

# ***Dmrt1* mutation causes a male-to-female sex reversal after the sex determination by *Dmy* in the medaka**

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**Abstract** *DMRT1*, which is found in many vertebrates, exhibits testis-specific expression during the sexual differentiation period, suggesting a conserved function of *DMRT1* in the testicular development of vertebrate gonads. However, functional analyses have been reported only in mammals. The current study focused on the *Dmrt1* function in the teleost medaka, *Oryzias latipes*, which has an XX–XY sex determination system. Although medaka sex is determined by

the presence or absence of the Y chromosome-specific gene *Dmy*, we demonstrated that in one *Dmrt1* mutant line, which was found by screening a gene-driven mutagenesis library, XY mutants developed into normal females and laid eggs. Histological analyses of this mutant revealed that the XY mutant gonads first developed into the normal testis type. However, the gonads transdifferentiated into the ovary type. The mutant phenotype could be rescued by transgenesis of the *Dmrt1* genomic region. These results show that *Dmrt1* is essential to maintain testis differentiation after *Dmy*-triggered male differentiation pathway.

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## **Abbreviations**

DM domain	Doublesex ( <i>dsx</i> ) and male abnormality-3 ( <i>mab-3</i> ) domain
ENU	<i>N</i> -ethyl- <i>N</i> -nitrosourea
<i>DMRT1</i>	Doublesex- and mab-3-related transcription factor 1
<i>Dmy</i>	DM domain gene on the Y chromosome

## **Introduction**

A DM domain gene, *DMRT1* (doublesex- and mab-3-related transcription factor 1), is required for human testis development (Raymond et al. 1999). In mice,

*Dmrt1* is expressed and required for both the germ cells and the Sertoli cells of the testes (Kim et al. 2007; Krentz et al. 2009; Matson et al. 2010). XY *Dmrt1*-null mutant mice are born as males with testes, but these gonads later undergo abnormal differentiation (Raymond et al. 2000). The avian Z-linked *DMRT1* is required for male sex determination in chickens (Smith et al. 2009). It is also involved in male development in other vertebrates including turtles, frogs, and teleost fish. The two sex-determining genes found in non-mammalian vertebrates, *Dmy* in medaka (Matsuda et al. 2002; Matsuda et al. 2007) and *DM-W* in the African frog (Yoshimoto et al. 2008; Yoshimoto et al. 2010), are *Dmrt1* homologues.

The teleost fish medaka *Oryzias latipes* has an XX–XY male heterogametic sex determination system like mammals. The key factor that recruits the bipotential gonad with the XY chromosome constitution to the testicular pathway was identified as being a DM-domain-related gene on the Y chromosome, namely *Dmy* or *dmrt1bY* (Matsuda et al. 2002, Nanda et al. 2002). *Dmy* mRNA and protein are expressed specifically in the somatic cells surrounding germ cells in the early gonadal primordium before morphological sexual dimorphism is observed. The first morphological sexual dimorphism is observed in the proliferation of germ cells. Therefore, the number of germ cells is an important indicator for distinguishing a bipotential gonad recruitment to the testicular or ovarian pathway in the early developmental stages.

In an inbred Hd-rR strain, the sexual dimorphism of germ cells between the XX and XY gonads has been apparent at stage 38; subsequently, germ cells entered mitotic arrest in XY (Satoh and Egami 1972; Kobayashi et al. 2004), whereas they entered meiosis in XX after hatching. *Dmy* dysfunction leads to complete male-to-female sex reversal. XY fish of *Dmy* mutants or with low-level expression of *Dmy* have developed to egg-laying females (Matsuda et al. 2002; Shinomiya et al. 2004; Otake et al. 2006). Furthermore, the germ cells in the hatching stage of the embryos that have been blocked for *Dmy* translation by *Dmy*-specific grip-NA become female-like (Paul-Prasanth et al. 2006). These results suggest that XY gonads with blocked *Dmy* function commit to the female pathway.

In medaka, *Dmrt1* is expressed in spermatogonium-supporting cells, which is the same lineage cells

expressing *Dmy*, after testicular differentiation (20–30 days after hatching) (Kobayashi et al. 2004). In the case of medaka, high-temperature treatment or steroid hormone treatment can induce XX sex reversal (Yamamoto 1958; Sato et al. 2005; Hattori et al. 2007). This means that a gonad lacking *Dmy* can develop into testis and that *Dmy* is not necessary for testicular development. *Dmy* works as a natural inducer for normal testicular development. *Dmrt1* appears to be a key gene in normal testicular development because in steroid hormone treatment-induced XX sex-reversed testes, *Dmrt1* is expressed in the Sertoli cell lineage, similar to the expression of *Dmy* in XY testes (Kobayashi et al. 2004). High temperatures have also induced *Dmrt1* expression in XX embryos (Hattori et al. 2007). However, it remains unclear whether *Dmrt1* is necessary for testicular development in medaka.

To examine *Dmrt1* function in the gonadal development of medaka, we screened an *N*-ethyl-*N*-nitrosourea (ENU)-based gene-driven mutagenesis library of medaka and found *Dmrt1* mutants. In one *Dmrt1* mutant line, XY individuals showed a male-to-female sex-reversal phenotype. The XY mutants developed into normal egg-laying females. Histological analyses of the developing gonads in the mutants revealed that by 10 days after hatching, the gonads of the XY mutants had already commenced development along the female pathway.

## Materials and methods

### Biological specimen

Two laboratory strains of medaka were used: the Cab strain and the inbred strain Hd-rR. The Cab strain was used to establish an ENU-based gene-driven mutagenesis library of medaka. The Cab strain is a sub-strain of the Carbio strain, which was established from fish obtained from Carolina Biological Supplies. Founder fish were repeatedly mutagenized with ENU and crossed with wild-type females, and the progeny were used to establish a permanently cryopreserved resource of 5,771 F<sub>1</sub> males (Taniguchi et al. 2006; Ishikawa et al. 2010). The Hd-rR strain was used for back-crossing and as a control in the gene expression analyses.

### Screening of *Dmrt1* mutants in a gene-driven mutagenesis library

A gene-driven mutagenesis library has sets of frozen sperm and genomic DNA that were prepared from 5,771 F<sub>1</sub> males. Polymerase chain reaction (PCR) products of the *Dmrt1* exon 2 region of 5,600 stock DNAs within 5,771 were screened using high resolution melting (HRM) analysis and sequencing. PrimeSTAR<sup>®</sup> Max DNA Polymerase (Takara Bio Inc., Shiga, Japan) was used as a DNA polymerase, and 0.83× LCGreen Plus (J. K. International, Tokyo, Japan) and 10% dimethylsulfoxide were added to the PCR reaction mix with the following primers: DM29f: 5'-CTG TAC AAG TGA ACC CCG CCT-3' and DM30r: 5'-GCG GTG TTT TGT TCG GTG-3'. PCR conditions were as follows: 5 min at 95°C followed by 30 cycles of 20 s at 96°C, 30 s at 55°C, and 30 s at 72°C, followed by 5 min at 72°C; and a final denaturing and re-annealing step (one cycle of 94°C for 30 s followed by rapid cooling to 28°C). The final step was included to redistribute DNA strands derived from mutant and wild-type alleles to maximize heteroduplex formation. After PCR, the plates were imaged in a 96-well LightScanner (Idaho Technology Inc., Salt Lake City, UT, USA) for HRM analyses as previously described (Taniguchi et al. 2006; Ishikawa et al. 2010).

The genomic DNAs showing melting curves that differed from those of the wild-type allele were selected as mutant candidates. The selected genomic DNA were purified using an ExoSAP-IT (General Electronics, Fairfield, CT, USA) and then used as a template for the sequencing reaction. The BigDye<sup>®</sup> Terminator v3.1 Cycle Sequencing Kit (Life Technologies Corporation, Carlsbad, CA, USA) with the primer 5'-GAA CCA CGG CTT CGT GTC T-3' was used for sequencing, which was performed using a 3100 genetic analyzer (Life Technologies Corporation).

### Genotyping

The genetic sex of each specimen was determined using genomic PCR using the common primers for *Dmrt1* and *Dmy* (Shinomiya et al. 2004). We used two methods to distinguish between the *Dmrt1* mutant alleles and the wild-type alleles. The first method was direct sequencing of the PCR product. We amplified the *Dmrt1* genomic region using the DM29f and

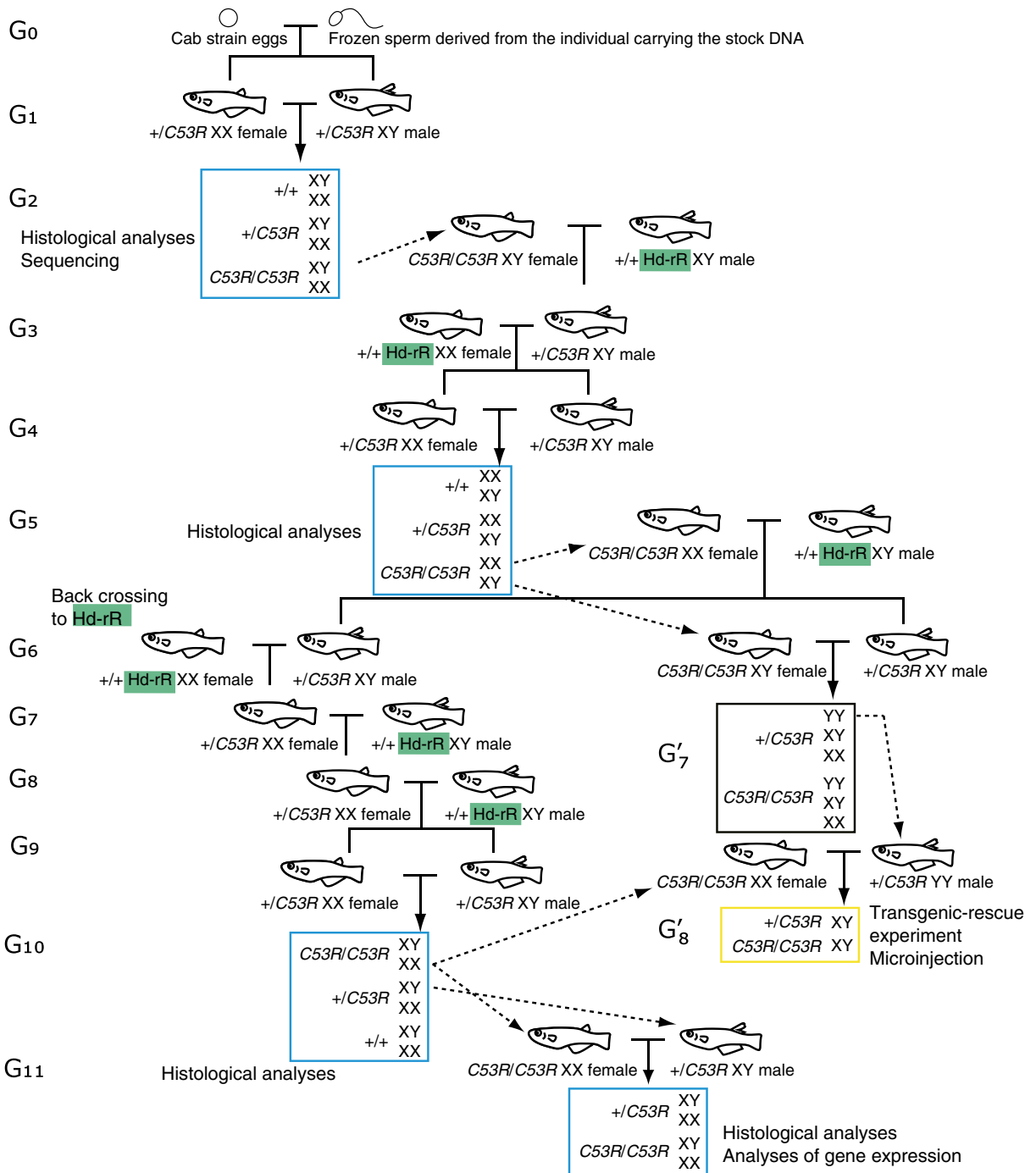
DM30r primers, and the PCR products were purified using ExoSAP-IT (General Electronics). The sequencing methods are described above. The other method was genotyping the *Dmrt1*<sup>C53R</sup> mutation using the TaqMan<sup>®</sup> probe and the ABI PRISM 7500 (Life Technologies Corporation) with the Multiplex PCR Kit (QIAGEN, Hilden, Germany) using the DM29f and DM30r primers and the TaqMan<sup>®</sup> probe 5'-FAM-CTG CCG CTG CGC CA-TAMRA-3' (2.0 μM), which was specific for the wild-type allele, and the VIC-TAMRA probe 5'-VIC-TGC CGC CGC GCC A-TAMRA-3' (1.6 μM), which was specific for the mutant allele. PCR conditions were as follows: 15 min at 95°C, followed by 40 cycles of 15 s at 95°C and 60 s at 60°C.

### *Dmrt1*<sup>C53R</sup> mating experiment

The mating scheme for the *Dmrt1*<sup>C53R</sup> lines is shown in Fig. 1. The G<sub>1</sub> generation was produced by in vitro fertilization of normal eggs with frozen sperm. Further, the G<sub>2</sub> generation was produced by sib-mating between heterozygous individuals from the G<sub>1</sub> generation. A homozygous XY mutant (*C53R/C53R*) of the G<sub>2</sub> generation that developed as a female was crossed with an inbred Hd-rR male to produce the G<sub>3</sub> generation. XY heterozygous individuals from the G<sub>3</sub> generation were again back-crossed with Hd-rR. In the G<sub>4</sub> generation, heterozygous males and females were sib-mated to obtain the G<sub>5</sub> generation.

An XX female with a genomic homozygous mutation (*C53R/C53R*) was crossed with an Hd-rR XY male to obtain the G<sub>6</sub> generation in which two types of mating were performed to obtain two distinct lines. The first line was used for back-crossing. A heterozygous XY of the G<sub>6</sub> generation was back-crossed with Hd-rR XX. Further, backcrosses were repeated two times. Heterozygous females of the G<sub>9</sub> generation were then mated with heterozygous males of the G<sub>9</sub> generation to obtain various combinations of *Dmrt1* and sex chromosome types of G<sub>10</sub> offspring for histological analyses 5 days after hatching. Homozygous XX from the resultant G<sub>10</sub> generation were crossed with heterozygous XY to obtain many mutant individuals for gene expression and histological analyses.

The other line was used to obtain all XY progeny for a transgenic rescue experiment. To this end, we mated mutant XY G<sub>5</sub> females with heterozygous XY G<sub>6</sub> males. Select heterozygous (+/*C53R*) YY males



**Fig. 1** Mating scheme. The mating scheme of *Dmrt1*<sup>C53R</sup> mutants. Details are provided in the “Materials and methods” section. The G<sub>2</sub>, G<sub>5</sub>, G<sub>10</sub>, and G<sub>11</sub> generations were used for the

histological analyses. The G<sub>11</sub> generation was also used for gene expression analyses. The G<sub>8</sub>' generation was used for the transgenic rescue experiment

were crossed with homozygous (*C53R/C53R*) XX females derived from the G<sub>10</sub> generation of a back-

crossed line to obtain all of the XY eggs for the microinjection.

## Histological analyses of gonads

For histological analysis of the gonads and counting of the germ cells, fish were dissected into head and body segments 5, 10, 15, 20, and 30 days after hatching. Body segments containing gonads were fixed for 3 h in Bouin's reagent and then embedded in paraffin. On the other hand, each dissected head was used for DNA extraction and genotyping according to the protocol listed in the "Genotyping" section. Cross-sections were cut serially at 5- $\mu$ m thicknesses and subjected to hematoxylin and eosin staining. All germ cells were counted for each fry at 5 days after hatching. After cell counting, the mean and standard error were calculated for each sex and the difference between the sexes was statistically evaluated using the paired *t* test.

## RT-PCR

To analyze testis- or ovary-specific gene expression, we extracted RNAs from *Dmrt1*<sup>C53R</sup> and wild-type (Hd-rR) individuals. Whole bodies of each individuals at the day of hatching or the gonads of each individuals dissected at 30 days after hatching were put into a 1.5-mL tube (ASSIST, Tokyo, Japan) with 300  $\mu$ L Buffer RLT (QIAGEN) and two zirconia beads with 3-mm diameters. They were homogenized in Micro Smash™ MS-100 (TOMY SEIKO CO., LTD, Tokyo, Japan) and then centrifuged for 3 min at 18,800 $\times$ *g* in a microcentrifuge. The supernatant was used for the RNA extraction, while the pellet was used for DNA extraction for genotyping. RNA was extracted using the RNeasy Mini Kit (QIAGEN), according to the manufacturer's protocol. Extracted RNA (200 ng of total RNA) was reverse-transcribed using a QuantiTect Reverse Transcription Kit (QIAGEN).

*Dmy* was amplified with the following primers (Otake et al. 2006): 5'-TGC CGG AAC CAC AGC TTG AAG ACC-3' and 5'-GCA TCT GCT GGT ACT GCT GGT AGT TG-3'. The PCR conditions were as follows: 5 min at 95°C, followed by 45 cycles of 20 s at 96°C, 30 s at 65°C, and 30 s at 72°C, followed by 5 min at 72°C. We analyzed the *Dmy* PCR products using the MultiNA Microchip Electrophoresis System (SHIMAZU, Kyoto, Japan). Quantitative gene expression analysis of the gonadal soma-derived factor (*Gsdf*) was performed using the ABI PRISM 7500 (Life Technologies Corporation) and QuantiTect SYBR Green PCR Kits (QIAGEN) following the protocols of Shibata et al. (2010). *Dmy* and *Dmrt1* at 30 days after hatching were amplified using the following primers: 5'-CTG ACA TGA GCA AGG AGA

AGC AG-3' and 5'-CGC TTG TGG CCT TTC AGC-3'. PCR conditions were as follows: 5 min at 95°C, followed by 30 cycles of 20 s at 96°C, 30 s at 65°C, and 30 s at 72°C, followed by 5 min at 72°C. PCR products were analyzed by using 3% agarose gel electrophoresis. *Foxl2* and *Cyp19a1* were amplified using the following primers: *Foxl2*, 5'-CGC CAC AGC AAA GCT CG-3' and 5'-TCG AGT GTC CAG TAA TTC CCC T-3'; and *Cyp19a1*, 5'-TGT GCT CAT TGT TGC TTG CAT C-3' and 5'-TAA TGC AAA ACA TTT GAT GAA TAA CAG TTC-3'. The PCR conditions were as follows: 5 min at 95°C, followed by 30 cycles of 20 s at 96°C, 30 s at 55°C, and 30 s at 72°C, followed by 5 min at 72°C. The *Foxl2* PCR products were electrophoresed on a 2% agarose gel, and the *Cyp19a1* PCR products were electrophoresed on a 1% agarose gel.

## Transgenesis

The transgene constructs for generation of the *Dmrt1* transgenic fish were prepared by sub-cloning the bacterial artificial chromosome (BAC) clone mCON075P1 containing the *Dmrt1* genomic region. We replaced the original BAC clone vector pBAC-*lac* backbone with the pCC1 vector (Epicentre Technologies, Madison, WI, USA). The pCC1 vector contains two types of replication origins. Under normal conditions, a single copy of this vector is present in a bacterium, whereas multiple copies are present in a bacterium in culture medium with induction solution. Green fluorescent protein (GFP) was used as a fluorescent reporter under the control of the medaka *ohvas* promoter (Tanaka et al. 2001). We constructed a recombinant plasmid where *vasa*-GFP was inserted into the pCC1 vector in an earlier study (Otake et al. 2010). In the present study, we amplified this region using PCR and the following primer sets: 5'-TAG CGA TGA GCT CGG ACT TCC ATT GTT CA-3' and 5'-CGT AAT CAT GGT CAT AGC TGT TTC CTG TGT-3'. The PrimeSTAR® Max DNA Polymerase (TakaraBio Inc.) was used for the PCR reaction, and the conditions were as follows: 5 min at 96°C, followed by 35 cycles of 10 s at 98°C, 5 s at 55°C, and 65 s at 72°C, followed by 3 min at 72°C. We joined the BAC clone to the amplified fragment using a Red/ET BAC Modification Kit (GeneBridge, Heidelberg, Germany), which enables the insertion of large sequences into BAC clones by homologous recombination.

TransforMax EPI300 electro-competent cells (Epicentre Technologies) were used for the transformation.

Recombinant clones were cultured and induced by the addition of the CopyControl Induction Solution (Epicentre Technologies) to generate high plasmid copy numbers, according to the manufacturer's instructions. The clones were purified using CsCl-ethidium bromide gradient ultracentrifugation. The BAC was injected into medaka single-cell-staged embryos of a cross between *Dmrt1*<sup>C53R/C53R</sup> XX females and *Dmrt1*<sup>+ /C53R</sup> YY males. Fertilized eggs were collected within 40 min of spawning. The attaching filaments were scraped off the chorion onto a piece of paper. For microinjection of the recombinant BAC clone, we used DNA at 2.0 ng/μL in Yamamoto's solution. The injected eggs were incubated at 26°C.

## Results

### Identification of a medaka *Dmrt1* mutation

We screened 5,600 stock DNAs of a gene-driven mutagenesis medaka library. A 278-bp PCR fragment of the *Dmrt1* gene containing a 5'-untranslated region

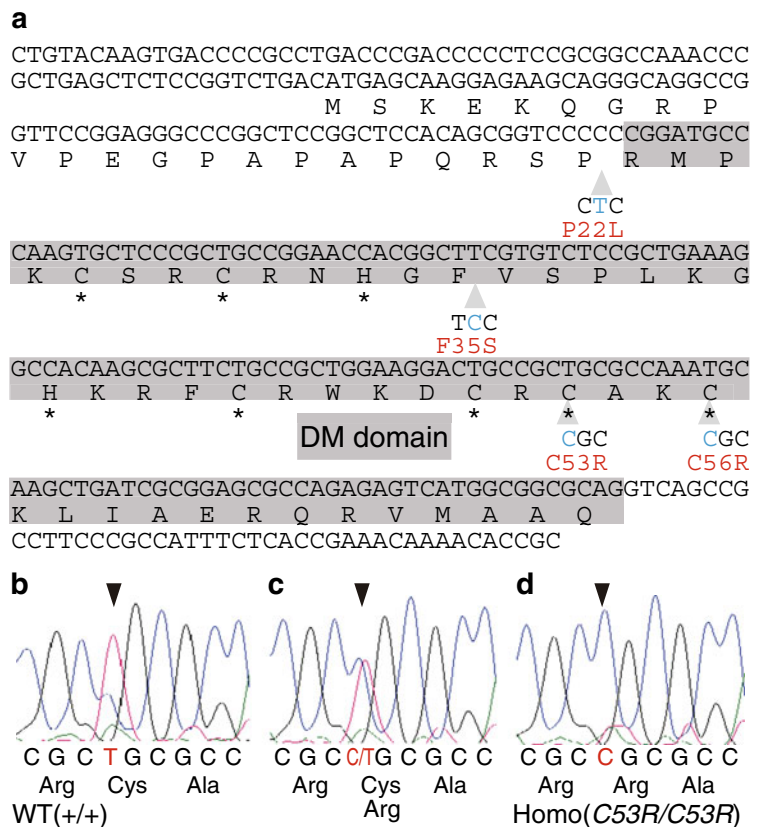
(47 bp), a translated region (207 bp), and an intron region (24 bp) was analyzed by HRM and sequencing. Four stock DNAs contained mutations in the translated region (Fig. 2a). Frozen sperm corresponding with the each stock DNA artificially fertilized normal eggs to obtain the G<sub>1</sub> generation. Heterozygous G<sub>1</sub> males and females were mated to obtain G<sub>2</sub> homozygous mutants.

We histologically analyzed gonads of these G<sub>2</sub> homozygous mutants (Table 1). In two mutant lines (*Dmrt1*<sup>P22L</sup> and *Dmrt1*<sup>F45S</sup>), we could not find differences with normal gonads in either sex. In another mutant line (*Dmrt1*<sup>C56R</sup>), the XY mutants had abnormal gonads. Most of them developed as males, but their testes contained a few oocytes, whereas a few of them developed female-like appearances and their gonads had many oocytes but no ovarian ducts and they did not lay eggs (Table 1).

In the other line (*Dmrt1*<sup>C53R</sup>), both XX and XY mutants had abnormal gonads with a low number of germ cells. We assumed that the phenotypes were dependent on other mutated loci located near the

**Fig. 2** *Dmrt1* mutations. **a**

The mutation positions on the DM domain in *Dmrt1* exon 2. Four missense mutations were found: P22L, F35S, C53R, and C56R. Asterisks show the conserved cysteine and histidine residues thought to be important in the zinc-finger motif. The gray arrowhead shows the mutated position. The chromatogram of a wild-type individual showed one peak for thymine in amino acid 53 (b), that of the heterozygous individual (*Dmrt1*<sup>+ /C53R</sup>) showed a double peak of cytosine and thymine (c), and that of the homozygous mutant (*Dmrt1*<sup>C53R/C53R</sup>) showed one peak of cytosine (d)



**Table 1** Gonadal phenotypes in the G<sub>2</sub> generation of *Dmrt1* mutant lines at 30 days after hatching

Sex chromosome type	<i>Dmrt1</i> alleles	Specimens (N)	Gonad type	
			Testis	Ovary
XX	<i>P22L/P22L</i>	18	0	18
XY	<i>P22L/P22L</i>	9	9	0
XX	<i>F45S/F45S</i>	10	0	10
XY	<i>F45S/F45S</i>	10	10	0
XX	<i>C56R/C56R</i>	5	0	5
XY	<i>C56R/C56R</i>	14	13 <sup>a</sup>	1
XX	<i>C53R/C53R</i>	5 <sup>b</sup>	–	–
XY	<i>C53R/C53R</i>	5 <sup>b</sup>	–	–

<sup>a</sup> Two of these testes contained a few oocytes

<sup>b</sup> In both XX and XY, three gonads had no germ cells, whereas two had a few oocytes

*Dmrt1*. Therefore, we bred >100 offspring to obtain recombinants between the locus and *Dmrt1*. We then found that one XY homozygous mutant and two XX homozygous mutants developed into egg-laying females. The XY mutant was mated to the Hd-rR strain to obtain heterozygous individuals (Fig. 1). The heterozygous individuals were then saved for the next generation. We then histologically analyzed the gonads of the G<sub>5</sub> generation obtained by mating heterozygous males with females. All of the XY homozygous mutants had ovaries. On the other hand, other combinations of sex chromosome type and *Dmrt1* alleles had normal gonads depending on the sex chromosome type (Table 2). The *Dmrt1* mutation was confirmed by genomic DNA sequencing (Fig. 2b–d).

**Table 2** Gonadal phenotypes in the G<sub>5</sub> generation of the *Dmrt1*<sup>C53R</sup> line at 30 days after hatching

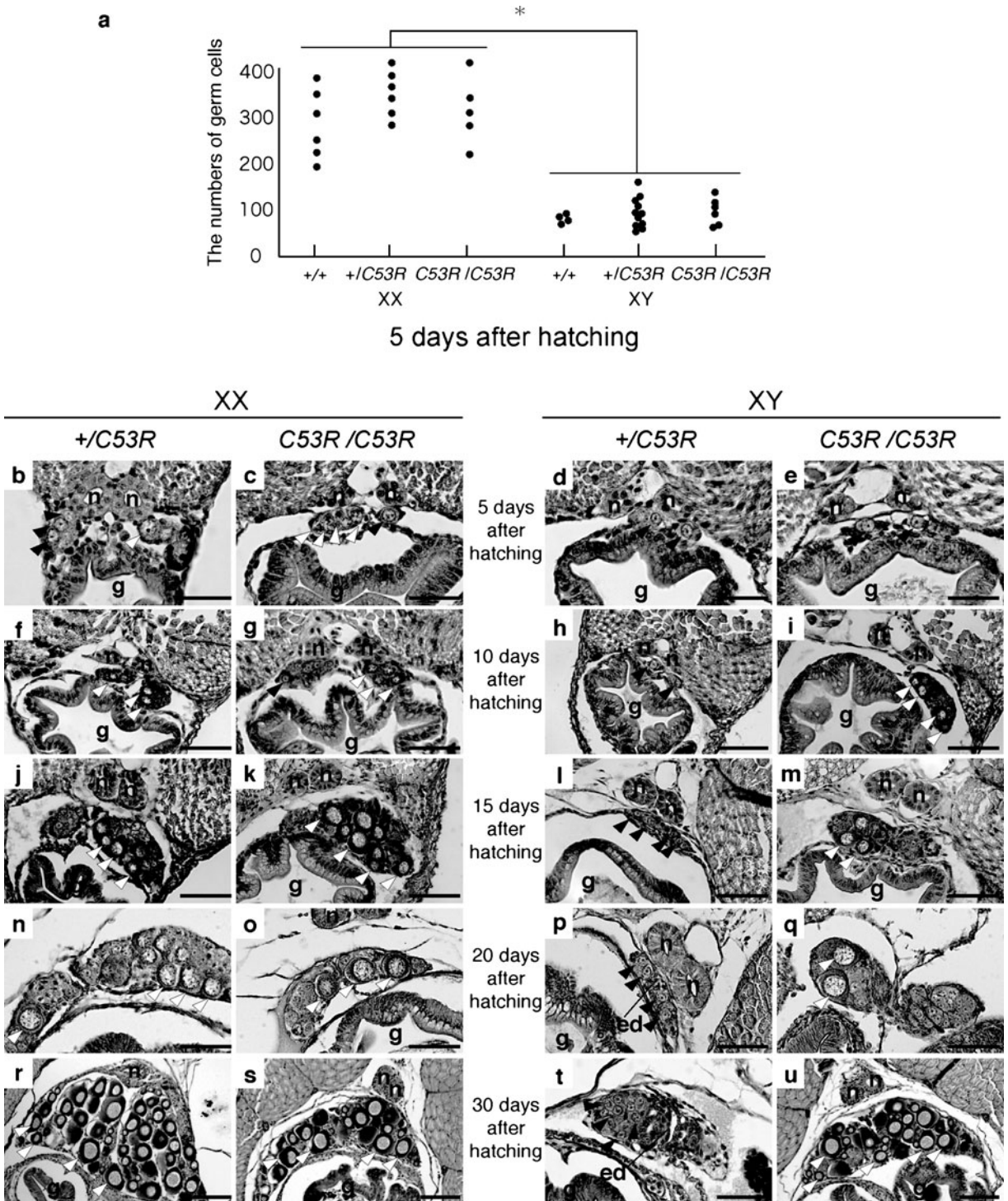
Sex chromosome type	<i>Dmrt1</i> alleles	Specimens (N)	Gonad type
XX	+/+	6	Ovary
XX	+/ <i>C53R</i>	5	Ovary
XX	<i>C53R/C53R</i>	5	Ovary
XY	+/+	5	Testis
XY	+/ <i>C53R</i>	7	Testis
XY	<i>C53R/C53R</i>	7	Ovary

## Mutant phenotypes

The genetic males (XY) of the *Dmrt1*<sup>C53R/C53R</sup> mutants developed into females. Therefore, to clarify when sex reversal occurs during gonadal differentiation, we histologically analyzed the gonadal phenotypes of the *Dmrt1*<sup>C53R</sup> mutants at 5, 10, 15, 20, and 30 days after hatching (Fig. 3, Table 3). At 5 days after hatching, we used the G<sub>10</sub> generation obtained by sib-mating the heterozygous (*Dmrt1*<sup>+/*C53R*</sup>) individuals from the G<sub>9</sub> generation, whereas at 10, 15, 20, and 30 days after hatching, we used the G<sub>11</sub> generation obtained by sib-mating the homozygous XX females (*Dmrt1*<sup>C53R/C53R</sup>) with the *Dmrt1*<sup>+/*C53R*</sup> XY males from the G<sub>10</sub> generation. Before obtaining the G<sub>9</sub> generation, we back-crossed the homozygous (*Dmrt1*<sup>C53R/C53R</sup>) or heterozygous (*Dmrt1*<sup>+/*C53R*</sup>) individuals six times with the Hd-rR strain to replace the genomic background with the inbred strain (Fig. 1). In a gene-driven mutagenesis library, it has been calculated that each sperm has approximately 2,300 ENU-induced mutations per genome (Taniguchi et al. 2006; Ishikawa et al. 2010). Furthermore, the mutagenesis library has been made using the Cab strain. We found some XX males in this strain.

Medaka germ cells become sexually dimorphic at stage 38, 2 days before hatching (Kobayashi et al. 2004). After hatching, the germ cells in the XY gonad (male type) enter mitotic arrest, whereas the germ cells in the XX gonad (female type) proliferate and enter meiosis (Sato and Egami 1972). Therefore, testicular morphogenesis is distinct from ovarian morphogenesis at 5 days after hatching in normal medaka development. Germ cell numbers at 5 days after hatching differed significantly between the XY and XX individuals; however, there were no significant differences between germ cell numbers in the *Dmrt1*<sup>C53R/C53R</sup> XY mutants and those in the wild-type XY individuals (Fig. 3a).

Histological analyses also demonstrated significant differences between the XY and XX gonads at 5 days after hatching (Table 3, Fig. 3b–e). XX gonads contained gonial germ cells and oocytes, whereas XY gonads contained only gonial germ cells. At 10 days after hatching, both heterozygous (*Dmrt1*<sup>+/*C53R*</sup>) and mutant (*Dmrt1*<sup>C53R/C53R</sup>) XX gonads were similar to the ovary type seen in normal development. These gonads contained gonial germ cells and numerous oocytes at different developing stages in



which the most advanced oocytes had entered the diplotene stage of meiotic prophase (Fig. 3f, g). Conversely, all of the *Dmrt1*<sup>+/C53R</sup> XY gonads (*N*=5) were similar to the developing testis in normal development (Fig. 3h),

whereas the *Dmrt1*<sup>C53R/C53R</sup> XY gonads (*N*=5) were similar to the developing ovary in normal development (Fig. 3i). At 15, 20, and 30 days after hatching, the XX and *Dmrt1*<sup>C53R/C53R</sup> XY gonads were observed to be



**Fig. 3** Histological analyses of the *Dmrt1*<sup>C53R</sup> mutant gonads. The number of germ cells in the *Dmrt1*<sup>C53R/C53R</sup> XY gonads resembled that in the wild-type (*Dmrt1*<sup>+/+</sup>) XY gonads (\**P*<0.01) at 5 days after hatching (a). The gonads at 5 (b–e), 10 (f–i), 15 (j–m), 20 (n–q), and 30 days (r–u) after hatching. The *Dmrt1*<sup>+/C53R</sup> and *Dmrt1*<sup>C53R/C53R</sup> XX gonads were the ovary type. The *Dmrt1*<sup>+/C53R</sup> XY gonads were the testis type; however, the *Dmrt1*<sup>C53R/C53R</sup> XY gonads were the testis type at 5 days after hatching but were the ovary type and contained oocytes at 10, 15, 20, and 30 days after hatching. Black arrowheads indicate gonial-type germ cells; white arrowheads indicate various sizes of oocytes at the diplotene stage. *n* nephric duct, *g* gut, *ed* efferent duct. Scale bar=40 μm

normally developing ovaries filled with many oocytes at various stages of the diplotene stage, with oocytes at the zygotene and pachytene stages and gonial germ cells (Fig. 3j–k, m–o, q–s, u). On the other hand, the *Dmrt1*<sup>+/C53R</sup> XY gonads, which contained gonial germ cells and somatic cells, were similar to the testis type seen in normal development (Fig. 3l, p, t). The somatic cells at 20 and 30 days after hatching formed testis-specific structures—efferent ducts. The *Dmrt1*<sup>C53R/C53R</sup> XY gonads appeared to have fewer oocytes than did the XX gonads.

All homozygous XY mutants developed into egg-laying females, while all heterozygous XY mutants developed into males. By mating these females with males, we could obtain YY individuals containing heterozygous and homozygous mutants in the *Dmrt1* locus. The heterozygous YY individuals developed into male with male secondary sexual characteristics and produced sperm, whereas homozygous YY mutants did not have male secondary sexual characteristics and did not lay eggs. The gonads of homozygous YY mutants contained a few developing oocytes without ovarian cavity (data not shown).

Next, we analyzed the gene expression profile of testis- and ovary-specific genes on the day of hatching and 30 days after hatching (Fig. 4). On the day of hatching, the expression of the testis-specific genes *Dmy* and *Gsdf* was examined by reverse transcriptase (RT)–PCR and real-time quantitative RT–PCR. All

XY individuals expressed *Dmy*, but XX individuals did not (Fig. 4a). The relative mRNA expression level of *Gsdf* in the *Dmrt1*<sup>C53R/C53R</sup> XY embryos was lower than that in the wild-type XY embryos but higher than that in all of the XX embryos (Fig. 4b).

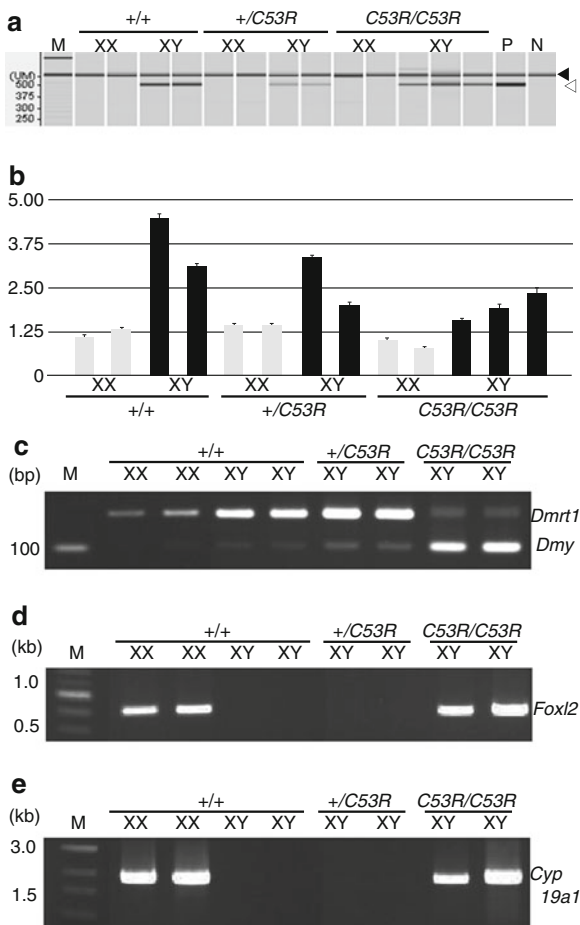
At 30 days after hatching, the expression of the testis-specific genes *Dmy* and *Dmrt1* and the ovary-specific genes *Foxl2* and *Cyp19a1* were also examined by RT–PCR. The *Dmrt1* expression was detected in all samples (Fig. 4c). Wild-type (*Dmrt1*<sup>+/+</sup>) XX and mutant (*Dmrt1*<sup>C53R/C53R</sup>) XY gonads showed lesser expression than did the wild-type XY and heterozygous (*Dmrt1*<sup>+/C53R</sup>) XY gonads. Conversely, *Dmy* expression was detected in only XY gonads. Moreover, the *Dmrt1*<sup>C53R/C53R</sup> XY gonads showed higher expression than did the other wild-type and *Dmrt1*<sup>+/C53R</sup> XY gonads. The wild-type XX gonads expressed both the ovary-specific genes, whereas the expression of these genes was undetectable in the wild-type XY gonads and in the *Dmrt1*<sup>+/C53R</sup> XY gonads (Fig. 4d, e). However, the *Dmrt1*<sup>C53R/C53R</sup> XY gonads expressed both the ovary-specific genes (Fig. 4d, e).

#### Phenotype of mutant carrying *Dmrt1* transgene

To determine whether the sex reversal of the *Dmrt1*<sup>C53R/C53R</sup> XY mutants was caused by the *Dmrt1*<sup>C53R</sup> mutation, we inserted an exogenous *Dmrt1* gene regulated by its own promoter into the genome of *Dmrt1*<sup>C53R/C53R</sup> XY mutant embryos. To this end, we constructed a recombinant BAC clone containing the medaka *Dmrt1* genomic region derived from an inbred Hd-rR strain and *vasa*-GFP driven by a medaka germ cell-specific *olvas* promoter (Tanaka et al. 2001) as a fluorescent reporter gene (Fig. 5a). The BAC was injected into medaka single-cell-staged embryos of a cross between *Dmrt1*<sup>+/C53R</sup> YY males and *Dmrt1*<sup>C53R/C53R</sup> XX females. The *Dmrt1*<sup>+/C53R</sup> YY male was obtained by crossing *Dmrt1*<sup>+/C53R</sup> XY males with *Dmrt1*<sup>C53R/C53R</sup> XY females (Fig. 5b). We obtained

**Table 3** Numbers of specimens measured using histological analyses and the phenotypes at 5, 10, 15, 20, and 30 days after hatching

	Sex chromosome type	<i>Dmrt1</i>	5	10	15	20	30
Ova ovary-type gonads, <i>Tes</i> testis-type gonads	XX	+/ <i>C53R</i>	6 Ova	5 Ova	5 Ova	5 Ova	5 Ova
	XX	<i>C53R/C53R</i>	5 Ova	5 Ova	5 Ova	5 Ova	5 Ova
	XY	+/ <i>C53R</i>	11 Tes	5 Tes	5 Tes	5 Tes	5 Tes
	XY	<i>C53R/C53R</i>	6 Tes	5 Ova	5 Ova	5 Ova	5 Ova



**Fig. 4** Gene expression analyses of the *Dmrt1*<sup>C53R</sup> mutants. **a** Expression of *Dmy* immediately after embryo hatching. The XY embryos expressed *Dmy* immediately after hatching, but the XX embryos did not. P (positive control), Hd-rR adult testis; N (negative control), distilled water. The black arrowhead shows the upper marker, which is used to correct the moving distance of DNA fragments with lower marker, which is the lower position (not shown), in the MultiNA system. The white arrowhead indicates the positions of the *Dmy* fragments. **b** The relative mRNA expression of *Gsdf* in the *Dmrt1*<sup>C53R/C53R</sup> XY embryos immediately after hatching was lower than that in the wild-type (Hd-rR) XY embryos but higher than that in all the XX embryos. **c** *Dmrt1* and *Dmy* expression in the gonads at 30 days after hatching. *Dmrt1* expression was detected in all samples. However, wild-type (Hd-rR) XX and *Dmrt1*<sup>C53R/C53R</sup> XY gonads showed lesser expression than did the wild-type (Hd-rR) XY and *Dmrt1*<sup>+/-</sup> XY gonads. Conversely, *Dmy* expression was detected only in XY gonads. However, the *Dmrt1*<sup>C53R/C53R</sup> XY mutant gonad showed stronger expression than did the other wild-type and *Dmrt1*<sup>+/-</sup> XY gonads. The expression of *Foxl2* (**d**) and *Cyp19a1* (**e**). At 30 days after hatching, the PCR products of ovary-specific genes were detected in the *Dmrt1*<sup>C53R/C53R</sup> XY gonads and wild-type XX gonads but not in the *Dmrt1*<sup>+/-</sup> XY or wild-type XY gonads

one germline-transmitting founder in the F<sub>0</sub> generation, a *Dmrt1*<sup>+/-</sup> XY male. The F<sub>0</sub> XY was crossed with a *Dmrt1*<sup>C53R/C53R</sup> XX female to obtain the F<sub>1</sub> generation. In the F<sub>1</sub> generation, 10 XY individuals and six XX individuals inherited the exogenous *Dmrt1*.

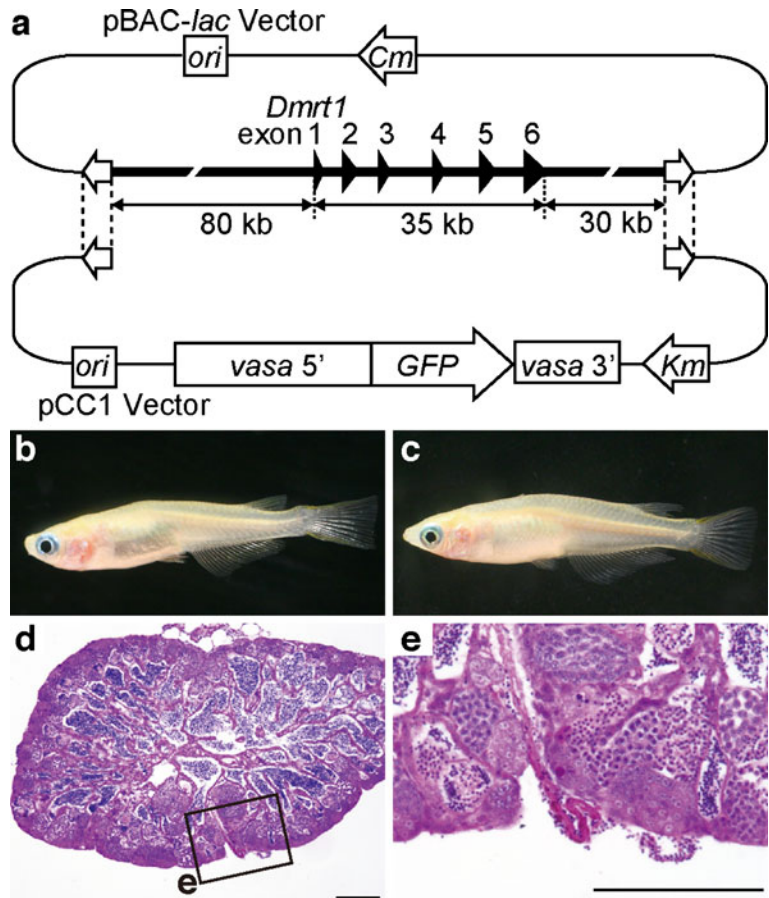
Genotyping of the *Dmrt1* allele in the F<sub>1</sub> individuals could not be achieved because the copy number of exogenous wild-type *Dmrt1* inherited in F<sub>1</sub> was significantly higher than that of endogenous *Dmrt1*, and hid the original *Dmrt1* allele in the genotyping assay in each F<sub>1</sub>. Genotypes of all F<sub>1</sub> were judged as being wild type. Therefore, we established an F<sub>2</sub> genotyping method in which we mated each of the 10 F<sub>1</sub> XY individuals with *Dmrt1*<sup>C53R/C53R</sup> XX females to obtain the next generation (F<sub>2</sub>). Since we could judge transgene inheritance using the GFP fluorescent from *vasa*-GFP in the F<sub>2</sub>, we could select F<sub>2</sub> individuals lacking the transgene to genotype for the *Dmrt1* allele. Based on the F<sub>2</sub> genotypes, we could judge the parental genotypes. Since *Dmrt1*<sup>C53R/C53R</sup> females were used for female parents, when we could not find *Dmrt1*<sup>+/-</sup> F<sub>2</sub> in the offspring, the F<sub>1</sub> parental male was deemed *Dmrt1*<sup>C53R/C53R</sup>. Nine of 10 F<sub>1</sub> XY individuals developed male secondary sex characteristics and were identified as being fertile.

The results from F<sub>2</sub> genotyping revealed that one of the nine F<sub>1</sub> XY individuals was homozygous mutant *Dmrt1*<sup>C53R/C53R</sup> and the remaining eight were *Dmrt1*<sup>+/-</sup> (Table 4). Furthermore, of the F<sub>2</sub> individuals that were obtained from crossing the F<sub>1</sub> *Dmrt1*<sup>C53R/C53R</sup> XY males (F<sub>1</sub> ID=9) with *Dmrt1*<sup>C53R/C53R</sup> XX females, no XY individuals carrying the transgene developed as females and 10 of 17 developed as males (Table 5). On the other hand, all XX individuals carrying the transgene were developed as females.

## Discussion

In this study, we identified and characterized a missense mutant of the medaka *Dmrt1* gene *Dmrt1*<sup>C53R</sup>. The heterozygous (*Dmrt1*<sup>+/-</sup>) XY gonads developed into testes, whereas the homozygous (*Dmrt1*<sup>C53R/C53R</sup>) XY gonads developed into ovaries. Furthermore, the *Dmrt1*<sup>C53R/C53R</sup> XY mutants were fertile, indicating that they underwent a complete male-to-female sex reversal. The influence of the *Dmrt1*<sup>C53R</sup> mutation on the XY individuals

**Fig. 5** Transgenic rescue experiment of *Dmrt1*<sup>C53R/C53R</sup> XY mutants. The structure of the bacterial artificial chromosome (BAC) clone containing the *Dmrt1* genomic region, which encompasses 35 kb of a six-exon region, 80 kb of the upstream region, and 30 kb of the downstream region (a). A pCC1 vector fragment encoding *vasa*-green fluorescent protein and a kanamycin resistance gene was replaced in the pBAC-*lac* vector region of the BAC construct using a homologous recombination system. The *Dmrt1*<sup>C53R/C53R</sup> XY mutants carrying the transgene developed into males. The *Dmrt1*<sup>C53R/C53R</sup> XY mutants have female secondary sexual characteristics (b), whereas the *Dmrt1*<sup>C53R/C53R</sup> XY mutants carrying the transgene are male (c) with a testis (d and e). Scale bar=100 μm



**Table 4** Genotyping of the F<sub>1</sub> males in the transgenic rescue experiment

F <sub>1</sub> male	F <sub>2</sub> individuals (N) <sup>a</sup>		F <sub>1</sub> male genotype
	C53R/C53R	+/C53R	
1	3	5	+/C53R
2	4	4	+/C53R
3	6	2	+/C53R
4	3	5	+/C53R
5	2	6	+/C53R
6	6	2	+/C53R
7	5	3	+/C53R
8	12	4	+/C53R
9	24	0	C53R/C53R
10	0	0	-

<sup>a</sup>F<sub>2</sub> individuals that did not inherit the transgene were obtained by crossing F<sub>1</sub> males with mutant *Dmrt1*<sup>C53R/C53R</sup> females

shows that *Dmrt1* is necessary for testicular development. To determine whether the sex reversal of the *Dmrt1*<sup>C53R/C53R</sup> XY mutants was caused by disrupted *Dmrt1* function, we inserted an exogenous *Dmrt1* gene into the *Dmrt1*<sup>C53R/C53R</sup> XY mutants. The resulting *Dmrt1*<sup>C53R/C53R</sup> XY “male” carrying the exogenous *Dmrt1* was fertile, revealing that *Dmrt1* is indispensable for testicular development in medaka. This function is the same as that of mouse *Dmrt1* (Raymond et al. 2000) and chicken *DMRT1* (Smith et al. 2009), suggesting the functional conservation of *DMRT1* among vertebrates.

**Table 5** Secondary sexual characteristics of F<sub>2</sub> progeny that inherited the transgene in the transgenic rescue experiment

Sex chromosome type	Male	Female	Not judged
XX	0	6	-
XY	10	-	7 <sup>a</sup>

<sup>a</sup>One of these gonads contained oocytes, whereas the others were immature and could not be decided on testes or ovaries

Steroid hormone or high temperature treatments have induced complete sex reversals. These facts have indicated that an XY bipotential gonad can develop into not only a testis but also a complete ovary in medaka (Yamamoto 1958; Sato et al. 2005; Hattori et al. 2007). Although our XY *Dmrt1* mutant finally developed into a female, in the early stage before 5 days after hatching, the germ cell number was similar to that of wild-type XY gonads, and the expression patterns of the testis-specific genes were also more similar to that of the wild-type XY gonads than the XX gonads. We found only gonial germ cells in XY mutant gonads at 5 days after hatching, whereas we found oocytes at the diplotene stage in mutant XY gonads that were similar to XX gonads at 10 days after hatching. These observations demonstrate that prior to 5 days after hatching, XY mutant gonads develop along the testis pathway. Conversely, by 10 days after hatching, the gonads of the *Dmrt1*<sup>C53R/C53R</sup> XY mutants have already commenced development along the female pathway. This delayed commencement of ovarian development may cause the low number of oocytes in XY mutant gonads compared to those in XX gonads.

An XY bipotential gonad develops into an ovary when fertilized XY eggs are immersed in estrogen-containing water. However, this estrogen treatment does not affect *Dmy* expression (Scholz et al. 2003; Suzuki et al. 2005). In these estrogen-treated XY gonads, significant increases of germ cell numbers have not been observed before hatching and at 1 and 2 days after hatching, whereas they have been confirmed at 3 days after hatching (Suzuki et al. 2005). On the other hand, *Dmy* dysfunction also leads XY bipotential gonads to the female pathway by blocking *Dmy* translation and mutations (Shinomiya et al. 2004; Otake et al. 2006; Paul-Prasanth et al. 2006). The germ cell numbers of these XY gonads are similar to those of XX gonads before hatching (Paul-Prasanth et al. 2006). Therefore, the transdifferentiation process from testis to ovary induced by *Dmrt1* mutation differs from that by estrogen treatment and that by *Dmy* dysfunction. The timing of transdifferentiation in *Dmrt1*-mutant XY gonads is later than that in estrogen-treated XY gonads.

At hatching, *Dmy* mRNA expression in heterozygous XY embryos was a little lower than those in wild-type and mutant XY embryos (Fig. 4a). However, the germ cell numbers of the heterozygous XY embryos

were not affected at 5 days after hatching (Fig. 3a), suggesting that *Dmy* function was not affected at the mRNA level. Heterozygous *Dmrt1* status may affect *Dmy* promoter activity.

Mutant XY gonads at 30 days after hatching were comparable to complete ovaries containing many oocytes at different stages of development and expressing ovary-specific genes (Figs. 3u and 4d, e). However, the testis-specific gene *Dmy* was expressed at a level that was markedly higher than those of wild-type or heterozygous XY gonads, whereas expression of the testis-specific gene *Dmrt1* was very low (Fig. 4c). This is not unusual because *Dmy* expression has been found in the follicle layer of estrogen-induced adult XY ovaries (Suzuki et al. 2005), suggesting that the *Dmy* acts on somatic cells surrounding germ cells regardless of testis or ovary status.

Three genes are known to be expressed with sexual dimorphism in somatic cells surrounding germ cells of XY gonads in medaka. The first gene is *Dmy*, whose mRNA has been detected in XY gonads at stage 36 by using in situ hybridization (Kobayashi et al. 2004). Expression of the second gene, *Gsdf*, at stage 37 is higher in XY gonads than in XX gonads (Shibata et al. 2010). The third gene is *Dmrt1*. However, in situ hybridization has not been able to distinguish between *Dmy* and *Dmrt1* because the hybridization probe for *Dmy* mRNA hybridizes both *Dmy* and *Dmrt1* mRNAs due to their high similarity (93% at the nucleic acid level) (Kobayashi et al. 2004). Although these XY-specific genes are expressed in somatic cells, morphological dimorphisms of somatic cells have not been found between XX and XY gonads.

Morphological sexual dimorphisms are first found in germ cells at this early developmental stage (Sato and Egami 1972; Kobayashi et al. 2004). The sexual dimorphism in the number of germ cells results in two distinct types of germ cell proliferation in gonadal sex differentiation: type I proliferation contributes to a linear increase in the number of germ cells in XY gonads, whereas type II proliferation sustains an exponential increase in germ cells in XX gonads, which lack the *Dmy* gene. In XX gonads, germ cells enter meiosis following type II proliferation (Saito et al. 2007). Since *Dmy* dysfunction causes female-type germ cell proliferation (Paul-Prasanth et al. 2006), it is assumed that a *Dmy* function is to inhibit type II proliferation of germ cells until 5 days after hatching. On the other hand, germ cells in *Dmrt1*-mutant XY

gonads, which have the *Dmy* gene, enter the female pathway at 10 days after hatching. Since the germ cells of normally developing XY gonads in this period are mitotically arrested, one of the presumed *Dmrt1* functions is also restraining type II proliferation of these germ cells.

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