MMEJ-assisted gene knock-in using TALENs and CRISPR-Cas9 with the PITCh systems

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Published online 17 December 2015; doi:10.1038/nprot.2015.140

Programmable nucleases enable engineering of the genome by utilizing endogenous DNA double-strand break (DSB) repair pathways. Although homologous recombination (HR)-mediated gene knock-in is well established, it cannot necessarily be applied in every cell type and organism because of variable HR frequencies. We recently reported an alternative method of gene knock-in, named the PITCh (Precise Integration into Target Chromosome) system, assisted by microhomology-mediated end-joining (MMEJ). MMEJ harnesses independent machinery from HR, and it requires an extremely short homologous sequence (5–25 bp) for DSB repair, resulting in precise gene knock-in with a more easily constructed donor vector. Here we describe a streamlined protocol for PITCh knock-in, including the design and construction of the PITCh vectors, and their delivery to either human cell lines by transfection or to frog embryos by microinjection. The construction of the PITCh vectors requires only a few days, and the entire process takes ~1.5 months to establish knocked-in cells or ~1 week from injection to early genotyping in frog embryos.

INTRODUCTION

Genome engineering technology using programmable nucleases provides a new avenue for generating gene knockouts, gene knock-ins and various genomic rearrangements such as chromosomal deletions, inversions, duplications and translocations[1]. To achieve such diverse genetic engineering, two main repair pathways of DNA DSBs have been commonly used (**Fig. 1**). Gene knockouts and chromosomal rearrangements often use non-homologous end joining (NHEJ), which is an error-prone repair pathway that does not require a repair template[2–4]. NHEJ can join the DNA ends generated by programmable nucleases with or without small insertions and deletions, which can cause a frameshift in an open reading frame. In addition, widely separated DNA ends can be joined by NHEJ, resulting in large deletions, inversions, duplications or translocations. NHEJ can occur throughout the cell cycle, whereas the activity of other repair pathways is limited to specific phases[5]. For gene knock-ins, HR has mainly been used. HR is a precise repair pathway, which is active during the late S/G2 phases, and it requires a repair template that harbors a long homologous sequence corresponding to the genomic DNA around the DSB site. The sister chromatid usually serves as the repair template for HR, but an exogenous targeting vector can also be used for the template, enabling gene knock-in. Although HR-mediated gene knock-in has been reported in several cells and organisms, such as human pluripotent stem cells[6,7] and mice[8], there remains room for advancements in gene knock-in strategies, especially in terms of promoting their efficiency and widening their applicability to various cells and organisms, including animal embryos with a low HR frequency.

The rapid and widespread development of genome editing methods has uncovered the substantial contribution of an alternative DSB repair pathway, MMEJ, to gene knockout by programmable nucleases (**Fig. 1**). When short microhomologies exist, both upstream and downstream of the DSB site, the two microhomologies can be annealed by MMEJ, often resulting in short deletions of the intervening sequence. MMEJ is known to be active during M–early S phases, which is when HR is inactive[5]. Efficiencies of NHEJ, HR and MMEJ in repairing DSBs are different between cell types and organisms, but many previous studies have reported frequent traces of MMEJ repair in the base-deleted alleles induced by simple expression of programmable nucleases[9–13], suggesting the superiority of this pathway in repairing DSBs generated by programmable nucleases. Particularly, Bae *et al.*[14] found that the frequencies of microhomology-associated deletions are 44.3% and 52.7% for transcription activator–like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9, respectively. Li *et al.*[15] also showed that more than 30% of the deletion alleles were joined by 3- to 5-bp microhomologies. Despite this, however, there had been no reports of the use of MMEJ for gene knock-in until we developed such systems.

Development of the PITCh systems

To use the MMEJ pathway for gene knock-in, we developed a novel knock-in system, which we named the PITCh system[16]. To date, HR- or NHEJ-mediated methods tend to have been adopted for the generation of gene knock-in (**Fig. 2a**,**b**; see 'Alternative

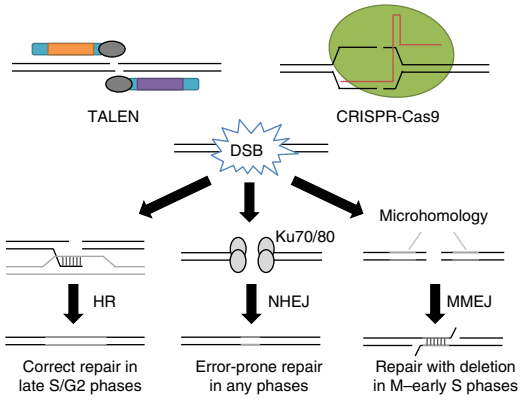


Figure 1 | A simplified schematic of DSB repair mechanisms induced by TALENs and CRISPR-Cas9. Three main pathways, HR, NHEJ and MMEJ, are shown here, but other repair mechanisms such as single-stranded annealing (SSA) and theta-mediated end joining (TMEJ) may also be involved[48]. Ku70/80 indicates the Ku70 and Ku80 heterodimer.

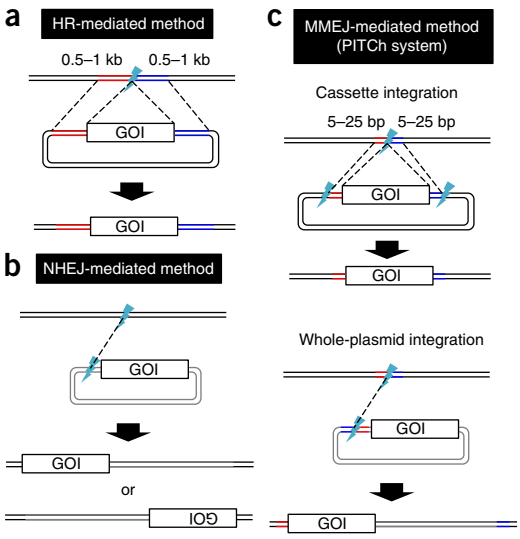


Figure 2 | General outlines of HR-, NHEJ- and MMEJ-mediated gene knock-in. (**a**) The HR-mediated method requires relatively long homology arms. (**b**) The NHEJ-mediated method does not require any homology arms, but the sequences around knock-in junctions are likely to be random. (**c**) The MMEJ-mediated method requires short microhomologies, and they are shown to be sufficient for a precise gene knock-in. GOI, gene of interest.

targeted knock-in methods' below). In contrast, the PITCh system generates DSBs in the donor vector (PITCh vector) and the genomic DNA using programmable nucleases; the donor sequence (either the whole plasmid or a specific gene cassette, depending on the experimental design) is then inserted into the genome by MMEJ, and it is stimulated by microhomologous DNA ends on the PITCh vector generated by the programmable nucleases (**Fig. 2c**).

PITCh systems harness very short (5–25 bp) microhomologous sequences as homology arms, which are easily added to the PITCh vector by PCR or by the insertion of annealed oligonucleotides. The addition of microhomologies is more convenient than construction of a targeting vector for HR-mediated genome engineering, which contains long (500–1,000 bp) homology arms. In addition, sticky ends produced by dimeric FokI-fused nucleases such as zinc-finger nucleases and TALENs are not required for MMEJ-mediated gene knock-in, unlike the NHEJ-mediated methods[17,18].

Development of TAL-PITCh. TALEN-mediated PITCh (TAL-PITCh) uses a spacer sequence between the left and right TALEN target sites as the microhomologies (**Fig. 3**). The left half of the spacer sequence should be a microhomology for the 5′ junction of the genomic target site, and the right half should be a microhomology for the 3′ junction. To generate corresponding microhomologies at the DNA ends of the exogenous DNA donor, the microhomologies comprising the spacer sequence of the TALEN target site on the TAL-PITCh vector is switched compared with the genomic target; i.e., the 5′ junction microhomology is on the right and the 3′ junction microhomology is on the left. As the optimal length of the spacer sequence is generally ~15 bp (refs. 19,20), the lengths of the 5′ and 3′ microhomologies are typically set to ~8 bp. After precise integration into the genome, two TALEN target sites remain in the 5′ and 3′ junctions, but they are no longer cut by TALENs because of the shortened spacer sequence.

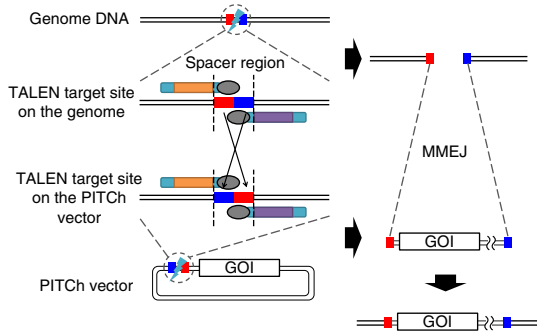


Figure 3 | A schematic of TAL-PITCh-mediated whole plasmid integration. The PITCh vector containing TALEN target sequence is linearized by TALENs and integrated into the genome via MMEJ. Red and dark blue boxes indicate the microhomologous sequence. GOI, gene of interest.

Development of CRIS-PITCh. In our original report of CRISPR-Cas9–mediated PITCh (CRIS-PITCh)[16], two or three different gene-specific guide RNAs (gRNAs) were required to generate microhomologies in the close vicinity of the Cas9 cleavage sites, depending on the experimental design: one targeting the genomic site and two targeting the 5′ and 3′ ends of the donor sequence in the PITCh vector (**Fig. 4a**). We have since updated the CRIS-PITCh system (CRIS-PITCh (v2)) to replace the gene-specific gRNAs targeting the PITCh vector with generic gRNAs (PITCh-gRNA)[21]. This was achieved by introducing generic PITCh-gRNA target sites upstream and downstream of the 5′ and 3′ microhomologies, respectively, and the lengths of the microhomologies were set to 20 bp (**Fig. 4b**). CRIS-PITCh (v2) is the focus of this protocol. To date, we have only published CRIS-PITCh (v2) data for whole plasmid integration in mammalian cultured cells, and PITCh-gRNA was designed to maximally eliminate off-target cleavages in various mammalian genomes, on the basis of the scores calculated by the CRISPR design tool (http://crispr.mit.edu/; **Supplementary Fig. 1**). Owing to the gRNA design differences, CRIS-PITCh (v2) requires distal MMEJ for precise integration, whereas the original CRIS-PITCh requires proximal MMEJ, with the MMEJ machinery trimming the extra bases outside the two microhomologies during the repair process. According to a recent report, the frequency of occurrence of distal MMEJ is higher than that of proximal MMEJ[22], which suggests that the modified system has increased efficiency . Interestingly, proximal and distal MMEJ systems have several mechanistic differences. For example, the ability to perform end resection does not affect the frequency of proximal MMEJ, whereas distal MMEJ is abolished in cells that lack resection-promoting proteins[23]. A further understanding of these two MMEJ systems might contribute to future improvements of the PITCh systems.

Overview of the procedure

In this protocol, we describe a procedure for CRIS-PITCh (v2)–mediated knock-in of the EGFP-2A-Puro cDNA cassette into any desirable gene locus in HEK293T cells. The entire workflow for PITCh-mediated gene knock-in in cultured cells is presented in **Figure 5**. In addition, a procedure for applying the TAL-PITCh system in *Xenopus laevis* embryos is described (**Box 1** and **Fig. 6**).

The CRIS-PITCh (v2) procedure is divided into three main stages: vector construction, cell culture and analysis. Two vectors need to be prepared: a programmable nuclease vector and a PITCh donor

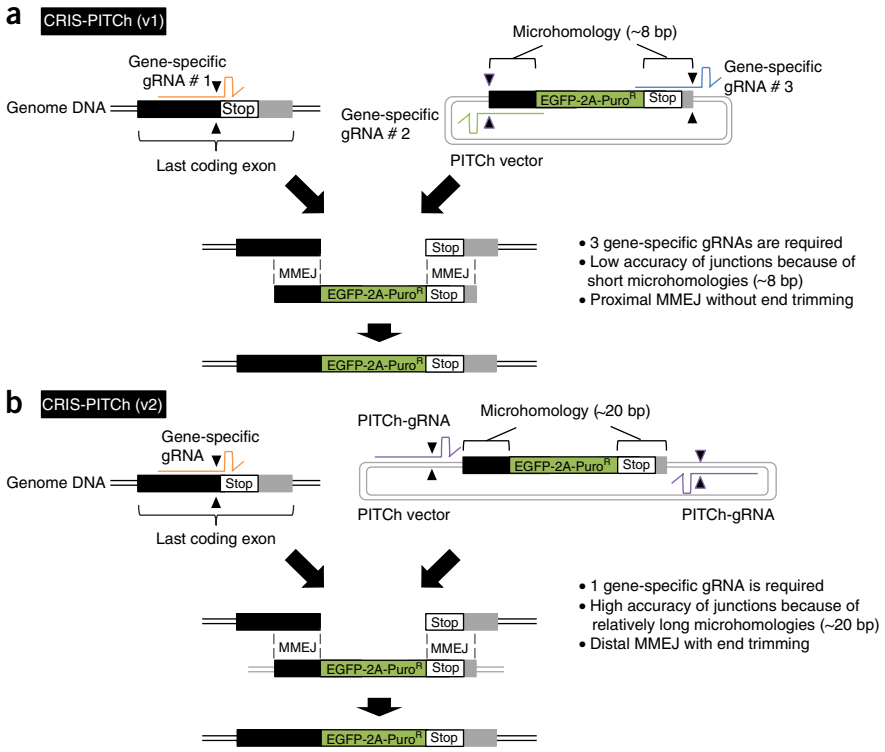


Figure 4 | The original and modified CRIS-PITCh systems (v1 and v2) for cassette knock-in. Both systems are used to insert an EGFP cDNA, followed by 2A-puromycin-resistance gene (Puro^R), into the C terminus of any desired gene locus. (**a**,**b**) Because CRIS-PITCh (v2) (**b**) has several advantages over the original version (**a**), including the requirement for fewer gRNAs and high accuracy of knock-in junctions, the procedures for CRIS-PITCh (v2) are mainly described in this protocol.

vector (Steps 1–25). These vectors are then co-transfected into cultured cells (Steps 26–29) and appropriate drug selection is performed (Steps 30 and 31). Successful gene knock-in can be confirmed at this stage by population analyses of PCR genotyping (Steps 32–42) and/or fluorescence observation (Steps 43–45). Subsequently, single-cell cloning followed by genotyping is performed (Steps 46–54).

Alternative targeted knock-in methods

HR-dependent insertion of exogenous donor DNA is a major strategy for gene knock-in. When programmable nucleases induce a DSB, the frequency of donor incorporation increases compared with the occurrence of spontaneous HR-mediated conventional gene targeting[24]. This strategy has been well established and applied in various cells and organisms[1], but the efficiency of gene knock-in is not always sufficient, because of the limited availability of the HR pathway[17,25]. In addition, construction of a targeting vector containing the left and right homology arms is time-consuming and laborious. Cloning-free single-stranded oligodeoxynucleotide (ssODN) has also been used as the repair template instead of a double-stranded DNA donor[26–28]. This is quite useful for small gene modifications, such as incorporating a single-nucleotide polymorphism or integrating a small tag (<100 bp), but it cannot be applied for inserting long gene cassettes. Alternatively, exogenous DNA can be incoporated using the NHEJ pathway[17,29,30]. However, this strategy has a tendency to induce mutagenic junctions caused by erroneous NHEJ. Obligate ligation-gated recombination (ObLiGaRe), which was developed by Maresca *et al.*[18], can incorporate the donor plasmid with high accuracy; however, this requires sticky-end ligation, and it cannot be adapted to the conventional CRISPR-Cas9 nuclease approach. Furthermore, the ObLiGaRe method has only been reported for whole plasmid integration in cultured cells, and the wider applicability is still unknown.

Advantages, limitations and applications of the PITCh systems

Compared with alternative targeted knock-in methods, the PITCh-ing strategy enables easy and versatile gene knock-in in various cells and animals with high efficiency (**Table 1**), with reliable junctions, and without any amplification of the genomic sequence for donor construction. The PITCh system has been proven to work well in both cultured cells and animals, including invertebrates and vertebrates[16,21]. On the basis of direct comparisons at a single genomic locus, the knock-in efficiency of TAL-PITCh is ~2.5-fold higher than the efficiency of HR-assisted gene knock-in in HeLa cells[16]. The protocols for TAL-PITCh and CRIS-PITCh provide a convenient and effective method of gene knock-in using programmable nucleases.

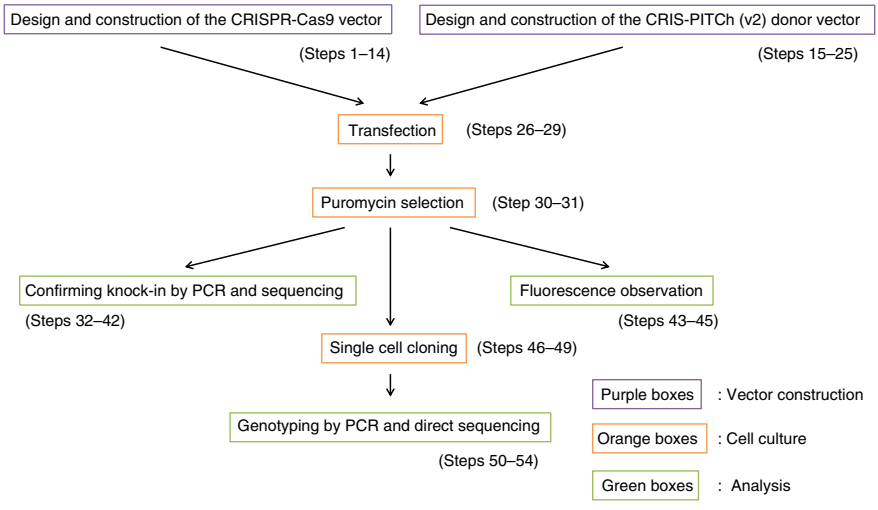


Figure 5 | General workflow of CRIS-PITCh (v2)-mediated gene knock-in in cultured cells. The experiments are classified into three groups—vector construction, cell culture and analysis—and some of them can be concurrently performed. Note that this workflow includes the minimal procedures to establish a knocked-in cell line, and additional analysis such as Southern blotting and copy number analysis are often required to confirm the detailed genotype.

Box 1 | TAL-PITCh in *Xenopus laevis* ● TIMING 1 week

REAGENTS

- Platinum Gate TALEN kit (http://www.addgene.org/TALEN/PlatinumGate/)
- mMESSAGE mMACHINE T7 Ultra transcription kit (Life Technologies, AM1345) ▲ CRITICAL The mMESSAGE mMACHINE T7 Ultra transcription kit contains a new type of cap analog, enhancing the translation efficiency of synthesized mRNA. Substitution of another transcription kit may affect the experimental outcome.
- Nuclease-free water (not diethylpyrocarbonate (DEPC)-treated; Life Technologies, cat. no. AM9937)
- *Xenopus laevis* (purchased from Hamamatsu Seibutu Kyozai or another supplier) ! CAUTION All animal experiments and husbandry should adhere to appropriate institutional ethics guidelines. General husbandry and manipulation techniques for *Xenopus* are described in Sive *et al.*[50].
- Human chorionic gonadotropin (HCG; Aska Pharmaceutical)
- 10× MMR (Marc's modified ringer) stock: this stock contains 1 M NaCl, 20 mM KCl, 10 mM $MgSO_4$, 20 mM $CaCl_2$, 50 mM HEPES and 1 mM EDTA; the pH is adjusted to 7.4 with NaOH, and then the solution is autoclaved. The stock can be stored at room temperature for up to 4 months.
- 50 mg/ml gentamicin sulfate solution (Wako, cat. no. 073-04914), dissolved in water. Store it at −20°C for up to 1 year.
- 0.1× and 0.3× MMR containing 50 μg/ml gentamicin, 0.2 μm filtered. Store it at 4 °C for up to 1 month.
- 2% (wt/vol) L-cysteine solution (Sigma-Aldrich, cat. no. C7352), dissolved in 0.1× MMR, adjusted to pH 7.8. Prepare just before use.
- 5% (wt/vol) Ficoll 400 solution (Sigma-Aldrich, cat. no. F4375), dissolved in 0.3× MMR. Store it at 4 °C for up to 1 month.
- 1% (wt/vol) UltraPure agarose (Life Technologies, cat. no. 16500100), melted in 0.3× MMR. Store it at 4 °C for up to 1 month.
- 20% (wt/vol) 3-aminobenzoic acid ethyl ester (MS-222, tricaine; Sigma-Aldrich, cat. no. A5040), dissolved in 0.1× MMR. Store it at 4 °C for up to 1 month.

EQUIPMENT

- RNeasy mini kit (Qiagen, cat. no. 70104)
- QIAquick PCR purification kit (Qiagen, cat. no. 28104)
- NANOJECT II microinjector (Drummond)
- Micromanipulator (Narishige M-152)
- Glass capillaries (Drummond 3-000-203-G/X)
- Glass or plastic Petri dish (100 mm)
- Puller (Narishige, PN-30)
- Incubator (temperature should be kept at 18 °C)
- Fluorescence stereoscope with charge-coupled device (CCD) camera (FZ10 (Leica) and DSVi1 system (Nikon) or equivalent)

PROCEDURE

Preparation of TAL-PITCh vector and TALEN mRNA for microinjection ● TIMING 2 d

▲ CRITICAL This procedure for making TALEN mRNA has been shown to work well for *Xenopus*[40].

1. Repurify the miniprepped PITCh vector, using a silica-based DNA purification kit such as the QIAquick PCR purification kit, to thoroughly remove endotoxin and RNase contamination.
2. Linearize the TALEN plasmid vectors using appropriate restriction enzymes. In our Platinum Gate TALEN system, XmaI (NEB) or SmaI (TaKaRa) are used. Then purify the linearized plasmids using the QIAquick PCR purification kit.
3. Make TALEN mRNA using an mMESSAGE mMACHINE T7 Ultra transcription kit according to the supplier's protocol. The polyA tailing should be performed. Next, purify the mRNA using silica-based RNA purification kits such as the RNeasy mini kit, and elute using non-DEPC-treated nuclease-free water. Typical yield of purified mRNA is up to 30 μg in the reaction volume of 20 μl.

■ PAUSE POINT Store it at −80 °C for up to 1 year. Do not repeatedly freeze-thaw the mRNA.

***Xenopus* egg injection ● TIMING 5 d**

! CAUTION Animal experiments should be performed according to the appropriate laws and regulations regarding animal ethics.

▲ CRITICAL For the details of egg preparation and the standard injection procedure, see the previously published protocol[50].

4. Inject 500 and 200 units of HCG into the female and the male frogs, respectively, 12–16 h before egg injection (i.e., the evening before the day of egg injection). Keep the frogs at 20 °C.
5. *Isolation of testis*. Deeply anesthetize the males using MS222 (tricaine), and then isolate the testis just before *in vitro* fertilization. Store the testis in a Microtube at 4 °C for up to 2 d.
6. *In vitro fertilization*. Squeeze eggs onto a glass Petri dish. Homogenize the testis with 500 μl of 0.1× MMR using a plastic pestle, and then mix this with the eggs on the dish. Incubate the mixture at room temperature for 10–20 min.
7. *Preparation of the mixture of mRNA and TAL-PITCh vector*. Co-inject a total of 500 pg of a paired TALEN mRNA against the target locus, and 50–100 pg of the PITCh vector into each egg. Use an injection volume of 4.6 nl per egg for *X. laevis*. Adjust the volume of the mixture with nuclease-free water according to your experimental scale. Load the mixture into a glass capillary needle and set this up on a Nanoject II microinjector.

▲ CRITICAL STEP Do not reuse the diluted mixture, and freshly prepare it before use.

▲ CRITICAL STEP The amounts and ratios of the TALEN mRNA and the PITCh vector depend on your target loci, and therefore they should be optimized in each experiment.

(continued)

Box 1 | (Continued)

8. *De-jelly the fertilized eggs*. Completely remove the jelly of the fertilized eggs using a 2% (wt/vol) cysteine solution (prepare fresh before use). This step should take no longer than 5 min. Next, wash the eggs three times with 0.1× MMR.
9. *Injection into eggs*. Transfer the de-jellied eggs into 5% (wt/vol) Ficoll solution on an agarose-coated dish. Inject the mixture into the animal pole of the eggs. The injection should be completed within 1 h after fertilization. Keep the eggs at 18 °C in the incubator until the blastula stage, and then transfer the cleaving eggs into 0.1× MMR containing gentamicin. Reporter activity may be observed if a promoterless vector carrying a fluorescent protein gene such as *EGFP* is knocked in. This will faithfully mimic the expression of the endogenous target gene in F_0 embryos (**Fig. 6a,b**).

The PITCh systems currently have some limitations, although future improvements may resolve these. In TAL-PITCh, the length of microhomology is restricted by the optimal range of the spacer sequence of the TALEN target site. In addition, TALEN sites remain at the 5′ and 3′ junctions after integration. These shortcomings could be overcome by using *de novo*–designed TALEN sites for the TAL-PITCh vector, similarly to the PITCh-gRNA sites in the CRIS-PITCh (v2) system. In both the original and the modified CRIS-PITCh, the requirement for a protospacer adjacent motif (PAM)—5′-NGG-3′ for the *Streptococcus pyogenes* Cas9—close to the genomic DSB site is potentially limiting. However, this problem can be mitigated by using either TALENs or a Cas9 from another species (with a different PAM) to induce genomic cleavage.

The TAL-PITCh system has currently been applied in cultured cells, silkworms and frogs[16]. The original and modified CRIS-PITCh system has been applied in cultured cells and zebrafish[16,21].

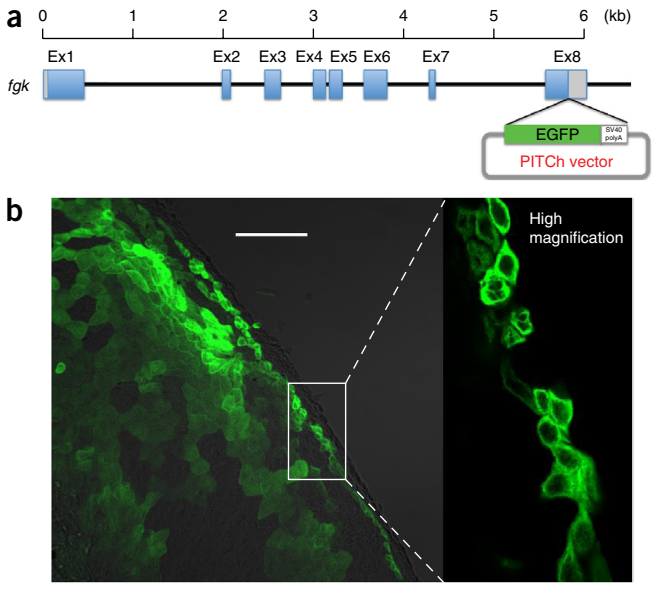


Figure 6 | *In vivo* visualization of endogenous keratin protein fused to EGFP in *Xenopus laevis*[16]. (**a**) A schematic illustration of TAL-PITCh–mediated EGFP knock-in into the C terminus of fin and gill keratin (*fgk*). A pair of TALENs targeting near the stop codon at the 8th exon *fgk* was designed. The PITCh donor vector contains a promoterless EGFP cDNA for C-terminal fusion. (**b**) Representative fluorescence images of an EGFP knock-in embryo at the tadpole swimming stage captured using a laser-scanning microscope. EGFP-tagged *fgk* illuminated the cytoskeletal pattern of keratin filaments in the fin-edge cells. Scale bar, 100 µm. All animal experiments were performed in accordance with the guidelines of Hiroshima University for the use and care of experimental animals.

Although further work is needed to clarify the exact applicability of PITCh systems in other organisms, it is expected that they will be applicable to a wide range of species, because of the advantageous properties of the MMEJ pathway. First, the MMEJ pathway is active during most periods of the cell cycle, whereas the activity of the HR pathway is restricted to the late S/G2 phases[5]. Second, traces of MMEJ repair are often observed in various animal species including rats[9], mice[11,12], *Drosophila*[10] and *Trypanosoma*[13], using programmable nucleases. Thus, the PITCh systems are expected to be versatile methods for easy and efficient gene knock-in. There are many potential applications of the PITCh systems, including the creation of reporter cell or animal lines, modeling of human diseases and the production of recombinant pharmaceutical proteins.

Experimental design

TALEN constructs for TAL-PITCh. For TAL-PITCh, a TALEN vector should be constructed only for the genomic target site. TALEN construction kits can be obtained from Addgene (https://www.addgene.org/TALEN/). We recommend using Platinum

TABLE 1 | Outline of reported PITCh-mediated gene knock-in in various cells and organisms.

Cell types or species	System	Gene locus	Efficiency[a] (%)	References
HEK293T cells	TAL-PITCh	*FBL*	67	16
	CRIS-PITCh (v1)	*FBL*	80	16
	CRIS-PITCh (v2)	*FBL*	ND	21
HeLa cells	TAL-PITCh	*ACTB*	67	16
CHO-K1 cells	TAL-PITCh	*HPRT1*	10–17	49
Silkworm	TAL-PITCh	*BLOS2*	17	16
Zebrafish	CRIS-PITCh (v2)	*Tyr*	77–85	21
	CRIS-PITCh (v2)	*krtt1c19e*	61	21
Frog	TAL-PITCh	*no29*	32	16
	TAL-PITCh	*fgk*	ND	16

[a]In cultured cells, efficiency is determined by PCR analysis of 5′ and 3′ junctions after drug selection and single-cell cloning. In silkworms, the percentage of knock-in in genotyped individuals at the G_1 generation is shown. In zebrafish, the percentage of 5′ junction-positive embryos is shown. In frogs, the percentage of the embryos showing full or half (i.e., the left or right half of the body) fluorescence expression is shown. ND, not determined.

TALENs created by the Platinum Gate TALEN kit (Addgene kit no. 1000000043)[31] to create highly active TALENs, but it should be possible to use other kits. A detailed protocol for constructing Platinum TALENs has been described previously[32], and it will not be provided here.

gRNA and Cas9 constructs for CRIS-PITCh. For the original CRIS-PITCh, two or three gRNAs are required for whole plasmid integration and backbone-free cassette integration, respectively. However, the CRIS-PITCh (v2) described herein requires a single custom gRNA targeting the intended genomic locus, which is designed according to previously published guidelines[33], and a commonly usable PITCh-gRNA. The basic CRISPR-Cas9 plasmids, which contain the Cas9 expression cassette and/or an empty gRNA cassette, can also be obtained from Addgene (https://www.addgene.org/CRISPR/). For better efficiency, Cas9 and multiple gRNA cassettes can be assembled into a single all-in-one vector using the multiplex CRISPR-Cas9 assembly system kit (Addgene kit no. 1000000055)[34] (**Fig. 7a**). Furthermore, the PITCh-gRNA– and Cas9-expressing vector for CRIS-PITCh

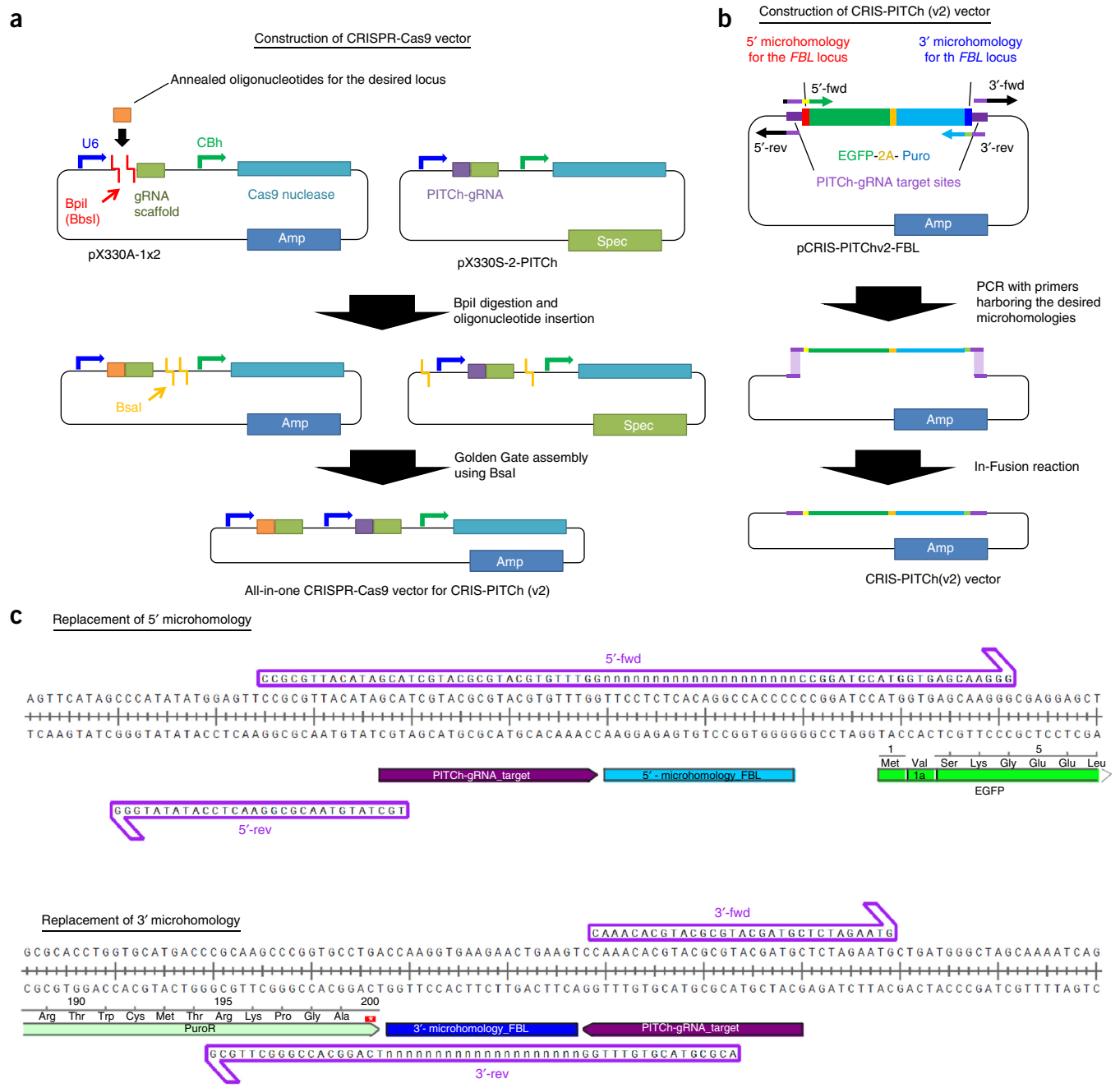


Figure 7 | A schematic illustration of vector construction for CRIS-PITCh (v2)–mediated gene knock-in in cultured cells. (**a**) Construction of an all-in-one CRISPR-Cas9 vector expressing Cas9 nuclease and two gRNAs, a target locus-specific gRNA and a generic PITCh-gRNA. Amp, ampicillin; Spec, spectinomycin; U6, human U6 promoter; CBh, chicken β-actin short promoter. (**b**) Construction of the CRIS-PITCh (v2) vector harboring the desired microhomologies. Arrows indicate primers. (**c**) Examples of primer design for In-Fusion cloning-mediated replacement of microhomologies. Variable sequences depending on the desired genomic target locus are indicated by *n*. These figures were generated using the SnapGene Viewer (http://www.snapgene.com/). fwd, forward; rev, reverse.

(v2), pX330S-2-PITCh, which is compatible with the multiplex CRISPR-Cas9 assembly system kit, is available from Addgene (plasmid no. 63670).

Donor construct for TAL-PITCh and CRIS-PITCh. Construction of PITCh vectors can be performed by PCR and In-Fusion cloning (Clontech) or by insertion of annealed oligonucleotides. A schematic illustration of constructing TAL-PITCh vector for whole-plasmid integration is shown in **Figure 8**. For TAL-PITCh, ~50-bp TALEN target site(s) should be added to the intended donor vector. For CRIS-PITCh (v2), an ~60-bp sequence containing the PITCh-gRNA target site(s) and 20-bp microhomologies should be added (**Fig. 7b**,**c**). As 50–60 bp of sequence can be added by synthetic oligo primers, genomic PCR is not necessary for PITCh vector construction, unlike the construction of the targeting vector for HR-mediated method. In addition, pCRIS-PITChv2-FBL, a PITCh donor vector for the C-terminal knock-in of EGFP-2A-Puro cDNA at the fibrillarin (*FBL*) locus, is also available from Addgene (plasmid no. 63672). This can be used not only as a positive control for PITCh knock-in experiments together with pX330A-FBL/PITCh (Addgene, plasmid no. 63671) but also as a PCR template for the CRIS-PITCh (v2) donor vector for EGFP-2A-Puro knock-in at any desired locus.

Activity validation of TALEN and CRISPR-Cas9 vectors. Nuclease activity of TALEN and CRISPR-Cas9 vectors should be validated before use for gene knock-in. There are two main types of activity validation assays currently available: episomal reporter assay and genomic cleavage detection. For the episomal reporter assay, single-stranded annealing assays using luciferase or GFP reporter genes have been widely used[20,35,36]. A traffic-light reporter assay can also be adopted[37]. To detect genomic cleavage, Cel-I (Surveyor) or T7 endonuclease I (T7EI) assays should be performed[38,39]. In addition, there are many options to estimate the mutation rate, such as restriction fragment length polymorphism (RFLP) analysis[40,41], heteroduplex mobility assay (HMA)[41,42] and high-resolution melting analysis (HRMA)[43].

Controls. As a positive control of transfection, first check the transfection efficiency using a simple fluorescent protein expression vector, such as GFP. As a negative control of PITCh knock-in, the TAL-PITCh or CRIS-PITCh vector should be transfected without co-transfecting TALEN or CRISPR-Cas9 vectors. If many puromycin-resistant colonies are observed in the absence of programmable nuclease vectors, it is an indication that random integration occurs very frequently. If random integration cannot be avoided by dose control of PITCh vectors, consider negative selection using the herpes virus thymidine kinase or diphthteria toxin genes.

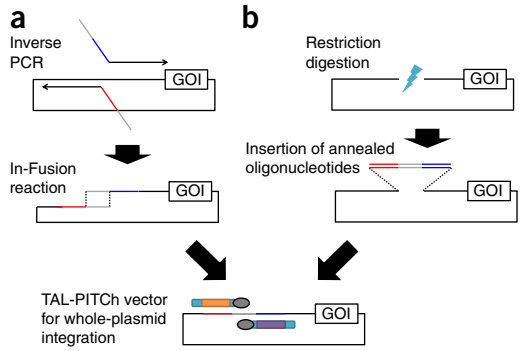


Figure 8 | Options for constructing the TAL-PITCh donor vector. (**a**) A TALEN target site on the TAL-PITCh vector can be added by inverse PCR and In-Fusion reaction. Red and blue lines indicate the left and right target sequence of TALENs (~18 bp each). Gray lines indicate the spacer region. (**b**) Another cloning method using restriction digestion and oligonucleotide insertion is shown. The DNA ends of the digested vector and the annealed oligonucleotides should be compatible. GOI, gene of interest.

Transfection, single-cell cloning and genotyping for cultured cell application. Transfection and single-cell cloning methods can be adapted from any other standard protocol using programmable nucleases published elsewhere[33,44,45]. In our protocol, the lipofection method using Lipofectamine LTX (Life Technologies) is used for transfection, and the limiting dilution method after puromycin selection is used for single-cell cloning. However, there will be circumstances when other transfection methods, such as electroporation or lipofection with other reagents, will be suitable. FACS could be used for single-cell cloning instead of limiting dilution. The PITCh system does not require long-range PCR for genotyping, because the microhomology sequence is extremely short. Thus, PCR genotyping is much easier than for HR-mediated gene knock-in.

mRNA synthesis, microinjection and genotyping for animal application. To produce knock-in animals using PITCh systems, microinjection into eggs is the most appropriate method. The quality of the TALEN or Cas9 mRNA and gRNA is crucial for successful and efficient knock-in. High-purity plasmids are also required to decrease the toxicity of DNA injection into eggs. The founder generation (F_0) usually displays somatic mosaicism, and therefore F_1 progeny that are heterozygous or homozygous for the mutant alleles should be obtained for further analysis.

MATERIALS

REAGENTS

Construction of the programmable nuclease vector for CRIS-PITCh (v2) in cultured cells

- pX330A-1x2 (Addgene, plasmid no. 58766). This vector contains a gRNA scaffold and the Cas9 coding region; it is used to assemble the locus-specific gRNA (by incorporation of gene-specific synthetic oligonucleotide) and to coexpress it with Cas9. See the **Supplementary Note** for the full plasmid sequence and **Figure 7a** for a schematic
- pX330S-2-PITCh (Addgene, plasmid no. 63670). This vector contains the PITCh-gRNA and the Cas9 coding region, and it is used to coexpress them. See the **Supplementary Note** for the full plasmid sequence and **Figure 7a** for a schematic
- pX330A-FBL/PITCh (Addgene, plasmid no. 63671). This vector contains the PITCh-gRNA, *FBL* gRNA and the Cas9 coding region, and it is used for positive control gene knock-in experiment along with pCRIS-PITChv2-FBL. See the **Supplementary Note** for the full plasmid sequence

- Synthetic oligonucleotides for the locus-specific gRNA template. These should be designed according to previously published guidelines[33]
- Primers for colony PCR screening: CRISPR-step2-F, 5′-GCCTTTTGC TGGCCTTTTGCTC-3′ and CRISPR-step2-R, 5′-CGGGCCATTTACC GTAAGTTATGTAACG-3′
- GenElute HP plasmid miniprep kit (Sigma, cat. no. NA0160)
- Quick ligation kit (New England Biolabs, cat. no. M2200)
- T4 DNA ligase reaction buffer (New England Biolabs, cat. no. B0202S)
- BpiI (Thermo Scientific, cat. no. ER1012)
- BsaI-HF (Life Technologies, cat. no. R0535)
- Taq DNA polymerase for colony PCR (BIOLINE HybriPol DNA Polymerase or equivalent)
- Ampicillin sodium (Wako, cat. no. 010-10371)
- Spectinomycin dihydrochloride pentahydrate (Sigma, cat. no. S4014)
- Luria broth base (Life Technologies, cat. no. 12795-027)
- Agar purified, powder (Nacalai Tesque, cat. no. 01056-15)
- X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside; Takara, cat. no. 9031)
- IPTG (Takara, cat. no. 9030)
- XL1-Blue supercompetent cells (Agilent Technologies, cat. no. 200236)
- Agarose S (Wako, cat. no. 313-90231)
- Tris-acetate-EDTA (TAE) buffer (Sigma-Aldrich, cat. no. T6025-1L)
- EtBr (ethidium bromide) solution (Wako, cat. no. 315-90051)
  ! CAUTION EtBr is mutagenic. Wear gloves when you are handling EtBr-containing gels and solutions.

Construction of the CRIS-PITCh (v2) donor vector

- pCRIS-PITChv2-FBL (Addgene, Plasmid no. 63672) or any desired donor vector. The donor vector contains the EGFP-2A-Puro cDNA cassette, which is flanked by microhomologies for the *FBL* locus and PITCh-gRNA target sites. See the **Supplementary Note** for the full plasmid sequence and **Figure 7b** for a schematic
- Primers for the addition of desired microhomologies by PCR. See **Figure 7b,c** for schematics. 5′-reverse (5′-TGCTATGTAACGCGGAACT CCATATATGGG-3′) and 3′-forward (5′-CAAACACGTACGCGTACGA TGCTCTAGAATG-3′) are generic primers. To design gene-specific primers, simply replace the 5′ and 3′ microhomologies in **Figure 7c** with the desired sequences
- Primers for sequencing the gene-specific microhomologies: donor-upstream-seq, 5′-GCCCTTAATTGTGAGCGGATAAC-3′ and donor-downstream-seq, 5′-CACCAGGGCAAGGGTCTG-3′
- High-fidelity DNA polymerase (Takara PrimeSTAR Max DNA Polymerase or equivalent)
- In-Fusion HD cloning kit (Clontech, cat. no. 639648)
- Wizard SV gel and PCR clean-up system (Promega, cat. no. A9281)

Mammalian cell culture

- HEK 293T cells (RIKEN Cell Bank, cat. no. RCB2202) **! CAUTION** The cell lines used in your research should be regularly checked to ensure that they are authentic, and that they are not infected with mycoplasma.
- DMEM, high glucose (Life Technologies, cat. no. 10313-039)
- FBS, qualified and heat-inactivated (Life Technologies, cat. no. 10438-034)
- Opti-MEM I reduced-serum medium (Life Technologies, cat. no. 11058-021)
- MEM non-essential amino acids solution, 100× (Life Technologies, cat. no. 11140-050)
- Penicillin-streptomycin, 100× (Life Technologies, cat. no. 15140-163)
- 0.25% (wt/vol) trypsin-EDTA (1×), Phenol Red (Life Technologies, cat. no. 25200-056)
- Lipofectamine LTX with Plus Reagent (Life Technologies, cat. no. 15338-100)
- 16% (wt/vol) paraformaldehyde (formaldehyde) aqueous solution (Electron Microscopy Sciences, cat. no. 15710) **! CAUTION** Paraformaldehyde is very toxic. It must be handled with gloves and safety glasses inside a chemical cabinet.
- Puromycin dihydrochloride (Wako, cat. no. 160-23151)
- PBS, tablet (Takara, cat. no. T900)

Genotyping analysis

- PCR primer for genomic PCR and DNA sequencing. For genotyping, gene-specific primers have to be designed. Around 300- to 500-bp fragments are typically amplified[16]. For DNA sequencing after TA cloning, universal primers such as T7 (5′-TAATACGACTCACTATAGGG-3′) and T3 (5′-GCAATTAACCCTCACTAAAGG-3′) can be used
- DNeasy blood and tissue kit (Qiagen, cat. no. 69504)
- KOD FX Neo (Toyobo, cat. no. KFX-201)
- TArget Clone Plus (Toyobo, cat. no. TAK-201)

EQUIPMENT

Construction of programmable nuclease and CRIS-PITCh (v2) vectors

- PCR tubes, 0.2 ml (Eppendorf, cat. no. 0030124359)
- Microtubes, 1.5 ml (Eppendorf, cat. no. 0030125150)
- Micro refrigerated centrifuge (KUBOTA, Model 3700 or equivalent)
- Thermal cycler (ASTEC, GeneAtlas G or equivalent)
- ASNOL sterilization plate (AS ONE, 1-7484-01)
- Mupid-2plus (Takara, M-2P)

Human cell experiments

- Falcon 50-ml conical centrifuge tubes (BD Biosciences, cat. no. 352070)
- 100-mm tissue culture dishes (Iwaki, cat. no. 3020-100)
- Non-tissue-culture plates, 96 well, flat-bottom with low evaporation lid (BD Biosciences, cat. no. 352070)
- Microplate 12 well with lid (Iwaki, cat. no. 2621351)
- Collagen type I–coated EZView LB culture plate 24-well glass-bottom plate (Iwaki, cat. no. 2891301) **▲ CRITICAL** HEK293T cells peel off easily from a glass-bottom plate. We strongly recommend using a collagen-coated plate.
- LUNA automated cell counter (Logos Biosystems, cat. no. L10001)
- 5-ml Corning Costar Stripette (Costar, cat. no. 4487)
- 10-ml Corning Costar Stripette (Costar, cat. no. 4488)
- BD Falcon Express Pipet-Aid (BD Biosciences, cat. no. 357590)
- Cellulose acetate, 0.20 μm (Advantec, cat. no. 25CS020AS)
- Terumo syringe, 50 ml (Terumo, cat. no. SS-50ESZ)
- CKX41 inverted microscope (Olympus)
- OLYMPUS FV1000D (Olympus)

REAGENT SETUP

Puromycin solution Dissolve 50 g of puromycin dihydrochloride in 50 ml of ddH_2O, and filter-sterilize it through cellulose acetate with a pore size of 0.20 μm. Dispense 500 μl to each Microtube, and store the tubes at −20 °C for up to 1 year. Perform all the operations on a clean bench.

4% (wt/vol) paraformaldehyde solution Dilute 10 ml of 16% (wt/vol) paraformaldehyde with 30 ml of ddH_2O, and work on a clean bench to filter-sterilize it through cellulose acetate with a pore size of 0.20 μm. Diluted solution can be stored at 4 °C for up to 1 week.

PROCEDURE

Construction of the CRISPR-Cas9 vector for CRIS-PITCh (v2) ● TIMING 1 week

▲ CRITICAL To perform TAL-PITCh-mediated gene knock-in in *Xenopus* embryos, follow the procedure in . See also **Figure 6**.

1| Prepare plasmid DNA for pX330A-1x2 and pX330S-2-PITCh using an appropriate miniprep kit, such as the GenElute HP plasmid miniprep kit. A schematic illustration of CRISPR-Cas9 vector construction for CRIS-PITCh (v2) is shown in **Figure 7a**.

▲ CRITICAL STEP In Golden Gate assembly, the purity of the plasmid DNA is very important. It is not guaranteed that any miniprep kit can be used. Note that the pX330A-1x2 plasmid is ampicillin resistant, whereas the pX330-S2-PITCh plasmid is spectinomycin resistant.

2| Anneal the synthesized oligonucleotides designed to target the desired genomic locus, and insert them into the pX330A-1x2 vector, according to the previously published protocol[33].

3| Mix the following components in a PCR tube:

Component	Amount (μl)	Final concentration
50 ng/μl pX330A-1×2_gene_X (with oligonucleotides inserted)	1.5	3.75 ng/μl
100 ng/μl pX330S-2-PITCh	1.5	7.5 ng/μl
10× T4 DNA ligase reaction buffer	2	1×
BsaI-HF	1	
Quick ligase	1	
ddH_2O	13	
Total	20	

4| Perform Golden Gate assembly in a thermal cycler under the following conditions: (37 °C, 5 min → 16 °C, 10 min) × 25 → 4 °C, ∞.

5| Take the tube from the thermal cycler and add the following reagents:

Component	Amount (μl)	Final concentration
10× NEBuffer 4	2.5	1×
10×BSA	2.5	1×
BsaI-HF	1	
Total	6	

▲ **CRITICAL STEP** This additional digestion results in nearly complete elimination of the formation of blue colonies containing intact vector in Step 7, by digesting the intact pX330A-1x2_gene_X plasmid. However, this step should be excluded to prevent overdigestion, if the total number of colonies is low.

6| Place the tube in the thermal cycler again, and run the following program: 37 °C, 30 min → 80 °C, 5 min → 4 °C, ∞.

7| *Transformation*. Transform the products into XL1-Blue chemically competent cells as follows: mix 2 μl of the products and 20 μl of XL1-Blue cells, and incubate the mixture on ice for 10 min. Perform heat-shocking at 42 °C for 30 s. Incubate the cells on ice again for 5 min. Plate the transformed bacteria onto an LB plate containing 100 μg/ml ampicillin with X-Gal/IPTG. Culture the bacteria overnight at 37 °C.
▲ **CRITICAL STEP** Unlike the original pX330 vector (Addgene, plasmid no. 42230), our pX330A vectors allow blue/white selection to efficiently screen the correctly assembled all-in-one plasmids.

8| The next day, perform colony PCR on 2–4 white colonies using the CRISPR-step2-F and CRISPR-step2-R primers, as described in Steps 9–12.

9| Mix the following components in PCR tubes:

Component	Amount per reaction (μl)	Final concentration
10× reaction buffer	0.8	1×
10 μM primer mixture (F+R)	0.32	0.4 μM
10 mM dNTP mixture	0.64	0.8 mM
50 mM $MgCl_2$	0.24	1.5 mM
HybriPol DNA polymerase	0.04	
ddH_2O	5.96	
Total	8	

10| For each reaction mix, pick a white colony, add it to a reaction mixture and stab it into another LB plate (replica plate). Place the replica plate at 37 °C and grow it overnight. The replica plate can be stored at 4 °C.

11| Place the reactions in a thermal cycler, and run the following program:

Cycle number	Denature	Anneal	Extend
1	95 °C, 30 s		
2–28	95 °C, 15 s	67 °C, 15 s	72 °C, 50 s
29			72 °C, 50 s

12| Run the PCR products on a 2% (wt/vol) agarose gel. Stain the gel with ethidium bromide and confirm the presence of the intended amplicon.
▲ **CRITICAL STEP** The intended amplicon includes one major band and one minor band; the latter is due to repetitive sequence in the amplicon. See the previous paper for details[34].
? TROUBLESHOOTING

13| Select positive clones from the replica plate (Step 10) and culture them with shaking overnight at 37 °C, in 3 ml of LB medium containing 100 μg/ml ampicillin.

14| Purify the plasmid using an appropriate miniprep kit.

Construction of the CRIS-PITCh (v2) donor vector ● TIMING 3 d
15| Prepare plasmid DNA for pCRIS-PITChv2-FBL using an appropriate miniprep kit. A schematic illustration of CRIS-PITCh (v2) vector construction is shown in **Figure 7b**,**c**.

16| By using a high-fidelity DNA polymerase, such as PrimeSTAR Max DNA polymerase, perform two separate PCRs—one to amplify the vector and one to amplify the insert, as illustrated in **Figure 7b**,**c**. For the vector, use generic 5′-reverse and 3′-forward primers; for the insert, use gene-specific primers containing the desired microhomologies. Set up the reaction mix as tabulated below:

Component	Amount per reaction (μl)	Final concentration
pCRIS-PITChv2-FBL (1 ng/μl)	0.5	0.05 ng/μl
10 μM primer mixture (F+R)	1	1 μM
PrimeSTAR MAX Premix (2×)	5	1×
ddH_2O	3.5	
Total	10	

▲ **CRITICAL STEP** Primers should be carefully designed, because the pCRIS-PITChv2-FBL contains two identical PITCh-gRNA target sequences, next to the 5′ and 3′ microhomologies.

17| Place the reactions in a thermal cycler and run an appropriate PCR program; an example is as follows:

Cycle number	Denature	Anneal	Extend
1	94 °C, 2 min		
2–36	98°C, 10 s	72 °C, 1.5 min	
37			72 °C, 4 min

18| Run each PCR product on a 1% (wt/vol) agarose gel, and then stain the gel using ethidium bromide solution.
? TROUBLESHOOTING

19| Excise the intended bands and collect them in Microtubes.

20| Purify the DNA using a Wizard SV gel and PCR clean-up system.

21| Ligate the two purified DNA fragments using the In-Fusion HD cloning kit.

22| Transform the product, and culture it overnight. See Step 7 for the detailed transformation procedure, although the blue/white selection is not needed in this step.

23| Pick 2–4 colonies, and culture them with shaking overnight at 37 °C, in 3 ml of LB medium containing 100 μg/ml ampicillin.

24| Purify the plasmid using an appropriate miniprep kit.

25| Confirm the addition of the microhomologies by DNA sequencing using the donor-upstream-seq and donor-downstream-seq primers.
? TROUBLESHOOTING

Cell culture and transfection ● TIMING 10 d
26| *Cell maintenance*. Culture HEK293T cells in 12 ml of DMEM, which contains 10% (vol/vol) FBS, 1× Penicillin-streptomycin and 1× non-essential amino acids, at 37 °C with 5% CO_2 in a 100-mm dish.

27| *Cell passage*. When cells become 80% confluent in a 100-mm dish, remove the medium with an aspirator, add 3 ml of trypsin and incubate it for 5 min at 37 °C. Subsequently, add 12 ml of DMEM and transfer the cell solution to a 50-ml Falcon tube. Centrifuge for 3 min at 880*g* at room temperature (18–22 °C). Discard the supernatant, add 5 ml of DMEM and mix by pipetting. 1 ml of cell solution should be added to a new 100-mm dish containing 11 ml of fresh DMEM.

28| The day before transfection, seed 2.5–5.0 × 10^5 cells into a 100-mm dish containing 12 ml of DMEM.

29| *Transfection*. Replace the medium in the dish with 7 ml of Opti-MEM before transfection. Add 1.2 μg of all-in-one CRISPR-Cas9 vector (from Step 14) and 0.6 μg of CRIS-PITCh (v2) vector (from Step 25) to a Microtube containing 500 μl of Opti-MEM. Add 30 μl of Lipofectamine LTX to another Microtube containing 500 μl of Opti-MEM, and incubate it for 5 min at room temperature. Then, mix the two solutions and incubate them for 30 min at room temperature. Add the mixture to the cultured cells and incubate the cells in a CO_2 incubator.
▲ **CRITICAL STEP** In our protocol, the amounts of plasmid DNA, lipofection reagent and seeding cells are much less than indicated in the manufacturer's instructions for Lipofectamine LTX. This is because high confluency often leads to unsuccessful drug selection. However, optimization may be needed if transfection efficiency is too low.
? TROUBLESHOOTING

30| The next morning, replace the medium with 12 ml of fresh DMEM. 72 h after transfection, replace the medium with 12 ml of DMEM containing 12 μg of puromycin (1 μg/ml) on a daily basis for 7 d.
▲ **CRITICAL STEP** The puromycin-resistance gene is driven by an endogenous promoter in this vector design. If the target gene is not active in the cell lines, the puromycin-resistance gene should be expressed in an independent expression cassette with a constitutive promoter. However, the use of an independent expression cassette with a constitutive promoter could lead to selection for clones with random integration.
? TROUBLESHOOTING

31| Replace the medium with 12 ml of DMEM without puromycin, and culture the cells for an additional few days until the colonies have grown enough for knock-in confirmation (Step 32 or Step 43) or single-cell isolation (Step 46).
▲ **CRITICAL STEP** Before proceeding to single-cell isolation, it is important to confirm the knock-in rate by genomic PCR followed by bacterial cloning and DNA sequencing (Steps 32–42) and/or fluorescence observation (Steps 43–45).

Knock-in confirmation ● TIMING 3 d
32| Collect puromycin-selected cells (from Step 31) in a 50-ml tube with 5 ml of fresh DMEM. Transfer 1 ml of cell solution into a new dish containing 11 ml of DMEM for further analyses such as fluorescence observation (Step 43) and single-cell cloning (Step 46). Centrifuge the remaining cell solution for 3 min at 880*g* at room temperature. Discard the supernatant and transfer the cells to a Microtube.

33| Extract genomic DNA from the cell pellet using a DNeasy blood and tissue kit, and perform genomic PCR with KOD FX Neo. Primers should be designed to separately amplify the 5′ and 3′ junctions of the knock-in cassette. Mix the following components in a PCR tube:

Component	Amount (μl)	Final concentration
2× PCR buffer for KOD FX Neo	5	1×
10 μM primer mixture (F+R)	1.2	1.2 μM
2 mM dNTP mix	2	0.4 mM
KOD FX Neo	0.2	
100 ng/μl genomic DNA	2	20 ng/μl
ddH_2O	0.5	
Total	10	

34| Place the reactions in a thermal cycler and run the following program:

Cycle number	Denature	Anneal	Extend
1	94 °C, 2 min		
2–36	94 °C, 30 s	68 °C, 30 s	
37			68 °C, 5 min

35| Run an aliquot of the PCR products on a 2% (wt/vol) agarose gel. Stain the gel with ethidium bromide and confirm the presence of the intended amplicon.

36| Clone the remaining PCR product from Step 34 into the pTA2 vector using TArget Clone Plus according to the manufacturer's instructions. The PCR product can directly be used for TA cloning without gel purification using this kit.

37| Transform the product into XL1-Blue chemically competent cells as in Step 7.

38| Perform colony PCR as in Steps 9–11, but use gene-specific primers.

39| Run the PCR products on a 2% (wt/vol) agarose gel and confirm the presence of the insert.

40| Culture the intended clones from the replica plate (from Step 38) with shaking overnight at 37 °C in 3 ml of LB medium containing 100 μg/ml ampicillin.

41| Purify the plasmid using an appropriate miniprep kit.

42| Sequence the plasmids with T7 and T3 primers and confirm the knock-in sequence.

Fluorescence observation ● TIMING 1 d

43| Collect an aliquot of cells (~5.0 × 10^4 cells) from Step 31 or 32, and transfer it to a collagen type I–coated EZView 24-well glass-bottom plate containing 1 ml of DMEM. Incubate the cells overnight in a CO_2 incubator.

44| Discard the medium and wash the cells twice with 1 ml of PBS. Fix the cells with 1 ml of 4% (wt/vol) paraformaldehyde in PBS and incubate them for 15 min at room temperature. Discard the paraformaldehyde/PBS and wash the cells three times with 1 ml of PBS. Add 1 ml of PBS and store the cells at 4 °C in the dark until fluorescence observation. Fluorescence observation should be performed on the same day or the next day.

45| Observe fluorescence and capture cell images with a 488-nm laser using a confocal laser-scanning microscope (Olympus FV-1000D).

▲ **CRITICAL STEP** The fluorescence intensity of the knocked-in gene encoding enhanced green fluorescent protein (*EGFP*) driven by an endogenous promoter is usually low compared with *EGFP* expressed under constitutive promoters such as cytomegalovirus (CMV) and CAG. Observe the fluorescence with a laser-scanning microscope preferably. When using a conventional fluorescence microscope, fluorescence observation and image capture should be conducted with higher exposure than usual.

Single-cell cloning ● TIMING 2–3 weeks

46| Collect an aliquot of cells from Steps 31 or 32, and count the cell number using a LUNA automated cell counter. Adjust to 1.5–2.0 × 10^3 cells/ml by adding DMEM.

▲ **CRITICAL STEP** When counting the cell number, low cell concentrations can give inaccurate measurements. Prepare highly concentrated cells (on the order of 10^6 or 10^7) and perform serial dilutions.

47| Mix 100 µl of the cells from Step 46 and 20 ml of DMEM, and transfer the mixture to a 50-ml tube (1.5–2 cells per 200 µl of DMEM).

48| Move 200 µl of the cells from Step 47 to each well of a 96-well plate. Incubate the plate overnight at 37 °C in a CO_2 incubator.

49| Check each well under a microscope to identify wells that contain only a single cell. Incubate the plate for ~7 d at 37 °C in a CO_2 incubator. When the cells become 80% confluent in a single well of the 96-well plate, transfer them to a 12-well plate. When the cells become 80% confluent in a single well of the 12-well plate, transfer them to a 100-mm dish.

? TROUBLESHOOTING

Genotyping clonal cells ● TIMING 1 d

50| Extract genomic DNA from the clonal cells using a DNeasy blood and tissue kit. As clonal cells are homogeneous, direct sequencing can be performed and bacterial cloning is not needed.

51| Perform genomic PCR as described in Steps 33 and 34.

52| Run the PCR products on a 2% (wt/vol) agarose gel. Excise the intended band and collect the gel fragments in Microtubes.

53| Purify the DNA from the gel fragments using a Wizard SV gel and PCR clean-up system according to the manufacturer's instructions.

54| Confirm the genotype by direct sequencing.

? TROUBLESHOOTING

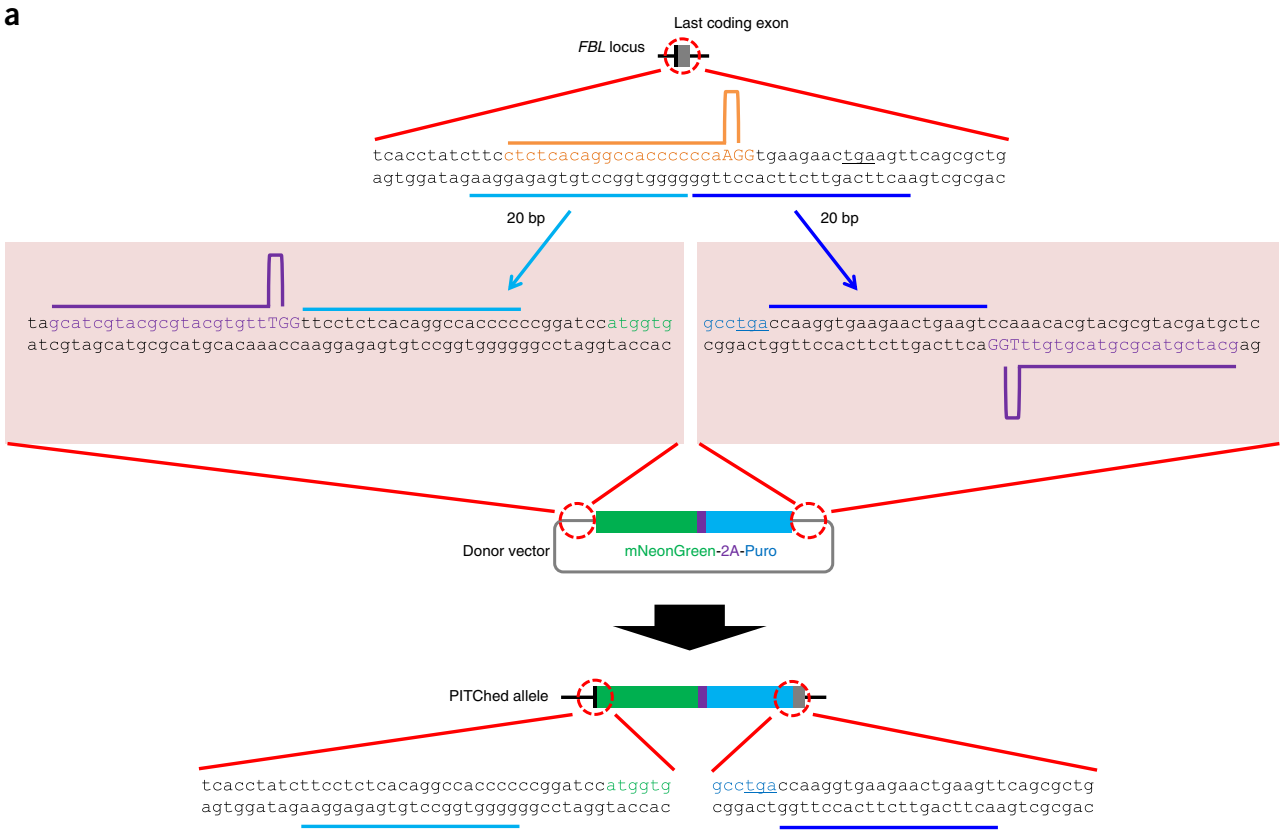
Troubleshooting advice can be found in **Table 2**.

TABLE 2 | Troubleshooting table.

Step	Problem	Possible reason	Possible solution
12	Unexpected band pattern	Failure of Golden Gate assembly or colony PCR	If Golden Gate assembly does not work, try simple digestion and ligation approaches. If colony PCR does not work, try AflIII/KpnI digestion for the insert check. An ~900-bp band will appear when two gRNA cassettes are correctly assembled
18	No amplification	Failed amplification because of long primers (>70 mer)	Try another high-fidelity DNA polymerase, such as KOD FX Neo. Alternatively, shorten the lengths of the primers (<70 mer) and divide the construction into two steps
25	Erroneous sequence	Improper PCR conditions	Decrease the cycle number of PCR amplification, and/or try another high-fidelity DNA polymerase
29	Low transfection efficiency	Improper transfection conditions	Increase the amount of plasmid DNA and/or lipofection reagent. Alternatively, try another transfection method (e.g., electroporation)
30	Puromycin selection does not work	Low concentration of puromycin and/or too-high cell confluency (>80%)	Raise the concentration of puromycin and/or perform cell passaging during selection to lower the confluency (>80%)
49	No proliferation from a single cell	Weak cell adhesion and/or malnutrition	Use collagen type I– or poly-L-lysine–coated 96-well plates, and/or use conditioned medium. Alternatively, use a cloning cylinder instead of a 96-well plate

● **TIMING**

Steps 1–14, construction of CRISPR-Cas9 vector for CRIS-PITCh (v2): 1 week
Steps 15–25, construction of CRIS-PITCh (v2) donor vector: 3 d
Steps 26–31, cell culture and transfection: 10 d
Steps 32–42, knock-in confirmation: 3 d
Steps 43–45, fluorescence observation: 1 d
Steps 46–49, single-cell cloning: 2–3 weeks
Steps 50–54, genotyping of clonal cells: 1 d
Box 1, application of TAL-PITCh in *Xenopus laevis*: 1 week (from injection to early genotyping)



b 5′ junction: 12/15 (precisely PITChed clones/total clones; 80%)

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TCATCACCTATCttcctctcacaggccaccccCCGGATCCATGG
TCATCACCTATCttcctctcacaggccaccccCCGGATCCATGG ×12
TCATCACCTATGttcctctcacaggccaccccCCGGATCCATGG
TCATCACCTATCttcctctcacaggccacccctcacaggccaccccCCGGATCCATGG +13
TCATCACTTATCttcctttcacaggccacccctgttttggttcctctcacaggccaccccCCGGATCCATGG +27
```

3′ junction: 7/14 (precisely PITChed clones/total clones; 50%)

```
CCCGGTGCCTGAccaaggtgaagaactgaagtTCAGCGCTGTCA
CCCGGTGCCTGAccaaggtgaagaactgaagtTCAGCGCTGTCA ×7
CC----------aaggtgaagaactgaagtTCAGCGCTGTCA -12
CCCGGTGCC--------gtgaagaactgaagtTCAGCGCTGTCA -8
CCCGGTCCCTGAccaaggtgaagaactgaagtTCAGCGCTGTCA
CCCGGTGCCTGCcaaggtgaagaactgaagtTCAGCGCTGTCA
CCCGGTGCCTGTccaaggtgaagaactgaagtTCAGCGCTGTCA
CCCGGTGCCAAccaaggtgaagaactgaagtTCAGCGCTGTCA
CCCGGTGCCTGAccaaggtgaagaagaactgaagtTCAGCGCTGTCA +3
```

c

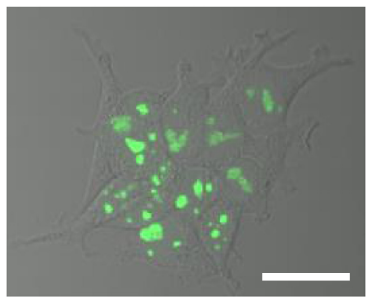

Figure 9 | CRIS-PITCh (v2)–mediated cassette knock-in in HEK293T cells. (**a**) A schematic illustration of CRIS-PITCh (v2)–mediated targeted integration at the human *FBL* locus. Orange letters indicate the locus-specific gRNA target site. Purple letters indicate the PITCh-gRNA target site. Light blue and blue bars indicate the microhomologies. The stop codon is underlined. (**b**) Sequences of the 5′ and 3′ junctions of knocked-in alleles. Bacterially cloned PCR products amplified from the genomes of puromycin-resistant cells before single-cell cloning were sequenced. The intended knocked-in sequence is shown at the top of each set of sequences, set off by a horizontal separator. Light blue and blue bars indicate the microhomologies. Red letters indicate precisely knocked-in alleles. Dashes indicate deletions. Blue letters indicate substitutions. Underlines indicate insertions. (**c**) A confocal laser-scanning microscopy image of cells after puromycin selection. Scale bar, 30 μm.

ANTICIPATED RESULTS

A detailed schematic design of CRIS-PITCh (v2)-mediated cassette integration at the *FBL* locus and the results of the sequencing analysis and fluorescence observation are shown in **Figure 9**. There are several factors that may affect the signal intensity of the fluorescent protein using the strategy illustrated in **Figure 9a**. First, the amount of fluorescent gene expression is determined by the expression level of the corresponding endogenous promoter. Second, post-transcriptional regulation mediated by an endogenous 3′ UTR can affect the expression level of the fluorescent protein, because the coding sequence alone is inserted just before the endogenous stop codon. We must also note that the openly available CRIS-PITCh (v2) vector, pCRIS-PITChv2-FBL, contains a conventional *EGFP* gene instead of the one encoding the ultra-bright mNeonGreen[46] used in **Figure 9**, although we have also confirmed the functionality of this pCRIS-PITChv2-FBL vector. Screening against random integrants is also important. If the targeted gene is expressed at low levels, the background frequency of random integrants will increase. It has been reported that by using programmable nucleases with high activity combined with efficient delivery methods, selection-free gene knock-in can be performed. For example, HR-mediated gene knock-in without drug selection has been achieved using the iCRISPR strategy in human embryonic stem cells[47]. However, we have only achieved gene knock-in with the use of puromycin selection, and it therefore remains to be confirmed whether the PITCh system can be applied without selection markers.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

ACKNOWLEDGMENTS The authors express their appreciation to A. Kawahara and Y. Hisano (University of Yamanashi, Yamanashi, Japan) for co-developing the modified PITCh system. We also thank H. Ochiai (Hiroshima University, Hiroshima, Japan) for sharing the synthesized mNeonGreen cDNA under the license agreement with Allele Biotechnology and Pharmaceuticals, Inc. This work was supported by the Japan Society for the Promotion of Science (25890014 to T.S., 25124708 to K.-I.T.S. and 26290070 to T.Y.), the Sasakawa Foundation (to S.N.), the Uehara Memorial Foundation (to T.S.) and the Ministry of Health, Labor, and Welfare of Japan (to T.Y.).

AUTHOR CONTRIBUTIONS T.S. organized and wrote the manuscript. S.N. performed the human cell experiments and wrote the manuscript concerning human cell procedures. Y.S. performed the frog experiments. K.-I.T.S. wrote the manuscript concerning frog procedures. T.Y. supervised the work.

COMPETING FINANCIAL INTERESTS The authors declare no competing financial interests.

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