学位論文

「生殖幹細胞アイデンティティにおけるDmrt1-Prkra-p53システム」

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著者の宣言

本学位論文は、著者の責任において実験を遂行し、得られた真実の結果に基づいて正確に作成したものに相違ないことをここに宣言する。

要旨

一般的に、脊椎動物の始原生殖細胞は、胚発生過程で未分化生殖巣に移動し、性決定後、卵巣あるいは精巣分化初期に卵原細胞もしくは精原細胞（雌雄生殖幹細胞）へと発生・分化すると考えられている。しかし、生殖巣分化初期の卵原細胞と精原細胞の形態学的あるいはエピゲノムレベルでの相違はほとんど明らかになっていない。さらに、卵原細胞と精原細胞のインテグリティに関わる分子基盤の知見も乏しい。本研究では、卵原細胞と精原細胞のアイデンティティあるいはゲノム保持を制御する分子機構を明らかにすることを目的として、両生類アフリカツメガエル（*X. laevis*）を用いて解析を行った。両生類を用いたのは、哺乳類では胎生期にほぼ減数分裂して卵母細胞になるため卵原細胞の解析が容易でないこと、そして、両生類の *X. laevis* を用いたのは、実験動物として繁殖が容易であり、またカエルでは唯一、性決定遺伝子が同定されているため、遺伝的雌あるいは雄個体の判定が可能であることが理由である。まず、生殖幹細胞でその生存に関与していることが知られている Dmrt1 (Doublesex and Mab3 Related Transcription factor) という転写因子の発現を、遺伝的雌雄判別した幼生および変態後の生殖巣を用いて、免疫組織学的解析を行った。その結果、幼生期の一次・二次卵原細胞と一次精原細胞は共に Dmrt1 を発現するが、変態後には、二次卵原細胞で発現が認められなかった。興味深いことに、幼生期の一次卵原細胞・一次精原細胞ともに、始原生殖細胞と同様に不定形な核および細胞の形態を呈していたが、変態後には一次卵原細胞のみ円形化が認められた。さらに、二重鎖 DNA 切断シグナルであるゲノム監査役のヒストンバリアント H2AX のリン酸化（γH2AX）を調べたところ、幼生期では卵原細胞・精原細胞で発現が見られたが、変態期では精原細胞のみ発現が認められた。これらのことから、Dmrt1 とγH2AX は、それぞれ生殖幹細胞としてのアイデンティティの維持あるいはゲノムインテグリティに関与すると考えられた。

次に、Dmrt1 の生殖幹細胞におけるアイデンティティの維持の分子基盤を明らかにすることを目的として、Dmrt1 結合タンパク質の同定を試みた。網羅的に解析するために、*X.*

laevis の生殖幹細胞を含む成体精巣の細胞抽出液から、抗 Dmrt1 抗体を用いて免疫沈降を行い、MS/MS 解析により３つの推定結合タンパク質 Phb2, Yb1 および Prkra を同定した。興味深いことに、Dmrt1 駆動のルシフェラーゼレポーターを用いた培養細胞系において、Prkra は Dmrt1 の転写活性を強く増強することが分かった。Prkra はがん抑制因子 p53 と関与が知られていたので、この Dmrt1 駆動のルシフェラーゼレポーター系において、p53 の影響を調べた。極めて興味深いことに、Prkra によって増強された Dmrt1 の転写活性は、p53 によって抑制されることが認められた。哺乳類マウスでの精原細胞では、p53 と Dmrt1 は精原細胞で拮抗的に機能することが知られていたが、このツメガエルでの結果は、脊椎動物の生殖幹細胞における p53-Dmrt1 システムの保存性を示唆すると共に、その拮抗作用は Prkra を介している可能性が新たに示された。

生殖幹細胞は、次世代に引き継がれる重要な細胞であり、様々なストレスや障害からゲノムが守られる必要がある。本研究では、生殖幹細胞において p53 とγH2AX はパトロール役としてゲノムを守ると共に、損傷細胞の除去に関わること、Dmrt1 は生殖幹細胞のアイデンティティに関わり、その機能は Prkra によって増強されるが、逆に p53 に抑制されることが示唆された。すなわち Dmrt1- Prkra -p53 システムは、生殖幹細胞インテグリティに重要な機能を果している可能性が考えられる。

Contents

1. Introduction

Sexual dimorphism normally occurs after gonadal sex determination in vertebrates. In the genetic sex-determining systems, gonadal sex is determined by sex-determining genes on the sex chromosome, such as mammalian Y-linked Sry, chicken Z-linked *Dmrt1*, the African clawed frog W-linked *Dm-W*, and teleost fish medaka Y-linked *dmy/Dmrt1* (Matsuda et al. 2002; Nanda et al. 2002; Smith et al. 2009; Yoshimoto et al. 2008). It is generally believed that these sex-determining genes are expressed in the somatic cells of gonads, leading to primary ovarian or testicular formation. After sex determination, primordial germ cells (PGCs) differentiate into primary oogonia or spermatogonia (female and male germ stem cells [GSCs]). However, the molecular and morphological differences between the female and male GSCs in many vertebrates remain unclear.

Dmrt1 (Doublesex and Mab3 Related Transcription factor) encodes a transcription factor characterized by the presence of a DNA-binding domain called the DM domain. Dmrt1 induces testis formation, and is required for somatic-cell masculinization, which leads to testis formation in various vertebrate species (Lambeth et al. 2014; Masuyama et al. 2012; Smith et al. 2009; Yoshimoto et al. 2010; Zhao et al. 2015). Its paralogs include the *X. laevis* and medaka sex-determining genes, *dm-W* and *dmy/Dmrt1bY*. In mice, Dmrt1-deficient Sertoli cells are reprogrammed into granulosa cells during postnatal development, indicating that Dmrt1 plays an important role in the regulation of somatic cell masculinization (Matson et al. 2011). In view of transcriptional control, Dmrt1 could repress transcription of five feminizing genes, *Foxl2, Esr1, Esr2, Wnt4* and *Rspo1* as a repressor, but activate three masculinizing genes *Ptgdr, Sox9*, and *Sox8* as an activator (Matson et al. 2011). Thus, *Dmrt1* might be a masculinizing master gene in vertebrates. *Dmrt1* also contributes to the development of both female and male germ cells in mice. Curiously, mouse Dmrt1 negatively controls meiosis in male germ cells by repressing *Stra8* transcription (Matson et al. 2010), but promotes meiosis in female germ cells by enhancing *Stra8* transcription (Krentz et al. 2011). However, there have been few reports investigating Dmrt1's functions in the GSCs of other vertebrate species.

Another factor involved in germ cell development is γH2AX, a phosphorylated form of the H2A variant, H2AX. γH2AX is induced by double-strand breaks (DSBs) in DNA, and contributes to DNA repair in mitotic cells. In several organisms, γH2AX is also involved in meiotic recombination and/or sex chromosome inactivation from the leptotene to diplotene stages in meiotic germ cells (Chicheportiche et al. 2007; Turner et al. 2004). To date, the presence of γH2AX has been demonstrated in spermatogonia (Blanco-Rodríguez 2009), but not in PGCs or oogonia. In addition, the function of γH2AX has not been understood in these GSCs yet.

To investigate the role of these two factors in the development of sexual dimorphism in GSCs and somatic cells in *X. laevis*, I performed immunostaining with antibodies against Dmrt1 and

γH2AX using genetically female (ZW) and male (ZZ) gonads from various stages of development. During the early stages of sex differentiation, Dmrt1 and γH2AX were expressed in both the ZW and ZZ GSCs. In vitellogenic ovaries, the nuclei and cell bodies of the female GSCs underwent condensation, and γH2AX expression was barely detected in the condensed nuclei after metamorphosis, but was still detected in the nuclei of male GSCs. Similarly, Dmrt1 expression in male GSCs continued during development, but was confined to only certain types of female GSCs.

Moreover, to understand the transcriptional regulation by Dmrt1, I identified Prkra as a Dmrt1-binding protein from *X. laevis* testis. Prkra strongly enhanced the transcriptional activity of Dmrt1 in transfected 293T cells. Interestingly, tumor suppressor p53 greatly repressed Dmrt1's transcriptional activity and the Prkra -enhanced activity in transfected 293T cells.

I discuss sexual dimorphism between female and male GSCs, and the potential functions of Dmrt1 and γH2AX in the GSCs during development, and then about the regulatory system of Dmrt1 together with p53 and Prkra.

2. Methods

2-1. **Animal care and use**

All of the experimental procedures involving *X. laevis* were approved by the Institutional Animal Care and Use Committee of Kitasato University. *X. laevis* frogs at various developmental stages were purchased from Watanabe Zoushoku (Yachiomachi, Japan) and maintained at 22 °C. Tadpole developmental stages were identified according to the descriptions by Nieuwkoop and Faber (Nieuwkoop and Faber 2006)

2-2. **Tissue sample preparation**

Gonadal tissues were isolated from *X. laevis* tadpoles and adults at different stages of development. Tissue samples were fixed in 4% paraformaldehyde solution (4% paraformaldehyde, 70 mM phosphate buffer [pH 7.3]) and embedded in paraffin. Paraffin sections (7 μm) were generated and used for immunohistochemical experiments.

2-3. **Real-time reverse transcription quantitative PCR (RT-qPCR)**

Total RNAs were isolated from the tissues of ZZ and ZW gonads at various stages of development, using the RNeasy Mini Kit (Qiagen, Venlo, Netherlands). RNA (1 μg) was reverse transcribed with the Transcriptor First Strand cDNA Synthesis Kit (Roche, Basel, Switzerland), according to the manufacturer's instructions. RT-qPCR was carried out using the SYBR Green Realtime PCR Master Mix (ToYoBo, Osaka, Japan). *Dmrt1*, *Stra8* or *Prkra* cDNA were amplified using the following primer pairs: 5'-GGGATTGCCAGTGCAAAAAG-3' (forward) and 5' -TTCCAGCATCAAGCAAGAGC-3' (reverse) or 5'-TACCTCAGCCAGGAGTGTG-3' (forward) and 5'-TGTCCATAGTCTGCTGGTAG-3' (reverse), 5'-CAGCTGCTGCATGAATTTG-3' (forward) and 5'-CTCTCCTAAGCTAGTTATGTCACC-3' (reverse), respectively.

2-4. **Antibodies**

The mouse monoclonal anti-VASA antibody and rabbit polyclonal anti-Dmrt1 antibody were produced using the *X. laevis* VASA-like protein and Dmrt1 as antigens, and were described elsewhere

(Komiya et al. 1994; Yoshimoto et al. 2010). The anti-phospho-Histone H2A.X (Ser139) and anti-BrdU (5-bromo-2'-deoxyuridine) rabbit polyclonal antibodies were purchased from EMD Millipore (Billerica, MA, USA) and Cell Signaling Technology (Beverly, MA, USA), respectively. Alexa 488- and 594-conjugated goat anti-mouse and anti-rabbit IgG antibodies were purchased from Invitrogen (Carlsbad, CA, USA).

2-5. **Immunohistochemistry**

The paraffin sections were deparaffinized in xylene followed by rehydration in a graded ethanol series. After washing in H_2O, the sections were boiled in 10 mM citrate buffer (pH 6.0) for antigen unmasking. The sections were then incubated overnight at 4 °C with anti-Dmrt1 (1:1000) and anti-VASA (1:500) antibodies, which were diluted in PBS (without calcium or magnesium) containing 0.2 % skim milk and 0.05 % Triton X-100. Alexa 488- and 594-conjugated goat anti- [mouse IgG (1:2000)] and anti-[rabbit IgG (1:2000)] anti- bodies (Invitrogen) were used to detect the primary antibodies. Images of 0.2 μm optical sections were captured and analyzed by the (Carl Zeiss Microscopy, Goettingen, Germany).

2-6. **BrdU incorporation**

Tadpoles or frogs were injected intraperitoneally with BrdU (diluted in 70 mM phosphate buffer) at 600 μg/g body weight, and sacrificed 4 or 24 h later. Tissue sections were deparaffinized and incubated in 1 M NaCl at 37 °C for 2 h, followed by neutralization in 0.1 M borate buffer (pH 8.5). The sections were blocked and then incubated overnight at 4 °C with anti-BrdU (1:200) and anti-Dmrt1 (1:10000) antibodies. The sections were washed, and then the signals were detected as described above.

2-7. **Immunogen preparation**

Bacterial expression vector pMALc2-Dmrt1 (130-336) was constructed by inserting the region encoding residues from 130 to 336 of *X. laevis* Dmrt1 into pMAL-c2 (New England Biolabs). The recombinant protein was produced in *E. coli* Rosetta (DE3) pLysS (Novagen) BL21(DE3), and purified using amylose resin (New England Biolabs), followed by elution with 10 mM maltose, according to the manufacturer's instructions. The purified protein was dialyzed with phosphate

buffered saline (PBS) and used as an immunogen.

2-8. **Production of mouse anti-Dmrt1 monoclonal antibodies**

Mouse monoclonal anti-Dmrt1 antibodies were generated based on the mouse medial iliac lymph node method (Sado et al. 2006). Briefly, the purified protein was injected into the tail base with Freund's complete adjuvant. Three weeks later, cells from the lymph nodes of the immunized mice were fused with mouse myeloma. The resulting hybridoma cells were plated onto 96-well plates and cultured in HAT (hypoxanthine aminopterin thymidine) selection medium. Monoclonal antibodies were purified from the hybridoma supernatants by ion-exchange chromatography.

2-9. **Immunoblotting**

Samples were ran by 10% SDS-PAGE and transferred to FluoroTrans 0.2 μm membrane (PALL). The membrane was blocked with 5% skim milk in PBS, incubated with purified monoclonal antibodies (1:400) or the 1:10000 anti-Dmrt1 rabbit polyclonal antibody (Fujitani et al. 2016) at 4 °C for overnight, and then washed with PBST (0.1% Tween-20 in PBS). Then it was incubated with anti-mouse or anti-rabbit IgG-HRP-conjugated secondary antibodies at room temperature for an hour, and then washed. Signals were detected using ImmunoStar LD substrate (Wako) and C-Digit (LI-COR). Both the anti-mouse and anti-rabbit HRP-conjugated antibodies were purchased from SIGMA, and diluted 1:20000).

2-10. **Immunoprecipitation (IP)**

Transfected 293T cells or testes dissected from one-year-old adult *X. laevis* were homogenized in RIPA buffer, followed by sonication. The cell extracts from a 35 mm dish with 1 μg of each anti-Dmrt1 monoclonal antibody or the testicular extracts (10 mg) with 100 μg of the anti-Dmrt1 monoclonal antibody 4F6 were mixed with 100 μl of EZveiw Red Protein G Affinity Gel (SIGMA), were incubated overnight at 4 °C . Mouse normal IgG (Santa Cruz Biotechnology; sc-2025) was used as a negative control. The gels were washed twice with RIPA buffer, and the denatured proteins were run by SDS-PAGE (Perfect NT Gel W, 10–20% acrylamide, 28 wells; DRC Co. Ltd.). Silver staining was performed by 2D-SILVER STSIN II (COSMO BIO; 423413) for MS/MS analysis.

2-11. **Enzymatic in-gel protein digestion**

The gels containing the interest bands were cut into small pieces, destained in 50% ACN/50 mmol/L NH4HCO3, washed with deionized water, dehydrated in 100% CAN, and dried in evaporator. The gel pieces were rehydrated in 25 mM Tris-HCl (pH 9.0)/20% ACN containing 50 ng/mL trypsin (sequencing grade; Roche) for 45 min. After unabsorbed solution was removed, the gel pieces were incubated in 50 mM Tris-HCl (pH 9.0) for 20 h at 37 °C. The solution was transferred to a new tube.

In addition, the remaining fragments were extracted in 5% formic acid/50% ACN for 20 min at room temperature, and transferred to the tube.

2-12. **Protein identification by LC-MS/MS analysis**

The digested peptides were desalted and separated by HPLC (the EASY-nLC 1000, Thermo Fisher Scientific) and analyzed by mass spectrometer (Q-Exactive mass spectrometer, Thermo Fisher Scientific). Then the proteins were identified by using the obtained data and *X. laevis* database.

2-13. **cDNA cloning and plasmid construction**

Phb2, *Yb-1*, and *Prkra* cDNAs were amplified from *X. laevis* adult testis cDNA by PCR using PrimeSTAR polymerase (TaKaRa) with primer sets *Phb2* (5'-GCTCAGAATTTAAAGGATTTTGC-3', 5'-TCACTTCTTTCCTTGTTTGAAAAC-3'), *Yb-1* (5'-AGCAGCGAGGTTGAAACAC-3', 5'-TTACTCAGCCCCGCCCTG-3') and *Prkra* (5'-TCCCAGGAGAGGTTTCCAG-3', 5'-TCACTTTTTAATACACATGATTTTTA-3'), respectively. PCR products were cloned into a vertebrate expression vector pcDNA3-S-Tag (Ito et al. 1999). Effector plasmids used for luciferace reporter assay pcDNA3-FLAG-p53 was cloned into a vertebrate expression vector pcDNA3-FLAG (Ito et al. 1999) by PCR using PrimeSTAR polymerase (TaKaRa) with the following primer pair (5'-GAACCTTCCTCTGAGAC-3', 5'-TCATTCCGAGTCGGGCTGTTC-3').

2-14. **Luciferase reporter assay**

24 hours before transfection, 293T cells were plated at 5×10^4 cells per well in 48-well plate. The cells were transfected with luciferase reporter plasmid p4xDmrt1-luc (Yoshimoto et al. 2010), effector plasmids, and *Renilla* luciferase vector pRL-SV40 (Promega) by PEI. After 24 hours, luciferase activities were measured by Luminocounter 700 (NITI-ON). Each firefly luciferase activity was normalized by *Renilla* luciferase activity using the dual luciferase assay system (Promega).

2-15. **Whole mount *in situ* hybridization**

Whole mount *in situ* hybridization for *X. laevis Prkra* mRNA was performed as described previously (Wada et al. 2017), using DIG-labeled sense or anti-sense probes from nucleotides 1-930 in genebank number NM_001086031.1.

2-16. **Statistical analysis**

Two-group, or multiple group comparisons were performed by Student's t-test, or one-way ANOVA followed by Tukey HSD test, respectively. Significance for all test was set at $p < 0.05$.

3. Results

3-1. **Dmrt1 is expressed at similar levels in ZZ and ZW gonads during early sex differentiation**

To examine the Dmrt1 expression during ZZ and ZW gonadal development after sex determination, I conducted quantitative RT-PCR (RT-qPCR) experiments using total RNA samples from pools of ZW and ZZ gonads at various developmental stages. At stage 50, just after sex determination, *Dmrt1* expression was slightly higher in the ZW gonads than in the ZZ gonads (Fig. 1). However, during most stages of tadpole development, it was expressed at similar levels in the ZW somatic cells actively proliferate in the ZZ and ZW gonads, which share a similar morphology. Female GSCs are located in the cortical region of the ZW gonads, while male GSCs are mainly located in the medulla in the ZZ gonads (Yoshimoto et al. 2008). During these stages, the nuclei of the ZZ and ZW GSCs were large and unstructured, similar to their morphologies in the PGCs of the female and male gonads before sex determination (Fig. 2A). To clarify the localization of Dmrt1 in the developing gonads, I performed immunohistochemical analysis using stage-56 tadpoles and an anti-Dmrt1 antibody, as well as an antibody to VASA, a germ-line-specific protein. Dmrt1 was highly expressed in the large, unstructured nuclei (green) in both the primary oogonia and spermatogonia, while VASA was expressed in the cytoplasm (red) of these cells (Fig. 2A). The strong expression of Dmrt1 in both the ZZ and ZW GSCs was consistent with the results of my RT-qPCR experiments (Fig. 1). Importantly, weaker, but substantial Dmrt1 expression was observed in the somatic cells surrounding the primary spermatogonia in the ZZ gonads at stage 56 (Fig. 2A, arrows). In contrast, there was no detectable Dmrt1 expression in the somatic cells of the ZW gonad. These findings supported a role for Dmrt1 in GSC development and a male-specific function in somatic cell masculinization, which leads to testicular development after sex determination (Yoshimoto et al. 2010).

3-2. **Primary oogonia exhibit nuclear and cell body morphological changes during tadpole development**

During the later stages of metamorphosis (stages 59–65), differences between male and female germ cell morphologies, but not tissue morphologies were observed. At stage 62, many cysts consisting of secondary oogonia, which were proliferating or entering their first meiosis, were observed in the ZW gonads by staining for Dmrt1 and VASA (Fig. 2B). Notably, the primary oogonia underwent significant changes in cell morphology: their cell bodies shrank and their large, distorted nuclei became more condensed, taking on a round or elliptical shape (Fig. 2A). However, they were still located in the cortex of the gonads. Dmrt1 was expressed in the primary oogonia (arrows in Fig. 2B)

and in subpopulations of secondary oogonia in the cysts, but was barely detected in the primary oocytes (Fig. 2).

In the ZZ gonads, the primary structure of the testis cord was evident at these stages. The primary spermatogonia were surrounded by slender somatic cells (pre-Sertoli cells). As expected, Dmrt1 was highly expressed in the primary spermatogonia, whereas its expression in the surrounding pre-Sertoli cells (arrowheads in Fig. 2B) was relatively low. The nuclei of the primary spermatogonia remained large and distorted (Fig. 2B).

3-3. **Meiotic female and male germ cells express little or no Dmrt1 after metamorphosis**

After metamorphosis, the female and male gonads (ovaries and testes) show distinct differences in gross morphology. In the ZW ovaries, a sequence of hollow segmental cords, called ovarial sacs is formed. At the microscopic level, diplotene oocytes during vitellogenesis are frequently observed in the region surrounding the ovarian cavities. However, the oogonia remain in the cortex. Immunohistochemical analysis of ovarian Dmrt1 expression two weeks after metamorphosis revealed that Dmrt1 was expressed in the primary oogonia with condensed nuclei (Fig. 3A). In contrast, there was no detectable Dmrt1 expression in the somatic cells or oocytes at various stages including diplotene (Fig. 3A). Analysis of the ZZ testes showed that their cephalocaudal dimensions were shorter after metamorphosis. Several types of cysts appeared during germ cell development, which consisted of synchronized secondary spermatogonia, spermatocytes, or spermatids. Dmrt1 was expressed in the primary spermatogina, secondary spermatogonia, and Sertoli cells (Fig. 3B), but not in the spermatocytes or spermatids in the immature testes one month after metamorphosis (data not shown) or in these cells in mature testes (Fig. 3B). Notably, the primary spermatogonia consisted of Dmrt1-high and -faint expressing cells (Fig. 3B, arrowhead and arrow, respectively).

3-4. **Dmrt1 expression is not directly involved in GSC proliferation**

Since Dmrt1 was expressed in some cysts consisting of synchronized secondary oogonia in immature ovaries and in some primary spermatogonia in the mature testis (Fig. 3), I sought to determine if there was a relationship between Dmrt1 expression and cell proliferation. I labeled proliferating cells in the immature and mature gonads by injecting BrdU into stage-62 tadpoles and adult frogs, and detecting the labeled cells with an anti-BrdU antibody. In the immature ovary, some, but not all of the Dmrt1-expressing secondary oogonia were BrdU-positive, and many Dmrt1-expressing primary oogonia were BrdU-negative (Fig. 4). In the adult testis, Dmrt1 expression in the secondary

spermatogonia was not strongly related to the degree of BrdU incorporation (Fig. 4). In addition, there was no relationship between Dmrt1 expression and BrdU incorporation in the primary spermatogonia.

3-5. A phosphorylated form of H2AX (γH2AX) and Dmrt1 are coexpressed in primary spermatogonia and oogonia

γH2AX, which is a phosphorylated form of the histone variant H2AX, is involved in DNA repair in most cells or meiosis in both female and male germ cells. γH2AX is also expressed in embryonic stem cells and neural stem cells in mammals (Banáth et al. 2009; Fernando et al. 2011). To examine the γH2AX expression in GSCs, I performed immunohistochemical analysis of ZZ and ZW gonads at various developmental stages using both anti-γH2AX and anti-Dmrt1 antibodies. γH2AX was markedly expressed in the primary spermatogonia and oogonia, but not in the somatic cells of the ZZ and ZW gonads at stage 53, just after sex determination (Fig. 5A). The colocalization of γH2AX and Dmrt1 was observed in the nuclei of the primary GSC cells.

3-6. γH2AX is differentially expressed in female and male GCSs after metamorphosis

In the ZW gonads at stage 62, the γH2AX signal was markedly expressed in the primary oocytes (Fig. 5A). The same pattern of γH2AX expression was observed at later stages of gonadal development. In contrast, γH2AX was expressed at similar levels in the primary spermatogonia at stages 53 and 62. In the adult testis, γH2AX was expressed in some cysts consisting of synchronized spermatocytes or secondary spermatogonia, as well as in the primary spermatogonia (Fig. 5B). Taken together, my findings suggested that Dmrt1 and γH2AX are coexpressed in primary and secondary spermatogonia throughout testicular development and that γH2AX is differentially expressed in female and male GSCs after metamorphosis.

3-7. Mouse monoclonal antibody 4F6 reacts specifically to *X. laevis* Dmrt1

To identify Dmrt1-associating proteins in *X. laevis* testes by proteome analysis, I made mouse monoclonal antibodies against the truncated C-terminal protein of *X. laevis* Dmrt1 from 130 to 336 a.a, which contained a specific region among DM domain family proteins. I examined specificity of twenty monoclonal antibodies to Dmrt1 by immunoblotting, immunoprecipitation (IP) and immunohistochemistry (IHC), and then selected the antibody 4F6. The results using 4F6 were shown in Fig. 6. 4F6 reacted specifically to overexpressed FLAG-tagged Dmrt1 in 293T cells on immunoblot

analysis (Fig. 6A). Immunoprecipitates by IP using 4F6 from the extract of 293T cells overexpressing FLAG-tagged Dmrt1 showed specific reaction with the anti-FLAG antibody (Fig. 6B). In addition, the analysis of IHC with 4F6 or the anti-Dmrt1 polyclonal antibody (Fujitani et al. 2016) on the section of adult testis revealed that both of the antibodies reacted to the exact same cells, that is, spermatogonia and Sertoli cells (Fig. 6C).

3-8. **Prkra has potential to enhance transcriptional activity of Dmrt1**

Immunoprecipitates by the anti-Dmrt1 monoclonal antibody 4F6 and normal mouse IgG as a negative control from *X. laevis* adult testes were separated by SDS-PAGE, followed by silver staining. I compared the staining patterns between the two IP samples, and observed seven bands specific to 4F6 (Fig. 7). Then, each band derived from 4F6 and its corresponding region derived from normal IgG were excised from the gels, and analyzed by MASS spectrometry. I identified 332 proteins from the IP sample by 4F6. Then, 124 proteins were selected as the 4F6-specific proteins, because the remaining 208 proteins were also found in the sample using normal IgG. I next focused on three proteins, Prohibitin 2 (Phb2), Y-box binding protein-1 (Yb-1), and Protein Kinase, Interferon-Inducible Double Stranded RNA Dependent (Prkra), which are all known to function in nuclei.

To clarify how Phb2, Yb-1, and Prkra are involved in Dmrt1 function, I investigated an effect of each protein on transcriptional regulation by Dmrt1 using luciferase reporter assay. Expression plasmids for each protein and Dmrt1 as well as a Dmrt1-driven luciferase reporter plasmid carrying four repeats of Dmrt1-binding sequence 5'-TTGATACATTGTTGC-3' (Yoshimoto et al. 2010) were co-transfected into 293T cells (Fig. 8). Exogenous expression of Phb2 had a little less effect and Yb-1 had more effect on luciferase activities driven by Dmrt1. In contrast, Prkra greatly enhanced the Dmrt1-driven activity in a dose-dependent manner (Fig. 8 and Fig. 9).

I also examined whether each protein could directly interact with Dmrt1 in cultured cells. After co-expression of Dmrt1 and S-tagged Phb2, Yb-1, and Prkra in 293T cells, the cell extracts were mixed with S-protein agarose. Then, the pull-down samples as well as the cell extracts were examined by Western blot analysis (data not shown). No signals for Dmrt1 that bound to Phb2, Yb-1, or Prkra could be detected, indicating the possibility of their indirect interactions with Dmrt1.

3-9. **p53 has the potential to repress transcriptional activity of Dmrt1**

Prkra was characterized as a negative regulator of p53 (Li et al. 2007). Then, I investigated the effects of p53 on transcriptional activity of Dmrt1 in the presence and absence of exogenous Prkra by luciferase reporter assay in co-transfected 293T cells (Fig. 9). The Dmrt1-driven luciferase activity enhanced by Prkra was strongly downregulated by p53 expression dose-dependently. Interestingly, even in the assay without exogenous Prkra, the Dmrt1-driven activity was also significantly repressed by p53 expression in a dose-dependent manner.

3-10. *Prkra* mRNA expresses in germ stem cells of the tadpole gonads

Because Prkra can contribute to upregulation of Dmrt1 function, I next investigated an expression profile of *Prkra* mRNA in developing gonads in *X. laevis*. I first performed RT-qPCR of *Prkra* transcripts during gonadal development (Fig. 10A). The *Prkra* transcripts showed no or a few significant differences between ZW and ZZ gonads from stage 50 just after sex determination to stage 65 when metamorphosis is almost completely finished. In addition, the transcripts of ZW or ZZ gonads exhibited uniform expression during tadpole development. In contrast, the amount of the *Prkra* mRNA gradually increased in adult testes from six weeks after metamorphosis to one to two years, which might be related to a prosperous spermatogenesis.

Next, to clarify topological distribution of the *Prkra* mRNA, I performed a whole mount *in situ* hybridization of stage 56 ZW and ZZ tadpole gonads, which started displaying sexual differences in morphology, using DIG-labeled *Prkra* RNA sense and anti-sense probes. To identify *Prkra*-expressing cells, I made their sections, followed by immunostaining with an anti-VASA antibody and nuclear staining with Hoechest 33258 (Fig. 10B). Germ stem cells are characterized as not only expression of VASA, but also faint staining of nuclei. Although almost no signals were detected in the case of the sense probe, the anti-sense probe seemed to hybridize the *Prkra* mRNA in both somatic and germ cells of the ZW and ZZ gonads. However, strong signals were observed in some germ stem cells indicated by arrowhead, of both sexes.

4. Discussion

The transcription factor *Dmrt1* has the potential to both activate and repress the expression of its target genes. In the somatic cell masculinization in mouse postnatal gonads, Dmrt1 activates the expression of *Sox8*, *Sox9*, and *Ptgdr*, and represses the transcription of *Foxl2*, *Esr1/2*, *Wnt4*, and *Rspo1* (Matson et al. 2011). In mouse germ cells, it positively and negatively regulates the expression of *Stra8*, which is involved in the premeiotic phases of oogenesis and spermatogenesis, respectively (Krentz et al. 2011; Matson et al. 2010). It has also been reported that Dmrt1 protects male GSCs against pluripotency and apoptosis in mice (Takashima et al. 2013b). In *X. laevis*, Dmrt1 was expressed in the primary oogonia and spermatogonia at an early stage of sex differentiation (Fig. 2), but its expression was reduced or absent in some subpopulations of secondary oogonia (Figs 2 and 3). Moreover, Dmrt1 expression was not involved in the proliferation of primary or secondary GSCs in either sex (Fig. 4). Collectively, these findings suggest that *X. laevis* Dmrt1 may be involved in maintaining GSC identity in male primary spermatogonia throughout development, and in female primary oogonia before metamorphosis, possibly by negatively or positively regulating apoptosis- and pluripotency-promoting genes or GSC-maintaining genes, respectively. Dmrt1 might also prevent entry into premeiotic S-phase in secondary spermatogonia by regulating the *stra8* expression in *X. laevis*, as it does in mice. The absence of Dmrt1 from some secondary oogonia in *X. laevis* indicates that it does not function as a meiotic regulator in these cells. The real-time PCR analysis revealed that the *Dmrt1* and *stra8* mRNAs shared similar expression patterns during gonadal development (Fig. 1). Interestingly, *stra8* was expressed in the ZW and ZZ gonads at stages 50 and 53, which have no premeiotic germ cells. Although it is assumed that Stra8 function is limited to meiosis in germ cells in mammals, Stra8 might play another role in gonadal development in *X. laevis*.

The phosphorylation of H2AX, which generates γH2AX, was originally identified as an early event after ionizing radiation-induced DNA DSBs (Rogakou et al. 1998). Meiotic DNA DSBs may also induce the generation of γH2AX. Here, I observed γH2AX expression not only in meiotic germ cells but also in female and male primary GSCs during early sex differentiation (Fig. 5). γH2AX was recently reported to be expressed in mammalian embryonic stem cells (ESCs), induced pluripotent stem cells, and neural stem cells (Banáth et al. 2009; Fernando et al. 2011; Turinetto et al. 2012) Turinetto and Giachino (Turinetto, Valentina 2015) suggested that γH2AX may contribute to the creation of specific chromatin structures in response to other cellular signals besides DNA damage. Thus, my results, in combination with previous findings, suggest that γH2AX may play an important role in maintaining stem cell identify by regulating epigenetic changes in various types of stem cells.

Recently, Nishimura et al. (Nishimura et al. 2015) reported that *foxl3* shows specific expression in female GSCs throughout development, which would determine female germ cell identity for oocytes in the teleost fish medaka. My experiments revealed substantial differences in morphology

and protein expression between female and male GSCs during gonadal development in *X. laevis*. I found that γH2AX was barely detectable in female GSCs after metamorphosis. I also observed that primary oogonia become more condensed as ovarian development progressed, whereas primary spermatogonia exhibited a similar morphology to the PGCs throughout testicular development (Fig. 2). The tendency of male GSCs to maintain the PGC phenotype, compared with the phenotypic changes exhibited by female GSCs, could involve epigenetic differences and/or differences in the ovarian and testicular environments. It will be interesting to examine whether these sexual dimorphisms are common in other vertebrate species. If these differences are conserved, they may represent a mechanism for reducing 'male-driven evolution', which refers to the higher number of cell divisions in the male germ line compared to the female germ line, and the higher prevalence of male germline mutations (Miyata et al. 1987; Shimmin, Chang, and Li 1993). In mice, correlation been p53 and Dmrt1 is involved in cell fate and identity of male GSCs (Takashima et al. 2013b). Accordingly, there might be stronger relationships between the DNA repair system and germ cell identity mediated through p53-γH2AX and Dmrt1 in males than in females in *X. laevis*. It will be intriguing to study how the relationships could protect germ cells from mutations or whether they are conserved in vertebrates.

For comprehending gonadal development including sex determination and differentiation in *X. laevis*, I previously identified *Dmrt1* and its W-linked paralog *Dm-W*, and characterized the former as a gene for testis formation and germ-cell development and the latter as a female sex-determining gene (Yoshimoto et al. 2010), (Yoshimoto et al. 2008), (Mawaribuchi, Musashijima, et al. 2017), (Fujitani et al. 2016), (Yoshimoto and Ito 2011), (Yoshimoto et al. 2006). I also reported molecular evolution of the *Dmrt1* family genes (Mawaribuchi, Musashijima, et al. 2017), (Mawaribuchi et al. 2012), (Mawaribuchi, Takahashi, et al. 2017). However, it remains unknown how Dmrt1 activates or represses transcription of its target genes in gonadal somatic cells and germ cells as a transcription factor. To understand the transcriptional machinery by Dmrt1, I tried to identify complex members with Dmrt1 in *X. leavis*. By analyzing immunoprecipitaes with the anti-Dmrt1 monoclonal antibody from extracts of adult testes, I selected three proteins, Phb2, Yb-1, and Prkra from more than one hundred identified proteins.

Unexpectedly, a protein-protein binding assay in co-transfected 293T cells indicated that each protein could not directly interact with Dmrt1 (data not shown). The result suggested that each protein might be indirectly associated with Dmrt1 through other Dmrt1-binding proteins. Phb2 is an intercellular communicator between nucleus and mitochondria, and suppresses transcription of target genes in nuclei (Bavelloni et al. 2015). In contrast, a transcription factor Yb-1 is involved in transcriptional machinery by interacting with other transcription factors including p53 (Okamoto et al. 2000). Unfortunately, exogenous expression of Phb2 or Yb-1 induced a few or no changes on Dmrt1-driven luciferase reporter assay (Fig. 8). It is possible that transcription driven by Phb2 or Yb-1

could be influenced by indirect interaction with Dmrt1.

In Dmrt1-driven luciferase reporter assay using transfected 293T cells, only Prkra enhanced the luciferase activity (Fig. 8 and Fig. 9). Prkra was known to be involved in p53 function (Bennett et al. 2012). Importantly, p53 significantly repressed the enhanced activity by Prkra (Fig. 9), maybe mediated through p53-Prkra and Dmrt1-Prkra interaction. I also found that p53 could moderately attenuate the Dmrt1-driven activity in the absence of exogenous expression of Prkra. Because I could not observe direct interaction between Dmrt1 and p53 in co-transfected 293T cells (data not shown), p53 might also indirectly participate in the Prkra-independent transcription by Dmrt1.

In what types of cells does Prkra enhance transcriptional activity by Dmrt1 or p53 repress its enhanced activity? What does the regulation by Prkra and/or p53 mean? *In situ* hybridization analysis showed that *Prkra* was highly expressed in female and male germ stem cells (oogonia and spematogonia) in tadpole gonads of *X. leavis* (Fig. 10B). This observation coincided with the expression pattern during gonadal development in ZW and ZZ tadpoles and adults (Fig. 10A). In *X. leavis* germ stem cells, Dmrt1 and a phosphorylated form of the histone variant H2AX (γH2AX) could contribute to the maintenance of their stem cell identity and participate in genome protection against double strand breaks, respectively (Fujitani et al. 2016). p53 has been described as "the guardian of the genome", because it plays important roles in cell cycle regulation, DNA repair, and apoptosis, leading to genome stability by preventing mutations or eliminating DNA-damaged, mutated cells. For the next generation, p53 functions to guarantee germ cell quality (Gebel et al. 2017). Takashima et al. (Takashima et al. 2013a) reported that, in mouse spermatogonial stem cells, *Dmrt1* depletion causes apoptosis, but both *Dmrt1* and *p53* depletion induces pluripotency, suggesting that p53 and Dmrt1 might play contrary and/or related roles in spermatogonial stem cells. In other words, the balance between Dmrt1 and p53 might maintain germ stem cell identity. Taken together, these findings suggest that Prkra might enhance Dmrt1 function for germ stem cell identity, but p53 negatively controls Dmrt1 function, leading to apoptosis in damaged, mutated germ stem cells. In addition, Yb-1, one of the three Dmrt1-interacting proteins identified in this study, could directly bind with p53 (Okamoto et al. 2000), as described above. It is possible that germ stem cell identity regulated by Dmrt1 and p53 might be mediated through not only Prkra, but also Yb-1.

Prkra has also been characterized as a dsRNA binding protein (Redfern et al. 2013) a RISC (RNA-induced silencing complex) member required for subsequent siRNA-mediated post-transcriptional gene silencing (Patel and Sen 1998), and an activator of protein kinase R (Pkr) also known as interferon-induced, dsRNA-activated protein kinase (Huang, Hutchuns, and Patel 2002). I cannot imagine how dsRNA/RISC is involved in Prkra-Dmrt1 interaction. Interestingly, Pkr associated with p53 (Cuddihy et al. 1999), and the Prkra-Pkr signaling in response to stress-inhibited p53 turnover, leading to G1 cell cycle arrest (Bennett et al. 2012). It will be interesting to clarify whether the two signaling modules, Prkra-Pkr-p53 and Dmrt1-Prkra-p53, have mutual relation in germ stem cells.

5. Future tasks

(1) Study of Dmrt1's function for germ stem cell (GSC) identity in *Xenopus* frogs

- Identification for target genes of Dmrt1 in GSCs by ChIP-sequence using the anti-Dmrt1 antibody
- Sexual dimorphism of GSCs mediated through Dmrt1
- Molecular mechanisms of Dmrt1's function as an activator or a repressor

(2) Study of Dmrt1-Prkra-p53 for GSC identity and genome integrity in GSCs in *Xenopus* frogs

- Prkra function by production of GSC-specific knockout individuals
- p53 function by production of GSC-specific knockout individuals
- Regulation by Dmrt1-Prkra-p53 in GSC identity and genome integrity

6. Acknowledgements

I greatly thank Dr. Asako Otomo for all data provided for Fig 1-5, Dr. Mikako Wada for cloning stra8, Mr. Yuto Nagayama and Dr. Taro Tachibana for providing DMRT1 monoclonal antibodies, Ms. Rika Kato, Dr. Yusuke Kawashima and Dr. Yoshio Kodera for MS/MS analysis, Dr. Tomoko Kato and Dr. Shuji Takada for providing *in situ* hybridization results of mouse testis, Dr. Kei Tamura for statistics analysis, Professor Nobuhiko Takamatsu for consulting, Professor Toshiyuki Miyashita for consulting, Dr. Jim Goddard for careful reading and comments to the manuscript, and special thanks to Dr. Michihiko Ito for consulting all part of the manuscript and being a great friend of mine.

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8. Research records

8-1. Publications

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5) Mawaribuchi S, Ikeda N, **Fujitani K,** Ito Y, Onuma Y, Komiya T, Takamatsu N, Ito, M. *"Cell-mass structures expressing the aromatase gene Cyp19a1 lead to ovarian cavities inXenopus laevis"* Endocrinology; Early Release, en.2014-1096 July 22, 2014

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8-2. Presentations

1) **藤谷和子**、岡野則仁、高松信彦、伊藤道彦
転写因子 DMRT1 の天然変性領域という観点からの転写活性化領域の分子進化
41th.Annual Meeting of the Molecular Biology Society of Japan, Yokohama 2018

2) 和田美加子、**藤谷和子**、回渕修二、田村啓、高松信彦、伊藤道彦
ツメガエル初期生殖巣形成における mass-in-line 構造の雌雄化機構
39th.Annual Meeting of the Molecular Biology Society of Japan, Yokohama 2016

3) 岡野則仁、**藤谷和子**、田村啓、荻田悠作、和田美加子、高松信彦、伊藤道彦
生殖細胞運命・オス化に関わる転写因子 DMRT1 の天然変性領域とオーダー領域による転写活性化領域の分子進化
39th.Annual Meeting of the Molecular Biology Society of Japan, Yokohama 2016

4) **Fujitani K.**; Otomo A.; Nagayama Y.; Tachibana T.; Kato T.; Takada S.; Kato R.; Kodera Y.; Takamatsu N.: Ito M.
Screening of DMRT1-associated factors which regulates transcription of downstream genes
37th.Annual Meeting of the Molecular Biology Society of Japan, Yokohama 2014

5) 武蔵島正人、回渕修二、**藤谷和子**、和田美加子、Min K. Park、高松信彦、伊藤道彦
四肢動物進化糧における精巣形成遺伝子 Dmrt1 の非コードエクソン１の消失
37th.Annual Meeting of the Molecular Biology Society of Japan, Yokohama 2014

6) **Fujitani K.**; Otomo A.; Nagayama Y.; Tachibana T.; Kato T.; Takada S.; Kato R.; Kodera Y.; Takamatsu N.: Ito M.
DMRT1-associated proteins in *Xenopus laevis* testes
Young Scientist Meeting of Sexual Development, Atami 2014

7) 回渕 修治，井筒 ゆみ，**藤谷 和子**，小宮 透，高松 信彦，伊藤 道彦
アフリカツメガエル Dmrt1 は生殖細胞の運命に関与する
36th.Annual Meeting of the Molecular Biology Society of Japan, Kobe 2013

8) 和田 美加子，**藤谷 和子**，回渕 修治，村田 恵理子，伊藤 道彦，高松 信彦
アフリカツメガエル初期雌雄生殖巣形成における雄化関与遺伝子の解析
36th.Annual Meeting of the Molecular Biology Society of Japan, Kobe 2013

9) Mawaribuchi, S.; **Fujtiani K**.; Ikeda N; Otomo A.; Takamatsu N.; Ito M.
A Steroidgenic Gene Cyp17a1 and Syp19a1 Showed Ovary Formation-type Expression Just After Sex-determination in *Xenopus Laevis*
35th.Annual Meeting of the Molecular Biology Society of Japan, Fukuoka 2012

10) **Fujitani,K**.; Otomo, A.; Ito, M.
Cell lineage-specific expression of DMRT1 during *Xenopus laevis* gonadal development
Sixth International Symposium on Vertebrate Sex Determination, Kona (Hawaii) 2012

11) 登上繊維・プルキンエ細胞間シナプス除去の臨界期での Stathmin によるプルキンエ細胞樹状突起内微小管の制御
大川　宜昭, **藤谷　和子**, 右島　理可, 日野　敏昭, 高部　美穂, 横山　峯介, 井ノ口　馨
三菱化学生命科学研究所
第 26 回　日本神経科学大会, 2003 年 7 月 23 日～25 日、名古屋国際会議場

12) 発達期小脳の選択的シナプス除去の分子機構：Stathmin によるプルキンエ細胞樹状突起内微小管の制御
大川　宜昭, **藤谷　和子**, 右島　理可, 日野　敏昭, 高部　美穂, 横山　峯介, 井ノ口　馨
三菱化学生命科学研究所
第 26 回　日本分子生物学会年会, 2003 年 12 月 10 日～13 日、神戸ポートアイランド

13) Stathmin によるプルキンエ細胞樹状突起内微小管の制御と登上繊維・プルキンエ細胞間シナプス除去への関与の可能性
藤谷　和子, 大川　宜昭, 右島　理可, 日野　敏昭, 高部　美穂, 横山　峯介, 井ノ口　馨
三菱化学生命科学研究所・井ノ口グループ
第 27 回日本神経科学大会・第 47 回日本神経化学会大会合同大会,　2004 年 9 月 21 日～23 日、大阪国際会議場

14) Stathmin regulates dendritic microtubule stability of Purkinje cells that might be important for the olivocerebellar synapse elimination during cerebellar development
N. Ohkawa, **K. Fujitani**, R. Migishima, T. Hino, M. Takabe, M. Yokoyama, K. Inokuchi
Mitsubishi Kagaku Institute of Life Science (MITILS)
The Society for Neuroscience 34th Annual Meeting, October 23-27, 2004, San Diego, CA.

15) mARD1-mNAT1 アセチルトランスフェラーゼ複合体による tubulin アセチル化を介した樹状突起の発達制御

大川　宜昭[1]，杉崎　俊一郎[1,2]，徳永　絵里[1]，**藤谷　和子**[1]，瀬藤　光利[1]，井ノ口　馨[1,2,3]

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第 28 回　日本分子生物学会年会，2005 年 12 月 7 日～10 日、福岡ヤフードーム

16) ARD1-NAT1アセチルトランスフェラーゼ複合体によるtubulinアセチル化を介した樹状突起の発達制御

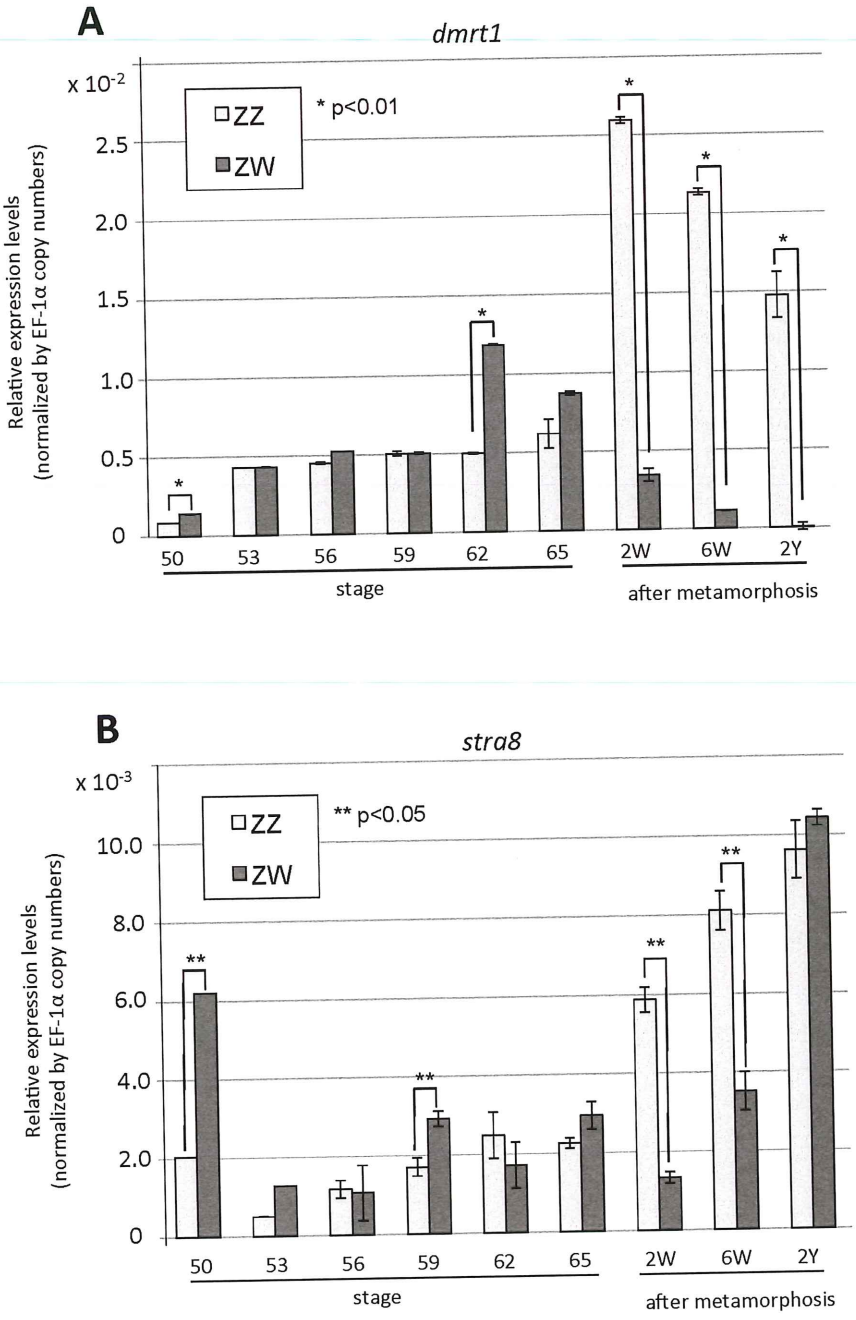
大川 宜昭[1]，杉崎 俊一郎[1]，徳永 絵理[1]，**藤谷 和子**[1]，瀬藤 光利[1]，井ノ口 馨[1, 2, 3]

[1] 三菱化学生命科学研究所，[2] 横浜国大・院・環境情報，[3] CREST・JST

Neuro 2007（第 30 回神経科学大会・第 50 回神経化学学会大会・第 17 回神経回路学会大会合同大会），2007 年 9 月 10 日～12 日

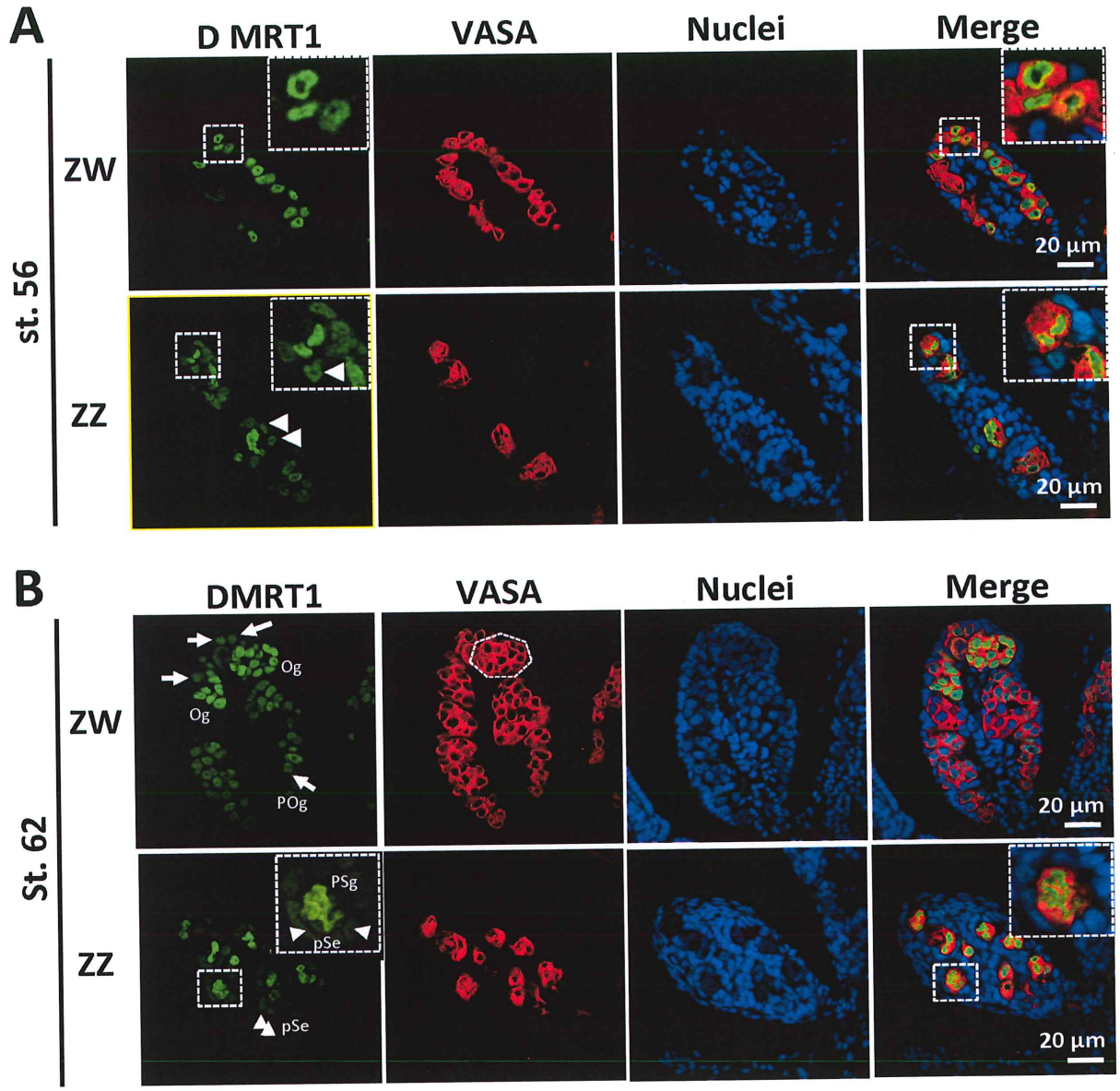
9. Figures

Fig.1



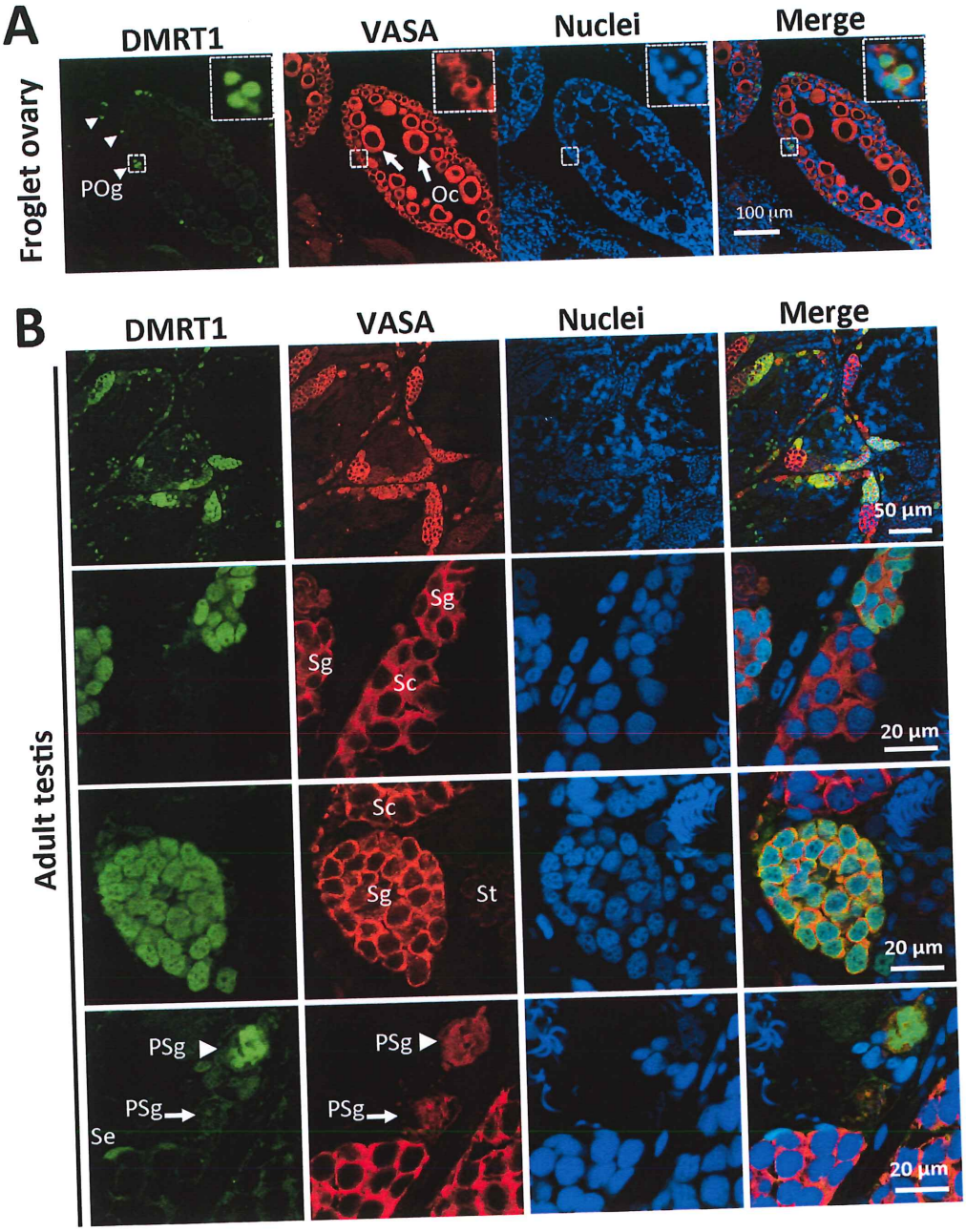
Real-time PCR analysis of dmrt1 and stra8 in ZZ and ZW gonads during sexual development. qPCR was performed for dmrt1 (A) or stra8 (B) using cDNAs derived from the total RNAs of three ZZ or ZW gonads from tadpoles at different stages of development and adult frogs. The dmrt1 or stra8 primer pairs were designed within common sequences shared between the two dmrt1 (dmrt1.L and dmrt1.S) and two stra8 cDNAs in X. laevis. W, weeks; Y, years.

Fig.2



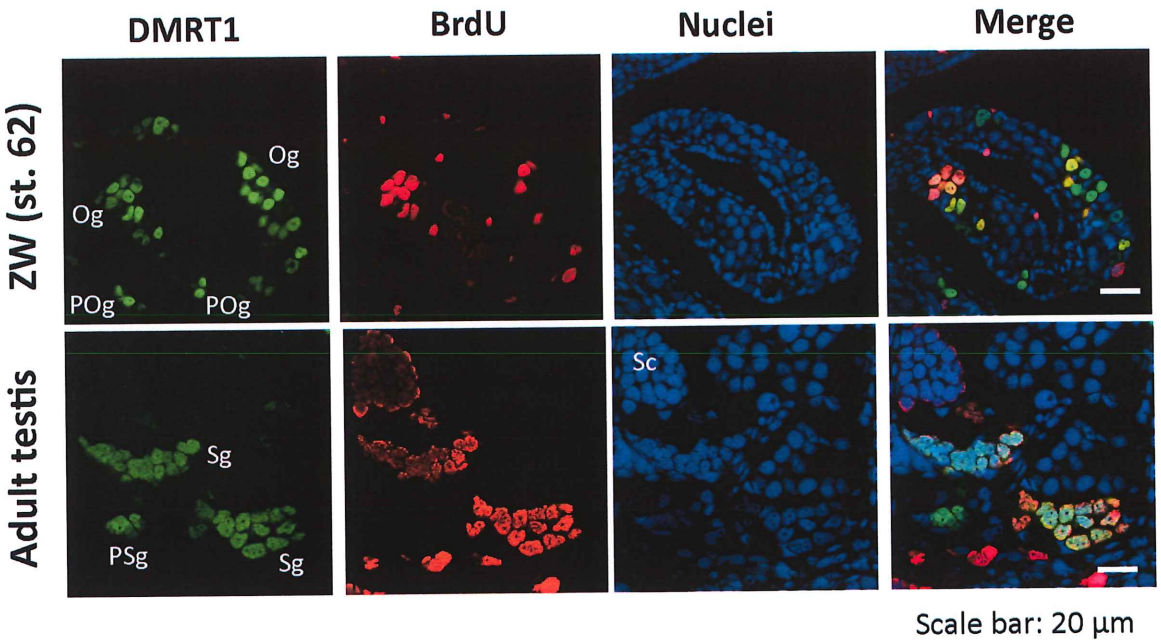
Distribution of Dmrt1 in ZZ and ZW gonads during tadpole development. Immunostaining with anti-Dmrt1 and anti- VASA antibodies was performed on gonadal sections from ZW and ZZ tadpoles at stages 56 (A) and 62 (B). Nuclei were stained with TOPRO-3. Oc, oocyte; Og, secondary oogonium; POg, primary oogonium; PSg, primary spermatogonium; pSe, pre-Sertoli cell. White arrowheads and arrows indicate Dmrt1-expressing pre-Sertoli cells and primary oogonia, respectively. Typical GSCs with somatic cells and their 2.59 magnification images are shown in the dashed squares. A dashed polygon indicates a typical cyst consisting of secondary oogonia.

Fig.3



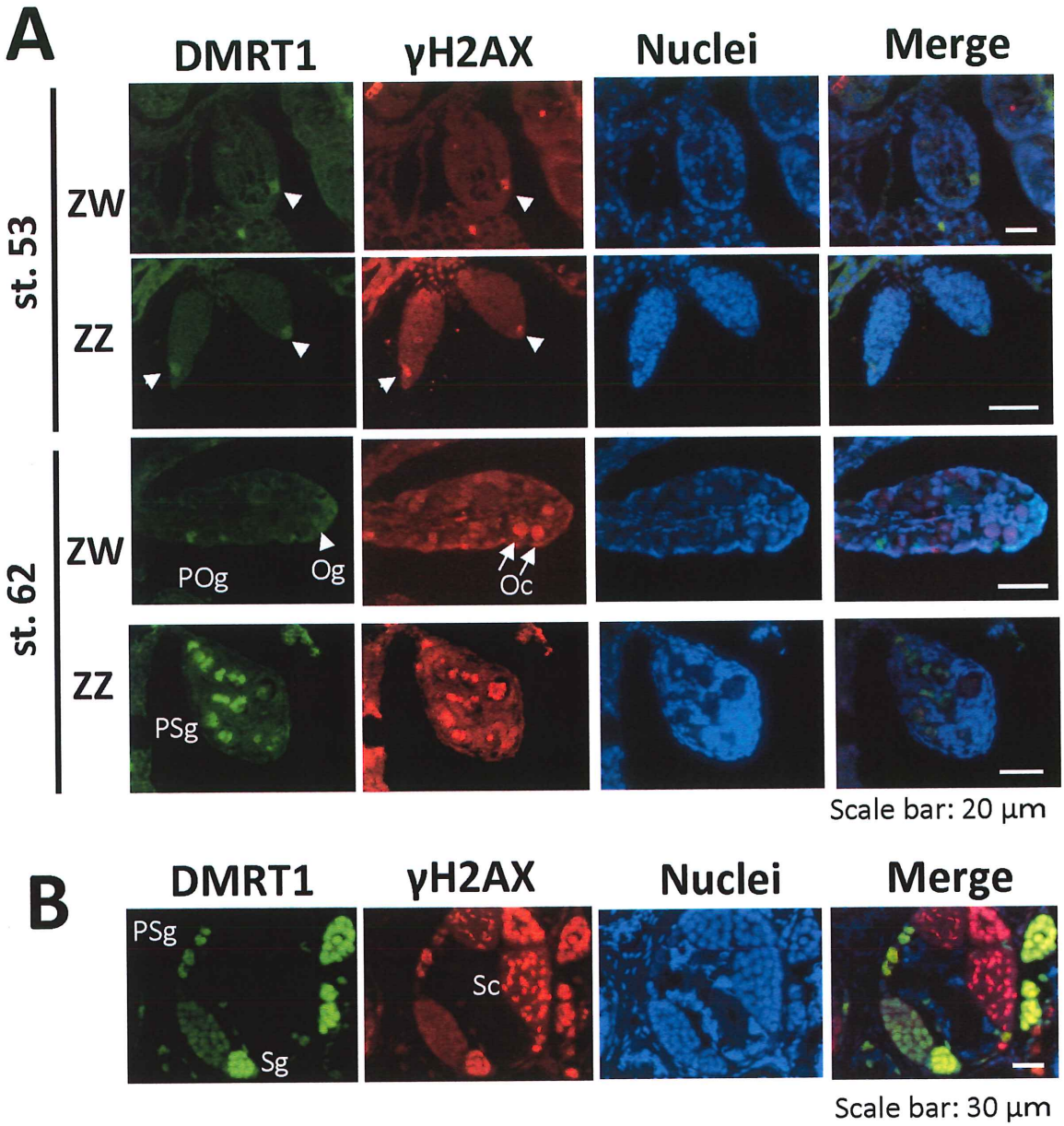
Distribution of Dmrt1 in the immature ovary and mature testis after metamorphosis. Immunostaining with anti-Dmrt1 and anti-VASA antibodies was performed using the immature ovary from a ZW frog 2 weeks after metamorphosis (A) and the mature testis of an adult ZZ frog (B). Nuclei were stained with TOPRO-3. Oc, Oocyte; POg, primary oogonium; PSg, primary spermatogonium; Se, Sertoli cell; Sg, secondary spermatogonium; Sc, spermatocyte; St, spermatid. pSe, pre-Sertoli cell. Dmrt1- expressing primary oogonia and their 59 magnification images are shown in the dashed squares. White arrows and arrowhead in (A) indicate primary oogonia and diplotene oocytes, respectively. White arrowheads in (B) indicate Dmrt1- expressing primary spermatogonium.

Fig.4



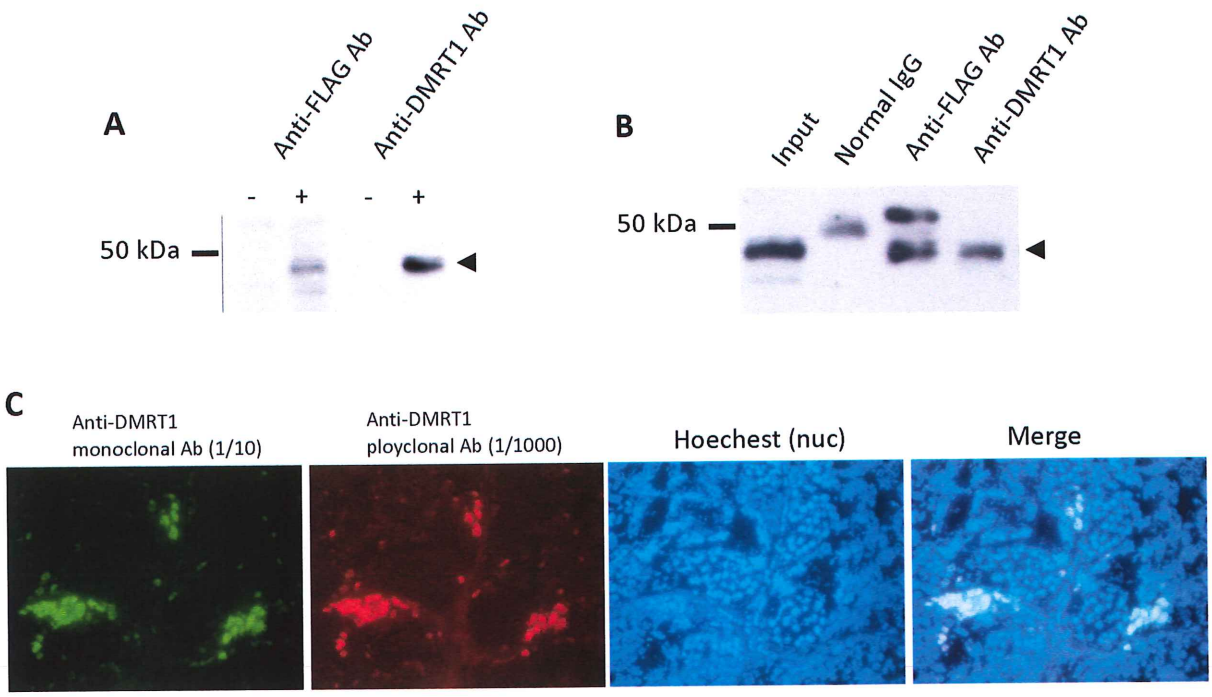
BrdU incorporation and Dmrt1 expression in the ZW developing ovary and ZZ adult testis. Immunostaining with anti-Dmrt1 and anti-BrdU antibodies was performed on the immature tadpole ovary at stage 62 and the mature testis of an adult frog. Nuclei were stained with TOPRO-3. Og, secondary oogonium; POg, primary oogonium; PSg, primary spermatogonium; Sg, secondary spermatogonium; Sc, spermatocyte.

Fig.5



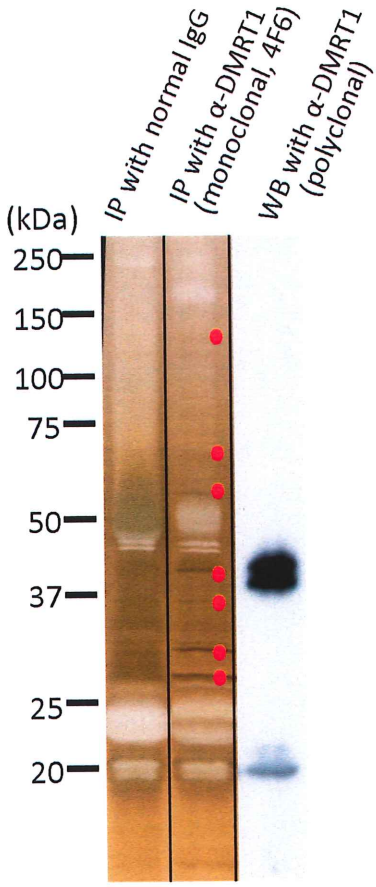
Distribution of γH2AX in ZZ and ZW gonads during development. Immunostaining with anti-Dmrt1 and anti-γH2AX antibodies was performed on gonadal sections from ZW and ZZ tadpoles at stages 53 and 62 (A) and on gonadal sections from a ZZ adult frog (B). Nuclei were stained with TO-PRO. Oc, oocyte; POg, primary oogonium; PSg, primary spermatogonium; Sg, secondary spermatogonium; Sc, spermatocyte. White arrowheads and arrows indicate Dmrt1- or γH2AX-positive primary GSCs and leptotene/zygotene oocytes, respectively.

Fig.6



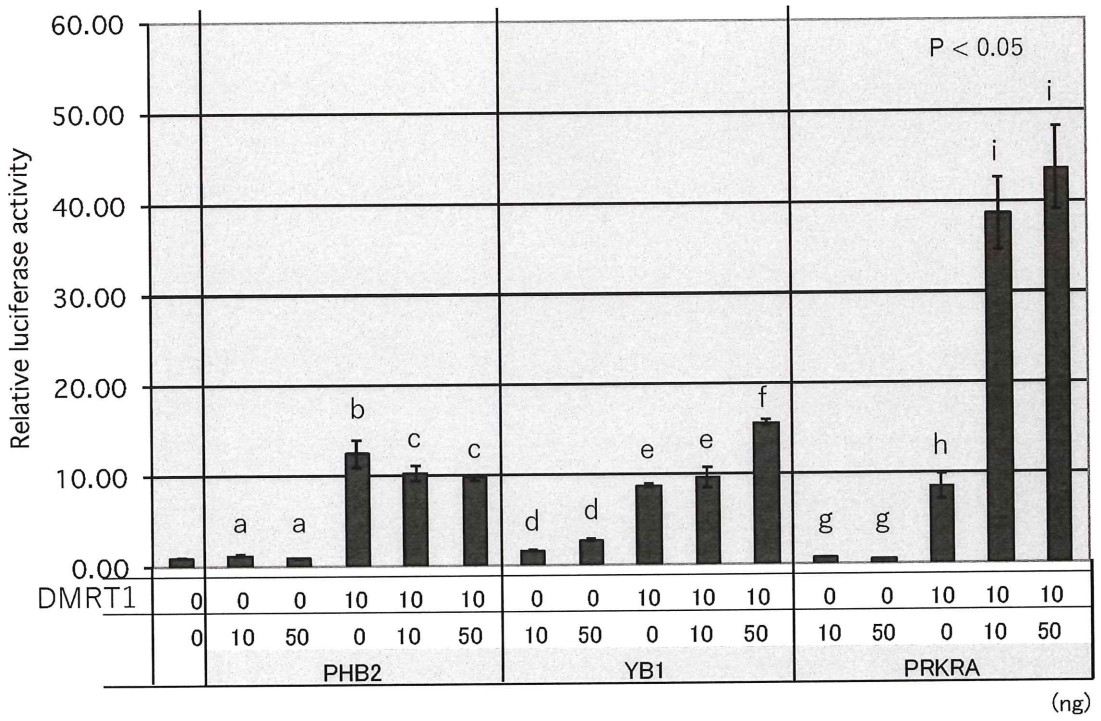
Immunoreaction of an anti-DMRT1 monoclonal antibody 4F6 against *X. laevis* DMRT1. (A) Immunoblot analysis using the anti-FLAG (M5) or anti-DMRT1 (4F6) monoclonal antibodies. pcDNA3-FLAG or pcDNA3-FLAG-DMRT1 was transiently transfected into 293T cells. Extracts of 293T cells were examined by immunoblotting with each antibody followed by a HRP conjugated anti-mouse IgG antibody. DMRT1 was detected as a single band at the same size by both antibodies (arrowhead). (B) Imminoprecipitation (IP) analysis with the anti-FLAG (M5) or anti-DMRT1 (4F6) monoclonal antibodies. pcDNA3-FLAG-DMRT1 was transiently transfected into 293T cells. The cell lysate was mixed with each antibody and pulled down with protein A/G agarose. IP extracts were examined by immunoblotting using the anti-DMRT1 polyclonal antibody(Fujitani et al. 2016) followed by a HRP conjugated anti-rabbit IgG antibody. (C) Immunohistochemical analysis using the anti-DMRT1 monoclonal antibody 4F6 (1/10) and anti-DMRT1 polyclonal antibodies (1/1000) (Fujitani et al. 2016). A frozen section of adult testis was stained with Hoechst 33258 for nuclei, and reacted with both the antibodies followed by Alexa 594-conjugated anti-mouse (red) and Alexa 488 anti-rabbit-IgG (green) antibodies. Both signals show the same staining patterns.

Fig.7



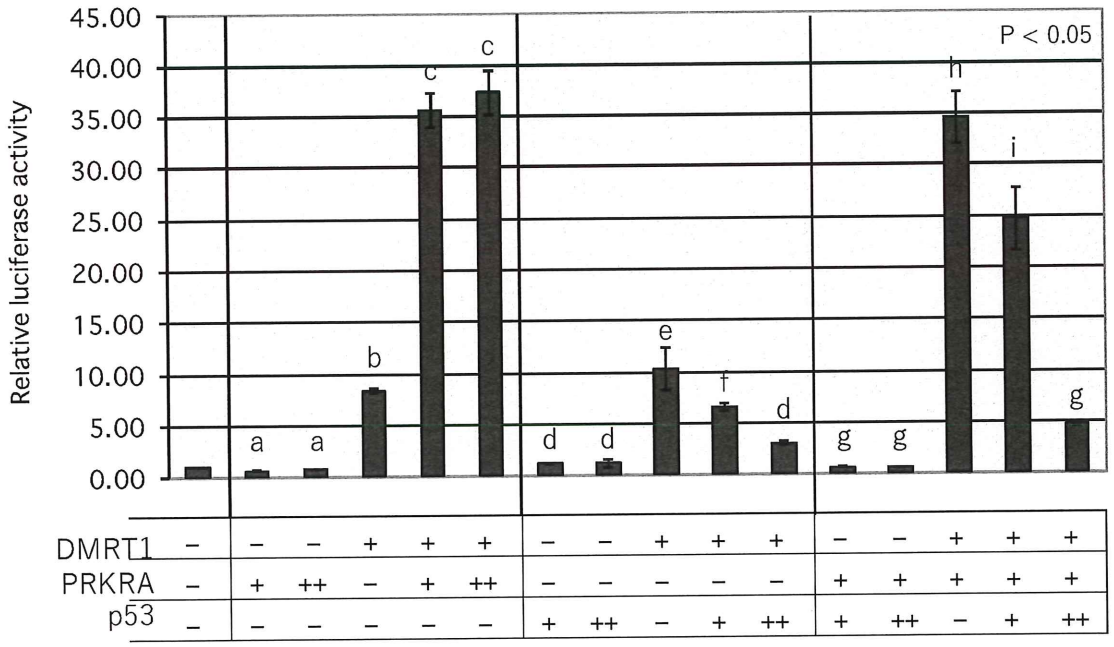
Silver staining of immunoprecipitates using the anti-DMRT1 monoclonal antibody 4F6 form *X. laevis* adult testes. Testis extracts were mixed with normal mouse IgG or the anti-DMRT1 antibody 4F6, and pulled down with protein A/G agarose. The immunoprecipitates were examined by silver staining (the right and middle lanes) or immunoblotted with an anti-DMRT1 polyclonal antibody (right lane). Seven 4F6-specific bands were excised, and examined by LC-MS.

Fig.8



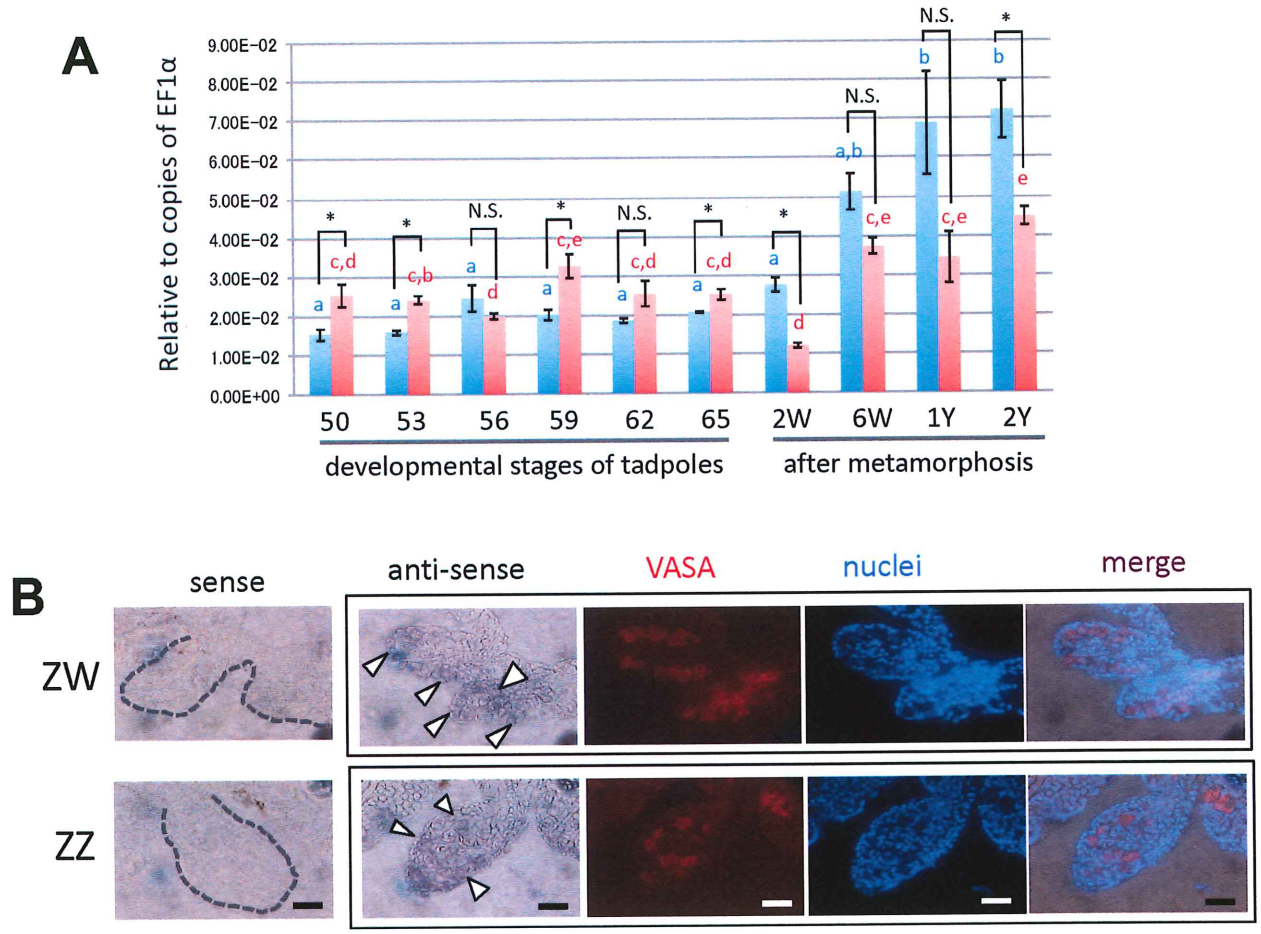
Effects of PHB2, YB1 and PRKRA on transcriptional activity by DMRT1 using luciferase reporter assay. 150 ng of DMRT1-driven firefly luciferase reporter plasmid (p4xDMRT1-luc), 10 ng of DMRT1 expression plasmid (pcDNA3-FLAG-DMRT1), and 10 ng renilla luciferase vector (pRL-TK-luc) as transfection internal control in the presence or absence of PHB2, YB1 or PRKRA expression plasmids (pcDNA3-FLAG- PHB2, YB1 or PRKRA) were transiently co-transfected into 293T cells, using 1.2 μg PEI MAX. Total amount of DNA was kept at 250 ng per each transfection with pcDNA3-FLAG empty vector. 24 hours after transfection, cell lysates were used to measure luciferase activity. Relative activity is shown as the fold increase compared with the value obtained with 250 ng of pcDNA3-FLAG empty vector. -, +, and ++ indicate 0, 3.3, and 20 ng, respectively. Values are expressed as mean ± SE, n = 3. The letters above the bars indicate results of Tukey HSD test following one-way ANOVA ($p < 0.05$).

Fig.9



Effects of PRKRA and/or p53 on transcriptional activity by DMRT1 using luciferase reporter assay. 150 ng of DMRT1-driven firefly luciferase reporter plasmid (p4xDMRT1-luc), 3.3 ng of DMRT1 expression plasmid (pcDNA3-FLAG-DMRT1), and 10 ng renilla luciferase vector (pRL-TK-luc) as transfection internal control in the presence or absence of PRKRA and/or p53 expression plasmids (pcDNA3-FLAG-PRKRA and/or -p53) were transiently co-transfected into 293T cells, using 1.2 μg PEI MAX. Total amount of DNA was kept at 250 ng per each transfection with pcDNA3-FLAG empty vector. 24 hours after transfection, cell lysates were used to measure luciferase activity. Relative activity is shown as the fold increase compared with the value obtained with 250 ng of pcDNA3-FLAG empty vector. -, +, and ++ indicate 0, 3.3, and 20 ng, respectively. Values are expressed as mean ± SE, n = 3. The letters above the bars indicate results of Tukey HSD test following one-way ANOVA ($p < 0.05$).

Fig.10



Expression of *Pact* mRNA in developing ZW and ZZ gonads. (A) Quantitative RT-PCR analysis of *Prkra* mRNA during gonadal development of ZW (red) and ZZ (blue) tadpoles and adults in *X. leavis*. cDNAs were synthesized using total RNAs from ZW and ZZ gonads at various stages of tadpoles after sex determination, and at 6 weeks, 1 year, and 2 years of frogs after metamorphosis, and then amplified by PCR using specific primer pairs as described in Table 1. W and Y show weeks and year(s), respectively. *EFα* was used for normalization. RT-qPCR data represent the mean (n=3) and SD. Values are expressed as mean ± SE, n = 3. Differences among stages were evaluated by one-way ANOVA followed by the Tukey HSD test ($p < 0.05$). Mean values without sharing the same letters are significantly different from each other. Sexual differences between ZZ and ZW gonads at each stage were evaluated or by Student's t-test (* $p < 0.05$). N.S., not significant. (B) Distribution of *Prkra* mRNAs on transverse sections of ZW and ZZ tadpole gonads at stage 56. Whole-mount *in situ* hybridization of the gonads with the attached mesonephros was performed with the *Prkra* sense or anti-sense RNA probe, followed by 7-μm sectioning transversely using a cryostat. The sections were treated with an anti-VASA monoclonal antibody for germ cells (red) and Hoechst 33258 for nuclei (blue). Note that the nuclei in germ cells were faintly stained by Hoechst 33258. Arrowheads indicate *Prkra*-expressing germ cells. Scale bars, 20μm.