One-step generation of conditional and reversible gene knockouts

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Loss-of-function studies are key for investigating gene function, and CRISPR technology has made genome editing widely accessible in model organisms and cells. However, conditional gene inactivation in diploid cells is still difficult to achieve. Here, we present CRISPR-FLIP, a strategy that provides an efficient, rapid and scalable method for biallelic conditional gene knockouts in diploid or aneuploid cells, such as pluripotent stem cells, 3D organoids and cell lines, by co-delivery of CRISPR-Cas9 and a universal conditional intronic cassette.

Analyzing gene function is a crucial step in advancing our understanding of normal physiology and disease pathogenesis. In cell-based models, loss-of-function studies require inactivation of both copies of the gene. Gene knockouts in cell lines have been achieved by loss-of-heterozygosity1 or serial gene targeting approaches3. The development of site-specific nucleases has greatly facilitated functional studies in cells because both copies of a gene can be efficiently inactivated in a single step3. Recently, the CRISPR-Cas9 gene editing technology4−7 has become the tool of choice for gene knockout studies owing to its simplicity and robustness. Cas9 nuclease is an RNA-guided nuclease that is highly efficient in inducing a double-strand break (DSB) at a genomic site of interest. These DSBs can be repaired by error-prone non-homologous end joining (NHEJ) to generate gene-inactivating mutations; or, in the presence of a donor template, the DSBs can be repaired by homology-directed repair (HDR) to generate more precise and complex alleles8. While simple constitutive knockouts are useful and informative, it is desirable to engineer conditional loss-of-function models, particularly for genes essential for cell viability or embryonic development. Here, we describe a simplified, one-step method for engineering conditional loss-of-function mutations in diploid cells.

Existing methods for engineering conditional mutations in cultured cells9−12 rely on the inclusion of a drug selection cassette that must be removed in a second step to ensure proper expression of the targeted conditional allele (Supplementary Fig. 1a,b). These methods were not designed for the generation of conditional loss-of-function models in a single step, particularly where the target gene is essential for cell growth or viability. To overcome these limitations, our strategy combines an invertible intronic cassette (FLIP), which is similar to COIN12, with high efficiency Cas9-assisted gene editing. Critically, the nonmutagenic orientation of the FLIP cassette expresses a puromycin resistance gene (puro8), allowing selection of correct nuclease-assisted targeting into the exon of one allele and simultaneous enrichment of cells that inactivate the second allele by nuclease-mediated NHEJ (Fig. 1a). Upon exposure to Cre recombinase, the FLIP cassette is inverted to a mutagenic configuration that activates a cryptic splice acceptor and polyadenylation signal (pA) and disrupts the initial splicing acceptor, resulting in the complete loss of gene function (Fig. 1b and Supplementary Fig. 2a). In contrast to COIN, which requires the removal of the drug selection cassette, our FLIP cassette permits the generation of conditional mutant cells in one step.

Initially we inserted a variant of the FLIP cassette containing a DsRed2 reporter in place of puro8 into a cytomegalovirus promoter−enhanced green fluorescent protein (CMV-eGFP) expression plasmid (CMV-eGFP[FLIP], Fig. 1c). Following transient transfection of HEK293 cells with CMV-eGFP[FLIP], both green and red fluorescence were observed, demonstrating that the FLIP cassette inserted in the non-mutagenic orientation is inert (Fig. 1d). This was further confirmed by flow cytometry analysis, showing a similar level of eGFP expression from both CMV-eGFP and CMF-eGFP[FLIP] (Supplementary Fig. 3). The Cre-recombined CMV-eGFP[FLIP] showed loss of eGFP expression, suggesting the inactivation of eGFP expression in the inverted, mutagenic orientation of the FLIP cassette (Fig. 1c,d).

Next, we employed CRISPR-Cas9 in mouse embryonic stem cells (mESCs) to introduce the puro8 FLIP cassette into one allele of β-catenin (Ctnnb1) via HDR and to simultaneously induce a frameshift mutation by NHEJ in the second Ctnnb1 allele (Fig. 1a, Supplementary Fig. 4a). β-catenin is important for the morphology and efficient self-renewal of mESCs13,14. A donor vector containing the puro8 FLIP cassette flanked by ~1-kb homology arms was inserted in exon 5 of Ctnnb1 by co-transfection of mESCs with Cas9 and gRNA expression plasmids. Following selection in puromycin, drug-resistant colonies were genotyped by PCR to confirm correct integration of the FLIP cassette and then assayed for NHEJ events in the second allele by Sanger sequencing (Supplementary

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Figure 1 | FLIP cassette strategy for bi-allelic conditional gene modification. (a) Schematic drawing of the FLIP cassette strategy for bi-allelic conditional gene modification. (b) The design of the FLIP cassette. HAL, homologous arm left; HAR, homologous arm right; PGK, phosphoglycerate kinase promoter. SD, splice donor; SA1, SA2, splice acceptor; purple triangles, LoxP sites; pink triangles, Lox5171 sites; BP1, BP2 (blue circles), branching point; pA, polyadenylation signal. (c) Schematic of the FLIP cassette containing a DsRed reporter gene. (d) Images of HEK 293 cells transfected with the FLIP cassette. Both eGFP and DsRed proteins are expressed (top row). After Cre recombination, the eGFP expression is disrupted, and only DsRed expression is maintained (bottom row). Scale bars, 400 µm.

Figs. 2b and 4b,c). From 64 clones, 14 (21.9%) were correctly targeted, among which 4 carried a frameshift mutation in the second allele (Supplementary Table 1). The recovery of Ctnnb1 compound mutant clones (FLIP targeted/NHEJ frameshift; FLIP/−) with wild-type morphology strongly suggests that the insertion of the FLIP cassette does not disrupt the function of β-catenin in the nonmutagenic orientation. Upon expression of Cre recombinase in Ctnnb1FLIP− clones, we observed a loss of β-catenin expression in cells (Fig. 2a, Supplementary Fig. 4d). Moreover, compared to control (Ctnnb1FLIP+) cells treated with Cre recombinase, the Ctnnb1FLIP− cells became scattered and lost their dome-like morphology (Fig. 2b). In addition, we performed quantitative RT-PCR analysis to determine the splicing efficiency of the FLIP intron in comparison to the neighboring intron 7 of β-catenin. Our data demonstrate highly efficient splicing of the FLIP intron. Thus the FLIP cassette is inert to gene activity in the nonmutagenic orientation (Supplementary Fig. 5 and Supplementary Table 2).

We additionally targeted Apc, Esrrb, Nfx1, Sox2, Tcf7l2, Trim13 and Trim37 in mESCs; ARID1A and TP53 in human HEK293 cells; and TP53 in human induced pluripotent stem cells (Supplementary Figs. 6–9). The FLIP intron targeting efficiency ranged from 19.8% to 40.6% in mESCs (Supplementary Table 1; note that nontargeted clones are a result of random integration of the puro cassette). Importantly, for all genes, FLIP/− clones were obtained (Supplementary Table 1, Supplementary Figs. 6–9). To induce gene knockout, a Cre-expressing plasmid was transfected to embryonic stem cell (ESC) clones with an average transfection efficiency >95% (Supplementary Fig. 10) and conditional inactivation of gene expression was confirmed by protein blot and immunofluorescence for Esrrb, Sox2, Trim13 and Trim37 (Supplementary Figs. 6d,h,i and 7m,q).

We further modified our FLIP intronic cassette to generate a reversible conditional allele. The region containing the cryptic splice acceptor and pA is flanked by two flipase recognition target (FRT) sites (Supplementary Fig. 11a, FLIP-Flp Excision (FLIP-FlpE)). When inserted into eGFP, the intronic FLIP-FlpE cassette permits the expression of eGFP, like the original FLIP cassette (Supplementary Figs. 3 and 11b). Upon Cre recombination, the FLIP-FlpE cassette turns into the mutagenic orientation, which blocks the eGFP expression. Next, the added FRT sites enable the mutagenic FLIP-FlpE cassette to be excised by Flp recombinase, thus allowing the revival of eGFP expression (Supplementary Fig. 11a,b). The FLIP-FlpE cassette was inserted in exon 5 of the mouse β-catenin allele. The Ctnnb1FLIP−FLIP− (FLIP-FlpE homozygote) mutant clone went through a series of recombinations, first by Cre and then by Flp. At each step, the mutant showed wild-type, mutant (after Cre) and again wild-type (after Cre and Flp) morphology, respectively (Supplementary Fig. 11e). Accordingly, we observed loss and gain of β-catenin expression (Supplementary Fig. 11f,g), suggesting that, with a simple modification, the FLIP intronic cassette can also be used for ‘switchable’ gene expression.

To extend our application, we inserted the FLIP-FlpE cassette into exon 16 of the mouse Apc allele in intestinal organoids expressing CreERT2 under the villin promoter (Supplementary Fig. 12a). Apc is a component of the destruction complex acting in the Wnt pathway, and its deletion causes hyperactive Wnt signaling and makes organoids adopt a cystic morphology. ApcFLIP−FlpE− clones (Supplementary Fig. 12b,c) initially showed budding morphology when cultured in standard ENR (Egf, Noggin, Rspordin) medium. Upon treatment with 4-hydroxytamoxifen (4-OHT) for Cre activation, the organoids adopt a cystic morphology due to the loss of Apc (Supplementary Fig. 12d). In addition to the application of CRISPR–FLIP to intestinal organoids, FLIP-targeted ESC clones can be used to generate other cell types—for example, mouse embryonic fibroblast (MEF) (Supplementary Fig. 13).
assembly of targeting vectors easy and scalable. The FLIP cassette is invariant and can be generically applied to any gene, including noncoding RNA genes. The CRISPR -FLIP technology is widely applicable to many diploid and aneuploid cell types, including mESCs, fibroblasts, 3D organoids, human induced pluripotent stem cells (hiPSCs) and cell lines (such as 293 cells).

METHODS
Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

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

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AUTHOR CONTRIBUTIONS

A.A.R., W.C.S. and B.-K.K. wrote the manuscript. A.A.-R., J.F., W.C.S. and B.-K.K. designed the FLIP cassette targeting vector. A.A.-R., W.C.S. and B.-K.K. designed and discussed the experiments. A.A.-R., R.C.M. and J.K. targeted mESCs and performed WB. A.A.-R. performed immunofluorescence. K.A. and A.M. targeted hiPSCs. A.M. targeted HEK 293 cells. A.A.-R. and A.M. performed FACS. A.A.-R. performed immunofluorescence. K.A. and A.M. targeted mESCs and discussed the experiments. A.A.-R., R.C.M. and J.K. targeted mESCs and performed WB. A.A.-R. performed immunofluorescence. K.A. and A.M. targeted hiPSCs. A.M. targeted HEK 293 cells. A.A.-R. and A.M. performed FACS. A.A.-R. performed the organoid experiments. S.F. and T.G. performed the bioinformatics analysis. K.T. derived Sox2hiPSCMEFs. J.C.R.S. supervised K.T. W.C.S. and B.-K.K. supervised the project.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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1. Mortensen, R.M., Conner, D.A., Chao, S., Geisterfer-Lowrance, A.A. & Seidman, J.G. Mol. Cell. Biol. 12, 2391–2395 (1992).
2. te Riele, H., Maandag, E.R., Clarke, A., Hooper, M. & Berns, A. Nature 348, 649–651 (1990).
3. Urnov, F.D. et al. Nature 435, 646–651 (2005).
4. Cong, L. et al. Science 339, 819–823 (2013).
5. Mali, P. et al. Science 339, 823–826 (2013).
6. Cho, S.W., Kim, S., Kim, J.M. & Kim, J.-S. Nat. Biotechnol. 31, 230–232 (2013).
7. Jinek, M. et al. Science 337, 816–821 (2012).
8. Sander, J.D. & Joung, J.K. Nat. Biotechnol. 32, 347–355 (2014).
9. Gu, H., March, J.D., Orban, P.C., Mossmann, H. & Rajewsky, K. Science 265, 103–106 (1994).
10. Abuin, A. & Bradley, A. Mol. Cell. Biol. 16, 1851–1856 (1996).
11. Tate, P.H. & Skarnes, W.C. Methods 53, 331–338 (2011).
12. Economides, A.N. et al. Proc. Natl. Acad. Sci. USA 110, E3179–E3188 (2013).
13. Anton, R., Kestler, H.A. & Küh, M. FEBS Lett. 581, 5247–5254 (2007).
14. Lyashenko, N. et al. Nat. Cell Biol. 13, 753–761 (2011).
15. Sato, T. et al. Gastroenterology 141, 1762–1772 (2011).
16. Dominski, Z. & Kole, R. Mol. Cell. Biol. 11, 6075–6083 (1991).
17. Burset, M., Seledtsov, I.A. & Solovyev, V.V. Nucleic Acids Res. 28, 4364–4375 (2000).
18. Chu, V.T. et al. Nat. Biotechnol. 33, 543–548 (2015).
ONLINE METHODS

DsRed FLIP cassette inserted in the eGFP cDNA. The FLIP cassette inserted in the middle of eGFP and containing a DsRed2 reporter gene was synthesized and ordered from GenScript. The split eGFP cDNA and the FLIP cassette were cloned into the mammalian expression vector pCDNA4TO (Invitrogen) using BamHI (R0136S, NEB) and XhoI (R0146S, NEB) for pre-recombined form. The vector was subsequently transformed into Cre-expressing bacteria (A111, Gene bridges) to generate the Cre-recombined form. Correct clones were confirmed with restriction digest BamHII (R0136S, NEB) and XhoI (R0146S, NEB) and Sanger sequencing. The FLIP-FlpE cassette was also synthesized and inserted into the same site of the eGFP expression vector.

FLIP cassette containing selection marker genes. The FLIP cassette was PCR amplified and cloned into Pjet1.2 vector (ThermoFisher Scientific, K131). Replacement of DsRed was done through restriction digest excision using EcoRI (R3101S, NEB) and Acc65I (R0599S, NEB), followed by insertion of PCR-amplified selection marker genes using Infusion cloning (639099, Clontech). The FLIP cassette including the selection marker gene was then transferred to the vector pUC118 (3318, Clontech) using the restriction enzymes ScaI (R0156S, NEB) and PstI (R0140S, NEB) and Mighty cloning (6027, Takara).

Addition of homologous arms to the FLIP cassette—FLIP-targeting vector generation. Homologous arms around an intron insertion site were amplified by high fidelity Phusion DNA polymerase (M0530S, NEB). After PCR product purification, both homologous arms and FLIP cassette-containing vector were mixed with the type II restriction enzyme SapI and T4 DNA ligase (M0202T, NEB). Then, we performed 25 cycles of the following two-step program: 37 °C for 2 min and 16 °C for 5 min. The reaction mixture was directly used for *Escherichia coli* transformation. DNA was extracted (27106, Qiagen) and analyzed with restriction digest to identify FLIP donor vectors that were correctly assembled.

Cas9 and gRNA plasmids. Cas9 (41815, Addgene) optimized for human codon and empty gRNA vector (41824, Addgene) were obtained from Addgene.

Cell culture. Human embryonic kidney (HEK) 293 cells. Human embryonic kidney 293 cells were cultured in media consisting of DMEM, high glucose (11965092, ThermoFisher Scientific) supplemented with 10% fetal bovine serum (ThermoFisher Scientific), 1x penicillin-streptomycin according to the manufacturer’s recommendation (P0781, Sigma). The cells were tested negative for mycoplasma.

Embryonic stem cells (ESCs). Murine E14 Tg2a embryonic stem (mES) cells were cultured feeder-free on 0.1% gelatin-coated dishes in serum + LIF + 2i (Chiron and PD03) composed of GMEM (G5154, Sigma), 10% fetal bovine serum (Gibco), 1x non-essential amino acids according to the manufacturer’s recommendation (11140, ThermoFisher Scientific), 1 mM sodium pyruvate (113-24-6, Sigma), 2 mM L-glutamine (25030081, ThermoFisher Scientific), 1x penicillin-streptomycin according to the manufacturer’s recommendation (P0781, Sigma) and 0.1 mM 2-mercaptoethanol (M7522, Sigma), 20 ng/ml murine LIF (Hyvonen lab, Cambridge), 3 μM CHIR99021 and 1 μM PD0325901 (Stewart lab, Dresden). BOBSC human induced pluripotent stem (hiPS) cells were cultured feeder-free on dishes coated with Synthema II (3535, Corning) in TeSR-E8 media (05940, Stem Cell Technologies). ESCs were kept in a tissue culture incubator at 37 °C and 5% CO2. Cells were split in a 1:10 – 1:15 ratio every 3–4 d depending on confluence. All cells were tested negative for mycoplasma.

Intestinal organoid culture. Mouse small intestinal organoids were cultured as previously described.

Cell electroporation. For targeting of mESCs, 1 × 10⁶ cells were collected and resuspended in magnesium- and calcium-free phosphate-buffered saline (D8537, Sigma). A total of 50 μg of DNA consisting of the targeting vector, Cas9 and gRNA in a 1:1:1 ratio were added to the cells and then transferred to a 4 mm electroporation cuvette (Bio-Rad). Electroporation was performed using the Bio-Rad Gene Pulser XCell’s (165-2660, Bio-Rad) exponential program and the following settings: 240 V, 500 μF, unlimited resistance. For targeting of human IPS cells, 2 × 10⁶ cells were dissociated with Accutase (SCR005, Millipore) and resuspended in nucleofection buffer (Solution 2, LONZA). A total of 12 μg of DNA, consisting of 4 μg Cas9 plasmid, 4 μg of each gRNA plasmid and 4 μg of targeting vector, was added to the cells and transferred to a 100 μl nucleofection cuvette (LONZA). Nucleofection was performed with the AMAXA Human Nuclease Factor Kit 2 (LONZA Cat # VPH-5022) using the B-016 program. The cells were plated and cultured for 1 d in TeSR-E8 media (05940, STEM CELL technologies) containing ROCK inhibitor (Y-27632, Stem Cell Technologies) to promote survival of transfected cells. For targeting of HEK293 cells, the cells were cultured until they reached 50–60% confluence. A total of 8 μg of DNA, consisting of targeting vector, Cas9 and gRNA in a 1:1:1 ratio, was transfected using Lipofectamine-2000 (11668019, Invitrogen) according to the manufacturer’s instructions.

Plasmid transfection. 1 μg of pCAGGS-Cre-IRES-Puro and/or pCAGGS-Flip-IRES-Puro plasmid vector and 3 μl of Lipofectamine-2000 (11668019, Invitrogen) were mixed according to the manufacturer’s protocol, applied to 2 × 10⁵ recently seeded (less than 30 min) cells/6-well and incubated overnight. Media was refreshed the following morning. Ctnmb1FLIP+ and Ctnmb1FLIP+CreERT2 clones were generated by transfecting 0.66 μg of PiggyBac CreERT2-expressing plasmid (with hygromycin 50 μg/ml) together with 0.33 μg of transposase using Lipofectamine-2000 as described above. Cre-recombinase was activated by adding 4-OHT with a final concentration of 1 μM for 48 h.

Protein blot. Following transfection, ESCs were cultured for 2–5 d and then lysed in buffer containing complete protease-inhibitor cocktail tablets (11697498001, Roche) and centrifuged at 13,000 rpm for 15 min at 4 °C. Protein concentration was measured with Bradford assay (5000204, Bio-Rad) and equal amounts were loaded on a 10% acrylamide gel and run at 120 V for 1.5–2 h. The proteins were subsequently transferred to an Immobilon-FL PVDF 0.45 μm membrane (IPFL0010, Millipore) at 90 V for 1 h, 15 min. The following primary antibodies and dilutions were used to detect the indicated proteins: Rabbit monoclonal antibody against β-catenin (1:1000, 8480S, Cell Signaling), mouse monoclonal against alpha-Tubulin antibody (1:5000, ab7291, Abcam),...
mouse monoclonal antibody against beta-actin (1:5000, ab8226, Abcam), mouse monoclonal antibody against Esrrb, (1:1000, PP-H6705-00, Bio-Techne), rat monoclonal antibody against Sox2, (1:500, 14-9811-80, eBioscience), mouse monoclonal antibody against Trim13 (1:500, sc-398129, Santa Cruz), mouse monoclonal antibody against Trim37 (1:500, sc-514828, Santa Cruz) and rabbit monoclonal against Vinculin (1:3000, ab19002, Abcam). The membrane was washed and the indicated secondary antibodies conjugated to horseradish peroxidase were applied: horse anti-mouse IgG (1:5000, Cell Signaling), goat anti-rabbit (1:5000, Cell Signaling) and goat anti-rat HRP conjugated (1:5000, SC2032, Santa Cruz). Detection was achieved using ECL prime western blotting detection system (RPN2133, GE Healthcare) (Supplementary Fig. 14).

**Immunofluorescence.** Cells were cultured in Ibid tissue culture dishes (IB-81156, Ibid) coated with 0.1% gelatin, washed twice with calcium and magnesium free PBS and fixed in 4% PFA for 20 min at room temperature (RT). The cells were permeabilized in 0.5% Triton X-100 (T8787, Sigma) in PBS for 15 min at RT. Subsequently, blocking was performed in 5% donkey serum (D9663, Sigma) and 0.1% Triton X-100 for 1 h at RT. The following primary antibodies in blocking buffer were applied for the indicated protein: Sox2 (1:500, 14-9811-80, eBioscience) and β-Catenin (1:1000, 4627, Cell Signaling). Primary antibodies were incubated overnight at 4 °C. Subsequently excess primary antibody was washed away and anti-rat Alexa Flour 594®-conjugated antibody (1:1000, A21209, Abcam) was added for Sox2 and incubated for 1 h at RT. Excess secondary antibody was washed away and DAPI (1:1000, D9542, Sigma) was added and incubated for 10 min at RT. Cells were washed and mounted in RapiClear (RCCS002, Sunjin lab).

**Chimeric embryo generation and ESC-derived fibroblast establishment.** Sox2FLIP/FLIP mESCs transfected with pPyCAG-eGFP-IRES-Zeo plasmid were aggregated. Chimeras were generated by standard aggregation using F1 embryos and transplanted into pseudopregnant recipient mice of C57BL/6J strain. E13.5 embryos were beheaded and dissected to remove all organs, including genital ridges. The remaining body was cut into small pieces, trypsinised and plated on gelatin in serum + LIF media containing selecting reagents. GFP expression confirms that the MEFs are derived from the Sox2FLIP/FLIP mESCs. All animal work was performed in accordance with Home Office guidelines and regulations at the University of Cambridge, UK.

**Quantitative RT-PCR.** Total RNA was extracted using RNwasy Mini kit (74104, Qiagen) with an on-column DNase digestion (79254, Qiagen). Reverse transcription was performed using 250 ng of RNA using M-MLV Reverse Transcriptase (M1701, Promega). Quantitative PCR reactions were performed in triplicates using iQ SYBR Green Supermix according to the manufacturer’s protocol (1708882, Bio-Rad), with the primers in Supplementary Table 2 and the StepOnePlus Real Time PCR System (Applied Biosystems). Average gene expression was normalized to exon 5 and error bars represent ± s.d.

**Flow cytometry analysis.** HEK 293 cells were cotransfected with eGFP, eGFP[FLIP] or eGFP[FLIPFlpE] and a BFP reporter as described previously, and harvested 24 h post transfection. mESCs were transfected with eGFP as describes above and harvested 48 h post transfection. Cells were analyzed using BD LSRFortessa (BD Biosciences) and Flowjo software.

**Data availability.** Mammalian expression plasmids are available at Addgene. pUC118-FLIP-Puro (#84538 for generation of conditional knockouts), pUC118-FLIP-FlpE-Puro (#84539 for generation of reversible conditional knockouts), pUC118-mCtnnb1-FLIP-Puro (#84540, FLIP vector for conditional knockout of Ctnnb1), pUC118-mCtnnb1-FLIP-FlpE-Puro (#84541, FLIP-FlpE vector for reversible conditional knockout of Ctnnb1) and gRNA-mCtnnb1 (#84542).

19. Yusa, K. et al. Nature **478**, 391–394 (2011).
20. Sato, T. et al. Nature **459**, 262–265 (2009).