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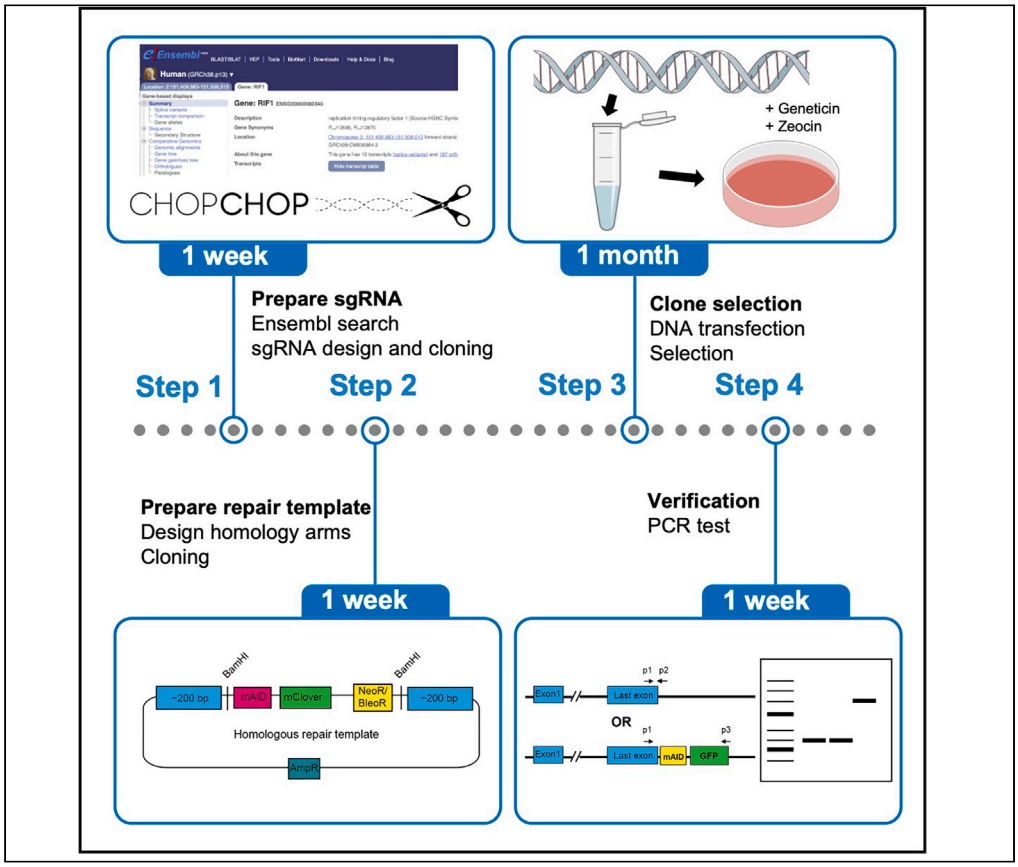
Protocol for biallelic tagging of an endogenous gene using CRISPR-Cas9 in human cells



We present here a protocol for biallelic tagging of an endogenous gene in human cells using CRISPR-Cas9 editing technology. Using RIF1 as an example, we describe tagging the gene with a mini-auxin-inducible degron and a green fluorescent protein at its C terminus. We detail steps for preparing and designing the sgRNA and homologous repair template, and clone selection and verification.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

C-terminal tagging of gene in human tissue culture cells using CRISPR-Cas9

Quick and efficient biallelic tagging on both alleles using two antibiotics

Simple cloning strategy for various tags and antibiotic-resistant genes

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Protocol for biallelic tagging of an endogenous gene using CRISPR-Cas9 in human cells

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SUMMARY

We present here a protocol for biallelic tagging of an endogenous gene in human cells using CRISPR-Cas9 editing technology. Using RIF1 as an example, we describe tagging the gene with a mini-auxin-inducible degron and a green fluorescent protein at its C terminus. We detail steps for preparing and designing the sgRNA and homologous repair template, and clone selection and verification.

For complete details on the use and execution of this protocol, please refer to Kong et al.[1]

BEFORE YOU BEGIN

The protocol below describes the steps to design the sgRNA targeting the region near the stop codon of *RIF1*, and to prepare the homologous repair templates with two different antibiotic selection markers to tag *RIF1* with a mini-auxin-inducible degron (mAID) and a green fluorescent protein (GFP) at its C-terminus. The required vectors can be purchased from Addgene. The sgRNA vector pX330-U6-Chimeric_BB-CBh-hSpCas9 is Plasmid #42230. Please refer to Ran et al. (2013)[2] for the information about this vector. The vector for C-terminal tagging with neomycin-resistant gene, pMK289 (mAID-mClover-NeoR) is Plasmid #72827. To generate the vector with the second antibiotic resistance gene, the NeoR gene is replaced with zeocin-resistant gene (pMK289-BleoR). Please refer to Natsume et al. (2016)[3] for the details about the tagging plasmids. Other C-terminal tagging plasmids with different tags and selection markers are also available in Addgene: https://www.addgene.org/browse/article/21321/.

To generate an inducible degradation cell line, your cells should express OsTIR1 that mediates the ubiquitination of mAID-tagged protein upon the addition of auxin. OsTIR1-expressing cells can be generated by transfecting your cells with pMK232 (CMV-OsTIR1-PURO, Plasmid #72834) or pMK243 (Tet-OsTIR1-PURO, Plasmid #72835), together with AAVS1 T2 CRIPR in pX330 (Plasmid #72833). Please refer to Natsume et al. (2016)[3] for the protocol of generating OsTIR1-expressing cells.

The protocol below describes the specific steps for using HCT116 cells, a human colorectal carcinoma cell line. However, we have also used this protocol in hTERT-RPE1 and DLD-1 cells. All these cell lines are diploid/near diploid. To achieve biallelic tagging, you are strongly recommended to use diploid or near-diploid cell lines.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit polyclonal anti-GFP (1:5000 dilution)	Abcam	Cat# ab290; RRID: AB_303395
Rabbit polyclonal anti-OsTIR1 (1:1000 dilution)	MBL	Cat# PD048; RRID: AB_2909494
Mouse monoclonal anti-α-Tubulin (1: 5000 dilution)	Sigma-Aldrich	Cat# 00020911; RRID: AB_10013740
Alexa Fluor 488 F(ab')2-goat anti-rabbit IgG (1:2000 dilution)	Thermo Fisher	Cat# A-11070; RRID: AB_2534114
Goat anti-rabbit IgG-HRP conjugate (1:2000 dilution)	Bio-Rad	Cat# 1706515; RRID: AB_11125142
Goat anti-mouse IgG-HRP conjugate (1:2000 dilution)	Bio-Rad	Cat# 1706516; RRID: AB_11125547
Chemicals, peptides, and recombinant proteins		
DOX: Doxycycline hyclate	Sigma-Aldrich	Cat# D9891
IAA: 3-Indoleacetic acid	Sigma-Aldrich	Cat# I2886
Lipofectamine 2000	Thermo Fisher	Cat# 11668-019
Zeocin	Thermo Fisher	Cat# R25001
Puromycin	Thermo Fisher	Cat# A11138-02
Geneticin	Thermo Fisher	Cat# 10131035
DMEM, high glucose	Thermo Fisher	Cat# 11965092
Fetal bovine serum	Thermo Fisher	Cat# 16000044
Penicillin-streptomycin (10,000 U/mL)	Thermo Fisher	Cat# 15140122
Trypsin-EDTA (0.25%), phenol red	Thermo Fisher	Cat# 25200072
Critical commercial assays		
NucleoSpin Plasmid Transfection-grade, Mini kit	Macherey-Nagel	Cat# 740490.250
NucleoBond Xtra Midi kit, transfection-grade	Macherey-Nagel	Cat# 740410.50
GeneJET Gel Extraction Kit	Thermo Fisher	Cat# K0691
DNA Ligation Kit, Mighty Mix	TaKaRa	Cat# 6023
DNeasy Blood & Tissue Kits	Qiagen	Cat# 69504
Q5 High-Fidelity 2× Master Mix	New England Biolabs	Cat# M0492L
FastDigest BpiI	Thermo Fisher	Cat# FD1014
T7 DNA ligase	New England Biolabs	Cat# M0318S
BamHI-HF	New England Biolabs	Cat# 3136S
ScaI-HF	New England Biolabs	Cat# R3122S
Quick CIP	New England Biolabs	Cat# M0525S
MAX Efficiency™ DH5α Competent Cells	Thermo Fisher	Cat #18258012
GeneJET Gel Extraction Kit	Thermo Fisher	Cat# K0691
Plasmid Plus Midi Sample Kit	Qiagen	Cat# 12941
QIAprep Spin Miniprep Kit	Qiagen	Cat# 27104
Experimental models: Cell lines		
HCT116	ATCC	Cat# CCL-247
HCT116: OsTIR1	Kong et al.[1]	N/A
HCT116: OsTIR1 RIF1-mAID-GFP	Kong et al.[1]	N/A
Oligonucleotides		
sgRNA targeting RIF1 last exon Forward: CACCGATACTAAATAGAATTTTCAT	IDT	N/A
sgRNA targeting RIF1 last exon Reverse: AAACATGAAAATTCTATTTAGTATC	IDT	N/A
U6 sequencing primer Forward: GAGGGCCTATTTCCCATGATTCC	IDT	N/A
P1 primer for PCR test: GTTGCTTTAGAAATTCCATTATC	IDT	N/A
P2 primer for PCR test: CCTTCAATTTTCTCTGAAAATAC	IDT	N/A
P3 primer for PCR test: TATGGCTGATTATGATCAGT	IDT	N/A
Recombinant DNA		
Plasmid: pX330-U6-Chimeric_BB-CBh-hSpCas9	Addgene	Cat# 42230
Plasmid: AAVS1 T2 CRIPR in pX330	Addgene	Cat# 72833
Plasmid: pMK289 (mAID-mClover-NeoR)	Addgene	Cat# 72827
Plasmid: pMK289-BleoR	Kong et al.[1]	N/A
Plasmid: px330 sgRIF1 C-terminus	Kong et al.[1]	N/A

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Plasmid: pUCIDT RIF1-mAID-Clover (Zeo)	Kong et al.[1]	N/A
Plasmid: pUCIDT-RIF1-mAID-Clover (Neo)	Kong et al.[1]	N/A
Plasmid: pMK243 (Tet-OsTIR1-PURO)	Addgene	Cat# 72835
Other		
Mr. Frosty™ Freezing Container	Thermo Fisher	Cat# 5100-0001

MATERIALS AND EQUIPMENT

Cell culture medium for HCT116 cells		
Reagent	Final concentration	Amount
Dulbecco's Modified Eagle Medium (DMEM)	N/A	445 mL
Fetal bovine serum	10%	50 mL
Penicillin-Streptomycin (10,000 U/mL)	100 U/mL	5 mL
Total	N/A	500 mL

Note: DMEM medium should be stored at 2°C–8°C. Fetal bovine serum should be stored at ≤ −10°C. Penicillin-Streptomycin should be stored at −5°C to −20°C. The shelf life of the reagents for the cell culture medium is 12 months from the date of manufacture. For culturing HCT116-OsTIR1 cells, the medium is supplemented with 1 μg/mL of puromycin.

STEP-BY-STEP METHOD DETAILS

Design of sgRNA and preparation of sgRNA expression vector

Timing: ~1 week

Design sgRNA sequence and clone it into pX330 vector. For general information of pX330 vector and sgRNA design, please refer to Ran et al. (2013).[2] Here, we specifically explain how to decide the sgRNA for C-terminal tagging and to clone it into the pX330 vector.

1. To obtain the sequence information of *RIF1*, go to Ensembl genome browser (https://www.ensembl.org).
   a. Search for your gene of interest (in this example: *RIF1*). Choose "Human" in "Species". Click "Go" then select "RIF1 (Human Gene)".
   b. Choose the Gold-colored transcript that is annotated as Ensembl canonical transcript.

   Note: The table shows different transcripts of RIF1.

      i. Click the Ensembl link in the "Protein" column, i.e., "2472aa".

   Note: The transcript information will then be displayed on the top.

      ii. Click the Ensembl link in "About this transcript" stating the number of exons, i.e., "36 exons" (Figure 1A).
   c. The "Exons" table is below the transcript table. Sequences of exons and introns of *RIF1* gene are shown. The nucleotides are heavily annotated. You can remove the annotations by clicking "Configurate this page" on the left (Figure 1B).
   d. In "Configure Page", click "show full intronic sequence" to get the full sequence information of all introns. Select "No" in "Show variants" to remove annotations of the nucleotides (Figure 1B).

Figure 1. Obtain sequence information from Ensembl genome browser

(A) Choose the correct transcript (arrow 1) and then click "exons" (arrow 2).

(B) To configure the display, click "Configure this page" (arrow 3). Show full intronic sequences (arrow 4) and remove all annotations (arrow 5).

(C) Identify the stop codon in the last exon (arrow 6).

2. Go to the last exon of *RIF1* and identify the stop codon (Figure 1C).

   Note: The 5′/3′ upstream/downstream sequences are in green. The untranslated regions in exons are in red. The coding sequences are in blue and capital letters. The intron sequences are in grey and small letters.

3. Copy ~100 bp sequence with the stop codon in the middle and search for sgRNA sequence using online sgRNA design website, e.g., CHOPCHOP v3[4]: http://chopchop.cbu.uib.no/ (trouble-shooting 1).

Note: If there are multiple sgRNAs available in the 100 bp region, you can choose the one with higher ranking, which is defined primarily by the number of off-targets and efficiency. In addition, you can choose the one with target cleavage site closer to the stop codon, as the recombination efficiency will be higher if the induced DNA break is closer to the stop codon.

4. Identify the sgRNA target site for *RIF1* C-terminal tagging: **CCC**ATGAAAATTCTATTTAGTAT.

Note: sgRNA will target the complementary strand, **CCC** is the PAM site. TAG is the stop codon. Therefore, the sgRNA sense sequence is 5′-ATACTAAATAGAATTTTCAT-3′.

5. Order a pair of DNA oligos in a commercial company (e.g., Integrated DNA Technologies), and add overhangs that are required for cloning into pX330:

5′-CACCGATACTAAATAGAATTTTCAT-3′ (sense)

5′-AAACATGAAAATTCTATTTAGTATC-3′ (antisense)

6. Resuspend the DNA oligos.
   a. Spin down the oligo tubes in a benchtop microcentrifuge with maximum speed (~16,000 *g*) for ~ 1 min.
   b. Pipette in the volume of 1× TE buffer (10 mM Tris-HCl, pH 8.0 and 1 mM EDTA) required for 100 μM.
7. Anneal the oligos by performing the following reaction:

Reagent	Final concentration	Amount
sgRNA oligo (sense)	10 μM	1 μL
sgRNA oligo (antisense)	10 μM	1 μL
10 × annealing buffer (500 mM NaCl, 500 mM HEPES pH 7.4)	1×	1 μL
ddH_2O	N/A	7 μL
Total	**N/A**	**10 μL**

Boil the mixture at 95°C for 5 min, then cool down the mixture stepwise: 70°C for 10 min, 50°C for 10 min, 37°C for 10 min, and finally 25°C for 10 min.

Note: You can do the cooling step in a PCR machine.

8. Dilute the annealed oligos in ddH_2O at 1:200, then clone it into pX330 vector by the following reaction:

Reagent	Final concentration	Amount
pX330	50 ng	1 μL
Annealed oligos (diluted)	N/A	1 μL
10 × Tango buffer	1×	1 μL
DTT (10 mM)	0.5 mM	0.5 μL
ATP (10 mM)	0.5 mM	0.5 μL
FastDigest BpiI	N/A	0.5 μL
T7 ligase	N/A	0.5 μL
ddH_2O	N/A	5 μL
Total	**N/A**	**10 μL**

Incubate the mixture in a PCR machine. Set the program at 37°C for 5 min then 21°C for 5 min, for 6 cycles.

9. Transform 1–2 μL of the mixture to DH5α competent cells following the manufacturer's instructions: https://www.thermofisher.com/order/catalog/product/18258012. Spread the transformation mixture to LB-agar plates with 50 μg/mL ampicillin.
10. Pick a few colonies to grow and mini-prep DNA using QIAprep Spin Miniprep kit following manufacturer's protocol: https://www.qiagen.com/us/products/discovery-and-translational-research/dna-rna-purification/dna-purification/plasmid-dna/qiaprep-spin-miniprep-kit
11. Use U6 primer (5′-GAGGGCCTATTTCCCATGATTCC-3′) to sequence the purified plasmids to check if the sgRNA sequence is successfully ligated.

Note: Sequence 5–6 plasmids from different clones, the percentage of successful plasmids is high, usually > 50% using the reaction in step 8.

⚠ CRITICAL: The target site of the sgRNA should be close to the stop codon, ideally within 50 bp. It is because the efficiency of the homologous recombination reduces when the Cas9-mediated double-stranded break is moving further apart from the stop codon.

Preparation of the homologous repair templates for endogenous tagging

Timing: ~1 week

In this step, we describe how to design the homology arms and how to clone the homology arms into the C-terminal tagging vectors with antibiotic resistance markers (i.e., pMK289 and pMK289-BleoR). For information about the tagging vector, please refer to Natsume et al. (2016).[3]

12. Go to the Ensembl Exons table of *RIF1*. Copy ~200 bp sequence before and ~200 bp sequence after the stop codon.

ATATAATTGATCCTGTTGCTTTAGAAATTCCATTATCCAAAAACCTTCTGGCACAGATTAGTGCTCT
TGCTCTTCAGCTGGATTCAGAAGATCTTCATAATTATTCAGGAAGCCAACTATTTGAAATGCACGA
GAAACTAAGTTGTATGGCAAACTCTGTAATAAAAAATCTACAGTCACGTTGGAGATCACCAT**CCC**
*ATGAAAATTCTATT**TAG**TAT*TTTCAGAGAAAATTGAAGGTTTTTTTAAACATCACTGGATTTCTTGAT
TGAGGAAACAAGTTCTGAAATAATAGCACAATTTCAAAGAAGAGACTCTTTGCAAAGTTGATAACA
TTTCAAACCCTGAAGGACAGTGACTTATTATGTAAGTTCAATTTTGTAAGTTCATTATGTAAGATCCT
TTTTTTTTTCATAATAT

Note: The target site of sgRNA is italicized, the PAM site and stop codon are in bold.

Note: The homology arm is usually 200–800 bp long. Increasing the length of homology arms is known to increase on-target insertion.[5] For gene targeting in HCT116 cells, we use 200 bp-long of homology arm. If you are using other cell lines, you may need to increase the length of the homology arms to increase the efficiency of recombination repair.

13. Replace the stop codon with a BamHI site:

ATATAATTGATCCTGTTGCTTTAGAAATTCCATTATCCAAAAACCTTCTGGCACAGATTAGTGCTC
TTGCTCTTCAGCTGGATTCAGAAGATCTTCATAATTATTCAGGAAGCCAACTATTTGAAATGCACG
AGAAACTAAGTTGTATGGCAAACTCTGTAATAAAAAATCTACAGTCACGTTGGAGATCACCATCC
CATGAAAATTCTATT**GGATCC**TATTTTCAGAGAAAATTGAAGGTTTTTTTAAACATCACTGGATTTCT
TGATTGAGGAAACAAGTTCTGAAATAATAGCACAATTTCAAAGAAGAGACTCTTTGCAAAGTTGAT
AACATTTCAAACCCTGAAGGACAGTGACTTATTATGTAAGTTCAATTTTGTAAGTTCATTATGTAAGA
TCCTTTTTTTTTTCATAATAT

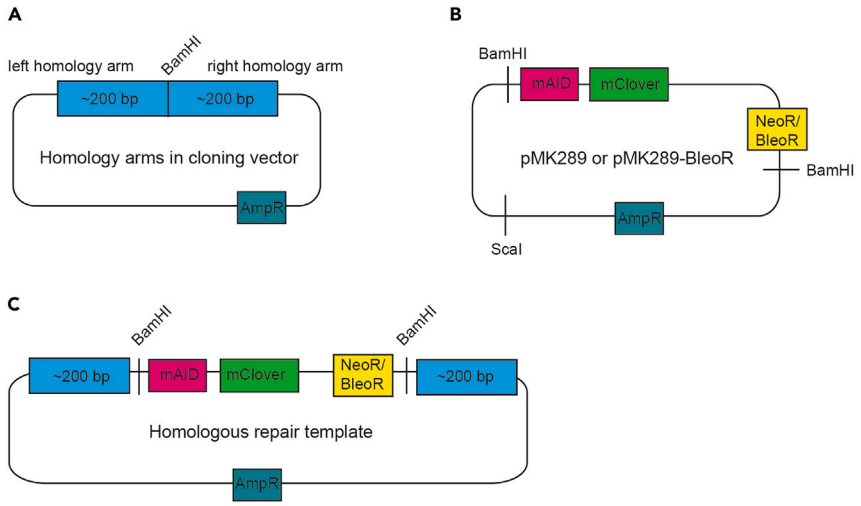

Figure 2. Cloning of the homologous repair templates

(A) The cloning vector with homology arms.

(B) pMK289 and pMK289-BleoR. pMK289 is available from Addgene. pMK289-BleoR is generated by replacing NeoR gene to BleoR gene.

(C) The correct homologous repair template.

⚠ CRITICAL: The homology arm sequences should not contain BamHI site (GGATCC). Mutate the BamHI site if any, but it has a potential to introduce cryptic splice site.

14. Check if "TAG" to "GGATCC" mutation would disrupt the binding of sgRNA to the target site. If the target site is still intact, mutate the PAM site in the repair template to 'NGT', 'NGA', or 'NGC' (troubleshooting 2).
15. Order the designed sequence in a cloning vector via a standard gene synthesis service (e.g., Thermo Fisher GeneArt gene synthesis).

Note: The cloning vector should not contain any BamHI site (Figure 2A).

16. Cut pMK289 and pMK289-BleoR with BamHI and ScaI with the following reaction.

Reagent	Final concentration	Amount
pMK289 or pMK289-BleoR	1 μg	1 μL
BamHI	N/A	1 μL
ScaI	N/A	1 μL
10 × CutSmart Buffer	1×	2 μL
ddH_2O	N/A	16 μL
Total	**N/A**	**20 μL**

17. There will be three DNA bands in DNA gel. The DNA band with ~3 kb in length is the fragment containing mAID, GFP and NeoR/BleoR.
    a. Cut and gel purify the ~3 kb band (Figure 2B) using the GeneJET Gel Extraction Kit (Thermo Fisher). Please follow the manufacturer's instructions: https://www.thermofisher.com/order/catalog/product/K0691.
    b. DNA is eluted with 30 μL of elution buffer.
18. Measure the concentration of the DNA by a NanoDrop.

19. Cut the plasmid containing the homology arms with BamHI with the following reaction:

Reagent	Final concentration	Amount
Plasmid with homology arms	1 μg	1 μL
BamHI	N/A	1 μL
10 × CutSmart Buffer	1×	2 μL
Quick CIP	N/A	1 μL
ddH_2O	N/A	16 μL
Total	**N/A**	**20 μL**

Note: Quick CIP is added to the reaction to remove the phosphates from the DNA ends. This will prevent self-ligation of the linearized vector.

20. Gel-purify the linearized plasmid and ligate it with the ~3 kb fragment from pMK289 or pMK289-BleoR with the following reaction:

Reagent	Final concentration	Amount
3 kb fragment from pMK289 or pMK289-BleoR	~ 3 ng/μL	30 ng
Linearized plasmid with homology arms	~ 1 ng/μL	10 ng
2 × DNA Ligation Mighty Mix	1×	5 μL
ddH_2O	N/A	depend on the volume of DNA
Total	**N/A**	**10 μL**

Incubate the reaction mix at 25°C for 10 min.

Note: The molar ratio between the insert (3 kb fragment from pMK289) and vector (linearized plasmid with homology arms) should be around 3:1.

21. Select bacterial clones with correct recombinant plasmids.
    a. Transform 1–2 μL of the ligation mixture to DH5α competent cells (see step 9).
    b. Pick several colonies, mini-prep DNA (see step 10).
    c. Check the plasmids by Sanger sequencing using a primer priming at the left homology arm (Figure 2C) (troubleshooting 3).
22. The correct plasmid will be transformed to DH5α competent cells again for midi-prep using Qiagen Plasmid Plus kit following the manufacturer's protocol: https://www.qiagen.com/be/products/discovery-and-translational-research/dna-rna-purification/dna-purification/plasmid-dna/qiagen-plasmid-plus-kits.

⚠ CRITICAL: If the stop codon is not within the target site of the sgRNA, the PAM site needs to be mutated to prevent cleavage by Cas9. Alternatively, mutations can also be introduced in the 20 bp protospacer to prevent cutting.

Generation of cells with endogenous tagging at *RIF1* gene

Timing: ~1 month

In this step, you need a cell line expressing OsTIR1 if you want the mAID-tagged protein to be degraded upon the addition of auxin. Please refer to Natsume et al. (2016)[3] for how to generate OsTIR1-expressing cells.

23. Quickly thaw a vial of frozen HCT116-OsTIR1 cells in a 37°C water bath for 1–2 min.
    a. Dilute the thawed cells with ~10 mL pre-warmed growth medium.

b. Plate them into a 10 cm cell culture dish.
c. Let them recover for several passages.

24. Seed ~2 × 10^5 cells to a 60 mm culture dish 24 h before DNA transfection. One hour before transfection, change the medium of the dish.
25. Set up the following transfection mix:

Reagent	Final amount	Amount
pX330 with *RIF1* sgRNA	1 μg	1 μL
pMK289 with *RIF1* homology arms	1 μg	1 μL
pMK289-BleoR with *RIF1* homology arms	1 μg	1 μL
Lipofectamine 2000	N/A	6 μL
Opti-MEM Reduced Serum Medium	N/A	250 μL
Total	**N/A**	**259 μL**

26. Incubate the transfection mixture at 25°C for 20 min before adding to the cells dropwise. Mix gently by rocking the dish back and forth.
27. 48 h later, trypsinize cells using 0.25% trypsin-EDTA solution and transfer them to a 100 mm plate with 10 mL medium. Add 100 μg/mL of zeocin and 500 μg/mL of geneticin to cells.

Note: The concentrations of zeocin (100 μg/mL) and geneticin (500 μg/mL) stated here are optimized for HCT116 cells. For other cell lines, the concentrations should be optimized. Similarly, the Lipofectamine 2000 transfection protocol described here works well in HCT116 cells (> 80% transfection efficiency), but if you are using different cells, the transfection protocol needs to be optimized.

28. Change medium every 3–4 days and add zeocin and geneticin continuously. After ~2 weeks, you can observe a few cell colonies grow on the dish.
29. Pick 15–20 colonies directly by using a 1000 mL pipette trip and transfer each cell colony to a well (with medium) in a 12-well plate (troubleshooting 4).

Note: Single-cell clones should be discernable via microscope. They should have round edges. Don't pick clones of multiple-cell origin. Please refer to Zhong et al. (2020)[6] for how to select single-cell clones.

Note: Alternative methods to isolate single-cell clones include single cell sorting using flow cytometry, or single cell seeding into 96 well plate.

30. Let the cells grow and expand. Then collect cells from each colony for verification to see if any of them has tagged biallelically (see below).
31. Make frozen stocks of each clone.
    a. Trypsinize cells and resuspend the cell pellet (~1–2 × 10^6 cells) with 1 mL of 90% FBS + 10% DMSO and transfer the cell suspension into cryogenic tubes.
    b. Place the cryogenic tubes in an isopropanol-filled freezing container and transfer it to −70°C for more than 16 h.

Note: The knock-in efficiency is usually quite high. For cell clones that survive two antibiotics selection, 10%–30% of them show successful knock-in.

Detect biallelic tagging in any of the clones

Timing: ~1 week

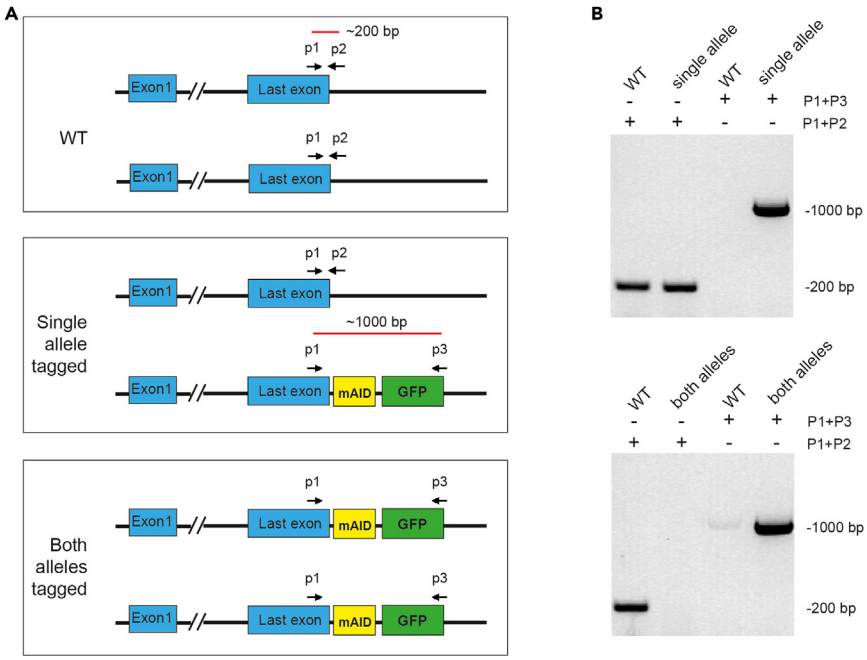

Figure 3. Verification of biallelic tagging by PCR

(A) Positions of primers P1, P2 and P3 designed to confirm biallelic tagging of *RIF1*.

(B) PCR results of WT cells, cells tagged only in one allele and biallelically tagged cells.

To detect successful tagging in any of the cell clones, you can first check the expression of the tagged proteins. In this case, you can detect if the cells express RIF1-GFP by western blot. However, there is a possibility that only one allele of *RIF1* is tagged. In this step, genomic DNA of the cells is extracted to allow verification by PCR to check if biallelic tagging is achieved.

32. Harvest ~1 × 10^6 cells and extract their genomic DNA using Qiagen DNeasy Blood & Tissue Kit following the manufacturer's protocol: https://www.qiagen.com/us/products/discovery-and-translational-research/dna-rna-purification/dna-purification/genomic-dna/dneasy-blood-and-tissue-kit.

Note: Purify genomic DNA from the parental cells as control.

33. Design and order three PCR primers, P1, P2 and P3 (Figure 3A).

Note: P1 primer (forward) is 5′-GTTGCTTTAGAAATTCCATTATC-3′, which primes ~200 bp before the stop codon of *RIF1*. P2 primer (reverse) is 5′-CCTTCAATTTTCTCTGAAAATAC-3′, which primes at the stop codon. P3 primer (reverse) is 5′-TATGGCTGATTATGATCAGT-3′, which primes at the end of GFP.

Note: As least one of the primer sites must be outside of the homology arm in the DNA repair plasmid. It is because even a small amount of episomal plasmid DNA will result in false positive result in the PCR test in cell clones that do not have successful tagging.

34. PCR using P1 and P2 primers gives ~200 bp products using genomic DNA from parental cells as the template, but no products will be formed using P1 and P3 primers (Figure 3A and 3B).
35. PCR using P1 and P2 primers gives no product using genomic DNA from biallelic tagged cells as the template, but ~1 kb products would be generated when using P1 and P3 primers.

Note: Successful tagging would disrupt P2 priming. If both ~200 bp and ~1 kb products can be seen, then the clone is tagged in only single allele (Figures 3A and 3B) (troubleshooting 5).

36. Set up the PCR reaction as follows:

Reagent	Amount
Genomic DNA	1 μL (~50 ng)
Q5® High-Fidelity 2× Master Mix	10 μL
P1	1 μL (500 nM)
P2 or P3	1 μL (500 nM)
ddH_2O	7 μL
Total	**20 μL**

37. PCR cycling condition:

Steps	Temperature	Time	Cycles
Initial Denaturation	98°C	30 s	1
Denaturation	98°C	10 s	25–35 cycles
Annealing	58°C	20 s	
Extension	72°C	20 s	
Final extension	72°C	2 min	1

Note: The extension rate of Q5 polymerase is ~20–30 sec per kb. For calculation of the annealing temperature, you can use the manufacturer's Tm calculator: https://tmcalculator.neb.com/

38. Run the PCR products in 1.2% agarose gel to detect the size of the products (Figure 3B).
39. Send the PCR products to Sanger sequencing to verify and confirm that the PCR products are indeed the expected tagging sequence.
40. To confirm the tagging is functional, treat cells with indole-3-acetic acid (IAA) at 250 μM to induce degradation of RIF1-mAID-GFP and then detect the protein by western blotting (see expected outcomes).
41. To show that the tagged protein is also functional, conduct immunofluorescence using anti-GFP antibody to detect if the tagged proteins display expected localization (See expected outcomes).

EXPECTED OUTCOMES

Once the RIF1-mAID-GFP cells are generated, we can detect the tagged endogenous RIF1 proteins by both western blot and immunofluorescence. The mAID-tagged RIF1 can readily be degraded upon the addition of indole-3-acetic acid (IAA) at 250 μM. Expression of RIF1-mAID-GPF, TIR1 and tubulin (loading control) can be detected using anti-GFP, anti-OsTIR1 and anti-α-tubulin antibodies, followed by detection with HRP-conjugated anti-rabbit or anti-mouse secondary antibodies (Figure 4A). Immunofluorescence using anti-GFP antibody and Alexa Fluor 488 anti-rabbit antibody shows that GFP-tagged RIF1 localizes to ultrafine anaphase bridges (UFBs) and DNA damage foci (Figure 4B and 4C), two sites that have been previously shown to be recognized by RIF1. Therefore, this protocol allows the successful generation of a cell model for loss-of-function and subcellular localization studies of the protein of interest.

Compared to other published protocols for bi-allelic tagging (e.g., Zhong et al. (2020),[6] Xu et al. (2021),[7] Guarnaccia et al. (2021)[8] and Ren et al. (2023)[9]), our protocol provides a simple and efficient C-terminal tagging method for easy-to-transfect cultured cells (such as HCT116)

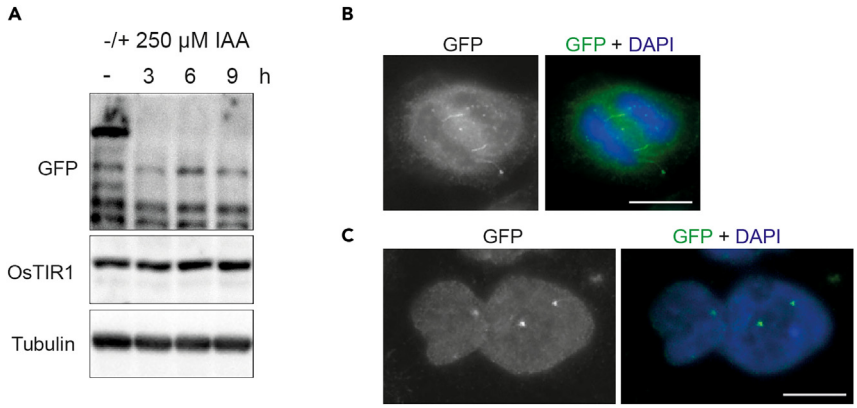

Figure 4. Confirmation of the endogenous tagged cells by western blot and immunofluorescence

(A) RIF1-mAID-GFP cells were treated with doxycycline and IAA and the tagged proteins are readily degraded.

(B) RIF1-mAID-GFP localizes on UFBs.

(C) RIF1-mAID-GFP localizes at DNA damage foci. Scale bars, 10 μm.

using the set of plasmids provided by Natsume et al. (2016).[3] Different tags (mAID, mCherry, GFP and 3xFLAG) and selection genes (Bsr, BleoR, NeoR and Hygro) can be used and the cloning procedure for the homology repair plasmids is the same, which is simply cut and paste. In addition, our protocol also provides information on how to generate cells in which rapid protein depletion can be induced.

LIMITATIONS

There are several limitations of this method. First, since two antibiotic resistance markers are used, the method only allows efficient tagging in diploid or near-diploid cells. If your cell line of interest is highly aneuploidy, there are likely more than two copies of a particular gene. Therefore, we suggest using diploid or near-diploid cell lines for the experiment. Second, the method only allows C-terminal tagging. Therefore, it is important for you to check if your protein of interest can remain functional after C-terminally tagging. Finally, the sgRNA needs to target the region very close to the stop codon. If there is no suitable sgRNA target site near the stop codon, you may need to design the sgRNA further apart from the stop codon, which may lead to low efficiency of recombination.

TROUBLESHOOTING

Problem 1

It is possible that there is no sgRNA sequence available in 100 bp region (related to step 3).

Potential solution

Increase the search to 200 bp sequence. But bear in mind that the efficiency of the homologous recombination will reduce when the Cas9-mediated double-stranded break is moving further apart from the stop codon.

Problem 2

It is possible that mutating PAM site to 'NGT', 'NGA', or 'NGC' would all lead to a missense mutation in the coding region (related to step 14).

Potential solution

If PAM site mutation is not feasible, you can mutate 2–3 nucleotides in the target sequence. Please make sure they are silent mutations. However, silent mutations may introduce unexpected alternations such as cryptic splice sites.

Problem 3

Since it is a ligation with one restriction enzyme digestion, around half of the ligation would be in reverse direction that cannot be detected by digestion with BamHI (related to step 21).

Potential solution

Design a sequencing primer in the left homology arm. The purified plasmids can then be verified by sequencing.

Problem 4

Sometimes, the single-cell colonies may be too close together and cannot be easily picked up by 1000 mL pipette tips. Also, there may be no clone after selection (related to step 29).

Potential solution

You can trypsinize the cells and count the cell number. Then dilute the cell solution to seed a single cell into each well of the 96-well plate. This ensures you obtain single-cell clones.

If there is no clone after antibiotic selection, one possibility is that the antibiotic concentrations you used are too high for your cells. The concentrations of the antibiotics should be optimized for your cell line, i.e., to find out the lowest concentration of the antibiotic that is just enough to kill untransfected cells. Another possibility is that the transfection efficiency is too low so no successful knock-in can be generated. You should optimize the condition for transfection, or use other transfection method such as nucleofection.

Problem 5

All the clones you get show both ~200 bp and ~1000 bp PCR products (related to step 35).

Potential solution

It is possible that some clones are mixed cell population instead of single-cell clones. You can reisolate single-cell clones by seeding single cell into 96-well plate, and test the newly isolated single-cell clones by PCR again.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Ying Wai Chan (gywchan@hku.hk).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate code and did not generate new datasets.

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AUTHOR CONTRIBUTIONS

N.K. and Y.W.C. developed and wrote the protocol.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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