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CRISPR/Cas9-mediated targeting of the *Rosa26* locus produces *Cre* reporter rat strains for monitoring *Cre/loxP*-mediated lineage tracing

Yuanwu Ma[1, 2], Lei Yu[1], Shuo Pan[1], Shan Gao[1], Wei Chen[1], Xu Zhang[1], Wei Dong[1, 2], Jing Li[1], Rui Zhou[1] Lan Huang[1], Yunlin Han[1], Lin Bai[1], Li Zhang[1,2], Lianfeng Zhang[1, 2]*

[1]Key Laboratory of Human Disease Comparative Medicine, NHFPC, Institute of Laboratory Animal Science, Peking Union Medicine College, Chinese Academy of Medical Sciences, Beijing 100021, China.

*Correspondence: Email: zhanglf@cnilas.org; Fax: +86-10-67776394; Tel: +8610-87778442. (Lianfeng Zhang)

[2]Neuroscience center, Chinese Academy of Medical Sciences, Beijing 100021, China.

Running title

Generating *Cre* reporter rat strains

Key words

CRISPR/Cas9, *Cre/loxP*, rat, *Rosa26*, *Cre* reporter

Abbreviations

CRISPR, clustered regularly interspaced short palindromic repeats; Cas, CRISPR-associated; sgRNA, single guide RNA; HR, homologous recombination;

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Abstract

The rat is an important laboratory animal for physiological, toxicological and pharmacological studies. CRISPR/Cas9 is a simple and efficient tool to generate precise genetic modifications in rats, which will promote the accumulation of rat genetic resources and enable more precise studies of gene function. To monitor *Cre/loxP*-mediated excision *in vivo*, we generated a *Cre* reporter rat strain (*Rosa26-imCherry*) by knockin of a *Cre* reporter cassette at *Rosa26* locus using CRISPR/Cas9. *Rosa26-imCherry* rats exhibited inducible expression of the *mCherry* cassette (*imCherry*) using the *Cre/loxP* system, whereas normal rats exhibited ubiquitous expression of *eGFP* but not *mCherry* in the whole body. Injection of *AAV9-Cre* into the hippocampus and skeletal muscle resulted in *mCherry* expression in virus infected cells. *Cre/loxP*-mediated *mCherry* expression was then evaluated by crossing *Rosa26-imCherry* rats with transgenic rats ubiquitously expressing *CAG-Cre*, heart-specific *a-MHC-Cre* transgenic rats, and liver-specific *Alb-Cre* knockin rats. Finally, using the established system the expression pattern of *Cre* driven by two endogenous gene promoters (*Wfs1-Cre* knockin rat, *Nestin-Cre* knockin rat) was traced. In summary, we demonstrated excision of the *loxP*-flanked allele in *Rosa26-imCherry* rats *via* activation of *mCherry* expression in the presence of *Cre* recombinase. This newly established *Rosa26-imCherry* rat strain represents a useful tool to facilitate *Cre*-expression pattern determination and tracing experiments.

Introduction

The rat is an important laboratory animal for studying physiological and molecular signal processes under normal or disease conditions. As a disease model, the rat offers many advantages over the mouse, especially in physiological, toxicological and pharmacological studies [1]. However, the genetic modified genetic resources have remained a bottleneck in rat studies because of the limitations of available genetic engineering techniques. ES-cell-based gene-targeting technology in rat, which was first established by Tong *et al* in 2010 allows the creation of precisely genetically modified rats [2]. However, genome manipulation in rat ES cells is inefficient and time-consuming. Newly developed genome-editing tools, such as zinc-finger nucleases (ZFNs) [3], transcription activator-like effector nucleases (TALENs) [4] and the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated 9 (Cas9) system have been effectively used to produce conventional and conditional knockout rats [5-8]. We previously demonstrated that CRISPR/Cas9 is a simple and efficient tool to generate floxed allele and gene knockin rats [8-9]. In the past three years, we have produced more than one hundred genetically modified

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rats in our laboratory (www.ratresource.com). These rapidly accumulating genetic resources will allow scientists to choose a rat or mouse model based on its biological characteristics rather than technical considerations [10-11].

Traditional gene knockout strategies are of limited utility for studies of the function of genes that are critical in embryogenesis and development. The conditional loss/gain of function of a gene of interest in a defined cluster of cells in a fixed manner can be used to study such genes. Several *Cre* reporter mouse strains have been widely used for the evaluation of *Cre* expression patterns and lineage tracing studies [12-16]. The CRISPR/Cas9 system provides an efficient tool to produce *Cre*-driven rat lines [9] and *Cre* tool resources are rapidly expanding (www.ratresource.com, http://www.rrrc.us/). However, a *Cre* reporter rat strain is not available. The *Rosa26* locus is the most frequently used locus to produce ubiquitous or controlled expression of a gene of interest in mice [13, 17-20]. Here, we used the CRISPR/Cas9 system to knockin a *Cre* reporter cassette into the rat proviral *Rosa26* locus. The reporter cassette consisted of two *loxP* sites flanking an *eGFP* expression cassette *mCherry* expression cassette, which allowed the deletion of the *eGFP* cassette and expression of *mCherry* sequentially in the presence of *Cre* recombinase. We then generated and evaluated several *Cre*-expressing rat strains, including ubiquitous and tissue-specific strains, and used these *Cre* reporter rat strains to trace the *Cre* expression patterns in two endogenous gene promoter-driven *Cre* knockin rat lines.

Results

Knockin of the *Cre* reporter cassette at the proviral *Rosa26* locus

We used a two-cut strategy to insert the *Cre* reporter cassette into the proviral *Rosa26* locus (Fig. 1a). A *Cre* reporter donor plasmid in which the *Cre* reporter cassette was flanked by two ~1 kb homologous arms was used as the template for CRISPR/Cas9-mediated homologous recombination. The *Cre* reporter cassette (5.3 kb), which contained two *loxP* sites, flanking the *eGFP* coding sequence with a triple repeat of *poly A* elements directed by a *CAG* promoter (cytomegalovirus (*CMV*) early enhancer element and chicken beta-actin promoter) [21], was placed in front of an *mCherry* expression cassette (Fig. 1a). Two sgRNAs, both located downstream of exon 1 of *Rosa26* (Fig. 1a, and Supplementary Fig. S1), were designed to target the *Rosa26* locus as described previously [9] (Table 1). Then, the two sgRNAs, the *Cre*

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reporter donor plasmid, and Cas9 protein were co-microinjected into one-cell fertilized eggs for genetic modification.

After microinjection and embryo transfer, we obtained 35 pups (Table 2). Two pairs of primers (upstream and downstream) were designed to detect HR-mediated *Cre* reporter cassette insertion. The primers were designed outside of the homologous arm to exclude random insertion (Fig. 1a). Thus, only genomic DNA with correct insertion of the *Cre* reporter cassette produced the desired PCR products. PCR and sequence analysis showed that 15 pups (42.9%) harbored the correct *Cre* reporter insertion (Fig. 1b, Table 2). We named the HR-mediated *Cre* reporter insertion rat as *Rosa26-imCherry* (inducible expression of *mCherry* using the *Cre/loxP* system) (www.ratresource.com). Next, we examined the reporter gene expression pattern in the *Rosa26-imCherry* rat strain. The *eGFP* and *mCherry* fluorophores have strong epifluorescence that can be easily visualized *eGFP* was ubiquitously expressed in embryo and adult organs, including the muscle, liver, intestine, kidney, testis, lung, brain, heart, and pancreas (Fig. 1c, 1d & 2a), whereas no *mCherry* fluorescence was detected (data not show). FACS revealed that *eGFP* was ubiquitously expressed in blood cells (Fig. 1e). After crossing the *Rosa26-imCherry* F_0 rat with wild-type SD rats, *Rosa26-imCherry* was stably transmitted to the offspring through the germline (data not shown). The strain was collected and now is available upon request (www.ratresource.com, SD. Rosa26(tm-imCherry)-GC/ILAS)

Activation of *loxP*-flanked *mCherry* by local injection of *adeno-associated virus9* (*AAV9*) carrying the *Cre* expression cassette

To evaluate the versatility of the *Rosa26-imCherry* rat, adeno-associated viral (*AAV*) vector of serotype 9 containing a *Cre* expression cassette (*AAV9-CAG-Cre*) (Hanbio Co.Ltd, China) was injected in the hippocampus and skeletal muscle of 8 week-old *Rosa26-imCherry* rats. Ten days after *AAV9-CAG-Cre* virus injection, the hippocampus and injected deltoid muscle were dissected from 4% paraformaldehyde-perfused animals and visualized by fluorescence microscopy. The *mCherry* expression was detected in infected cells in the brain and deltoid muscle (Fig. 3a-k). Thus, *mCherry* expression was activated in *Rosa26-imCherry* rats by injecting *adeno-associated virus9* (*AAV9*) carrying a *Cre* expression cassette.

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Ubiquitous activation of *loxP*-flanked *Rosa26-imCherry* by crossing with a *Cre* strain

To verify that the *mCherry* expression could be induced in the *Rosa26-imCherry* rat strain by crossing with a rat strain ubiquitous expressing *Cre*, a *CAG* promoter-driven *Cre* transgenic rat (Fig. 4a) was generated by pronucleus injection. Four potential founder rats (#1, #2, #6, #13) with *Cre* insertion in the genome were identified by PCR using the primers Cre-F/Cre-R (Fig. 4b, Table 2). The four potential founders were all crossed with *Rosa26-imCherry*, and the double-positive (*CAG-Cre/Rosa26-imCherry*) rats were examined by fluorescence stereoscopy and fluorescence microscopy (Fig. 4c). Founder #2 of the *CAG-Cre* transgenic rats expressed *Cre* recombinase in the whole body, and ubiquitous *mCherry* expression was activated by deletion of the *eGFP* cassette. Amplication and sequencing of the *Cre*-mediated recombination in double-positive (*CAG-Cre/Rosa26-imCherry*) rat (Table 3) confirmed precise recombination between the two *loxP* sites in the double-positive rat (Fig. 2b & 2c). The *CAG-Cre* transgenic rat strain was available upon request (www.ratresource.com, SD. Tg(CAG-Cre)-GC/ILAS).

Heart-specific activation of *loxP*-flanked *Rosa26-imCherry* expression

The *α*-myosin heavy chain (*α-MHC*) promoter has been widely used for cardiac-specific gene modification [22-23]. We obtained sixteen pups after pronuleus microinjection to generate an *α-MHC-Cre* transgenic rat (Fig. 5a). PCR using the primers of Cre-F/Cre-R detected insertion of the target fragment in the genome in five potential founder rats (#1, #4, #7, #10, #16) (Fig. 5b, Table 2). The five potential founders were each crossed with *Rosa26-imCherry*, and the double-positive (*α-MHC-Cre/Rosa26-imCherry*) rats were analyzed by fluorescence stereoscopy and fluorescence microscopy (Fig. 5d-f). Two (founders #4, #16) of five potential founders exhibited heart tissue-specific *mCherry* expression. This establishment of heart-specific *Cre* transgenic rat strains indicates that a gene of interest can be modified in a heart tissue-specific manner and that *mCherry* expression in *Rosa26-imCherry* rats can be activated in a time- and tissue-specific manner. The *α-MHC-Cre* transgenic rat strain was collected and available upon request (www.ratresource.com, SD. Tg(α-MHC-Cre)-GC/ILAS).

Liver-specific activation of *loxP*-flanked *Rosa26-imCherry*

The *albumin* (*Alb*) gene is specifically expressed in hepatocyte cells [24-26]. We used the rat endogenous *albumin* (*Alb*) promoter to generate *Alb-Cre* knockin rats (Fig. 6a). The *Cre* coding sequence was fused to the last codon of the *Alb* gene using a "self-cleaving" 2A peptide from porcine teschovirus-1 (*P2A*) [27]

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(Fig. 6a). This peptide mediates a co-translational cleavage event that will release the *Alb* and *Cre* recombinase protein products [28-31]. The *Alb-Cre* knockin rat was established by CRISPR/Cas9. In brief, two sgRNAs localized at exon 14 and downstream of exon 14 of the *Alb* gene were used for targeting. After microinjection and embryo transfer, 10 pups were born and three rats (#3, #9, and #10) showed correct *Cre* insertion as indicated by genotyping and sequencing (Fig. 6b & 6c, Table 2). The potential founder (#3) was crossed with the *Rosa26-imCherry* rat, and the double-positive (*Alb-Cre/Rosa26-imCherry*) rats were analyzed by fluorescence stereoscopy and fluorescence microscopy (Fig. 6d-f). The endogenous *Alb* promoter drove the *Cre* expression and induced *mCherry* expression specifically in liver hepatocyte cells without leaky expression in other tissues in the double-positive rats. These results suggest that *Alb-Cre* knockin rat strains can be used to modify a gene of interest in a liver-specific manner and that *mCherry* expression in *Rosa26-imCherry* rats can be activated in a tissue-specific manner. The *Alb-Cre* rat strain was collected and now is available upon request (www.ratresource.com, SD. $Alb^{(tm\text{-}Cre)}$-GC/ILAS).

Tracing the *Wfs1* expression pattern in *Rosa26-imCherry* rats

Wolframin ER transmembrane glycoprotein (*Wfs1*) is a nuclear gene responsible for Wolfram syndrome [32]. However, the molecular functions of *Wfs1* and the mechanism by which *Wfs1* mutation induces Wolfram syndrome remain unclear [33-36]. To detect the expression pattern of *Wfs1* in rat, we generated a *Cre* knockin rat using CRISPR/Cas9 as described previously [9] (Fig. 7a). The *Cre* coding sequence was inserted immediately after the start codon in the exon 2 of *Wfs1*. Two sgRNAs localized upstream of exon 2 and downstream of exon 2 of the *Wfs1* gene were used for gene targeting. After microinjection and embryo transfer, 9 pups were born, and four rats (founder rats #1, #3, #5, and #8) showed correct *Cre* insertion as indicated by genotyping and sequencing analysis (Fig. 7b & 7c, Table 2). The potential founder #1 was crossed with the *Rosa26-imCherry* rat, and the *Wfs1* expression pattern in the double-positive (*Wfs1-Cre/Rosa26-imCherry*) rats was detected by tracing *mCherry* expression using fluorescence stereoscopy and fluorescence microscopy (Fig. 7d). The results demonstrated that *Wfs1* was ubiquitously expressed in the rats. (Fig. 7d). The *Wfs1-Cre* rat strain was collected and now is available upon request (www.ratresource.com, SD. $Wfs1^{(tm\text{-}Cre)}$-GC/ILAS).

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Tracing the expression pattern of *Fabp2* in *Rosa26-imCherry* rats

Fatty acid binding protein 2 (Fabp2) play roles in fatty acid absorption and the intracellular transport of dietary long-chain fatty acids, but the pattern of expression of *Fabp2* remains unclear [37-38]. We previous work, we established a *Fabp2-Cre* knockin rat strain [39]. We crossed the *Fabp2-Cre* rat with the *Rosa26-imCherry* rat and the double-positive (*Fabp2-Cre/Rosa26-imCherry*) rats were examined by fluorescence stereoscopy and a fluorescence microscopy. Consistent with a previously report [38], *mCherry* expression was detected in epithelial cells of the small intestine (Fig. 8). We also detected the *mCherry* signal in some liver cells (Fig. 8). The *mCherry* expressing cells in liver requires further characterization.

Discussion

The *Rosa26* locus is widely used to produce ubiquitous or controlled expression of a gene of interest in mice [13, 17-20]. The *Cre/loxP* system is the tool most frequently used in mice to control gene expression in a temporal and spatial manner [40-41]. The *loxP*-flanked *LacZ*, and *eGFP* mouse line are well-established for monitoring *Cre*-mediated excision and tracing *in vivo* [14, 41]. Newly developed genome editing tools, especially the CRISPR/Cas9 system, have been proven effective in generating genetically modified rats and promoting the accumulation of rat genetic resources [5-8, 10-11]. Tools for the evaluation of *Cre* expression in rat are urgently required.

In this context, a *Rosa26-imCherry* rat strain was established by knockin of a *Cre* reporter cassette driven by a *CAG* promoter into at the *Rosa26* locus using CRISPR/Cas9, which allowed *Cre/loxP*-mediated *eGFP* deletion and *mCherry* expression in the presence of *Cre* recombinase (Fig. 1 & 2). We first evaluated the *Rosa26-imCherry* rats by injecting *AAV9* carrying *Cre* expression cassette into the hippocampus and skeletal muscle (Fig. 3), which revealed that *mCherry* expression was effectively activated by *Cre* recombinase. Subsequent evaluation of *mCherry* expression in the offspring of *Rosa26-imCherry* rats crossed with a ubiquitous *Cre* expression transgenic rat strain (*CAG-Cre*) (Fig. 4), a heart-specific *Cre* transgenic rat strain (*α-MHC-Cre*) (Fig. 5), and a liver tissue-specific *Cre* knockin rat strain (*Alb-Cre*) (Fig. 6) suggested that *mCherry* was strictly expressed in *Cre*-expressing tissues. Furthermore, we used the

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Rosa26-imCherry rat to trace the expression pattern of *Wfs1* and *Fabp2* in rat using *Wfs-Cre* and *Fabp2-Cre* knockin rats. *Wfs1* has been reported to be expressed in the brain, heart and pancreatic insulin-producing β-cells [15, 42]. However, our results showed that *mCherry* was expressed in the whole body. The rat *Cre* reporter system is performed in an irreversible; once labeled, a rat will carry the system throughout its lifespan. This shortcoming can be circumvented using temporally controlled *Cre* lines. For example, *Cre* expression can be controlled controlled *via* a promoter is selectively expressed during embryonic development. Fusion of *Cre* recombinase to the human estrogen receptor (ER) allows *Cre* recombinase activity to be induced by estrogen 17β-oesradiol, the anti-estrogen tamoxifen, or its active metabolite 4-hydroxy-tamoxifen (4-OHT) [43-46]. *Fabp2* is an abundant cytosolic protein in small-intestine epithelial cells, and its polymorphism is associated with increased body mass index (BMI), impaired lipid metabolism and cardiovascular disease [38, 47-48]. Our results showed the *Fabp2* was expressed not only in small-intestine epithelial cells but also in some cells in the liver. Taken together, these results show that the established *Rosa26-imCherry* rat and *Fabp2-Cre* rat are valuable tools for lineage tracing and *Cre* expression determination.

In conclusion, we have established a *Cre* reporter strain- the *Rosa26-imCherry* rat. In this rat *eGFP* is expressed in whole body, and *mCherry* expression can be activated in cells expressing the *Cre* recombinase. Crosses with a *Cre*-expressing strains or infection with a *Cre*-containing *AAV9* virus, confirm the function of the reporter strains. This system can be used to easily monitor the *Cre* expression in living tissues or to trace lineage and their descendants in rat development. Furthermore, modified forms of *Cre* such as Cre^{ERT2} can be used to achieve inducible recombination in the cell for tracing the lineage of cells in a temporal or spatial manner during embryogenesis or adulthood.

Materials and Methods

Animals

All rats used in this study were bred in an AAALAC-accredited facility with *ad libitum* access to food and water. All rat experiments were approved by the Animal Care and Use Committees of the Institute of Laboratory Animal Science of Peking Union Medical College (ILAS-GC-2015-001).

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DNA constructs

The sgRNA plasmids were constructed based on the pUC57-sgRNA vector (Addgene, 51132) as described previously [8]. The oligonucleotides for the sgRNA construction are listed in Table 1. All donor plasmids were constructed based on the Brown Norway rat genomic sequence (assembly Rnor_6.0) and performed by *Taihe Biotech* (China). The donor template sequences were cloned into the pGSI plasmid (Supplementary Fig. S2).

Microinjection mixture preparation

The sgRNAs were prepared using MEGAshortscript T7 Transcription kit (Ambion, AM1354) as before [8]. Cas9 protein was prepared as before [8]. For microinjection, Cas9 protein (30 ng/μl) and sgRNAs (10 ng/μl/each) were used. All solutions used for Cas9 mRNA and sgRNA preparation were RNase-free.

Cas9/sgRNA injection into fertilized rat eggs

All rats used in this study were Sprague Dawley (SD) background and purchased from Beijing Vital River Laboratories. The rats were housed in the animal center under standard conditions. The microinjection process was performed as described[8]. In brief, zygotes were obtained from female donor rats mated with SD males after treatment with pregnant mare serum gonadotropin (PMSG, Sigma-Aldrich) and human chorionic gonadotropin (hCG, Sigma-Aldrich). Microinjections were performed in fertilized eggs using a Nikon microinjection system under standard conditions. Microinjections were performed both in the cytoplasm and male pronucleus as described [8]. After microinjection, the injected zygotes were transferred to pseudopregnant SD rats (20-30 zygotes per pseudopregnant SD rat).

Genomic DNA preparation and genotyping

Genomic DNA was extracted from the tails of 7-day-old rats using phenol-chloroform and recovered by alcohol precipitation. In brief, each rat tail was dissolved in SNET (20 mM Tris-Cl, 5 mM EDTA, 400 mM NaCl, 1% SDS, 0.4 mg/ml Proteinase K) at 55℃ for at least 4h. The lysis mixture was extracted with an equal volume of phenol/chloroform, followed by an equal volume of chloroform. The aqueous phase was recovered, and the precipitate the genomic DNA was precipitated by adding 2.5 volumes of alcohol. The genomic DNA was dissolved in TE buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA). The PCR primers

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were designed according to the target sequence and insertion sequence. The PCR primers used for genotyping the genetically modified rats are listed in Table 3. The PCR products were sub-cloned for sequencing analysis using a TA cloning kit (TaKaRa).

Recombinant *AAV9-Cre* virus preparation

The *AAV* vector plasmids contained a *Cre* expression cassette directed by a *CAG* promoter. The recombinant *AAV* virus was produced by transient transfection of HEK293 cells with the vector plasmid and helper plasmid. This step was performed by *Hanbio* (China). The final viral vector titers were in the range of $1 \sim 5 \times 10^{12}$ vg/ml. The vector serotype used was *AAV9*.

Intracranial stereotaxic injection and muscle injection

Rats were anesthetized by intraperitoneal injection of pentobarbital (Sigma) at 40 mg/kg of body weight. Five microliter of viral vector (*AAV9-CAG-Cre*) was injected using a stereotaxic apparatus (*RWD Life Science*, China) with a micropipette at a flow rate of 0.5 μl/min. The coordinates used for the hippocampus injection were bregma 3 mm, lateral 2 mm and dura 2.8 mm. The injection was performed on either the left or right side of the brain. Five microliters of viral vector (*AAV9-CAG-Cre*) was injected into the deltoid muscle using a micropipette at a flow rate of 0.5 μl/min. After injection, the micropipette was left in place for at least 5 min.

Histological procedure

Rats were anesthetized by intraperitoneal injection of pentobarbital and sequentially perfused with 0.9% saline and 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS). The collected tissues were fixed in 4% PFA overnight and then incubated in 30% sucrose in PBS until settling to the bottom. Frozen sections (10 μm) were washed three times with PBS and mounted under glass coverslips using mounting medium with DAPI. Sections were imaged with a fluorescence microscope (4× and 10× objectives) (Leica).

Generation of *CAG-Cre* transgenic rats

The gene coding *Cre* recombinase was inserted downstream of the *CAG* promoter to produce the plasmid *pCAG-Cre* (Fig. 4a). The *pCAG-Cre* plasmid was linearized with *Sal* I and purified for microinjection. Rats carrying the *CAG-Cre* transgene were identified by PCR analysis of tail genomic DNA using the *Cre*

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specific primers Cre-F and Cre-R. The positive transgenic rats were crossed with *Rosa26-imCherry* rats or maintained by mating with SD rats.

Generation of the *α-MHC-Cre* transgenic rat

The gene encoding *Cre* recombinase coding was inserted downstream of the *α-MHC* promoter (α-myosin heavy chain promoter) to produce the plasmid *pα-MHC-Cre* (Fig. 5a). The *pα-MHC-Cre* plasmid was linearized with *Not I* and purified for microinjection. Rats carrying the *α-MHC-Cre* transgene were identified by PCR analysis of tail genomic DNA using the *Cre*-specific primers Cre-F and Cre-R. The positive transgenic rats were crossed with *Rosa26-imCherry* rats or maintained by mating with SD rats.

Cre/*loxP*-mediated recombination *in vivo*

To detect *Cre/loxP*-mediated recombination activity *in vivo*, the *Cre* rat lines was were crossed with *Rosa26-imCherry* rats. The offsprings that were double-positive for *Cre* and *Rosa26-imCherry* were sampled for fluorescence observation. *Cre*-mediated recombination in double-positive rats (*Rosa26-imCherry/ CAG-Cre*) was detected using primer R-Rosa26-delgfp-F and R-Rosa26-delgfp-R (Table 3). The resultant PCR products were sequenced to assess *Cre*-mediated precise recombination.

Germline transmission

Germline transmission of genetic modifications in rats was confirmed by offspring genotyping. The selected potential founder rats were crossed with wild-type SD rats. Germline transmission was considered successful when genotype of the offspring was identical to that of the founder parent.

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AUTHOR CONTRIBUTIONS

L. Z. and Y. M. designed experiments. Y. M., Y. L. S. P., X. Z., W. C., W. D., J. L., R. Z., L. H., Y. H., L. B., and L. Z. performed the experiments. Y. M., and X. Z. collected the data and performed the data analysis. Y. M. wrote the manuscript. L. Z. revised the manuscript.

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COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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Supporting Information

Supplementary Fig. S1: The genome coordinates of rat Rosa26 locus.

Supplementary Fig. S2: Sequences of plasmids *Donor-Rosa26-imCherry, pCAG-Cre, pα-MHC-Cre, Donor-Alb-P2A-Cre, Donor-Wfs1-Cre* and *pGSI*.

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Table 1 The oligonucleotides used for generating sgRNA expression vectors

R-Rosa26-EA-gRNA up	5'-TAGGAGCCATGGCCGCGTCCGG-3'
R-Rosa26-EA-gRNA down	5'-AAACCCGGACGCGGCCATGGCT-3'
R-Rosa26-EB-gRNA up	5'-TAGGACGGGCGGTCGGTCTGAG-3'
R-Rosa26-EB-gRNA down	5'-AAACCTCAGACCGACCGCCCGT-3'
R-Alb-EA-gRNA up	5'-TAGGTGTGATGTGTTTAGGCTA-3'
R-Alb-EA-gRNA down	5'-AAACTAGCCTAAACACATCACA-3'
R-Alb-EB-gRNA up	5'-TAGGCTCTAGGTTGGGCTAACA-3'
R-Alb-EB-gRNA down	5'-AAACTGTTAGCCCAACCTAGAG-3'
R-Wfs1-EA-gRNA up	5'-TAGGGAACTCTAGGCTGCCCTG-3'
R-Wfs1-EA gRNA down	5'-AAACCAGGGCAGCCTAGAGTTC-3'
R-Wfs1-EB-gRNA up	5'-TAGGATCCCCCAGCCCTCAGAG-3'
R-Wfs1-EB gRNA down	5'-AAACCTCTGAGGGCTGGGGGAT-3'

Table 2 Summary of *Rosa26-imCherry* and genetic modified *Cre* expression rats

Vector	Injected zygotes	Transferred embryos (Recipients)	Newborns (Dead)	Positive Rats
Donor-Rosa26-imCherry	227	210 (7)	35 (0)	15
pCAG-Cre	123	102 (3)	14 (0)	4
pα-MHC-Cre	110	94 (3)	16 (0)	5
Donor-Alb- Cre	164	131 (4)	10 (0)	3
Donor-Wfs1-Cre	173	140 (5)	9 (0)	4

Cas9 protein (30 ng/μl) and sgRNA (10 ng/μl/each) targeting *Rosa26*, *Alb*, or *Wfs1* with a donor plasmid for each gene were injected into fertilized eggs. The oligonucleotides used for sgRNAs plasmid construct were shown in Table 1. The donor plasmid sequence was shown in supplementary Fig. S2. For transgenic rat preparation, 3 ~ 5ng/μl of linearized plasmid (*pα-MHC-Cre*/ *pCAG-Cre*) were injected into fertilized eggs. The genetic modified *Cre* rats were identified by PCR. Primers used for detection were shown in Table 3.

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Table 3 The primers for amplifying and sequencing CRISPR/Cas9-induced modifications

Name	Sequence (5'-3')	Amplicon
R-rosa-HR-upF1	GGGTGGCGAAGGTAATGTCT	1491 bp
R-rosa-HR-upR1	GAAAGTCCCTATTGGCGTTACTATG	
R-rosa-HR-Fd1	ATCATCAAGGAGTTCATGCGCTTC	2399 bp
R-rosa-HR-Rd1	CATTAACAGGAAATGGCTCAGTTTATAAATG	
Cre-F	TACTGACGGTGGGAGAATG	437 bp
Cre-R	CTGTTTCACTATCCAGGTTACG	
mCherry-F	TGAAGGTGACCAAGGGTG	399 bp
mCherry-R	TCTTGACCTCAGCGTCGT	
R-Alb-Creki-upF	GGATAACTGCTTCGCCACTGAG	1698 bp
Cre-R	CTGTTTCACTATCCAGGTTACG	
Cre-F	TACTGACGGTGGGAGAATG	1136 bp
R-Alb-Creki-downR	CAAAGTCTTCCATTATCACCCAATTC	
R-Wfs1-Creki-upF	CAAAAAACAATGCAGTGACTCCTG	2493 bp
Cre-R	CTGTTTCACTATCCAGGTTACG	
Cre-F	TACTGACGGTGGGAGAATG	2292 bp
R-Wfs1-Creki-downF	TGGGTCAGAGTGGTCATTGATG	
R-Rosa26-delgfp-F	CAGGAAGGAAATGGGCGG	2725 bp (no Cre); 460 bp (with Cre)
R-Rosa26-delgfp-R	ACCCTTGGTCACCTTCAGCTTG	

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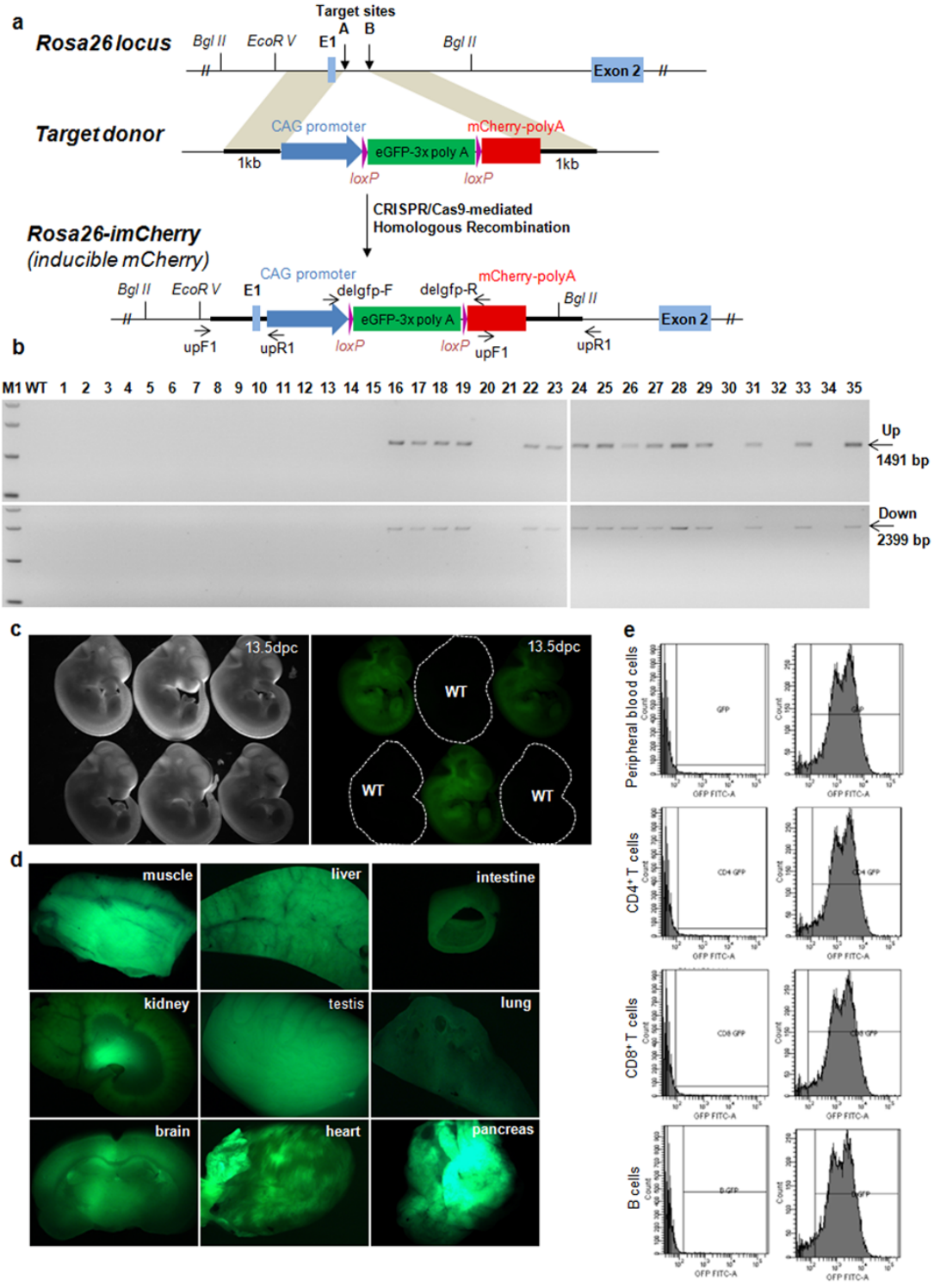

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Figure 1 Generation of *Rosa26-imCherry* knockin rats using the CRISPR/Cas9 system.

(a) Schematic overview of CRISPR/Cas9-mediated knockin of the *Cre* reporter cassette at the *Rosa26* locus in rat. The *Cre* reporter cassette contains a *CAG* promoter (cytomegalovirus (*CMV*) early enhancer element and chicken beta actin promoter), two *loxP* sites flanking the *eGFP* expression cassette, and the *mCherry* expression cassette (inducible *mCherry*, *imCherry*). The expression of *mCherry* occurs only in the presence of *Cre* recombinase.

(b) PCR amplification of the sgRNA:Cas9-mediated *Cre* reporter cassette knockin at the endogenous *Rosa26* locus. Up: PCR amplification of the genomic junction with the left homologous arm and part of the *Cre* reporter cassette. Down: PCR amplification of the genomic junction with the right homologous arm and part of the *Cre* reporter cassette. The primers used for PCR amplification are indicated in (a) and shown in Table 3. The PCR amplicons were cloned and sequenced for insertion analysis. M1: DL2, 000 (TaKaRa).

(c) In *Rosa26-imCherry* rats, *eGFP* was ubiquitously expressed in 13.5 dpc embryos, as observed by fluorescence stereoscopy.

(d) *eGFP* was ubiquitously expressed in adult organs in *Rosa26-imCherry* rats. *eGFP* expression was observed in 8-week-old male rats using a fluorescence stereoscopy.

(e) *eGFP* was ubiquitously expressed in blood cells, including B cells and T cells, as detected by FACS.

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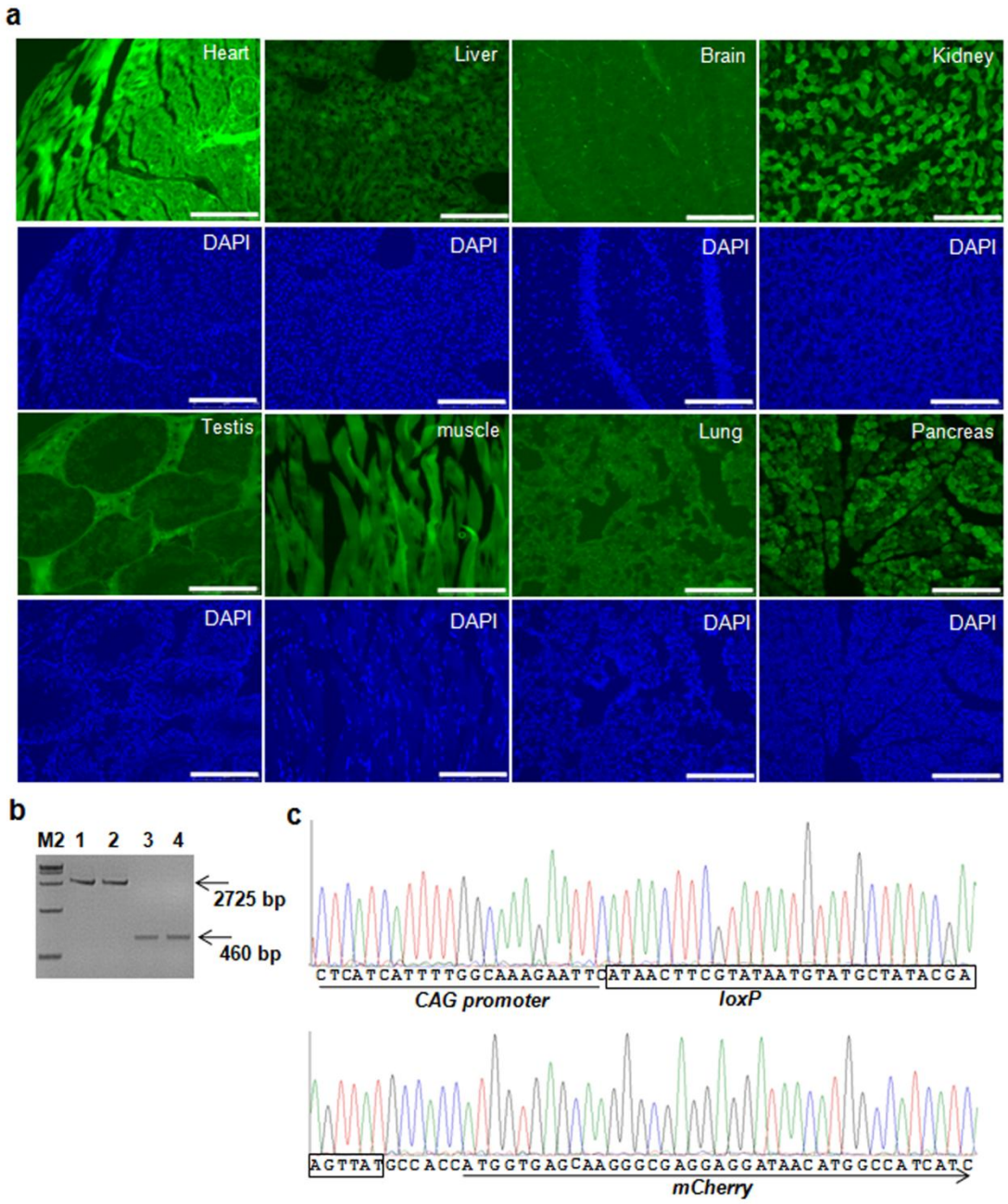

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Figure 2 Characterization of *Rosa26-imCherry* knockin rats.

(a) *eGFP* was ubiquitously expressed in all tissues, as observed by fluorescence microscopy. DAPI (4',6-diamidino-2-phenylindole) was used to visualize nuclei. Bar = 250 μm; original magnification, ×100.

(b) PCR amplification of *Cre*-mediated recombination in double-positive rats (*CAG-Cre/Rosa26-imCherry*). The primers used for PCR amplification are indicated in (a) and shown in Table 3. M2: DL15, 000 (TaKaRa); 1, 2: *Rosa26-imCherry* rat control; 3, 4: double-positive rat (*CAG-Cre/ Rosa26-imCherry*).

(c) Chromatographs from the sequencing confirming precise *Cre*-mediated recombination between the two *loxP* sites.

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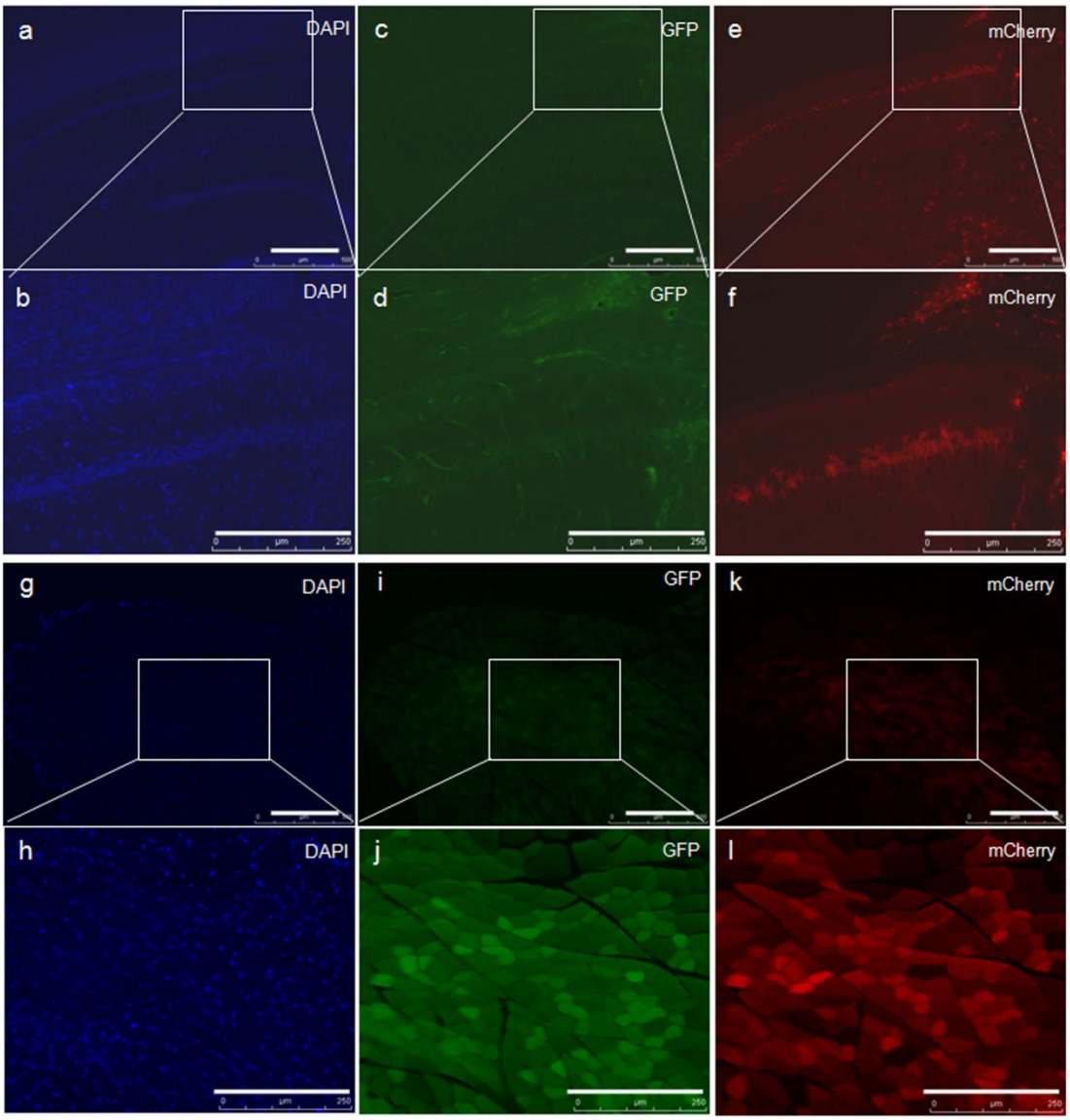

Figure 3 Activation of *mCherry* expression in *Rosa26-imCherry* rats by injection of *adeno-associated virus type 9 (AAV9)-CAG-Cre* virus.

(a-f) Frozen sections of brains collected from 8-week-old *Rosa26-imCherry* rats ten days after injection with *AAV9-CAG-Cre*.

(g-k) Sections of deltoid muscle collected from 8-week-old *Rosa26-imCherry* rats ten days after injection with *AAV9-CAG-Cre*. The *AAV9-CAG-Cre* virus was purchased from *Hanbio* (China). Bar = 250 μm; original magnification, ×40 (a, c, e, g, i, k), ×100 (b, d, f, h, j, l).

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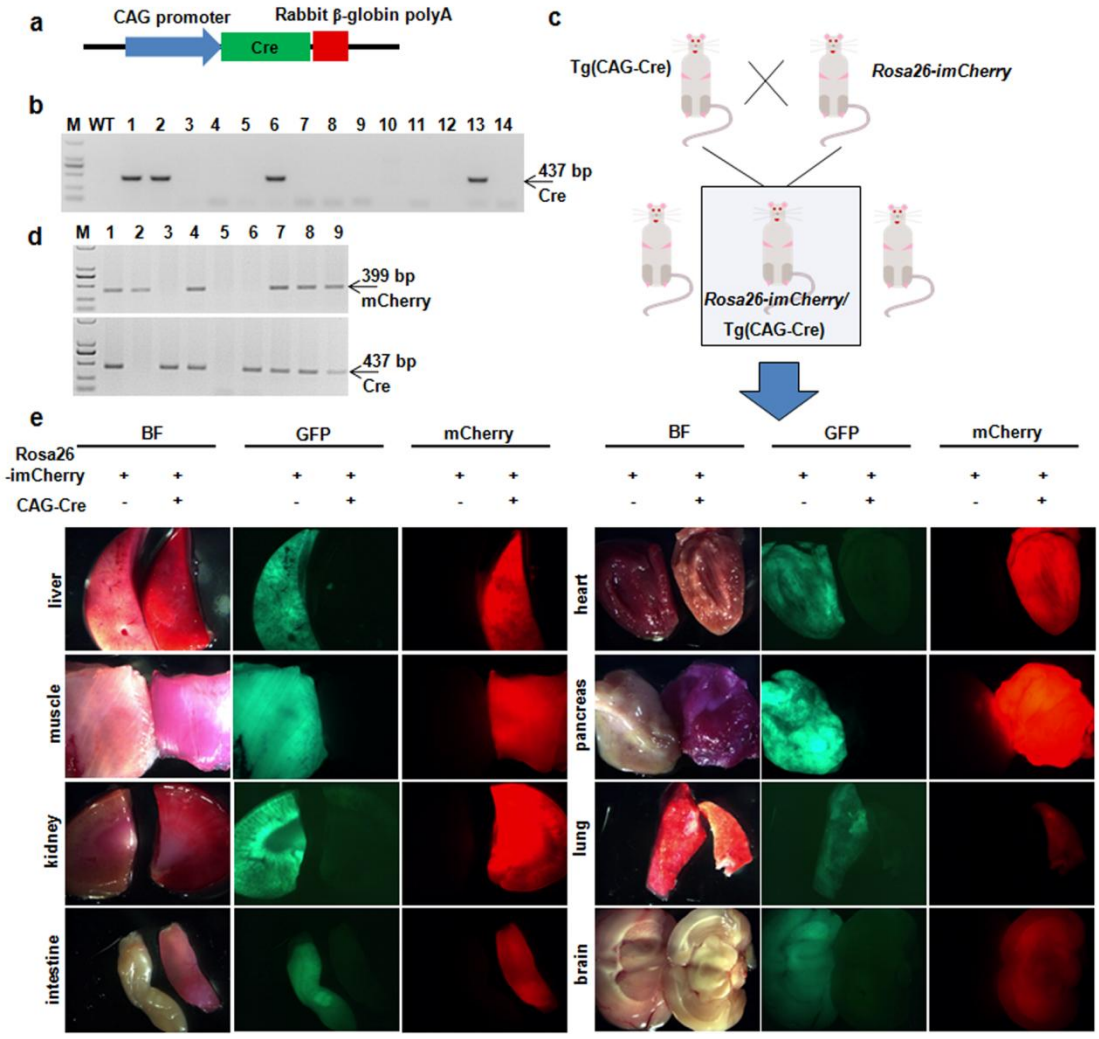

Figure 4 Ubiquitous activation of *mCherry* expression by crossing with a *CAG-Cre* transgenic rat.

(a) The ubiquitous *Cre* expression plasmid (*pCAG-Cre*) was produced by inserting the *Cre* coding sequence downstream of the *CAG* promoter in the *CAG* expression vector. *CAG-Cre* transgenic rats were generated by microinjection of linearized *pCAG-Cre*.

(b) *CAG-Cre* transgenic rats were genotyped using the primers Cre-F and Cre-R listed in Table 3.

(c) Tg(*CAG-Cre*)/ *Rosa26-imCherry* double-positive rats were generated by mating Tg(*CAG-Cre*) rats with *Rosa26-imCherry* rats.

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(d) The offspring generated by crossing Tg(*CAG-Cre*) rats with *Rosa26-imCherry* rats were genotyped using Tg(*CAG-Cre*) specific primers and *Rosa26-imCherry*-specific primers.

(e) Ubiquitous *mCherry* expression was observed in double-positive rats. In double-positive rat #2, ubiquitousl expression of *mCherry* but not *eGFP* expression was detected. BF: bright field.

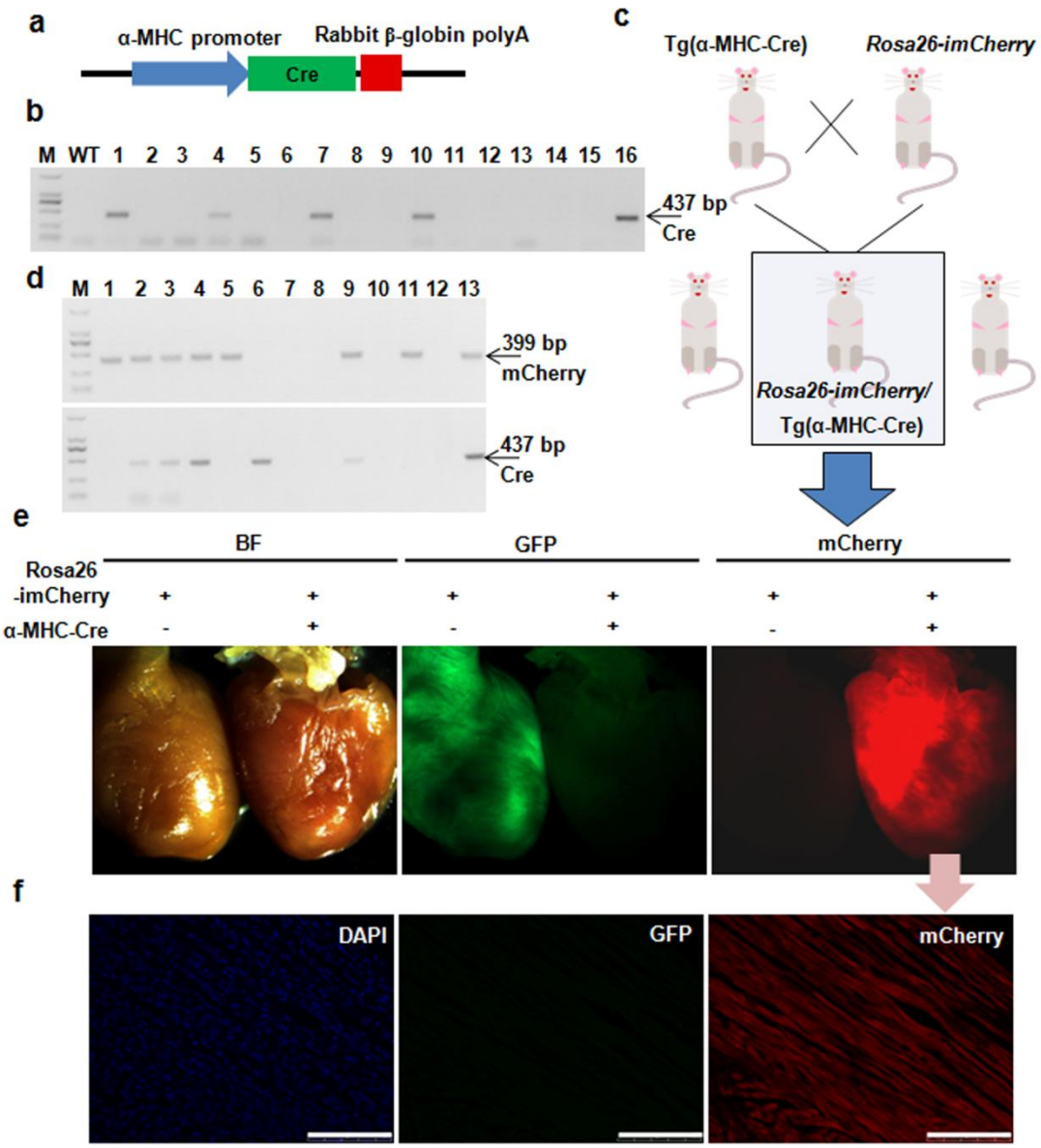

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Figure 5 Specifical activation of the *mCherry* in heart tissue by crossing with an *α-MHC-Cre* transgenic rat.

(a) The heart-specific *Cre* expression plasmid (*pα-MHC-Cre*) was produced by inserting the *Cre* coding sequence downstream of the *α-MHC* promoter (α-myosin heavy chain promoter) in the *α-MHC* expression vector. The *α-MHC-Cre* transgenic rat was generated by microinjection of linearized *pα-MHC-Cre*.

(b) *α-MHC-Cre* transgenic rats were genotyped using the primers Cre-F and Cre-R listed in Table 3.

(c) Tg(*α-MHC-Cre*)/*Rosa26-imCherry* double positive rats were generated by mating Tg(*α-MHC-Cre*) rats with *Rosa26-imCherry* rats.

(d) Offspring produced by crossing Tg(*α-MHC-Cre*) rats with *Rosa26-imCherry* rats were genotyped using Tg(*α-MHC-Cre*) specific primers and *Rosa26-imCherry*-specific primers.

(e-f) *mCherry* expression was specifically observed in the heart in double-positive rats. Double-positive founder #4 and founder #16 exhibited specific *mCherry* expression in heart tissue. *mCherry* expression was detected in founder #4 detected using a fluorescence stereoscope (e) and fluorescence microscope (f). The images were obtained in the ventral view. Bar = 250 μm; original magnification, ×100 (f); BF: bright field.

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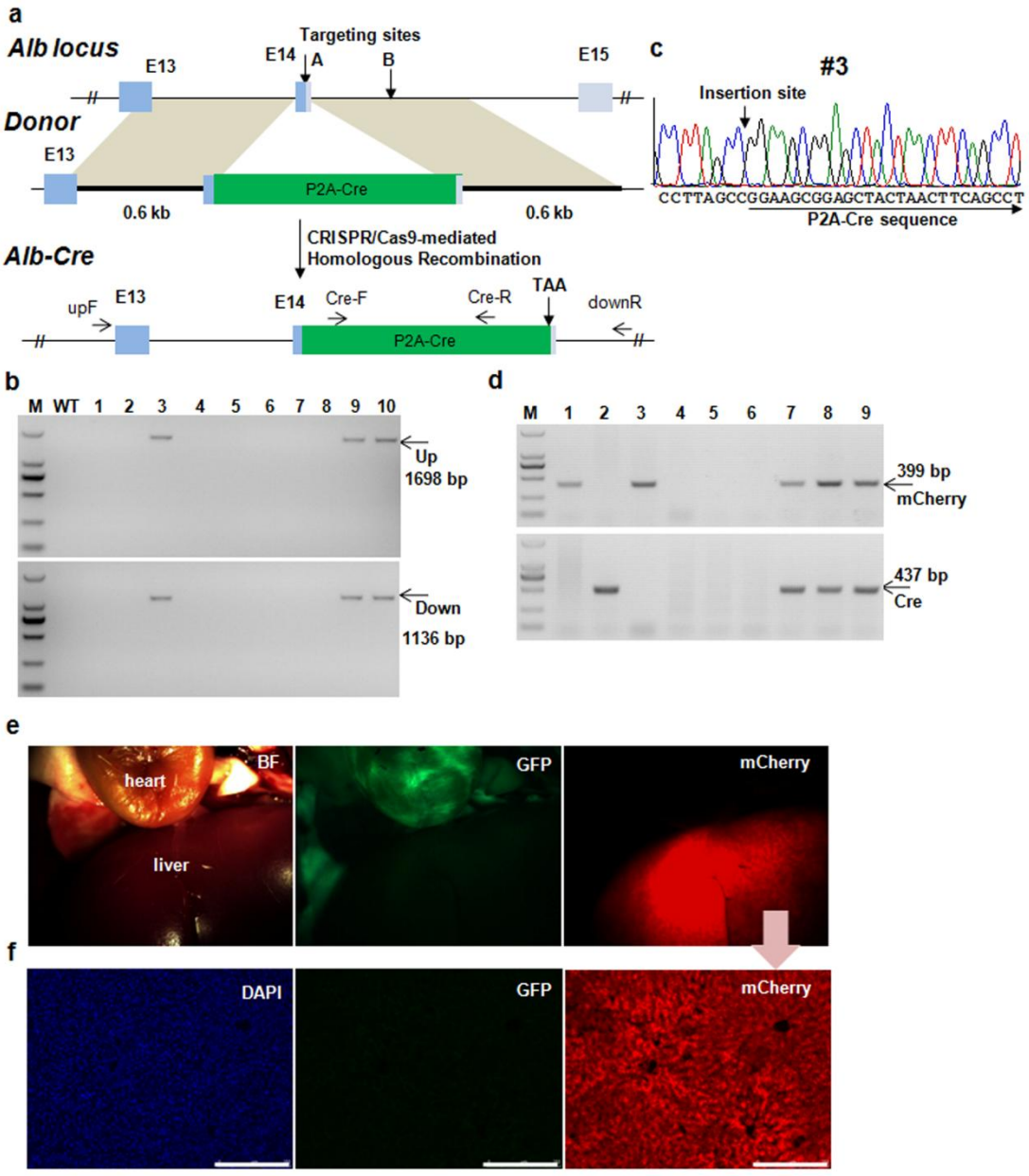

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Figure 6 Specific activation of *mCherry* expression in liver tissue by crossing with an *Alb-Cre* knockin rat.

(a) Schematic overview of the strategy for generating the *albumin* (*Alb*)-*Cre* knockin rat using CRISPR/Cas9. The two sgRNAs-targeting sites (A and B) are indicated by black arrows. The *Cre* coding sequence was fused to the last codon of the *Alb* gene via a *P2A* sequence. The donor plasmid contained two ~0.6-kb homologous arms on both sides of the *P2A-Cre* insertion sequence. *P2A*: "Self-cleaving" 2A peptide from porcine teschovirus-1.

(b) PCR amplification of the sgRNA:Cas9-mediated *Cre* knockin at the endogenous *Alb* locus. Up: PCR amplification of the genomic junction with the left homologous arm and part of the *Cre* cassette (R-Alb-Creki-upF/Cre-R). Down: PCR amplification of the genomic junction with the right homologous arm and part of the *Cre* cassette (Cre-F/ R-Alb-Creki-downR). The two amplicons contained a region of sequence overlap. The primers used for PCR amplification are indicated in (a) and described in Table 3.

(c) Chromatographs from the sequencing files (*Alb-Cre*: #3) showing the precise integration of the *P2A-Cre* Sequence into the endogenous locus.

(d) Genotyping of offspring produced by crossing *Alb-Cre* knockin rats with *Rosa26-imCherry* rats.

(e-f) *mCherry* expression was observed specifically in the liver in double-positive rats (#7, #8, #9). *mCherry* expression was detected in double-positive rat #7 using a fluorescence stereoscope (e) and fluorescence microscope (f), respectively. (e) The images were obtained in the ventral view. Bar = 250 μm; original magnification, ×100 (f); BF: bright field.

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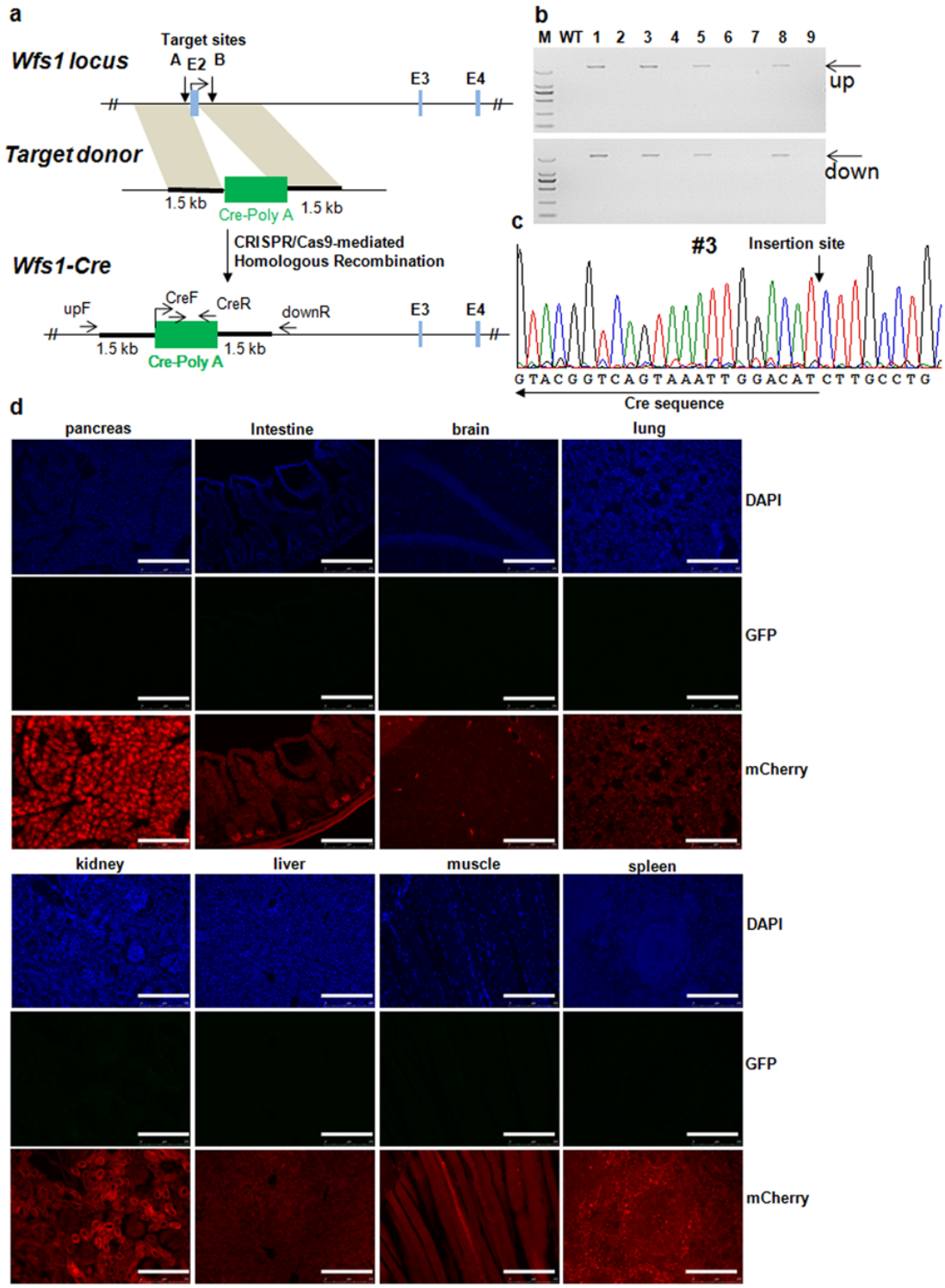

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Figure 7 Tracing the *Wfs1* expression pattern by crossing *Rosa26-imCherry* with *Wfs1-Cre* knockin rats.

(a) Schematic overview of the strategy for generating the *Wfs1-Cre* knockin rat using CRISPR/Cas9. The two sgRNAs-targeting sites (A and B) are indicated by black arrows. The *Cre-poly A* sequence was fused to the start codon of the *Wfs1* gene. The donor plasmid contained two ~1.5-kb homologous arms on both sides of the *Cre-poly A* insertion sequence.

(b) PCR amplification of the sgRNA:Cas9-mediated *Cre* knockin at the endogenous *Wfs1* locus. Up: PCR amplification of the genomic junction with the left homologous arm and part of the *Cre* cassette (R-Wfs1-Creki-upF/Cre-R). Down: PCR amplification of the genomic junction with the right homologous arm and part of the *Cre* cassette (Cre-F/R-Wfs1-Creki-downF). The two amplicons contained a region of overlapping sequence. The primers used for PCR amplification are indicated in (a) and described in Table 3.

(c) Chromatographs from the sequencing (*Wfs1-Cre*: #3) showing precise integration of the *Cre* sequence into the endogenous locus.

(d) *mCherry* expression was ubiquitously detected in adult organs by fluorescence microscopy. Bar = 250 μm; original magnification, ×100 (d).

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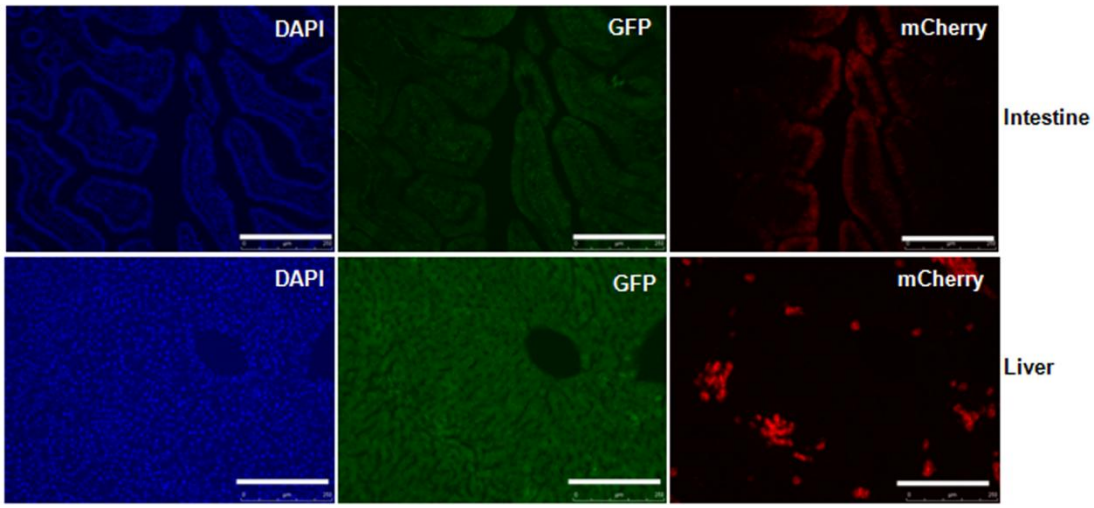

Figure 8 Tracing the *Fabp2* expression pattern by crossing *Rosa26-imCherry* with *Fabp2-Cre* knockin rats.

The *Rosa26-imCherry* rat strain was crossed with previously established *Fabp2-Cre* knockin rats. The double-positive rats (*Rosa26-imCherry/Fabp2-Cre*) exhibited *mCherry* expression in small-intestine epithelial cells and some liver cells. Bar = 250 μm; original magnification, ×100.

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