Primordial germ cell-specific expression of eGFP in transgenic chickens

Yota Hagihara[1]*, Yuya Okuzaki[1,2]*, Kazuma Matsubayashi[1], Hidenori Kaneoka[1]**, Takayuki Suzuki[2,3], Shinji Iijima[1], and Ken-ichi Nishijima[1,2,3]

[1]*Department of Biomolecular Engineering, Graduate School of Engineering, Nagoya University, Furo-cho, Chikusa-ku, Nagoya, 464-8603, Japan*

[2]*Avian Bioscience Research Center, Graduate School of Bioagricultural Sciences, Nagoya University, Furo-cho, Chikusa-ku, Nagoya, 464-8601, Japan*

[3]*Laboratory of Avian Bioscience, Graduate School of Bioagricultural Sciences, Nagoya University, Furo-cho, Chikusa-ku, Nagoya, 464-8601, Japan*

*These authors contributed equally

**Corresponding author: Department of Biomolecular Engineering, Graduate School of Engineering, Nagoya University, Chikusa-ku, Nagoya, 464-8603, Japan

Fax: +81 52 789 3221

E-mail: kaneoka@chembio.nagoya-u.ac.jp

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Abstract

PR domain zinc finger protein 14 (PRDM14) plays an essential role in the development of primordial germ cells (PGCs) in mice. However, its functions in avian species remain unclear. In the present study, we used CRISPR/Cas9 to edit the *PRDM14* locus in chickens in order to demonstrate its importance in development. The eGFP gene was introduced into the *PRDM14* locus of cultured chicken PGCs to knockout *PRDM14* and label PGCs. Chimeric chickens were established by a direct injection of eGFP knocked-in (gene-trapped) PGCs into the blood vessels of Hamburger-Hamilton stages (HH-stages) 13-16 chicken embryos. Gene-trapped chickens were established by crossing a chimeric chicken with a wild-type hen with very high efficiency. Heterozygous gene-trapped chickens grew normally and SSEA-1-positive cells expressed eGFP during HH-stages 13-30. These results indicated the specific expression of eGFP within circulating PGCs and gonadal PGCs. At the blastodermal stage, the ratio of homozygous gene-trapped embryos obtained by crossing heterozygous gene-trapped roosters and hens was almost normal; however, all embryos died soon afterward, suggesting the important roles of *PRDM14* in chicken early development.

Keywords

Chicken; CRISPR/Cas9; Primordial germ cells; PRDM14; eGFP

1. Introduction

Transgenic chickens have been constructed by many groups, including ours, using retro- or lentiviral vectors (Kamihira et al., 2005; McGrew et al., 2004; Motono et al., 2010; Zhu et al., 2005). However, in most cases, the germinal transmission of transgenes has been very low, which makes the establishment of transgenic chickens a labor- and time-consuming process. Rapid advances in genome editing technology, particularly that using transcription activator-like effector nuclease (TALEN) and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9), have provided a simple and convenient process for the establishment of transgenic chickens using cultured primordial germ cells (PGCs). *DDX4*- and *ovomucoid*-knockout chickens have already been established by these technologies (Oishi et al., 2016; Taylar et al., 2017). Thus, PGCs are very important for chicken genetic engineering; however, their growth and differentiation mechanisms have not yet been elucidated in detail, thereby limiting the efficiency of manipulations.

PR domain zinc finger protein 14 (PRDM14) is a key regulator of germinal fate and is expressed in PGCs. In mice, *Prdm14* has been shown to function together with *Prdm1* (encoding Blimp1) and *Tfap2* (encoding AP2γ), and repress mesodermal genes in PGCs. In addition, the co-expression of these factors in P19 embryonal carcinoma cells has been suggested to induce PGC-like cells in an *in vitro* model (Kurimoto et al., 2008; Magnúsdóttir et al., 2013; Yamaji et al., 2008). However, limited information is

currently available on the function of chicken *PRDM14*. We previously reported that the higher expression of *PRDM14* was specific to PGCs, except for pluripotent cells at the blastodermal stage and neural plate cells at Hamburger Hamilton stage (HH-stage) 4, as well as to the adult testis. Furthermore, *PRDM14* was essential for the proper development of PGCs *in vivo* (Okuzaki et al., 2019). However, the role of chicken *PRDM14* in early embryonic development remains unknown. We herein report the establishment of an eGFP knocked-in (gene-trapped) chicken targeting the *PRDM14* locus using the CRISPR/Cas9 system. The specific expression of eGFP in PGCs was observed in heterologous gene-trapped embryos. Furthermore, an analysis of its homozygous descendants demonstrated that *PRDM14* is indispensable for early embryonic development.

2. Results and discussion

To clarify the function of *PRDM14* using gene-trapped chickens, we designed a genome-editing strategy. CRISPR/Cas9-mediated homologous recombination was used to target the *PRDM14* locus; the eGFP gene containing the stop codon was inserted immediately downstream of the initiation codon of *PRDM14* based on the sequence we previously reported (Okuzaki et al., 2019). The targeting vector had a 797-bp sequence containing a 5’-untranslated region plus a coding sequence of 12 nucleotides as the

5’-homology arm, and a 779-bp sequence that contained a part of exon 1 and the entire sequence from intron 1 to exon 3 as the 3’-homology arm. The eGFP gene flanked by additional HindIII linker sequences was inserted between these homologous arms (Fig. 1a). Three single-guide RNAs (sgRNAs) were designed based on the 5’-end region of the *PRDM14* coding sequence using CRISPR-direct (https://crispr.dbcls.jp) and one sgRNA sequence was selected based on their recombination activity checked by a single-strand annealing assay. No candidate sequence for off-target sites was predicted by CRISPR-direct for sgRNA.

Cas9 and sgRNA expression and targeting vectors were simultaneously introduced into cultured chicken PGCs, established from a male embryo, by electroporation. Five days post-transfection, eGFP-positive cells were collected using a cell sorter. The percentage of eGFP-positive cells was 1.25%. This percentage increased to approximately 77% by the first sorting and the copy number of eGFP in bulk cells was approximately 0.7. After two-cycle purification by a cell sorter, the fraction of eGFP-positive cells increased to approximately 93% (Fig. 1b). We also isolated several clones and their copy numbers of eGFP were almost 1 (data not shown), suggesting that a single copy of the eGFP gene was introduced into the diploid genome. This was confirmed by PCR using a specific primer. Since homologous recombination resulted in a 33-bp deletion in exon 1, a primer was designed in this deletion sequence for the specific amplification of the original allele (Fig. 1a). Amplification from the original

allele was observed in all samples, while the left and right arms of the transgene were amplified from the PGC clones, but not from wild-type PGC (Fig. 1c). Thus, all of the PGC clones obtained were heterozygous gene-trapped cells. The lack of homozygous gene-trapped clones was consistent with our previous findings showing that *PRDM14* was required for the proliferation of PGCs *in vivo* (Okuzaki et al., 2019).

eGFP-positive PGCs purified by flow cytometry (not cloned) were transplanted into developing 2.5-day embryos as a preliminary trial. After a 3.5-day *in vitro* culture of grafted embryos, the percentages of SSEA-1^{+}eGFP^{+} cells in the gonad were similar or higher than SSEA-1^{+}eGFP^{-} cells (Fig. 2a). The replacement rates in grafted embryo #1, #2, and #3 were 85.7, 52.6, and 81.5%, respectively, suggesting that manipulated PGCs settled in the gonads with high efficiency. We obtained 6 male germline chimeric chickens from the *in vitro* culture of manipulated embryos. After sexual maturation, the copy number of the transgene in the sperm was analyzed by qPCR. The copy number varied by individuals and one chicken (c835) showed the highest copy number of approximately 0.9 (Fig. 2b). Following the artificial insemination of a wild-type hen with c835 semen, 18 transgenic chickens out of 84 descendants were obtained (Fig. 2c), while we could not obtain transgenic chicken from other chimeric roosters. Their copy numbers of eGFP were almost 1 (data not shown) and sequence analyses of genomic DNA from the blood cells of a G1 transgenic chicken showed that the eGFP gene was inserted into the expected site.

In order to confirm the expression of eGFP, the blood of 2.5-day embryos from G1 chickens was analyzed using a flow cytometer. SSEA-1^{+}eGFP^{+} cells were detected in transgenic chickens, but not in wild-type blood cells (Fig. 3a), suggesting that circulating PGCs (cPGCs) expressed eGFP. In the genital ridges of 3.5-day embryos from a G1 chicken, we detected eGFP-positive cells using a fluorescence microscope (Fig. 3b). Flow cytometric analyses showed that eGFP-positive cells were also positive for SSEA-1 in the genital ridge of a transgenic chicken; however, SSEA-1^{+}eGFP^{+} cells were not detected in those of wild-type chickens (Fig. 3c). These results suggested that PGCs in the genital ridge expressed eGFP. In addition, SSEA-1^{+}eGFP^{+} cells were detected in the gonadal cells of a 6.5-day transgenic chicken by flow cytometric analyses, but not in wild-type embryos (Fig. 4a). Fluorescent images of frozen-sectioned gonads showed that eGFP fluorescence was mainly observed in the cortex of gonads (Fig. 4b), which corresponds to the site of accumulation of PGCs in this stage (Ayers et al., 2013). Immunostaining showed that the majority of SSEA-1-positive cells in the gonads expressed eGFP, suggesting that eGFP was specifically expressed in the PGCs of the gonads (Fig. 4c), but not in SSEA-1-negative cells, which contain both somatic and differentiated germ cells at this stage. Collectively, these results show that eGFP is expressed in PGCs in embryonic blood and the gonads of G1 transgenic chicken, reflecting the expression pattern of endogenous *PRDM14*. Therefore, PGCs can be detected by eGFP fluorescence.

In order to investigate the function of *PRDM14* in embryonic development, G2 descendants were obtained by mating between G1 hens and roosters. The appearance of wild-type (*PRDM14*$^{+/+}$) as well as *PRDM14* heterozygous (*PRDM14*$^{+/eGFP}$) and homozygous gene-trapped (*PRDM14*$^{eGFP/eGFP}$) chickens was analyzed at various developmental stages. *PRDM14*$^{eGFP/eGFP}$ embryos were detected at the blastodermal stage at a slightly lower frequency than Mendelian predictions (Table 1). Although eGFP-fluorescence was below the detection limit in the blastoderms of either *PRDM14*$^{+/eGFP}$ or *PRDM14*$^{eGFP/eGFP}$ embryo by flow cytometric analyses (data not shown), a qRT-PCR analysis showed that the expression level of eGFP in *PRDM14*$^{+/eGFP}$ embryos was 50% that in *PRDM14*$^{eGFP/eGFP}$. *PRDM14* in *PRDM14*$^{+/eGFP}$ embryos was 50% that in the wild-type, and *PRDM14* was not detected in *PRDM14*$^{eGFP/eGFP}$ embryos, as expected (Fig. 5). However, after a 2.5-day incubation, *PRDM14*$^{eGFP/eGFP}$ embryos did not survive (Table 1), suggesting the important role of *PRDM14* in the early development of chickens. Furthermore, we did not observe any *PRDM14*$^{eGFP/eGFP}$ embryos after HH-stage 3 (data not shown). This was unexpected because mice lacking *Prdm14* were previously reported to be sterile, but viable (Yamaji et al., 2008), and the knockout of *PRDM14* in Zebrafish did not cause early embryonic lethality, but resulted in motor neuron maturational arrest (Liu et al., 2012). We previously showed that *PRDM14* was expressed in cells other than PGCs, such as in the blastoderm (Okuzaki et al., 2019), and the loss of *PRDM14* expression in these cells

may have caused early embryonic lethality in *PRDM14*$^{eGFP/eGFP}$ chickens. The mechanisms of the lethality, such as perturbed expression of pluripotency-related genes, are remained to be elucidated.

We previously demonstrated that *PRDM14* was essential for the proliferation of PGCs after the blastoderm because the number of PGCs was decreased by *in vivo* injection of replication-competent retrovirus vector containing shRNAs against *PRDM14* (Okuzaki et al., 2019). We failed to confirm that *PRDM14* knockout decreased PGCs because of the early embryonic lethality of *PRDM14*$^{eGFP/eGFP}$. Further detailed studies, such as those using an inducible and/or cell-specific knockout system for *PRDM14* expression, are needed to clarify the stages of PGCs and/or the cells at specific stages that critically required *PRDM14* expression for proper development.

3. Methods

3.1. Chickens and eggs

Fertilized eggs (White Leghorn) were purchased from Nisseiken or Japan Layer. All animal experiments were performed according to the Ethical Guidelines for Animal Experimentation of Nagoya University.

3.2. PGC culture

Blood containing cPGCs was collected from embryos at HH-stages 13-16 and cells from a single embryo were cultured in PGC medium containing KO-DMEM (Thermo Fisher Scientific) supplemented with B-27 supplement (Thermo Fisher Scientific, 1×), L-glutamine (Wako, 2 mM), non-essential amino acids (Wako, 1×), 0.1 mM 2-mercaptoethanol (Wako), nucleotides (Thermo Fisher Scientific, 1×), 100 U/ml penicillin G (Wako), 100 μg/ml streptomycin (Wako), 22 U/ml sodium heparin (Wako), 1.2 mM sodium pyruvate (Thermo Fisher Scientific), chicken serum (0.2%), activin A (Shenandoah Biotechnology, 25 ng/ml), BMP4 (Peptide Institute, 25 ng/ml), and FGF2 (PeproTech, 4 ng/ml). These PGC preparations were derived from a male embryo and almost all cells were SSEA-1- and chicken Vasa homolog (CVH)-positive (Okuzaki et al., 2019).

3.3. Plasmid construction

The plasmid expressing Cas9 and sgRNA was generated by inserting annealed and phosphorylated oligonucleotides corresponding to the targeting sequence (Table S1) into the BbsI site of pX330 (Addgene). In the construction of the targeting vector, the genomic DNA sequence of *PRDM14* was amplified by PCR from the chicken genome as a template with the primers (Table S1). The amplified fragment was cloned into pGL3-basic (Promega). The eGFP coding sequence was then inserted immediately after the start codon of *PRDM14* in-frame to disrupt the PRDM14 protein.

3.4. Transfection and single-cell cloning of PGCs

Cas9/sgRNA expression and targeting vectors were introduced into 1.5×10^5 cells of cultured PGCs by electroporation using the electroporator NEPA21 (NEPAGENE). PGCs were then transferred to PGC medium (Whyte et al., 2015) containing 10 ng/ml Y27632 (Wako) in a 24-well plate. After a one-week culture, eGFP-positive cells were isolated using the flow cytometer FACSJazz (BD Bioscience) and then transferred to a 96-well plate for cloning.

3.5. Genomic DNA analysis

Genomic DNA was purified from PGCs and chicken semen using Mag Extractor-Genome- (TOYOBO). To confirm the presence of the knock-in fragment, targeting sites were amplified with several sets of primers (Table S1), and the copy number of eGFP was measured by LightCycler 96 (Roche Diagnostics) using the genomic DNA of the eGFP transgenic chicken as a control (Motono et al., 2010).

In some experiments, the sex of embryos at days 2.5 –6.5 was determined by PCR of genomic DNA from the body using the primers specific to XhoI repeat sequence on W chromosome as described (Motono et al., 2008).

3.6. Analysis of grafted PGCs in gonads of 5.5-day recipients

To assess the proportion of donor-derived PGCs that had migrated and settled in the gonads of the recipients, 5,000 eGFP-positive PGCs were injected into the blood vessels of HH-stages 13-16 recipient embryos in bulk as described previously (Motono et al., 2010). After an incubation for 3.5 days, recipient embryos were washed with phosphate-buffered saline (PBS) and the gonads were dissected. Cells from the gonads were incubated with an anti-SSEA-1 antibody (Santa Cruz Biotechnology) at 4˚C for 1 h. After washing with PBS, cells were incubated with phycoerythrin (PE)-labeled goat anti-mouse IgM (Santa Cruz Biotechnology) at 4˚C for 30 min. The percentages of SSEA-1^{+}eGFP^{+} cells were measured using a flow cytometer.

3.7. Production of germline chimeric chickens

To produce germline chimeras, approximately 5,000 eGFP-positive PGCs were injected into male embryos as described above. Embryos were cultured and allowed to hatch as previously reported (Motono et al., 2010).

3.8. Progeny test

Germline chimeric chickens were mated with wild-type hens after sexual maturation. Genomic DNA from the blood of offspring was extracted using Easy DNA Extraction kit Version 2 (KANEKA). The primers for PCR are shown in Table S1. All heterozygous gene-trapped chickens used in this research were descendants of c835

chimeric chicken.

Heterozygous gene-trapped chicken line (*PRDM14*$^{+/eGFP}$) will be deposited at Avian Bioscience Research Center and made available to the research community upon request.

3.9. Observation of eGFP expression in PGCs of heterozygous G1 transgenic chickens by a flow cytometric analysis and fluorescent microscopy

Blood containing cPGCs was collected from the 2.5-day embryos of G1 transgenic chickens, and cells were indirectly stained with an anti-SSEA-1 antibody. The percentages of SSEA-1^{+}eGFP^{+} cells were measured using a flow cytometer. To examine the localization of eGFP-positive PGCs, the genital ridges of 3.5-day embryos were observed from the ventral aspect by a fluorescence microscope (Leica Microsystems).

3.10. Immunohistochemistry

The gonads of 6.5-day embryos were dissected and fixed with 4% paraformaldehyde (PFA) at 4˚C for 2 h. The gonads were treated stepwise with 10, 20, and 30% sucrose in PBS for 1 h at each concentration on ice and then embedded in a compound for frozen sections (Leica Microsystems). Samples were sectioned by a cryostat (Leica Microsystems) for immunohistochemistry. Cells in frozen sections were permeabilized by a treatment with 0.2% Triton X-100 (Wako) at room temperature for

20 min, and then blocked with 3% bovine serum albumin (BSA) in PBS for 30 min. Samples were incubated at 4°C overnight with primary antibodies (anti-GFP (Sigma) or anti-SSEA-1) followed by secondary antibodies (anti-mouse IgG-Alexa Fluor 488 (Thermo Fisher Scientific) or anti-mouse IgM-PE) at room temperature for 2 h. After an incubation with 100 ng/ml of DAPI in PBS for 3 min, fluorescent images were taken with an upright microscope (Leica Microsystems).

3.11. qRT-PCR

Total RNA was extracted from blastodermal cells using the ReliaPrep RNA Cell Miniprep System (Promega), and the ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO) were used for cDNA synthesis. Real-time PCR was performed using LightCycler 96 and Thunderbird qPCR Mix (TOYOBO). LightCycler amplification involved pre-denaturation at 95°C for 60 s, followed by 40 cycles of 95°C for 3 s, 60°C for 10 s, and 72°C for 30 s. The primers used are listed in Table S1.

Conflicts of Interest

The authors declare no conflicts of interest associated with this manuscript

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Figure legends

FIGURE 1

Targeting of the chicken *PRDM14* locus in PGCs. (a) Structure of CRISPR/Cas9 targeting sites in *PRDM14*. The upper part of the diagram shows the *PRDM14* wild-type allele. The first 4 exons are shown as boxes, and the 5'-untranslated region is shown by a white box. The position of the deleted sequence by recombination and the sgRNA targeting site are indicated by a hatched box and arrowhead, respectively. The bottom part shows the structure of the knocked-in allele. The left and right homology arms (HA, dotted squares) and the eGFP coding sequence with a stop codon are indicated. The primer sets for the original allele were shown in black arrows, and for the left and right arms in gray and blanked arrows, respectively. (b) Flow cytometric analyses of eGFP fluorescence after the reporter cassette was knocked into the *PRDM14* locus. A flow cytometric analysis was performed after the two-round sorting of eGFP-positive PGCs. (c) Targeted gene-trapping using the reporter cassette was detected by genomic PCR in eGFP knocked-in PGC clones. WT, wild-type genome.

FIGURE 2

Generation of *PRDM14* gene-trapped G1 transgenic chickens. (a) Flow cytometric analysis of the gonadal cells of 5.5-day recipients after grafting eGFP knocked-in PGCs.

a and b gates indicate SSEA-1^{+}eGFP^{-} and SSEA-1^{+}eGFP^{+} cells, respectively. The control was an ungrafted embryo. (b) Copy numbers of the eGFP sequence in the semen of germline chimeric chickens. The genomic DNAs of eGFP transgenic chickens (copy number of 1 (Motono et al., 2010)) were used as a standard. Data are the mean ± standard error of 5 (c828, c834 and c835) or 2 (c830 and c831) different samples. c833 was from a single sample. (c) Detection of the eGFP reporter cassette in the offspring of chimeric chicken c835. Genomic DNAs were extracted from the blood of hatched chicks and subjected to PCR using eGFP-specific primers. *GAPDH* was amplified as an internal control. PC (positive control) is the genome of an eGFP transgenic chicken. Typical results of PCR analyses were shown.

FIGURE 3

Detection of eGFP expression in G1 embryonic PGCs. (a) Flow cytometric analysis of 2.5-day embryonic blood. Blood cells were stained with the anti-SSEA-1 antibody. a and b gates indicate SSEA-1^{+}eGFP^{-} and SSEA-1^{+}eGFP^{+} cells, respectively. (b) Distribution of eGFP-PGCs in the genital ridges of 3.5-day transgenic embryos. The eGFP fluorescence of dissected trunks as observed from the ventral aspect. Genital ridge regions were surrounded by dotted lines. (c) Flow cytometric analysis of eGFP-PGCs in the genital ridges of 3.5-day transgenic embryos. a and b gates indicate SSEA-1^{+}eGFP^{-} and SSEA-1^{+}eGFP^{+} cells, respectively.

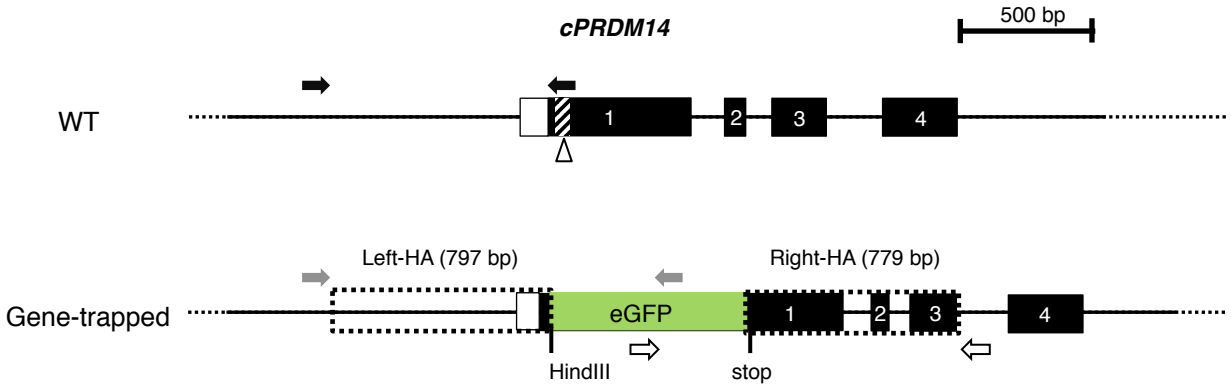
FIGURE 4

PGC-specific expression of eGFP in gonads of transgenic embryos. (a) Flow cytometric analysis of eGFP-PGCs in the gonads of 6.5-day transgenic embryos. Gonadal cells were stained with an anti-SSEA-1 antibody. a and b gates indicate SSEA-1^{+}eGFP^{-} and SSEA-1^{+}eGFP^{+} cells, respectively. (b) Distribution of eGFP-PGCs in the gonads of 6.5-day transgenic embryos compared with a non-transgenic control. (c) Immunohistochemistry of 6.5-day transgenic gonads. Frozen sections were stained with the anti-eGFP antibody and anti-SSEA-1 antibody.

FIGURE 5

Expression of *eGFP* and *PRDM14* in *PRDM14* gene-trapped embryos. RNAs were purified from the blastodermal cells of *PRDM14* gene-trapped embryos and subjected to qRT-PCR. The expression levels of *eGFP* and *PRDM14* are shown as relative expression levels against *GAPDH*. Data are the mean ± standard error (N=19 (+/+), 23 (+/eGFP), 7 (eGFP/eGFP)). * indicates significant differences by the Student's *t*-test ($p<0.01$).

(a)


cPRDM14
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WT
1
2
3
4
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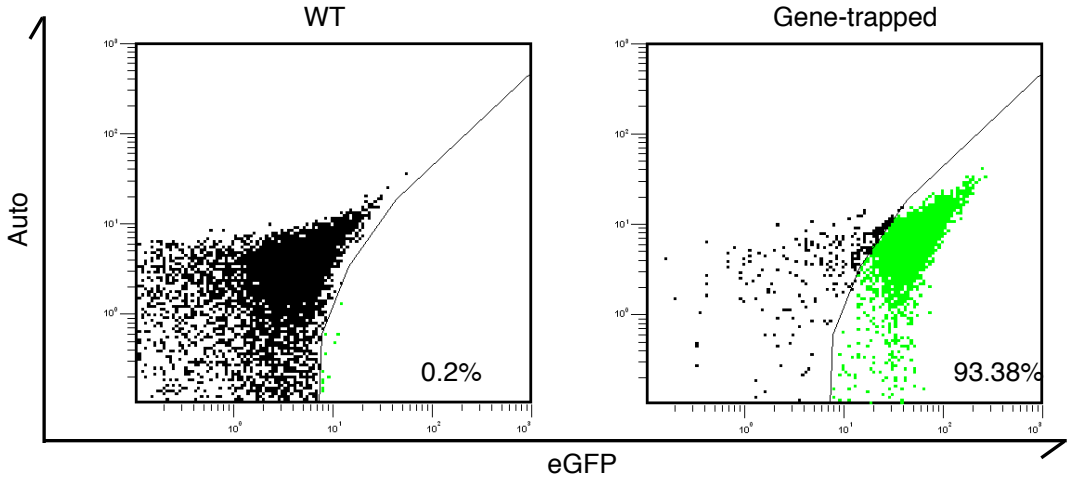
Right-HA (779 bp)
Gene-trapped
eGFP
1
2
3
4
HindIII
stop

(b)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(c)

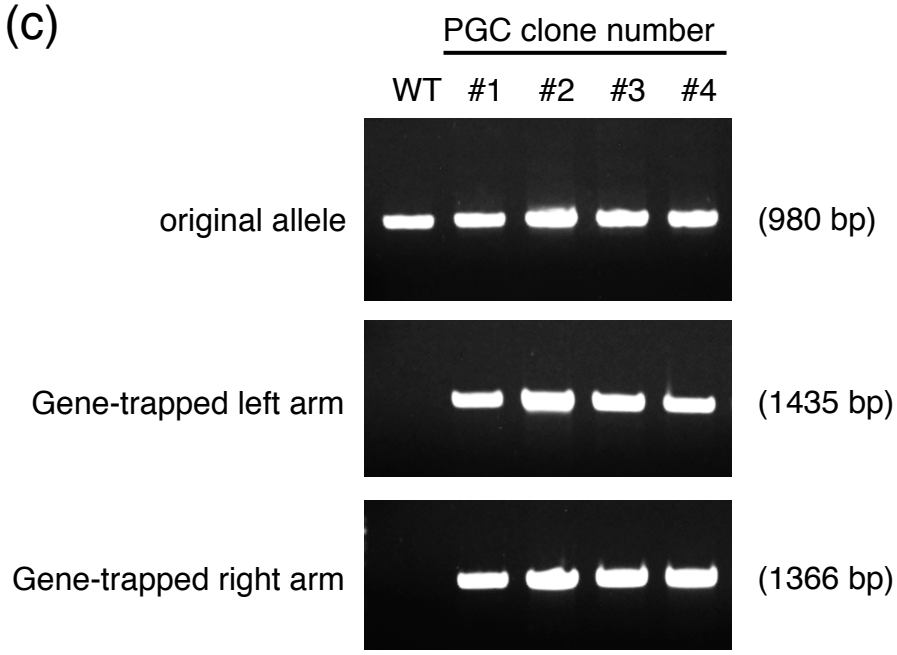
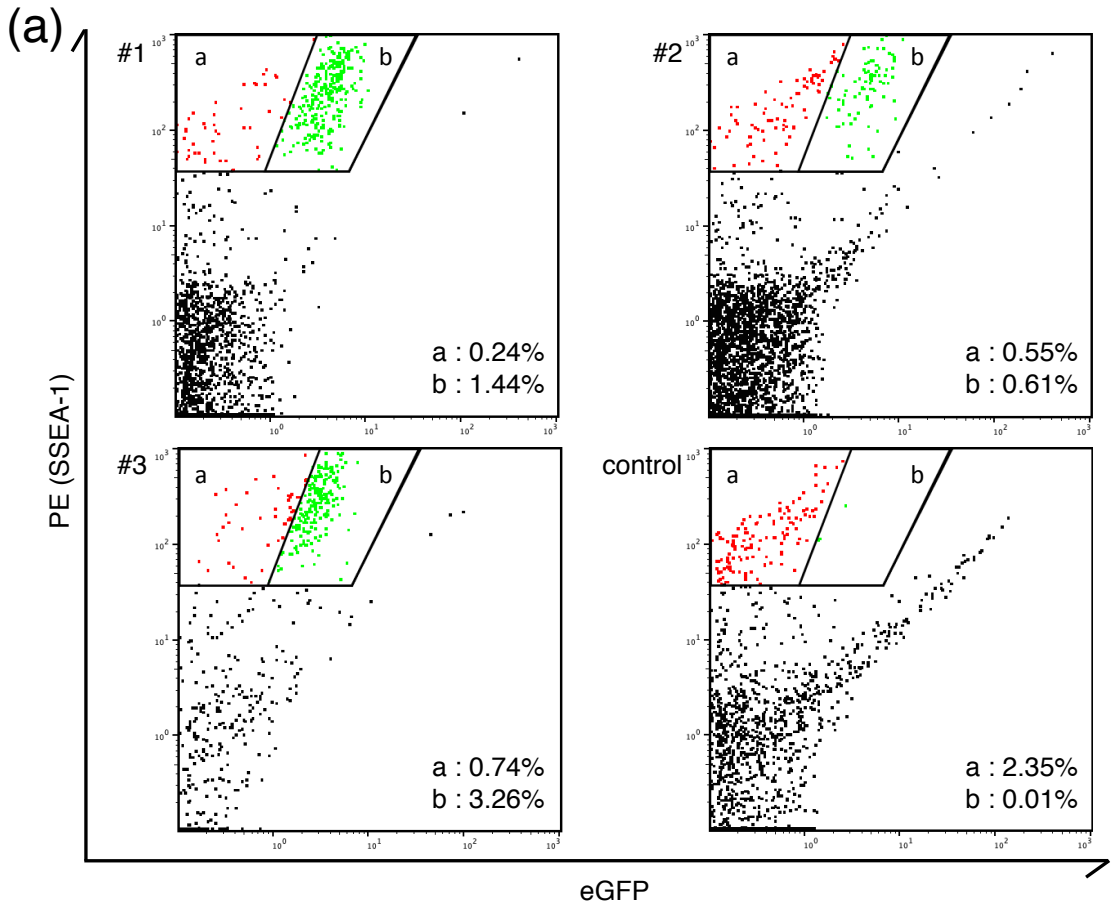
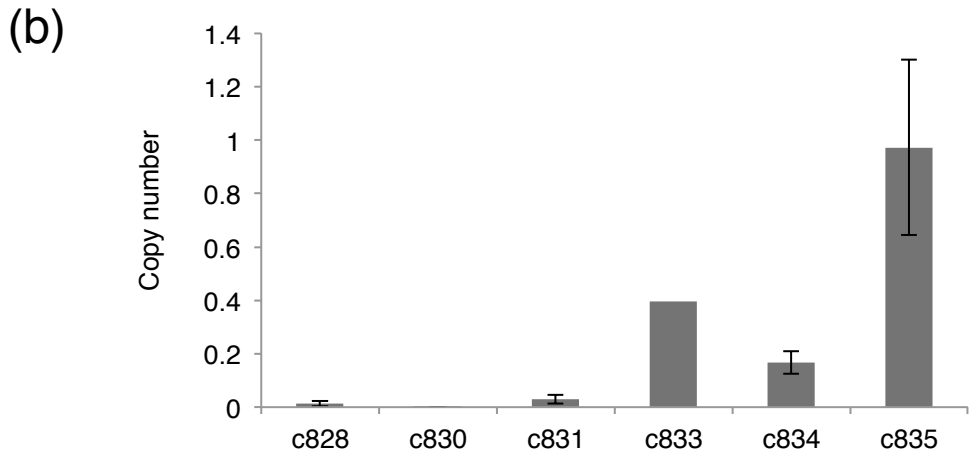

PGC clone number
WT
#1
#2
#3
#4
original allele
(980 bp)
Gene-trapped left arm
(1435 bp)
Gene-trapped right arm
(1366 bp)

Fig. 1


(a)
#1
a
b
a : 0.24%
b : 1.44%
#2
a : 0.55%
b : 0.61%
#3
a : 0.74%
b : 3.26%
control
a : 2.35%
b : 0.01%
PE (SSEA-1)
eGFP


(b)
Copy number
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1.2
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0.6
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c828
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c831
c833
c834
c835

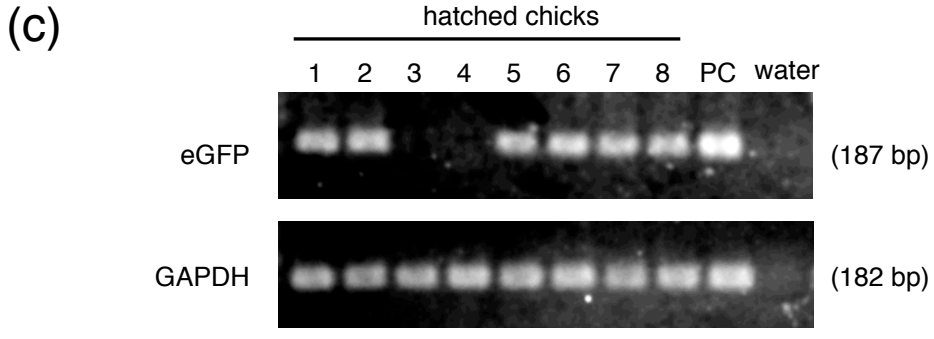

(c)
hatched chicks
1 2 3 4 5 6 7 8 PC water
eGFP
(187 bp)
GAPDH
(182 bp)

Fig. 2

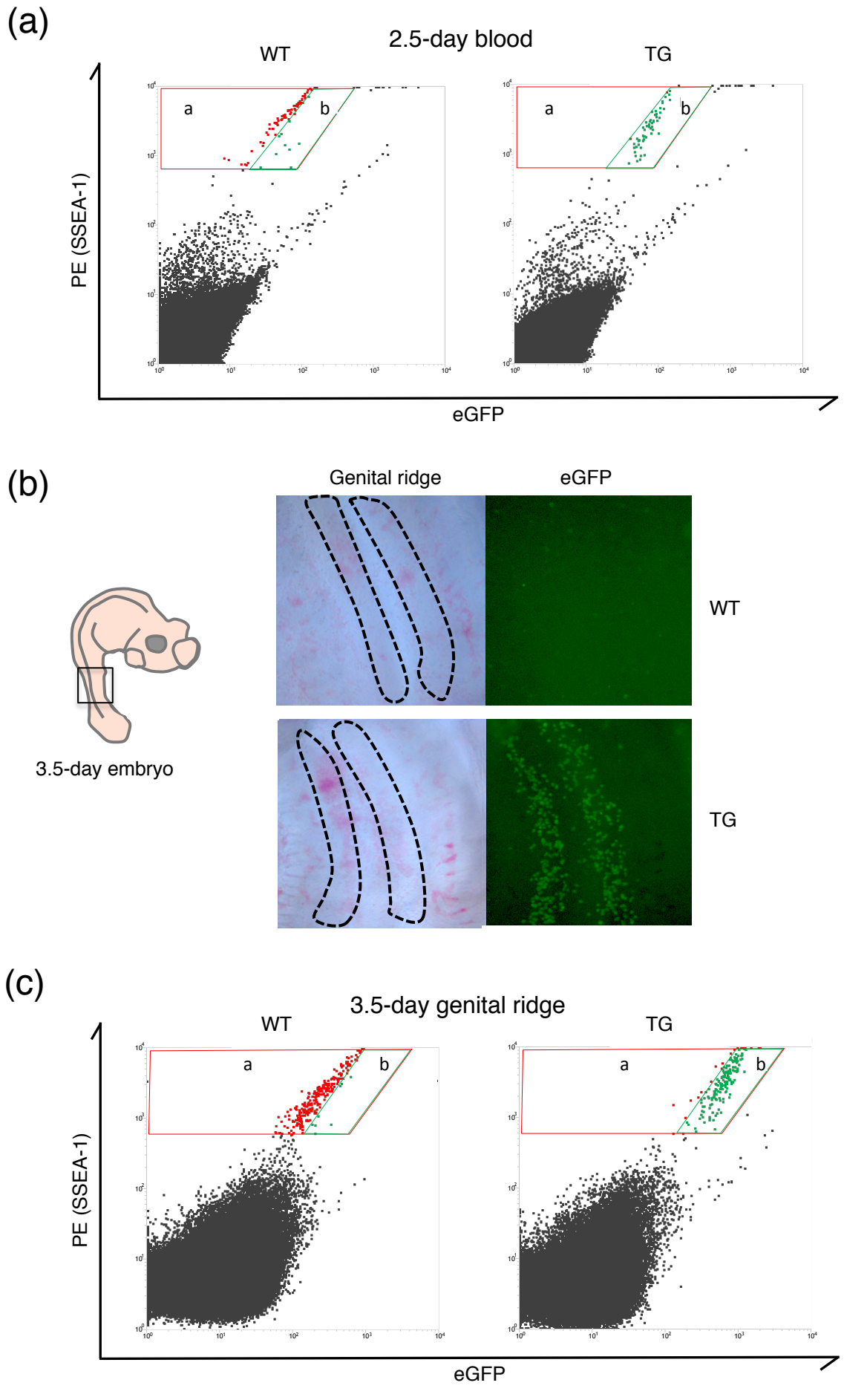
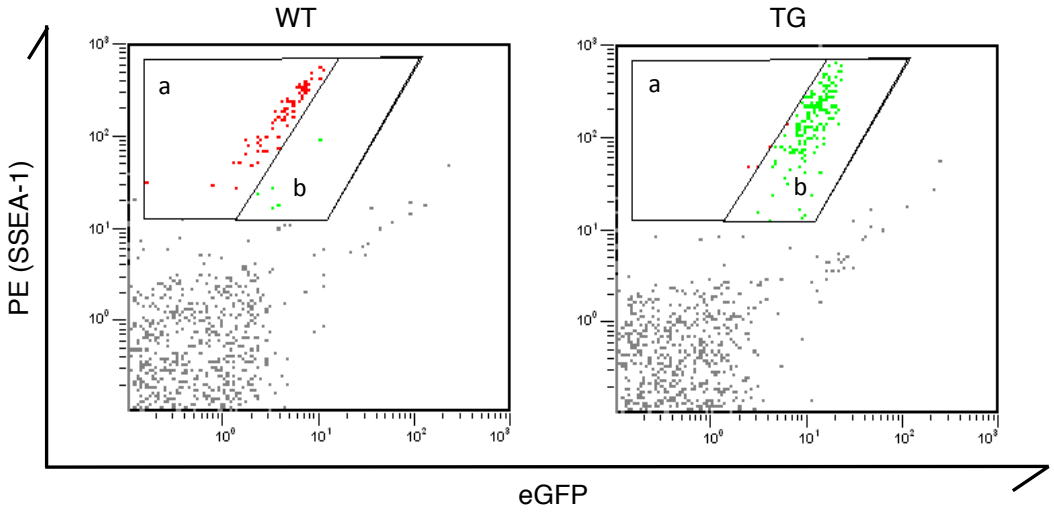
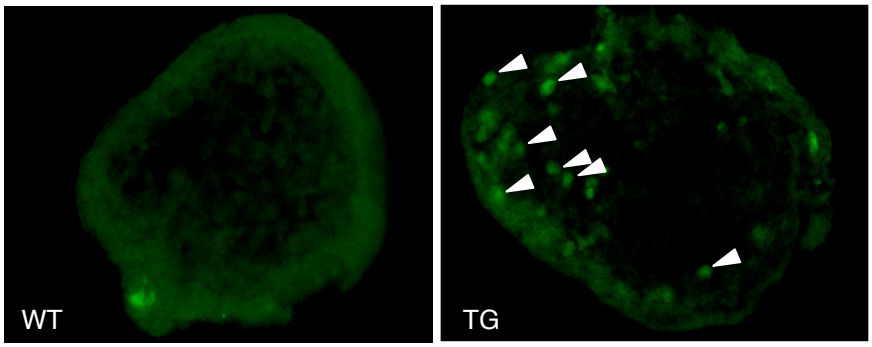

(a)
2.5-day blood
WT
TG
a
b
a
b
PE (SSEA-1)
eGFP
(b)
Genital ridge
eGFP
WT
TG
3.5-day embryo
(c)
3.5-day genital ridge
WT
TG
a
b
a
b
PE (SSEA-1)
eGFP

Fig. 3

(a)


6.5-day gonad
WT
TG
a
b
a
b
PE (SSEA-1)
eGFP

(b)


WT
TG

(c)

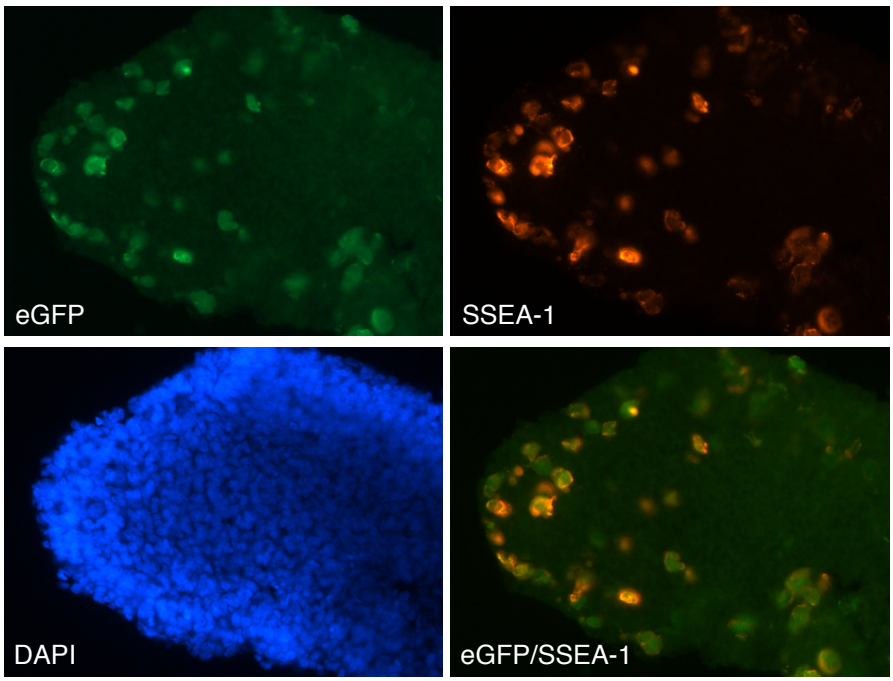

eGFP
SSEA-1
DAPI
eGFP/SSEA-1

Fig. 4

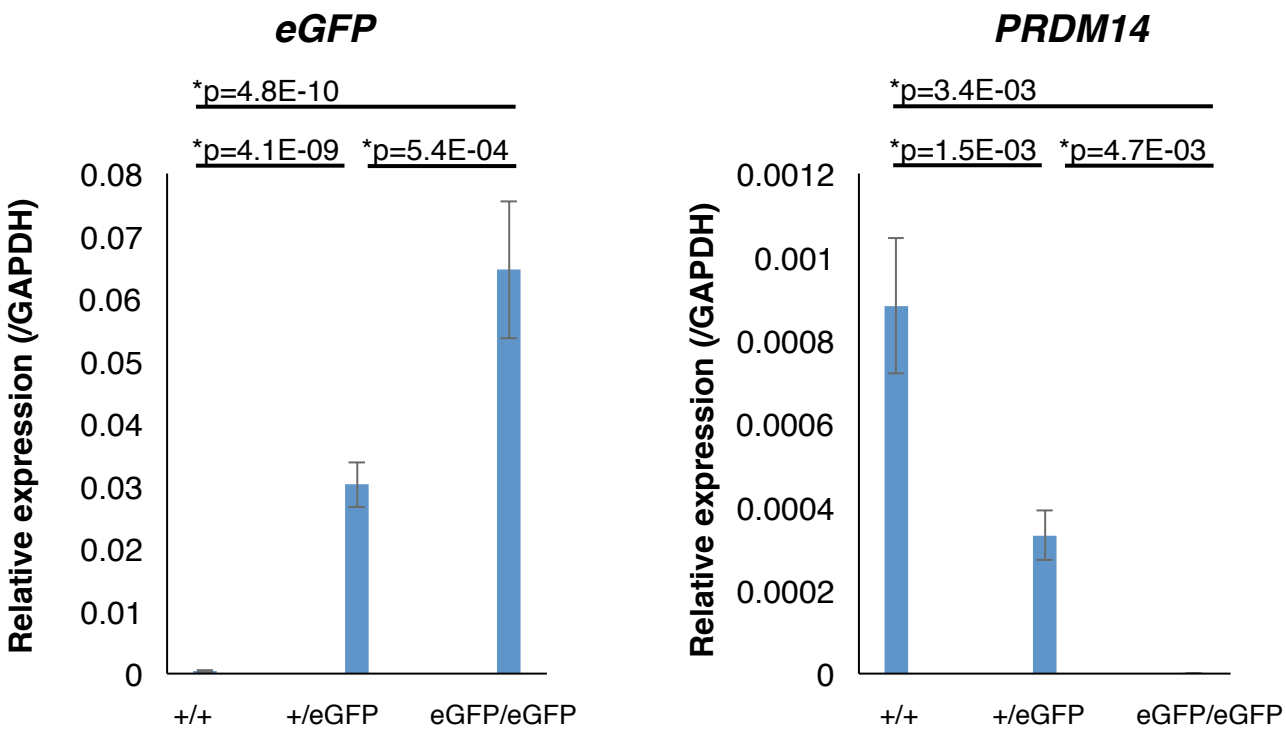

eGFP
*p=4.8E-10
*p=4.1E-09
*p=5.4E-04
Relative expression (/GAPDH)
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
+/+
+/eGFP
eGFP/eGFP
PRDM14
*p=3.4E-03
*p=1.5E-03
*p=4.7E-03
Relative expression (/GAPDH)
0.0012
0.001
0.0008
0.0006
0.0004
0.0002
0
+/+
+/eGFP
eGFP/eGFP

Fig. 5

Table 1: Genotyping of PRDM14 in G2 embryos

	PRDM14$^{+/+}$ embryo (%)	*PRDM14*$^{+/eGFP}$ embryo (%)	*PRDM14*$^{eGFP/eGFP}$ embryo (%)
blastoderm	27 (33.3%)	38 (46.9%)	16 (19.8%)
2.5 days	9 (29.0%)	22 (71.0%)	0
3.5 days	12 (32.4%)	25 (67.6%)	0

Supplementary Table 1: Primer List

Primers for HA cloning and sgRNA		
PRDM14 genome	Dir	CATGGATCCAAATCAGGGCTTTGGAGGATGGC
	Rev	CATCTCGAGCTCCCACATCAGCGAGTTGTCG
PRDM14 sgRNA	Dir	CACCGCGTCCGTGGCGTCCGTGGC
	Rev	AAACGCCACGGACGCCACGGACGC

(Restriction enzymes recognition sites were underlined)

Primers for qRT-PCR and genomic DNA analysis		
GAPDH	Dir	GGGCACGCCATCACTATC
	Rev	GTGAAGACACCAGTGGACTCC
eGFP	Dir	CGGCAACTACAAGACCCGC
	Rev	GAAGTTCACCTTGATGCCGTTC
PRDM14*	Dir	TACAGCGACTCCTCGCCTTT
	Rev	GTCCGTGGCGTCCGTG

*, This primer set amplifies only endogenous PRDM14.

Primers for confirming genome editing		
original allele	Dir	CACAGCTCAGGCTCAGGGTTTC
	Rev	CGAGCGCGTCCGTGGCGT
left arm**	Rev	GAAGTTCACCTTGATGCCGTTC
right arm	Dir	CGGCAACTACAAGACCCGC
	Rev	TCAGCTGCCCGTACTCGAAGAC

**, used in combination with direct primer for original allele.