# Studies on germ cell sex in medaka

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**2014**

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## General Introduction

Animals that reproduce sexually develop either testes or ovaries, the functional organs that produce sperm or eggs, respectively. Sperm and eggs are the only cells that can create the next generation and are derived from cells of common origin called germ cells. The essential step in the decision to produce either sperm or eggs is called sex determination. In vertebrates, sex determination genes have been identified in several species and are known to be expressed in gonadal somatic cells (Hattori et al., 2012; Kamiya et al., 2012; Koopman et al., 1990; Matsuda et al., 2002; Nanda et al., 2002; Smith et al., 2009; Yano et al., 2012; Yoshimoto et al., 2010). Influenced by these somatic cells, germ cells become either sperm or eggs. However, how the sperm–egg fate of germ cells is determined remains unknown at the molecular level. The objective of this study was to unveil the mechanism by which germ cells acquire sexual differences and identity, using the model fish medaka (*Oryzias latipes)*.

In female mammals, all germ cells enter meiosis and undergo differentiation into oocytes before birth. On the other hand, in males, the germ cells show mitotic arrest at the fetal stage and recommence mitotic division after birth (e.g. Kocer et al., 2009). Thus, it is generally believed that entering meiosis and mitotic arrest at the fetal stage are signs of female and male sexual identity in germ cells, respectively. However, meiosis is an essential process for gametogenesis in both males and females, and moreover, oocyte formation occurs even without meiotic entry (Dokshin et al., 2013). Therefore, meiosis alone is not sufficient for the determination of sexual identity.

In fish, on the other hand, mitotic oogonia and spermatogonia are present in the ovaries and testes, respectively. Recently, a niche structure called a germinal cradle was identified in medaka ovaries; it was shown to contain germline stem cells (Nakamura et al., 2010). Therefore, in medaka, germline stem cells in the ovaries supply eggs throughout the lifetime, as in the testes. Interestingly, reciprocal transplantation experiments in fish using testicular and ovarian cells have revealed that germ cells from adult gonads are able to develop into either sperm or eggs, depending on the sex of the host gonad (Okutsu et al., 2006; Wong et al., 2011; Yoshizaki et al., 2010). In medaka, germ cells derived from testes can colonize ovaries and undergo oogenesis (unpublished data). These results suggest that germline stem cells are sexually undifferentiated or not yet fixed in fish. Thus, the sexual identity of germ cells is likely determined at some point early in the processes of gametogenesis, either during cyst formation or during the entrance of meiosis. In medaka, the comparison of several stages of germ cell development between sexes is possible through the lifetime (Nishimura and Tanaka, 2014). Therefore, the medaka is a good model for investigation of the acquisition of sexual differences and identity in germ cells.

The medaka is a gonocoristic fish whose sex is determined by XX and XY chromosomes. On the Y chromosome, the male sex determination gene, *DMY*/*dmrt1bY*, has been identified (Matsuda et al., 2002; Nanda et al., 2002). In medaka, the gonadal primordium is formed at the embryonic stage (st.) 33, and in XY gonads, *DMY*/*dmrt1bY* begins to be expressed in gonadal somatic cells at this stage (Nishimura et al., 2014). The first sign of sex differentiation can be observed in germ cells (Fig. 1). By st.35, the

onset of gonadal sex differentiation, both XX and XY germ cells undergo stem-type self-renewal division, in which germ cells divide completely to generate two isolated daughter cells surrounded by supporting cells (type I division). In the absence of *DMY*/*dmrt1bY*, immediately following st.35, a subset of XX germ cells shifts to cystic division, in which germ cells divide synchronously with intercellular bridges (type II division). Type II division is gametogenesis-committed, and it is followed by meiosis and oogenesis near the hatching stage (Nishimura and Tanaka, 2014; Saito et al., 2007). In contrast, in the presence of *DMY*/*dmrt1bY*, XY germ cells continue to undergo type I division slowly after st.35. In XY germ cells, type II division and spermatogenesis occur more than 30 days after hatching (Nishimura and Tanaka, 2014; Satoh and Egami, 1972). Therefore, in medaka, spermatogenesis occurs much later than oogenesis.

From these observations, I first hypothesized that the transition from type I to type II division at the embryonic stage is essential for the egg fate decision in XX germ cells and is suppressed by the action of *DMY*/*dmrt1bY* in XY somatic cells. In Chapter 1, based on this hypothesis, I used microarray data to analyzed the global gene expression profiles of XX and XY germ cells and somatic cells at st.33, the onset of *DMY*/*dmrt1bY* expression, and st.35, immediately prior to the shift from type I to type II division of XX germ cells. Contrary to the expectations, most of the genes showing sexual differences in germ cells at st.35 were also identified at st.33. I identified a novel gene, *Sdgc* (Sex chromosome–dependent Differential expression in Germ Cells), transcripts of which were highly enriched in early XY germ cells. Analysis of *Sdgc* in germ cells revealed a novel mechanism by which sexually different characters arise at the cellular

level. However, this study did not reveal how germ cells acquire sexual identity after their somatic sex is determined.

In Chapter 2, to further explore the candidate genes that affect the sexual identity of germ cells, I analyzed the global gene expression profiles of type I, type II germ cells, and meiotic oocytes using RNAseq. This experiment was based on the hypothesis that the egg fate decision is made in XX germ cells at some point before they enter meiosis. I successfully identified the gene for the sex determination in germ cells and demonstrate the mechanism of germline sex determination at the molecular level for the first time in vertebrates.

## Chapter 1: Analysis of a novel gene, *Sdgc,* reveals sex chromosome–dependent differences of medaka germ cells prior to gonad formation

### 1.1 Introduction

In vertebrates, the master sex–determination genes identified so far are expressed in the gonadal somatic cells (supporting cells) (Hattori et al., 2012; Kamiya et al., 2012; Koopman et al., 1990; Matsuda et al., 2002; Nanda et al., 2002; Smith et al., 2009; Yano et al., 2012; Yoshimoto et al., 2010). Therefore, it is generally accepted that sex determination first occurs in the gonadal somatic cells during development. Following this step, under the influence of the somatic cells, the sex of germ cells is determined, and these cells are then fated to develop into either sperm or eggs.

In mice, when germ cells are isolated from XY gonads at 11.5 days post–coitum (dpc) and reaggregated with XX somatic cells from urogenital ridges, the XY germ cells behave like XX female germ cells and enter meiosis. By contrast, germ cells from XY gonads at 12.5 dpc mitotically arrest as prospermatogonia in response to the same treatment (Adams and McLaren, 2002; McLaren, 2000). This observation indicates that by 12.5 dpc, germline sex is largely determined by the action of the sex determination gene, *Sry,* in the gonadal somatic cells.

Somatic cells also exert a strong influence on germ cell sex determination in teleost fish, as demonstrated by the reciprocal transplantation of testicular and ovarian germ cells. In rainbow trout and zebrafish, isolated oogonia and spermatogonia from mature gonads can recolonize the undifferentiated gonads of embryos and produce

either sperm or eggs, depending on the sex of the surrounding somatic cells (Okutsu et al., 2006; Wong et al., 2011; Yoshizaki et al., 2010).

In medaka, *Oryzias latipes*, the sex determination gene on the Y chromosome, *DMY*/*dmrt1bY* (Matsuda et al., 2002; Nanda et al., 2002), so far was reported to start to be expressed in gonadal somatic cells immediately after the gonadal primordium forms at stage 33 (st.33), and suppresses the shift of germ–cell proliferation modes from a self–renewal type of division (type I division) towards a gametogenesis–committed cystic type of division (type II division) at st.35 (Fig. 1). The shift from type I to type II division is critical for ovary formation (Nakamura et al., 2012a; Nakamura et al., 2012b; Saito et al., 2007; Tanaka et al., 2008).

Taken together, the evidence enumerated above indicates that in vertebrates, the gonadal somatic cells control the sex of germ cells and determine whether they develop into sperm or eggs. In this study, I found that medaka germ cells exhibit sexually different characters even before the formation of the gonadal primordium. The differences at this early stage are controlled in a germ cell–autonomous manner by the number of Y chromosomes, but not by the expression of the sex determination gene protein, DMY/dmrt1bY. Thus, these findings reveal a novel mechanism by which sexually different characters arise at the cellular level.

## 1.2 Materials and Methods

### *Animals*

All the treatments of animals in this research followed the guideline of National

Institute for Basic Biology and were approved by the Institutional Animal Care and Use Committee of National Institutes of Natural Sciences. The OKcab strain and *sox9b*-DsRed/*olvas*-EGFP transgenic medaka (Nakamura et al., 2010) were used in this study. To produce all XX progeny, XX males, which had been treated with 100 ng/ml 11-ketotestosterone during the embryonic development, were crossed with XX females. To produce all XY progeny, first, XY females were generated by treatment with 200 ng/ml estradiol and were crossed with XY males. As a consequence, approximately 25% of the progeny were YY males, which were subsequently crossed with XX females to generate all XY progeny. All XX and XY progenies were used for the isolation of germ and somatic cells by fluorescence activated cell sorting (FACS), *in situ* hybridization, cell culture and qPCR experiments as described below. Hd-rR-II1 strain was also used for qPCR experiment. To ablate germ cells, we injected 2000 ng/µl *cxcr4*-morpholino (MO) and 1000 ng/µl *nanos3*-MO into one– or two–cell stage embryos (Kurokawa et al., 2006; Kurokawa et al., 2007).

***Fluorescence activated cell sorting (FACS) and microarray analysis***

All XX and all XY progeny of *sox9b*-DsRed/*olvas*-EGFP transgenic medaka were used in this study. Approximately 500 gonadal fragments, which included intestines, gonads and body trunks, were dissected from st.33 and st.35 embryos and were digested with 0.2% collagenease (Worthington) and 1% trypsin (Worthington) in Leibovitz's L15 for 2 hr at 29°C. Then, the cell suspension was resuspended with L15 supplemented with 10% FBS to stop the digestion reaction, and was filtered through 35 µm cell strainers.

*olvas*-EGFP and *sox9b*-DsRed positive cells were isolated by FACS (Beckman Coulter, COULTER EPICS ALTRA) according to fluorescence intensity and size. Total RNA was extracted from 8000–10000 cells using an RNAqueous–Micro Kit (Ambion), followed by cRNA labeling using a Low Input Quick Amp Labeling Kit (Agilent) according to the manufacturer's instructions. Hybridization of cRNA targets was performed on a 60–mer oligo microarray (Agilent, Design ID: 027381). The microarray experiments were done using three independent biological replicates. Scanned data were processed by Feature Extraction (Agilent) and further analyzed by the Subio platform.

***In situ hybridization and immunohistochemistry***

Whole–mount *in situ* hybridization and immunohistochemistry were performed as previously described (Aoki et al., 2008; Nakamura et al., 2006). A cDNA clone for *Sdgc* (clone name: olte54h09) was obtained from NBRP medaka (http://www.shigen.nig.ac.jp/medaka/). For immunohistochemistry, anti-OLVAS (medaka Vasa antibody, 1:100, rat)(Aoki et al., 2008) or anti-GFP (1:100, mouse; Clontech) was used as primary antibodies that were detected with Alexa 488-conjugated secondary antibodies (1:100).

***Generation of DMY/dmrt1bY reporter transgenic medaka lines***

A bacterial artificial chromosome (BAC) transgenic method that uses homologous recombination was employed to generate a *DMY/dmrt1bY-EGFP*-3'UTR reporter construct as previously described (Nakamura et al., 2008). Specifically, the targeting

DNA fragment for recombination was prepared to include the *DMY*/*dmrt1bY*-3'UTR downstream of the EGFP open reading frame. After homologous recombination, this fragment was inserted immediately downstream of the translation initiation site of the *DMY*/*dmrt1bY* gene in BAC clone DMY H1. Microinjection of BAC clone DNA was performed as previously described (Nakamura et al., 2008). For early germ cell tracking, one–cell stage embryos were injected with *mCherry*:*nos3*-3'UTR (mCherry ORF fused to the 3'UTR of *nanos3* ) capped RNA as previously described (Herpin et al., 2007).

***Chimeric analysis***

*sox9b*-DsRed/*olvas*-EGFP transgenic embryos (Nakamura et al., 2010) were used as hosts, and OKcab embryos were used as donors. To eliminate the host's germ cells, 2000 ng/µl *cxcr4*-MO and 1000 ng/µl *nanos3*-MO were injected into one– or two–cell stage embryos (Kurokawa et al., 2006; Kurokawa et al., 2007). The transplantation procedure was performed as previously described (Nakamura et al., 2012b).

***Quantitative PCR (qPCR)***

Germ cells are located at the lateral sides of the intestine in st.30 embryos. In each experiment, approximately 50 tissue fragments containing germ cells were dissected from XX and XY embryos, and total RNA was extracted using Isogen (Nippon Gene). For the experiment using *cxcr4*/*nanos3*-MO-treated embryos and chimeric embryos, 20–30 *olvas*-EGFP-negative embryos (no endogenous germ cells) were used. For extracting RNA from adult gonads, three testes and one ovary were used in each

experiment. After total RNA extraction, cDNA was produced using SuperScript III (Invitrogen) or ReverTra Ace® qPCR RT Kit (TOYOBO). Quantification was performed with the ABI StepOnePlus Real–Time PCR system (Applied Biosystems) and SYBR green (TOYOBO). The primers used in this study are shown in Table 1. The amount of target gene normalized to the *β-actin* or *olvas* (for chimeric analysis) expression and relative to the XX gonads was calculated using the $\Delta\Delta C_T$ method. All qPCR reactions were performed in triplicate. Three independent experiments using independent pools of normal gonads and two independent experiments using independent pools of morpholino–treated gonads and chimera gonads were performed.

***Sequence analysis and identification of SNPs in the Sdgc genomic region***

To identify SNPs in the genomic region of the *Sdgc* gene, the genomic fragments were amplified and sequenced using DNA from OKcab (XX: n = 4, XY: n = 4) and Kaga (XX: n = 4, XY: n = 6) inbred lines. From OKcab DNA, 535 bp genomic fragments, including an intron, were amplified and sequenced by q-Sdgc-F and -R primers (Table 1). From Kaga DNA, 346 bp genomic fragments were amplified and sequenced using gSdgc-F and q-Sdgc-R primers. As a result, 14 SNPs were observed in the 346 bp amplicon (Fig. 11).

***Linkage analysis of Sdgc***

For single nucleotide polymorphism (SNP) genotyping, XX Kaga and XY Hd-rR-II1 were crossed to generate the F1 progeny, which were subsequently intercrossed to

obtain F2 progeny (Kimura et al., 2012). Genomic DNA from the F2 progeny (n=94) was amplified using gSdgc-F and q-Sdgc-R. Then, the PCR products were diluted 100–fold and used as templates for nested PCR, the products of which were further genotyped via High Resolution Melting analysis using a LightScanner® (*Idaho* Technology) (Table 3). Locus order was determined together with M–markers (Kimura et al., 2012) by AntMap (http://lbm.ab.a.u–tokyo.ac.jp/~iwata/antmap/).

***Knock–down of DMY/dmrt1bY and Sdgc by gripNA***

GripNA (Active Motif) was designed to target the translation initiation site of *DMY*/*dmrt1bY* and *Sdgc* at the sequence CATGTCAGCCCGGGGAGC (Paul-Prasanth et al., 2006) and CTCGGGTTGGTTTTGCAT, respectively. The underlined CAT is complementary to the ATG start codon. For qPCR analysis and cell culture experiments, 0.5mM *DMY*/*dmrt1bY*-gripNA and 0.25mM *Sdgc*-gripNA were injected into one– or two–cell stage embryos. 0.1mM human-CREB gripNA was injected as a control.

***Overexpression and rescue experiments of Sdgc***

*Sdgc* ORF was amplified using FLAG-tagged primers and inserted into a *Sal*I site of the pBLSK+ PTV1-2A-mCherry vector (Nakamura et al., 2012b). *Olvas*-3'UTR was inserted into downstream of the mCherry by InFusion (Clontech) to generate *FLAG*:*Sdgc*:*2A*:*mCherry*:*olvas*-3'UTR. Capped RNA was synthesized with T7 promoter using mMESSAGE mMACHINE kit (Ambion). For overexpression, 200ng/µl of the mRNA was injected into XX embryos. For the rescue experiment, 200ng/µl of

the mRNA and 0.25mM *Sdgc*-gripNA were injected into XY embryos. The subsequent culture experiments were described below.

***Overexpression of DMY/dmrt1bY***

The construction of *EGFP*:*nos3*-3'UTR (EGFP ORF fused to the 3'UTR of *nanos3* ) was described previously (Kurokawa et al., 2006). Based on *EGFP*:*nos3*-3'UTR construct, *DMY*/*dmrt1bY* ORF was inserted into downstream of *EGFP* by In-Fusion (Clontech) to generate *EGFP*:*DMY*:*nos3*-3'UTR. Capped RNA was synthesized with SP6 promoter using mMESSAGE mMACHINE kit (Ambion). 50ng/µl of each construct was injected into one– or two–cell stage embryos.

***Mitotic activity of germ cells in culture***

Approximately 200 tissue fragments containing germ cells at st.30 were dissociated using 0.1% trypsin and 0.1% collagenase in PBS at 29°C for 1 hr. The cells were resuspended in L15 containing 10% FBS to stop the enzymatic activity. Next, GFP-positive germ cells were isolated by FACS (Beckman Coulter, COULTER EPICS ALTRA) using the enrich mode. Approximately $5.0 \times 10^3$ XX and $7.8 \times 10^3$ XY germ cells were successfully isolated. For the preparation of somatic cells, tails from XX and XY embryos were separately dissociated using trypsin and collagenase under the same conditions described above. Germ cells and somatic cells were divided into two tubes and mixed in the following combinations: germ XX–soma XX, germ XX–soma XY, germ XY–soma XX and germ XY–soma XY. The cell mixtures were divided into three

portions and plated on collagen–coated cover slips that had been placed in a six–well dish. After a two–hour incubation, 2 ml of Medium #1 (Table 2) was added to each dish. After a 24 hr incubation at 26°C, the culture medium was changed to include 25 μM EdU, and cells were cultured for an additional 24 hr, then fixed in 4% paraformaldehyde and analyzed by immunohistochemistry. For knockdown and overexpression experiment, 30–40 tissue fragments containing germ cells at st.30 were dissociated as described above. Then, the cell suspension was divided into two portions and plated on collagen–coated cover slips. Cell culture and EdU treatment were performed as described above.

## 1.3 Results

### *Germ cell–specific expression of a novel transcript, Sdgc*

To study gene expression in germ cells and gonadal somatic cells during gonadal sex differentiation, we used a *sox9b*–DsRed/*olvas*–EGFP transgenic medaka (Nakamura et al., 2010), and isolated EGFP–positive germ cells and DsRed–positive supporting cells from XX and XY embryos by fluorescence activated cell sorting (FACS) at st.33 and st.35. Microarray expression profiling revealed that several transcripts exhibited sexually different expression levels in germ cells by st.33. Among them, a novel transcript, *olte54h09* (NCBI accession: FS527139; named *Sdgc* after its function, Sex chromosome–dependent Differential expression in Germ Cells), was highly enriched in XY germ cells relative to XX germ cells (Fig. 2A,B). Quantitative PCR (qPCR) analysis of *Sdgc* and other gonadal markers confirmed that *Sdgc* was

already upregulated in XY embryos by st.33 (Fig. 2C). This observation raised the possibility that germ cells exhibit sexually different gene expression even before the expression of *DMY*/*dmrt1bY* begins in gonadal somatic cells (Fig. 2D).

To confirm this possibility, we performed qPCR and *in situ* hybridization for *Sdgc* in st.30 embryos. At st.30, the gonad is not yet formed, and the germ cells are located laterally to the forming hindgut; from this position, they subsequently move towards the prospective gonadal area on the dorsal side (Nakamura et al., 2006). *Sdgc* expression was detected as clusters on the lateral sides, and was stronger in XY embryos than in XX embryos (Fig. 3A–D). The expression levels of *Sdgc* were more than 2–fold higher in XY embryos than in XX embryos (Fig. 3I). When endogenous germ cells were removed by inhibiting germ–cell migration using *nanos3*/*cxcr4*–morpholinos (Kurokawa et al., 2006; Kurokawa et al., 2007), the *Sdgc* signal totally disappeared (Fig. 3E–I). Together with microarray data showing higher enrichment in isolated germ cells, these observations indicate that *Sdgc* is expressed in germ cells.

The *Sdgc* transcript encodes a putative protein of 142 amino acids (Fig. 4). Eleven repeats of QGPPAQ(E)GR constitute the majority of the protein, and no known domains were found in the sequence. The 5'UTR was found on three different scaffolds, but none of those scaffolds had yet been mapped to medaka linkage groups (LG). When we performed qPCR analysis of *Sdgc* in a different strain, Hd-rR-II1, expression was also upregulated in XY embryos (Fig. 5). Therefore, upregulation of *Sdgc* in XY germ cells is not specific to the OKcab strain.

We then examined *Sdgc* expression in adult testes and ovaries. *Sdgc* was highly enriched in testes as compared to ovaries (Fig. 6A). In testes, the signal was more abundant in type B spermatogonia than in type A spermatogonia, but was absent in spermatocytes (Fig. 6B,C). In ovaries, the transcripts were only present at extremely low levels in early stage oocytes (Fig. 6D). No expression was detected in oogonia (Fig. 6E), consistent with the sexually different expression in germ cells at the early embryonic stages.

***Expression of Sdgc depends on Y chromosome number in germ cells***

Next, we examined the expression levels of *Sdgc* using medaka with different Y–chromosome constitution and found that *Sdgc* expression levels increased with the copy number of the Y chromosome (Fig. 7A). We then asked whether the upregulation of *Sdgc* was due to the presence of a Y chromosome in germ cells or in somatic cells. To address this question, we generated chimeric medaka by transplanting XY germ cells into XX hosts and vice versa (Nakamura et al., 2012b), using *sox9b*–DsRed/*olvas*–EGFP transgenic line as hosts and non–transgenic strains as donors. Prior to manipulation, the host germ cells with EGFP fluorescence were ablated by *nanos3*/*cxcr4*–morpholino injection. *Sdgc* expression levels in XY hosts with XX germ cells resembled that of XX hosts with XX germ cells. By contrast, the expression of *Sdgc* in both XX and XY hosts with XY germ cells was more than 2–fold higher than in the hosts with XX germ cells (Fig. 7B). The sex of the host did not correlate with any statistically significant difference in the *Sdgc* expression level in germ cells. These

results clearly indicate that up–regulation of *Sdgc* depends on the presence of a Y chromosome in germ cells, but is independent of the genetic sex of the surrounding somatic cells, suggesting that sexually different expression occurs as a germ cell–autonomous event.

***DMY/dmrt1bY–independent expression of Sdgc in germ cells***

The only differences identified to date in the two medaka sex chromosomes reside at the sex–determination locus, leading us to speculate that *DMY*/*dmrt1bY* may up–regulate the *Sdgc* gene in XY germ cells in a germ cell–autonomous manner. Interestingly, *DMY*/*dmrt1bY* transcripts were detected in XY germ cells at both st.33 and st.35 by microarray (Fig. 2D). The germ–cell expression of *DMY*/*dmrt1bY* was further confirmed by *in situ* hybridization in st.30 embryos. The *DMY*/*dmrt1bY* signal was also detected, only in XY embryos, as single clusters on both lateral sides of the forming hindgut, where it was colocalized with expression of OLVAS, a marker of germ cells (Fig. 8A–C). When endogenous germ cells were removed by *cxcr4*/*nanos3* morpholino treatment, the *DMY*/*dmrt1bY* signal disappeared (Fig. 8D). To further investigate *DMY*/*dmrt1bY* expression in germ cells, we generated a *DMY*/*dmrt1bY*–EGFP reporter transgenic medaka, in which *DMY*/*dmrt1bY* expression could be visualized by EGFP driven by regulatory elements of *DMY*/*dmrt1bY*. *DMY*/*dmrt1bY* reporter expression in germ cells was first detected as early as st.28 and persisted until around st.31 (Fig. 9A–C). Together, these data indicate that *DMY*/*dmrt1bY* is expressed in germ cells before formation of the gonadal primordium.

To determine whether *Sdgc* is directly regulated by DMY/dmrt1bY protein, we knocked down *DMY*/*dmrt1bY* using synthetic antisense oligonucleotides (gripNA). Specifically, we used a *dmy*–gripNA described previously (Paul-Prasanth et al., 2006), for which the specificity had already been confirmed by *in vitro* translational suppression and *in vivo* analysis. We also confirmed that this gripNA effectively decreased green fluorescence in embryos of *DMY*/*dmrt1bY*–EGFP reporter transgenic medaka (Fig. 10A,B). Although the reporter fluorescence was dramatically reduced, *Sdgc* expression levels did not change in gripNA–injected embryos relative to normal embryos at st.30 (Fig. 10C). Overexpression of *DMY*/*dmrt1bY* also had no effect on the expression level of *Sdgc* (Fig. 10D,E). These results demonstrate that upregulation of *Sdgc* is not due to a direct effect of DMY/dmrt1bY protein expression, but rather is critically dependent on the presence of a Y chromosome in germ cells.

***Sdgc is mapped closely to the DMY/dmrt1bY locus on the sex chromosomes***

We suspected that upregulation of *Sdgc* may have been due to a conformational difference or presence of single–nucleotide polymorphisms (SNPs) between the X and Y chromosomes. If this is true, then *Sdgc* should be located on the sex chromosomes. To test this possibility, we mapped *Sdgc* using the M–markers (Kimura et al., 2012) (Fig. 11 and Table 3). We found that both *Sdgc* and a sex–linked marker (*SL1*) flanking *DMY*/*dmrt1bY* (Matsuda et al., 2002; Nanda et al., 2002) are located between the *MID0122* and *MID0123* linkage markers (Fig. 12). Thus, *Sdgc* maps near the region where *DMY*/*dmrt1bY* resides on the sex chromosomes (LG1).

Interestingly, in the Kaga strain, 11 SNPs found in the intronic region of *Sdgc* were heterozygous only in XY specimens (Fig. 11), further supporting the idea that the gene is located near *DMY*/*dmrt1bY*, where homologous recombination should occur less frequently. Because *Sdgc* expression was detected in XX germ cells, we reasoned that *Sdgc* must be present on both the X and Y chromosomes, but that the two alleles have different expression.

***A cell–autonomous and DMY/dmrt1bY–independent sex difference in the mitotic activity of isolated germ cells***

Finally, we investigated whether germ cells behaved differently between the two sexes, using an *in vitro* culture system. First, germ cells dissociated from embryonic tissue at st.30 were plated onto culture dishes together with dissociated somatic cells. Then, the mitotic activity of germ cells was examined in the two different media by monitoring incorporation of EdU (Fig. 13 and Table 2). Under both culture conditions, the rate of EdU incorporation in XY germ cells was higher than in XX germ cells (Fig. 13C,D). To exclude the possibility that the sex of the dissociated somatic cells might affect the mitotic activity of germ cells, we isolated XX or XY germ cells at st.30 by FACS and cultured them on XX or XY feeder cells, respectively, derived from somatic cells of the tail region at st.30. After 24 hr in culture, EdU treatment was performed for an additional 24 hr to observe the mitotic activity of the germ cells (Fig. 13B). Again, the rate of EdU incorporation in XY germ cells was significantly higher than in XX germ cells, and this difference was not dependent on the sex of the somatic cells (Fig.

14A). This observation suggests that an intrinsic sexual difference within germ cells contributes to the difference in germ–cell mitotic activity.

To examine the relationship between cell–autonomous gene expression and sexually different mitotic activity, we knocked down *DMY*/*dmrt1bY* and *Sdgc* and performed culture experiments on the resultant cells. *DMY*/*dmrt1bY* knockdown had no effect on the mitotic activity of XY germ cells (Fig. 14B), whereas knockdown of *Sdgc* significantly reduced mitotic activity (Fig. 14C), an effect that could be rescued by overexpression of *Sdgc* (Fig. 14C). Knockdown of *Sdgc* had no effect on XX germ cells (Fig. 14D), but *Sdgc* overexpression increased the mitotic activity of XX germ cells (Fig. 14D). Collectively, these results suggest that *Sdgc* confers higher mitotic activity upon XY germ cells relative to XX germ cells *in vitro*. This observation further supports the idea that the cell–autonomous acquisition of sexually different characters in germ cells is independent of DMY/dmrt1bY protein expression.

**1.4 Discussion**

In this study, we examined the gene expression and behavior of germ cells before gonadal primordium formation in medaka. We found that *Sdgc* and the sex–determination gene *DMY*/*dmrt1bY* were expressed in germ cells at this early stage. However, although *Sdgc* expression was upregulated only when a Y chromosome is present in germ cells, it was unaffected by *DMY*/*dmrt1bY* knockdown or overexpression. This sexually different expression was induced in a germ cell–autonomous manner. In addition, early XX and XY germ cells exhibited different mitotic activities *in vitro*.

Together, these data show that XX and XY germ cells possess different characters even before gonadal primordium formation and the onset of sex determination by gonadal somatic cells.

***Two possible models for inducing cell–autonomous sexually different characters of germ cells***

The master male sex–determining gene evolved relatively recently (5-10 million years ago) from a duplicated copy of *dmrt1* (Kondo et al., 2003; Matsuda, 2003). This evolutionary process was associated with the insertion of a transposable element called *Izanagi* that confers a novel expression pattern upon *DMY*/*dmrt1bY* (Herpin et al., 2010). Unexpectedly, in our study, sexually different characters of germ cells were not impaired by knockdown and overexpression of *DMY*/*dmrt1bY*. Instead, upregulation of *Sdgc* and increased mitotic activity of germ cells *in vitro* were dependent on the presence of the Y chromosome in germ cells. In addition, *Sdgc* mapped close to *DMY*/*dmrt1bY* on sex chromosomes and produced higher mitotic activity in XY germ cells than in XX germ cells *in vitro*. Although the possibility that the transcriptional level of *DMY*/*dmrt1bY* affects *Sdgc* expression cannot be completely ruled out, our findings suggest two possible models for inducing cell–autonomous, sexually different characters of germ cells.

First, the sexually different characters may be caused at the chromosomal level. Previous studies (Kondo et al., 2006; Kondo et al., 2004; Takeda, 2008) showed that the male sex–determining gene *DMY*/*dmrt1bY* was inserted into one homologue of a

chromosome pair that became the X and Y. Outside the inserted fragment and where *Sdgc* is located, X and Y are largely homologous and have the same gene content. Thus, *Sdgc* was most likely already on this chromosome pair, and both copies might have been expressed at the same level prior to the insertion event. It is likely that insertion of *DMY*/*dmrt1bY* and/or subsequent changes are related to restriction of recombination near the *Sdgc* locus (Kondo et al., 2001; Takeda, 2008), resulting in accumulation of DNA fragments such as repetitive sequences. Recent analyses suggest that these elements affect the structural change of chromatins through a small RNA–related mechanism and/or epigenetic regulation (e.g. Grewal and Moazed, 2003; Slotkin and Martienssen, 2007). The structural changes caused by these fragments may have facilitated and maintained the expression difference between the X and Y copies, leading to sexually different characters of germ cells. As for development of *Sdgc*, however, we cannot exclude the possibility that a high–expression version of *Sdgc* was inserted near *DMY*/*dmrt1bY,* and that this linkage has been conserved.

Second, infrequent recombination may result in single–nucleotide polymorphism (SNP) accumulation in the intergenic or non–coding region near *Sdgc* and *DMY*/*dmrt1bY* locus. This effect could be due to the possibility that either *DMY*/*dmrt1bY*–hitchhiked *Sdgc* or the insertion process itself of *DMY*/*dmrt1bY*. Both cases, can cause sex–specific differences in the X and Y chromosomsal regions around the insertion site and consequently in different transcriptional activity between the X and Y chromosome copies of *Sdgc*. Consistent with this, we identified sex–specific SNPs around *Sdgc*. Importantly, in either model, structural changes of chromosome or

different SNPs, *Sdgc* could potentiate sexually different cell behavior, as evidenced by the reduction of mitotic activity of XY germ cells by *Sdgc* knockdown *in vitro*.

***Sex chromosome based sexual differences in germ cells prior to gonad formation in mammals***

Regarding the events that occur differently on the X and Y chromosome prior to gonad formation in vertebrates, X–chromosome reactivation is initiated during the development of primordial germ cells in mice (Sugimoto and Abe, 2007). However, it is still not known whether this reactivation occurs in a germ cell–autonomous manner or instead is affected by surrounding somatic cells (McLaren, 2003). Our chimeric analysis provides good evidence that germ cells in vertebrates could exhibit cell–autonomous, sexually different characters that are independent of somatic cells.

***Dominant regulation of germ cell sex by somatic cells***

No obvious effect of *Sdgc* knockdown on germ cells was observed during late developmental stages of sex differentiation *in vivo*. One possibility is that mitotic activity of germ cells is predominantly regulated by somatic cells at that stage, thus cell–autonomous effects may be overridden. It is also possible that the *Sdgc*-gripNA, which was injected at the one– or two–cell stage, is not sufficiently active by the sex differentiation stage, which occurs late during development. Further investigation will be necessary to resolve this issue.

Knockdown of *DMY/dmrt1bY* did not affect expression of *Sdgc*. *DMY/dmrt1bY* expression was also detected in the *Sdgc*-knockdown XY embryos at the

onset of sex determination by somatic cells (Fig. 15A,B). Thus, given that *DMY*/*dmrt1bY* functions as a protein, *Sdgc* seems to function in a pathway independent of *DMY*/*dmrt1bY*.

***Sex chromosome differentiation and sexual plasticity***

Our findings provide insight into the relationship between sex chromosome differentiation and sexual plasticity at the cellular level. Many studies indicate that medaka (*Oryzias latipes*) sex chromosome arose relatively recently (Herpin et al., 2010; Kondo et al., 2004; Myosho et al., 2012; Takehana et al., 2007) and is not as differentiated as in mammals, in which sexual plasticity is much reduced in germ cells (e.g. Koopman et al., 1991; Lavery et al., 2011), whereas our results indicate that the newly arisen sex chromosomes can confer cell–autonomous, sexually different characters upon early stage germ cells. These results may indicate that the medaka sex chromosomes are on the evolutionary path to developing genes that influence sexually different characters and sexual plasticity of germ cells.

## Chapter 2: Unveiling the mechanism of the sperm–egg fate decision in germ cells

### 2.1 Introduction

Sex determination is an essential step for germ cells to develop into either sperm or eggs. The sex determination genes in somatic cells have been identified in several vertebrates (Graves, 2013; Kikuchi and Hamaguchi, 2013) and affect the sexual phenotypes of germ cells (McLaren, 2000; Murray et al., 2010). However, it is still totally unknown how germ cells determine their sexual fate in vertebrates. Two questions remained to be answered: 1) at which stage of germ cell development is the sexual fate decision made? 2) what is the sex determination gene in germ cells?

To explore the candidate gene affecting the sexual identity of germ cells after the somatic sex is determined, I analyzed the global gene expression profiles of type I, type II germ cells, and meiotic oocytes using RNAseq. This experiment was based on the hypothesis that the egg fate decision is made in XX germ cells at some point before they enter meiosis. I identified *SDiG* as an essential gene for sperm–egg fate decision and demonstrated how germ cells acquire sexual identity through analysis of *SDiG* function.

### 2.2 Materials and Methods

#### *Animals*

All treatments of animals in this study followed the guidelines of the National Institute for Basic Biology and were approved by the Institutional Animal Care and Use

Committee of National Institutes of Natural Sciences. The OKcab strain and *sox9b*-DsRed/*olvas*-EGFP transgenic medaka (Nakamura et al., 2010) were used in this study.

***Fluorescence Activated Cell Sorting (FACS)***

For isolating type I germ cells, XX and XY embryos at st.35 were used. For isolation of type II germ cells and meiotic oocytes, XX larvae at 5 days post hatching (dph) were used. The gonadal fragments were digested with 0.2% collagenease (Worthington) and 1% trypsin (Worthington) in Leibovitz's L15 for 2 hr at 29°C. Then, the cell suspension was resuspended with L15 supplemented with 10% FBS to stop the digestion reaction, and was filtered through 35 μm cell strainers. *olvas*-EGFP positive cells were isolated by FACS (Beckman Coulter, COULTER EPICS ALTRA) according to fluorescence intensity and size. Total RNA was extracted from 10000-15000 cells (st.35 germ cells) and 30000-50000 cells (type II and meiotic germ cells) using an RNAqueous®–Micro Kit (Ambion). The quality and quantity of RNA were determined by Agilent RNA 6000 Pico Assay kit with Agilent 2100 Bioanalyzer (Agilent).

***RNAseq analysis***

120-200ng total RNA was used for preparation of RNAseq library. rRNA was removed using Ribo-Zero Magnetic Gold Kit (epicentre), then RNAseq libraries were constructed using Script-Seq v2 RNA-Seq Library Preparation Kit (epicentre) according to the manufacturer's instruction. Sequence read pools were generated using illumina

Genome Analyzer IIX (st.35 germ cells) and illumina HiSeq2000 (type II and meiotic oocytes) and were mapped on medaka genome using Tophat (http://ccb.jhu.edu/software/tophat/index.shtml). The expression level of genes was determined by Cufflinks (http://cufflinks.cbcb.umd.edu/). The reads per kb per million reads (RPKM) method was used to calculate gene expression levels.

***In situ hybridization, immunohistochemistry, and histology***

Whole–mount *in situ* hybridization and immunohistochemistry were performed as previously described (Aoki et al., 2008; Nakamura et al., 2006). A cDNA clone for *SDiG* was obtained from NBRP medaka (http://www.shigen.nig.ac.jp/medaka/). SDiG polyclonal antibody was generated by immunizing rabbits with C-terminal peptide. For detection of SDiG protein, samples were treated with 5 mg/ml proteinase K (Roche) and 2N HCl for 15 min, respectively, prior to blocking. Can Get Signal solution B (TOYOBO) was used as the immunoreactive reagent (1:100). In addition, serum or antibodies specific for the following proteins were used: medaka OLVAS (1:100)(Aoki et al., 2008), EGFP (1:100; mouse; Life Technologies), and DsRed (1:100; rabbit; Life Technologies). Secondary reagents were Alexa Fluor 488–, 568–, and 647–conjugated antibodies (1:100; Molecular Probes). For PAS staining, whole ovaries and testes were fixed in Bouin solution, and 4-µm–thick plastic sections were prepared using Technovit® 8100 (Heraeus Kulzer). The staining procedures were performed as previously described (Quintero-Hunter et al., 1991).

***EdU treatment***

St.35 embryos were treated with 100 μM EdU for 72 hours in balanced salt solution (BSS) (Yang and Tiersch, 2009), followed by EdU detection using the Click-iT EdU labeling kit (Invitrogen) and immunohistochemistry for SDiG and OLVAS.

***TALEN-induced mutagenesis***

TALEN target sites of *SDiG* were searched using the TALEN Targeter program (https://tale-nt.cac.cornell.edu/node/add/talen) (Doyle et al., 2012) using the following parameters: spacer length of 15–18 bp, repeat array of 16–18 bp, and upstream base of T only. TALEN assembly followed a modified version (Sakuma et al., 2013) of the original protocol (Cermak et al., 2011). TALEN plasmids were linearized by *Not*I digestion and used as templates for *in vitro* RNA synthesis with the mMESSAGE mMACHINE® T7 transcription kit (Life technologies). TALEN mRNAs (250 ng/μl left and right) were injected into one- or two-cell stage embryos. The F0 founders were crossed with *sox9b*-DsRed/*olvas*-EGFP transgenic and non-transgenic medaka. Mutant alleles, NΔ17 and FHΔ8, were identified from the F1 adults. Unless otherwise indicated, all experiments were performed using the NΔ17 mutants (Fig. 21).

***Chimeric analysis***

*SDiG*$^{-/-}$ or *SDiG*$^{+/-}$; *sox9b*-DsRed/*olvas*-EGFP transgenic embryos were used as donors, and non-transgenic embryos were used as hosts. The transplantation procedure was performed as previously described (Nakamura et al., 2012b). Embryos with EGFP-positive germ cells were screened at st.35 and raised to 20 dph.

***Rescue construction***

*The olvas* promoter (5.1 kb) (Tanaka et al., 2001) and the 3'UTR (Kurokawa et al., 2006) were amplified from a fosmid (GOLWFno476_m14) containing the *olvas* gene and inserted upstream and downstream of EGFP in hsGBA-NKm vector (Nakamura et al., 2008) using In-Fusion (Takara) (Fig. 24). The resultant vector was further amplified, which was inserted downstream of the *SDiG* locus in the BAC. The resultant rescue construct, which allowed germline integration of the transgenes to be monitored, was injected into progeny of $SDiG^{+/-}$ females crossed with $SDiG^{-/-}$ males (non-transgenic line) at the one-cell stage. Embryos with EGFP-positive germ cells were screened at st.35 and raised to 15 dph.

***RT-PCR***

Total RNA was extracted from adult gonads of wild-type, $SDiG^{+/-}$, and $SDiG^{-/-}$ medaka using TriPure Isolation Reagent (Roche). Two testes and one ovary were used in each experiment. cDNA was produced from 800 ng total RNA using SuperScript III (Invitrogen) and used as the template for RT-PCR. PCR conditions and primer sets for *dmrt1*, *foxl2*, *p45011β*, and *aromatase* were described previously (Kurokawa et al., 2007). Two independent experiments were performed using independent pools of gonads.

***Artificial fertilization***

A gonad was dissected from 2- or 3-month-old $SDiG^{-/-}$ XX or wild-type XY medaka

and minced with forceps to suspend sperm in 90 µl balanced salt solution (BSS) (Yang and Tiersch, 2009). The resultant sperm were subsequently inseminated to 10–27 eggs in each experiment. The inseminated eggs were incubated at room temperature for 15 min, with gentle mixing by pipetting every 5 min. After replacing the BSS, fertilization rate was assessed by monitoring activation of the egg membrane. The eggs were incubated in BSS at 29°C until hatching. The hatching rate was calculated based on the number of fertilized eggs.

***Fertility determination of female SDiG$^{-/-}$ mutants***

Six pairs of 3-month-old *SDiG*$^{+/-}$ and $^{-/-}$ XX females were crossed with a *SDiG*$^{+/-}$ XY male. Eggs were collected every day for 3 days. The spawned and fertilized eggs were counted each day, and these counts were used as indicators of fecundity. The hatching rate was calculated based on the number of fertilized eggs.

***Fadrozole (FAD) and tamoxifen (TAM) treatment***

To block estrogen production or signaling, 15 dph larvae from *SDiG*$^{+/-}$ XX and $^{-/-}$ XY parents were kept in 1 L of water containing 100 ng/ml (0.38µM) fadrozole or 0.1 µM, and 0.5 µM tamoxifen (TAM) for 20 days. All larvae treated with 2.5 µM TAM were dead 1 day after the treatment was initiated. The water was changed every day. After 5 days under normal conditions, *SDiG*$^{-/-}$ XX gonads at 40dph were examined by immunohistochemistry.

***E2 treatment***

Embryos (0–7 dpf) were treated with 200 ng/ml E2, and larvae were treated with 100 ng/ml E2 for 5 days. The water was changed every day. After treatment, the larvae were kept under normal conditions for 1 week and examined by immunohistochemistry at 12 dph.

## 2.3 Results

To perform the RNAseq analysis, germ cells at different stages of development were isolated from *olvas*-EGFP transgenic medaka by FACS. XX and XY type I germ cells at stage (st.) 35 were isolated from the EGFP-positive fraction (Fig. 16A). While type II germ cells in 5 days post hatching (dph) XX larvae were enriched in the small cell-size population in the EGFP-positive fraction (88.6%), meiotic oocytes at the pachytene stage were enriched in the large cell-size population of the EGFP-positive fraction (77.0%) (Fig. 16B). Then, the global gene expression profiles of these isolated cells were analyzed using RNAseq. I found that a transcript named *SDiG* (Sex Determination in Germ Cells) was highly enriched in type II germ cells and showed dimorphic expression pattern during the gonadal development. Here, I examined the expression and function of *SDiG* in medaka.

***SDiG*/SDiG *show dimorphic expression during gonadal development***

*SDiG* transcripts and SDiG protein (*SDiG*/SDiG) were first detected in germ cells of both XX and XY embryos at st.35, the time of onset of gonadal sex differentiation (Fig. 17A–D, and Fig. 18A–D). After this stage in XX gonads, germ

cells can be categorized into two types according to their division (Nishimura and Tanaka, 2014; Saito et al., 2007)(Fig. 19A). Type I is stem-type self-renewal division, in which germ cells divide completely to generate two isolated daughter cells surrounded by supporting cells. Type I includes both mitotically active and quiescent germ cells (Nakamura et al., 2012a). Type II is cystic division, in which germ cells divide synchronously with intercellular bridges. Type II is gametogenesis-committed division, followed by meiosis and oogenesis. In XY gonads, type I germ cells and very few type II germ cells are observed until 1 month after hatching.

*SDiG*/SDiG appeared in a subset of mitotically active type I germ cells, but not in quiescent type I germ cells, at st.35 onwards in both XX and XY gonads (Fig. 19, A,B, and Fig. 20). In XX gonads, the signals continued to be detected in type II germ cells, but disappeared in meiotic germ cells and oocytes (Fig. 17 E–G and Fig. 19B). Whereas *SDiG*/SDiG were detected throughout gonadal development in XX fish, they disappeared in all germ cells of XY fish by 10 dph (Fig. 17, Fig. 18 and Fig. 19C), suggesting that *SDiG*/SDiG expression was associated with the oogenesis.

***SDiG is responsible for suppressing the initiation of spermatogenesis in germ cells***

To examine the function of *SDiG* in the gonadal sex differentiation, we generated TALEN-induced mutants of *SDiG*. We designed two types of TALENs targeting *SDiG* and obtained two different mutant alleles (NΔ17 and FHΔ8), which contain frameshifts causing premature truncation (Fig. 21A). Antiserum recognizing the C-terminus of SDiG protein detected the protein in both heterozygous (+/–) mutants but

failed to detect it in both homozygous (–/–) mutants (Fig. 21B–D), indicating that *SDiG* was successfully disrupted by TALEN. One week after hatching, a stage at which oocytes are formed in wild-type XX gonads (see Fig. 19B), the *SDiG*$^{-/-}$ XX gonads had no oocytes, but were instead filled with cystic and meiotic germ cells (Fig. 22A–C), suggesting that commitment to type II division and meiosis is not affected by loss of *SDiG*. Surprisingly, instead of oocytes, spermatid-like cells were present at the periphery of gonads in both mutant lines (NΔ17 in Fig. 22A,D, and FHΔ8 in Fig. 21E); these cells expressed *protamine* (Fig. 22E). At the same stage (1 week after hatching) in normal XY gonads, only type I germ cells are present (see Fig. 18J), and spermatogenesis does not begin until puberty, which occurs later than 1 month after hatching. This indicates that, in *SDiG*$^{-/-}$ XX mutants, precocious spermatogenesis begins much earlier than puberty in wild-type males.

Chimeric analysis revealed that *SDiG*$^{-/-}$ germ cells completed spermatogenesis in wild-type XX gonads (Fig. 22F and Fig. 23), indicating that the phenotype is due to loss of *SDiG* function in germ cells but is not affected by somatic cells. Furthermore, introduction of a bacterial artificial chromosome (BAC) containing *SDiG* allele restored the formation of oocytes in *SDiG*$^{-/-}$ XX larvae (Fig. 22G and Fig. 24). Collectively, these data indicate that *SDiG* is responsible for suppressing the onset of spermatogenesis in female medaka germ cells.

***Sperm are filled in histologically functional ovaries***

To characterize the gonadal structures producing sperm in the XX mutants,

we performed *in situ* hybridization and RT-PCR using sex-specific markers. Throughout gonadal development from 10 dph to 30 dph and in adult gonads of *SDiG*$^{-/-}$ XX fish, the female markers *foxl2* and *aromatase* were expressed in somatic cells surrounding spermatogenic cells, whereas the male markers *dmrt1* and *p45011β* were absent, reflecting the normal female expression pattern in somatic cells (Fig. 25A, Fig. 26A–F, and Fig. 27A–F). In adults, the secondary sex characteristics, which are distinguished by the morphology of dorsal/anal fins and urogenital papilla (Kurokawa et al., 2007), were of the female type in the XX mutants (Fig. 25B,C). Consistent with this, the *SDiG*$^{-/-}$ XX gonads exhibited morphologically ovarian structures characterized by an ovarian cavity on the dorsal side and a stromal compartment on the ventral side (Fig. 25D,E,J). In wild-type ovaries, a thin multilayered tissue called the germinal epithelium separates the ovarian cavity from the stromal compartment. In addition, the germinal cradle, a unit in which germline stem cells, cystic germ cells, and early diplotene oocytes are surrounded by *sox9b*-expressing cells (Fig. 25D), is laid between the epithelial cells of germinal epithelium and the basement membrane bordering the stromal compartment (Fig. 25F,K) (Nakamura et al., 2010). In *SDiG*$^{-/-}$ XX gonads, spermatogenesis proceeded within the expanded germinal epithelium (Fig. 25G,J,K). Early stages of spermatogenic cells were inclined to localize on the basal side of the expanded germinal epithelium underlain by basement membrane, and were surrounded by *sox9b*-expressing cells, forming a structure similar to the germinal cradles (Fig. 25E,J,K). Notably, a subset of these germ cells expressed *nanos2*, a marker of germline stem cells (Nakamura et al., 2010), suggesting that germline stem cells were established

in the absence of *SDiG* function (Fig. 27G–I).

In wild-type testes, spermatogenesis proceeds synchronously in cysts composed of Sertoli cells surrounding spermatogenic cells at the same stage. The cysts are arranged along a distal-to-proximal axis, so that the spermatogonia are located most distally and matured spermatids are released into the efferent duct in the most proximal position (Fig. 25I). In the expanded germinal epithelium of *SDiG*$^{-/-}$ XX gonads, however, the distal-to-proximal arrangement of the cysts was not observed, so that various stages of spermatogenic cells were adjacent to each other (Fig. 25H). This observation suggests that feminized somatic cells are not properly able to form cysts enclosing masculinized germ cells, as Sertoli cells would otherwise do in wild-type testes. Occasionally, oocytes appeared in between spermatogenic cells in the expanded germinal epithelium (Fig. 25E), which subsequently exited as follicles into the stromal compartment (Fig. 25G), as seen in follicle formations of the wild-type ovary (Fig. 25J,K) (Nakamura et al., 2010). Collectively, the expanded germinal epithelium in *SDiG*$^{-/-}$ XX gonads harbors germline stem cells and is able to support follicle formation but cannot organize the cysts in a distal-proximal direction. Any morphological trait of efferent ducts was not observed, possibly leading to the large accumulation of spermatogenic cells within the germinal epithelium of *SDiG*$^{-/-}$ XX gonads. We conclude that *SDiG*$^{-/-}$ XX mutants undergo spermatogenesis in histologically functional ovaries.

***SDiG$^{-/-}$ XX gonads produce both functional sperm and eggs***

To investigate whether the sperm produced by *SDiG*$^{-/-}$ XX mutants were functional, artificial insemination was performed. The gonads from 2- or 3-month-old XX mutants were minced with forceps to suspend sperm in medaka Ringer's solution, and the resultant sperm were subsequently inseminated into ovulated eggs of wild-type females. Approximately 50% of the eggs were successfully fertilized (Fig. 28A), as confirmed by the activation of the egg membrane (Fig. 28C,D). The fertilized eggs exhibited normal development, more than 95% of which hatched (Fig. 28B,E). Therefore, the *SDiG*$^{-/-}$ XX mutant produced functional sperm. Furthermore, *SDiG*$^{-/-}$ XX mutants spontaneously spawned a few fertile eggs, although their fertility, as assessed by the number of spawned eggs, the fertilization rate, and hatching rate, was lower than that of the *SDiG*$^{+/-}$ mutants (Fig. 28F–H).

***The oocyte formation in SDiG$^{-/-}$ XX mutants is independent of estradiol***

The presence of eggs in *SDiG*$^{-/-}$ XX mutants suggest the existence of a *SDiG*-independent pathway controlling oocyte formation. The oocytes did not appear until around 20–30 dph (Fig. 26G–I) in the mutants, a stage at which an ovarian cavity starts to form due to the action of estradiol (E2) (Suzuki et al., 2004). However, inhibition of *aromatase* activity and E2 signaling by the administration of fadrozole (FAD) and tamoxifen (TAM), respectively, did not block oocyte formation in the mutants (Fig. 29). On the other hand, administration of E2 during the embryonic and larval stages of both XX and XY *SDiG*$^{-/-}$ mutants did not induce the precocious oocyte

formation observed in E2-treated *SDiG*$^{+/-}$ XY larva (Fig. 30). These findings suggest that, after derepression of spermatogenesis upon loss of *SDiG* expression, oocytes are formed in an E2-independent manner.

## 2.4 Discussion

In this study, I showed that *SDiG* is an essential factor for the sperm–egg fate decision in medaka, and that suppression of the initiation of spermatogenesis is a key aspect of germline sex determination. The determination likely occurs prior to the onset of meiosis and is independent of the processes of cyst formation and meiotic entry, supporting the previous notion that mitosis/meiosis and the sperm-egg fate decision are dissociable in worms and mice (Dokshin et al., 2013; Morgan et al., 2013). Interestingly, in *SDiG*$^{-/-}$ XX mutants, spermatogenesis occurred much earlier than male puberty. In wild-type males, germ cells lose *SDiG* expression but do not initiate precocious spermatogenesis. This suggests the existence of mechanism posing the initiation of spermatogenesis until the onset of puberty in wild-type males.

### *Relationship between somatic and germline sex determination*

This study provides insight into the relationship between somatic and germline sex determination in medaka. *SDiG* transcripts and SDiG protein can initially be detected not only in XX germ cells but also in XY germ cells, even after expression of *DMY*/*dmrt1bY*. This suggests that *DMY*/*dmrt1bY* is not able to repress the expression

of *SDiG*/SDiG in germ cells. Instead, the signals may suppress the expansion of *SDiG*/SDiG-positive cells, as the number of these cells was not increased in XY gonads as compared to XX gonads. After hatching, *SDiG*/SDiG-positive cells had completely disappeared by 10 dph. This observation suggests other *DMY*/*dmrt1bY* downstream signals may suppress the expression of *SDiG*/SDiG and/or eliminate the *SDiG*/SDiG-positive cells. Therefore, dual regulation of suppression of the number of *SDiG*/SDiG-expressing cells and *SDiG*/SDiG expression is essential for male development. Overall, the default sexual feta of germ cells is to be females, and in males, the program is suppressed by the action of sex determination genes in somatic cells. A similar system of sex determination has also been observed in mammals. In mice, entering meiosis at the fetal stage is an indication of female sexual identity in germ cells, whereas blocking the initiation of meiosis is an indication of male sexual identity (e.g. Kocer et al., 2009). Interestingly, both XX and XY germ cells initially express the meiotic gene, *Scp3* (Chuma and Nakatsuji, 2001; Di Carlo et al., 2000). This suggests that in mice, both XX and XY germ cells are initially fated to be female. In the absence of sex determination signals, XX germ cells continue to express meiotic genes and enter meiosis. In the presence of SRY, male somatic cells suppressed meiosis and established a male sexual identity. Therefore, cell non-autonomous suppression of the default female program in germ cells during male development is conserved among animals and may be a fundamental mechanism of gonadal sex differentiation.

***Regulation of ovarian development by germ cells***

The formation of an ovarian structure filled with sperm provides insight into how germ cells regulate ovarian development. In medaka, germ cell ablation results in female-to-male sex reversal in XX fish (Kurokawa et al., 2007). In contrast, overproliferation of germ cells leads to male-to-female sex reversal in XY fish (Morinaga et al., 2007; Nakamura et al., 2012a). These results indicate that germ cells are necessary and sufficient for ovarian formation, and the existence of germ cell-derived factors (feminizing factors) that maintain and/or promote ovarian structures is predicted (Nishimura and Tanaka, 2013). However, it remains unknown which germ cell stage is critical for the ovarian development in medaka. In zebrafish, oocytes have been proposed to be critical for the maintenance of ovarian structures, because oocyte ablation in adult ovaries leads to sex reversal to testes (Dranow et al., 2013). In *SDiG*$^{-/-}$ mutants, oocytes did not appear until 20 dph–30 dph, but expression of *aromatase* and *foxl2* was maintained in somatic cells. Although the involvement of very few oocytes in the maintenance of female-specific genes is not entirely exclusive in *SDiG*$^{-/-}$ mutants, I propose two possibilities for the maintenance of ovarian structures by germ cells. First, all germ cells, irrespective of developmental stage or sexual identity, have the potential to produce feminizing factors. In this case, feminizing factors should be somehow blocked in masculinized somatic cells during male development. Second, germ cells that have the potential to express *SDiG*/SDiG produce the feminizing factors. Spermatogenic cells in *SDiG*$^{-/-}$ ovaries have the ability to express *SDiG*/SDiG, since *SDiG*$^{-/-}$ mutants containing a *SDiG*-EGFP reporter transgene, in which *SDiG*/SDiG

expression can be visualized by EGFP production driven by regulatory elements of *SDiG*, express EGFP in germ cells (unpublished data). In contrast, in wild-type testes, SDiG protein and the *SDiG*-EGFP reporter are not detectable. Therefore, the same mechanism involved in suppressing SDiG expression may also act to suppress feminizing factors in germ cells, enabling somatic cells to form testicular structure after the initiation of germ cell proliferation in males.

## General Discussion

Generally, the sex of germ cells is determined largely by the sex of somatic cells. In particular, in some species of fish, including medaka, the sexual identity of germ cells is totally dependent on the surrounding somatic cells and not at all on the sex chromosome constitution of germ cells (Okutsu et al., 2006; Shinomiya et al., 2002; Yoshizaki et al., 2010). Once the sex of somatic cells is determined, germ cells acquire sexual identity and develop either into sperm or eggs. However, little is known about the intrinsic factors affecting germ cell sex.

Here, I identified two intrinsic factors that affect germ cell sex in medaka. First, analysis of *Sdgc* revealed that medaka germ cells show cell-autonomous sex differences in gene expression and cell behavior, even before the somatic sex is determined. Interestingly, the sexual differences depend on the presence of a Y chromosome but not on the expression of the sex determination gene, *DMY*/*dmrt1bY*. This finding reveals a novel mechanism by which sexually different characters arise at the cellular level, even in animals in which sex is determined by genes. However, the sexual different characters prior to gonad formation seemed not to have a critical effect on the sexual identity of germ cells after gonad formation, possibly due to the dominant role of somatic cells in determining germ cell sex and/or the high sexual plasticity of medaka germ cells. Therefore, I further investigated the factors affecting the sexual identity of germ cells after somatic sex determination occurs. I identified *SDiG* as an essential gene for the sexual identity of germ cells. Through analysis of *SDiG* function, I revealed that suppressing the initiation of spermatogenesis is essential for acquisition

of a sexual identity in medaka germ cells. Surprisingly, once the suppression is released in females by disrupting *SDiG*, germ cells initiate spermatogenesis in an ovarian environment. This result suggests that, once the sexual identity of germ cells is established, germ cells could initiate gametogenesis independent of somatic sex. I would like to discuss how germ cells in other organisms acquire sexual differences and identity, and compare them with those in medaka. I would also like to discuss how somatic sex-independent gametogenesis occurs in natural environments.

***Chromosome-dependent sexual differences and identity***

Whereas the somatic cells have dominant roles in determining germ cell sex, sex chromosomes are also known to play an autonomous role in the sexual differences and sexual identity of germ cells in several gonochoristic animals (Casper and Van Doren, 2006; Murray et al., 2010). For example, in fruit flies (*Drosophila melanogaster*), the presence of two X chromosomes initiates cell-autonomous expression of *sxl* in primordial germ cells during migration to the gonads, which is essential for subsequent egg development (Hashiyama et al., 2011). Germ cells with only one X chromosome do not express functional *sxl,* and thus are unable to form oocytes in a female somatic environment. Conversely, XX germ cells are unable to form sperm in a male somatic environment (Casper and Van Doren, 2006; Murray et al., 2010). Sex chromosomes are also known to be important for the sexual development of germ cells in vertebrates. In mice, XX germ cells tend to behave as male when surrounded by male somatic cells, but some XX germ cells in close proximity to the

mesonephric rete region enter meiosis (female trait at the fetal stage), which is not observed in XO or XY germ cells (McLaren, 1981). Furthermore, although XX germ cells in a male somatic environment die shortly after birth, XO germ cells in a testis can initiate spermatogenesis (Koopman et al., 1991; McLaren, 1995; Murray et al., 2010). In contrast, XY germ cells in a female somatic environment produce a smaller number of oocytes than XX or XXY germ cells (Lavery et al., 2011; Mahadevaiah et al., 1993). In fruit flies and mice, the sex chromosomes are well differentiated, so that expression of chromosome-specific genes and/or the dosage effect of these genes likely confer cell-autonomous sexual differences and identity to germ cells, which are essential for proper gametogenesis in the corresponding somatic background.

However, in medaka, sex chromosomes have newly arisen via transposition of a sex determination gene to an autosome (Herpin et al., 2010; Kondo et al., 2004), and the only distinguishing feature so far identified in medaka sex chromosomes is the presence of a sex determination locus (*DMY*/*dmrt1bY*). Furthermore, reciprocal transplant experiments have shown that both XX and XY germ cells are able to differentiate into functional sperm and eggs, depending on the surrounding somatic sex (Shinomiya et al., 2002; unpublished data). Therefore, in medaka, the sex chromosomes are not likely to be sufficiently differentiated to confer the cell-autonomous sexually differences and identity that trigger germ cells to undergo the sex-specific gametogenesis. However, the analysis of *Sdgc* provides mechanistic insight into how germ cells could acquire sexual differences via the newly arisen sex chromosomes. *Sdgc* is cell-autonomously upregulated in XY germ cells compared to XX germ cells and

affects the sexual different cell behavior of germ cells. Interestingly, *Sdgc* is mapped near the sex determination locus on both the X and Y chromosomes. Recombination appears to be suppressed near this locus, as several sex chromosome-specific SNPs have been identified in the *Sdgc* locus, suggesting that sex chromosome differentiation initiate from the loci near the sex determination gene. This differentiation may alter the activity of regulatory elements of *Sdgc*, leading to the differential expression of *Sdgc* between XX and XY germ cells. Therefore, medaka sex chromosomes may be on the evolutionary path to developing genes that influence cell-autonomous sexually different characters in germ cells.

***The mechanism for acquisition of sexual identity in germ cells***

Here, I have demonstrated that suppression of the initiation of spermatogenesis is essential for acquisition of sexual identity in female germ cells in medaka. Interestingly, negative regulation of the opposite sex for acquisition of sexual identity is observed in sex determination of germ cells in *Caenorhabditis elegans.* TRA-2 activity (oogenic program) relative to FEM-3 activity (spermatogenic program) has been suggested to be crucial factor controlling the sperm–egg fate decision (Ellis and Schedl, 2007; Zanetti and Puoti, 2013). TRA-2 promotes oogenic program by directly inactivating FEM-3 activity. However, in males, a male-specific somatic-derived factor, HER-1, negatively regulates TRA-2 activity, permitting high levels of FEM-3 activity to induce spermatogenesis. The sexual identity of germ cells in medaka may be controlled by a similar mechanism. In male medaka, *DMY*/*dmrt1bY*

downstream signals in somatic cells may suppress the expression of *SDiG*/SDiG in germ cells, allowing the expression of genes essential for the spermatogenic program. However, in the absence of *DMY*/*dmrt1bY*, germ cells in females maintain expression of *SDiG*/SDiG to suppress the spermatogenic program, which in turn induces oogenesis. Therefore, elucidating the upstream negative regulators of *SDiG* derived from male somatic cells and the downstream targets of *SDiG* in female germ cells may provide further insight into how germ cells acquire sexual identity in medaka.

***Somatic sex-independent gametogenesis in hermaphroditic animals***

Germ cells undergo either spermatogenesis or oogenesis in close contact with somatic cells of the corresponding sex. Thus, gametogenesis usually occurs under condition in which the somatic sex and the germ cell sex are the same. Contrary to this notion, another surprising phenomenon observed in this study is that spermatogenesis can progress in a female somatic environment, as shown in the *SDiG* mutants. One attractive notion arising from this study is that once the sexual identity has been established, germ cells can maintain their identity and undergo gametogenesis, independent of the somatic sex. Importantly, the somatic-sex independent gametogenesis is likely to be observed during normal reproduction of hermaphroditic animals. In *C. elegans*, XX hermaphrodites initially undergo spermatogenesis and switch to oogenesis during a later stage of development. Interestingly, the spermatogenesis occurs in a female somatic environment and is activated by suppression of the oogenic program (TRA2 activity) in germ cells (Ellis and Schedl,

2007; Zanetti and Puoti, 2013). A similar phenomenon has also observed in hermaphrodite fish. Most species of fish show sequential hermaphroditism, in which an individual undergoes a male phase and a female phase sequentially, depending on developmental stages or social cues (e.g. Devlin and Nagahama, 2002; Kobayashi et al., 2013). In anemone fish (*Amphiprion* species), oogenesis first occurs in an immature ovary. Then, testicular differentiation occurs, and an individual first develop as a functional male. As a consequence, during the functional male phase, anemone fish possess both mature testes and immature ovaries (Miura et al., 2003). Interestingly even during male phase with high levels of androgen (Miura et al., 2008), the germ cells can undergo oogenesis as well as spermatogenesis in the gonads, probably in preparation for the subsequent female phase (Brusle-Sicard and Reinboth, 1990; Godwin, 1994). Oogonia established during the immature stage may retain their sexual identity and undergo oogenesis even in a male environment. Although the mechanism that permits simultaneous oogenesis and spermatogenesis in a single gonad is not well understood, it is intriguing to speculate whether regulation of *SDiG* in germ cells plays a pivotal role in sperm–egg fate decision in these hermaphrodite fish.

***Fundamental mechanisms of sex determination in somatic and germ cells***

In mammalian somatic cells, antagonism between the SOX9/FGF9-regulated and Wnt4/RSPO1/β-catenin-regulated pathways is a key element in the establishment of sexual identity during embryonic development, while antagonism between FOXL2 and DMRT1/SOX9 is important for maintenance of sexual identity in adults (Kim et al.,

2006; Matson et al., 2011; Tavosian, 2013; Uhlenhaut et al., 2009). In other words, sexual identity in somatic cells is established and maintained by suppressing the opposite sex. The model of sex determination in somatic cells may also extend to germ cells. This study shows that *SDiG* in germ cells suppresses the initiation of spermatogenesis during the oogenesis, suggesting that similar antagonism may be present in the establishment and/or maintenance of sexual identity in germ cells. Furthermore, the progression of spermatogenesis in ovarian environment in *SDiG* XX mutants also suggests that, even if this antagonism in germ cells results in the establishment of the sex opposite to that in somatic cells, germ cells could initiate the gametogenesis independent of the somatic sex. In gonochoristic animals, the sex-specific signals derived from somatic cells may create an imbalance between male and female signals in germ cells, allowing gametogenesis under conditions in which the somatic and germ cell sexes are the same. On the other hand, in hermaphroditic animals, additional signals, which may be derived from the environments and/or germ cells themselves, may transiently antagonize the somatic-derived signals, allowing gametogenesis under conditions in which the somatic and germ cell sexes are different. Therefore, it is ultimately germ cells that instruct the adaptation of the sexual fates in gametogenesis, and somatic and/or environmental signals are "cues" that create the imbalance between female and male signals in germ cells. Further elucidating the molecular machinery regulating the sexual balance in germ cells will provide greater insight into the establishment of germ cell sex and development of reproductive systems.

**Acknowledgements**

First of all, I would like to give big thanks to my supervisor Dr. Minoru Tanaka for valuable advices and supports in this study. I am grateful to Dr. Satoru Kobayashi and Ms. C. Noda for the FACS sorting, Dr. Shuji Shigenobu, Dr. Taro L. Saito, Dr. Jun Yoshimura, Dr. Shinichi Morishita and Dr. Tatsuya Tsukahara for supporting the microarray analysis, Dr. Toshihiro Kawasaki, Dr. Noriyoshi Sakai for teaching me cell culture experiments, Dr. Kiyoshi Naruse, Dr. Tetsuaki Kimura and Ms. Ikuyo Hara for genetic mapping of *Sdgc*, Dr. Tetsuya Sato, Dr. Yasuyuki Ohkawa and Dr. Mikita Suyama for RNAseq analysis, and Dr. Amaury Herpin and Manfred Schartl for the generation *DMY/dmrt1bY*-EGFP transgenic medaka. cDNAs and BAC used in this studies were provided by NBRP medaka. I am also grateful to people in Lab of Molecular Genetics for Reproduction, especially Dr. Yasuhiro Yamamoto and Ms. Ikuko Watakabe for supporting my experiments and Ms. Kinoshita and Mr. Suzuki for fish maintenance. Finally, I would like to give a special thank to Shuhei Nakamura, the previous lab member, for teaching me basic techniques essential for this study.

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**Figures 1-30**

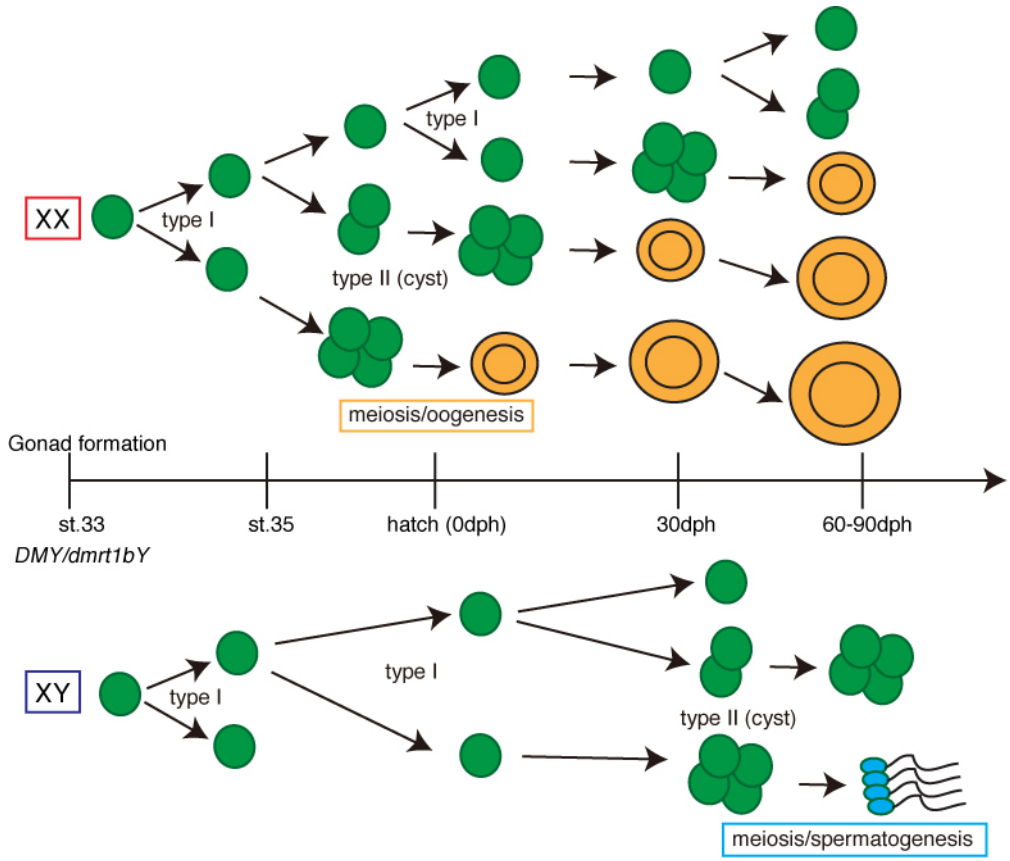



**Figure 1. Schematic representation of gametogenesis during gonadal development in medaka.** In medaka, the gonad is formed at st.33. By st.35, both XX and XY germ cells undergo stem-type self-renewal division (type I). In the absence of *DMY/dmrt1bY*, immediately following st.35, a subset of XX germ cells initiates cystic division (type II), followed by meiosis and oogenesis near hatching stage. On the other hand, in the presence of *DMY/dmrt1bY*, XY germ cells continue to undergo type I division slowly after st.35. In XY germ cells, type II division and spermatogenesis occur more than 30 days after hatching (dph).

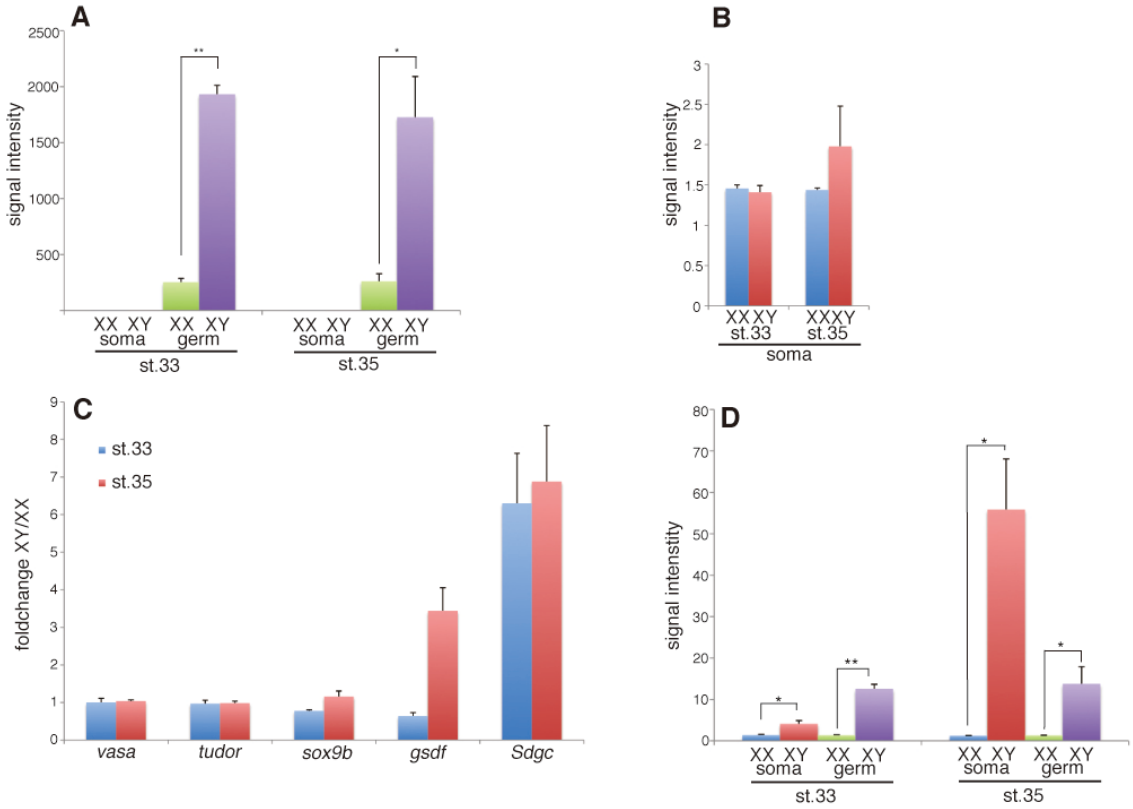



**Figure 2. *Sdgc* is enriched and *DMY/dmrt1Y* transcripts are detected in XY germ cells as determined by microarray analysis.** (**A**, **B** and **D**) microarray analysis (n=3) (**A**) Signal from the novel gene, *Sdgc*, is highly enriched in XY germ cells as compared to XX germ cells at both st.33 and st.35. (**B**) In the gonadal somatic cells, the signal intensity of *Sdgc* is extremely low, and no sexual difference is present. (**C**) qPCR analysis of *Sdgc* and gonadal marker expression during gonad formation. The y-axis indicates the fold change of expression levels in XY compared to XX embryos. The expression levels were normalized to *β-actin* (n=3). (**D**) *DMY/dmrt1bY* is detected in XY germ cells at st.33. By the time the gonad forms as bilateral structures at st.35, *DMY/dmrt1Y* expression is abundant in XY gonadal somatic cells but still persists in germ cells. The y-axis indicates the raw signal intensity of microarray data. *$p<0.05$, **$p<0.01$, Student's *t*-test, n = 3. Values are expressed as the mean ± s.e.m.

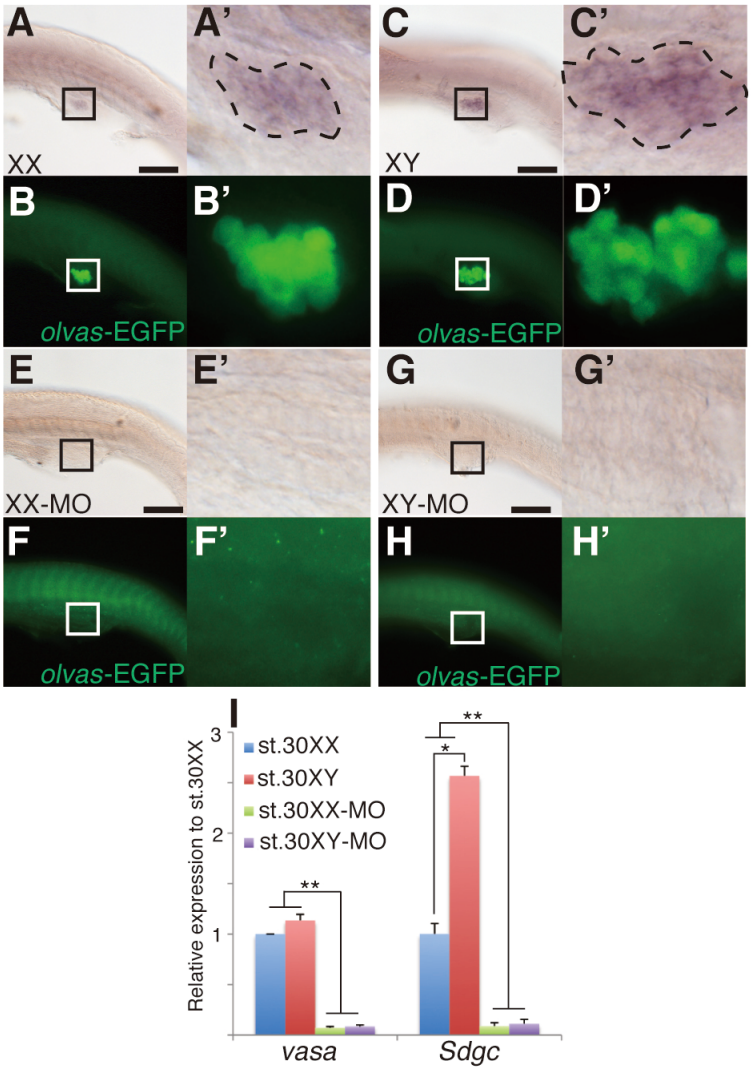



**Figure 3. *Sdgc* is expressed specifically in germ cells and upregulated depending on Y chromosome number in germ cells.** (**A** to **D**) Expression analysis of *Sdgc* by *in situ* hybridization and immunohistochemistry with anti-EGFP using *olvas*-EGFP transgenic embryos at st.30. Purple signals (A' and C', dotted lines) are observed in clusters of germ cells (B' and D', *olvas*-EGFP: green) lateral to developing hindgut. (**E** to **H**) When germ cells were ablated by morpholinos, the *Sdgc* signal disappears (E, G) along with the germ cell marker (F, H). Bars represent 100 µm. (**I**) qPCR analysis of *Sdgc* using embryos with and without (MO) germ cells at st.30. The y–axis indicates the expression levels relative to those observed in st.30 XX control embryos. The expression levels were normalized to *β-actin* (n = 2). $*p < 0.05$, $**p < 0.01$, Student's *t*–test. Values are expressed as the mean ± s.e.m.

```
        10        20        30        40        50        60
GAGAAAGCGGCAAAGCAGCAACAAGTGACAACCCGAAGCAGCATCGTTGAGCAAAGATGC
    scaffold804: 41833-41858                scaffold2884: 11492-11521    M  Q
    scaffold4140: 5809-5834

        70        80        90       100       110       120
AAAACCAACCCGAGCAGGGGCCGCCGGCCCAGGGGCGGCAGGGGCCGCCGGCCCAGGGGC
  N  Q  P  E  Q  G  P  P  A  Q  G  R  Q  G  P  P  A  Q  G  R

       130       140       150       160       170       180
GGCAGGGGCCGCCGGCCCAGGGGCGGCAGGGGCCGCCGGCCCAGGGGCGGCAGGGGCCGC
  Q  G  P  P  A  Q  G  R  Q  G  P  P  A  Q  G  R  Q  G  P  P

       190       200       210       220       230       240
CGGCCCAGGGGCGGCAGGGGCCGCCGGCCCAGGGGCGGCAGGGGCCGCCGGCCGAGGGGC
  A  Q  G  R  Q  G  P  P  A  Q  G  R  Q  G  P  P  A  E  G  R

       250       260       270       280       290       300
GGCAGGGGCCGCCGGCCCAGGGGCGGCAGGGGCCGCCGGCCCAGGGGCGGCAGGGGCCGC
  Q  G  P  P  A  Q  G  R  Q  G  P  P  A  Q  G  R  Q  G  P  P

       310       320       330       340       350       360
CGGCCCAGGGGCGGCAGGGGCCGCCGGCCCAGGGGCGGCCACTTGTTTGGCACAGTGCTG
  A  Q  G  R  Q  G  P  P  A  Q  G  R  P  L  V  W  H  S  A  V

       370       380       390       400       410       420
TCAACAGAGCTCGTGAAGAACAGCGTAAGGCTTCCATGAATAAAGTTATCGTTCGGAGGC
  N  R  A  R  E  E  Q  R  K  A  S  M  N  K  V  I  V  R  R  L

       430       440       450       460       470       480
TGTTGCCCCTCGCCACCTCGGAGGAGGAGCGGGCCCGCCTCATCATGGTTTATGGGCCCC
  L  P  L  A  T  S  E  E  E  R  A  R  L  I  M  V  Y  G  P  P

       490       500       510       520       530
CATAAAAAAATAAAAGAAATATTTGTATAAGGTCATGTTCTGTGACAAAAGTTGAATG
```

**Figure 4. Structure and characterization of *Sdgc* cDNA.** Nucleotide sequence and deduced amino acid sequence of *Sdgc*. Putative initiation ATG and stop TAA codons are boxed. Polypeptide repeats (QGPPAQ(E)GR) are underlined. The 5'UTR regions mapped on medaka genome are double underlined.

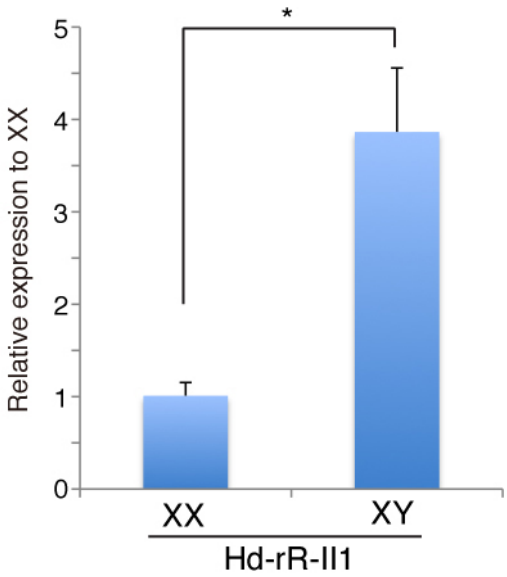



**Figure 5. qPCR analysis of *Sdgc* in Hd-rR-II1 strain.** The expression level of *Sdgc* is higher in XY compared to XX embryos at st.30. The expression levels were normalized to *olvas* (n=2). *$p$<0.01, Student's *t*-test. Values are expressed as the mean ± s.e.m.

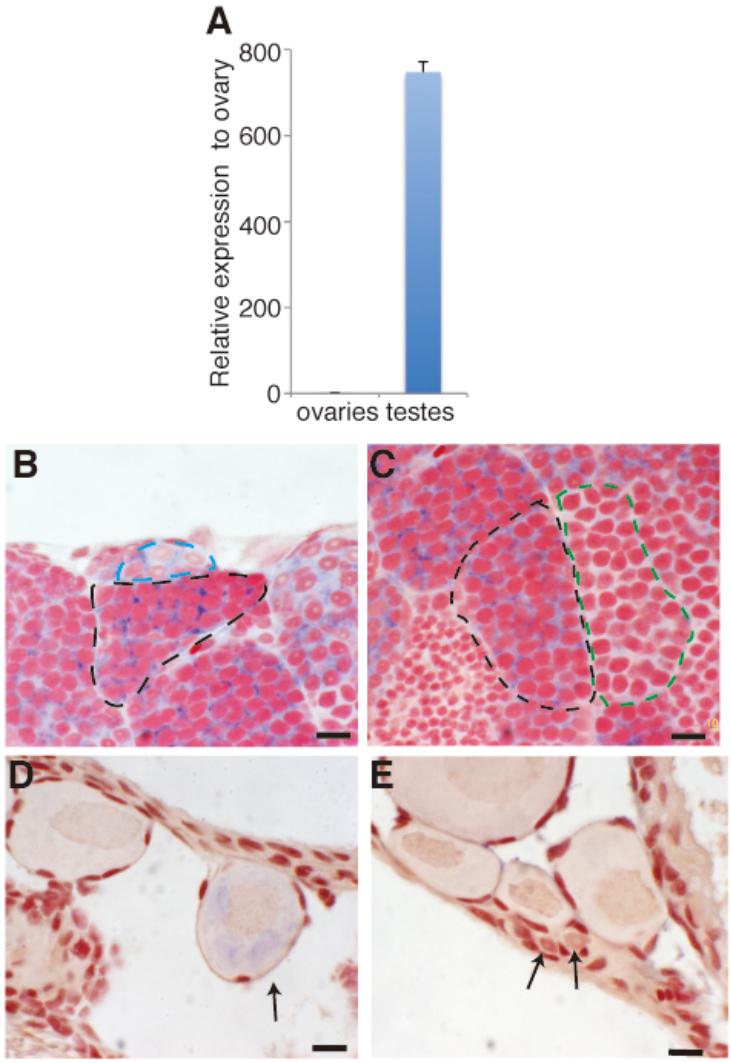



**Figure 6. Expression of *Sdgc* in adult gonads.** (**A**) *Sdgc* is highly enriched in testes as compared to ovaries. The y-axis indicates the expression levels of *Sdgc* in testes relative to ovaries normalized by *olvas* expression (n = 3). Values are expressed as the mean ± s.e.m. (**B**) *Sdgc* expression is detected in type A (blue dotted line) and type B spermatogonia (black dotted line) but not in spermatocytes (**C**, green dotted line). (**D**) In ovaries, *Sdgc* is weakly detected in the early stage of oocytes (arrow). (**E**) *Sdgc* signal is not detected in oogonia (arrows). Bars represent 10μm.

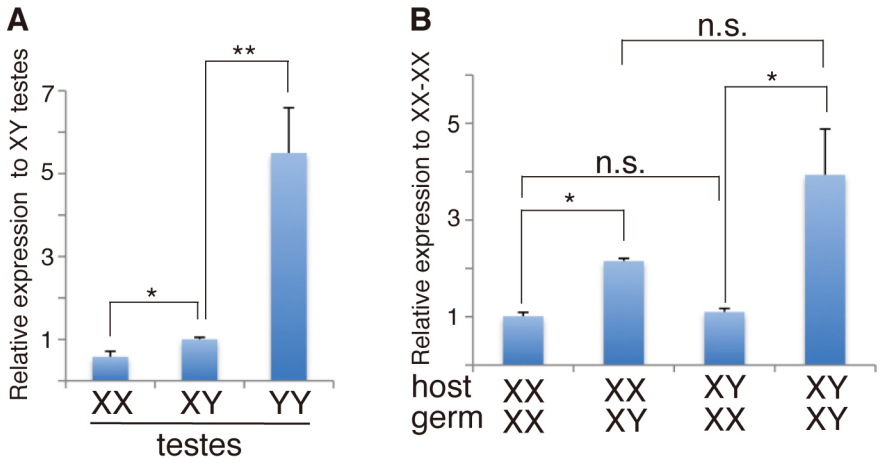



**Figure 7. *Sdgc* is upregulated depending on Y chromosome number in germ cells.** (**A**) *Sdgc* expression levels increased in parallel with the number of Y chromosomes in testes. The y–axis indicates the expression levels of *Sdgc* in XX and YY testes relative to those observed in XY testes; signals were normalized to *olvas* (n = 3). (**B**) qPCR analysis of chimeric embryos at st.30. The y–axis indicates the expression levels relative to those observed in XX embryos with XX germ cells (n = 2). Note that the expression levels of *Sdgc* are not affected significantly by the sex of host. *$p < 0.05$, **$p < 0.01$, Student's *t*–test. Values are expressed as the mean ± s.e.m.

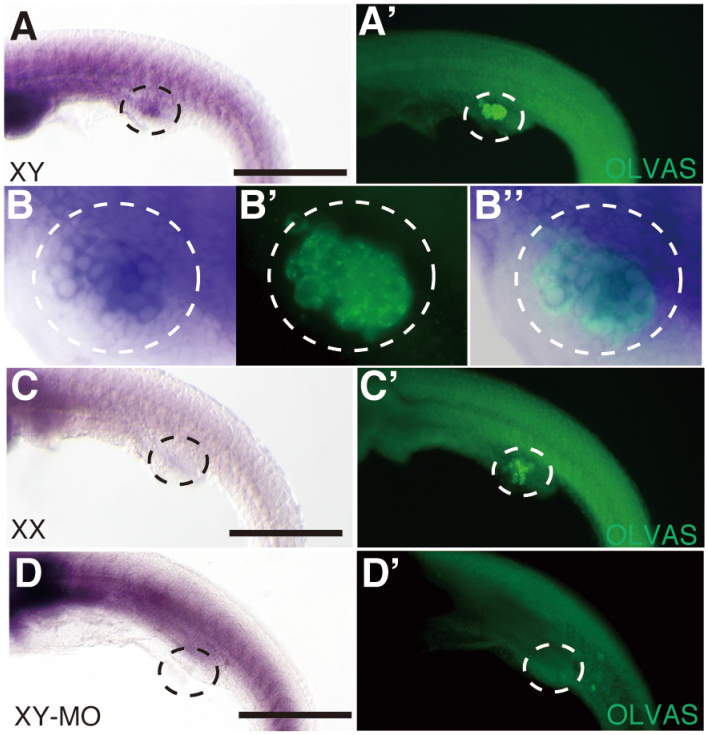



**Figure 8. *DMY/dmrt1bY* is expressed in germ cells before formation of the gonadal primordium.** (**A** to **D**) Lateral views of embryos at st.30. The anterior portion is on the left. (**A**) *in situ* hybridization for *DMY/dmrt1bY* and immunohistochemistry to detect OLVAS expression using XY embryos at st.30. *DMY/dmrt1bY* signal (purple; dotted line) is detected at the lateral sides of the intestine where germ cells are located at st.30. (**B**) The *DMY/dmrt1bY* signal co–localizes with the germ cells marker, OLVAS (green; dotted line). (**C**) The *DMY/dmrt1bY* signal is not detected in the OLVAS-positive germ cells (green; dotted line) of XX embryos. (**D**) When germ cells are ablated by the morpholinos (XY–MO), the *DMY/dmrt1bY* signal disappears. Bars represent 200μm.

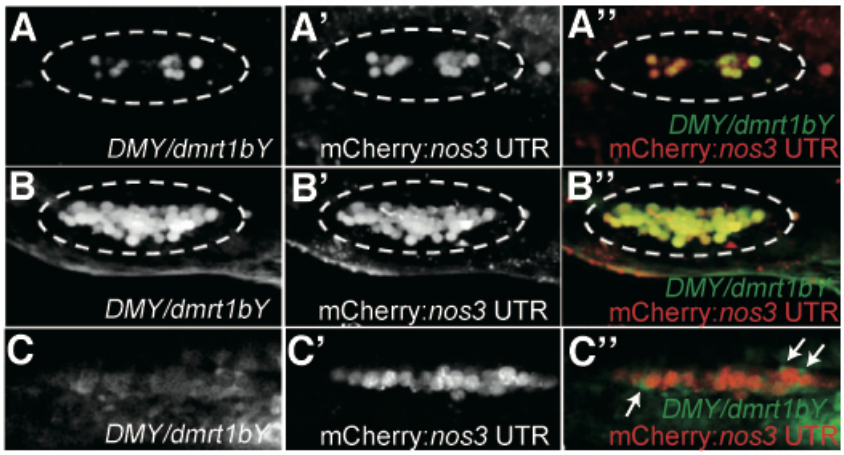



**Figure 9. Detection of *DMY/dmrt1bY* expression in germ cells of *DMY/dmrt1bY*-EGFP reporter transgenic medaka.** (**A**) *DMY/dmrt1bY* expression is first detected in clustered germ cells located within the lateral plate mesoderm at st.28. Expression is co-localized with the fluorescence of the germ cell-specific marker (mCherry:*nos3*-3'UTR). (**B**) Germ cell-specific expression of *DMY/dmrt1bY* is observed until st.30–31. (**C**) By st.33, while germ cell expression becomes weaker, additional *DMY/dmrt1bY* expression is detected in the somatic cells directly surrounding the germ cells (arrows).

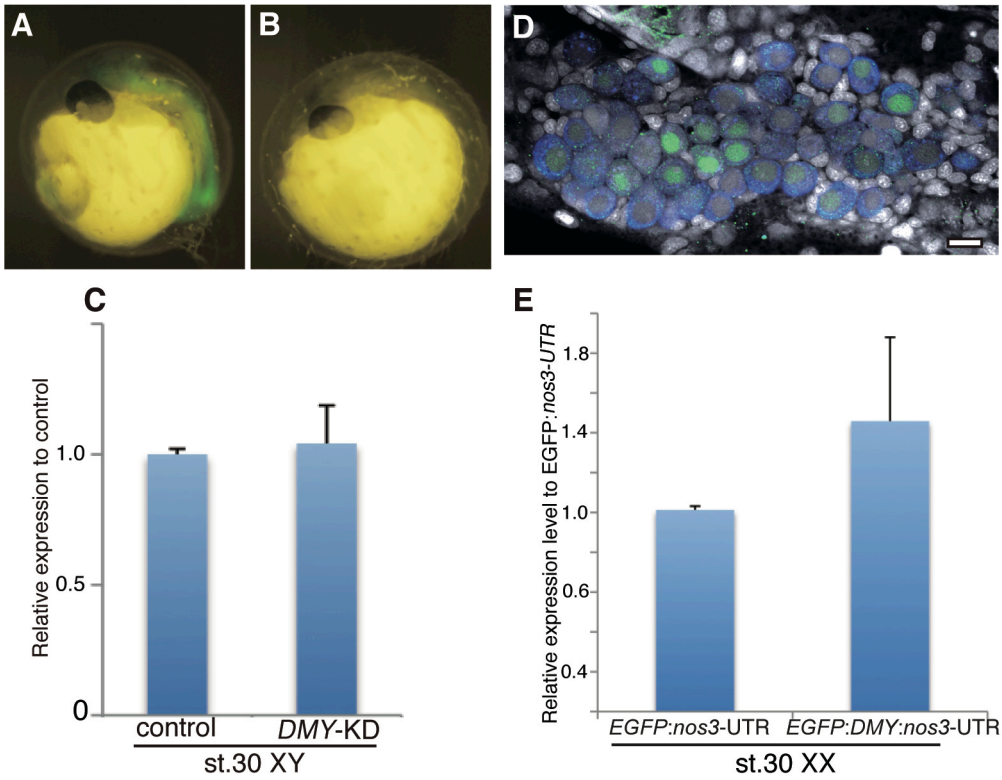
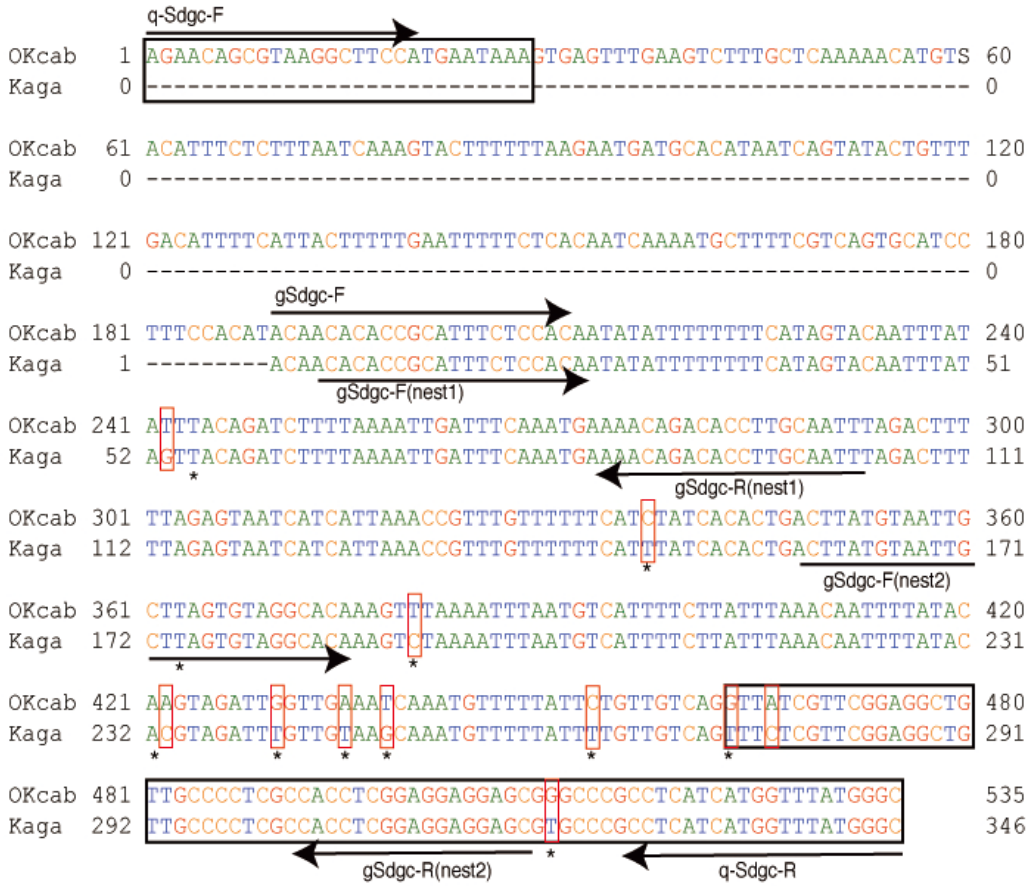



**Figure 10. Knock-down or overexpression of *DMY/dmrt1bY* in germ cells did not alter the expression level of *Sdgc*.** (**A** and **B**) Injection of gripNA targeting *DMY/dmrt1bY* (*dmy*-gripNA) into *DMY/dmrt1bY*-EGFP embryos. (**A**) Uninjected controls show EGFP fluorescence in a whole body (EGFP–positive: n = 27, EGFP–negative: n = 0). (**B**) EGFP fluorescence level is reduced by *dmy*-gripNA injection (EGFP–positive: n=3, EGFP–negative: n = 31) (**C**) qPCR analysis of XY embryos injected with *dmy*-gripNA (*DMY*-KD, n=3). The expression level of *Sdgc* does not differ between *dmy*-gripNA injected embryos and control embryos. (**D**) Immunohistochemistry of EGFP and DMY fusion protein (EGFP:DMY) using *EGFP*:*DMY*:*nos3*-3'UTR injected embryos at st.33. Note that EGFP:DMY signal (green) was localized to the nuclei of germ cells (blue). Bar represents 10 µm. (**E**) qPCR analysis of embryos injected with *EGFP*:*nos3*-3'UTR (control) and *EGFP*:*DMY*:*nos3*-3'UTR. The expression of *Sdgc* was not altered by *DMY/dmrt1bY* expression in XX germ cells (n=3). Values are expressed as the mean ± s.e.m.




| position | 55 | 148 | 174 | 191 | 232 | 241 | 246 | 249 | 264 | 274 | 321 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Kaga: XX | T/T | T/T | T/T | C/C | A/A | T/T | T/T | G/G | T/T | T/T | T/T |
| Kaga: XY | T/A | T/C | T/G | C/T | A/G | T/A | T/A | G/T | T/C | T/G | T/G |

**Figure 11. Identification of SNPs in the genomic region of *Sdgc* and primer sets for linkage analysis.** 14 SNPs (red boxes and asterisks) were identified between OKcab and Kaga strains when the genomic amplicon was sequenced with gSdgc-F and q-Sdgc-R primers. The alignment is based on the sequence of XX/XY OKcab and XX Kaga. Asterisks indicate the heterozygous SNPs found in XY Kaga (see the table below). The position of the heterozygous SNPs in the table is based on the Kaga sequence. The purpose of each primer is indicated in Table 1. Exons are boxed (black). For linkage analysis, PCR products were amplified by gSdgc-F and q-Sdgc-R primers, and then used as templates for nested PCR. Next, two PCR products amplified by nested primers (nest1 and nest2) were genotyped via High-Resolution Melting analysis.

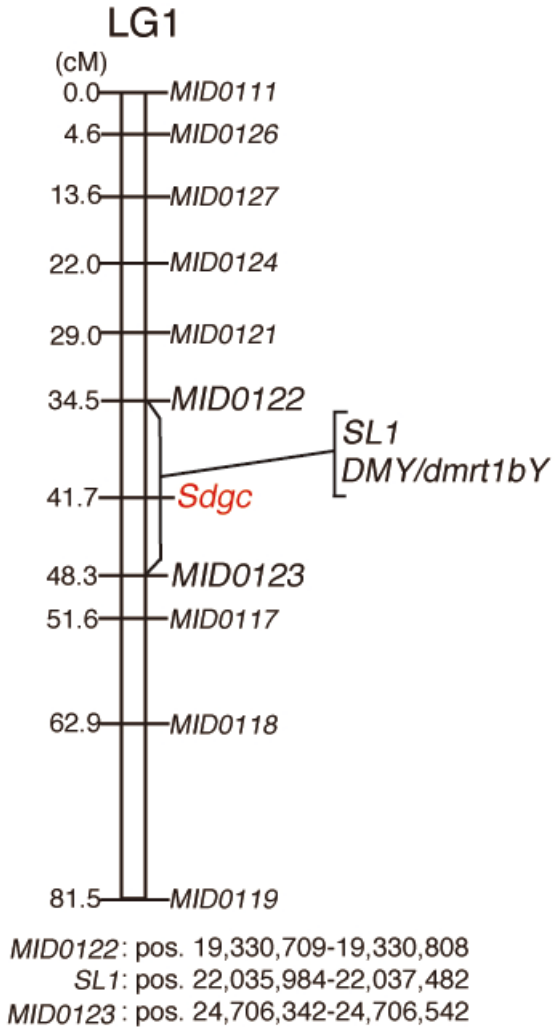



**Figure 12. Linkage analysis of *Sdgc*.** *Sdgc* was mapped between *MID0122* and *MID0123* on LG1, which are the sex chromosomes (X and Y) in medaka. The physical map revealed that the sex-linked marker, *SL1*, which closely maps to *DMY*/*dmrt1bY*, is also located between the same *MID* markers (see the position (pos.) below). Thus, *Sdgc* resides near the *DMY*/*dmrt1bY* locus on the sex chromosomes. Note that *DMY*/*dmrt1bY* and *SL1* are not used in the present linkage analysis and the order of *Sdgc*, *SL1,* and *DMY*/*dmrt1bY* is not determined.

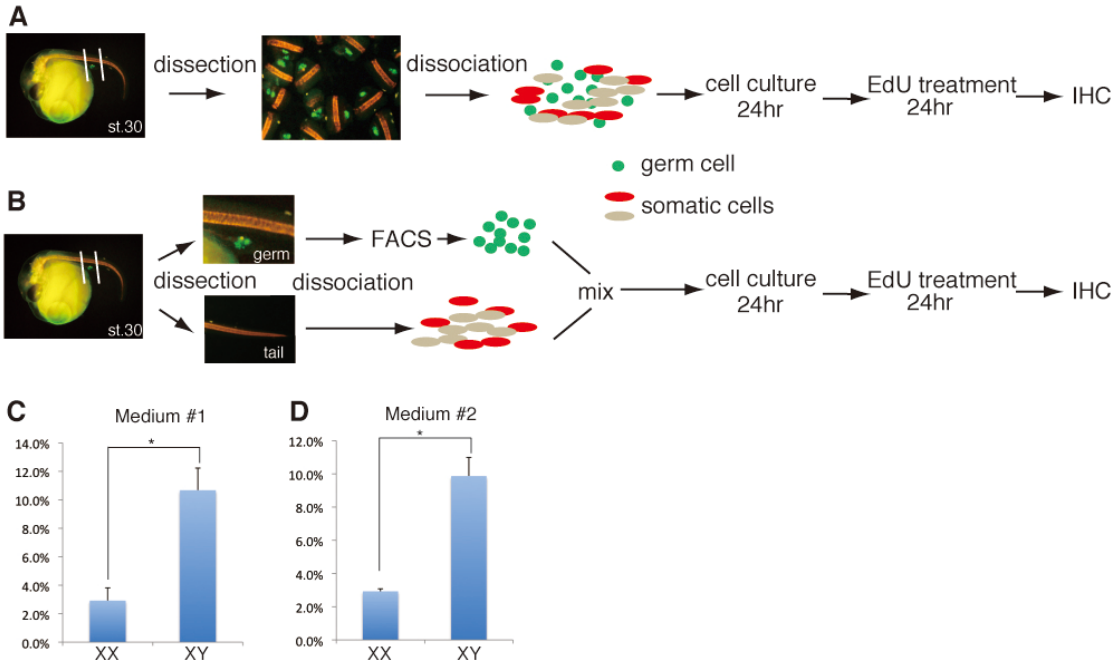



**Figure 13. Sexually different germ cell mitotic activity in XX and XY germ cells *in vitro*.** (**A**) Schematic representation of the *in vitro* culture experiment. (**B**) Experimental procedure of the *in vitro* culture using FACS isolated germ cells. (**C** and **D**) The EdU incorporation rate of germ cells cultured in Medium #1 (C, n = 4) or Medium #2 (D, n = 3). See Table 2 for the components of each medium. *$p < 0.05$, Student's *t*-test. Values are expressed as the mean ± s.e.m.

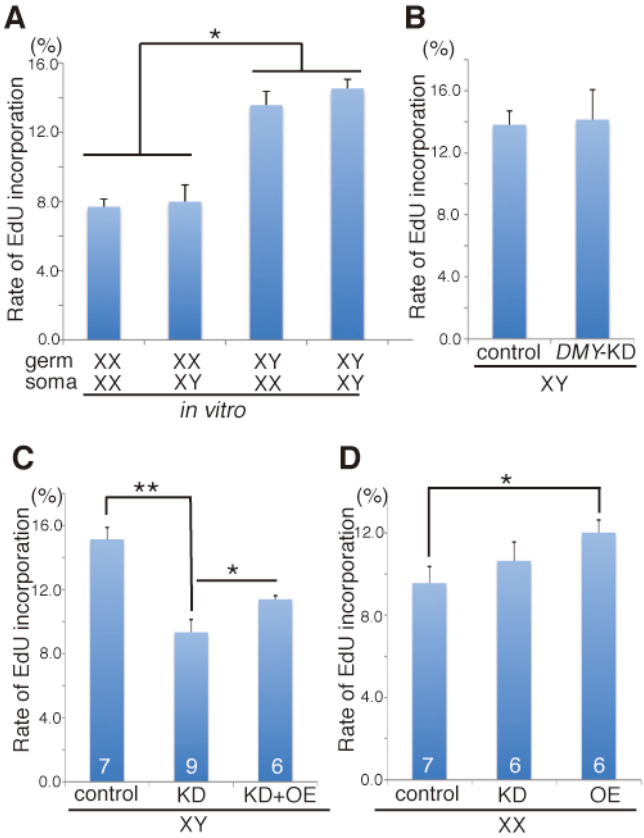



**Figure 14. Sexually different mitotic activity observed in cultured germ cells.** (**A**) An *in vitro* analysis of EdU–positive germ cells reveals that XY germ cells incorporate EdU at a higher rate as compared to XX germ cells, irrespective of the sex of the somatic cells (Germ–Soma: XX–XX vs XY–XX: $p$ = 0.00286, XX–XY vs. XY–XX: $p$ = 0.00404, XX–XX vs. XX–XY: $p$ = 0.794, XY–XX vs. XY–XY: $p$ = 0.369, Student's $t$–test, n = 3). (**B**) Knockdown of *DMY*/*dmrt1bY* by injection of 0.5 mM *dmy*-gripNA (*DMY*-KD) does not affect the mitotic activity of germ cells *in vitro*. As a control, 0.1 mM human-CREB gripNA was injected. n=4 (**C** and **D**) Knockdown of *Sdgc* by 0.25 mM *Sdgc*-gripNA (KD) significantly decreased EdU incorporation in XY germ cells (C) but not in XX germ cells (D). Overexpression of *Sdgc* by injecting 200ng/µl mRNA of *FLAG*:*Sdgc*:*2A*:*mCherry*:*olvas*-3'UTR (the expression of *Sdgc* can be localized in germ cells, OE) in XX germ cells (D) and the rescue experiment (KD+OE) by injecting both *Sdgc*-gripNA and the mRNA in XY germ cells (C) significantly increased the EdU incorporation. Each number in the bar indicates the number of culture dishes examined. Student's $t$–test, *$p$<0.05, **$p$<0.01. All values (%) are expressed as the mean ± s.e.m.

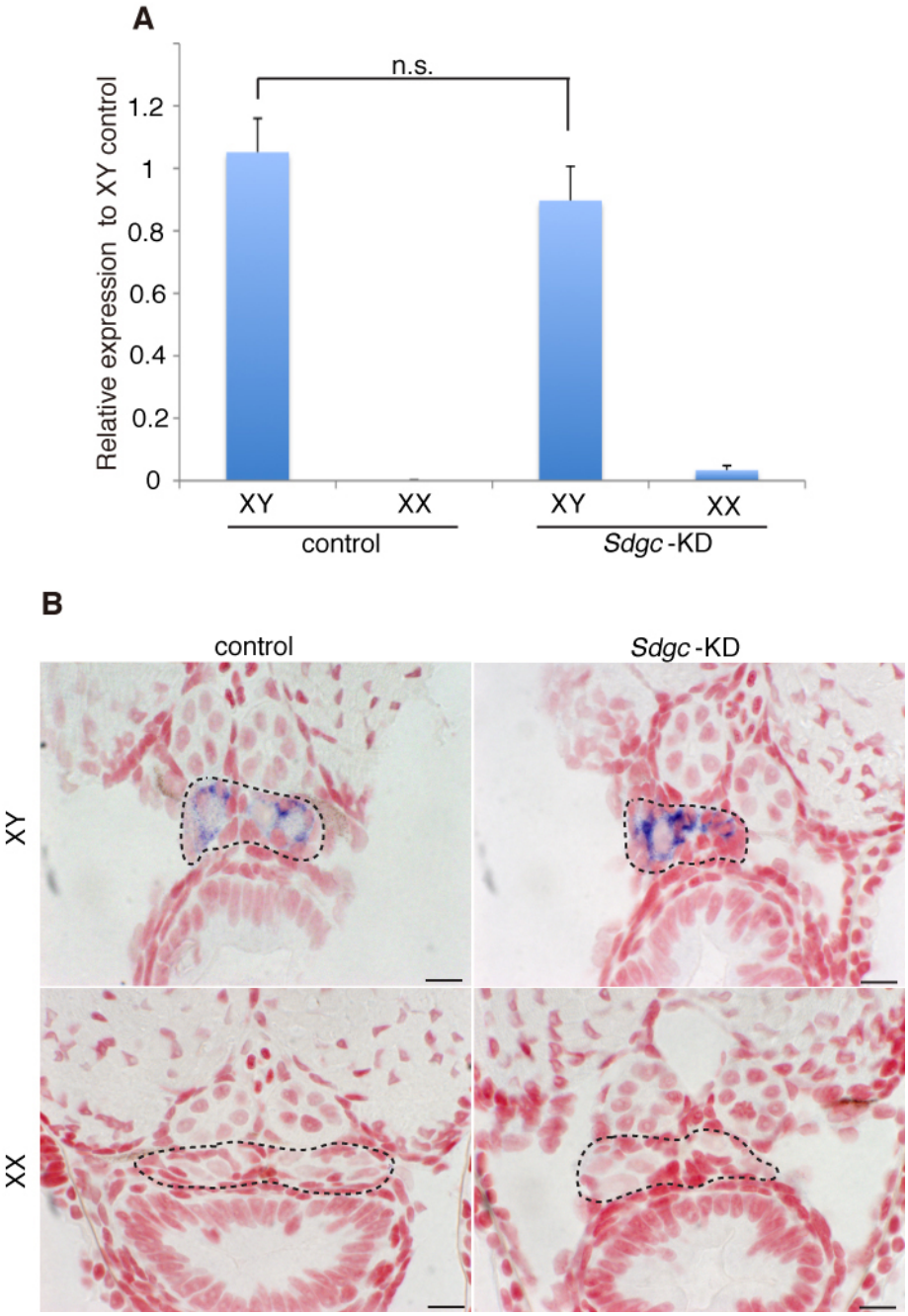



**Figure 15. Knockdown of *Sdgc* did not alter the expression of *DMY/dmrt1bY* during gonadal sex differentiation.** (**A**) qPCR analysis of *DMY/dmrt1bY* using *Sdgc*-knockdown (Sdgc-KD) XY embryos at st.35. The expression level of *DMY/dmrt1bY* did not differ significantly from that of control XY embryos. The expression levels were normalized to *β-actin* (n=3). Values are expressed as the mean ± s.e.m. (**B**) *DMY/dmrt1bY* expression was normally detected in gonadal somatic cells of *Sdgc*-knockdown XY embryos at st.35 by *in situ* hybridization (purple signals in dotted line). Bars represent 10μm.

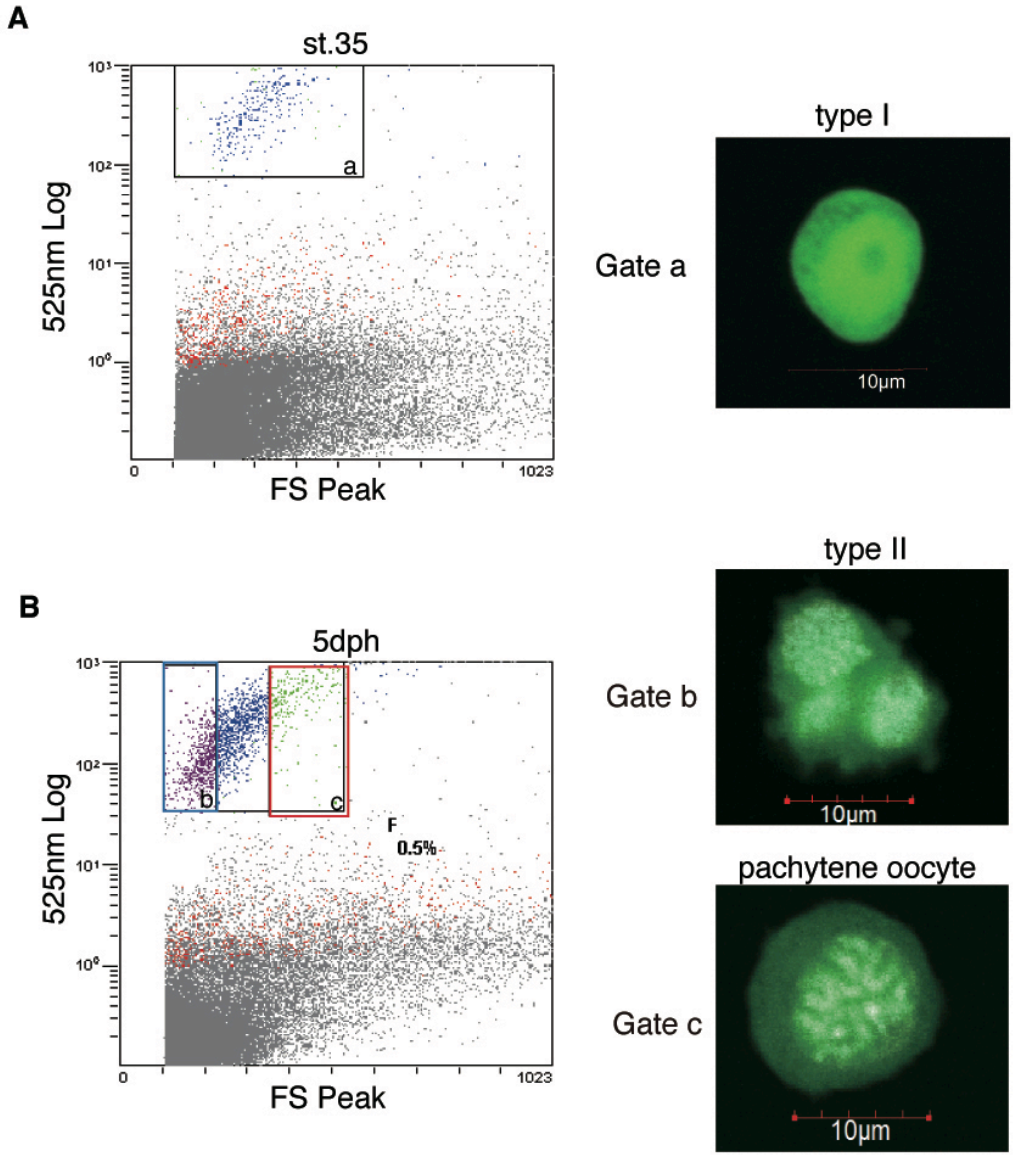



**Figure 16. Isolation of type I and type II germ cells and pachytene oocytes by a fluorescence-activated cell sorter (FACS).** (**A**) Isolation of type I germ cells from st.35 embryos. Type I germ cells were isolated from the EGFP positive fraction (gate a, black box). (**B**) Isolation of type II germ cells and pachytene oocytes from 5 dph larvae. Type II germ cells were enriched in the small cell-size population (Gate b, blue box) of the EGFP-positive fraction (black box). Pachytene oocytes were enriched in the large cell-size population (Gate c, red box) of the EGFP-positive fraction. The x-axis indicates forward scatter light intensity. The y-axis indicates EGFP fluorescence intensity.

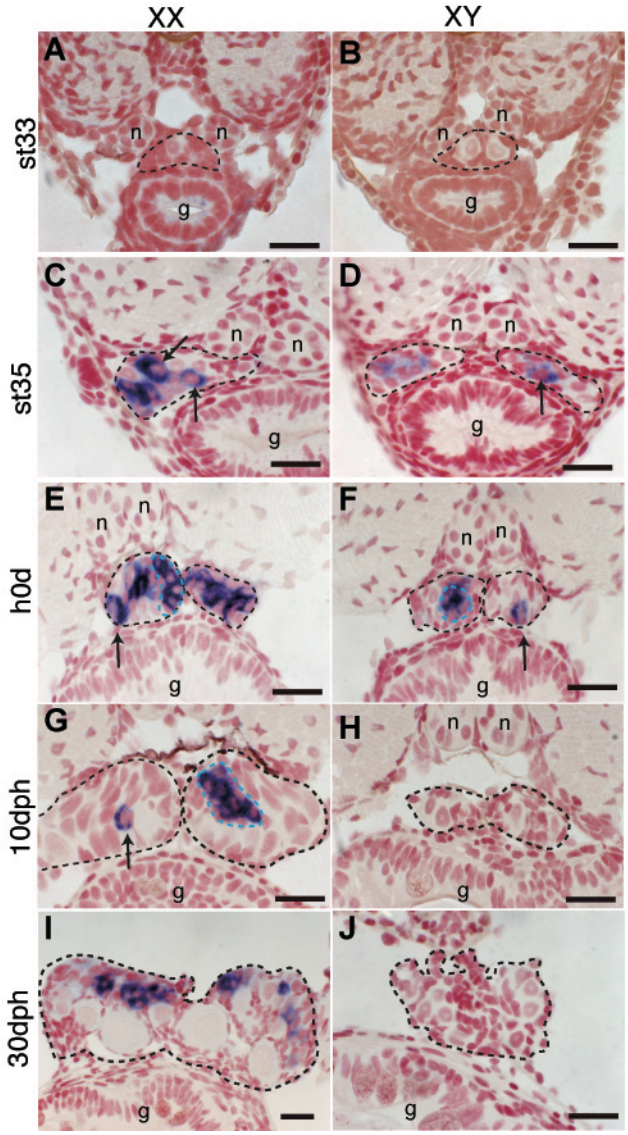



**Figure 17. *SDiG* expression analysis by *in situ* hybridization during gonadal development.** Cross-sections of the gonadal region. The gonad is encircled by black dotted lines. (**A** and **B**) No *SDiG* signal is detected in either XX or XY gonads at st.33, the onset of gonadal formation. (**C** and **D**) *SDiG* signals are detected in a subset of germ cells (arrows) in both XX and XY at st.35. (**E** and **D**) Signals were detected in single isolated type I germ cells (arrows) and cystic type II germ cells (blue dotted lines) in both XX and XY embryos at the hatching stage (h0d). (**G** to **J)**, Whereas *SDiG* signals disappear by 10 dph in XY gonads (H and J), they are continuously detected in germ cells afterwards in XX gonads (G and I). Scale bars: 20 µm. (n) nephric duct. (g) hindgut.

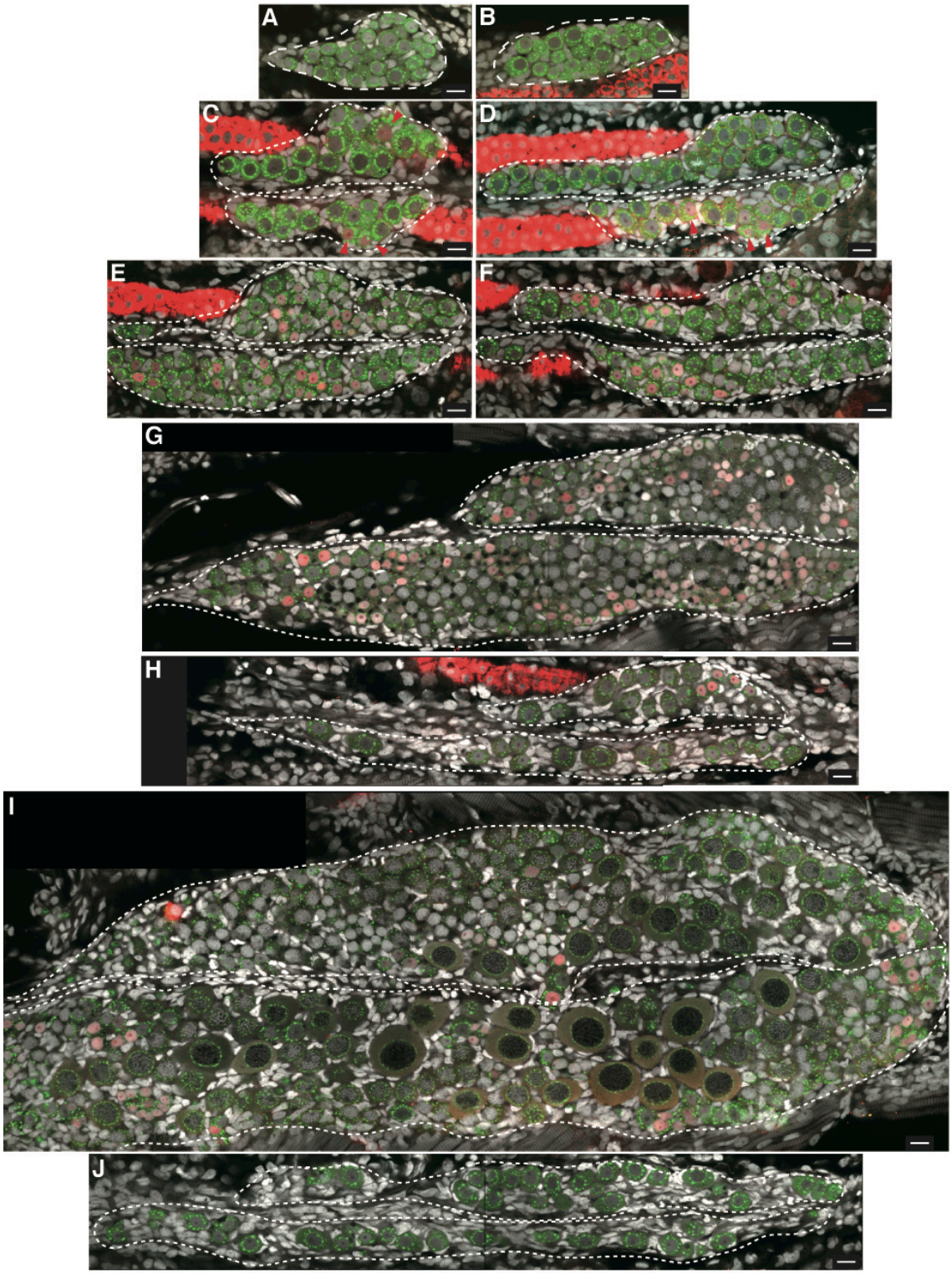

A
B
C
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**Figure 18. SDiG expression by immunohistochemistry during gonadal development.** Ventral view of gonads (dotted lines). Germ cells (green) are detected by OLVAS expression. (**A** and **B**) SDiG protein is not detected in either XX (A) or XY (B) gonads at st.33. (**C** and **D**) SDiG signals (red) start to be detected in nuclei of germ cells (red arrowheads) at st.35 in both XX (C) and XY (D). (**E**, **G** and **I**) In XX gonads, SDiG-positive germ cells are continuously detected from h0d (E) onwards (5dph in G and 10dph in I). (**F**, **H** and **J**) In XY gonads, SDiG-positive germ cells are present at h0d (F) and 5 dph (H) but disappear by 10 dph (J), when only type I germ cells are present. Note that non-specific signals in nephric ducts (strong red behind the gonads) are detected by the SDiG-antibody. Scale bars: 10 μm.

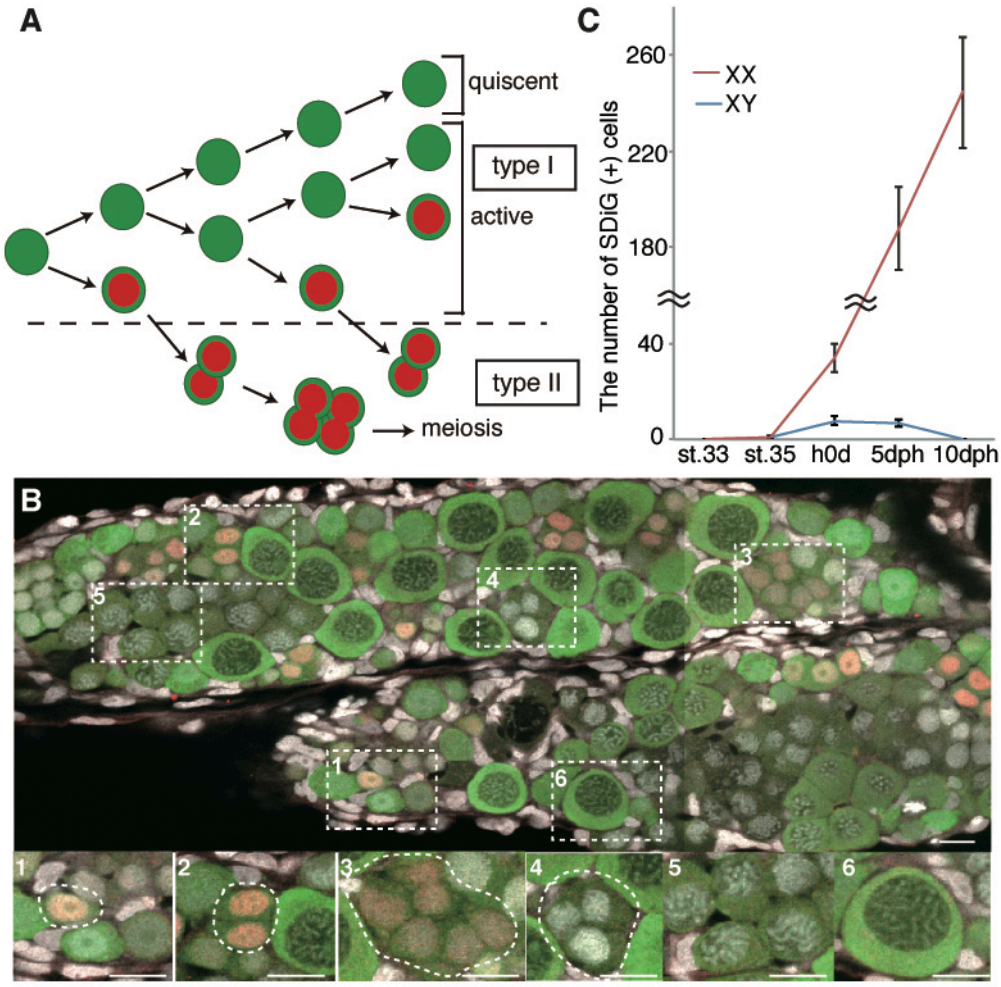


**Figure 19. Dimorphic expression of SDiG during gonadal development.** (**A**) Schematic representation of type I and type II division and expression of SDiG protein (red) in germ cells (green). 'Active' and 'quiescent' indicate mitotic states. (**B**) Ventral view of the gonad, observed by a confocal laser microscopy. SDiG expression (red) in XX gonad at 7 dph. SDiG signals are detected in a subset of type I (**1**) and all mitotic type II (**2**: 2-cell cyst, **3**: 8-cell cyst) germ cells, but not in meiotic germ cells (**4**: zygotene, **5**: pachytene) or diplotene oocytes (**6**). Scale bars: 10 µm. (**C**) The number of SDiG-positive cells during gonadal development in XX and XY gonads. St.33 XX: n = 8; XY: n = 8. st.35 XX: n = 15; XY: n = 15; h0d (hatching stage) XX: n = 19; XY: n = 20. 5 dph XX: n = 13, XY: n = 13. 10 dph XX: n = 12; XY: n = 13. Values are expressed as means ± s.e.m.

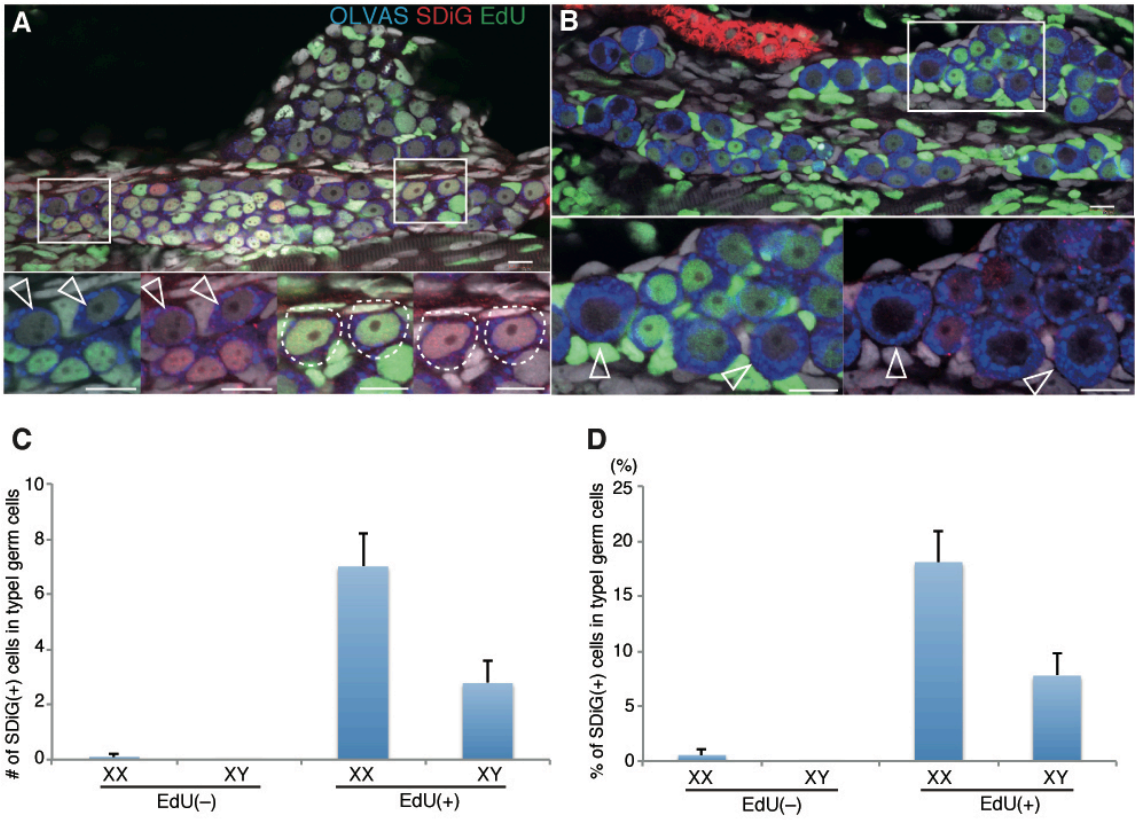


**Figure 20. SDiG is expressed in mitotically active type I germ cells.** Long-term EdU treatment from st.35 for 72 hr until hatching (h0d). (**A**) XX gonad at h0d. Insets show EdU (green) and SDiG (red) double-positive type I (right, dotted lines) and SDiG double-negative type I (left, arrowheads) germ cells. (**B**) XY gonad at h0d. The inset shows EdU and SDiG double-negative germ cells (arrowheads). (**C** and **D**) The number (C) and rate (D) of SDiG-positive cells in mitotically quiescent [EdU(–)] and active [EdU(+)] type I germ cells at h0d. XX: n = 10, XY: n = 14. Scale bars: 10µm.

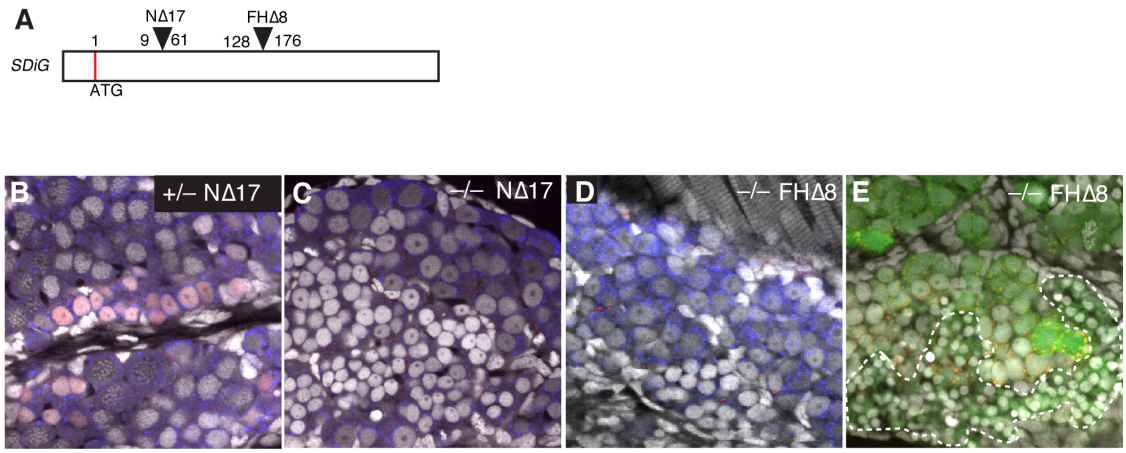


**Figure 21. Design of TALEN targeting *SDiG*.** (**A**) *SDiG* gene and target sites of TALENs (arrowheads). The numbers indicate the base positions starting at the adenine nucleotide of the initiation codon. Deletions of 17 bp (NΔ17) and 8 bp (FHΔ8) were obtained. (**B** and **D**) SDiG signals (red) are detected in $SDiG^{+/-}$ mutants (B), but not in $SDiG^{-/-}$ mutants for both NΔ17 (C) at 7 dph and FHΔ8 (D) at 20 dph. Blue cells are germ cells, detected by the presence of OLVAS. (**E**) Spermatogenesis (white dotted-line) is observed in $SDiG^{-/-}$ XX mutants for FHΔ8 at 20 dph. Green cells (*olvas*-EGFP) are germ cells. Scale bars: 10 µm.

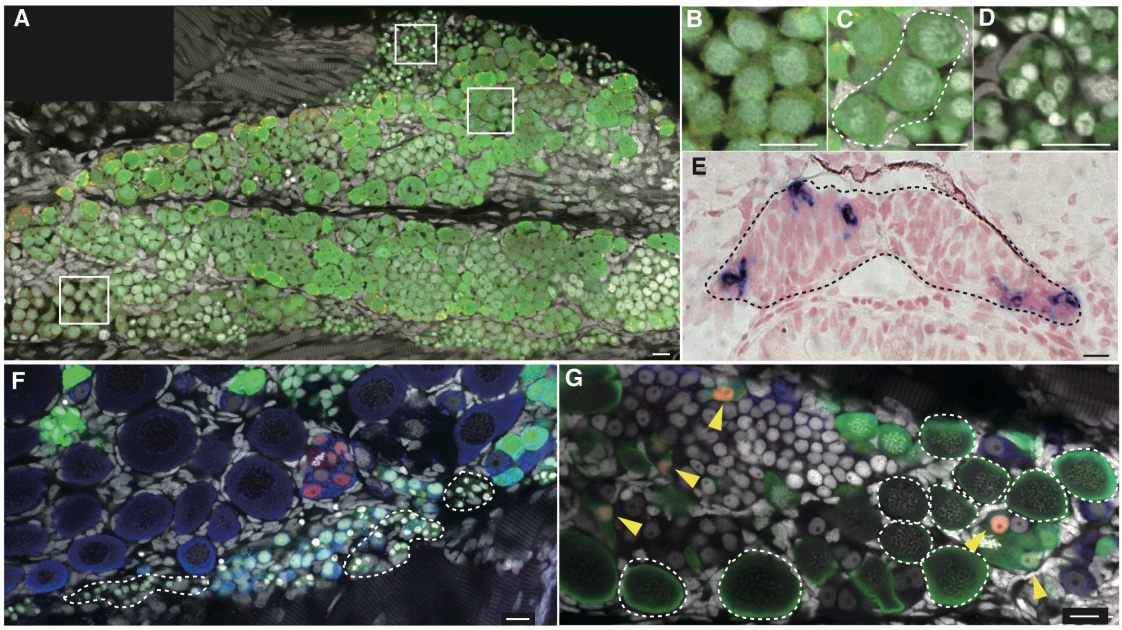


**Figure 22. *SDiG* is responsible for suppressing initiation of spermatogenesis in medaka germ cells.** (**A** to **D**) Ventral view of a *SDiG*$^{-/-}$ XX gonad at 7 dph. The white boxes are magnified in (**B** to **D**). Green cells (*olvas*-EGFP) represent germ cells. Meiotic germ cells at zygotene (B) and pachytene (C, dotted line) stages, and spermatid-like cells (D). Red dots in germ cells represent OLVAS protein. (**E**) Detection of *protamine* (purple signals) in *SDiG*$^{-/-}$ XX gonad at 10 dph. (**F**) Chimeric analysis reveals that EGFP-positive *SDiG*$^{-/-}$ germ cells progress spermatogenesis (dotted lines) in wild-type XX gonad at 20dph. (**G**) Introduction of *SDiG* transgene in *SDiG*$^{-/-}$ XX mutants rescues oocyte formation (dotted lines) at 15dph. EGFP-positive cells represent the transgene-integrated germ cells. The yellow arrowhead indicates SDiG protein (red signal) derived from the transgene. Blue cells in (**F**) and (**G**) are germ cells detected by OLVAS protein. Scale bars: 10μm. Scale bars: 10 μm.

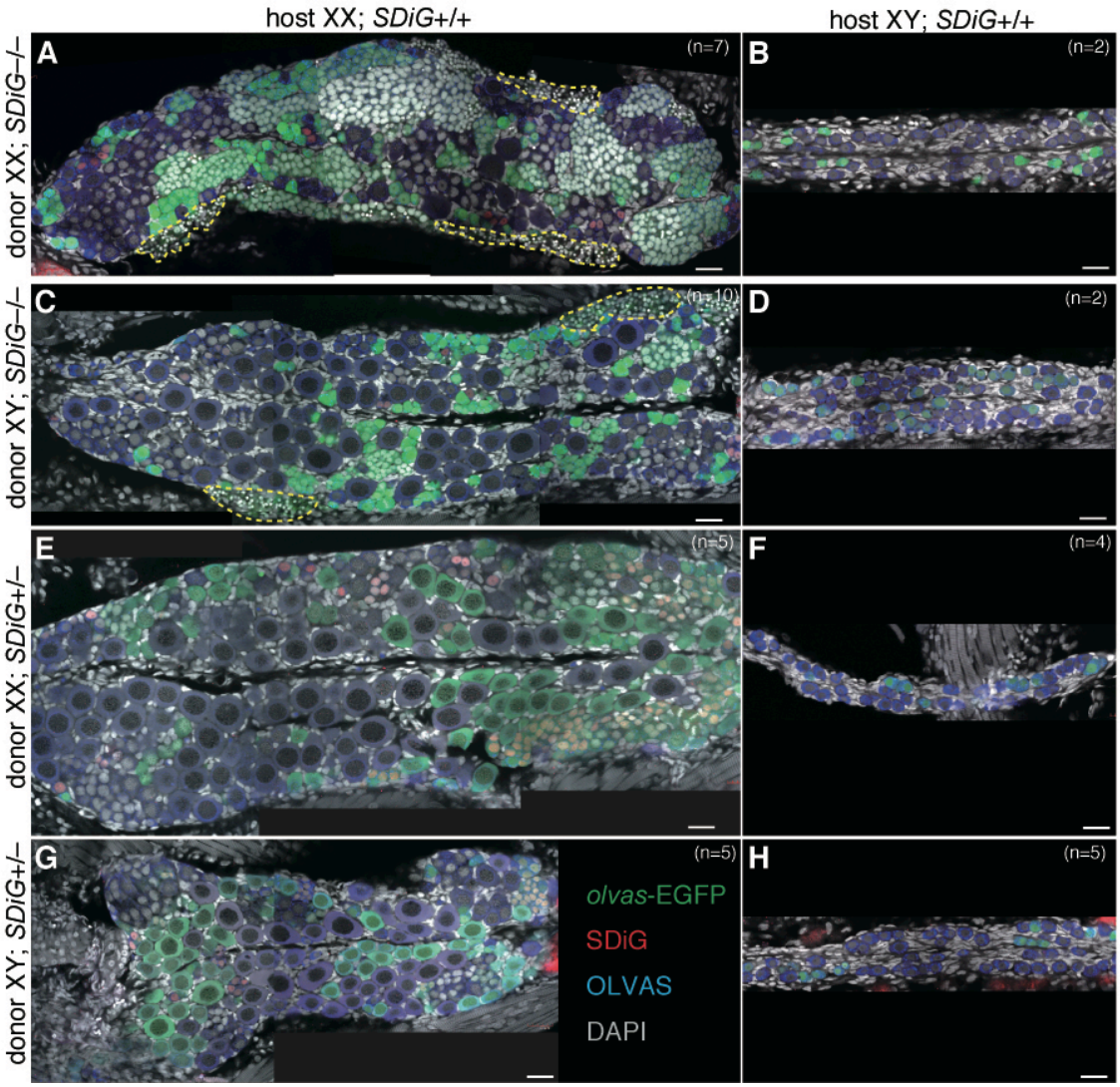


**Figure 23. *SDiG*$^{-/-}$ germ cells progress spermatogenesis in wild-type XX gonads.** Ventral view of chimeric gonads at 20 dph. *SDiG*$^{+/-}$ or $^{-/-}$ cells were transplanted into wild-type embryos at the blastula stage. Germ cells derived from donors expressed EGFP under control of the *olvas* promoter (green, *olvas*-EGFP). (**A** to **D**) *SDiG*$^{-/-}$ germ cells in XX (A and C) and XY (B and D) wild-type gonads at 20 dph. Whereas both XX (A) and XY (C) donor germ cells (green) undergo spermatogenesis (yellow dotted lines) in XX gonads, host germ cells (blue) undergo oogenesis. On the other hand, spermatogenesis does not initiate in XY gonads at this stage (B and D). (**E** to **H**) *SDiG*$^{+/-}$ germ cells in wild-type XX (E and G) and XY (F and H) gonads. Donor germ cells (green) behave in the same way as host germ cells (blue) do. Scale bars: 20μm.

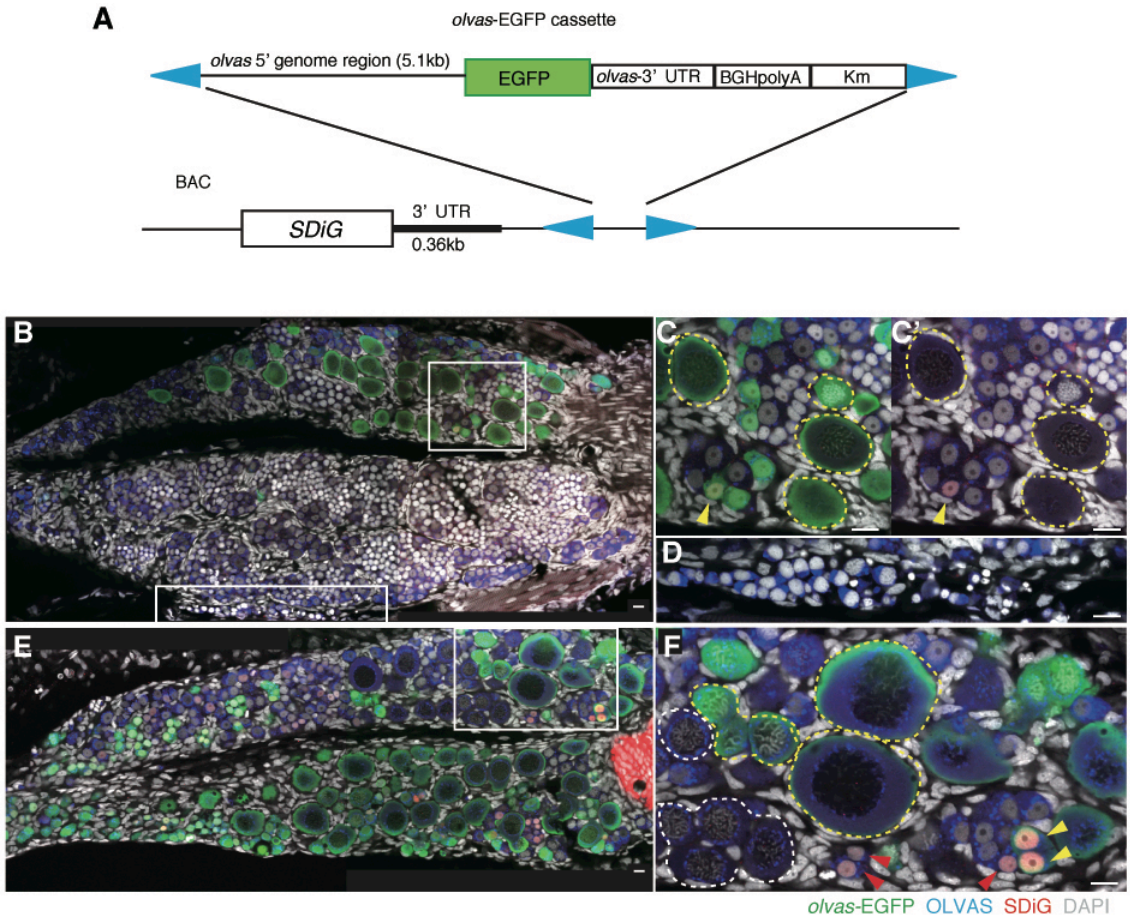


**Figure 24. *SDiG*-rescue experiments. (A)** *SDiG*-rescue construction. An *olvas*-EGFP cassette in which EGFP was expressed under the control of a 5.1 kb promoter and 3'UTR of *olvas* was inserted by homologous recombination into the BAC containing *SDiG*. The resultant BAC was injected into $SDiG^{+/-}$ or $SDiG^{-/-}$ embryos. In this system, germ cells that integrated the transgene are labeled with EGFP. (**B** to **D**) Oocyte formation (dotted lines) in $SDiG^{-/-}$ XX gonad with the rescue transgenes at 15 dph. The white boxes in (B) are magnified in (C) and (D). The arrowhead in (C) indicates SDiG protein derived from the transgene. EGFP-negative germ cells undergo spermatogenesis (D). (**E** and **F**) $SDiG^{+/-}$ XX gonad with rescue transgenes at 15 dph. The white box is magnified in (F). Both EGFP-positive (yellow-dotted lines) and -negative (white-dotted lines) oocytes are observed. Red and yellow arrowheads indicate SDiG protein in EGFP-negative and -positive germ cells, respectively. n = 2. Scale bars: 10 µm.

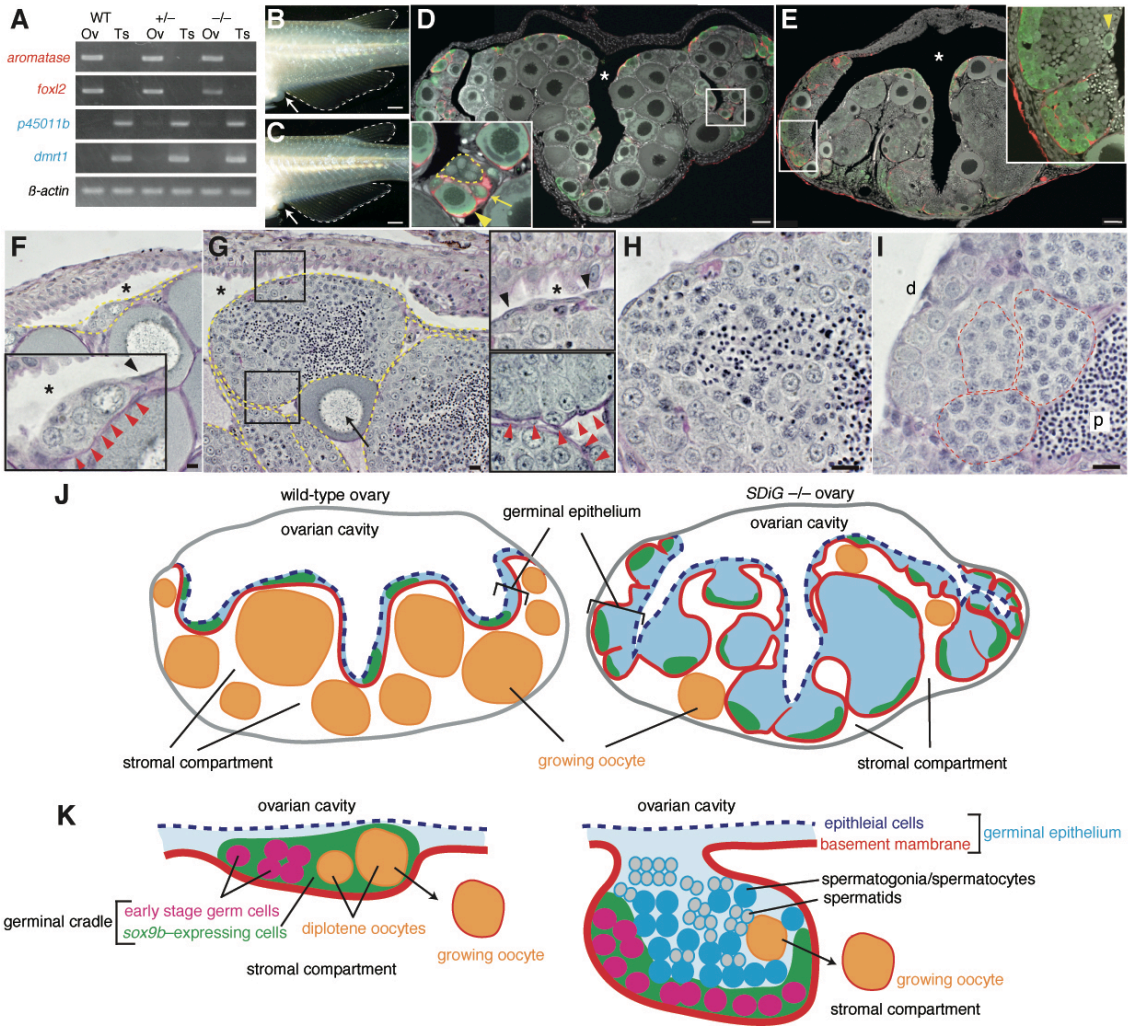
A
WT
+/–
–/–
Ov Ts Ov Ts Ov Ts
aromatase
foxl2
p45011b
dmrt1
ß-actin
B
C
D
E
F
G
H
I
d
p
J
wild-type ovary
ovarian cavity
germinal epithelium
SDiG –/– ovary
ovarian cavity
stromal compartment
growing oocyte
stromal compartment
K
ovarian cavity
germinal cradle
early stage germ cells
sox9b–expressing cells
diplotene oocytes
stromal compartment
growing oocyte
ovarian cavity
epithleial cells
basement mambrane
germinal epithelium
spermatogonia/spermatocytes
spermatids
growing oocyte
stromal compartment

**Figure 25. Progression of spermatogenesis in a female gonadal environment.** (**A**) RT-PCR analysis. Ov: ovary, Ts: testis. (**B** and **C**) Female secondary sex characteristics in XX wild-type (B) and *SDiG*$^{-/-}$ (C) adult fish, as characterized by the shape of dorsal/anal fins (dotted lines) and urogenital papilla (arrowheads). Scale bars: 1 mm. (**D** and **E**) Cross-sections of adult gonads with immunohistochemistry. (**D**) Wild-type ovary. The inset shows a germinal cradle, in which a germline stem cell (arrow), cystic germ cells (yellow dotted line), and a diplotene oocyte (arrowhead) are surrounded by *sox9b*-expressing cells (red). (**E**) *SDiG*$^{-/-}$ XX gonad. The inset shows expanded germinal epithelium in which *sox9b*-expressing cells (red) surround germ cells (green). The yellow arrowhead indicates an oocyte arising in between spermatogenic cells. Scale bars: 50 µm. (**F** to **I**) Cross sections of adult gonads subjected to PAS staining. (**F**) Wild-type ovary. The inset shows the germinal cradle between basement membrane (red arrowheads) and epithelial cells (black arrowhead) of the germinal epithelium (yellow dotted-lines). (**G**) *SDiG*$^{-/-}$ XX gonad. Spermatogenesis progresses within the expanded germinal epithelium (yellow dotted-lines) that resides between the basement membrane (lower-inset, red arrowheads) and epithelial cells (upper-inset, black arrowheads). The arrow indicates an oocyte that has exited from the germinal epithelium to the stromal compartment. Asterisks in (D to G) indicate ovarian cavities. (**H** to **I)** Spermatogenesis proceeds as units of cysts (I, red dotted lines) from distal (d) to proximal (p) position in wild-type testis (I), but the arrangement of cysts is not observed in the *SDiG*$^{-/-}$ XX gonad (H). Scale bars: 10 µm. **(J** and **K)** Schematic representation of cross-sections of wild-type and *SDiG*$^{-/-}$ ovaries. (**J**) Wild-type ovary (left) and *SDiG*$^{-/-}$ ovary (right). (**K**) Germinal cradle (left) and expanded germinal epithelium (right). In the wild-type ovary, the germinal epithelium harbors the germinal cradles containing the germline stem cells, cystic germ cells, and early diplotene oocytes. As folliculogenesis proceeds, diplotene oocytes exit through the basement membrane towards the stromal compartment where oocyte growth and maturation occur. In the *SDiG*$^{-/-}$ ovary, early-stage germ cells (red cells in the right panel K), surrounded by *sox9b*-expressing cells, start spermatogenesis. As a result, sperm expand and fill the germinal epithelium. A few oocytes (orange cells in the right panel K) also develop in the germinal epithelium, possibly in an estrogen-independent manner. These oocytes exit from the germinal epithelium to form follicles, as seen in folliculogenesis of the wild-type ovary.

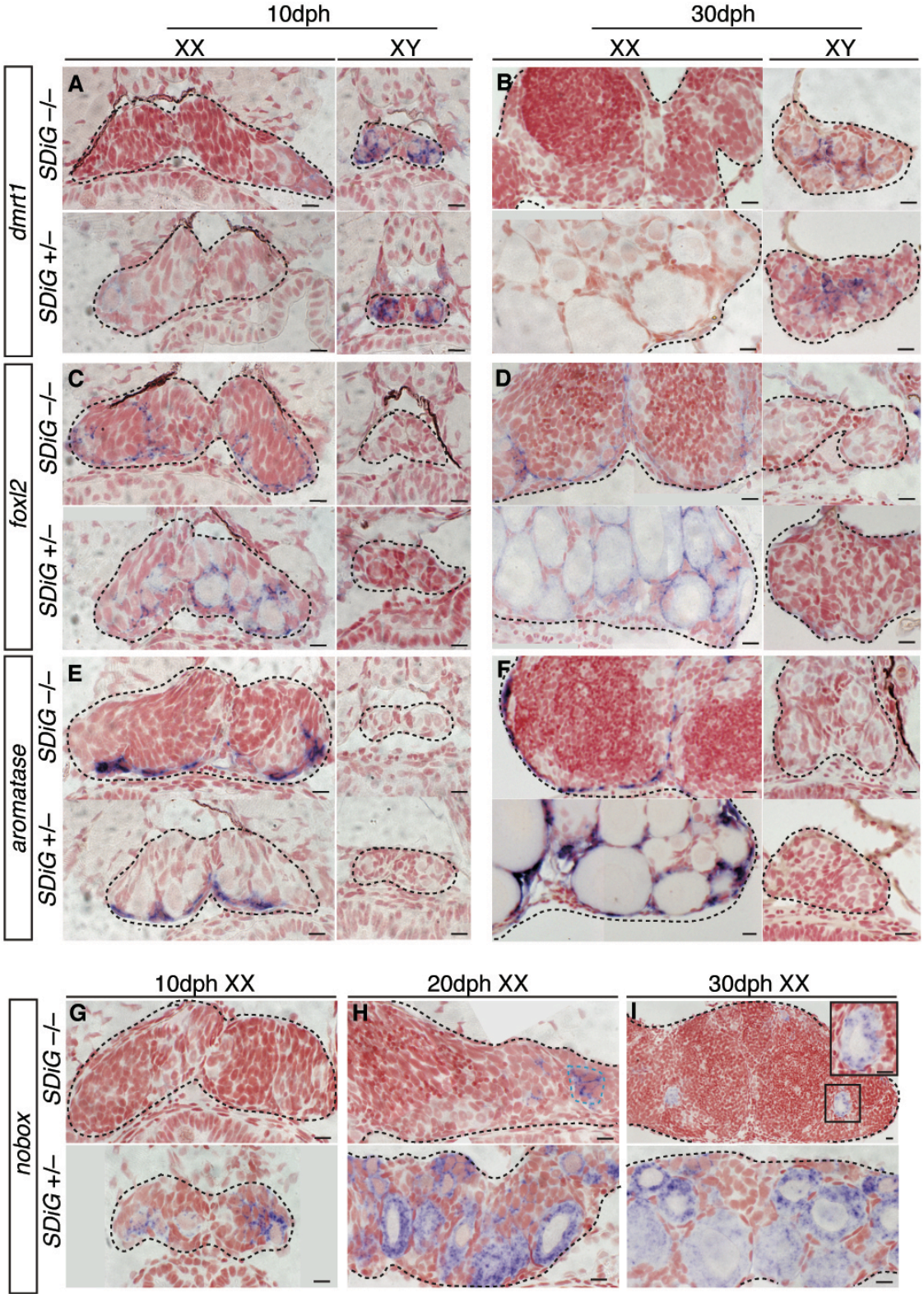
10dph
30dph
XX
XY
XX
XY
dmrt1
SDiG −/−
SDiG +/−
A
B
foxl2
SDiG −/−
SDiG +/−
C
D
aromatase
SDiG −/−
SDiG +/−
E
F
10dph XX
20dph XX
30dph XX
nobox
SDiG −/−
SDiG +/−
G
H
I

**Figure 26**. ***In situ* hybridization for sex marker genes during gonadal development.** Cross-sections of gonads are encircled by black dotted lines. Signals of each gene are indicated in purple. (**A** and **B**) Expression analysis of *dmrt1*, a male marker gene, at 10 dph (A) and 30 dph (B). *dmrt1* is expressed in XY gonadal somatic cells. (**C** and **D**) Expression analysis of *foxl2*, a granulosa marker gene, at 10 dph (C) and 30 dph (D). *foxl2* is expressed in somatic cells surrounding spermatogenic cells and oocytes of *SDiG*$^{-/-}$ and *SDiG*$^{+/-}$ XX gonads, respectively. (**E** and **F**) Expression analysis of *aromatase*, estrogen synthetase, at 10 dph (E) and 30 dph (F). The *aromatase* signals appear on the ventral side of gonads in both *SDiG*$^{-/-}$ and $^{+/-}$ XX larva at 10 dph, and subsequently surround spermatogenic cells and oocytes at 30 dph. (**G** to **I**) Expression analysis of *nobox*, an oocyte marker gene, at 10 dph (G), 20 dph (H) and 30 dph (I). In *SDiG*$^{+/-}$ XX gonads, the *nobox* signals in oocytes are observed from 10 dph. On the other hand, no signals are detected in *SDiG*$^{-/-}$ gonads at 10 dph. The signals appear in a subset of cystic germ cells (blue dotted line in H) at 20 dph and a very few oocytes (inset in I) at 30 dph. $n \geq 3$. Scale bars: 10 μm.

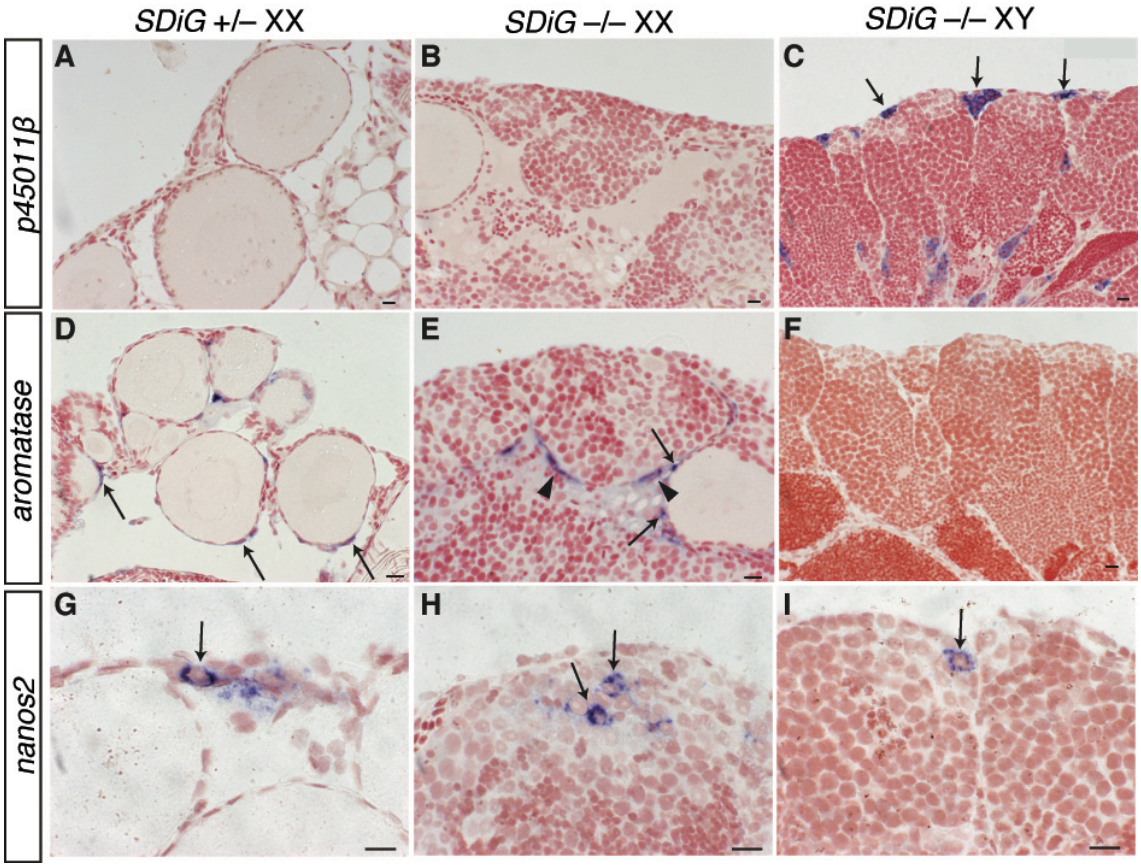


**Figure 27. *In situ* hybridization for sex-marker and stem-marker genes in adult gonads.** Cross-sections of adult gonads. Signals for each gene are indicated in purple. (**A** to **C**) Expression analysis of *p45011β*, 11-ketotestosterone synthetase, in the adult gonads. *p45011β* is only expressed in interstitial cells of testes (C, arrows). (**D** to **F**) Expression analysis of *aromatase*. The *aromatase* signals are detected in somatic cells surrounding oocytes of both *SDiG*$^{+/-}$ and $^{-/-}$ ovaries (D and E, arrows), and in somatic cells surrounding the expanded germinal epithelium filled with spermatogenic cells in *SDiG*$^{-/-}$ ovaries (E, arrowheads). (**G** to **I**) Expression analysis of *nanos2*, a marker of germline stem cells. The *nanos2* signals are detected in a subset of germ cells (arrows) in the germinal cradle in *SDiG*$^{+/-}$ ovary (G), expanded germinal epithelium in *SDiG*$^{-/-}$ ovary (H), and lobule of *SDiG*$^{-/-}$ testis (I). n = 2. Scale bars: 10 μm.

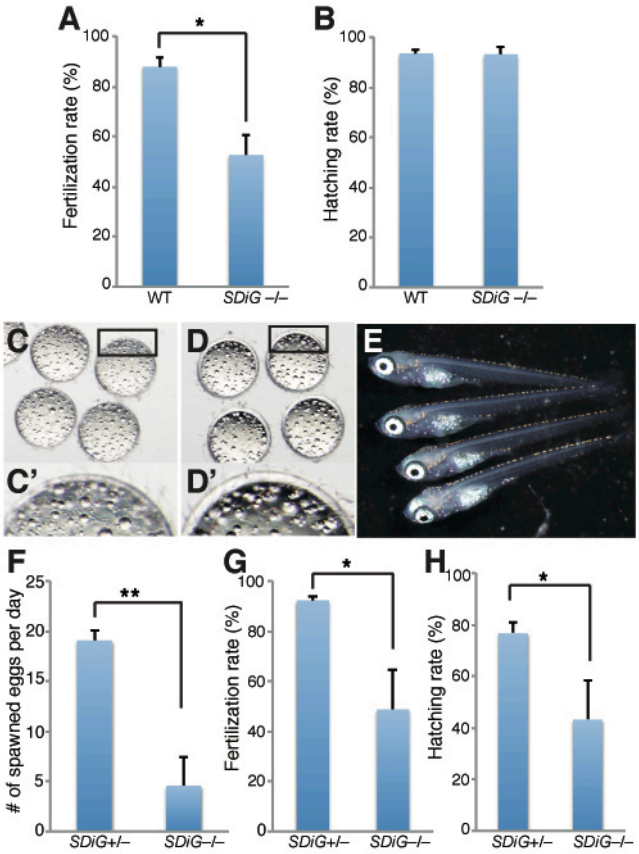


**Figure 28. *SDiG*$^{-/-}$ XX gonads produce both functional sperm and eggs. (A)** Fertilization rate of artificial insemination using sperms from wild-type testes (n=3) and *SDiG* –/– ovaries (n=8). In each experiment, one gonad was used. (**B**) Hatching rate of the fertilized eggs in (A). (**C**) Unfertilized eggs. (**D**) Fertilized eggs by sperms derived from *SDiG* –/– ovaries. The activated egg membrane is shown in (D'). (**E**) Hatched embryos from the eggs in (D). (**F**) The average number of spawned eggs per day by *SDiG* +/– and –/– XX females which are paired with *SDiG* +/– XY males. n=6 (6 pairs). (**G**) Fertilization rate of eggs. (**H**) Hatching rate of the fertilized eggs in (G). Statistics by two-tailed student *t*-test: * $p<0.05$, ** $p<0.001$.

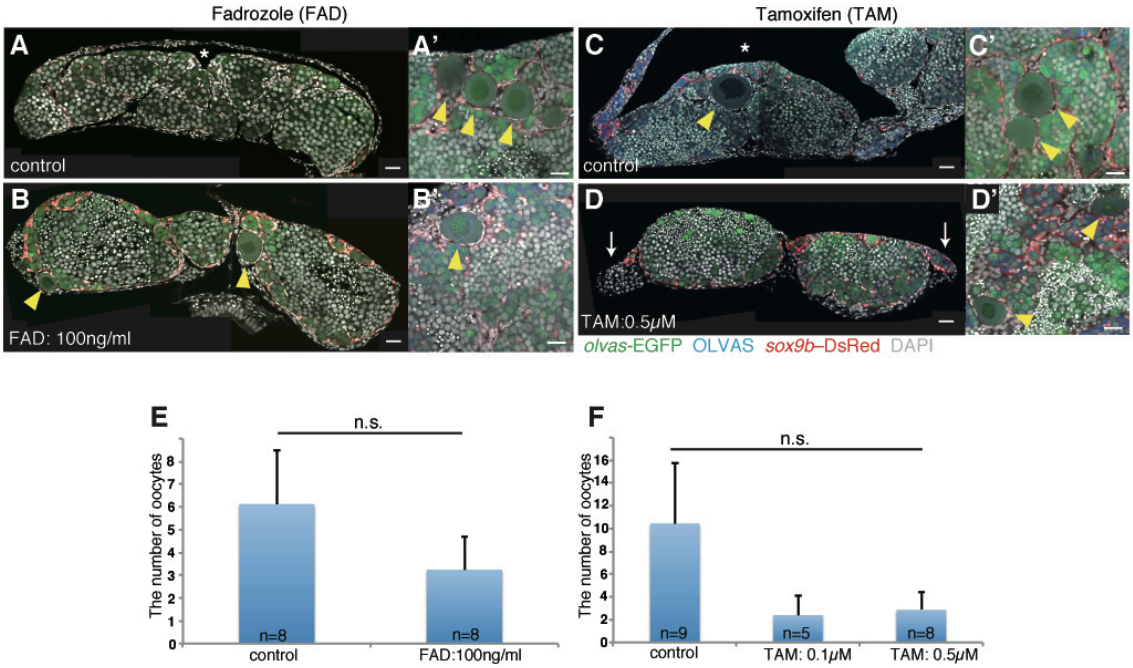


**Figure 29. Inhibition of estrogen production and signaling does not block oocyte formation in *SDiG*$^{-/-}$ gonads.** Larvae were treated with an aromatase inhibitor, fadrozole (FAD), and an estrogen receptor antagonist, tamoxifen (TAM), from 15 dph for 20 days. (**A** to **D**) Cross section of gonads. (**A'** to **D'**) Ventral view of gonads. (**A** and **B**) FAD-untreated (A**,** control) and FAD-treated (B) *SDiG*$^{-/-}$ XX gonads at 40 dph (5 days after completion of the treatment). (**C** and **D**) TAM-untreated (C, control) and TAM treated (D) *SDiG*$^{-/-}$ XX gonads at 40 dph. Note ovarian cavities (asterisks in A and C) develop in control gonads, but not in drug-treated gonads (B and D), indicating that E2 production and activity are blocked by the drug treatments. Arrows in (D) indicate the primordium of gonadal elongation for the formation of ovarian cavity. Arrowheads indicate diplotene oocytes. (**E** and **F**) Quantitation of oocyte number in FAD- (E) and TAM- (F) treated gonads. Scale bars: 20 μm.

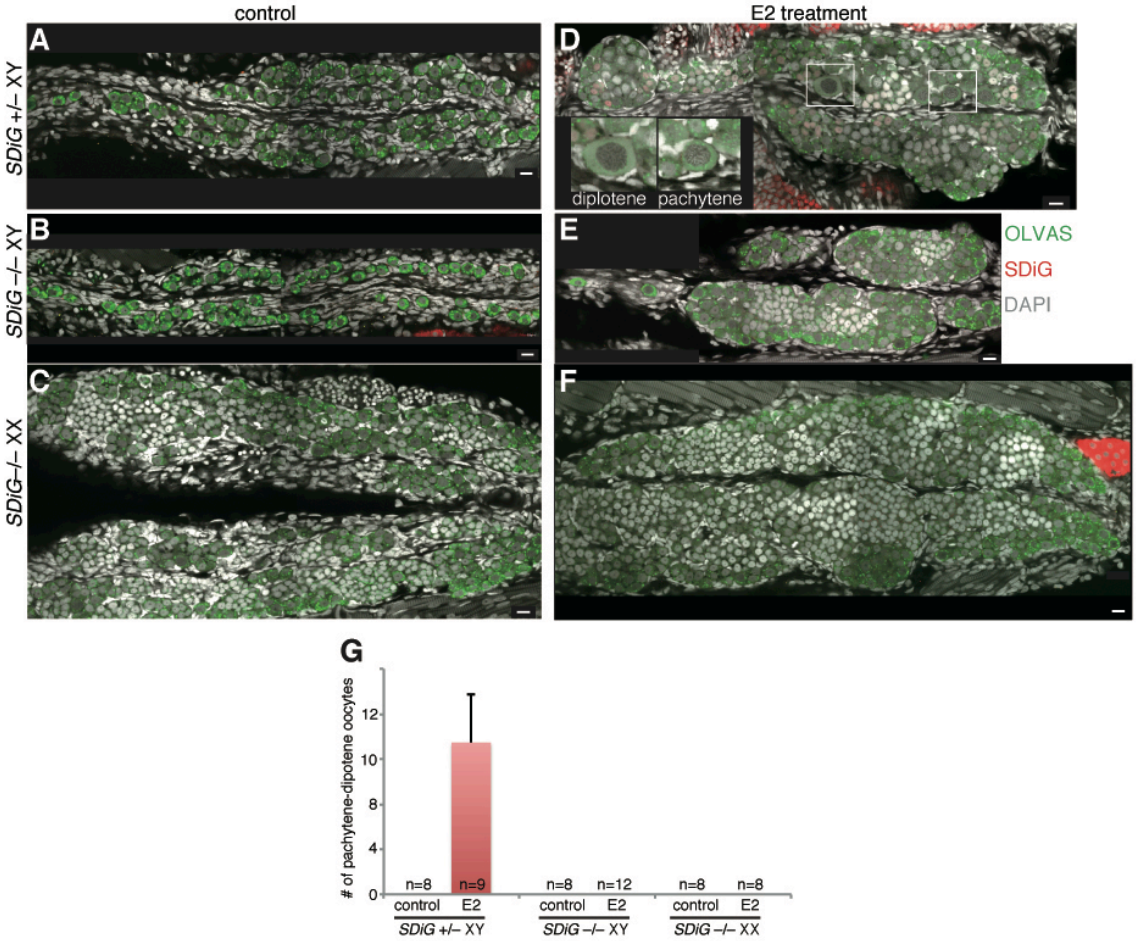


**Figure 30. Estrogen (E2) treatment during gonadal development does not induce oocytes in *SDiG*$^{-/-}$ gonads.** Fertilized eggs were treated with E2 until 5 dph. (**A** to **F**) E2-untreated (control) and E2-treated gonads at 12 dph (1 week after completion of the treatment). Oocytes formation is induced in E2-treated *SDiG*$^{+/-}$ XY gonads (A and D, insets), but not in both *SDiG*$^{-/-}$ XY (B and E) or XX (C and F) gonads. (**G**) Quantitation of the number of pachytene and diplotene oocytes (insets in D). Scale bars: 10 µm.

**Tables 1-3**

**Table 1. Primers used in Chapter 1.**

| Primer Name | Sequence (5'-3') | Purposes |
|---|---|---|
| q-vasa-F | GATTTCCGCTCAGGCAAGTG | q-PCR |
| q-vasa-R | GTCAATGGTGTTGGGCAGGT | q-PCR |
| q-beta-actin-F | TGGCGCTTGACTCAGGATTT | q-PCR |
| q-beta-actin-R | GCAGATGCCTGGGGTGTTTA | q-PCR |
| q-tudor-F | GCGTCTGTTGCAGCTTCCTT | q-PCR |
| q-tudor-R | ACCGAAACACCTGCTGCACT | q-PCR |
| q-sox9b-F | TTGGCCAGACAGCCAATGTT | q-PCR |
| q-sox9b-R | TCTCTGTTGACCCTGTTGGCTT | q-PCR |
| q-gsdf-F | TCCATGGCCACCGAGGTCTT | q-PCR |
| q-gsdf-R | CCGAGGAATTGCAGAGAGCACA | q-PCR |
| q-dmy-F | ACCCTGACCTACCGCTCCAT | q-PCR |
| q-dmy-R | CGCAGCTTTTCCTCATTTGG | q-PCR |
| q-Sdgc-F | AGAACAGCGTAAGGCTTCCA | q-PCR, genomic DNA sequence |
| q-Sdgc-R | GCCCATAAACCATGATGAGG | q-PCR, genomic DNA sequence, HRM |
| gSdgc-F | ACAACACACCGCATTTCTCC | genomic DNA sequence, HRM |
| gSdgc-F(nest1) | ACACACCGCATTTCTCCA | HRM, genotyping |
| gSdgc-R(nest1) | AATTGCAAGGTGTCTGTTT | HRM, genotyping |
| gSdgc-F(nest2) | ACTTATGTAATTGCTTAGTGTAGGC | HRM, genotyping |
| gSdgc-R(nest2) | GCTCCTCCTCCGAGGTG | HRM, genotyping |
| MID0123-F | ATCGTCTTTTGATCTATTAGAAAAGC | genotyping |
| MID0123-R | TCCTGCTCTGTGCTTTGG | genotyping |

F: forward, R: reverse, nest: primers used for nested PCR.

**Table 2. Components of culture media.**

| Components | Medium#1 | Medium#2 |
|---|---|---|
| Leibovitz L15 | + | + |
| Hepes (10mM, pH 7.9) | + | + |
| Penicillin (50U/ml) | + | + |
| Streptomycin (50µg/ml) | + | + |
| Kanamycin (100µg/ml) | + | + |
| Glutamax (2mM) | + | + |
| Fetal bovine serum | 10% | 5.0% |
| Bovine serum albumine | | 0.5% |
| Embryonic Extract | | 3 embryos/ml |

# Table 3. The genotyping panel of *Sdgc*

data type f2 intercross A=HdrR/HdrR B=Kaga/Kaga H=HdrR/Kaga

94 148 0

| | |
|---|---|
| *Sdgc | H A H A A H H H A H A H H A H B H H H B H B B A H H B H A H H A H H B H H A B H H B H H A - A H H H H B H H A H B H B H H B B B H H H B B H B A H H B H A H H A A H A H B A A H A H H H H A |
| *MID0111 | H H H A B H H H A H A A H A B H A A H H H B H A A H B H B B B H H H B H A A H H B B H B H H B A H B B B H H A H H H A A H B A H A H H B B H H A A H B H B H H A H H A B H H B H H H A A H H |
| *MID0127 | H H A A B H H H A H A A H A B H A A H H H B H A A H B H B B B A B H B H A A B H B B A H A H H H H B H B H H A H H H H A A B A B A B H B B H H A A B B H A H H A A H A H H H A H H H A H B H |
| *MID0124 | H H A A A H H H A H A H H A H H A H B H H B H A A H B H B H B A B H B H A A B A B B A H A H H H H B H B H H A H H H H A H B H B H B A B B H B A A B B H A H H A A H A H H A A H H H A H B H |
| *MID0122 | H A A A A H H H A H A H H A H B A H H B H B B A H H B H B H H A H H B H H A B A H B H H A B H H H H H B H H A H B H H H H B B B H H A B B A B A H B B H A H H A A H A H B A A H H H H H B A |
| *MID0117 | H A H A A H B H A H H H H A H B H H H B H H B A H H B H B A A A H H B H H A B H H B H H H B A H H H H B H A H B B H B H A B B H H H H B B A B A H H B A H H H A A H A H B A A H A A H H H A |
| *MID0118 | H A H A H H B B A H H H A A B B H H H B H H A A H H B H B A A A A H B H H H B H H H A H H H A H H A H B H A A B B H B H A B H H B H H B B H B A A H B A H H H A A H A H B A A H H A H H H A |
| *MID0119 | A A H A H H B B A H A H A A H B H B H H H H A A H H B H B A H H A A H A A B B H H A A H H A H H H H H B H A H B H H B B A H H H H H H H B B B A A H B A H H A A A H A H B A A H B A H H B H |
| *MID0211 | H A A H B H H B A B H H B B H A B A H B H H A H B B B H H B A H A A H H A A H H H H H B A A H B B H A H A A A B H H H B H H H H A H H H B H H A H B H A A B B H B H H A H H H H A B B A H A |
| *MID0221 | A H H H B H H B H B H H H H A A B A H B H A A A B B B H B B H H H A H B A A B H H B B B H H B B B H A B A H A H B H H B H H A H A H H B B H H A H H A H A H B H B H H H H H H H A B B A H A |
| *MID0229 | A H H H B A H B H B H H H H A A B H H B H A H A B B B B H H H H H A H B A A B B H B B B H H H B B H H B A H A H B A H H H H A H A H H B B H A A H H A H A H B H B A H H H H H B A B B A H H |
| *MID0215 | A H H H B A H B H H H A A H A A H H H B H A H A B H B B H B H H H A H H A A B B H B B B H H H B B A H B A H H H B A H H H H A A H H H B B H A H H H H H A H B H H A B H B H H B H B B A B H |
| *MID0216 | A H H H B A B B H H H A A B A A H H H B H A H H B H H B H B B H H A H A H A A H B B B B H H B B A H B A H H H B A H H H H B A B H H B B H A H A H H H A B B H H A H H B H H B H B B H H H |
| *MID0231 | A H H A B A B H H H H H A H H A H H H B H A A H B A A B H B B H H A H A B A A H B H B H B H H B B H H B A H H H B A H B H B B A B B H H B A A H A A H A A H B B H A B H B B H B B B B H H H |
| *MID0220 | A H H H H A B H H A H H A H H H H H H B H H A H H A A B H B B H B A H A B H A H B A B H B H H H B H H B A B H H B H H B H H B A B B H H B H A H A A H A H H H H H A B H H B B B B B B H H |
| *MID0311 | H A B B H B B H B B A H H H B A B A H B A A B H H H B H H B A H B H A A H H H H B B B B A H H H H A H B B A H H H B B B A A H H H H H H H A B B B H H H H A H B H A A H H A B A A H H A B A |
| *MID0321 | H A A B H B B H B B A H A H B A B A A H A A B H H H H H H B A H B H A A A H H H B B H B A A H H H A H B H H H H H B H B A A B H B H A H H A B H H H H H H A H B A A H A H A H H H H H A B H |
| *MID0316 | H A A H A B B H B H A H A H B H B A A H A A B H H H H A H B A A B A A A A H H A B B H H H A H A B H H B H H H H B B B B H A B H B H A A H H B H H A H H H A A B A A H A H H B H H H A H H A |
| *MID0322 | H A A H A B B H H H A B A H H H B A A H A A B H A H H A A B A A B A A A H H A A B B A H H A H A B H B B H H B H B B B H H H B H B H A A A H B H B A B A B A H B A H H A H H B H H H A H H A |
| *MID0323 | H A A H B H H H H B A B A H H H B H A H H A B H H H A A A H A H B H H A H B A A H A A H H A H A B H H B H H B H B B B H B H H H H H H H A H B B B A B A H H H B A H H A B H B H H H A H H A |
| *MID0318 | H A A A B H H H A B A B H B H H H H A H H A H H H H A H A A A H B H B H H B A H H A A H H H H A B H H B H A B H B B B H B H H H H H H H A H B B H B B A H H H H A H H H B H B H H H A B H A |
| *MID0421 | H B B H H B A H H B H A H H A B H H A B H B A B H B H H H B H H H H A H B B H B H H H H A H H A A B B H B H H H H A H H H B H A B H A B H H H H H A H A H B H H H A H B H B H H H H A A A H |
| *MID0412 | H H B H H H A H H B H A H H A A H H H H H B B B H B H H B B H H H H H H B B H B H H H H H A H A H B H H B H H H B A H H H B H A H H A B H H B H H H H A H B H H H A H B H B H H H H A A A H |
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