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Mice Mutant in the DM Domain Gene *Dmrt4* Are Viable and Fertile but Have Polyovular Follicles[▽]

Jorune Balciuniene, Vivian J. Bardwell, and David Zarkower*

Department of Genetics, Cell, and Developmental Biology, University of Minnesota, Minneapolis, Minnesota 55455

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Proteins containing the DM domain, a zinc finger-like DNA binding motif, have been implicated in sexual differentiation in diverse metazoan organisms. Of seven mammalian DM domain genes, only *Dmrt1* and *Dmrt2* have been functionally analyzed. Here, we report expression analysis and targeted disruption of *Dmrt4* (also called *DmrtA1*) in the mouse. *Dmrt4* is widely expressed during embryonic and postnatal development. However, we find that mice homozygous for a putative null mutation in *Dmrt4* develop essentially normally, undergo full sexual differentiation in both sexes, and are fertile. We observed two potential mutant phenotypes in *Dmrt4* mutant mice. First, ovaries of most mutant females have polyovular follicles, suggesting a role in folliculogenesis. Second, 25% of mutant males consistently exhibited copulatory behavior toward other males. We also tested potential redundancy between *Dmrt4* and two other gonadally expressed DM domain genes, *Dmrt1* and *Dmrt7*. We observed no enhancement of gonadal phenotypes in the double mutants, suggesting that these genes function independently in gonadal development.

Regulators of sex determination and sexual differentiation are generally not conserved between phyla. An apparent exception that has emerged in the past few years involves genes related to the *Drosophila* sexual regulator *Doublesex* (*Dsx*) (28). Genes sharing a novel DNA binding motif identified in Dsx, the DM domain, have been shown to regulate sexual differentiation in insects and nematodes (1, 4, 12, 19, 22). In particular, DSX and the nematode protein MAB-3 have been shown to control several analogous aspects of sexual differentiation (1, 26) and regulate some analogous genes (2, 27). Moreover, the male isoform DSX-M can functionally replace MAB-3 in *Caenorhabditis elegans* (19). These results have raised the possibility that this gene family has an ancient and conserved function in sexual development.

Mammals have seven DM domain-encoding genes, but functional analysis has been reported for only two. The first, *Dmrt1*, is expressed only in the gonad and is required in mice for several aspects of testicular differentiation (18). *Dmrt1* is expressed testis specifically in a variety of vertebrates with different primary sex determination mechanisms, and a recently duplicated copy of *Dmrt1* (*DMY*/*Dmrt1bY*) has become the Y-linked testis-determining gene in the Medaka fish (14, 16). By contrast, *Dmrt2* does not appear to play a role in gonadal development or sexual differentiation. *Dmrt2* null mutant mice have defects in segmentation and die perinatally of lung defects but do not have obvious defects in gonadal development or signs of incomplete sexual differentiation at the time of death (21).

Based on the few examples studied so far, it is unclear whether DM domain genes comprise a family of general developmental regulators (e.g., *Dmrt2*) or are primarily involved in sexual differentiation (e.g., *Dsx*, *mab-3*, and *Dmrt1*). To better define the functional repertoire of these genes in mammals, we conducted a mutational analysis of the murine *Dmrt* genes. Here we report the targeted disruption of *Dmrt4*. *Dmrt4* is widely expressed in embryos and adults. *Dmrt4* mutants are viable and fertile, with no obvious anatomical defects, but ovaries of mutant females have elevated numbers of polyovular follicles. Thus, *Dmrt4* appears to play a role in folliculogenesis. In addition, 25% of mutant males attempted to copulate with other males, suggesting a possible behavioral abnormality.

MATERIALS AND METHODS

Animals. Mice were maintained under controlled temperature and a 12-h dark/12-h light cycle; protocols were approved by the University of Minnesota Institutional Animal Care and Use Committee. Animals were of a mixed C57BL/6J and 129/S1 background, unless indicated otherwise. Pups were weaned at 21 to 28 days and group housed by sex except as noted. Behavioral tests were conducted during hours 4 to 8 of the light cycle.

Generation of *Dmrt4* mutant animals. *Dmrt4* was disrupted by homologous recombination in CJ7 embryonic stem (ES) cells using the targeting vector pJB15; details of the targeting vector are available on request. Southern blotting probes for identification of targeted ES cells were made by PCR using the following DNA primers: for the 5′ probe (SP1), 5′-GAGTTTCTGTGTCACCA GCA-3′ (forward) and 5′-TGATGCTCTACTTTCCTGAA-3′ (reverse); for the 3′ probe (SP2), 5′-TTATGATGCGTTATGTAGTC (forward) and 5′-GATAA GTAATTCCATCCCAA-3′ (reverse).

A correctly targeted ES cell clone was injected into C57BL/6J blastocysts, and the resulting chimeras were bred to generate animals heterozygous for the floxed allele $Dmrt4^{loxP}$. Subsequently, the DM domain was deleted by breeding to β-actin Cre recombinase mice (11) (gift of Mark Lewandoski) to generate animals carrying the deleted putative null allele $Dmrt4^{\Delta}$.

Genotyping. Genotyping of the *Dmrt4* wild-type and deleted alleles was performed by PCR using a mixture of four primers: P1, 5′-GAGAAAGATTCAT CCTCCCT-3′; P2, 5′-AGATCTGCAGTTTTGACAAC-3′; P3, 5′-GAGCCGG TCAGTCCCAACTT; and P4, 5′-CCGGTTTCCTGTGCAAGAAC-3′. PCR conditions were 94°C for 5 min and 35 cycles of 94°C for 45 s, 52°C for 45 s, and 72°C for 1 min, with a final extension step at 72°C for 10 min.

RT-PCR. Tissues were harvested and stored in liquid nitrogen prior to RNA extraction. Total RNA was extracted from adult mouse tissues using Trizol reagent according to the manufacturer's protocol (Invitrogen Corporation). cDNA was synthesized using a Superscript II polymerase kit (Invitrogen). Reverse transcription-PCR (RT-PCR) primers for genes tested were designed to

* Corresponding author. Mailing address: Department of Genetics Cell Biology and Development, 6-160 Jackson Hall, 321 Church St. SE, Minneapolis, MN 55455. Phone: (612) 625-9450. Fax: (612) 626-7031. E-mail: zarko001@umn.edu.

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span an intron. Primers were as follows: 5′-GGAGCCGGTCAGTCCCAACT-3′ (forward) and 5′-AATGTAGTCTCTGGCCCAC-3′ (reverse) for *Dmrt4*; 5′-CA TTCAGAGAGAAAGATCGC-3′ (forward) and 5′-GAGAAAGACCTGGGA CTGTC-3′ (reverse) for the alternative *Dmrt4* RNA initiated from upstream (*altDmrt4*). PCRs were performed using the following conditions: 94°C for 5 min and 35 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s, with a final extension step at 72°C for 10 min. Primers and PCR conditions for *Hprt* cDNA were as previously described (10).

Histology. Organs and tissues from adult animals were fixed in 10% neutral buffered formalin and were paraffin embedded. Sectioning and hematoxylin-eosin staining were performed by the University of Minnesota Cancer Center Histopathology Core using standard protocols. Whole-mount immunohistochemistry was performed on embryonic day 11.5 (E11.5) embryos fixed with 4% paraformaldehyde (5) using anti-neural cell adhesion molecule (NCAM) antibody (AB5032; Chemicon International) at a dilution of 1:500. Necropsy was performed by the University of Minnesota Cancer Center Histopathology Core, which sectioned and examined major organs.

Skeletons of wild-type and *Dmrt4* mutant pups at postnatal day 1 were stained with alizarin red and alcian blue as previously described (5).

Fertility test. We established long-term breeding pairs for two homozygous mutant and two wild-type females with CD1 males as well as two mutant and two wild-type males with CD1 females, starting at 8 to 10 weeks of age. Numbers and sizes of litters were recorded for 1 year.

Rotarod test. Motor coordination and balance were assessed by performance on a rotarod. After an initial 5-min trial on a rotarod rotating at a constant speed of 5 rpm, a 5-min test session was performed with acceleration from 4 rpm to 40 rpm. The trial and the first test were conducted on the same day at least 1 h apart. A second test was performed on the following day.

Buried food retrieval test. General olfactory function was investigated based on the ability to find hidden food. Food was withheld from male and female test animals for 16 h prior to the test to increase motivation. Asteroid-shaped Cheetos (Frito-Lay) were randomly buried 3 cm under clean bedding, and the test animal was placed into the cage. Time required to retrieve the Cheeto was recorded.

Intermale behavior test. This test was based on a "resident-intruder" paradigm. Sexually naive 8- to 12-week-old males (wild type, heterozygous, or *Dmrt4* homozygous mutant) were used as "residents." Resident males were individually housed for 1 week prior to the test session, with bedding unchanged for 3 to 4 days to allow establishment of territory. Eight-week old males of the passive A/J strain were then introduced as "intruders." The intruder was placed into the resident's cage for a maximum of 10 min, and the session was filmed. The intruder was immediately removed if an aggressive attack occurred. Recordings were coded prior to analysis of behaviors. We scored the latency to the first aggressive attack and the number and duration of all nonaggressive mounts. A mount was scored as strongly sexual if a resident approached an intruder from behind, grasped it with its front paws, and exhibited a strong spinal curvature with rapid pelvic thrust.

Male olfactory preference test. The bedding preference test was essentially as previously described (3). The test was performed in a large Plexiglas cage (45 by 25 cm by 21 cm deep) containing three color-coded bowls (7.5-cm diameter) placed 15 cm apart. Sexually naïve 8- to 12-week-old males of each genotype were tested. On two consecutive days prior to the test, the animals were habituated for 10 min in the test cage containing bowls filled with clean bedding. For testing, bowls were filled with clean bedding or soiled bedding was collected immediately prior to testing from group-housed adults of each sex whose cages had not been changed for 1 week. Test sessions were video recorded, and time spent investigating each bedding type was assessed by an investigator blind to the content of each bowl.

Maternal aggression and pup retrieval tests. Sexually mature females were paired with wild-type males for 10 days and then housed singly. Litters were culled to six pups, and maternal aggression was tested on postpartum days 6 to 8. To avoid injuries, pups were removed from the cage just before the test. A wild-type "intruder" male was placed into the cage for 10 min. The intruder was a group-housed male of a mixed background. The test sessions were video recorded. The number of females that exhibited aggression toward the intruder was recorded. Immediately after the maternal aggression test, pups were returned to the home cage in a scattered manner. The time to retrieve the pups to the nest was recorded.

Statistical analysis. One-way analysis of variance (ANOVA) was used to analyze significance of differences between the means of three genotypic groups for the majority of the measurements (rotarod, food retrieval, bedding preference test, etc). A chi-square test was used to calculate *P* value for differences in incidence of male-male mounting among the three genotypic groups.

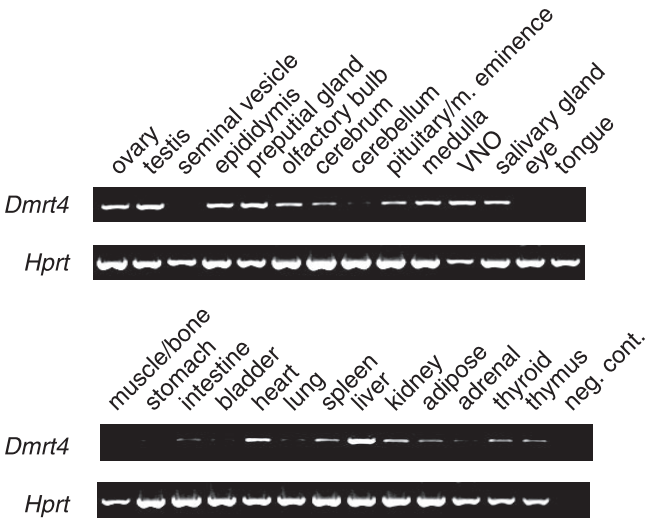

FIG. 1. *Dmrt4* adult mRNA expression. RT-PCR analysis of cDNA from the adult tissues indicated, performed as described in Materials Methods. Equal amounts of cDNA were amplified with primers directed against *Dmrt4* and *Hprt*. m. eminence, median eminence; neg. cont., negative control (no cDNA template).

RESULTS

***Dmrt4* mRNA is widely expressed in adult tissues.** During embryogenesis murine *Dmrt4* mRNA is expressed in a variety of tissues including testis, ovary, brain, heart, and kidney (10). Postnatal expression of murine *Dmrt4* has not been described, so we assayed expression in adult tissues by RT-PCR (Fig. 1). As in the embryo, *Dmrt4* mRNA was expressed in most adult tissues tested. The highest mRNA expression was observed in ovary, testis, epididymis, preputial gland, vomeronasal organ (VNO), liver, salivary glands, and heart. *Dmrt4* also is expressed throughout the brain, with higher expression in the olfactory bulbs and medulla.

Conditional targeting of *Dmrt4*. To test the function of *Dmrt4*, we disrupted the gene by homologous recombination in ES cells, as diagrammed in Fig. 2. The DM domain has been shown to be essential for all functions of several genes containing this motif (4, 18, 19). We therefore deleted exon 1, which contains the DM domain, as well as 1.2 kb upstream of the translational start site (including the 5′ untranslated region, transcriptional start, and proximal promoter) and 450 bp of intron 1. The resulting mutant allele is designated $Dmrt4^{\Delta}$.

RT-PCR confirmed that $Dmrt4^{\Delta/\Delta}$ homozygotes do not express the major DM domain-encoding *Dmrt4* mRNA (data not shown). However, a *Dmrt4* cDNA lacking the DM domain (from E14.5 retina) has been described (BU924300), and the genomic sequences encoding this RNA were not removed by our deletion. This alternative RNA initiates ~9 kb upstream of *Dmrt4* exon 1, presumably under control of an alternative upstream promoter, and splices into *Dmrt4* exon 2, bypassing the DM domain. We investigated the expression of this RNA in tissues of wild-type and $Dmrt4^{\Delta/\Delta}$ adults by RT-PCR (Fig. 3). In wild-type animals, the DM domain-less RNA was detectable only in testis and medulla oblongata (brain stem). However, in $Dmrt4^{\Delta/\Delta}$ mutants the alternative RNA was ectopically expressed in all brain regions tested, including the olfactory bulbs

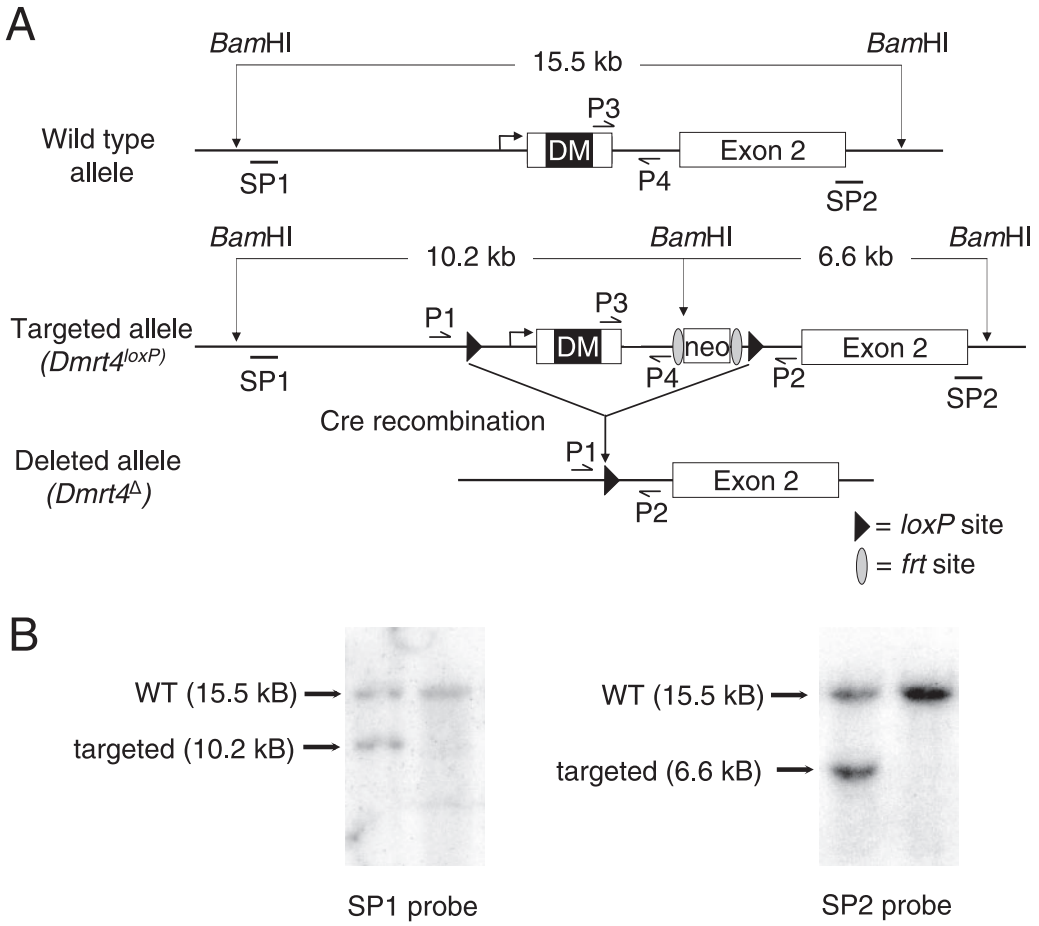

FIG. 2. Disruption of *Dmrt4* by homologous recombination. (A) Targeting strategy. Homologous recombination of targeting vector pJB15 inserts a *loxP* site (recognition site for Cre DNA recombinase; black triangles) upstream of the *Dmrt4* transcriptional start site and a second *loxP* site into intron 1. A neomycin positive selection cassette is inserted into intron 1 and is flanked by *frt* sites (gray ovals), which are recognized by Flpe DNA recombinase. Homologous recombination generates the targeted allele $Dmrt4^{loxP}$ and introduces a new BamHI site, as indicated. Breeding to Flpe-expressing mice can delete the neomycin cassette (not shown). Breeding to Cre-expressing mice deletes sequences between the *loxP* sites, generating the putative null allele $Dmrt4^{\Delta}$. SP1 and SP2 denote probes used for Southern analysis of neomycin resistant clones. P1, P2, P3, and P4 are PCR primers used to genotype animals. (B) Homologously targeted ES cell clone. Southern blots of ES cell DNA digested with BamH1 and probed with SP1 (left panel) and SP2 (right panel) probes. Both probes detect the 15.5-kb wild-type BamHI fragment from the untargeted *Dmrt4* allele. In each panel the first lane contains DNA from a correctly targeted clone, as indicated by the presence of a novel 10.2-kb BamHI fragment detected by SP1 and a novel 6.2-kb BamHI fragment detected by SP2. The second lane contains DNA from a control ES cell clone. WT, wild type.

and medulla. It is unknown whether the alternative RNA is translated, but in principle it could encode a 39-amino-acid peptide containing amino acids 251 to 290 of Dmrt4. Based on these results we can conclude that the *Dmrt4* mutation we generated should eliminate all DM domain-dependent functions of the gene. However, we cannot exclude the possibility that the mutant allele may retain DM domain-independent activity or have neomorphic activity.

***Dmrt4* mutants are viable and fertile.** Interbreeding of $Dmrt4^{\Delta/+}$ heterozygotes produced homozygous progeny at the expected frequency, and these animals were of normal size and had no obvious physical abnormalities. As a test of fertility we bred two wild-type and two mutant animals of each sex for 12 months. There was no significant difference in the numbers of litters, average litter sizes, or the lengths of the fertile period between mutant and wild-type males. For females, the average number of litters was 6.5 ± 0.7 for wild type and 9.5 ± 0.7 for $Dmrt4^{\Delta/\Delta}$, with the mutants continuing to produce litters longer than the wild-type females. There was no difference in average numbers of pups per litter between wild-type (7.8 ± 2.2) and mutant females (8.4 ± 0.9). These results indicate that fertility of *Dmrt4* mutants of both sexes is not significantly compromised.

Polyovular follicles in *Dmrt4* mutant ovaries. To look for more subtle phenotypes, we performed full necropsy on an adult wild-type and *Dmrt4* mutant littermate of each sex, including histological examination of all major organs. For tissues and organs with high *Dmrt4* mRNA expression, we examined a larger number of animals. No histopathological abnormalities were evident in most adult tissues examined, including testis, epididymis, salivary gland, and VNO (Fig. 4). However, most *Dmrt4* mutant ovaries sectioned (seven of eight) contained at least one polyovular follicle (Fig. 5) and, in some cases, more than one (Fig. 5D). Most of these were biovular (Fig. 5B to D), but we observed one with five oocytes (Fig. 5A). We also observed polyovular follicles in

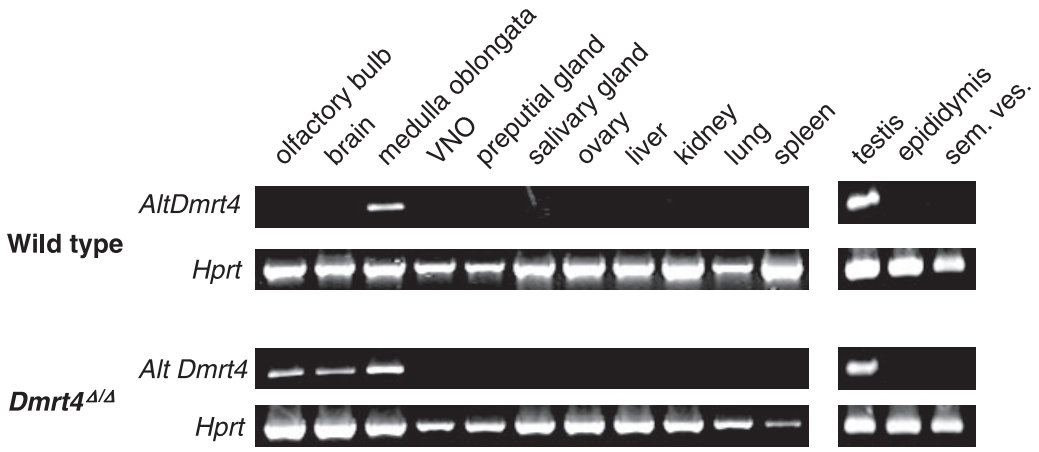

FIG. 3. Expression of *Dmrt4* RNA from an alternative upstream promoter. RT-PCR of cDNA from indicated wild-type and $Dmrt4^{\Delta/\Delta}$ adult tissues, amplified with primers specific for the alternative *Dmrt4* transcript (*altDmrt4*), as described in Materials and Methods, and *Hprt* primers as a positive control. sem. ves., seminal vesicle.

two of six heterozygous ovaries sectioned, whereas we did not observe polyovular follicles in the ovaries of six wild-type females of the same mixed genetic background. These results suggest that our *Dmrt4* mutant allele is haploinsufficient or semidominant for this phenotype.

Skeletal and motoneural development and function. Because *Dmrt2* is required for skeletal patterning, we evaluated skeletal development of *Dmrt4* mutants, comparing skeletal preparations from wild-type and homozygous littermates at postnatal day 1 (Fig. 6). There were no apparent defects in bone or cartilage development. To assess function of the musculoskeletal system and balance, we performed a rotarod test. This revealed no significant difference between wild-type (n = 18), heterozygous (n = 15), or homozygous mutant (n = 23) animals in average latency to falling off the rotarod (111.8 s, 127.3 s, and 103.9 s, respectively; P = 0.2, one-way ANOVA test).

Normal olfactory epithelium formation and general olfaction. *Dmrt4* has been reported to regulate neurogenesis in the olfactory placode during *Xenopus* development, based on morpholino depletion experiments in embryos (6). The olfactory placode and pit form normally in *Dmrt4* mutant mice, and histological examination of adult olfactory tissues revealed no obvious defects. To visualize sensory neurons, we stained embryos at the 35-somite stage with an anti-NCAM antibody. Clustered neurons were present in the sensory epithelium in both wild-type and mutant (Fig. 7), and we did not observe the severe deficit in neurogenesis found in *Xenopus*. To assay general olfactory function, we tested for the ability to locate hidden food. There was no significant difference in average time required to retrieve buried Cheetos between wild type (163 s; n = 21), heterozygotes (279 s; n = 20), and homozygotes (209 s; n = 36) (P = 0.15, one-way ANOVA test). These results indicate that *Dmrt4* is not essential for neurogenesis of olfactory pit epithelium or general olfaction in the mouse, though we cannot exclude mild or transient effects.

Male-male sexual interaction. During husbandry of $Dmrt4^{\Delta/\Delta}$ animals, we noted several episodes of sexual mounting by mutant males on other males with which they were housed. We therefore examined intermale behavior further by conducting a resident-intruder assay (Materials and Methods). We tested 17 wild-type, 27 heterozygous, and 16 homozygous mutant males for interaction with wild-type intruder males of the passive A/J strain. There was no significant difference in latency of investigation by residents of intruders (P = 0.24, one-way ANOVA test), with average latencies of 17 s, 18 s, and 31s for wild type, heterozygotes and homozygotes, respectively. Likewise, the incidence of aggression by the residents did not differ significantly between the three genotypes (59%, 59%, and 44% for wild type, heterozygotes, and homozygotes, respectively). Latency to attack also was similar (310 s, 260 s, and 299 s, respectively; P = 0.6, one-way ANOVA test).

The total frequency of mounting was similar between genotypes, but we observed qualitative differences. Instances of a resident mounting an intruder in any manner were observed in 24% of wild-type, 7% of heterozygous, and 31% of homozygous animals (P = 0.2, chi-square test). The average duration of mounts was 0.027 s/min, 0.016 s/min, and 0.055 s/min for wild type, heterozygotes, and homozygotes, respectively (P = 0.2, one-way ANOVA test). Homozygous mutants differed in the occurrence of mounts scored as clearly sexual (involving clasping with both forelimbs, strong spinal curvature, and rapid pelvic thrusting). None of the wild-type animals exhibited mounting of this type, whereas 2/27 of heterozygotes and 4/16 of homozygotes did (P = 0.05, chi-square test). Although the proportion of animals exhibiting same-sex sexual mounting was low, individuals exhibiting this behavior did so consistently upon repeated testing.

Olfactory function and sexual preference. Olfaction and sexual behavior are strongly linked in mice (9, 20). As described earlier, we observed high *Dmrt4* expression in olfactory tissues including the VNO, which is implicated in male sexual behavior (9, 20, 24). Because we also observed a possible abnormality in sexual behavior, we further investigated olfactory function and sexual preference.

Although the main and accessory olfactory epithelia appeared histologically normal, we considered the possibility that *Dmrt4* might mediate pheromone response by regulating genes required for vomeronasal function. We used RT-PCR to assess mRNA expression of several VNO receptor mRNAs (V1ra1,

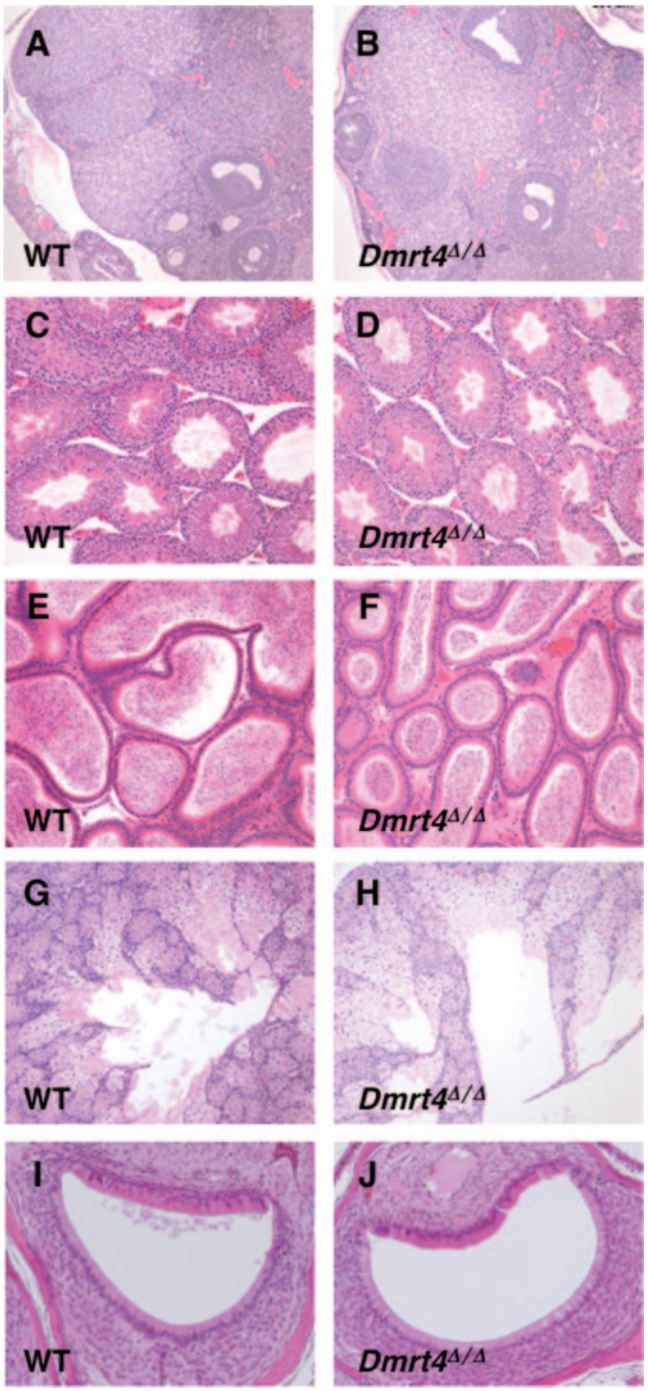

FIG. 4. Histology of wild-type and $Dmrt4^{\Delta/\Delta}$ tissues. Left column shows hematoxylin-eosin-stained sections of wild-type (WT) organs, and right column shows $Dmrt4^{\Delta/\Delta}$ tissues. Tissues are ovary (A and B), testis (C and D), epididymis (E and F), salivary gland (G and H), and VNO (I and J).

V1Ra2, V1rC25, and V2r3) (24) and two G proteins ($G_o\alpha$ and $G_\alpha i2$) (13, 17) involved in olfaction. All of the tested mRNAs were expressed at comparable levels in wild-type and *Dmrt4* mutant VNOs (not shown).

As a test of sexual preference, we assayed the selectivity of males for fresh bedding versus female- or male-soiled bedding. We tested eight wild-type, eight heterozygous, and four *Dmrt4* homozygous mutant animals. As shown in Table 1, males of all genotypes showed a similar preference for female-soiled bedding over fresh or male-soiled bedding. From these data we conclude that *Dmrt4* mutants have relatively normal olfactory detection of pheromones as well as food (described above).

Maternal aggression. In addition to male-specific behavior, we also assayed two aspects of female-specific behavior: maternal aggression and pup retrieval. The percentage of mothers displaying aggression toward a male intruder was similar for wild type (43%; $n = 7$) versus mutants (40%; $n = 10$). Likewise, we observed no differences in time required to retrieve four pups removed from the nest between wild-type (45 s; $n = 7$) and $Dmrt4^{\Delta/\Delta}$ (49 s; $n = 10$) mothers ($P = 0.7$, one-way ANOVA test).

Testing redundancy with other *Dmrt* genes. *Dmrt4* expression overlaps that of several other DM domain genes, including two whose expression is gonad-specific, *Dmrt1* and *Dmrt7*. We tested whether *Dmrt4* might function redundantly with either gene by analyzing the gonadal phenotypes of double mutants. Both *Dmrt1* and *Dmrt7* have defects in testicular differentiation (18; also unpublished results), and we looked for more severe phenotypes in double mutants. Doubly heterozygous *Dmrt4*; *Dmrt1* and *Dmrt4*; *Dmrt7* breeding pairs produced pups of all genotypes at the expected ratios, and litter sizes were normal. Males homozygous for *Dmrt4* and either *Dmrt1* or *Dmrt7* mutations had testicular histology indistinguishable from that of the *Dmrt1* or *Dmrt7* single mutants (data not shown). From the lack of additive phenotypes, we conclude that *Dmrt4* is unlikely to function redundantly with *Dmrt1* or *Dmrt7* in the gonad.

DISCUSSION

We have generated a targeted mouse mutation in *Dmrt4*, removing the DM domain as well as promoter and intronic sequences. This mutation does not abolish all transcription from the *Dmrt4* locus but eliminates all protein isoforms containing the DM domain. Despite its widespread embryonic and postnatal expression, we find that *Dmrt4* is not required for viability or fertility, at least on a mixed genetic background.

Our analysis revealed two potential phenotypes in *Dmrt4* mutants. The first was the presence of polyovular follicles in most mutant ovaries. The origin of polyovular follicles is poorly understood, but it is likely that they arise during folliculogenesis by the incorporation of multiple oocytes into primordial follicles. The defect in *Dmrt4* mutants may therefore reflect inappropriate germ cell-granulosa cell interaction. In this regard, we speculate that *Dmrt4* might play a role in the perinatal ovary roughly analogous to that of *Dmrt1* in the perinatal testis, where *Dmrt1* is required for proper intercalation of gonocytes among Sertoli cells (18). Polyovular follicles occur infrequently in mice and other rodents (25); but they can be induced in mice by forced expression of inhibin-α (15) or by administration of exogenous estrogen (8), and their abundance can be modulated by gonadotropins (7). Because estrogen can affect polyovular follicle formation, we assayed mRNA expression of the estrogen receptors ERα and ERβ but did not observe a difference in *Dmrt4* mutant ovaries (not shown). The conditional *Dmrt4* mutation we generated might allow a more detailed dissection

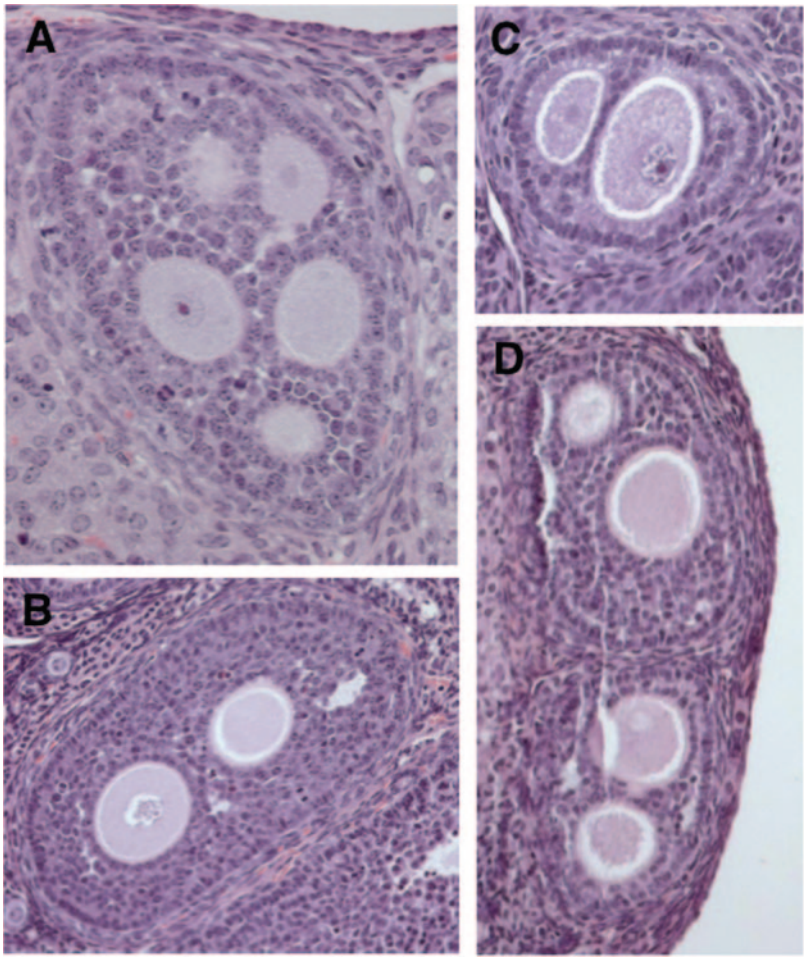

FIG. 5. Polyovular follicles in *Dmrt4* mutant ovaries. Shown are examples of polyovular follicles from *Dmrt4* mutant adult female ovaries. (A) Penta-ovular follicle. (B and C) Biovular follicles. (D) Pair of biovular follicles.

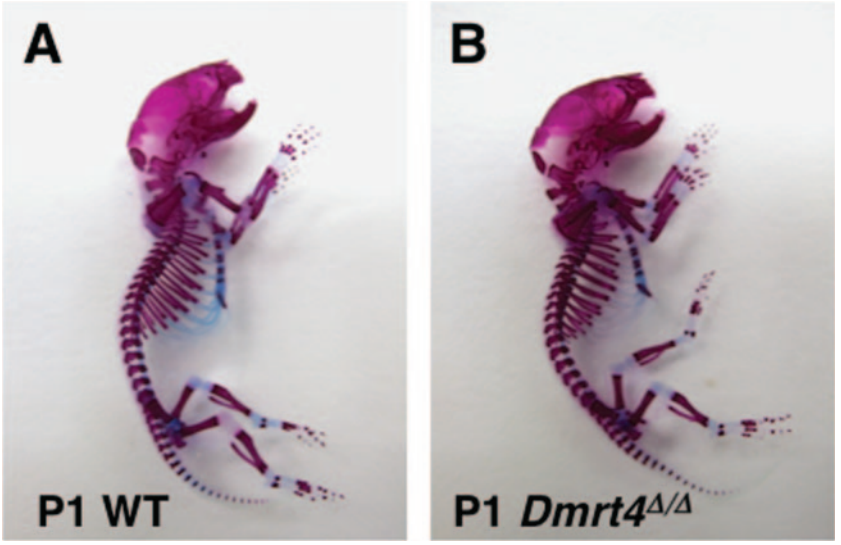

FIG. 6. Skeletal development of *Dmrt4* mutant embryos. Skeletal preparations of 1 day postnatal (P1) wild-type (WT) and *Dmrt4* mutants are shown.

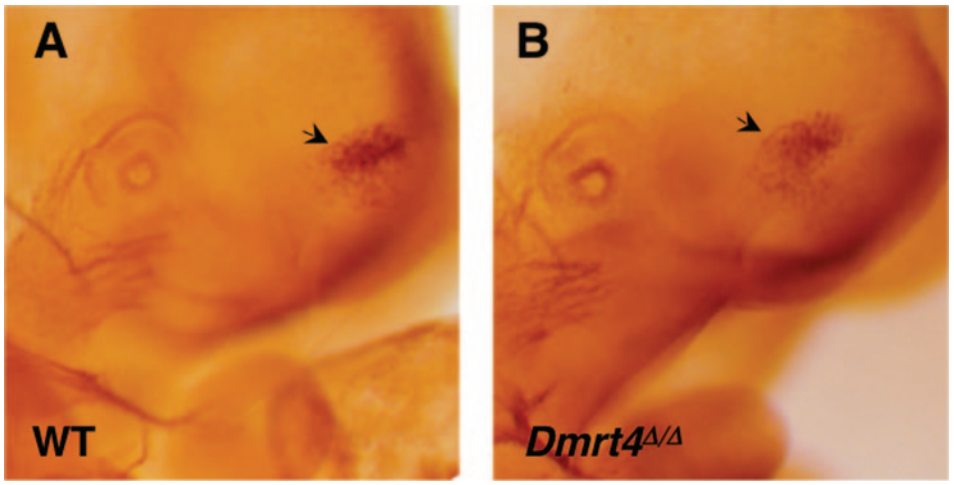

FIG. 7. Olfactory neurons in *Dmrt4* mutant embryos. Whole-mount immunohistochemistry with NCAM antibody staining of E11.5 embryos is shown. An arrow indicates the position of nasal placode olfactory epithelium. Both embryos are at the ~35-somite developmental stage.

of where and when *Dmrt4* expression is required for normal follicle development.

A second potential phenotype we observed was sexual mounting of males by some mutant males. Bedding preference tests indicated that *Dmrt4* mutant males retain a preference for female soiled bedding and that mutant males do mate with females, so the behavior we observed is more likely to represent lower selectivity rather than reversed mate preference. We examined *Dmrt4* mutants for defects in structures and functions linked to sexual behavior, in particular, in the olfactory system, but did not find any. The nonselective mounting behavior we observed resembles that of males mutant in the VNO putative ion channel TRP2 (24). However, *TRP2* mutants lack male-male aggression, which was not significantly effected in *Dmrt4* mutants. Only a minority of mutant males exhibited clearly sexual mounting of other males, but the behavior when present was reproducible and was never observed in wild-type siblings. The mice we tested were on a mixed genetic background, which may affect the penetrance of the phenotype. It will be important to retest *Dmrt4* mutants for this behavior after extensive breeding onto defined backgrounds. If the male-on-male sexual behavior is robust on an inbred background, it may be possible to identify the anatomical focus of the defect by cell-type-specific gene targeting with the conditional *Dmrt4* allele.

In *Xenopus*, morpholino oligonucleotide depletion of *Dmrt4* or overexpression of a C-terminally truncated Dmrt4 protein reduces proliferation of NCAM-expressing neurons in the olfactory placode during embryonic development (6). Moreover, forced expression of *Dmrt4* was sufficient to induce expression of neurogenic markers in cultured *Xenopus* explants. In the mouse, however, the reduction in olfactory placode neurons, if any, was modest during the equivalent period (Fig. 7), and the adult olfactory epithelium of mutants was histologically normal. We also found that a number of markers of olfactory neurons are expressed normally and that mutants have normal olfactory detection of food and pheromones.

There are several possible explanations for this discrepancy. First, the effects of *Dmrt4* depletion in *Xenopus* on adult olfactory placode-derived tissues have not been reported, so it is possible that the reduced embryonic neurogenesis observed in the frog is transient. Second, *Dmrt4* expression differs between frog and mouse (6, 10), and the *Xenopus Dmrt4* expression pattern more closely resembles that of murine *Dmrt3* (23). It may be, therefore, that the functions of these genes have shifted along with their expression patterns during vertebrate evolution. Third, because *Dmrt4* expression overlaps that of *Dmrt3* in the olfactory placode, it is possible that the two genes function redundantly in mice. It will be important to test the phenotype of *Dmrt3*; *Dmrt4* double mutants, and we are currently pursuing those experiments. The extensive expression of *Dmrt4* makes genetic redundancy a possibility in a number of tissues, and it will be important to test this as mutations in other *Dmrt* genes become available.

In summary, we have used ES cell homologous recombination to create a conditional allele and a deleted allele of the murine *Dmrt4* gene and have characterized the phenotype of the deleted allele. We observed defects in the ovary and possibly in male sexual behavior, and these merit more in-depth study in the future, particularly on better-defined genetic backgrounds. However, we can conclude from these studies of mice of mixed background that, despite its widespread expression, *Dmrt4* is not essential for normal viability and fertility.

TABLE 1. Results of test of sexual preference

Genotype	Avg time (s) investigating bedding[a]		
	Fresh bedding	Male-soiled	Female-soiled
WT[b]	30	86	126
$Dmrt4^{\Delta/+}$	35	89	105
$Dmrt4^{\Delta/\Delta}$	43	95	134

[a] *P* values by one-way ANOVA are 0.29, 0.93, and 0.26 for fresh, male-soiled, and female-soiled bedding, respectively.
[b] WT, wild type.

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