001, unpaired Student's t-test.

### Statistical Analysis

All statistical values of culture cells (mES cell and N2A cell, Figure 6A) and neuron cells (Figure 4D) were presented as mean ± SEM. Differences between datasets were judged to be significant at * P < 0.05, ** P < 0.01, *** P < 0.001, unpaired Student's t-test.

For embryos, number above each bar showed total embryos (mouse embryos, Figures 1C, 1F, and 6B; human embryos, Figure 5F) or blastomeres (Figure 5E). Differences between datasets were judged to be significant at * P < 0.05, ** P < 0.01, *** P < 0.001, $\chi 2$-test.

**Developmental Cell, Volume *45***

## Supplemental Information

# Tild-CRISPR Allows for Efficient and Precise Gene Knockin in Mouse and Human Cells

**Xuan Yao, Meiling Zhang, Xing Wang, Wenqin Ying, Xinde Hu, Pengfei Dai, Feilong Meng, Linyu Shi, Yun Sun, Ning Yao, Wanxia Zhong, Yun Li, Keliang Wu, Weiping Li, Zi-jiang Chen, and Hui Yang**

# Figure S1.

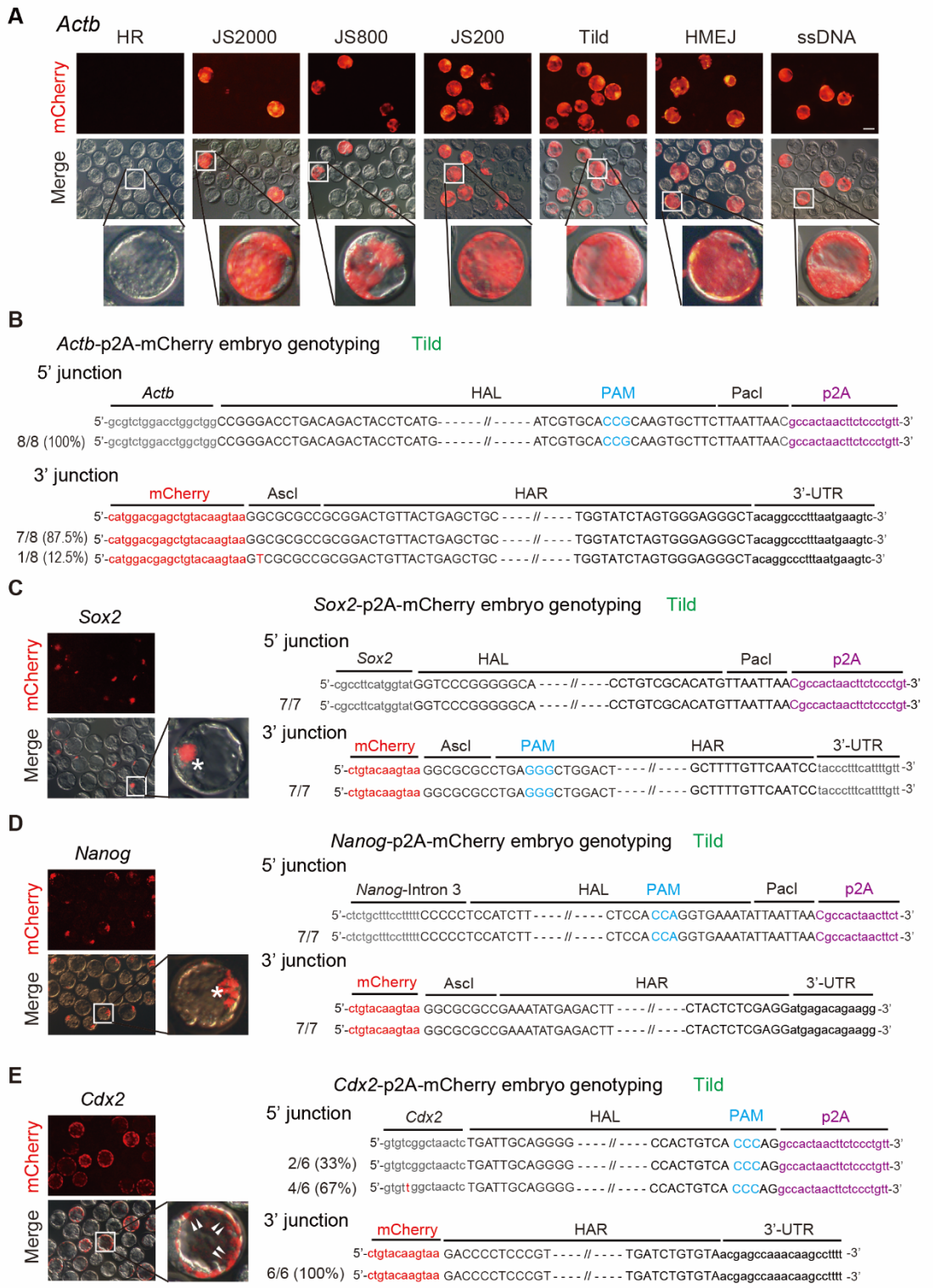

A
Actb
HR
JS2000
JS800
JS200
Tild
HMEJ
ssDNA
mCherry
Merge
B
Actb-p2A-mCherry embryo genotyping
Tild
5' junction
Actb
HAL
PAM
PacI
p2A
5'-gcgtctggacctggctggCCGGGACCTGACAGACTACCTCATG- - - - - // - - - - - ATCGTGCACCGCAAGTGCTTCTTAATTAACgccactaacttctccctgtt-3'
8/8 (100%) 5'-gcgtctggacctggctggCCGGGACCTGACAGACTACCTCATG- - - - - // - - - - - ATCGTGCACCGCAAGTGCTTCTTAATTAACgccactaacttctccctgtt-3'
3' junction
mCherry
AscI
HAR
3'-UTR
5'-catggacgagctgtacaagtaaGGCGCGCCGCGGACTGTTACTGAGCTGC - - - - // - - - - TGGTATCTAGTGGGAGGGCTacaggccctttaatgaagtc-3'
7/8 (87.5%) 5'-catggacgagctgtacaagtaaGGCGCGCCGCGGACTGTTACTGAGCTGC - - - - // - - - - TGGTATCTAGTGGGAGGGCTacaggccctttaatgaagtc-3'
1/8 (12.5%) 5'-catggacgagctgtacaagtaaGTCGCGCCGCGGACTGTTACTGAGCTGC - - - - // - - - - TGGTATCTAGTGGGAGGGCTacaggccctttaatgaagtc-3'
C
Sox2
mCherry
Merge
Sox2-p2A-mCherry embryo genotyping
Tild
5' junction
Sox2
HAL
PacI
p2A
5'-cgccttcatggtatGGTCCCGGGGGCA - - - - // - - - -CCTGTCGCACATGTTAATTAACgccactaacttctccctgt-3'
7/7 5'-cgccttcatggtatGGTCCCGGGGGCA - - - - // - - - -CCTGTCGCACATGTTAATTAACgccactaacttctccctgt-3'
3' junction
mCherry
AscI
PAM
HAR
3'-UTR
5'-ctgtacaagtaa GGCGCGCCTGAGGGCTGGACT- - - - // - - - - GCTTTTGTTCAATCCtacccttttcattttgtt-3'
7/7 5'-ctgtacaagtaa GGCGCGCCTGAGGGCTGGACT- - - - // - - - - GCTTTTGTTCAATCCtacccttttcattttgtt-3'
D
Nanog
mCherry
Merge
Nanog-p2A-mCherry embryo genotyping
Tild
5' junction
Nanog-Intron 3
HAL
PAM
PacI
p2A
5'-ctctgctttccttttttCCCCCTCCATCTT - - - - // - - - - CTCCACCAGGTGAAATATTAATTAACgccactaacttct-3'
7/7 5'-ctctgctttccttttttCCCCCTCCATCTT - - - - // - - - - CTCCACCAGGTGAAATATTAATTAACgccactaacttct-3'
3' junction
mCherry
AscI
HAR
3'-UTR
5'-ctgtacaagtaa GGCGCGCCGAAATATGAGACTT - - - - // - - - -CTACTCTCGAGGatgagacagaagg-3'
7/7 5'-ctgtacaagtaa GGCGCGCCGAAATATGAGACTT - - - - // - - - -CTACTCTCGAGGatgagacagaagg-3'
E
Cdx2
mCherry
Merge
Cdx2-p2A-mCherry embryo genotyping
Tild
5' junction
Cdx2
HAL
PAM
p2A
5'-gtgtcggctaactcTGATTGCAGGGG - - - - // - - - - CCACTGTCA CCCAGgccactaacttctccctgtt-3'
2/6 (33%) 5'-gtgtcggctaactcTGATTGCAGGGG - - - - // - - - - CCACTGTCA CCCAGgccactaacttctccctgtt-3'
4/6 (67%) 5'-gtgttggctaactcTGATTGCAGGGG - - - - // - - - - CCACTGTCA CCCAGgccactaacttctccctgtt-3'
3' junction
mCherry
HAR
3'-UTR
5'-ctgtacaagtaa GACCCCTCCCGT - - - - // - - - - TGATCTGTGTAacgagccaaacaagcctttt -3'
6/6 (100%) 5'-ctgtacaagtaa GACCCCTCCCGT - - - - // - - - - TGATCTGTGTAacgagccaaacaagcctttt -3'

**Figure S1.** Tild-CRIPSR-mediated targeted integration in mouse embryos.

(**A**) Representative fluorescence images of gene-edited mouse embryos at blastocyst stage using different gene targeting donors at the *Actb* locus. Cas9 mRNA, sgRNA, and each donor vector (HR, JS2000, JS800, JS200, Tild, HMEJ, or ssDNA) were injected into mouse zygotes and the injected zygotes were cultured into blastocyst stage for fluorescence observation. Square, blastocysts shown at a higher magnification on the bottom panel.

(**B**) Sequence analysis of mCherry$^{+}$ blastocysts at the *Actb* loci in mouse embryos using Tild donor. PCR products amplified from individual blastocyst on 5' and 3' junction sites were sequenced. Upper, homology arm; purple, p2A; red, mCherry; blue, PAM sequence; HAR or HAL, right or left homology arm. Dashed lines mark the region omitted for clarity.

(**C-E**) Representative fluorescence images and sequence analysis of gene-edited mouse embryos at blastocyst stage using Tild-CRISPR-mediated targeting at the *Sox2* (**C**), *Nanog* (**D**), and *Cdx2* (**E**) loci. Square, blastocysts shown at a higher magnification on the right panel; Asterisk, ICM; arrowheads, trophoblast. PCR products amplified from individual mCherry$^{+}$ blastocyst on 5' and 3' junction sites were sequenced. Upper, homology arm; purple, p2A; red,

mCherry; blue, PAM sequence; HAR or HAL, right or left homology arm. Dashed lines mark the region omitted for clarity.

Related to Figure 1.

**Figure S2.**

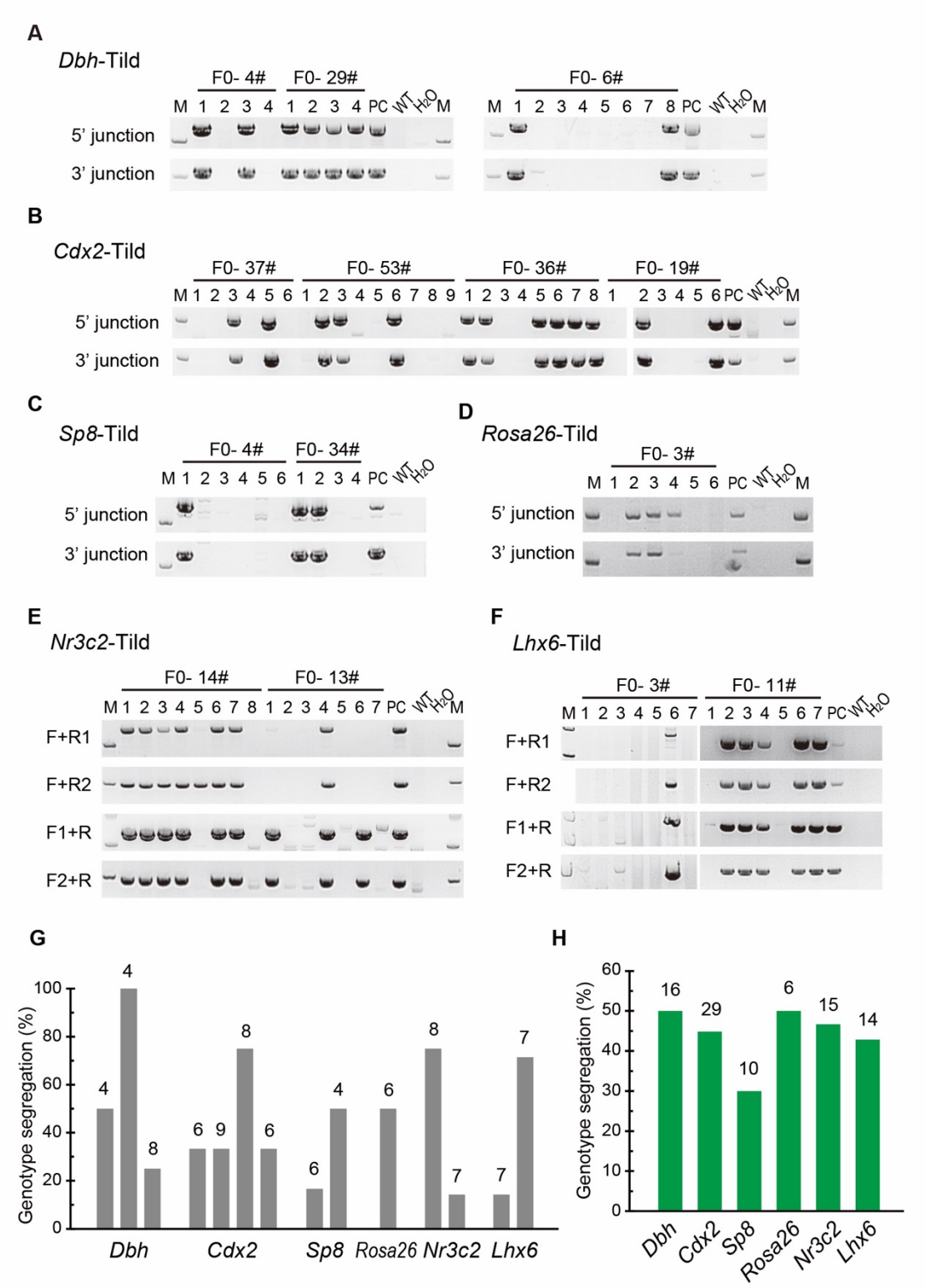

A
Dbh-Tild
F0- 4#
F0- 29#
F0- 6#
M 1 2 3 4 1 2 3 4 PC WT H2O M
M 1 2 3 4 5 6 7 8 PC WT H2O M
5' junction
3' junction
B
Cdx2-Tild
F0- 37#
F0- 53#
F0- 36#
F0- 19#
M 1 2 3 4 5 6 1 2 3 4 5 6 7 8 9 1 2 3 4 5 6 7 8 1 2 3 4 5 6 PC WT H2O M
5' junction
3' junction
C
Sp8-Tild
F0- 4#
F0- 34#
M 1 2 3 4 5 6 1 2 3 4 PC WT H2O
5' junction
3' junction
D
Rosa26-Tild
F0- 3#
M 1 2 3 4 5 6 PC WT H2O M
5' junction
3' junction
E
Nr3c2-Tild
F0- 14#
F0- 13#
M 1 2 3 4 5 6 7 8 1 2 3 4 5 6 7 PC WT H2O M
F+R1
F+R2
F1+R
F2+R
F
Lhx6-Tild
F0- 3#
F0- 11#
M 1 2 3 4 5 6 7 1 2 3 4 5 6 7 PC WT H2O
F+R1
F+R2
F1+R
F2+R
G
Genotype segregation (%)
100
80
60
40
20
0
4 4 8
6 9 8
6 4
6 8 7
7 7
Dbh
Cdx2
Sp8
Rosa26
Nr3c2
Lhx6
H
Genotype segregation (%)
60
50
40
30
20
10
0
16
29
10
6
15
14
Dbh
Cdx2
Sp8
Rosa26
Nr3c2
Lhx6

**Figure S2.** Genotype segregation analysis of founder mice generated by Tild-CRIPSR.

(**A-F**) Genotyping analysis of offspring from founder mice at the *Dbh* (**A**), *Cdx2* (**B**), *Sp8* (**C**), *Rosa26* (**D**), *Nr3c2* (**E**), and *Lhx6* (**F**) loci.

(**G**) Genotype segregation analysis of individual founder mice.

(**H**) Overall genotype segregation rate at various loci.

Related to Figure 2, 3 and Table 1.

# Figure S3.

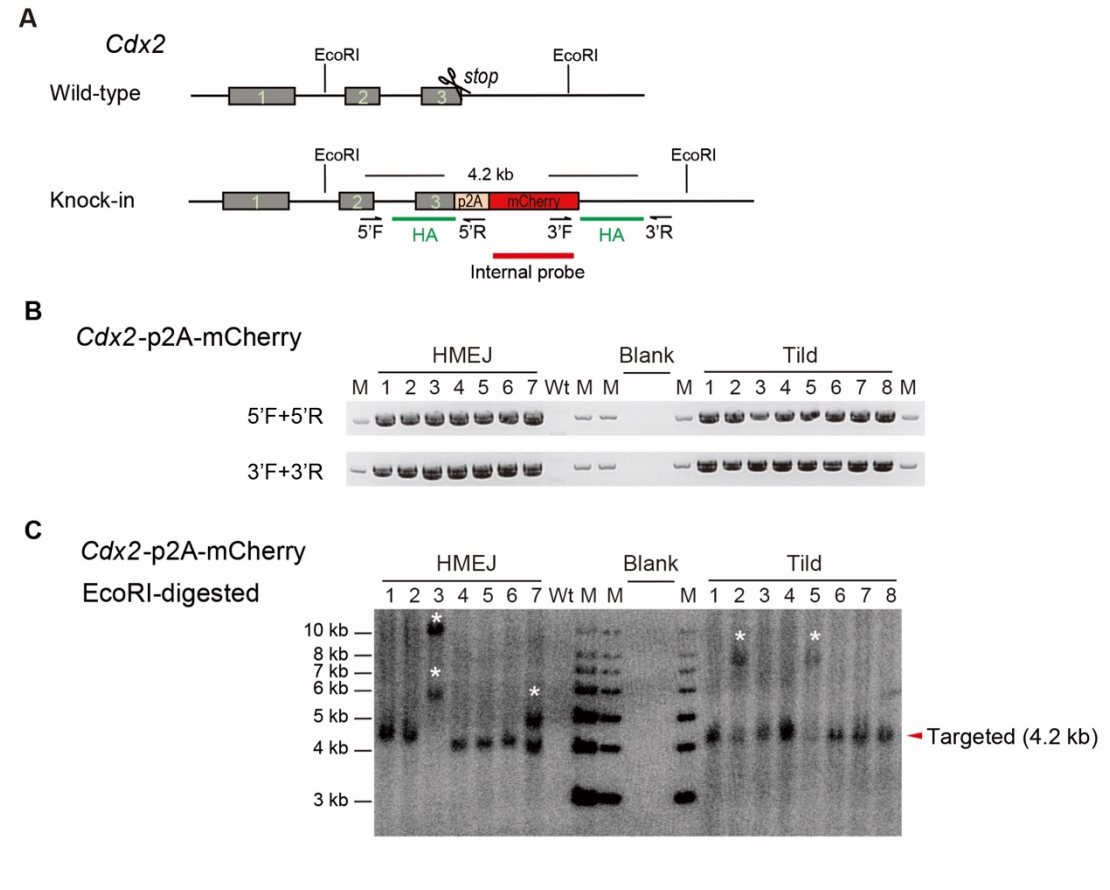

A
Cdx2
Wild-type
EcoRI
stop
EcoRI
1
2
3
Knock-in
EcoRI
4.2 kb
EcoRI
3
p2A
mCherry
5'F
HA
5'R
3'F
HA
3'R
Internal probe
B
Cdx2-p2A-mCherry
HMEJ
Blank
Tild
M 1 2 3 4 5 6 7 Wt M M M 1 2 3 4 5 6 7 8 M
5'F+5'R
3'F+3'R
C
Cdx2-p2A-mCherry
EcoRI-digested
HMEJ
Blank
Tild
1 2 3 4 5 6 7 Wt M M M 1 2 3 4 5 6 7 8
10 kb
8 kb
7 kb
6 kb
5 kb
4 kb
3 kb
Targeted (4.2 kb)

**Figure S3.** Southern blot analysis of *Cdx2*-p2A-mCherry mice generated by Tild-CRIPSR.

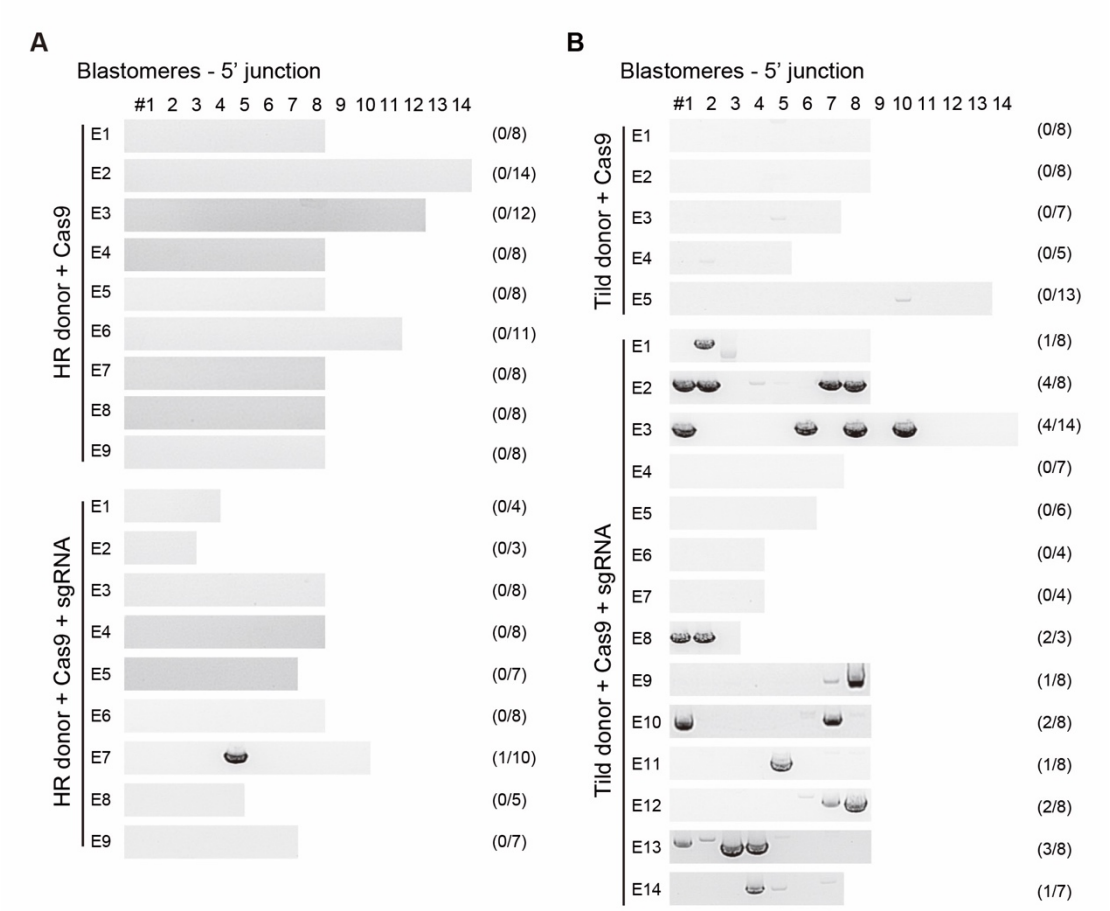
(**A**) Schematic overview of experimental design. Internal mCherry probe were indicated by red bars. Green bar, homology arm; F/R, forward/reverse primer.

(**B**) Genotyping results of *Cdx2*-p2A-mCherry fetus or mice used for southern blot. Seven knock-in fetus generated by HMEJ-based method and eight knock-in mice generated by Tild-CRIPSR were randomly selected.

(**C**) EcoRI-digested genomic DNA from *Cdx2*-p2A-mCherry mice were hybridized with internal mCherry probe. Internal mCherry probe expected fragment size: WT = N/A, Targeted = 4.2 kb. White asterisk, random integration. M, marker.

Related to Figure 2.

# Figure S4.




**C**

Wild-type embryos single cell

5' junction

*Oct4*-intron2 | HAL (*Oct4*-intron2-exon3-intron3-exon4-intron4) | PAM *Oct4*-intron4

6/19 5'-ggggcaggggcagacTACCCTCACCCATGAAGAGGAG - - - - - - - - - // - - - - - - - - - - -AGCTCTGGCTCCTCCAACTGCTctagggctgttggct-3'

13/19 5'-ggggcaggggcagacTACCCTCACCCATGAAGAGGAG - - - - - - - - - // - - - - - - - - - - -AGCTCTGGCTCCTCCAACTGCTctagggctgttggct-3'

(+CT,SNP)

**D**

*Oct4*-p2A-EGFP-ployA single cell

5' junction

*Oct4* | HAL | (ggg → gcc) PAM | intron4-exon5 | p2A

5'-ggggcaggggcagacTACCCTCACCCATG - - - - - // - - - - - GCTCCTCCAACTGCTctagccctgttggct - - - - - // - - - - - cccatgcattcaaacgccactaact-3'

HR 1/1 5'-ggggcaggggcagacTACCCTCACCCATG - - - - - // - - - - - GCTCCTCCAACTGCTctagccctgttggct - - - - - // - - - - - cccatgcattcaaacgccactaact-3'

Tild 10/21 5'-ggggcaggggcagacTACCCTCACCCATG - - - - - // - - - - - GCTCCTCCAACTGCTctagccctgttggct - - - - - // - - - - - cccatgcattcaaacgccactaact-3'

11/21 5'-ggggcaggggcagacTACCCTCACCCATG - - - - - // - - - - - GCTCCTCCAACTGCTctagccctgttggct - - - - - // - - - - - cccatgcattcaaacgccactaact-3'

(+CT,SNP)

**Figure S4.** Genotyping analysis of Tild-CRIPSR-mediated targeted integration at *OCT4* locus in human embryos.

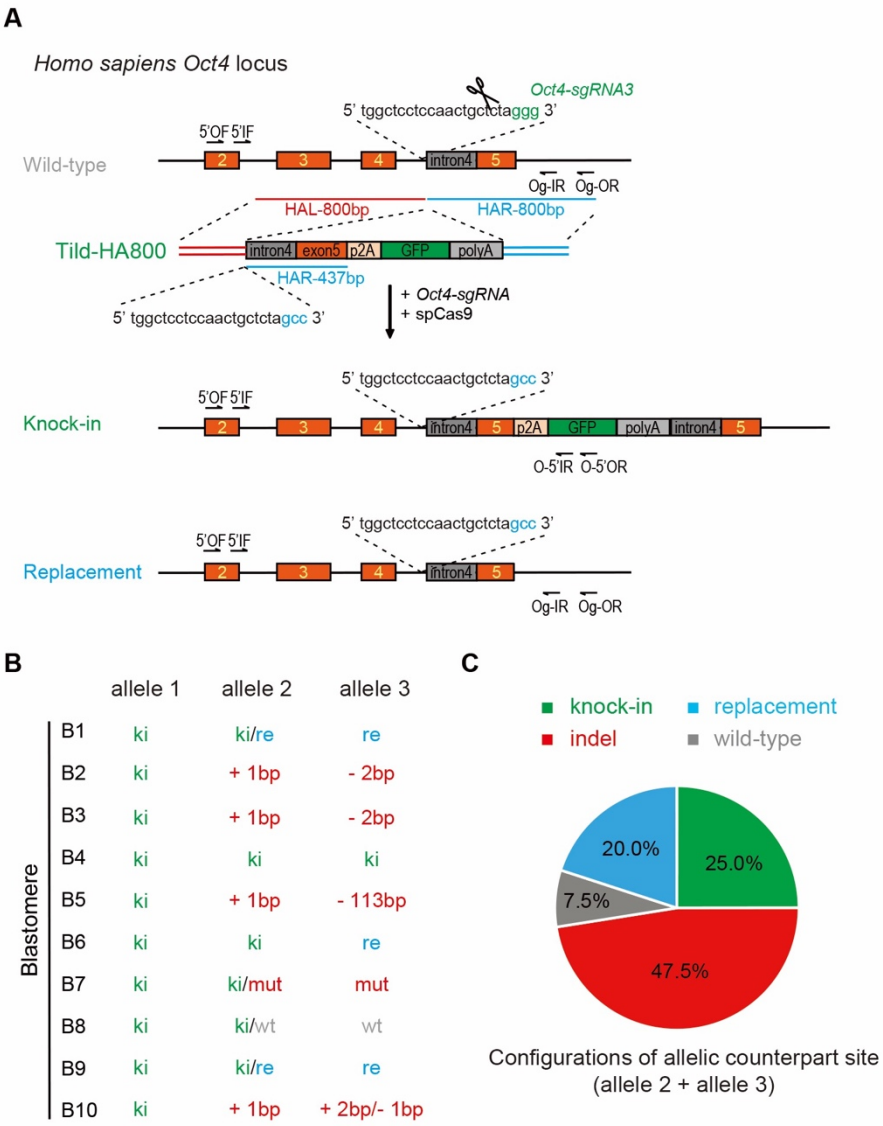
(**A-B**) Gel analysis of blastomeres in mosaic embryos generated by HR-based method and Tild-CRISPR. PCR products of 5' junction sites were amplified with 5'OF/O-5'OR and 5'IF/O-5'IR, shown in Figure S5A.

(**C**) Sequencing analysis of single blastomeres from wild-type embryos. Among 19 single blastomeres, 6 showed wild-type and 13 showed single nucleotide polymorphism (SNP) with additional "CT". PCR products were amplified with 5'OF/Og-OR and 5'IF/Og-IR, shown in Figure S5A.

(**D**) Sequencing analysis of single blastomere with *OCT4*-p2A-GFP-polyA knock-in using HR-based method and Tild-CRISPR. PCR products amplified from individual human blastomeres of 5' junction sites were sequenced. "ggg to gcc", replace PAM sequence ggg of sgRNA to gcc to avoid recutting. Upper, homology arm; blue, PAM sequence. Dashed lines mark the region omitted for clarity.

Related to Figure 5.

# Figure S5.




B

| Blastomere | allele 1 | allele 2 | allele 3 |
|---|---|---|---|
| B1 | ki | ki/re | re |
| B2 | ki | + 1bp | - 2bp |
| B3 | ki | + 1bp | - 2bp |
| B4 | ki | ki | ki |
| B5 | ki | + 1bp | - 113bp |
| B6 | ki | ki | re |
| B7 | ki | ki/mut | mut |
| B8 | ki | ki/wt | wt |
| B9 | ki | ki/re | re |
| B10 | ki | + 1bp | + 2bp/- 1bp |

**Figure S5.** Genotype analysis of the allelic counterpart site at the *OCT4* locus in human embryos using Tild-CRIPSR-mediated targeted integration.

(**A**) Schematic overview of sgRNA targeting site and p2A-GFP-polyA targeted integration at the *Homo sapiens OCT4* locus. The sgRNA targeted sequence was showed on the top and the PAM sequence was labeled in green. Knock-in, the allele with p2A-GFP-polyA targeted integration. Replacement, PAM sequence ggg of sgRNA was replaced to gcc, but without p2A-GFP-polyA targeted integration. Single blastomeres were performed with primary PCR using nested outer primer (5'OF, Og-OR and O-5'OR) and secondary PCR using nested inner primer (Knock-in: 5'IF + O-5'IR, Non-targeted: 5'IF + Og-IR). The PCR products were TA cloned and subjected to Sanger sequencing analysis. HAL/HAR, left/right homology arm; green, PAM sequence.

(**B** and **C**) Genotype analysis of the alleles in gene-edit embryos at the *OCT4* locus. Knock-in (ki), the allele sequencd with p2A-GFP-polyA targeted integration. Replacement (re), the allele sequencd with PAM sequence ggg of sgRNA was replaced to gcc, without p2A-GFP-polyA targeted integration. Wild-type (wt), the allele with no editing. Indel, the allele with insertion, deletion or point mutation.

Related to Figure 5.

# Figure S6.

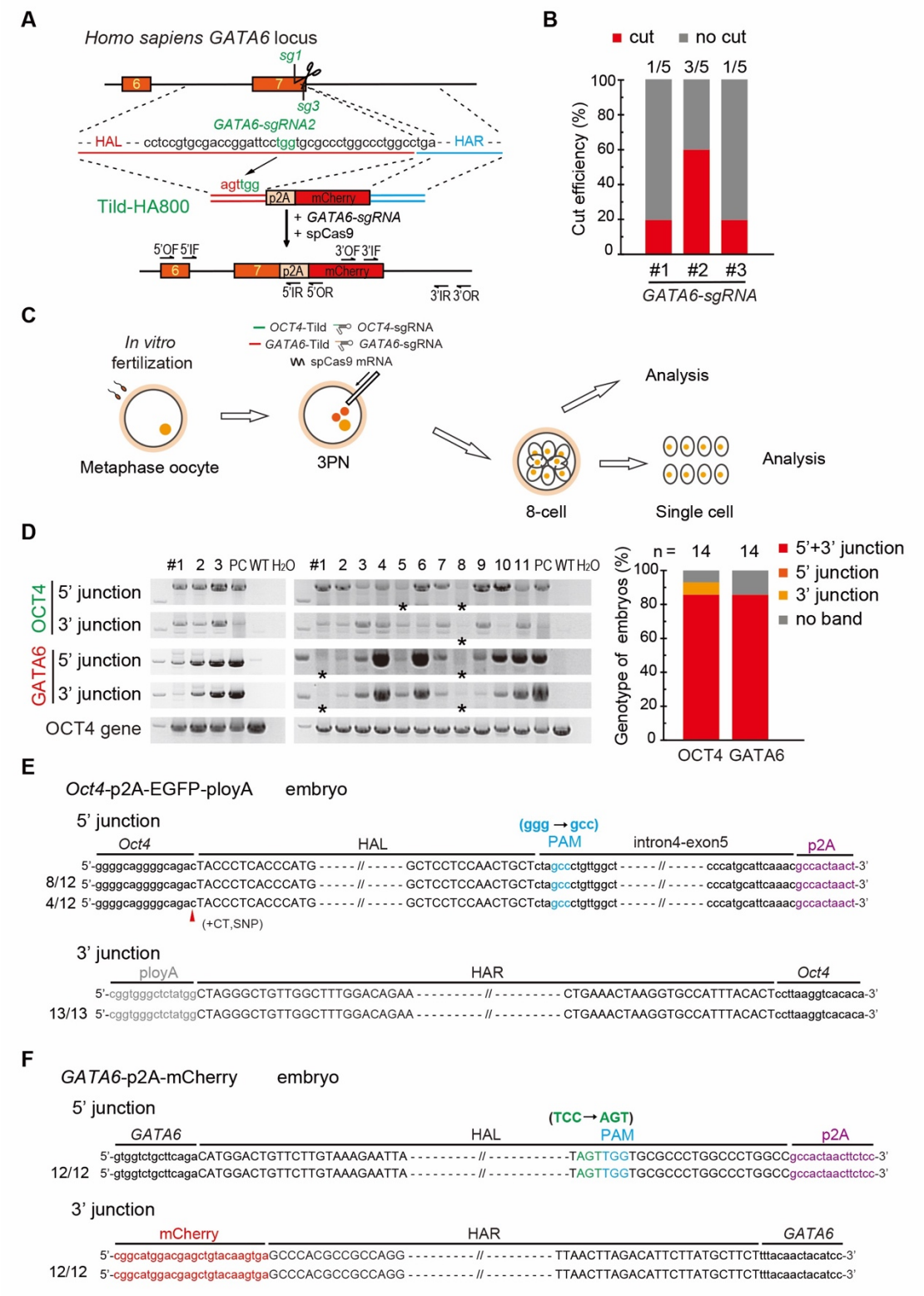

A
Homo sapiens GATA6 locus
sg1
sg3
GATA6-sgRNA2
HAL - - cctccgtgcgaccggattcctggtgcgccctggccctggcctga - - HAR - -
agttgg
Tild-HA800
p2A
mCherry
+ GATA6-sgRNA
+ spCas9
5'OF 5'IF
3'OF 3'IF
5'IR 5'OR
3'IR 3'OR
B
cut
no cut
1/5 3/5 1/5
Cut efficiency (%)
#1 #2 #3
GATA6-sgRNA
C
OCT4-Tild
OCT4-sgRNA
GATA6-Tild
GATA6-sgRNA
spCas9 mRNA
In vitro fertilization
Metaphase oocyte
3PN
Analysis
8-cell
Single cell
Analysis
D
#1 2 3 PC WT H2O #1 2 3 4 5 6 7 8 9 10 11 PC WT H2O
OCT4
5' junction
3' junction
GATA6
5' junction
3' junction
OCT4 gene
n = 14 14
5'+3' junction
5' junction
3' junction
no band
Genotype of embryos (%)
OCT4 GATA6
E
Oct4-p2A-EGFP-ployA embryo
5' junction
(ggg → gcc)
Oct4
HAL
PAM
intron4-exon5
p2A
5'-ggggcaggggcagacTACCCTCACCCATG - - - - - // - - - - - GCTCCTCCAACTGCTctagccctgttggct - - - - - // - - - - - cccatgcattcaaacgccactaact-3'
8/12 5'-ggggcaggggcagacTACCCTCACCCATG - - - - - // - - - - - GCTCCTCCAACTGCTctagccctgttggct - - - - - // - - - - - cccatgcattcaaacgccactaact-3'
4/12 5'-ggggcaggggcagacTACCCTCACCCATG - - - - - // - - - - - GCTCCTCCAACTGCTctagccctgttggct - - - - - // - - - - - cccatgcattcaaacgccactaact-3'
(+CT,SNP)
3' junction
ployA
HAR
Oct4
5'-cggtgggctctatggCTAGGGCTGTTGGCTTTGGACAGAA - - - - - - - - - // - - - - - - - - - CTGAAACTAAGGTGCCATTTACACTccttaaggtcacaca-3'
13/13 5'-cggtgggctctatggCTAGGGCTGTTGGCTTTGGACAGAA - - - - - - - - - // - - - - - - - - - CTGAAACTAAGGTGCCATTTACACTccttaaggtcacaca-3'
F
GATA6-p2A-mCherry embryo
5' junction
(TCC → AGT)
GATA6
HAL
PAM
p2A
5'-gtggtctgcttcagaCATGGACTGTTCTTGTAAAGAATTA - - - - - - - - - // - - - - - - - - - -TAGTTGGTGCGCCCTGGCCCTGGCCgccactaacttctcc-3'
12/12 5'-gtggtctgcttcagaCATGGACTGTTCTTGTAAAGAATTA - - - - - - - - - // - - - - - - - - - -TAGTTGGTGCGCCCTGGCCCTGGCCgccactaacttctcc-3'
3' junction
mCherry
HAR
GATA6
5'-cggcatggacgagctgtacaagtgaGCCCACGCCGCCAGG - - - - - - - - - // - - - - - - - - - TTAACTTAGACATTCTTATGCTTCTttacaactacatcc-3'
12/12 5'-cggcatggacgagctgtacaagtgaGCCCACGCCGCCAGG - - - - - - - - - // - - - - - - - - - TTAACTTAGACATTCTTATGCTTCTttacaactacatcc-3'

**Figure S6.** Tild-CRIPSR-mediated doubly-targeted integration in human embryos.

(**A**) Schematic overview of Tild-CRIPSR-mediated gene targeting strategy at the *Homo sapiens GATA6* locus. A p2A-mCherry reporter was designed to be fused with the last codon of the *GATA6* gene. HAL/HAR, left/right homology arm; green, PAM sequence; OF/OR, outer forward/reverse primer; IF/IR, inner forward/reverse primer.

(**B**) Cleavage efficiencies of each sgRNA targeting *GATA6* in human embryos. Number above the bar, total embryos analysis. Each indicated sgRNA, together with Cas9 mRNA, was injected into human tri-pronuclear zygotes and cultured into 8-cell embryos for genotyping.

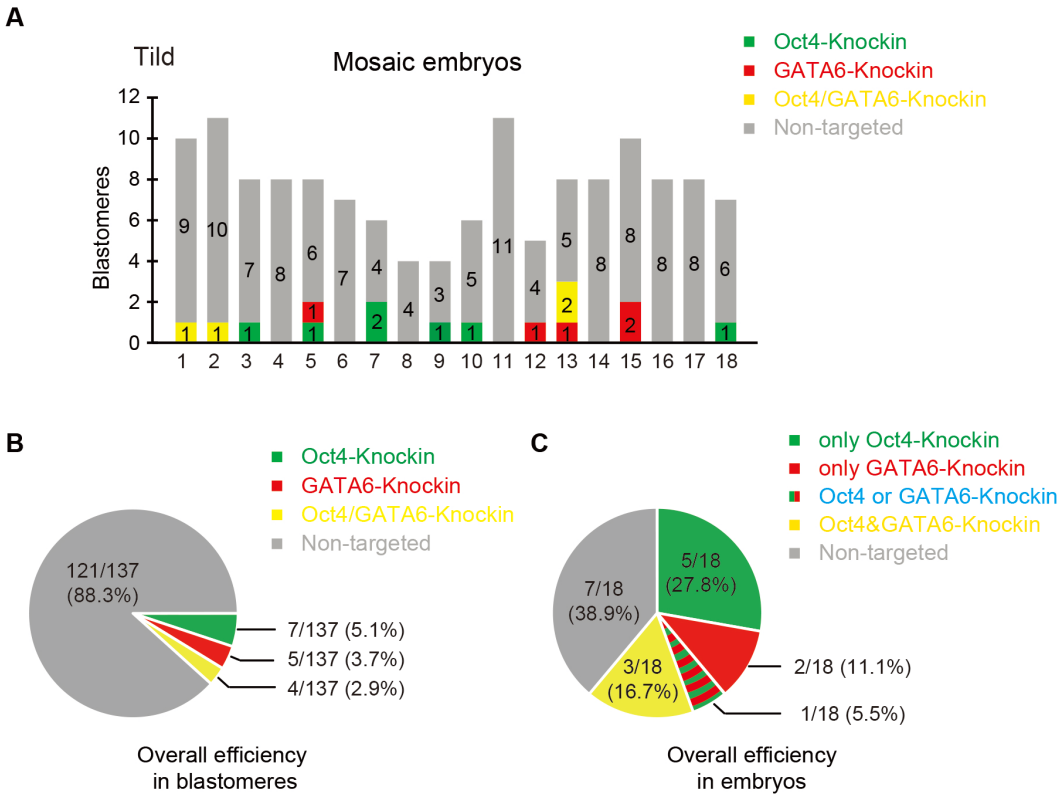
(**C**) Experimental design. *In vitro* fertilization (IVF) was performed on human oocyte and tri-pronuclear (3PN) embryos were injected with a mixture of *OCT4*/*GATA6*-Tild, *OCT4*/*GATA6*-sgRNA and spCas9 mRNA 24 hours post IVF. The injected embryos were cultured into 8-cell stage for genotyping analysis.

(**D**) Genotyping analysis of injected embryos. PCR products amplified from individual human embryos at day 3 of 5' and 3' junction sites were sequenced. Primers are listed in Table S1. *, samples failed to be amplified. PC, positive control from HEK-293T

cells with *OCT4*-p2A-GFP-polyA or *GATA6*-p2A-mCherry knock-in. Number above the bar on the right panel, total embryos analysis. (**E-F**) Sequencing analysis of injected embryos with *OCT4*-p2A-GFP-polyA and *GATA6*-p2A-mCherry knock-in. Band-positive PCR products amplified from all individual 8-cell human embryos of 5' and 3' junction sites were sequenced. Number, total sample analysis. "ggg to gcc", replace PAM sequence ggg of sgRNA to gcc to avoid recutting. "TCC to AGT", replace 3 bases (TCC) adjacent to PAM sequence to AGT to avoid recutting. Upper, homology arm; grey, polyA; blue, PAM sequence. Dashed lines mark the region omitted for clarity.

Related to Figure 5.

# Figure S7.




D

*Oct4*-p2A-EGFP-ployA single cell

5' junction

*Oct4* HAL (ggg→gcc) PAM intron4-exon5 p2A

5'-ggggcaggggcagacTACCCTCACCCATG - - - - - // - - - - - GCTCCTCCAACTGCTctagccctgttggct - - - - - // - - - - - cccatgcattcaaacgccactaact-3'

1/11 5'-ggggcaggggcagacTACCCTCACCCATG - - - - - // - - - - - GCTCCTCCAACTGCTctagccctgttggct - - - - - // - - - - - cccatgcattcaaacgccactaact-3'

10/11 5'-ggggcaggggcagacTACCCTCACCCATG - - - - - // - - - - - GCTCCTCCAACTGCTctagccctgttggct - - - - - // - - - - - cccatgcattcaaacgccactaact-3'

(+CT,SNP)

*GATA6*-p2A-mCherry single cell

5' junction

*GATA6* HAL (TCC→AGT) PAM p2A

5'-ataacgtggtctgcttcagaCATGGACTGTTCTTGTAAAG - - - - - - - - - // - - - - - - - - - - -CCGGATAGTTGGTGCGCCCTGGCCCTGGCCgccactaacttctcc-3'

9/9 5'-ataacgtggtctgcttcagaCATGGACTGTTCTTGTAAAG - - - - - - - - - // - - - - - - - - - - -CCGGATAGTTGGTGCGCCCTGGCCCTGGCCgccactaacttctcc-3'

**Figure S7.** Tild-CRIPSR-mediated doubly-targeted integration in single human blastomeres.

(**A**) Blastomere genotyping outcomes in mosaic embryos generated by Tild-CRISPR method. PCR products amplified from single blastomeres of 5' junction sites at the *OCT4* and *GATA6* loci were sequenced. Primers are listed in Table S1. Yellow, *OCT4*/*GATA6* doubly-targeted knock-in manner; Green, *OCT4*-targeted knock-in manner; Red, *GATA6*-targeted knock-in manner.

(**B**) Overall efficiency in blastomeres using Tild-CRISPR.

(**C**) Various genotype of mosaic embryos using Tild-CRISPR.

(**D**) Sequencing analysis of single blastomere with *OCT4*-p2A-GFP-polyA and *GATA6*-p2A-mCherry knock-in using Tild-CRISPR method. PCR products amplified from individual human blastomeres of 5' junction sites were sequenced.

Related to Figure 5.