Transgenic mouse lines expressing the 3xFLAG-dCas9 protein for enChIP analysis

Toshitsugu Fujita, Fusako Kitaura, Asami Oji, Naoki Tanigawa, Miyuki Yuno, Masahito Ikawa, Ichiro Taniuchi, and Hodaka Fujii*

Toshitsugu Fujita, Hodaka Fujii
Department of Biochemistry and Genome Biology, Hirosaki University Graduate School of Medicine, Zaifu-cho 5, Hirosaki, Aomori 036-8562, Japan

Toshitsugu Fujita, Fusako Kitaura, Naoki Tanigawa, Miyuki Yuno, Hodaka Fujii
Chromatin Biochemistry Research Group, Combined Program on Microbiology and Immunology, Research Institute for Microbial Diseases, Osaka University, Yamadaoka 3-1, Suita, Osaka 565-0871, Japan

Asami Oji, Masahito Ikawa
Genome Information Research Center and Department of Experimental Genome Research, Research Institute for Microbial Diseases, Osaka University, Yamadaoka 3-1, Suita, Osaka 565-0871, Japan

Ichiro Taniuchi
Laboratory for Transcriptional Regulation, RIKEN Center for Integrative Medical Sciences, 1-7-22 Suehiro-cho, Tsurumi-ku, Yokohama City, Kanagawa 230-0045, Japan

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*Corresponding author

E-mail: hodaka@hirosaki-u.ac.jp

Department of Biochemistry and Genome Biology, Hirosaki University Graduate School of Medicine, Zaifu-cho 5, Hirosaki, Aomori, 036-8562 Japan

Abstract

We developed the engineered DNA-binding molecule-mediated chromatin immunoprecipitation (enChIP) technology to isolate specific genomic regions while retaining their molecular interactions. In enChIP, the locus of interest is tagged with an engineered DNA-binding molecule, such as a modified form of the clustered regularly interspaced short palindromic repeats (CRISPR) system containing a guide RNA (gRNA) and a catalytically inactive form of Cas9 (dCas9). The locus is then affinity-purified to enable identification of associated molecules. In this study, we generated transgenic mice expressing 3xFLAG-tagged *Streptococcus pyogenes* dCas9 (3xFLAG-dCas9) and retrovirally transduced gRNA into primary $CD4^+$ T cells from these mice for enChIP. Using this approach, we achieved high yields of enChIP at the targeted genomic region. Our novel transgenic mouse lines provide a valuable tool for enChIP analysis in primary mouse cells.

Introduction

Identification of molecules associated with a genomic region of interest *in vivo* is an essential step in understanding the regulatory mechanisms underlying that region's functions. To this end, we previously developed engineered DNA-binding molecule-mediated chromatin immunoprecipitation (enChIP) technology to isolate genomic regions of interest along with their interacting molecules (Fujita *et al.* 2013; Fujita & Fujii 2013). In enChIP, the locus of interest is tagged with engineered DNA-binding molecules, such as transcription activator-like (TAL) proteins (Bogdanove & Voytas 2011) or a variant of the clustered regularly interspaced short palindromic repeats (CRISPR) system (Harrison *et al.* 2014; Wright *et al.* 2016) containing a guide RNA (gRNA) and a catalytically inactive form of Cas9 (dCas9). The tagged locus is then affinity-purified to enable identification of associated molecules. Locus-tagging can be achieved in cells by expressing engineered DNA-binding molecules (Fujita *et al.* 2013; Fujita & Fujii 2013, 2014b; Fujita *et al.* 2015; Fujita *et al.* 2016a; Fujita *et al.* 2017b), or *in vitro* using recombinant or synthetic engineered DNA-binding molecules (Fujita & Fujii 2014a; Fujita *et al.* 2016b; Fujita *et al.* 2017a) Combination of enChIP with mass spectrometry (MS), RNA sequencing, and next-generation sequencing (NGS) enables identification of proteins (Fujita *et al.* 2013; Fujita & Fujii 2013, 2014b), RNAs (Fujita *et al.* 2015), and other genomic regions (Fujita *et al.* 2017a; Fujita *et al.* 2017b) that interact with specific loci of interest in a non-biased manner.

To perform locus-tagging in primary cells, it is necessary to express both dCas9 and gRNA by transduction or other methods. However, the low transduction efficiency of some cell lineages results in a low percentage of cells expressing both dCas9 and gRNA. To resolve this technical issue, we generated transgenic (Tg) mouse lines expressing 3xFLAG-tagged *Streptococcus*

69 *pyogenes* dCas9 (3xFLAG-dCas9), either constitutively or inducibly. To facilitate their use in
70 various experimental contexts, expression of the tagged dCas9 and/or a reporter green fluorescent
71 protein (GFP) can be flexibly induced or abolished by Cre- or FLPe-mediated site-specific
72 recombination events that delete expression-modulating cassettes. We anticipate that these novel
73 Tg mouse lines will serve as a powerful tool for efficient enChIP analysis in primary cells.

Results and Discussion

Generation of Tg mouse lines expressing 3xFLAG-dCas9

To facilitate enChIP analysis using primary mouse cells, we generated two Tg mouse lines expressing 3xFLAG-dCas9 (Fig. 1A, B). One line, 3xFLAG-dCas9-IRES-EGFP, harbors 3xFLAG-dCas9 and IRES-EGFP in the *Rosa26* locus (Fig. 1A). In the other line, 3xFLAG-dCas9 and IRES-EGFP are present at the *Rosa26* locus, but expression of 3xFLAG-dCas9 can be induced by Cre-mediated deletion of the STOP cassette (along with the *neo*^{r} gene), and EGFP expression can be disrupted by FRT-mediated deletion of the IRES-EGFP cassette (Fig. 1B (i)). 3xFLAG-dCas9/CTV (neo$^+$/ GFP$^+$) mice were crossed with CAG-Cre mice (Matsumura *et al.* 2004) to delete the STOP cassette and *neo*^{r} gene, yielding 3xFLAG-dCas9/CTV (GFP$^+$) mice (Fig. 1B (ii)). 3xFLAG-dCas9/CTV (GFP$^+$) mice can be further crossed with CAG-FLPe mice (Schaft *et al.* 2001) to delete the IRES-EGFP cassette, yielding 3xFLAG-dCas9/CTV mice (Fig. 1B (iii)). Targeted integration of transgenes was confirmed by PCR (Fig. 1C). All mice were viable and fertile with normal litter sizes and did not exhibit any morphological abnormalities. Expression of EGFP was observed throughout the body, including thymocytes and splenocytes (Fig. 2A), and 3xFLAG-dCas9 was detected in nuclear extracts (NE) of thymocytes (Fig. 2B). In conventional enChIP using primary cells from mice, it is necessary to transduce both tagged dCas9 and gRNA. To compare the number of Tg mice required for enChIP with that required for conventional enChIP, we isolated CD4$^+$ T cells from a wild-type C57BL/6 mouse and transduced them with a retroviral plasmid expressing 3xFLAG-dCas9 (3xFLAG-dCas9/MSCV-EGFP). As shown in Supplementary Figure S1, the transduction efficiency of 3xFLAG-dCas9/MSCV-EGFP was about 10%. Considering that all cells express 3xFLAG-dCas9 in our Tg mice (Fig. 2A), this

means that 10 times more mice are required for conventional enChIP than for enChIP using our Tg mice. Thus, our Tg mouse lines have the advantage of reducing the number of mice required for enChIP, as well the time and effort needed to perform enChIP analysis in primary mouse cells.

enChIP analysis using primary CD4$^+$ T cells

Next, we performed enChIP analysis using primary cells from the Tg mice (Fig. 3A). For these experiments, CD4$^+$ T cells were purified from 3xFLAG-dCas9-IRES-EGFP mice and 3xFLAG-dCas9/CTV (GFP$^+$) mice and activated with anti-CD3 and anti-CD28 Abs. The activated cells were transduced with a retroviral vector expressing gRNA targeting the c-*myc* promoter (m-c-*myc* gRNA #1/pSIR-hCD2) or a negative control vector (pSIR-hCD2), and 2 days later, hCD2$^+$ cells were isolated and expanded in media containing IL-2. Cells were fixed with formaldehyde and subjected to enChIP analysis using anti-FLAG Ab. Yields of enChIP were monitored by real-time PCR. As shown in Figure 3B and C, efficient enrichment of the c-*myc* promoter region, but not irrelevant loci (*Gapdh, Pax5*), was detected in samples expressing gRNA targeting the c-*myc* locus. By contrast, no enrichment was observed for samples in the absence of gRNA. The yields of enChIP were comparable between 3xFLAG-dCas9-IRES-EGFP mice and 3xFLAG-dCas9/CTV (GFP$^+$) mice. These results demonstrate that primary cells from these Tg mice can be used for enChIP analysis.

Conclusions

We generated Tg mouse lines expressing 3xFLAG-tagged dCas9 and retrovirally transduced gRNA targeting a genomic locus into primary $CD4^+$ T cells from these mice. Using this approach, high yields of enChIP could be achieved. Thus, these Tg mouse lines represent a useful tool for enChIP analysis in primary mouse cells. The injection of adenovirus-mediated gRNA (Platt *et al.* 2014) into these Tg mice should also enable the isolation of genomic regions of interest from mouse tissues without the need for primary cell cultures. In addition, the Tg mouse strains generated in this study could be used for CRISPR interference (CRISPRi) experiments (Qi *et al.* 2013) using primary mouse cells. However, in enChIP analysis, such CRISPRi effects might be problematic. Therefore, it would be better to choose gRNA target sequences that bind to the CRSIPR complex without interfering with the functions of the target genomic regions.

Experimental procedures

Plasmids

To construct pCAG1.2-PM, a modified pCAGGS plasmid (Niwa *et al.* 1991) was digested with *Sac*I. Two oligonucleotides, *Mlu*I–*Pme*I oligo-S (27551) and *Mlu*I–*Pme*I oligo-A (27552), were annealed, phosphorylated, and inserted into the digested plasmid, yielding two plasmids, pCAG1.2-PM (*Pme*I–*Mlu*I) and pCAG1.2-MP (*Mlu*I–*Pme*I), distinguished by the orientations of the oligonucleotides. To construct 3xFLAG-dCas9/pCAG1.2-PM, pCAG1.2-PM was digested with *Eco*RV and *Not*I. 3xFLAG-dCas9/pMXs-puro (Addgene #51240) was digested with *Pac*I, blunted, and further digested with *Not*I. The vector backbone and the coding sequence of 3xFLAG-dCas9 were purified by agarose gel electrophoresis and ligated. To construct 3xFLAG-dCas9-IG/pCAG1.2-PM, 3xFLAG-dCas9/pCAG1.2-PM and 3xFLAG-dCas9/pMXs-IG (Addgene #51258) were digested with *Not*I and *Sal*I, respectively. After blunting, the plasmids were further digested with *Asc*I. The vector backbone and the coding sequence of 3xFLAG-dCas9 were purified by agarose gel electrophoresis and ligated. To construct 3xFLAG-dCas9-IG/pSKII-ROSA, pSKII-ROSA26arm0.5kb-zeo was digested with *Bam*HI and *Sal*I, and 3xFLAG-dCas9-IG/pCAG1.2-PM was digested with *Mlu*I and *Pac*I. After blunting, the vector backbone and the coding sequence of 3xFLAG-dCas9 were purified by agarose gel electrophoresis and ligated.

To construct 3xFLAG-dCas9/CTV, CTV (Addgene #15912) was digested with *Asc*I, and 3xFLAG-dCas9/pMXs-puro was digested with *Pac*I and *Not*I. After blunting, the vector

backbone and the coding sequence of 3xFLAG-dCas9 were purified by agarose gel electrophoresis and ligated.

To construct 3xFLAG-dCas9/MSCV-EGFP (Addgene #82613), the MSCV-EGFP plasmid (DeKoter *et al.* 1998) was digested with *Hpa* I and ligated with the coding sequence of 3xFLAG-dCas9, which was derived from 3xFLAG-dCas9/pMXs-puro by digesting with *Pac* I and *Not* I followed by blunting with DNA Blunting Kit (Takara).

To construct a gBlock targeting the mouse c-*myc* promoter, the oligonucleotides mcMYC promoter 1 sense (27822) and mcMYC promoter 1 antisense (27823) were annealed, phosphorylated, and inserted into gRNA cloning vector *Bbs*I ver. 1 digested with *Bbs*I. To construct m-c-*myc* gRNA #1/pSIR, the gBlock was excised with *Xho*I and *Hin*dIII and inserted into *Xho*I/*Hin*dIII-cleaved pSIR (Clontech). To construct a retroviral vector for expression of gRNA against the mouse c-*myc* promoter (m-c-*myc* gRNA #1/pSIR-hCD2), pSIR-hCD2 (Addgene #51143) and m-c-*myc* gRNA #1/pSIR were digested with *San*DI and *Hin*dIII. The vector backbone and insert were purified by agarose gel electrophoresis and ligated.

Oligonucleotides used for construction of plasmids are shown in Table 1.

Mice

Embryonic stem (ES) cells (EGR-101) (Fujihara *et al.* 2013) were transfected with linearized 3xFLAG-dCas9-IG/pSKII-ROSA, as described previously (Oji *et al.* 2016). ES cells retaining the transgene in the *Rosa26* locus were injected into blastocysts (ICR × ICR) to generate chimeric mice. The chimeric mice were crossed with B6D2F1 mice to generate heterozygous

3xFLAG-dCas9-IRES-EGFP mice (strain name: B6D2-*Gt(ROSA)26Sor*$^{em1(CAG\text{-}3XFLAG\text{-}dCas9,\text{-}EGFP)Osb}$) (RIKEN BioResource Center RBRC09976; Kumamoto University Center for Animal Resources and Development (CARD): CARD ID 2531).

B6JN/1 ES cells (Moriyama *et al.* 2014), derived from a hybrid between C57BL/6J and C57BL/6N, were electroporated with 3xFLAG-dCas9/CTV plasmid linearized with *Asi*SI. To generate chimeric mice, ES cells containing the *neo*^{r} gene, transgene, and *GFP* gene in the *Rosa26* locus were injected into blastocysts (Balb/c × Balb/c) by the animal facility group at RIKEN IMS. The chimeric mice were crossed with C57BL/6 mice to generate heterozygous 3xFLAG-dCas9/CTV (neo^{+}/GFP^{+}) mice (strain name: C57BL/6-*Gt(ROSA)26Sor*$^{tm1(CAG\text{-}3XFLAG\text{-}dCas9,\text{-}EGFP)Hfuj}$). The 3xFLAG-dCas9/CTV (neo^{+}/GFP^{+}) mice were crossed with CAG-Cre mice (Matsumura *et al.* 2004) to generate 3xFLAG-dCas9/CTV (GFP^{+}) mice (strain name: C57BL/6-*Gt(ROSA)26Sor*$^{tm1.1(CAG\text{-}3XFLAG\text{-}dCas9,\text{-}EGFP)Hfuj}$). The 3xFLAG-dCas9/CTV (GFP^{+}) mice can be crossed with CAG-FLPe mice (Schaft *et al.* 2001) to generate 3xFLAG-dCas9/CTV mice (strain name: C57BL/6-*Gt(ROSA)26Sor*$^{tm1.2(CAG\text{-}3XFLAG\text{-}dCas9)Hfuj}$).

All animal experiments were approved by the Institutional Animal Care and Use Committee at Research Institute for Microbial Diseases, Osaka University.

Genotyping

196 For genotyping, genomic DNA was extracted and subjected to PCR with KOD FX (Toyobo).
197 PCR conditions were as follows: heating at 94°C for 2 min; followed by 38 cycles of 98°C for 10
198 s, 62°C for 30 s, and 68°C for 4 min. Primers used for genotyping PCR are shown in Table 1.
199
200 **Cell staining and flow cytometry**
201 Thymi and spleens were isolated from euthanized mice and used to prepare single cells. For
202 surface staining, cells were stained for 30 minutes at 4°C with fluorochrome-conjugated
203 antibodies (Abs): fluorescein isothiocyanate (FITC)-conjugated mouse CD4 (130-102-541,
204 Miltenyi) and phycoerythrin (PE)-conjugated human CD2 (hCD2) (347597, BD Bioscience).
205 Flow cytometric analysis was performed on a FACSCalibur (BD Biosciences) and analyzed with
206 the FlowJo software (TreeStar).
207
208 **Immunoblot analysis**
209 NE were prepared with NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher
210 Scientific). Aliquots of NE (10 µg) were subjected to immunoblot analysis with anti-FLAG M2
211 Ab (F1804, Sigma-Aldrich), as described previously (Fujita & Fujii 2013).
212
213 **Transduction of gRNA and isolation of transduced cells**
214 Transduction of retroviral expression plasmids into primary $CD4^+$ T cells was performed as
215 described previously (Naoe *et al.* 2007). Briefly, $CD4^+$ T cells were purified from spleens using
216 the Mouse $CD4^+$ T cell isolation kit (Miltenyi, 130-104-454). Purified $CD4^+$ T cells were
217 activated with anti-CD3 Ab (3 µg/ml, 145-2C11, 553057, BD Pharmingen) and anti-CD28 Ab (3
218 µg/ml, 37.51, 553295, BD Pharmingen). A retroviral expression plasmid was transfected into
219 Plat-E cells (Morita *et al.* 2000) along with pPAM3 (Miller & Buttimore 1986) using

Lipofectamine 3000 (Invitrogen) to produce retroviral particles. Activated $CD4^+$ T cells were transduced with the retroviral particles by the spin infection method (Naoe *et al.* 2007). After culturing for 2 days in RPMI complete media containing mouse IL-2 (20 ng/ml, 402-ML, R & D Systems), transduced cells were analyzed by flow cytometry. $hCD2^+$ cells were purified using human CD2 MicroBeads (130-091-114, Miltenyi) and used for enChIP analysis.

enChIP real-time PCR

enChIP real-time PCR was performed as described previously (Fujita & Fujii 2013) with some modifications. Briefly, the $CD4^+$ T cells (ca. 1×10^6) were crosslinked with 0.1% formaldehyde in RPMI complete media at 37°C for 10 min. After quenching and washing with PBS, the chromatin fraction was extracted and fragmented by sonication. The sonicated chromatin was used for enChIP using 2 µg of anti-FLAG M2 Ab. DNA was purified using ChIP DNA Clean & Concentrator (Zymo Research) and subjected to real-time PCR. Primers used in the analysis are shown in Table 1.

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Abbreviations: enChIP, engineered DNA-binding molecule-mediated chromatin immunoprecipitation; CRISPR, clustered regularly interspaced short palindromic repeats; dCas9, catalytically inactive form of Cas9; gRNA, guide RNA; GFP, green fluorescent protein; MS, mass spectrometry; NGS, next-generation sequencing; Tg, transgenic.

Conflicts of interests

T.F. and H.F. have patents on enChIP ("Method for isolating specific genomic region using molecule binding specifically to endogenous DNA sequence"; patent number: Japan 5,954,808; patent application number: WO2014/125668). T.F. and H.F. are founders of Epigeneron, Inc.

Availability of data and materials

All data generated or analyzed during this study are included in the published article. Tg mice generated in this study can be obtained from RIKEN BioResource Center and Kumamoto University Center for Animal Resources and Development (CARD).

Authors' contributions

H.F. designed and performed experiments (design and construction of transgenes, flow cytometric analysis, immunoblot analysis, transduction of retroviruses), wrote the manuscript, and directed and supervised the study. T.F. and M.Y. performed enChIP analyses. N.T. constructed the retrovirus vector expressing gRNA targeting the c-*myc* locus, and performed transduction of gRNA retroviruses and enChIP analysis. F.K. and M.Y. maintained the mouse colony. F.K. screened ES cells and genotyped mice. A.O. and M.I. performed CRISPR-mediated knock-in of 3xFLAG-dCas9-IRES-EGFP transgenes. I.T. generated ES cells harboring the 3xFLAG-dCas9/CTV transgene, chimeric mice, and knock-in mice.

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330

Figure legends

Fig. 1. Generation of Tg mouse lines expressing 3xFLAG-dCas9. (**A**) Scheme of the targeted locus of 3xFLAG-dCas9-IRES-EGFP. (**B**) Scheme of the targeted loci of (i) 3xFLAG-dCas9/CTV (neo^{+}/GFP^{+}); (ii) 3xFLAG-dCas9/CTV (GFP^{+}); and (iii) 3xFLAG-dCas9/CTV. (**C**) Genotyping PCR of Tg mouse lines.

Fig. 2. Expression of 3xFLAG-dCas9. (**A**) Expression of GFP in thymocytes and splenocytes from Tg mice. Fluorescence in the FL-1 channel (GFP) is shown for C57BL/6 mice (WT: black) and Tg mice (Tg: green). Numbers represent mean fluorescence intensities (MFI) in the FL-1 channel. (**B**) Expression of 3xFLAG-dCas9 in thymocytes from Tg mice. Expression of 3xFLAG-dCas9 was detected by immunoblot analysis with anti-FLAG Ab. Coomassie Brilliant Blue (CBB) staining is shown as a protein loading control.

Fig. 3. enChIP analysis of CD4^{+} T cells from 3xFLAG-dCas9 Tg mice. (**A**) Scheme of enChIP analysis using Tg mouse lines. (**B**) Positions of primer sets used for enChIP real-time PCR. Green letters: primer positions for c-myc (1); red letters: primer positions for c-myc (2); yellow highlight: gRNA target site; underline: PAM. (**C**) Yields of enChIP analysis for the target site. Error bars represent differences between duplicate analyses. N.D.: not detected. Irrelevant loci (*Gapdh* and *Pax5*) were analysed as negative control loci.

351 **TABLE**

352

353 **Table 1. Oligodeoxyribonucleotides used in this study**

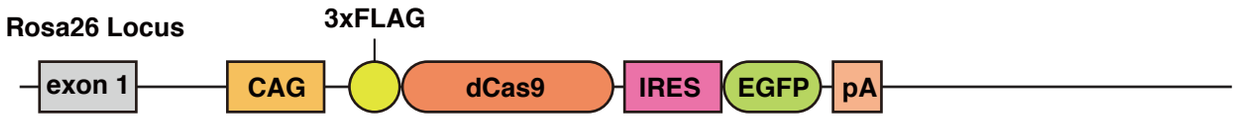
Number	Name	Sequence (5' → 3')	Experiments
27551	*Mlu*I–*Pme*I oligo-S	cacgcgtgtttaaacgagct	Construction of pCAG1.2-PM and pCAG1.2-MP
27552	*Mlu*I–*Pme*I oligo-A	cgtttaaacacgcgtgagct	Construction of pCAG1.2-PM and pCAG1.2-MP
27699	ROSA-5' TF2	ctcagagagcctcggctagg	Genotyping of all Tg mouse lines
27644	Cas9-R	cagggcgataagattaccaaac	Genotyping of 3xFLAG-dCas9-IRES-EGFP
26013	oIMR0013	cttgggtggagaggctattc	Genotyping of 3xFLAG-dCas9/CTV (neo^+/GFP^+)
28110	Cas9-R 27643	gtaggctttctgcctccttcact	Genotyping of 3xFLAG-dCas9/CTV (neo^+/GFP^+) and 3xFLAG-dCas9/CTV (GFP^+)
27822	mcMYC promoter 1 sense	caccgcgcccgggacgtgcgtgacg	Construction of gRNA targeting c-*myc* promoter: sense strand
27823	mcMYC promoter 1 antisense	aaaccgtcacgcacgtcccgggcgc	Construction of gRNA targeting c-*myc* promoter: antisense strand
28157	mcMYC-0.62k-F	tatacgtggcagtgagttgctga	enChIP real-time PCR [c-*myc* (1)]
28158	mcMYC-0.44k-R	ctctaaggctggggaaaacagaa	enChIP real-time PCR [c-*myc* (1)]
28222	mc-myc_-0.1k-F	ctgaggctcctcctcctcttc	enChIP real-time PCR [c-*myc* (2)]
28223	mc-myc_-0.1k-R	cccttccccacctctctctattt	enChIP real-time PCR [c-*myc* (2)]
26574	mGAPDH-prom-F	gggttcctataaatacggactgc	enChIP real-time PCR (*Gapdh*)
26575	mGAPDH-prom-R	agcatccctagacccgtacagt	enChIP real-time PCR (*Gapdh*)
28091	mPax5-ChIP-prom-F	gacctatggaggttgcaattgag	enChIP real-time PCR (*Pax5*)
28092	mPax5-ChIP-prom-R	agcaagtggttttgaaccctgta	enChIP real-time PCR (*Pax5*)

354

355

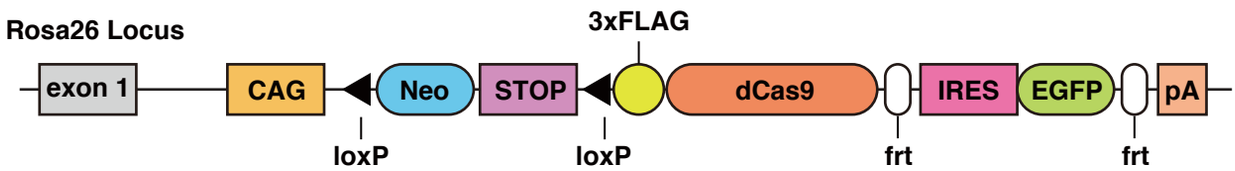
Figure 1

A

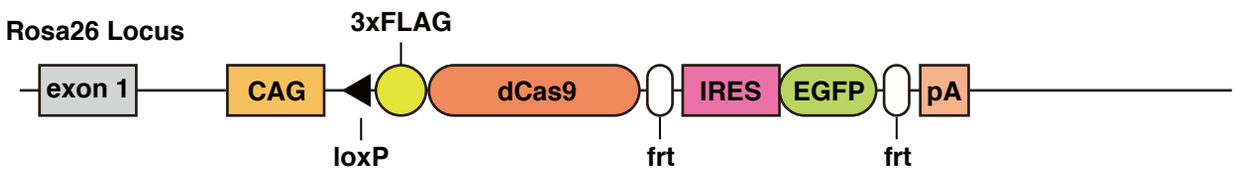


B

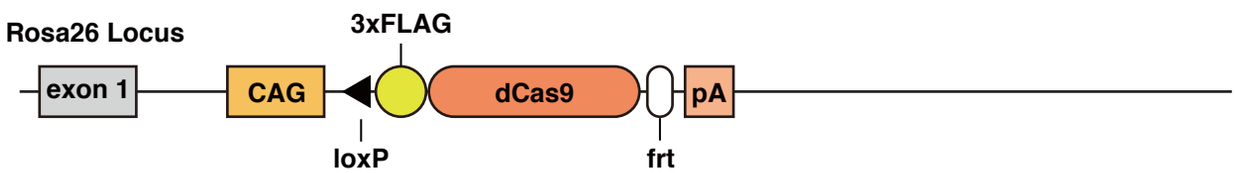
(i) 3xFLAG-dCas9/CTV (neo$^+$/GFP$^+$)



(ii) 3xFLAG-dCas9/CTV (GFP$^+$)

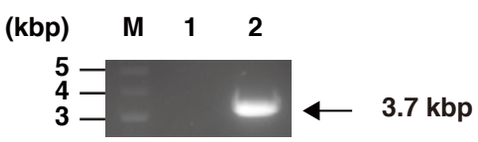


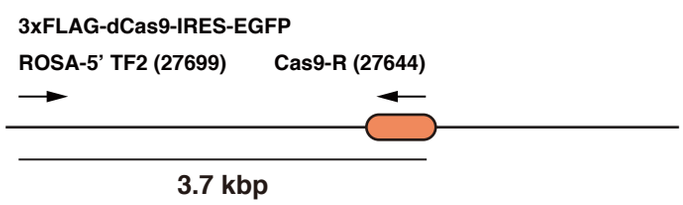
(iii) 3xFLAG-dCas9/CTV



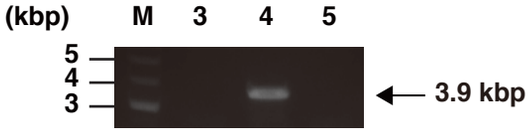
C

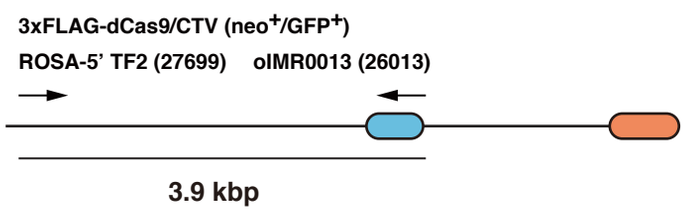
(a) ROSA-5' TF2 (27699) x Cas9-R (27644)



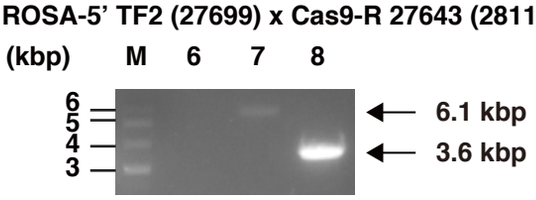


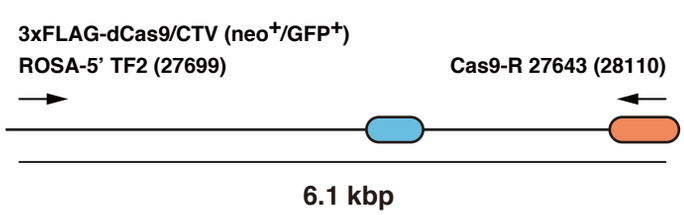
(b) ROSA-5' TF2 (27699) x oIMR0013 (26013)

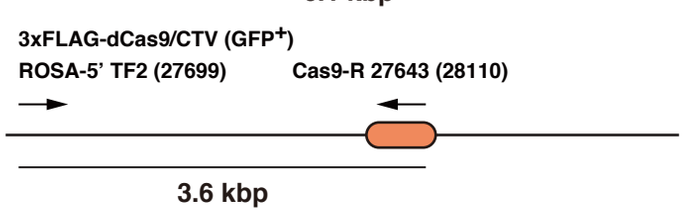




(c) ROSA-5' TF2 (27699) x Cas9-R 27643 (28110)







lanes

1, 3, 6: C57BL/6

2: 3xFLAG-dCas9-IRES-EGFP

4, 7: 3xFLAG-dCas9/CTV (neo$^+$/GFP$^+$)

5, 8: 3xFLAG-dCas9/CTV (GFP$^+$)

Figure 2

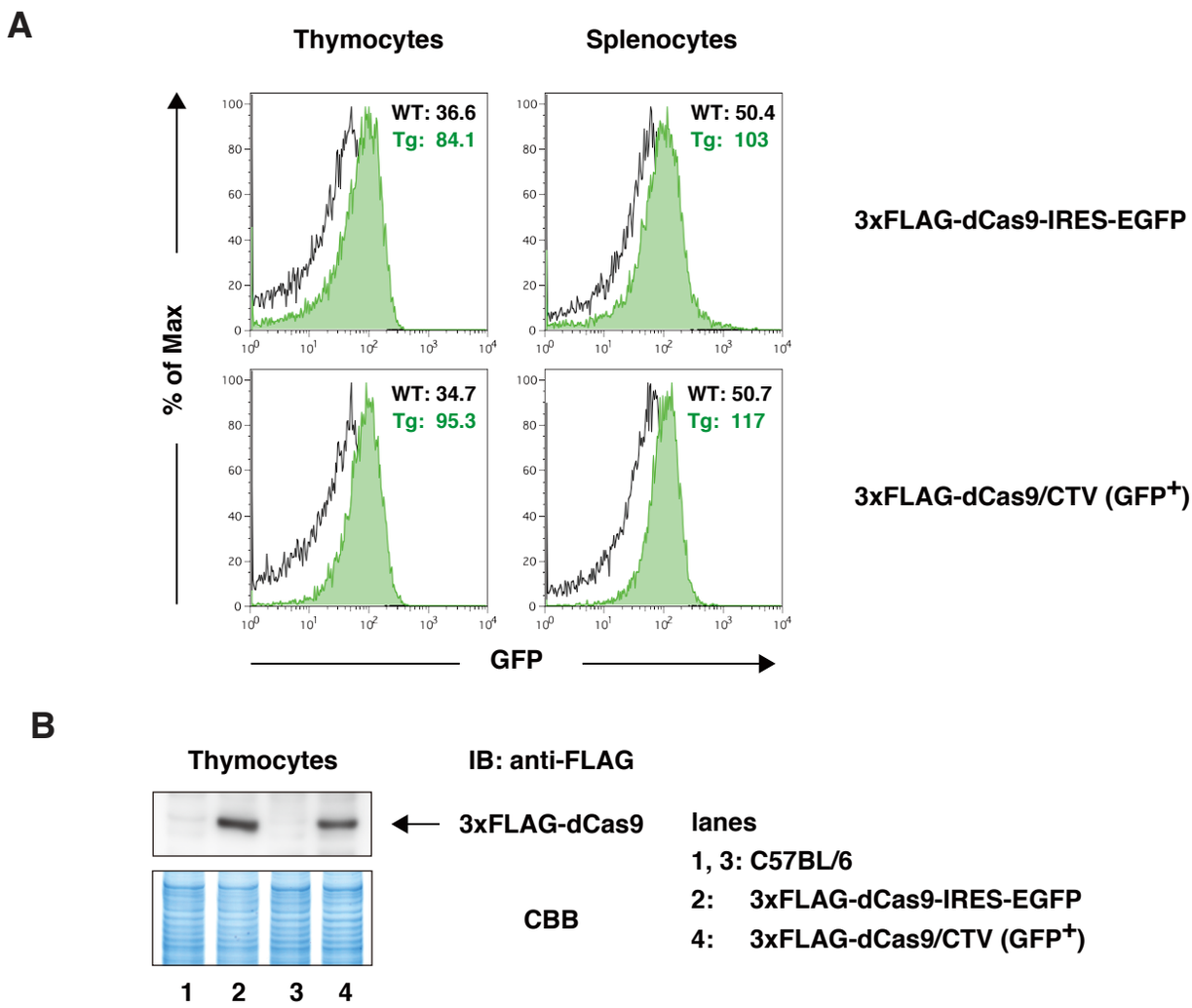


Figure 3