PRDM14 and BLIMP1 control the development of chicken primordial germ cells

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ABSTRACT

The differentiation of primordial germ cells (PGCs) is a fundamental step in development. PR domain-containing protein 14 (PRDM14) and B lymphocyte-induced maturation protein 1 (BLIMP1) play pivotal roles in mouse PGC specification. In the present study, we assessed the roles of chicken orthologs of PRDM14 and BLIMP1 in PGC development. *PRDM14* and *BLIMP1* were expressed in blastodermal cells and PGCs. The *in vivo* knockdown of *PRDM14* or *BLIMP1* by introducing a replication-competent retroviral vector expressing shRNAs to the blastodermal stage of embryos reduced the number of SSEA-1 or chicken vasa homolog-positive PGCs on day 5.5-6.5. Since the inhibition of Activin receptor-like kinase 4/5/7 in cultured PGCs reduced the expression of *PRDM14*, *BLIMP1,* and *NANOG*, and that of MEK inhibited *PRDM14* expression, the expression of these genes seems to be controlled by Activin A and FGF2 signaling. Overall, *PRDM14*, *BLIMP1,* and *NANOG* seem to be involved in the self-renewal of PGCs in cultured PGCs and embryos.

Keywords: chicken, primordial germ cells, PRDM14, BLIMP1, CVH

1. Introduction

The development of germinal cells, particularly that of primordial germ cells (PGCs), is an important issue for developmental and reproductive biology. In the chicken, PGCs appear at the center of the area pellucida of blastoderms at Eyal-Giladi and Kochav (EK)-stage (Eyal-Giladi and Kochav, 1976) X as SSEA-1-positive cells (Karagenç et al., 1996). After the development of the primitive streak, they translocate anteriorly to the germinal crescent (Howarth, 1995). They then migrate through the vesicular system to the genital ridge, at which they develop to gonadal germ cells and finally to germ cells. Several germ cell-specific genes have been identified, such as *CVH* (chicken vasa homologue) (Tsunekawa et al., 2000) and *DAZL* (deleted in azoospermia-like) (Kito et al., 2010; Rengaraj et al., 2010). Maternally inherited RNA-protein granules (the germplasm) have been supposed to specify germinal cells in the chicken, and the CVH protein is considered to localize to the chicken germplasm (Tsunekawa et al., 2000).

In mice, PGCs can be detected at the extraembryonic mesoderm as alkaline phosphatase-positive cells at approximately E7.5 (Ginsburg et al., 1990). They then migrate through the developing hindgut and colonize the embryonic gonads (Richardson and Lehmann, 2010). B lymphocyte-induced maturation protein 1 (BLIMP1, also known as PRDM1) and PR domain-containing protein 14 (PRDM14) were previously shown to be critical for PGC specification (Kurimoto et al., 2008; Ohinata et al., 2005; Yamaji et al., 2008). Using cultured epiblast-like cells, the overexpression of *Prdm14* was found to be sufficient for the induction of differentiation to PGCs although the combination of *Prdm14*, *Blimp1,* and *Tfap2c* facilitated this process more efficiently (Nakaki et al., 2013). In the chicken, the involvement of PRDM14 and BLIMP1 in the development of PGCs has not yet been clarified.

Chicken PGCs isolated from embryos have been cultured long-term *in vitro* using STO or BRL cells as the feeder layer (Song et al., 2014; van de Lavoir et al., 2006). Whyte et al. recently reported that FGF2, Activin A, and insulin were sufficient for the self-renewal of chicken PGCs without feeder cells in a defined medium, and that PGCs cultured under these conditions were germline-competent. Activin A can be replaced by BMP4 under certain conditions but BMP4 was inefficient for the clonal growth of PGCs (Whyte et al., 2015). Their cognate-signaling pathways were also elucidated:

ERK (FGF2) and SMAD2 (Activin A) were activated upon growth factor stimulations to support the proliferation of PGCs.

In the present study, we investigated the possible roles of PRDM14 and BLIMP1 in chicken PGC development and found that these factors regulate PGC development by supporting PGC self-renewal.

2. Results

2.1. Characterization of the chicken PRDM14 gene

Since the precise structure of the chicken *PRDM14* gene, particularly that of the 5'-untranslated region, has not yet been elucidated, we performed 5'-RACE and nested PCR to identify the 5' terminus of the gene and found that the transcriptional initiation site resided 85 bp upstream of the initiation codon. A DNA sequence analysis revealed that exon 1 encompassed from +1 (demonstrated in the present study) to +520, which was longer than the predicted exon 1 sequence in the NCBI database (gene ID 100858709) (Fig. S1A). The present results also showed that predicted exon 2 in the database was composed of two shorter exons. The amino acid sequence of this region differed from that of the database (Fig. S1B), but was consistent with that of quail (gene ID: 107310217), suggesting that the exon-intron structure elucidated in this study was legitimate. The locations of further downstream exons were consistent with those of the chicken database. In brief, the chicken PRDM14 protein was composed of 486 amino acids, and the PR/SET domain and C-terminal Zn-finger domains (6 repeats) were well conserved, whereas the N-terminal region was divergent between the mouse and chicken (Fig. S1C). A deletion mutant that lacked N-terminal 165 amino acid residues was constructed. Although an eGFP-fusion protein of full-length PRDM14 was located in the nucleus when expressed in mammalian cells, that of the truncated form of PRDM14 was detected both in nucleus and cytoplasm (Fig. S2). This result suggested that the variable N-terminal region contained a nuclear localization signal, although we cannot detect a typical consensus signal sequence. A similar N-terminal function was observed with mouse PRDM14 (Burton et al., 2013).

2.2. Expression of PRDM14 and BLIMP1 in the chicken embryos

Since *Prdm14* expressed specifically in PGCs comparing to neighboring somatic cells in mice (Kurimoto et al., 2008), the expression of *PRDM14* in chicken embryos was analyzed. RNAs were isolated from embryos at EK-stages X (blastodermal cells) to XIII and subsequent Hamburger and Hamilton (HH) stages (Hamburger and Hamilton, 1951) 1 to 3, and *PRDM14* mRNA levels were measured by qRT-PCR. Figure 1A shows expression levels in embryos at each developmental stage. *PRDM14* mRNA levels decreased between EK-stages X and XII, and those at EK stage XII onwards were less than 10% of those at EK-stage X. In adult chickens after sexual maturation and one-week chick organs, qRT-PCR analyses showed that the expression of *PRDM14* was very low (less than 1% of EK-stage X embryos) (Fig. S3A, B), except for the testis of adult chickens. We then examined the expression of *PRDM14* in chicken PGCs. Chicken PGCs were isolated as SSEA-1-positive cells (Karagenç et al., 1996) from the blood of 52 h embryos (circulating PGCs, cPGCs), the genital ridge of 3-day embryos (grPGCs), and the gonads of 5.5-day embryos (gPGCs) by a cell sorter. Almost 100% of SSEA-1-positive cells were CVH-positive by immunostaining (data not shown). *PRDM14* mRNA levels detected by qRT-PCR were very high in cPGCs at 52 h, and gradually decreased with development (Fig. 1B). Although blastodermal cells expressed *PRDM14*, its mRNA level was approximately 30% that of cPGCs (Table S1). *PRDM14* was not expressed in SSEA-1-negative somatic cells from the genital ridge (gr SSEA1$^-$ in Fig. 1B) or gonadal cells (g SSEA1$^-$ in Fig. 1B).

The space temporal expression of *PRDM14* was also detected using *in situ* hybridization. Densely stained cells were observed in the center of the area pellucida in EK stage X embryos. At this stage, the number of PGCs is considered to be approximately 25-45 (Karagenç et al., 1996); however, markedly higher numbers of stained cells were observed, suggesting that cells other than PGCs expressed *PRDM14* (Fig. 2A). At HH-stage 4, stained cells were detected in the germinal crescent and in neural plate (Fig. 2B). With development, cells in the genital ridge were stained at HH-stage 17 (Fig. 2C). The localization of stained cells in the germinal crescent at HH-stage 4 and genital ridge at HH-stage 17 coincided with that reported for chicken PGCs (Howarth, 1995). Thus, PRDM14 was strongly expressed in PGCs although the expression was observed in several cell types other than PGCs in very early stages of development. A recent study reported that chicken PGCs expressed higher levels of

PRDM14 than embryonic stem (ES)-like cells (Zhang et al., 2015).

BLIMP1 is known to be expressed by many cell types in the chicken although its level was found to be low and varied in somatic cells (Wan et al., 2014). In the present study, *BLIMP1* was strongly expressed in cPGCs and grPGCs, and its mRNA level in PGCs decreased with development (Fig. 1C). Its mRNA level in blastoderms was approximately 55% that of cPGCs (Table S1), and a certain expression level was observed in somatic cells at HH-stages 16-18 (gr SSEA-1$^-$ in Fig. 1C) and HH-stages 25-28 (g SSEA-1$^-$ in Fig. 1C).

2.3. In vivo knockdown of PRDM14 and BLIMP1 in the chicken blastoderm

The result showing that *PRDM14* and *BLIMP1* were strongly expressed in chicken PGCs suggests that these two genes play particular roles in chicken PGC development. To investigate this possibility, shRNAs against these genes were introduced into blastoderms *in vivo* using a replication competent retroviral RCAN vector (Morgan and Fekete, 1996). To increase the efficiency of the knockdown, three different shRNA sequences were tandemly aligned in the vectors under the control of different chicken Pol III promoters that have frequently been used for the expression of shRNA (Cummins et al., 2011; Kudo and Sutou, 2005) (Fig. 3A). These shRNAs effectively repressed the exogenous expression of *PRDM14* and *BLIMP1* when transfected to DF-1 chicken fibroblasts (Fig. 3B, C). After the injection of RCAN viruses to the subgerminal cavity of blastoderms, embryos were incubated at 38°C. Successful viral vector infection was confirmed by the expression of viral *Gag-Pol* in the mesonephros (data not shown). On day 5.5 (for SSEA-1) or 6.5 (for CVH), the gonads were isolated and the numbers of PGCs as SSEA-1 and CVH-positive cells were counted. Although PGC numbers varied slightly depending on individual embryos, statistical differences were observed between PRDM14-knockdown and control embryos into which shRNAs against the ovalbumin gene (*OVAL*) were introduced. When shRNAs against *PRDM14* were infected, the percentages of SSEA-1-positive cells against total gonadal cells were lower than the control embryos (Fig. 3D and Fig. S4A). Similar results were obtained with CVH-positive cells on day 6.5 (Fig. 3E and Fig. S4B). The percentage of CVH-positive cells (on day 6.5) was higher than that of SSEA-1-positive cells (on day 5.5) because of differentiation to SSEA-1-negative germ cells. The size and apparent

morphology of the gonads were not affected by the knockdown of *PRDM14* (Fig. S5), suggesting that the formation of gonads was normal. In the course of our research, the apparent difference between male and female embryos was not observed in the percentages of surviving PGCs (Fig. S6). Furthermore, the percentage of SSEA-1-positive cells toward total gonadal cells on day 5.5 with control shRNA was similar to that of non-manipulating embryos (2-4% at the same developmental stage). We collected the remaining SSEA-1-positive cells after the viral vector infection and evaluated the expression of PGC-related genes. Although viral *Gag-Pol* mRNAs were detected in all samples (data not shown), the expression of *PRDM14*, *BLIMP1*, *NANOG*, *CVH*, and *DAZL* of *PRDM14*-knockdown PGCs was slightly less than that of ovalbumin-knockdown control PGCs (Fig. S7). Based on these results, we assumed that a fraction of PGCs survived because of inefficient knockdown.

We then analyzed the impact of *BLIMP1* on the development of PGCs in similar experiments. The numbers of SSEA-1- and CVH-positive cells decreased following the knockdown of *BLIMP1* (Fig. 3F, G and Fig. S4), and surviving cells expressed similar levels of *NANOG* and slightly lower levels of *BLIMP1*, *PRDM14*, *CVH*, and *DAZL* to the control (Fig. S7), although the viral vector was successfully infected. The results shown in Fig. 3 suggest that *PRDM14* and *BLIMP1* were involved in PGC development *in vivo*. However, we cannot rule out the possibility that the knockdown of these genes affected the proper differentiation of somatic cells, with a decrease in PGC numbers being a secondary effect, because *PRDM14* appeared to be expressed in blastodermal cells other than PGCs (Fig. 2A) and a low level of *BLIMP1* was expressed in somatic cells (Fig. 1C).

2.4. Knockdown of PRDM14 and BLIMP1 in cultured PGCs

In order to gain insights into the physiological functions of PRDM14 and BLIMP1 in PGC development, the siRNAs of these genes were introduced into cultured PGCs. In these experiments, we obtained PGCs from one embryo and expanded them for more than 100 generations without cell cloning. Two lots of long-term cultured PGCs were used in knockdown experiments (PGC#1 derived from a male embryo and #2 from a female embryo) (Fig. S8A). We confirmed that PGC#1 differentiated to sperm in host chickens after grafting and successfully produced offspring (Hagihara et al.,

unpublished results). These cultured PGCs were SSEA-1-positive (Fig. S8B) and the expression of *PRDM14*, *CVH*, and *DAZL* was similar to or stronger than that in gonadal PGCs (gPGCs). The expression of *BLIMP1* and *NANOG* in #1 was similar to that in gPGCs, but was weaker in #2 (Fig. S8C).

Two different siRNAs against *PRDM14* were introduced into PGCs by electroporation. Electroporation provided better results for the introduction of siRNAs to PGCs than transfection with various lipofection reagents; however, efficiency was still low. The expression of *PRDM14* decreased to less than 50% of the original level (Fig. 4A) and the expression of *NANOG* was almost 60% that of the control (Fig. 4A), suggesting that *NANOG* was at least partly controlled by PRDM14. On the other hand, the levels of *CVH*, *DAZL*, and *BLIMP1* did not significantly change. The PGC preparation derived from the female embryo (#2) provided a similar expression pattern to PGC #1. To clarify whether PRDM14 transactivates *NANOG* expression directly as a transcriptional regulator, a reporter assay and chromosome immunoprecipitation (ChIP) assay were conducted with the promoter-flanking region up to -3000 bp. However, we did not obtain clear results to show the transactivation of the *NANOG* promoter by PRDM14 (Fig. S9).

When two different siRNAs against *BLIMP1* were introduced into PGCs, the expression of *BLIMP1* decreased to less than 40% of control-siRNA cells. *CVH* and *DAZL* were also not affected by the knockdown of *BLIMP1*. *NANOG* expression levels decreased to between 25 and 70% of the control, suggesting that BLIMP1 also affected *NANOG* expression (Fig. 4B). In addition, *PRDM14* mRNA levels decreased to less than 50% of the control in both PGC preparations (Fig. 4B). These results suggest that the knockdown of *PRDM14* did not affect the expression of *BLIMP1*, whereas the knockdown of *BLIMP1* decreased *PRDM14* levels, which indicates that BLIMP1 partially regulates the expression of *PRDM14*. A reporter assay was performed to confirm the involvement of BLIMP1 in the control of *PRDM14* expression. As shown in Fig. 5A, full-length and N-terminal and SET domain-less BLIMP1 stimulated *PRDM14* promoter activity, while Zn finger-less BLIMP1 did not show transactivation, suggesting that BLIMP1 was involved in the expression of *PRDM14* as a transcriptional regulator. In addition, BLIMP1 also appeared to activate the *NANOG* promoter based on the results of the reporter assay (Fig. 5B). The results of reporter assays were supported by those of ChIP assays showing that BLIMP1 bound to upstream regions of

PRDM14 (around -2.5 kb from the transcription start site) and *NANOG* (around -1.0 kb from the translation initiation site) but not *GAPDH* (the coding region) (Fig. 5C, D, E). Since we were unable to perform rescue experiments against siRNA knockdown because of the low transfection efficiency, we cannot exclude the possibility that the results of siRNA experiments were artifacts of transfection. However, the results of reporter assays support BLIMP1 controlling the expression of *PRDM14* and *NANOG*.

2.5. Growth signals affect the expression of PRDM14 and BLIMP1

Activin A, insulin, and FGF2 are known to be sufficient for the proliferation of PGCs in a defined medium (Whyte et al., 2015). To clarify whether FGF2 and Activin A and their cognate signals (ERK and SMAD2/3, respectively) affect the expression of *PRDM14*, *BLIMP1*, and *NANOG*, the MEK inhibitor PD0325901 and ALK4/5/7 inhibitor A83-01 were added to the culture medium of PGCs. In both cases, the inhibitors repressed the phosphorylation of the downstream signaling molecules (ERK with PD0325901 and SMAD2 with A83-01, Fig. 6A, B). As shown in Fig. 6C, PD0325901 down-regulated the expression of *PRDM14*. It is difficult to assess the physiological effects of the inhibitor on *NANOG* mRNA levels because mRNA levels appeared to gradually decrease by 12 h and subsequently increase. The inhibitor did not exert obvious effects on *BLIMP1* mRNA. With A83-01, the expression of *BLIMP1* and *NANOG* decreased in a parallel manner and *PRDM14* mRNA appeared to decrease with a lag period of approximately 12 h (Fig. 6D). This delay in the down-regulation of *PRDM14* may be a secondary effect of the decline in BLIMP1, as suggested by the finding that BLIMP1 controlled the expression of *PRDM14* (Fig. 4B). If this is the case, crosstalk between Activin A and FGF2 signals may fine-tune the expression of *PRDM14*. In the mouse, a similar observation was reported that the maintenance and up-regulation, but not induction of *Prdm14* were controlled by BLIMP1 in PGCs from a transgenic reporter analysis and single cell cDNA analysis (Kurimoto et al., 2008).

2.6. Effects of PRDM14 and BLIMP1 overexpression in blastodermal cells on the expression of germ cell-specific genes

The present results showed that PRDM14 and BLIMP1 played critical roles in PGC

proliferation and possibly in the maintenance of potential pluripotency. Since PRDM14 and BLIMP1 are transcriptional regulators, we investigated whether these proteins affect the expression of germ cell-specific genes. The *PRDM14* and *BLIMP1* genes were ectopically expressed in cultured blastodermal cells. Blastodermal cells were cultured in the presence of a MEK inhibitor (PD0325901), GSK-3β inhibitor (CHIR99021), and leukemia inhibitory factor (LIF), which are known to support the growth of mouse ES cells (ES medium). Blastodermal cells proliferated very well in ES medium. The expression levels of *BLIMP1*, *NANOG*, and *PRDM14* appeared to be high, at 30-80% those of cPGCs before cultivation (Table S1). *CVH* and *DAZL* were also expressed, but at levels that were less than 3% those of cPGCs (Table S1). The expression of *BLIMP1* remained constant after the 3-day culture in ES medium (Table S1). On the other hand, the expression levels of *PRDM14*, *NANOG*, *CVH*, and *DAZL* decreased to 1-6% those of fresh blastodermal cells after the 3-day culture (Table S1). One day after seeding, blastodermal cells were transfected with a *PRDM14* expression vector that contained the eGFP gene (Fig. S10A). eGFP-positive cells were collected by a cell sorter two days after the transfection in order to analyze the expression of germ cell-specific genes. The expression of *CVH* and *DAZL* was up-regulated by seven- and two-fold, respectively (Fig. 7A). These results suggested that *PRDM14* affects the expression of germ cell-specific genes, although the absolute levels of the *CVH* and *DAZL* transcripts were markedly lower than those of cPGCs. The overexpression of *PRDM14* did not affect the level of endogenous *BLIMP1* (Fig. 7A). When the BLIMP1-eGFP vector (Fig. S10C) was transfected into blastodermal cells, the expression levels of *CVH* and *DAZL* increased by approximately nine- and four-fold, respectively (Fig. 7B). To exclude the possibility that cytokines and inhibitors in the medium affected the expression of these genes, cells were cultured in the DMEM without these factors, which was similar to "differentiation medium" that caused an exit from the pluripotent state of ES towards several types of cells (Lavial et al., 2009). We confirmed that the expression levels of *PRDM14, CVH,* and *DAZL* after the culture in differentiation medium were reduced to 2-12% those in fresh blastodermal cells (Table S1). Following the transfection of the expression vector for either *PRDM14* or *BLIMP1*, the up-regulation of *CVH* and *DAZL* was similar to that in ES medium (Fig. S11). The expression of *PRDM14* was facilitated almost 50-fold by the expression of BLIMP1 (Fig. S11)**.** Overall, the expression of *CVH* and *DAZL* was stimulated to a certain extent

in the blastoderm by the ectopic expression of *PRDM14* and *BLIMP1*. On the other hand, DF1 fibroblasts did not express *CVH* or *DAZL* under similar conditions (data not shown).

In order to obtain further evidence for the involvement of PRDM14 and BLIMP1 in the expression of *CVH* and *DAZL*, an *in vitro* reporter assay was conducted with the *CVH* promoter. 293FT cells were co-transfected with a plasmid containing a 2700 bp full-length or several deleted *CVH* promoters and a *PRDM14* expression plasmid. As shown in Fig. 8A, the fragments containing 600 bp or longer sequences from the initiation codon facilitated luciferase activity. To confirm the transcriptional activation of PRDM14 to the *CVH* promoter, truncated forms of PRDM14 were also applied. Deletions in the N-terminal or PR/SET domain of PRDM14 partially decreased luciferase activity, whereas the complete loss of activity was observed with a Zn finger-less mutant (Fig. 8B), suggesting binding to the DNA fragment through the Zn finger domain of PRDM14. A ChIP assay was also conducted with an anti-PRDM14 antibody with cultured PGCs. A DNA fragment between -423 to -320 from the initiation codon, which resides just upstream of supposed exon 1, was amplified from immunoprecipitated DNA (Fig. 8C), suggesting that PRDM14 bound to the *CVH* promoter-flanking region in cultured PGCs. The *GAPDH* gene was not precipitated (Fig. 8D). These results are consistent with those of the reporter assay. We also performed an *in vitro* reporter assay for the *DAZL* promoter and found that the promoter-proximal region of *DAZL* (up to 3000 bp from the initiation codon) exhibited enhanced reporter activity of approximately three-fold that of PRDM14 (Fig. 8E). Taken together with the ChIP assay on cultured PGCs that showed the binding of PRDM14 to the -1.2 kb region of the *DAZL* promoter (Fig. 8F), PRDM14 may bind to the promoter-proximal regions of *DAZL* and enhance promoter activity. A similar reporter assay was performed with BLIMP1. BLIMP1 enhanced *DAZL* promoter activity, whereas the obvious up-regulation of the *CVH* promoter was not observed (Fig. S12A, B). The binding of BLIMP1 to the promoter-proximal region of *DAZL* (around -1.2 kb from the initiation codon) was confirmed by the ChIP assay with cultured PGCs (Fig. S12C). These results suggested that BLIMP1 bound to the *DAZL* promoter and transactivated its expression, but did not activate the *CVH* promoter as long as the 2.7 kb fragment was used. Since the BLIMP1-binding sites localized in the far upstream region of promoters around -10 kb in many cases (Mitani et al., 2017), there still remains the possibility that the

transcription factor may bind to a far upstream region. Overall, BLIMP1 potentially activates *DAZL* expression and PRDM14 transactivates both *CVH* and *DAZL* possibly as a transcriptional regulator. However, we cannot rule out the possibility that other transcription factor(s) control the expression of these genes in cooperation with PRDM14 and BLIMP1 under physiological conditions because the rate of activation in the reporter assays appeared to be relatively low. Indeed, predicted transcription factor binding sites other than PRDM14 were reported with the *CVH* promoter-flanking region (Jin et al., 2017).

3. Discussion

In mice, extensive analyses have been conducted on the specification of PGCs, and PRDM14 as well as BLIMP were identified as key regulators for PGC specification. BLIMP1 represses the somatic mesodermal program represented by *Hoxb1* gene expression (Kurimoto et al., 2008; Ohinata et al., 2005) and PRDM14 facilitates the reacquisition of pluripotency and epigenetic reprogramming (Kurimoto et al., 2008; Yamaji et al., 2008). In the present study, we demonstrated that the *in vivo* knockdown of *PRDM14* and *BLIMP1* after hatching decreased the numbers of PGCs (Fig. 3), suggesting that both genes play pivotal roles in PGC development after specification since the specification event occurs before hatching. FGF2 and Activin A are essential for the clonal growth of chicken PGCs without feeder cells (Whyte et al., 2015). We found that the expression of *PRDM14* and *BLIMP1* was controlled by FGF2 and Activin A signals, respectively, in cultured PGCs (Fig. 6), suggesting that these genes are involved in the self-renewal of PGCs. This function of PRDM14 and BLIMP1 appears to be one of the reasons for the decreased PGC number in *in vivo* knockdown experiments, as shown in Fig. 3. In addition, the expression of *NANOG* was controlled by Activin A because the Smad2/3 inhibitor repressed expression. *Nanog* is a potent pluripotency-related gene in mammals (Chambers et al., 2007). The involvement of this gene in PGC differentiation and survival has been reported in mice. The absence of *Nanog* results in the failed maturation of PGCs (Chambers et al., 2007) and induces apoptotic cell death (Yamaguchi et al., 2009). The expression of *Nanog* can induce PGC-like cells from epiblast-like cells, which are derived from naive pluripotent ES cells (Murakami et al., 2016). These findings indicate the importance of NANOG in

mouse PGC development. In the chicken, the NANOG protein is localized in epiblast and germ cells in early embryos (Nakanoh et al., 2015) and was supposed to be required for maintaining the pluripotency of chicken ES-like cells (Lavial et al., 2007).

It was speculated that Activin/TGF-β signaling controls the expression of *NANOG* in cultured chicken PGCs (Whyte et al., 2015). This notion is consistent with previous findings. *NANOG* is controlled by the Activin/TGFβ signal in human ES cells (Vallier et al., 2009; Xu et al., 2008). The expression of *NANOG* in chicken epiblasts is modulated by the Activin/TGF-β signal (Shin et al., 2011). We also showed that Activin A induced *NANOG* expression in cultured PGCs. However, in contrast to the present results, a previous study reported that inhibitors of the Activin A/TGF-β signal did not affect *NANOG* expression in chicken PGCs (Shin et al., 2011). One of the possible reasons for this discrepancy seems to be differences in the experimental set-up because they treated early-stage embryos with the inhibitor, whereas we used cultured cells. Thus, we cannot rule out the possibility that other signaling pathways that differ from Activin A/TGF-β induced *NANOG* expression in the embryonic body.

Since PRDM14 and BLIMP1 as well as NANOG are transcriptional regulators in mammals, it is reasonably assumed that one of the functions of PRDM14 and BLIMP1 in the chicken may be the regulation of pluripotency-related genes. In mice, the expression of *Nanog*, *Sox2*, *Pou5f1* (*Oct3/4*), and *Prdm14* is controlled by so-called super-enhancers and these transcription factors themselves form interconnected-autoregulatory loops (Hnisz et al., 2013). This means that the expression of *Prdm14* and *Nanog* is mutually dependent. In mice, several super-enhancers were identified in the *Nanog* locus and regulated the expression of *Nanog* and several pluripotency-related genes located in the vicinity (Blinka et al., 2016; Whyte et al., 2013). Furthermore, PGC-like cells induced from epiblast-like cells by the expression of *Nanog* started to express *Prdm14*, *Blimp1*, and *Tfap2C* and the binding of the NANOG protein to the putative enhancer regions of *Prdm14* and *Blimp1* was observed (Murakami et al., 2016). On the other hand, the up-regulation of *Nanog* was observed in PRDM14-induced PGCs (Nakaki et al., 2013). These findings also suggested that the expression of *Nanog*, *Prdm14*, and *Blimp1* is interconnected. Since the inhibition of SMAD2/3 reduced the expression of *PRDM14*, *BLIMP1*, and *NANOG* (Fig. 6) and the knockdown of *PRDM14* and *BLIMP1* affected the expression of *NANOG* (Fig. 4), these PGC-related genes may mutually control their expression, possibly through a

super-enhancer.

BLIMP1 appears to control *NANOG* expression as a transcriptional regulator based on the results of the reporter assay and ChIP assay, suggesting that BLIMP1 directly controls *NANOG* expression. Previous studies showed that BLIMP1 formed complexes with various co-repressors and acted primarily as a transcription repressor (John and Garrett-Sinha, 2009). Contrary to this widely accepted function, BLIMP1 appeared to activate *NANOG* and *PRDM14* expression in the present study. It is important to note that BLIMP1 was shown to interact with the BAF complex and activate gene expression during the B cell to plasmablast transition (Minnich et al., 2016). Thus, BLIMP1 is reasonably assumed to act as both an activator and repressor. To confirm the precise control mechanism of *NANOG* in relation to BLIMP1 and PRDM14 in the chicken, extensive analyses of the *NANOG* locus, including the identification of enhancers and transcription factors binding to these sequences, are necessary.

In mammals, germinal fate is specified by signaling molecules at relatively later stages of development (Extavour and Akam, 2003). PRDM14 and BLIMP1 are involved in PGC specification in mice. On the other hand, the maternally inherited germplasm, which possibly contains CVH (Tsunekawa et al., 2000), specifies germinal cells in the chicken. Since the exogenous expression of *CVH* induces several germ cell-specific genes in chicken ES cells, *CVH* was expected to be a key regulator of PGC differentiation and specification (Lavial et al., 2009). In the present study, we found that PRDM14 and BLIMP1 up-regulated *DAZL* and *CVH* expression in cultured blastodermal cells, which is partly supported by the results of the reporter and ChIP assays. Although these proteins potentially affected the expression of *CVH* and *DAZL*, their physiological importance remained unclear. We speculate that PRDM14 and BLIMP1 are not critical transcription factors that induce germ cell-specific gene expression.

Our results did not suggest the involvement of PRDM14 or BLIMP1 in the specification of the germinal fate in the chicken, as reported for mammals. However, these proteins appear to be involved in the regulation of proliferation and germ cell-specific genes in chicken PGCs. Further studies, particularly those in very early developmental stages, are needed to gain insights into the roles of PRDM14 and BLIMP1 in PGC development.

4. Materials and methods

4.1. Cell culture

293FT (Thermo Fisher Scientific, Tokyo, Japan) and DF-1 (chicken fibroblast, ATCC CRL12203) cells were maintained in DMEM high glucose (Sigma-Aldrich, St Louis, MO, USA) containing 10% fetal bovine serum (FBS) (Biological Industries, Kibbutz Beit Haemek, Israel), 100 U/ml penicillin G, and 100 μg/ml streptomycin (P&S).

4.2. Chickens and eggs

Chickens (White Leghorn) and fertilized eggs were purchased from Nisseiken (Tokyo, Japan) or Japan Layer (Gifu, Japan). Organs obtained from adult or newborn chickens were minced and washed with phosphate-buffered saline (PBS), and RNA was isolated. All animal experiments were performed according to the ethical guidelines for animal experimentation of Nagoya University.

4.3. Molecular cloning of chicken PRDM14

Regarding 5'-RACE, total RNA was extracted using ISOGEN II (Nippon Gene, Tokyo, Japan) and reverse-transcribed by ReverTra Ace (Toyobo, Osaka, Japan) with phosphorylated chicken *PRDM14* primer (Table S2 and Fig. S1A). Primers were designed based on cDNA sequences in the NCBI database (gene ID: 100858709). The cDNA-RNA hybrid was digested with RNase H (Takara, Shiga, Japan) at 37°C for 1 h and ligation was conducted with T4 RNA ligase (Takara) at 15°C overnight. Nested PCR was performed using KOD Fx Neo (Toyobo). The primers used are listed in Table S2. The amplified 5'-DNA fragment of chicken *PRDM14* was cloned into pBluescript II and its DNA sequence was elucidated. In the cloning of full-length *PRDM14* cDNA, the 5'-primer was designed based on the DNA sequence of the 5'-fragment obtained by 5'-RACE, and cDNA was amplified from the cDNA of blastodermal cells as a template and cloned into pcDNA4A (Thermo Fisher Scientific).

4.4. PGC isolation and flow cytometric analyses

PGCs were isolated essentially as described previously (Motono et al., 2008). Briefly, cells from blood (HH stages 13-16), the genital ridge (HH stages 16-18), and

the gonads (HH stages 27-28) were incubated with an anti-SSEA-1 antibody (SC21702, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4 °C for 1 h. After washing with PBS, cells were incubated with phycoerythrin-labeled goat anti-mouse IgM (SC3768, Santa Cruz Biotechnology) at 4 °C for 30 min. Stained cells were suspended in PBS containing 1% FBS, then sorted using a flow cytometer (EPICS ALTRA, Beckman-Coulter, Brea, CA, USA or FACSJazz, BD Biosciences, San Jose, CA, USA).

In long-term cultures, blood containing cPGCs was collected from 52 h embryo (HH-stage 13-15), and cells from a single embryo were cultured in KnockOut DMEM (Thermo Fisher Scientific) supplemented with B-27 supplement (Thermo Fisher Scientific, 1×), L-glutamine (Wako, 2 mM), 1× non-essential amino acids (Wako), 0.1 mM 2-mercaptoethanol (Wako), nucleotides (Thermo Fisher Scientific, 1×), P&S, sodium heparin (Wako, 0.2%), 1.2 mM sodium pyruvate (Thermo Fisher Scientific), chicken serum (0.2%), Activin A (Shenandoah Biotechnology, Warwick, PA, USA, 25 ng/ml), BMP4 (Peptide Institute, Osaka, Japan, 25 ng/ml), and FGF2 (PeproTech, Rocky Hill, NJ, USA, 4 ng/ml), as previously reported (Whyte et al., 2015). PGCs were enriched and the majority of cells were CVH- and SSEA-1-positive after 5 cycle passages.

4.5. qRT-PCR

Total RNA was extracted using ISOGEN II, and reverse-transcribed by ReverTra Ace. The ReliaPrep RNA Cell Miniprep System (Promega, Madison, WI, USA) and ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo) were used for RNA isolation and cDNA synthesis from PGCs and blastodermal cells that had been transfected and purified by the flow cytometer. Real-time PCR was performed using LightCycler (Roche Diagnostics, Mannheim, Germany) or LightCycler 96 (Roche Diagnostics) 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 S2.

4.6. In situ hybridization

Regarding the detection of chicken *PRDM14* mRNA, an antisense RNA probe was synthesized using full-length *PRDM14* coding sequence (1461 bp) DNA and an RNA synthesis kit (Roche), and then labeled with digoxigenin. Whole-mount *in situ*

hybridization was performed as previously described (Yamamoto-Shiraishi and Kuroiwa, 2013).

4.7. In vivo knockdown of PRDM14 and BLIMP1

Chicken U6-1, U6-4, and H1 promoters were amplified by PCR using the genomic DNA of chicken blood cells as a template. The primers used are shown in Table S2. The amplified promoter sequences were cloned into pBluescript II. Three short hairpin RNAs (shRNAs) against chicken *PRDM14*, *BLIMP1*, and *OVAL* (ovalbumin gene) were designed using siDirect (http://sidirect2.rnai.jp/) as shown in Table S2. Three *PRDM14* shRNAs were ligated to different promoters and tandemly arranged in pBluescript II to express shRNAs simultaneously under the control of different promoters. Similar *BLIMP1* and *OVAL* shRNA expression cassettes were constructed. These shRNA expression cassettes were included in the RCAN vector (pRCAN(A)-3×) (Morgan and Fekete, 1996). The efficiency of knockdown was confirmed by transfecting 400 ng of either the knockdown vector with *PRDM14* or *BLIMP1* expression vector (Fig. S10A, B) into 1×10^5 cells of DF-1 by a transfection reagent (Viafect, Promega). Forty-eight hours post-transfection, cells were harvested and the expression of *PRDM14* or *BLIMP1* was analyzed by Western blotting. In the propagation of the RCAN viral vector, chicken embryonic fibroblast cells were transfected with the vector DNA using FuGENE-HD (Promega). After two cycles of serial passages, 5 ml of DMEM high glucose containing 1% FCS was added to a confluent cell culture in 100 mm dishes. Medium was collected after 24 and 48 h and filtrated through a 0.45 μm membrane filter, followed by concentration by centrifugation at 30,000 rpm for 3 h. Precipitates containing the viral vector were suspended in DMEM and stored at -80 °C. In *in vivo* knockdown experiments, the sharp end of a freshly laid egg was cut by a diamond cutter and 2 μl of viral vector solution was injected into the subgerminal cavity. The eggshell was sealed with a PTFE membrane (MILLI WRAP, Merck Millipore, Burlington, MA). Eggs were incubated sealed side down at 38°C under 60% humidity with a rocking angle of 90° every hour. After 5.5 or 6.5 days, embryos were sacrificed and embryonic gonads were isolated. The gonads were then dissociated, permealized (if needed), and stained with an anti-SSEA-1 or CVH (Motono et al., 2008) antibody. The ratio of SSEA-1- or CVH-positive cells to total gonadal cells was analyzed using a flow cytometer. In qPCR

analyses, PGCs were isolated and purified by MACS (Miltenyi Biotech, Bergisch Gladbach, Germany) as SSEA-1-positive cells.

4.8. Knockdown of PRDM14 and BLIMP1 in cultured PGCs

One hundred and fifty picomoles of *PRDM14*- or *BLIMP1*-specific siRNAs (Hokkaido System Science, Sapporo, Japan) and control siRNA (Sigma-Aldrich) were introduced into 1.5×10^5 cells of cultured PGCs by electroporation using an NEPA21 electroporator (NepaGene, Chiba, Japan). Cells were then cultured for 48 h and the expression of germ cell-specific and pluripotency-related genes was analyzed by qRT-PCR. The sequences of siRNAs used are shown in Table S2. Since the knockdown efficiencies of PRDM14 and BLIMP1 were not sufficient in some experimental trials, possibly because of inefficient gene delivery, experiments in which *PRDM14* and *BLIMP1* expression (cDNA level) was reduced to less than 50% of the control were used in analyses.

4.9. Construction of reporter plasmids and assay

DNA fragments of approximately 2700, 1600, 600, and 200 bp (*CVH*), and approximately 3000 bp (*DAZL*, *PRDM14*, and *NANOG*) upstream of the initiation codons were amplified by PCR using chicken genomic DNA extracted from blood as a template and KOD plus Neo (TOYOBO). The primers used were shown in Table S2. Amplified DNAs were cloned into pGL3-basic (Promega). 293FT cells were seeded at 1.5×10^4 cells per well in a 96 well plate. After 24 h, cells were transfected with 100 ng of pcDNA4A/FLAG-PRDM14_eGFP or pcDNA4A/FLAG-BLIMP1_eGFP (Fig. S10A, C), 100 ng of the luciferase vector, and 5 ng of pGL4.74 (Promega) using Lipofectamine 2000 (Thermo Fisher Scientific). Cells were cultured for 24 h and luciferase activity was then analyzed with the Dual-Glo Luciferase Assay System (Promega). Luciferase activity in cells transfected with the control plasmid (pGL3-basic) was set as 1.

4.10. ChIP assay

PGCs cultured in 100 mm dishes were used for one assay. ChIP assays were conducted as previously reported (Inayoshi et al., 2005) with a house-made anti-PRDM14 antibody and anti-BLIMP1 antibody (#ab13700, Abcam, Cambridge,

UK), and control IgG (mouse IgG1 (G3A1), Cell Signaling Technology, Danvers, MA, USA; goat anti-mouse IgG (A90-116A), Bethyl Laboratories, Montgomery, TX, USA). The primers used for qPCR were listed in Table S2. The enrichment levels in the immunoprecipitates relative to control IgG (set as 1) are shown. As a control, binding to *GAPDH* gene was also analyzed (anti-BLIMP1, Fig. 5E; anti-PRDM14, Fig. 8D).

4.11. Forced expression of PRDM14 and BLIMP1 in blastodermal cells

Blastodermal cells were harvested from freshly laid eggs (EK-stage IX-XI) with a standard procedure, and maintained in ES medium (KnockOut DMEM containing 20% KnockOut Serum replacement (KSR) (Thermo Fisher Scientific), 2.5% chicken serum, P&S, 1× nucleosides (Merck Millipore), 2 mM L-glutamine, 1× non-essential amino acids, 0.1 mM 2-mercaptoethanol, LIF (Wako), 1 μM PD0325901 (Wako), 3 μM CHIR99021 (Wako), and 10μM Y27632 (Wako)). After a one-day culture, cells were transfected with pcDNA4A/FLAG-PRDM14_eGFP or pcDNA4A/FLAG-BLIMP1_eGFP (Fig. S10A, C) by FuGENE-HD. Cells were then incubated for 48 h and eGFP-positive cells were isolated using a flow cytometer and subjected to qRT-PCR. Cells were also cultured in the differentiation medium (DMEM containing 10% FCS, 1× non-essential amino acids, P&S, and 10 μM Y27632) as reported previously with slight modifications (Lavial et al., 2009).

4.12. Inhibition of Activin A and FGF2 signaling

Cells were cultured with 25 ng/ml of Activin A, 4 ng/ml of FGF2 in the presence of each inhibitor (1 μM PD0325901 or 1 μM A83-01 (Wako)), or vehicle (DMSO, 0.15%). To confirm the inhibition of the corresponding cognate signal, cells were harvested 1 h after the stimulation, and phosphorylated ERK (pERK), phosphorylated SMAD2 (pSMAD2), and β-ACTIN were detected by Western blotting. A rabbit anti-pERK antibody (#9101, Cell Signaling Technology), rabbit anti-pSMAD2 antibody (#3108, Cell Signaling Technology), or mouse anti-β-ACTIN antibody (6D1, Medical & Biological Laboratories, Nagoya, Japan) was used as the primary antibody and goat anti-rabbit IgG-HRP (#458, Medical & Biological Laboratories) or goat anti-mouse IgG-HRP (#330, Medical & Biological Laboratories) as the secondary antibody. β-ACTIN was used as the loading control. In the expression analysis of *PRDM14*, *BLIMP1*, and *NANOG*, cells were collected at defined periods, and qRT-PCR was

performed as described above.

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Online supplementary material

Fig. S1 shows structure of chicken *PRDM14* gene. Fig. S2 shows the nuclear localization of full-length but not N-terminal-deleted PRDM14. Fig. S3 shows the expression of *PRDM14* in chicks and matured chickens. Fig. S4 shows original flow cytometric data associated with Fig. 3. Fig. S5 shows the morphology of embryonic gonads infected with RCAN vectors. Fig. S6 shows the lack of sex differences in the effects of RCAN viruses. Fig. S7 shows the gene expression of the remaining PGCs after *in vivo* knockdown (related to Fig. 3). Fig. S8 shows the gene expression of long-term cultured PGCs. Fig. S9 shows the results of luciferase and ChIP analyses on the *NANOG* promoter by PRDM14. Fig. S10 shows the vector constructs to express PRDM14 and BLIMP1 (related to Figs. 3, 5, 7, 8 and Fig. S9, S11, S12). Fig. S11 shows changes in blastodermal gene expression by the overexpression of PRDM14 or BLIMP1 in differentiation medium. Fig. S12 shows the results of luciferase and ChIP analyses on the *DAZL* promoter by BLIMP1. Table S1 shows gene expressions of blastodermal cells and cPGCs. Table S2 shows primers, siRNAs and shRNA used in this study.

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Figure legends

Fig. 1. Expression of *PRDM14* and *BLIMP1*. (A) RNAs were purified from the indicated developmental stages of embryos and subjected to qRT-PCR. The expression level of *PRDM14* is represented as a relative expression level against *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH)*. Data are the mean ± standard error of 3 different samples. (B, C) RNAs were purified from SSEA-1-positive cells as PGCs, then subjected to qRT-PCR. The expression levels of *PRDM14* (B) and *BLIMP1* (C) are represented as relative expression levels against *GAPDH*. Expression was also measured with SSEA-1-negative cells (gr or g SSEA-1$^-$) as somatic cells. Data are the mean ± standard error of six (B) or three (C) different samples.

Fig. 2. Whole-mount *in situ* hybridization of *PRDM14* mRNA in developing embryos. (A-C) Whole mount *in situ* hybridization against *PRDM14* was performed with the indicated stages of embryos. Typical images of at least 4 different samples are shown. In B and C, arrows show the PRDM14-positive cells, which are most likely PGCs. In B, cells in neural plate were also stained (arrowheads).

Fig. 3. *In vivo* knockdown of *PRDM14* and *BLIMP1* in developing embryos. (A) Structure of the RCAN viral vector expressing shRNA against *PRDM14*, *BLIMP1*, or *OVAL*. *gag/pol/env*: Rous sarcoma virus structural genes, LTR: long terminal repeat, cU6-1 and cU6-4: chicken U6 promoters, cH1: chicken H1 promoter. Drawmaps are not in scale. (B, C) The shRNA expression plasmids and expression plasmids of the knockdown targets (FLAG-PRDM14 (B) or GFP-BLIMP1 (C)) were co-transfected into DF-1 cells. After a 48-h incubation, cells were lysed and the expression levels of each protein were examined by Western blotting with an anti-FLAG (B) or anti-GFP (C) antibody. β-ACTIN was used as an internal control. (D-G) RCAN virus solution was injected into EK-stages IX to XI embryos. After a 5.5-day (D, F) or 6.5-day (E, G) incubation, embryonic gonads were harvested. Cells were then analyzed using a flow cytometer with an anti-SSEA-1 (D, F) or anti-CVH (E, G) antibody. A box-and-whisker plot shows both the summary statistics and distribution of the primary data. Lines and boxes denote the median and 25th and 75th percentile levels, respectively, while bars represent maximum and minimum data, except for outliers (indicated as white circles).

N=21 (sh*OVAL*), 28 (sh*PRDM14*), and 20 (sh*BLIMP1*) for SSEA-1; N=17 (sh*OVAL*), 22 (sh*PRDM14*), and 26 (sh*BLIMP1*) for CVH. Data from 2-5 independent experiments are collected. * and ** indicate significant differences by the Mann-Whitney U test ($p<0.05$ and $p<0.01$, respectively). Original flow cytometric data are shown in Fig. S4.

Fig. 4. Knockdown of *PRDM14* and *BLIMP1* in cultured PGCs. (A, B) siRNAs against *PRDM14* (A) and *BLIMP1* (B) were electroporated to cultured PGCs. The expression levels of pluripotency-related and germ cell-specific genes were examined by qRT-PCR. The expression levels in control siRNA-treated cells were set as 1. Data are the mean ± standard error of 3-6 independent experiments.

Fig. 5. Regulation of *NANOG* and *PRDM14* promoters by BLIMP1. (A) A luciferase reporter plasmid with the *PRDM14* promoter was co-transfected into 293FT cells with BLIMP1 or its mutant expression plasmids. Data are the mean ± standard error of 4 independent experiments. (B) A luciferase reporter plasmid with the *NANOG* promoter and BLIMP1 expression plasmid were co-transfected into 293FT cells. Data are the mean ± standard error of 4 independent experiments. (C, D, E) ChIP assays against BLIMP1 were performed with cultured PGCs. The enrichment of DNA in the *PRDM14* (C; between 2484 to 2581 bp upstream of the transcription start site) or *NANOG* (D; between 978 to 1068 bp upstream of the initiation codon) sequence was analyzed by qRT-PCR. The *GAPDH* (between 1520 to 1702 bp downstream of the transcription start site) sequence was also analyzed as a control (E). Data are the mean ± standard error of 4 independent experiments.

Fig. 6. Inhibition of growth-associated signaling in cultured PGCs. (A, B) Cultured PGCs were seeded into medium containing FGF and Activin A in the absence of BMP4. Cells were stimulated for 1 h in the presence of PD0325901 or A83-01, lysed and the phosphorylation of signaling molecules was examined by Western blotting with an anti-pERK (A) or anti-pSMAD2 (B) antibody. β-ACTIN was used as an internal control. Typical images of 3 different experiments are shown. (C, D) Cultured PGCs were treated with PD0325901 (C) or A83-01 (D) for the indicated period. RNAs were then purified and subjected to qRT-PCR. The expression levels of *PRDM14*, *BLIMP1*, and *NANOG* were normalized by *GAPDH* and represented as relative expression levels

against the DMSO control. Data are the mean ± standard error of 5-6 independent experiments.

Fig. 7. Overexpression of *PRDM14* or *BLIMP1* in cultured blastodermal cells under ES medium. (A, B) Blastodermal cells collected from EK-stages IX to XI were inoculated into 24 well plates. After 24 h, a FLAG-PRDM14/eGFP (A) or FLAG-BLIMP1/eGFP (B) expression plasmid (Fig. S10A, C) was transfected. Cells were cultured for another 48 h, and eGFP-positive cells were sorted by a flow cytometer. RNAs were then purified and the expression levels of the *CVH*, *DAZL*, *NANOG*, and endogenous *BLIMP1* and *PRDM14* genes were examined by qRT-PCR. Expression levels in control vector-transfected cells are set as 1. Data are the mean ± standard error of seven (A) or six (B) independent experiments.

Fig. 8. Regulation of *CVH* and *DAZL* promoters by PRDM14. (A) A luciferase reporter plasmid with various lengths of *CVH* promoter-proximal sequences and a PRDM14 expression plasmid were co-transfected into 293FT cells. Data are the mean ± standard error of 4 independent experiments. (B) A luciferase reporter plasmid with a full-length *CVH* promoter, and expression plasmids for PRDM14 or its truncated mutants were co-transfected into 293FT cells. Data are the mean ± standard error of 4 independent experiments. (C, D) ChIP assays using an anti-PRDM14 antibody were performed with cultured PGCs. Enrichment of DNA in the *CVH* promoter (between 320 to 423 bp upstream of the initiation codon) or *GAPDH* coding region was measured. Data are the mean ± standard error of 3 independent experiments. (E) A luciferase reporter plasmid with the *DAZL* promoter and PRDM14 expression plasmid were co-transfected into 293FT cells. Data are the mean ± standard error of 4 independent experiments. (F) ChIP assay using an anti-PRDM14 antibody. The enrichment of *DAZL* sequences (between 1140 to 1237 bp upstream of the initiation codon) was detected by qRT-PCR. Data are the mean ± standard error of 3 independent experiments.

Fig. S1. Structure of chicken PRDM14. (A) Structure of the chicken *PRDM14* gene in comparison with that reported in the database (NCBI gene ID: 100858709). The exon-intron structure and primers used for cloning were shown. (B, C) The amino acid

sequence of chicken PRDM14 in comparison with that reported in the database (gene ID: quail, 107310217; human, 63978; mouse, 383491). The predicted domain structure was also shown. Black shading and white boxes show matched amino acids between the three and two sequence datasets, respectively.

Fig. S2. Nuclear localization signal of PRDM14. (A) Schematic presentation of the expression vectors for GFP-fused PRDM14. (B) 293FT cells were transfected with PRDM14 vector and localization was examined after a 2-d culture. Representative results of 3 independent experiments are shown.

Fig. S3. The expression of *PRDM14* in the chicken. (A, B) RNAs from one-week chick (A) and adult chicken (B) organs were subjected to qRT-PCR. The expression levels of *PRDM14* are represented as relative expression levels against *GAPDH*. Data are the mean ± standard error of six different chickens (three males and three females), except for the testis, oviduct, and ovary (N=3).

Fig. S4. *In vivo* knockdown of *PRDM14* and *BLIMP1* in developing embryos. RCAN virus solution was injected into EK-stages IX to XI embryos. (A) After a 5.5-day incubation, the percentages of SSEA-1^+ cells in embryonic gonads were measured using a flow cytometer. (B) After a 6.5-day incubation, the percentages of CVH^+ cells in embryonic gonads were investigated. Numbers indicate the percentages for the PGC fraction (boxed region) against total gonadal cells. Typical images are shown.

Fig. S5. Morphology of embryonic gonads on day 5.5. Typical images for the gonads of embryos that had been injected with RCAN viruses are shown. The gonads are indicated by arrowheads.

Fig. S6. Lack of apparent sex differences in the percentage of the PGC fraction after the knockdown of *PRDM14* by RCAN viral vector. Several embryos were simultaneously analyzed for PGC percentages using a flow cytometer and sex by genomic PCR to amplify female-specific *Xho* I repeats in the W chromosome (Motono et al., 2008). Mix, embryos without analysis of sex.

Fig. S7. Expression of pluripotency-related and germ cell-specific genes in surviving cells after the *in vivo* knockdown of *PRDM14* or *BLIMP1*. SSEA-1-positive gonadal cells from embryos infected with the RCAN virus were collected and the expression levels of representative genes were analyzed by qRT-PCR. Data are the mean ± standard error (N=16 (sh*OVAL*), 12 (sh*PRDM14*), 11 (sh*BLIMP1*)).

Fig. S8. Expression of pluripotency-related and germ cell-specific genes in cultured PGCs. (A) Detection of the female-specific sequence in the W chromosome in various cultured PGC preparations including #1 and 2. (B) SSEA-1 staining of cultured PGCs. A typical image is shown. (C) RNAs from freshly isolated or cultured PGCs were subjected to qRT-PCR and the expression levels of representative genes were shown as relative expression levels against *GAPDH*. Data are the mean ± standard error of 3 different samples, except for the expression of *PRDM14* in cPGCs and gPGCs (N=6).

Fig. S9. Regulation of *NANOG* promoter by PRDM14. (A) A luciferase reporter plasmid and a PRDM14 expression plasmid were co-transfected into 293FT cells. Data are the mean ± standard error of 3 independent experiments. (B) ChIP assay using an anti-PRDM14 antibody. The weak enrichment of *NANOG* sequences (between 978 to 1068 bp upstream of the initiation codon) was detected by qRT-PCR. Data are the mean ± standard error of 4 independent experiments.

Fig. S10. Structure of the expression vector used to confirm siRNA knockdown or the ectopic expression of *PRDM14* or *BLIMP1*.

Fig. S11. Overexpression of *PRDM14* or *BLIMP1* in cultured blastodermal cells under differentiation medium. Blastodermal cells collected from EK-stages IX to XI were transfected with the FLAG-PRDM14/eGFP, FLAG-BLIMP1/eGFP expression plasmid (Fig. S10A, C) after 24-h culture in DMEM without cytokines. Cells were cultured for another 48 h, and eGFP-positive cells were then sorted using a flow cytometer as in Fig. 7. Gene expression levels were examined by qRT-PCR. Expression levels in control vector-transfected cells are set as 1. (A) Transfection of the PRDM14 vector. (B) Transfection of the BLIMP1 vector. Data are the mean ± standard error of 5 independent experiments.

Fig. S12. Regulation of *CVH* and *DAZL* promoters by BLIMP1. (A, B) A luciferase reporter plasmid with *CVH* (A) or *DAZL* (B) promoter and a BLIMP1 expression plasmid were co-transfected into 293FT cells. Data are the mean ± standard error of 4 independent experiments. (C) ChIP assays with a BLIMP1 antibody were performed with cultured PGCs. The enrichment of DNA in the *DAZL* promoter region (between 1140 to 1237 bp upstream of the initiation codon) was detected by qRT-PCR. Data are the mean ± standard error of 4 independent experiments.

Table S1. Expression of pluripotency-related and germ cell-specific genes in blastodermal cells and cPGCs.

Table S2. Primers, siRNAs, and shRNAs used in the present study.

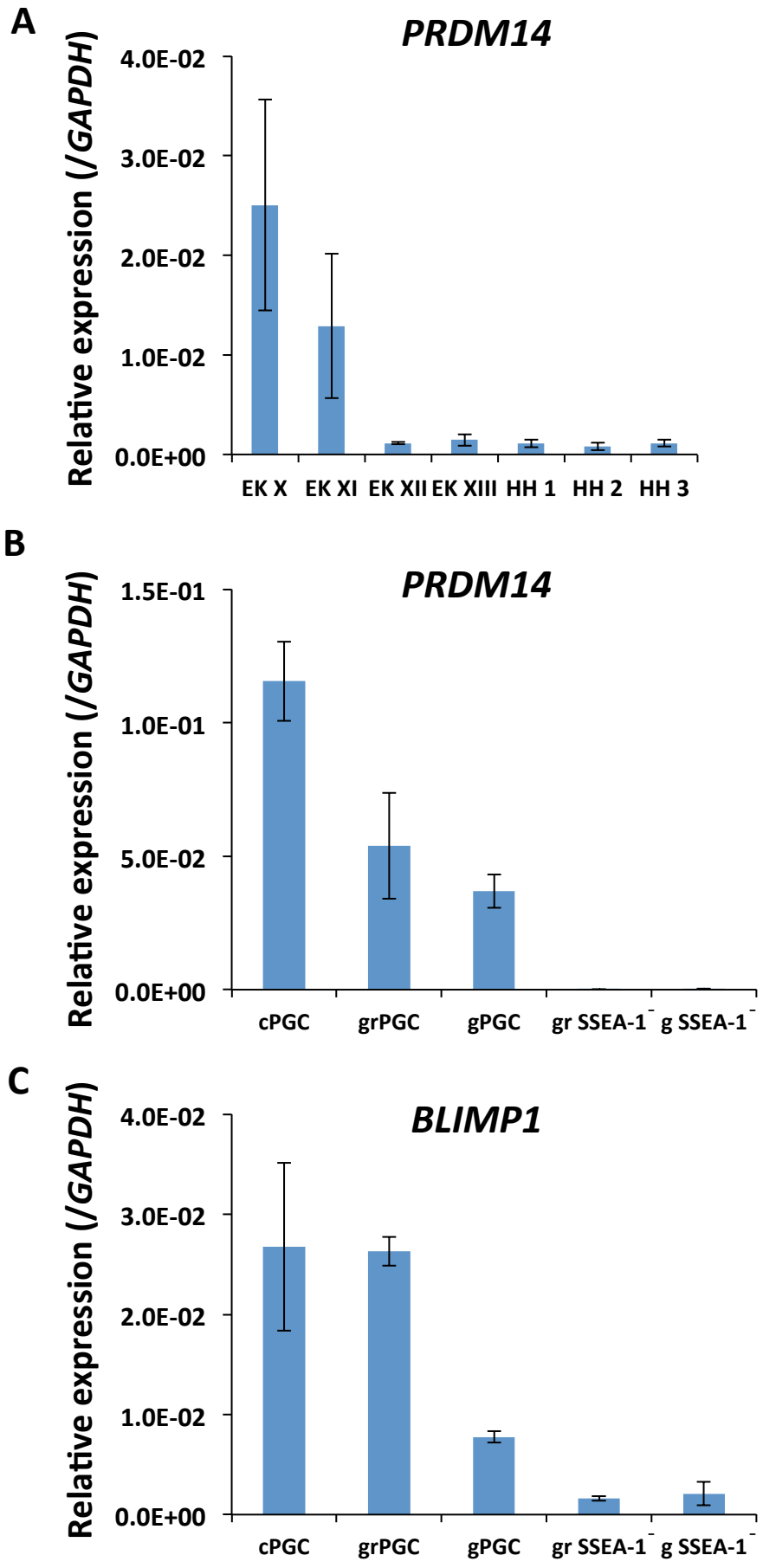


Fig. 1. Okuzaki et al.

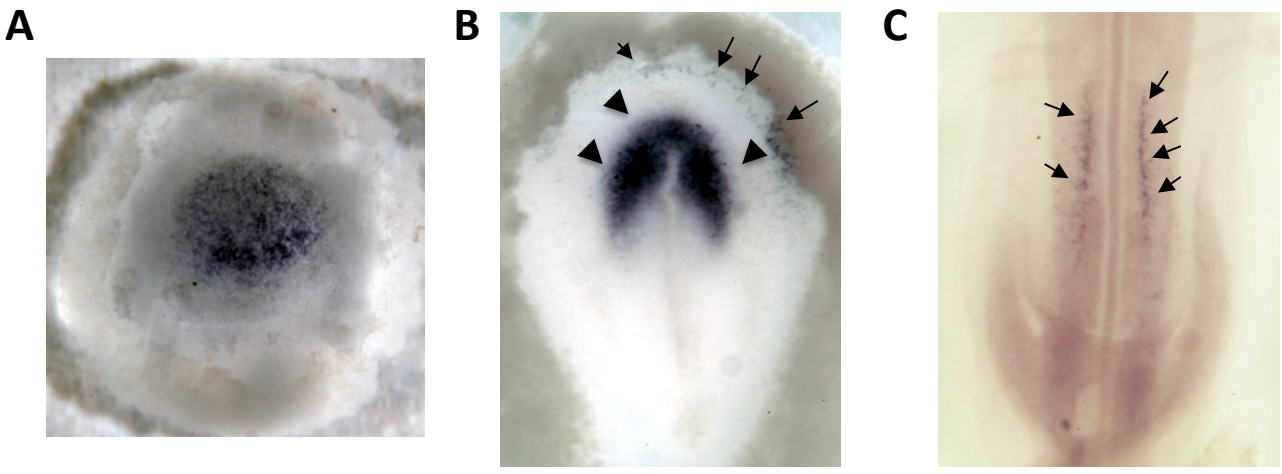


Fig. 2. Okuzaki et al.

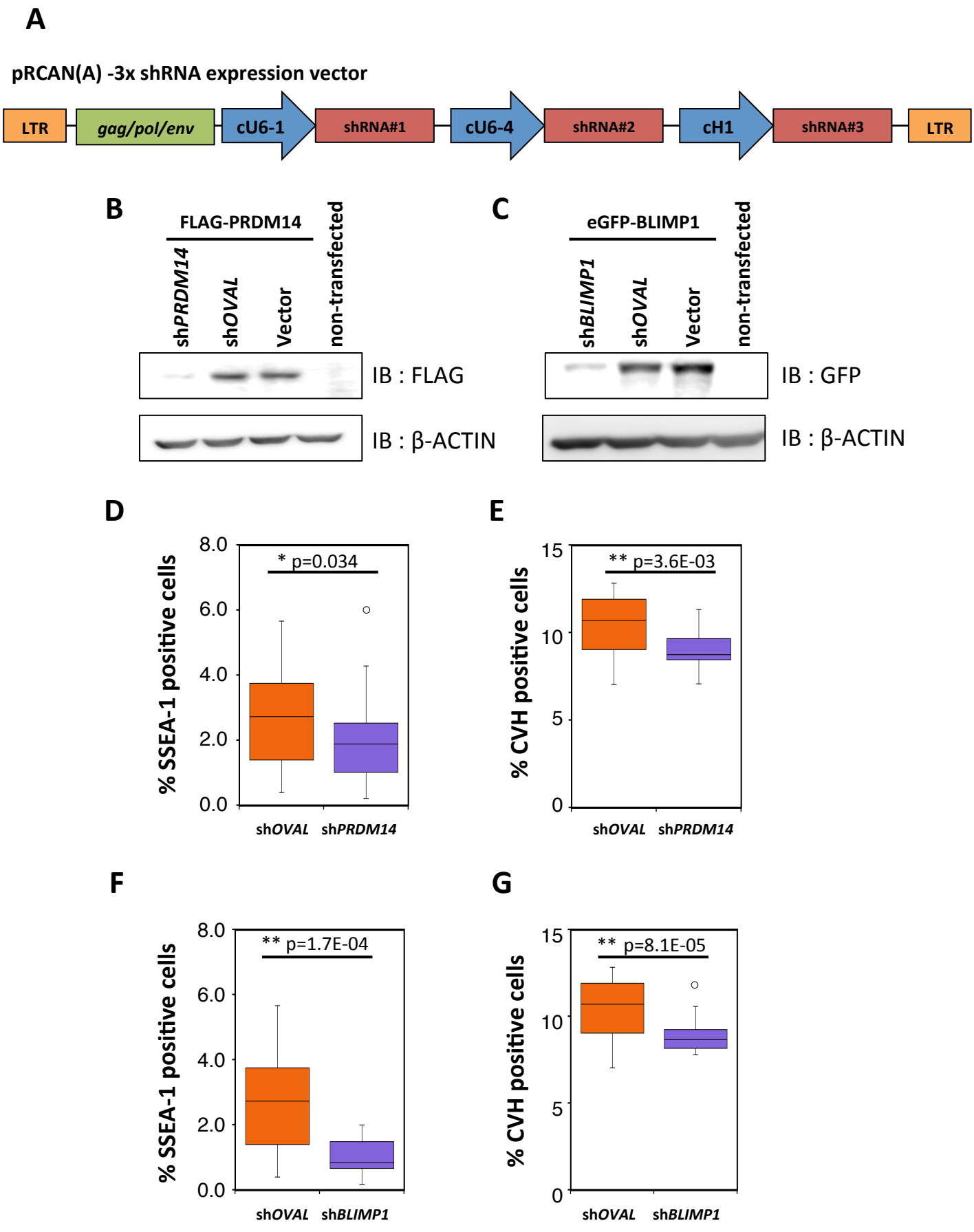
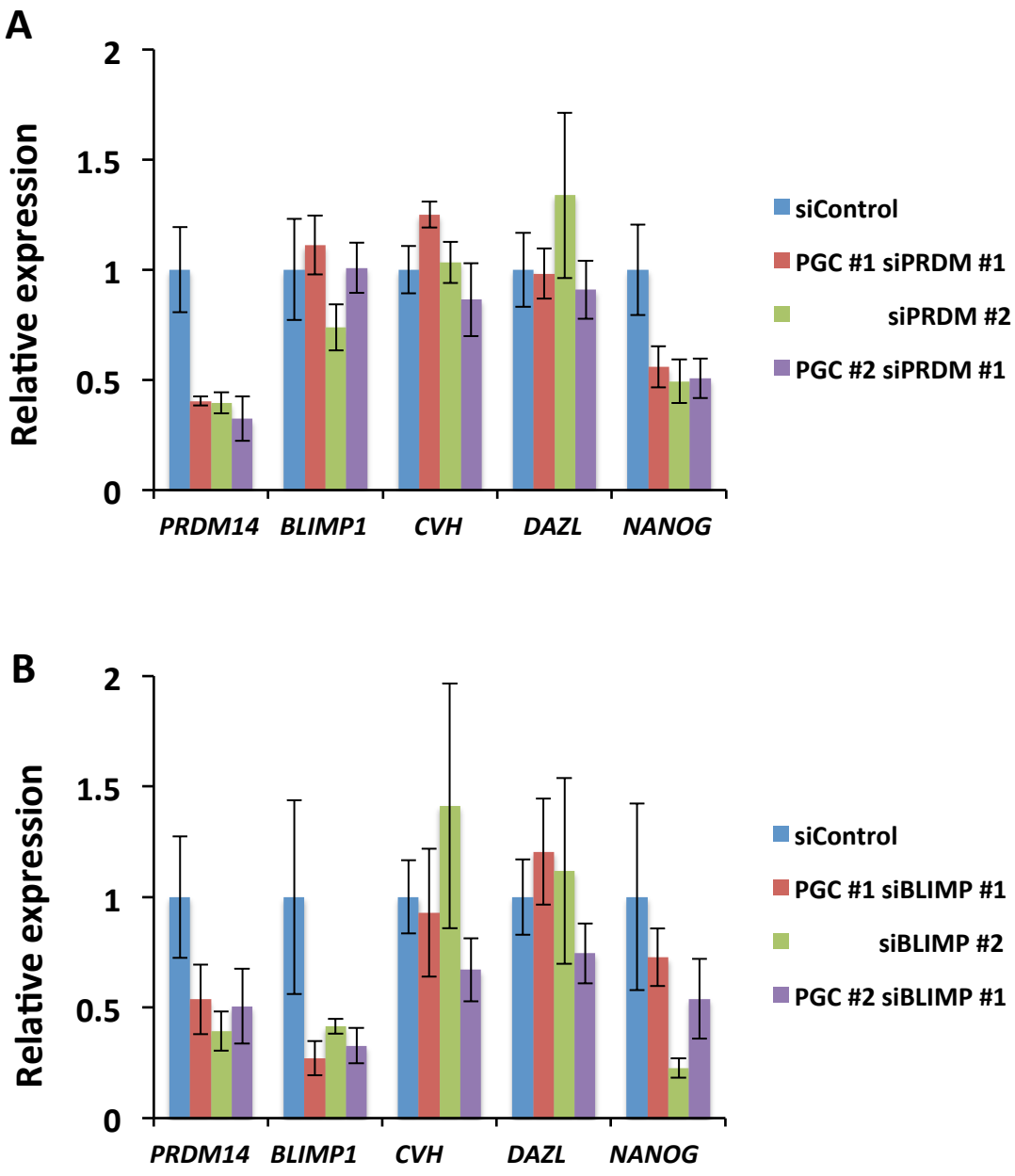


Fig. 3. Okuzaki et al.



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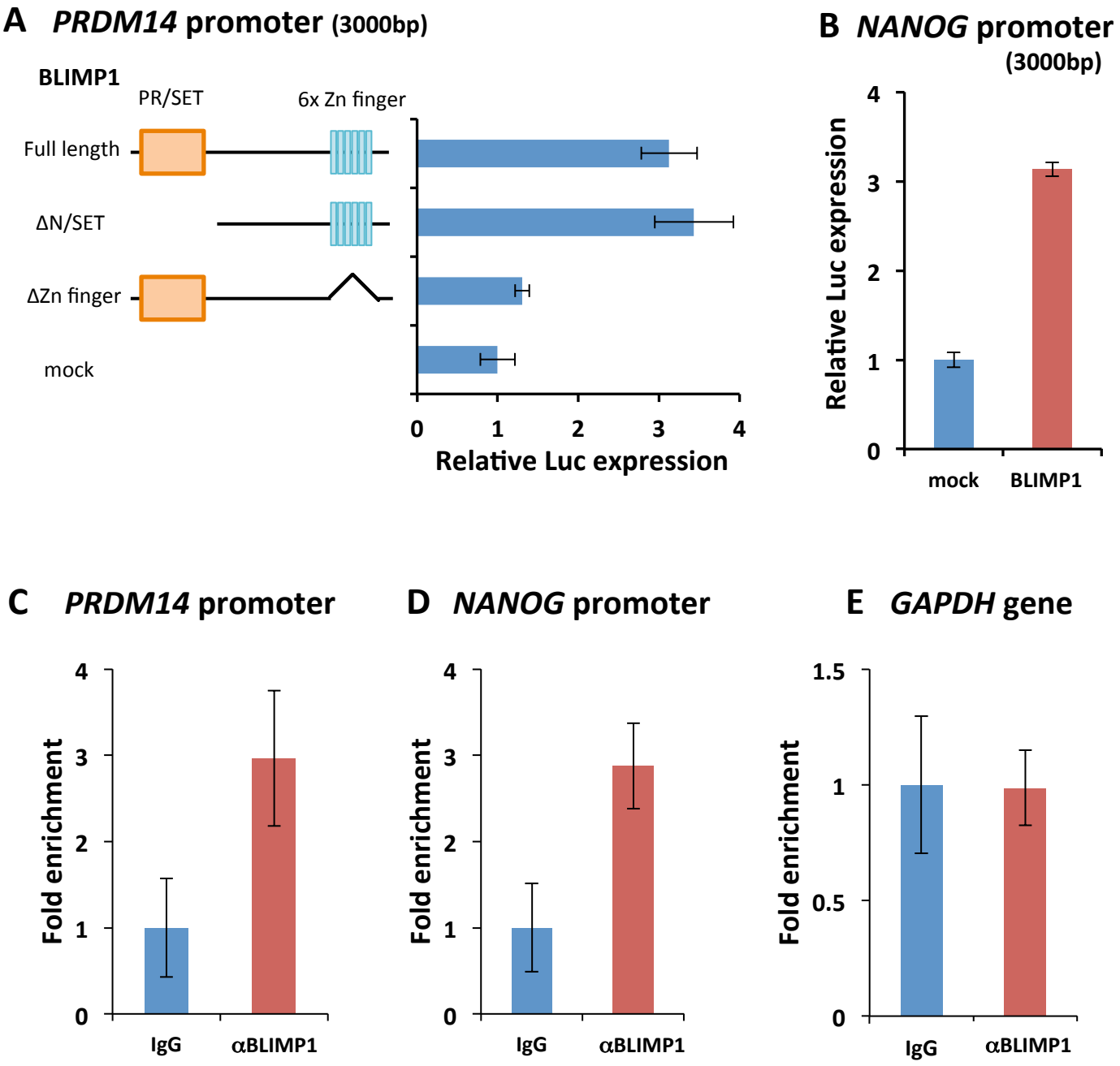
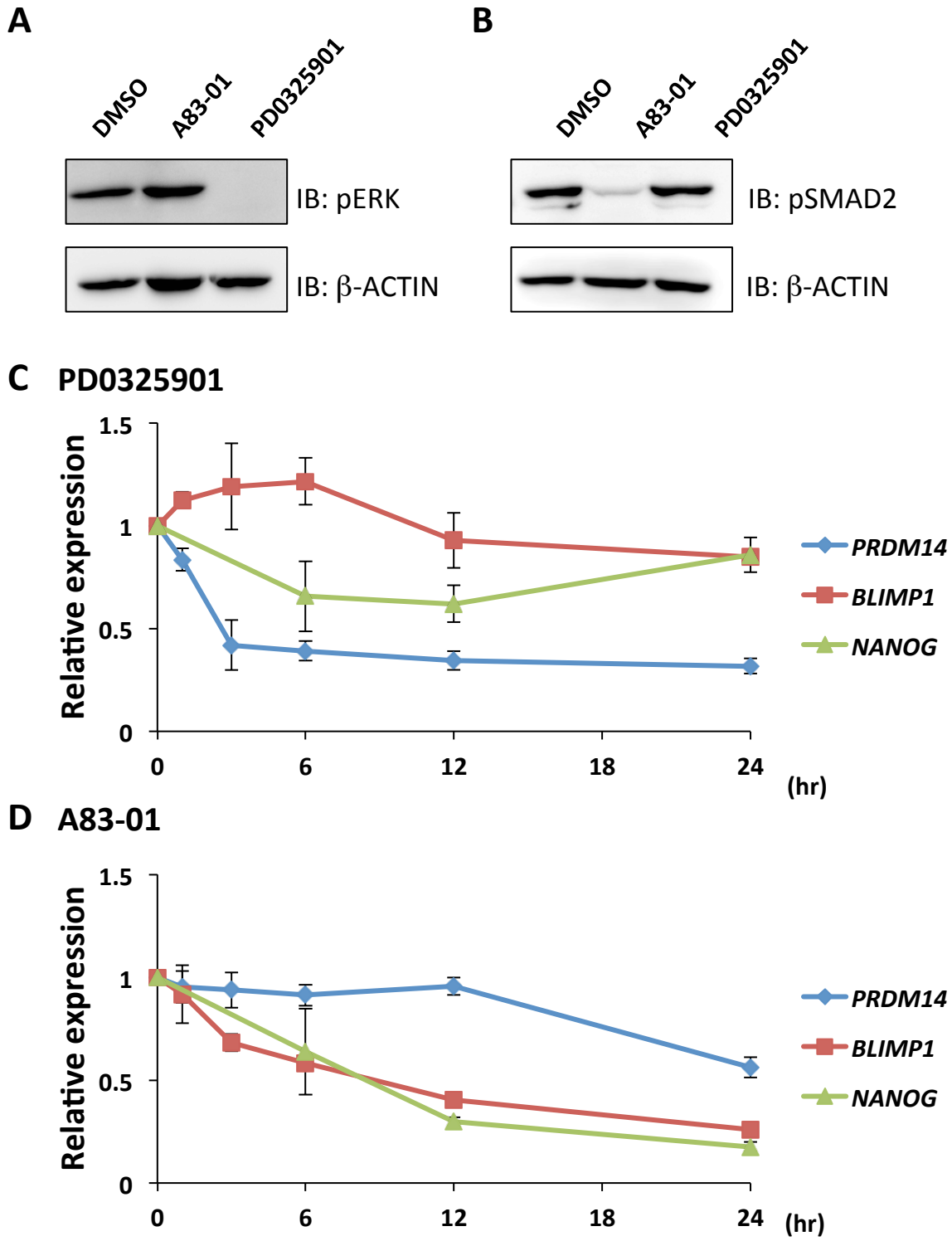


Fig. 5. Okuzaki et al.



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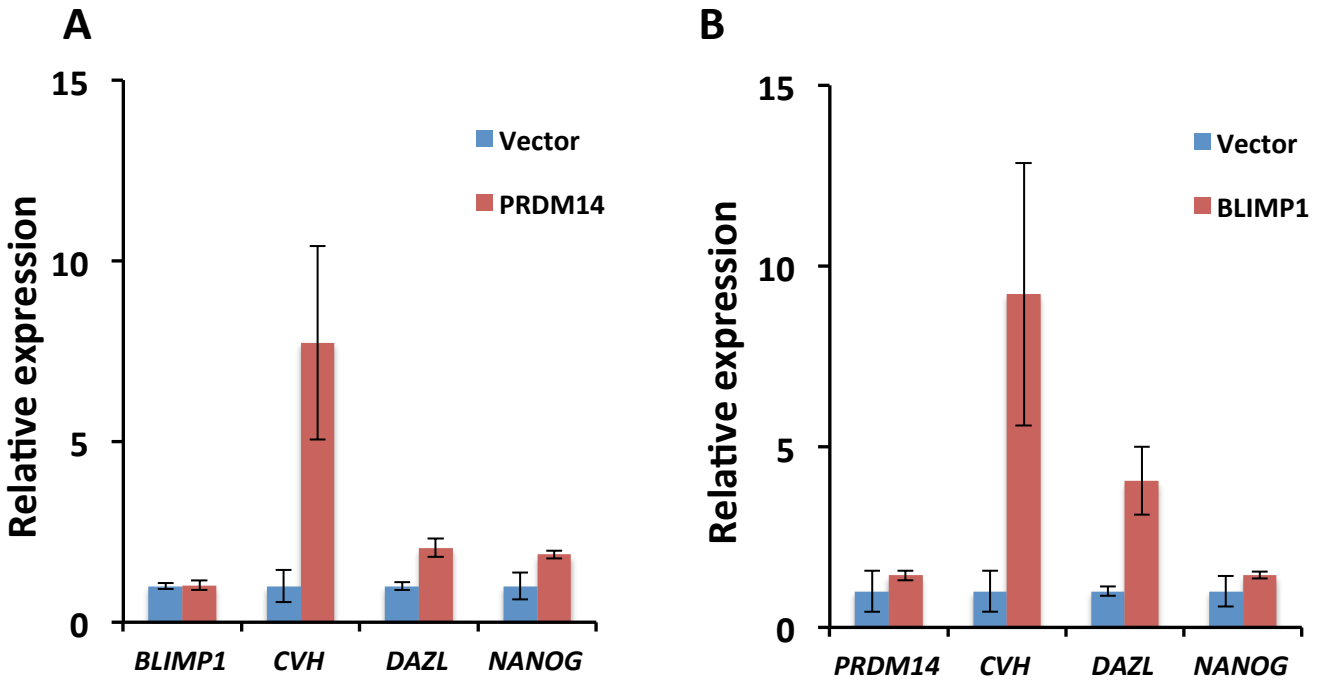

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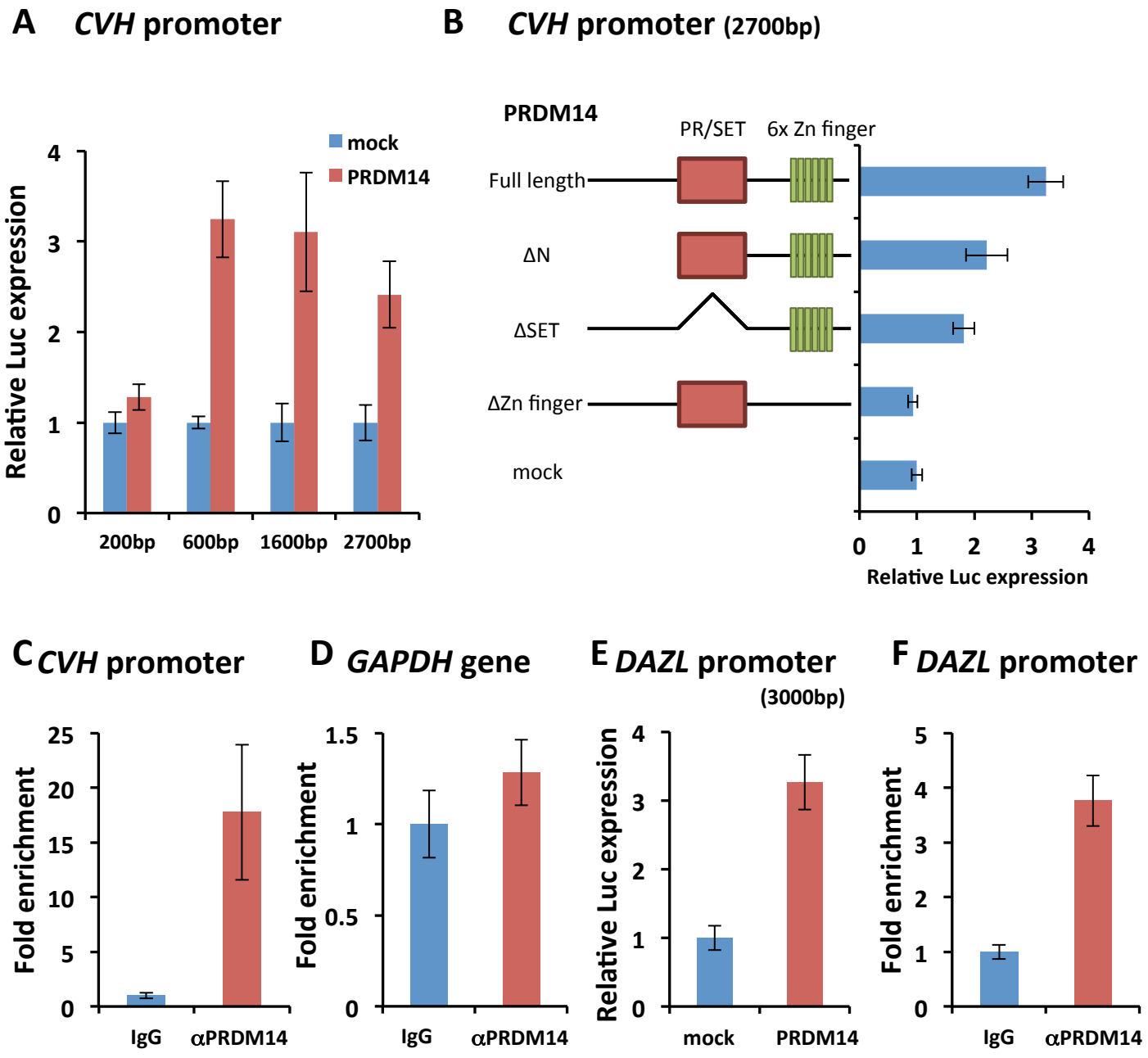


Fig. 8. Okuzaki et al.

Fig. S1. Okuzaki et al.

B

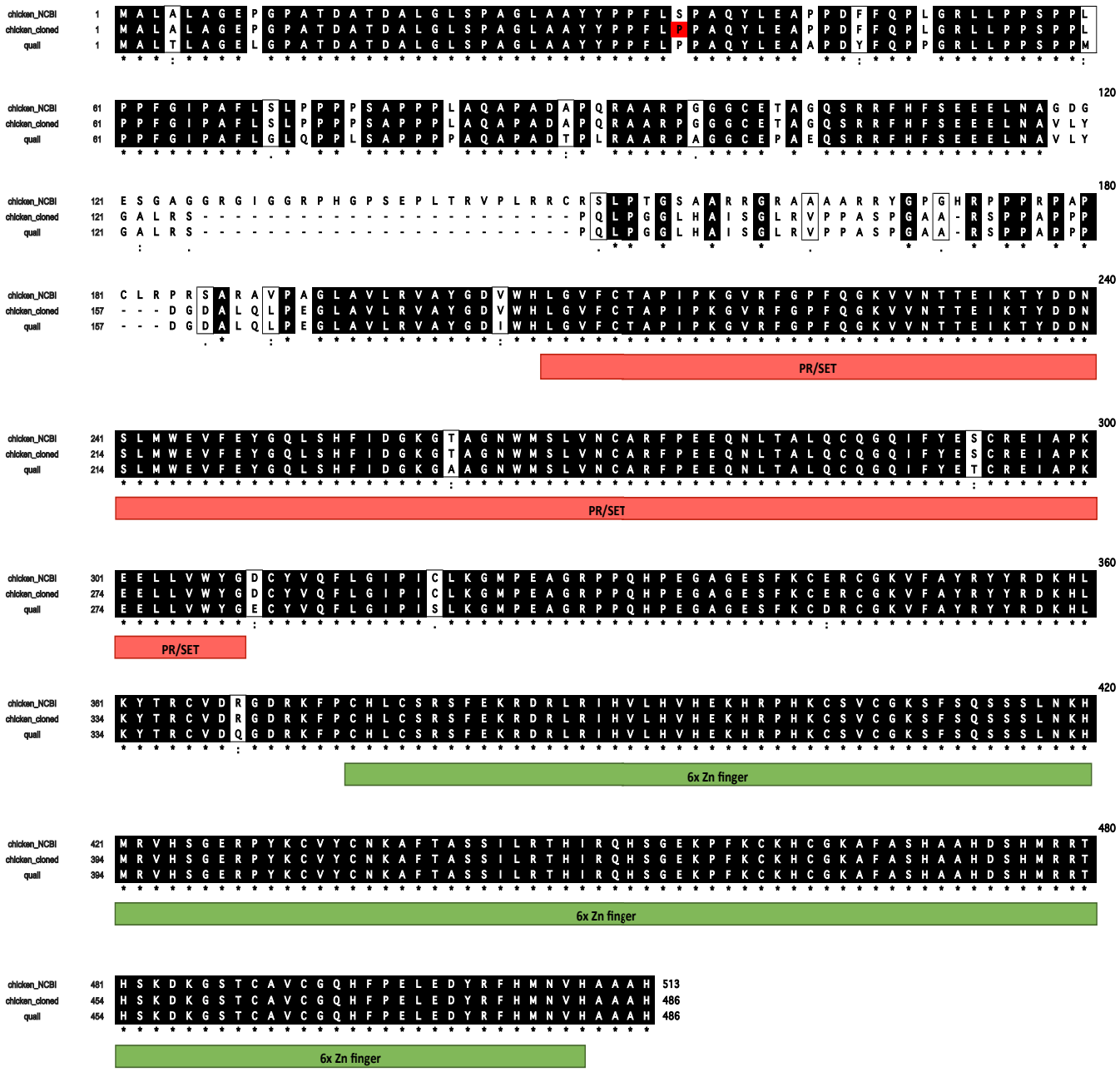


Fig. S1. Okuzaki et al.

C

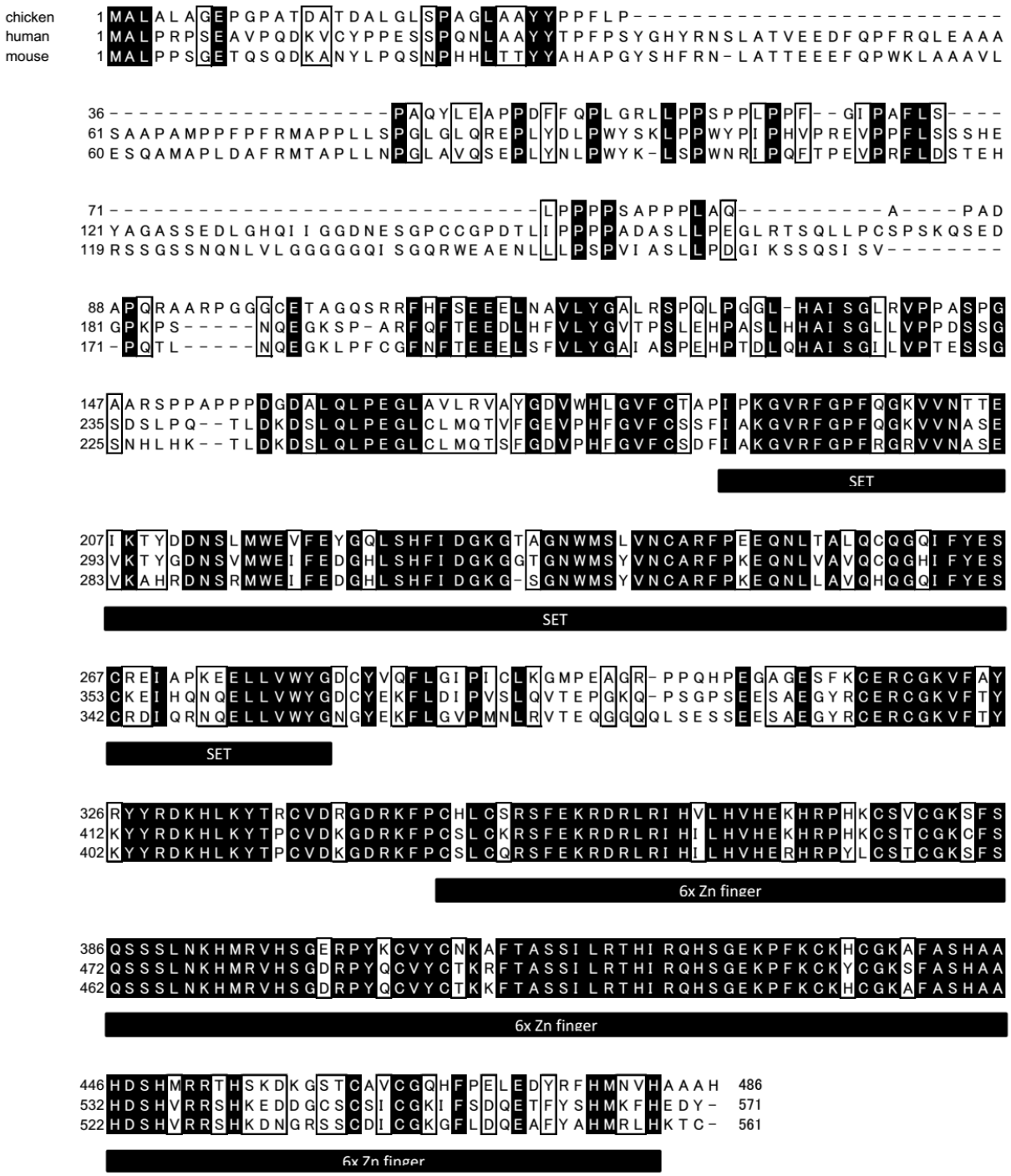
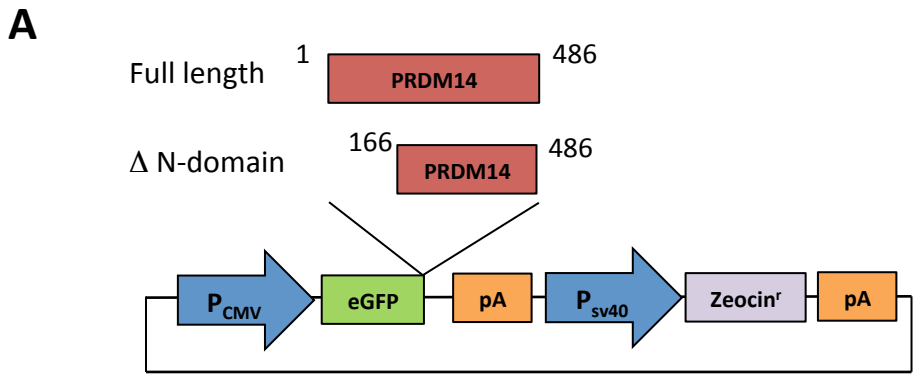


Fig. S1. Okuzaki et al.



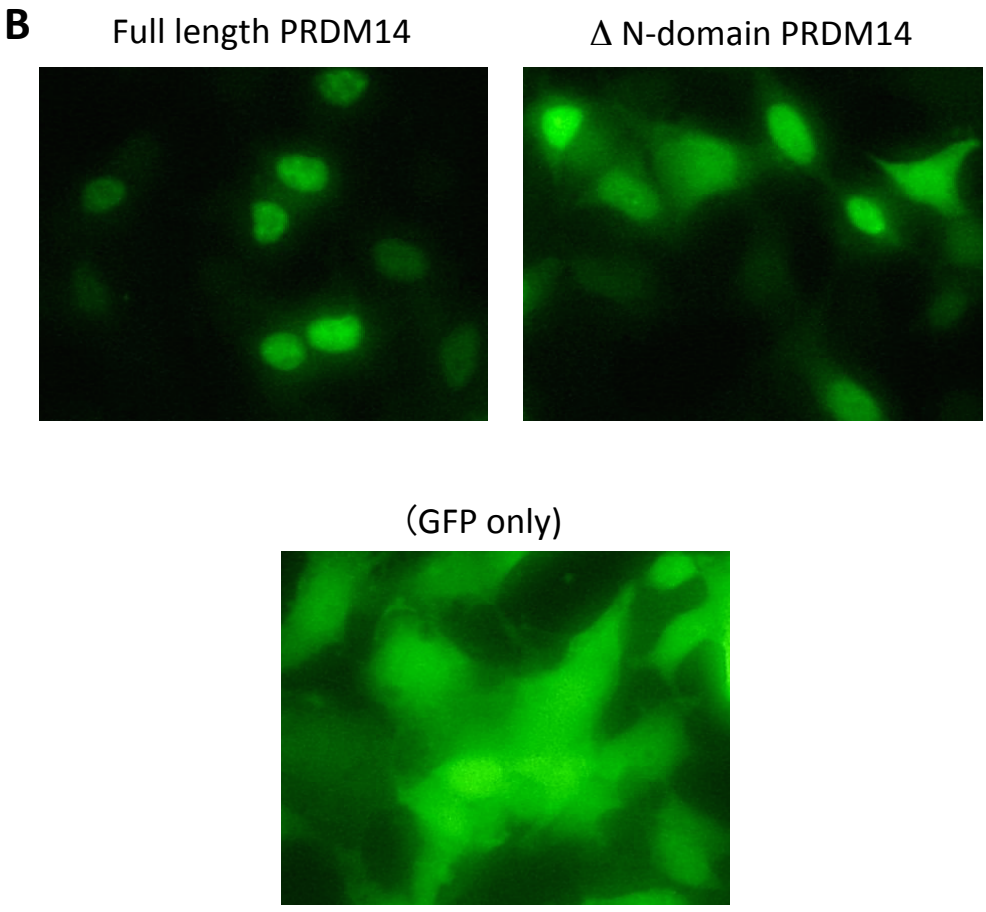
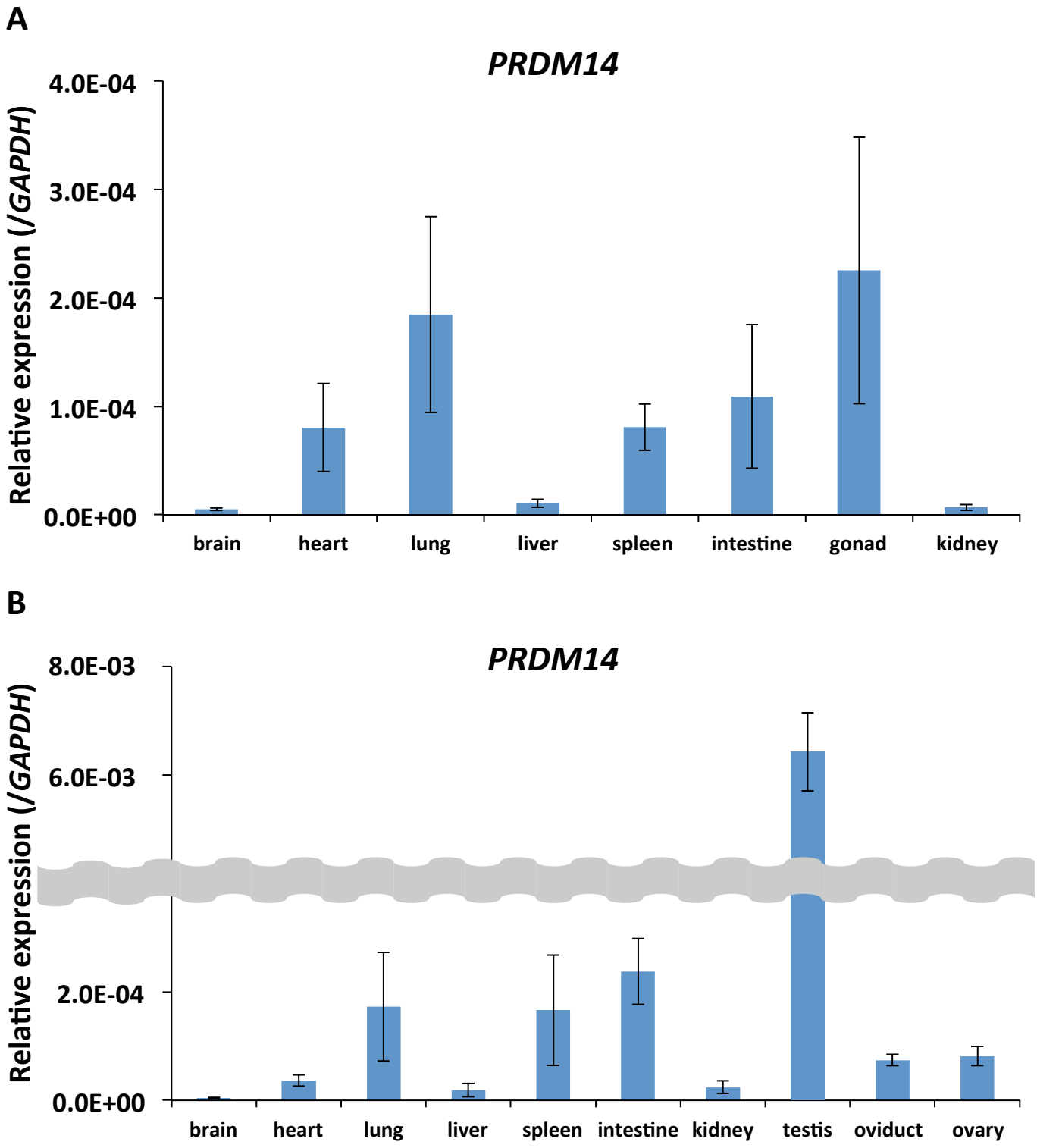


Fig. S2. Okuzaki et al.



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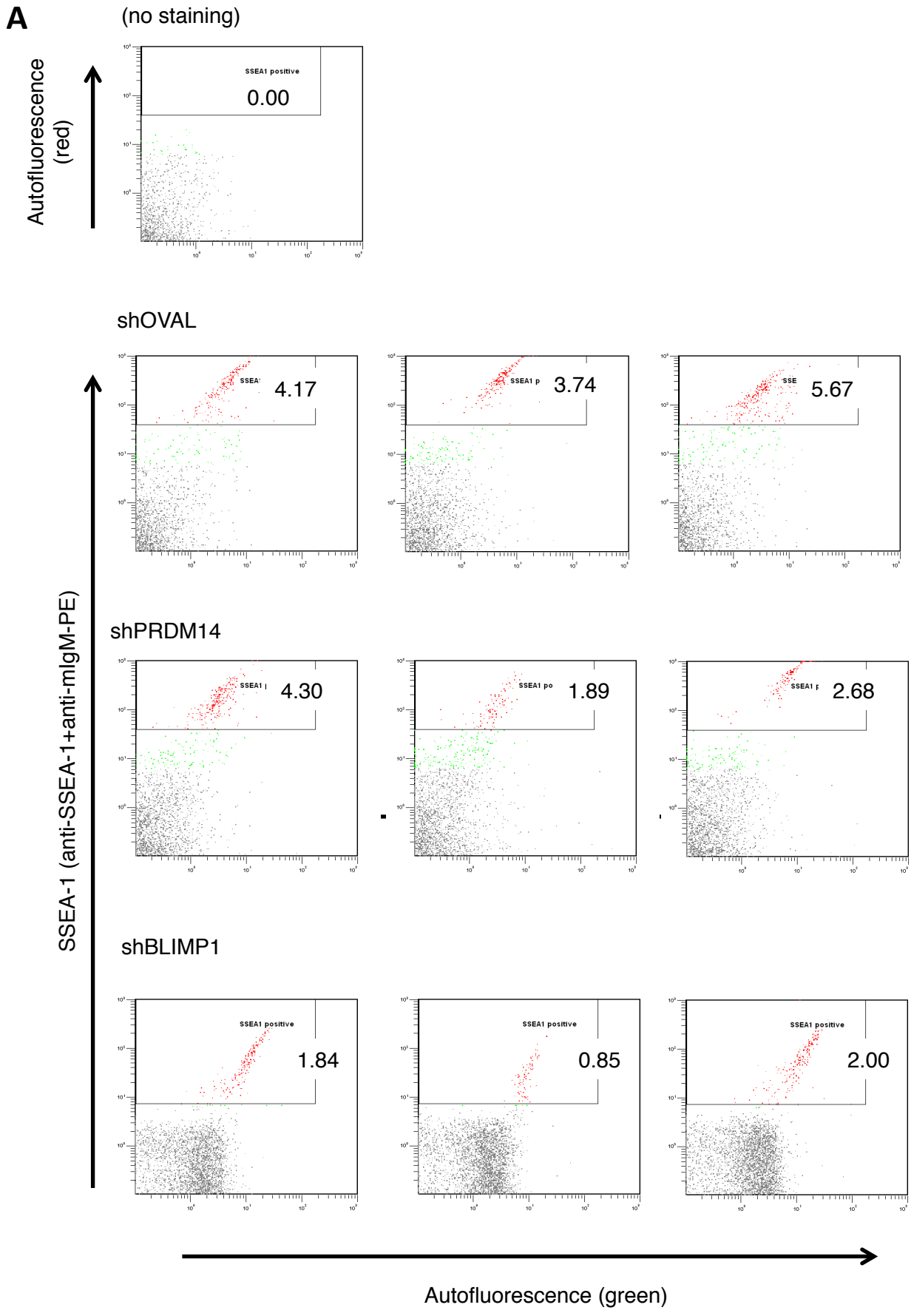

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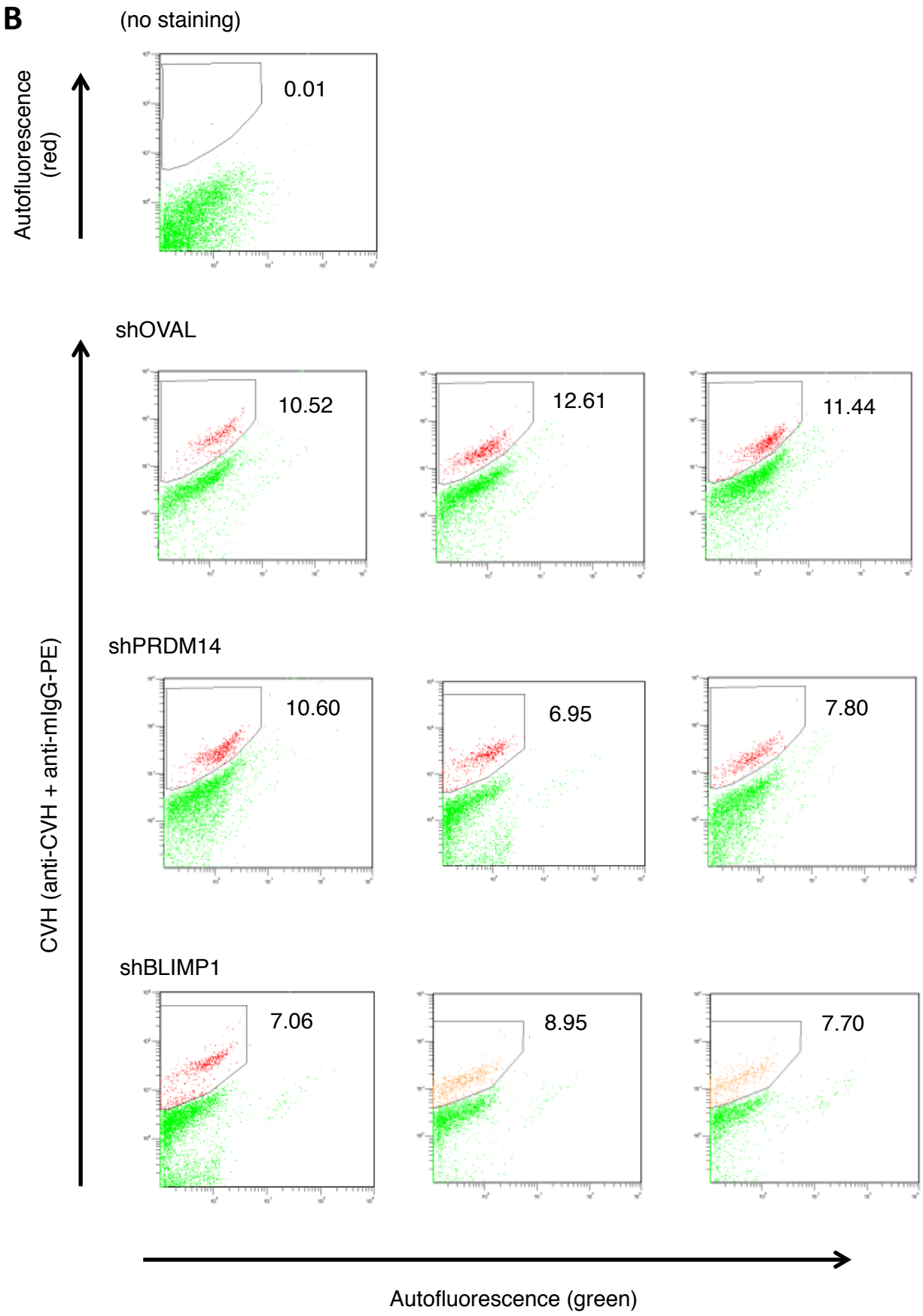
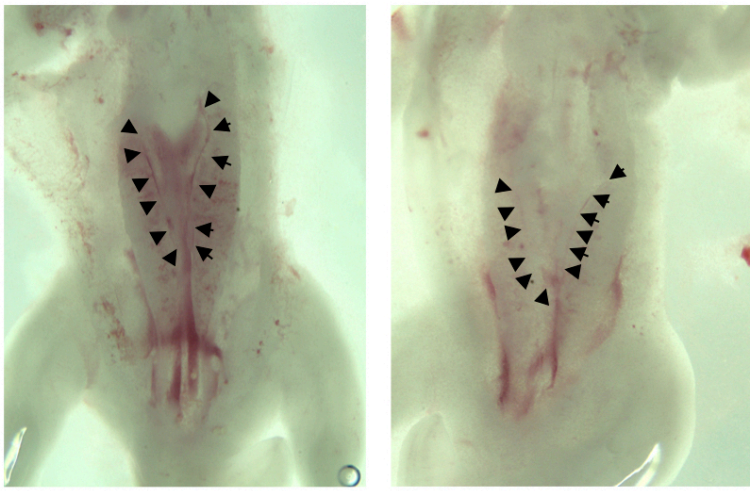
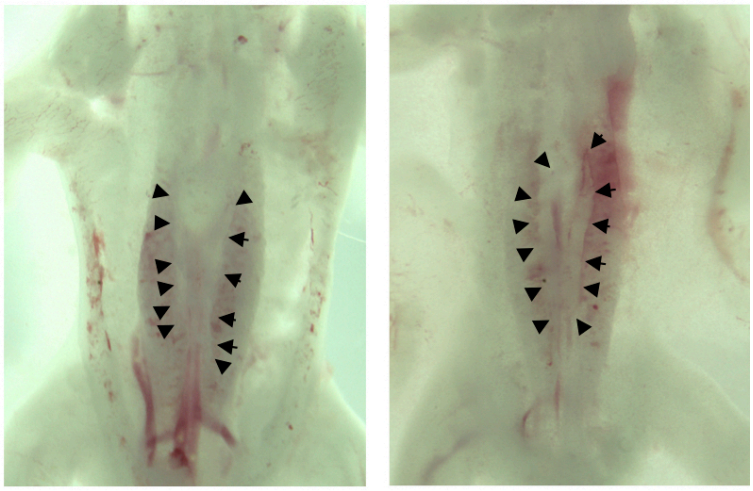


Fig. S4. Okuzaki et al.

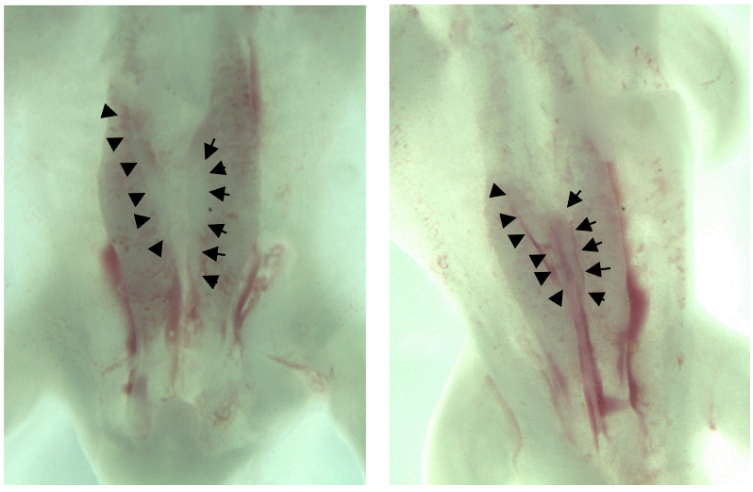
sh*OVAL*



sh*PRDM14*



sh*BLIMP1*



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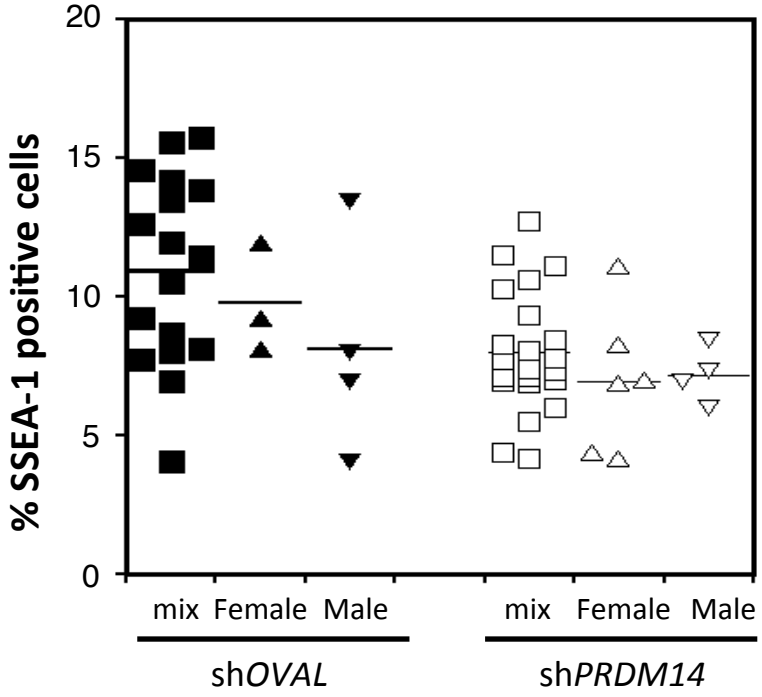
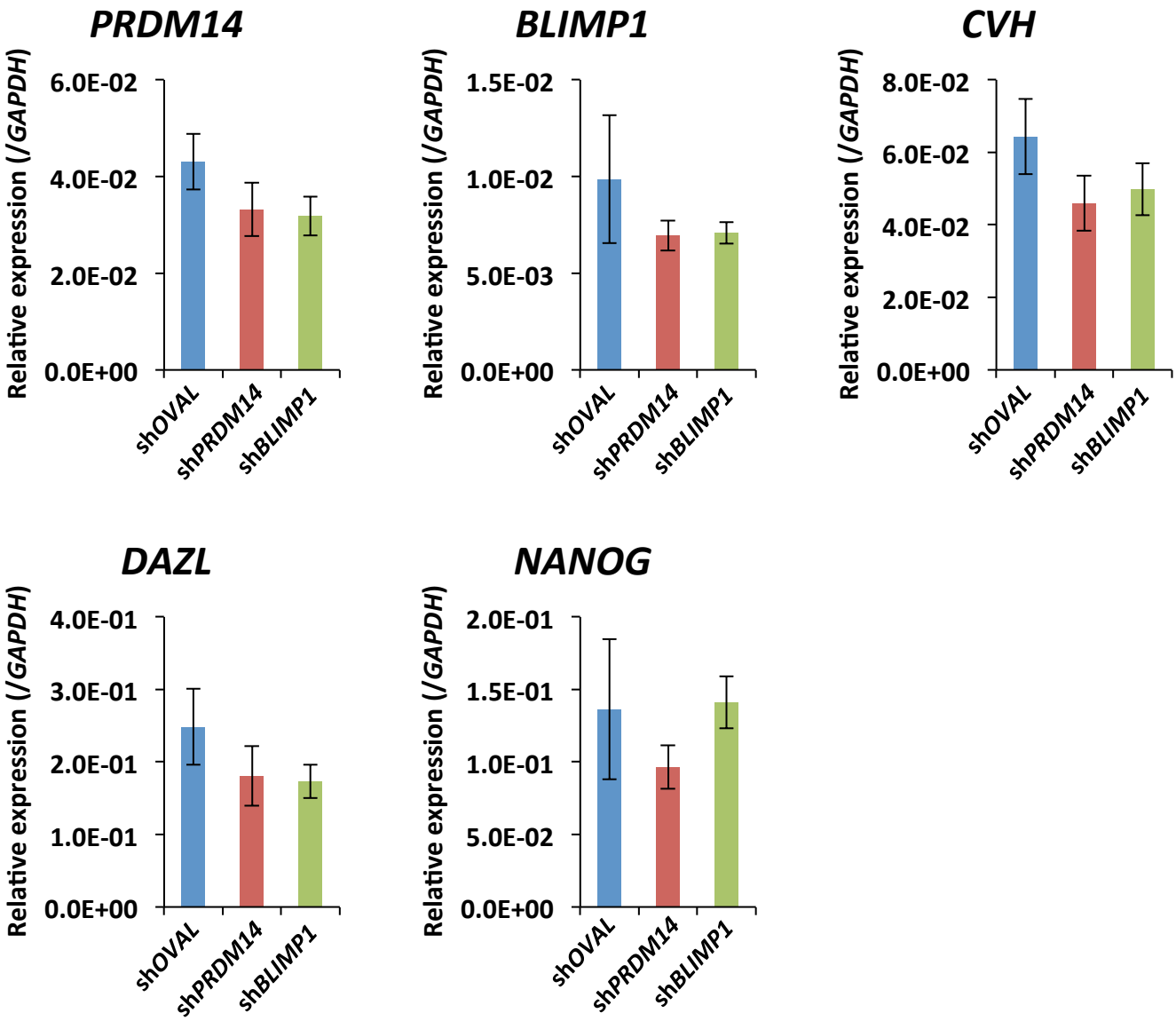


Fig. S6. Okuzaki et al.



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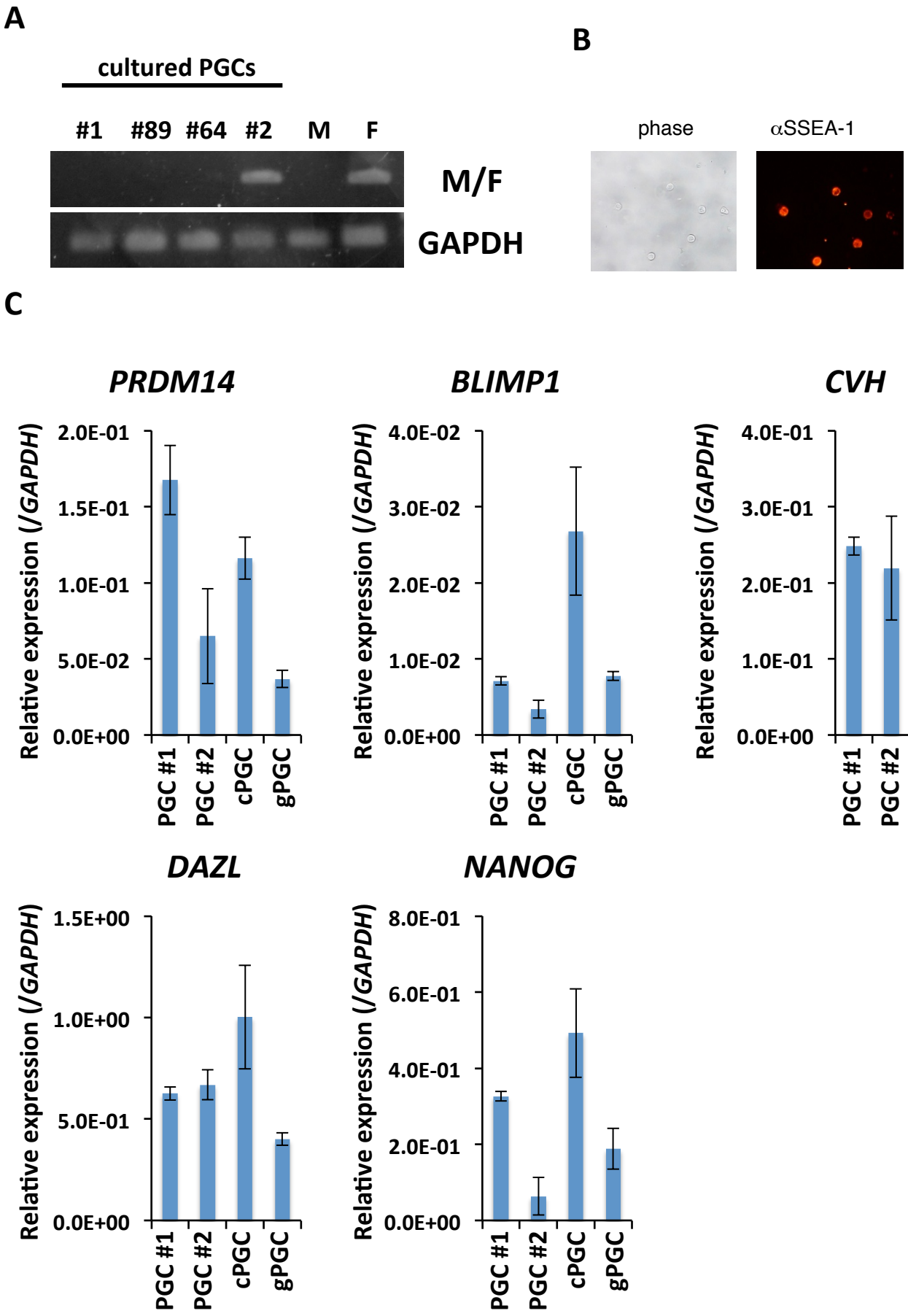


Fig. S8. Okuzaki et al.

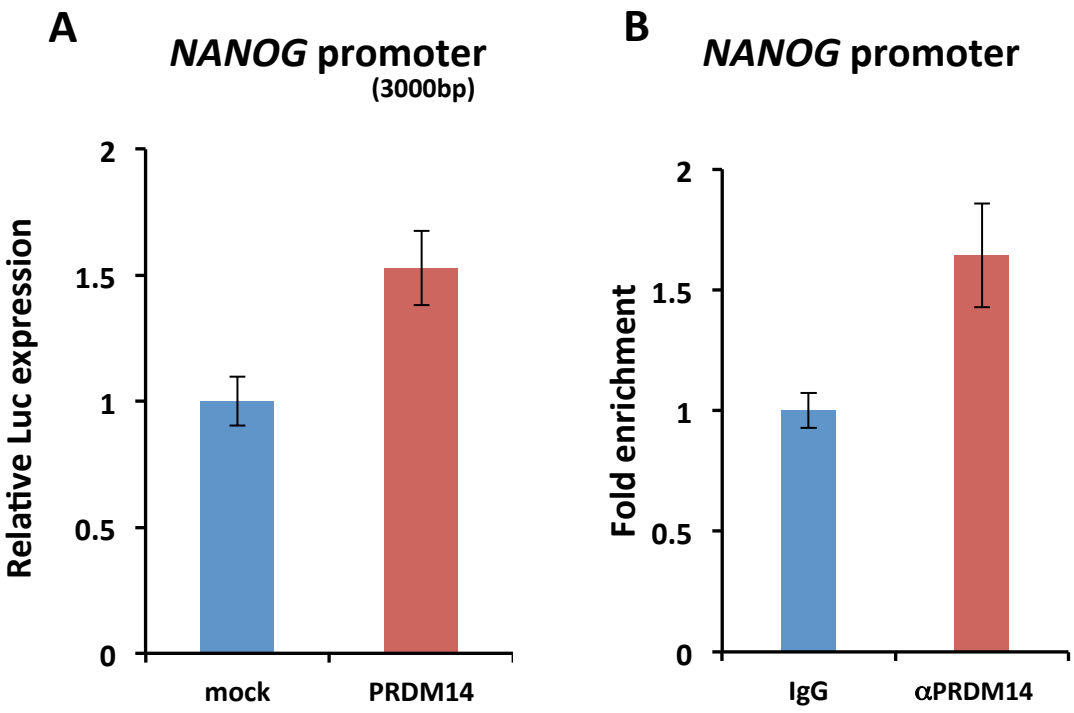
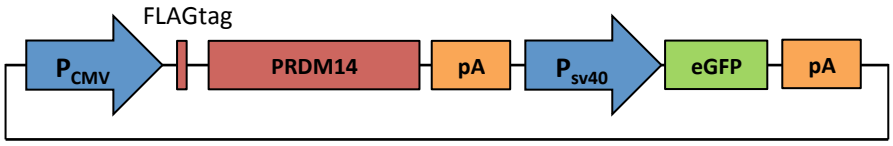
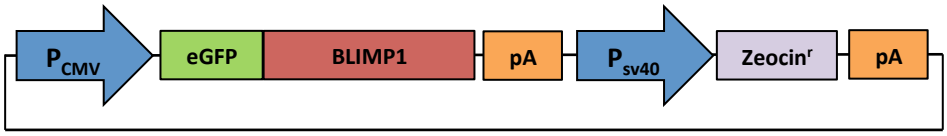


Fig. S9. Okuzaki et al.

A pcDNA4A/FLAG-PRDM14_eGFP



B pcDNA4A/eGFP-BLIMP1_Zeo



C pcDNA4A/FLAG-BLIMP1_eGFP

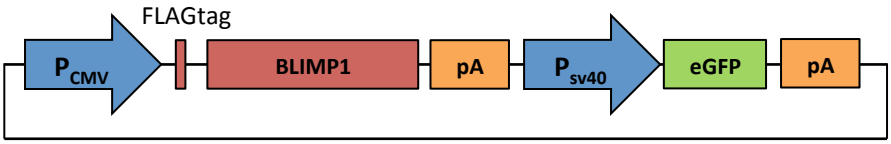


Fig. S10. Okuzaki et al.

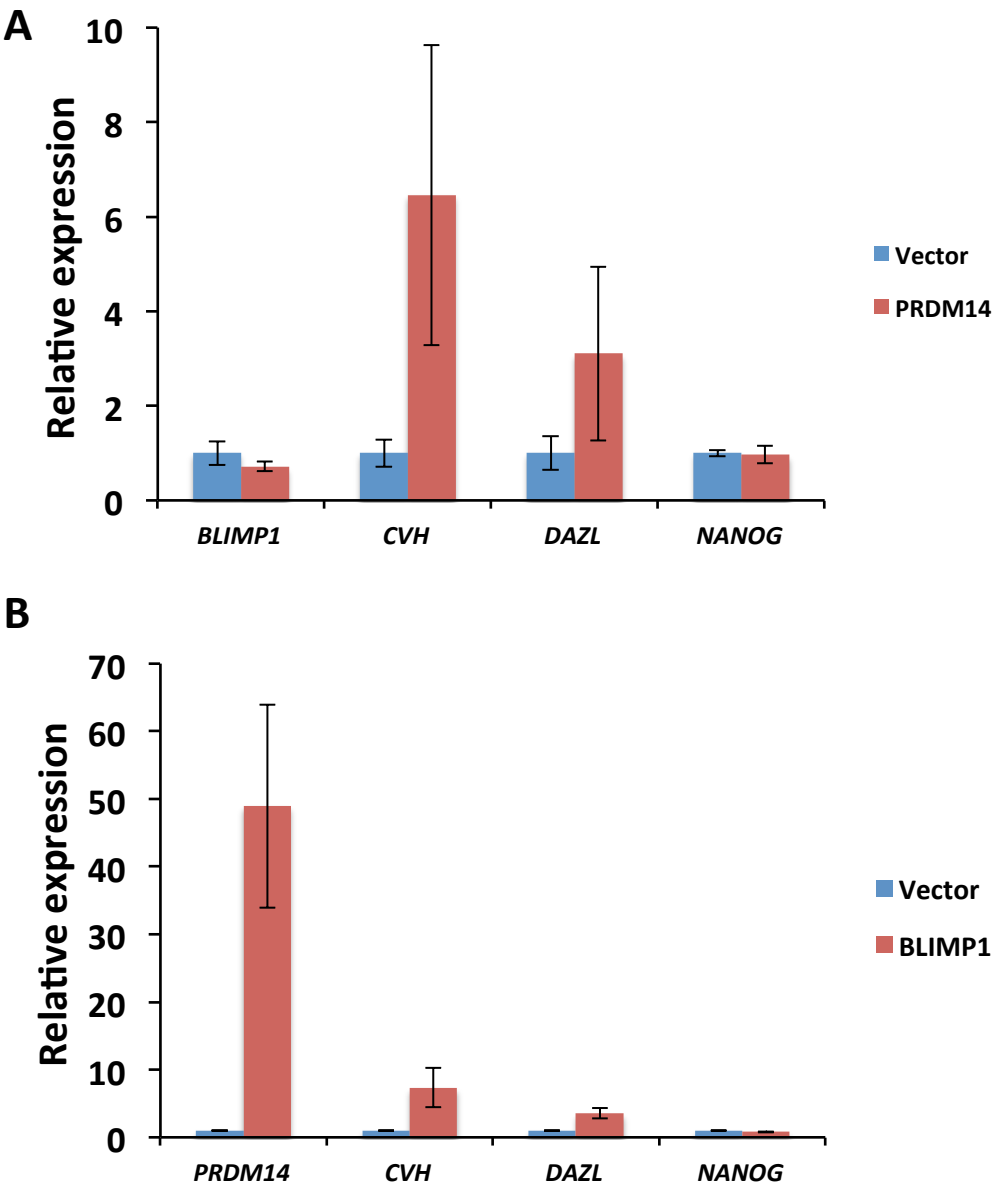


Fig. S11. Okuzaki et al.

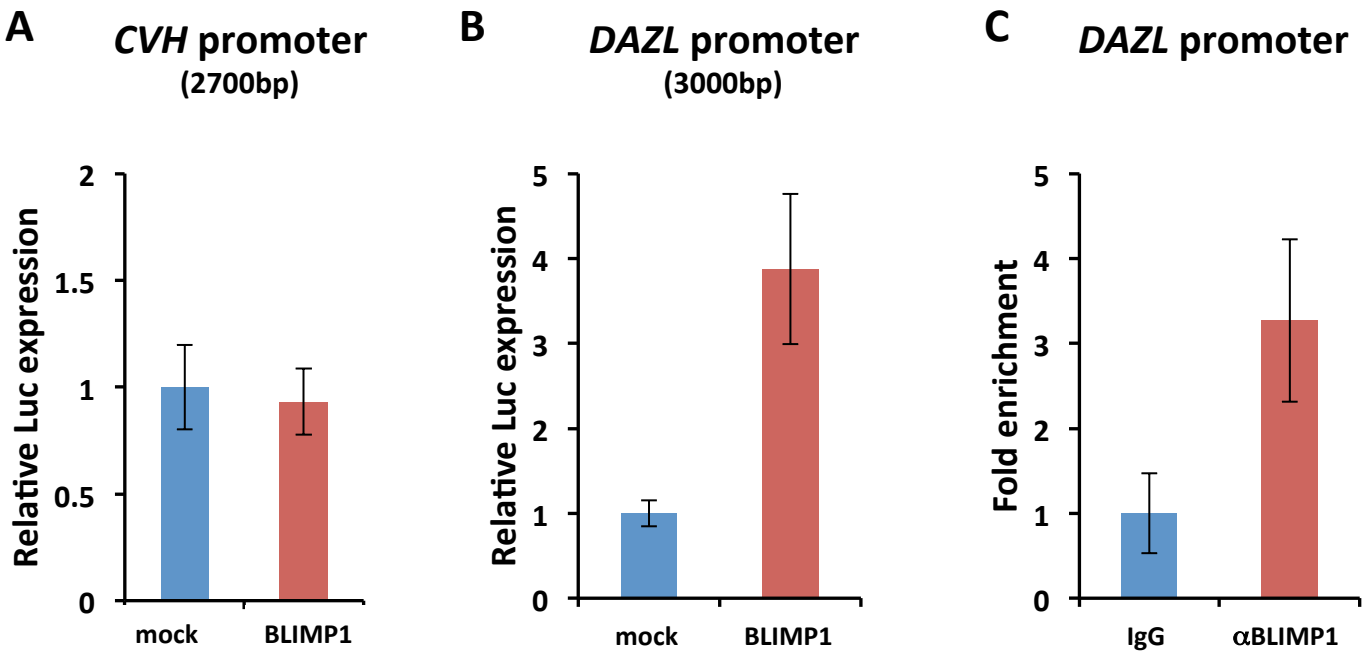


Fig. S12. Okuzaki et al.

Table S1. Expression of pluripotency-related and germ cell-specific genes in blastodermal cells and cPGCs.

Gene	Expression (/GAPDH x 10^{-2})			
	cPGC	Blastodermal cells		
		Before culture	After 3-d culture	
			ES medium	Differentiation medium
PRDM14	11 ± 1.3*	3.4 ± 0.3	0.03 ± 0.01	0.06 ±0.03
BLIMP1	2.7 ± 0.8*	1.5 ± 0.1	1.3 ± 0.0	7.3 ± 3.4
CVH	16.9 ± 4.6	0.49 ± 0.01	0.01 ± 0.00	0.06 ± 0.03
DAZL	100 ± 26	0.82 ± 0.03	0.05 ± 0.01	0.05 ±0.00
NANOG	49 ± 12	38 ± 2	1.9 ± 0.2	8.4 ±0.95

Freshly isolated or cultured blastodermal cells (EK-stages IX-X) were analyzed. Blastodermal cells were cultured in ES medium or differentiation medium for 3 days. The expression levels in sorted cPGC are also shown. Data are represented as mean ± SE of 3-6 independent determination. *, data from Fig. 1.

Table S2. Primers, siRNAs and shRNAs used in this study.

Primers for 5'RACE		
PRDM14 specific RT primer	Rev	CGTCGATGAAGTG
5'RACE 1st PCR	Dir	CCAAGGCAAAGTGGTCAACACC
	Rev	AGCGGACTCCTTTGGGGATG
5'RACE 2nd PCR	Dir	GATGAATTCACGACGACAACTCGCTGATGTG
	Rev	AAAGCGGCCGCCAAACGTCCCCGTAGGCCAC

(Restriction enzyme recognition sites are underlined)

Primers for Cloning (expression vector)		
PRDM14	Dir	CATGGATCCATGGCGCTGGCCCTGGCCGG
	Rev	CATGCGGCCGCCTAATGAGCAGCAGCGTGGACGTTCATG
BLIMP1	Dir	CATAGATCTATGGACATGGAGGATGCTGACATG
	Rev	CATGCGGCCGCTTAAGGGTCCATTGGTTCAACTGTTTC
chicken U6-1 promoter	Dir	CATACTAGTACCAAAACTCTGAAGAAACGA
	Rev	CATGGATCCCGAATATCTCTACCTCCTAG
chicken U6-4 promoter	Dir	CATACGCGTAGCGCCGCGGGGAATTG
	Rev	CATGGATCCCAAGCCCAGGTGTCTCTCGG
chicken H1 promoter	Dir	CATAAGCTTGGCACCAGACCCATTCACAC
	Rev	CATGGATCCACGCACTTTGTGGAAGTGCC

(Restriction enzyme recognition sites are underlined)

Primers for Cloning (luciferase assay vector)		
PRDM14 promoter 3000bp	Dir	CATAGATCTGTCCCTCAACGCAACGTTTCGA
PRDM14 promoter Reverse	Rev	CATGGATCCGCCGGGGGGCAGCAGGTAG
CVH promoter 2700bp	Dir	CATGCTAGCAACTTGAGCCTGCCATAATCAGAGC
CVH promoter 1600bp	Dir	CATGCTAGCAGCCACTCCAACTCTGCTTCC
CVH promoter 600bp	Dir	CATGCTAGCACAGCCACTGTAGCACGTGAGGAG
CVH promoter 200bp	Dir	CATGCTAGCGTGTGGCGCGGAGCGGAGCGCTG
CVH promoter Reverse	Rev	CATCTCGAGGCGAATGACCTGCAGGACCAGGAGCAC
DAZL promoter 3000bp	Dir	CATCTCGAGGCCGAAAGAGAAGGCTCAGGG
DAZL promoter Reverse	Rev	CATGGATCCTACGCAGAAAACTCTCGAAGACGAAGG
NANOG promoter 3000bp	Dir	CATCTCGAGATCCAGCAGTACAAGCTCCGAAGC
NANOG promoter Reverse	Rev	CATAAGCTTGGTCGGGACGACACCTCCAGCCG

(Restriction enzyme recognition sites are underlined)

Primers for qPCR (for expression analysis)		
GAPDH	Dir	GGGCACGCCATCACTATC
	Rev	GTGAAGACACCAGTGGACTCC
PRDM14	Dir	TGCAATAAGGCGTTCACAGC
	Rev	TCCTCCAGCTCTGGGAAGTG
CVH	Dir	TGACTTATGTCCCCCCTCCT
	Rev	GTAATGGTGCTGGAGGGTCA
DAZL	Dir	AGAATGTGCTGTCCCAGAGC
	Rev	CAAAGGTGTTCCTCAGACGG
NANOG	Dir	CAGCAGACCTCTCCTTGACC
	Rev	TTCCTTGTCCCACTCTCACC
BLIMP1	Dir	AGTGACAATGCCGACAAGTTGG
	Rev	ATTTCCCTTCTGAGCACGGTCA
BLIMP1	Dir	CGCATCAAACTGCAAAGAGGACAC
(confirmation of knockdown)	Rev	CCGTATCGCTGGTATAGATCTCTCC
RCAN *Gag-Pol*	Dir	GGGAGTCATCCAGTCAAACAACGT
	Rev	CCACCACCGGCCAATCAGTAG
RCAN *RT*	Dir	CCGCCTCATCAGCGATAGTCG
	Rev	AGACGTGAAGCAGGACCCGTTA
Male/Female check	Dir	CCCAAATATAACACGCTTCACT
	Rev	GAAATGAATTATTTTCTGGCGAC

Primers for qPCR (for ChIP)		
GAPDH gene	Dir	GGGCACGCCATCACTATC
	Rev	GTGAAGACACCAGTGGACTCC
DAZL prmoter	Dir	AGGAAGGTGCTACCACTACGCT
	Rev	GGGCGATGTGTGCCGTAACC
CVH promoter	Dir	ACTAAGAGCACTAGCGCCAC
	Rev	CAATGGGAGGGCGAATCGT
PRDM14 promoter	Dir	CAGCACTGCCTGTCACATCATG
	Rev	TGCCAGTGGCAAGATGCATGC
NANOG promoter	Dir	GCAACTCCCTGCCACTACCTAGC
	Rev	CCTCCCCAGCTTAGTCATGATGAG

siRNA

si*PRDM14* #1	UCAUGUGGAAGCGGUAGUCCU
	GACUACCGCUUCCACAUGAAC
si*PRDM14* #2	AUUGCAGUAGACGCAUUUGUA
	CAAAUGCGUCUACUGCAAUAA
si*BLIMP1* #1	GAGUUAUAAGCAAAGAGUACA
	UACUCUUUGCUUAUAACUCCA
si*BLIMP1* #2	CAGAUAUGUCAAUUAUGAAGU
	UUCAUAAUUGACAUAUCUGAA

shRNA

sh*PRDM14* #1	CGACAAACACCTCAAGTACACGGCTTCCTGTCACCGTGTACTTGAGGTGTTTGTC
sh*PRDM14* #2	CCCCTACAAATGCGTCTACTGCGCTTCCTGTCACGCAGTAGACGCATTTGTAGGG
sh*PRDM14* #3	CGACTACCGCTTCCACATGAACGCTTCCTGTCACGTTCATGTGGAAGCGGTAGTC
sh*BLIMP1* #1	CGACAATATCGACTTAACGTCCGCTTCCTGTCACGGACGTTAAGTCGATATTGTC
sh*BLIMP1* #2	CGATGATTTGCGTAAGAACTATGCTTCCTGTCACATAGTTCTTACGCAAATCATC
sh*BLIMP1* #3	CGAGTTATAAGCAAAGAGTACAGCTTCCTGTCACTGTACTCTTTGCTTATAACTC
sh*OVAL* #1	CGACAAATGGAATTATCAGAAAGCTTCCTGTCACTTTCTGATAATTCCATTTGTC
sh*OVAL* #2	CGAACCTATCAACTTTCAAACAGCTTCCTGTCACTGTTTGAAAGTTGATAGGTTC
sh*OVAL* #3	CGAAGATCAAAGTGTACTTACCGCTTCCTGTCACGGTAAGTACACTTTGATCTTC

(loop portion are shown in red)