Dmrt1, a gene related to worm and fly sexual regulators, is required for mammalian testis differentiation

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The only molecular similarity in sex determination found so far among phyla is between the *Drosophila doublesex* (*dsx*) and *Caenorhabditis elegans mab-3* genes. *dsx* and *mab-3* contain a zinc finger-like DNA-binding motif called the DM domain, perform several related regulatory functions, and are at least partially interchangeable in vivo. A DM domain gene called *Dmrt1* has been implicated in male gonad development in a variety of vertebrates, on the basis of embryonic expression and chromosomal location. Such evidence is highly suggestive of a conserved role(s) for *Dmrt1* in vertebrate sexual development, but there has been no functional analysis of this gene in any species. Here we show that murine *Dmrt1* is essential for postnatal testis differentiation, with mutant phenotypes similar to those caused by human chromosome 9p deletions that remove the gene. As in the case of 9p deletions, *Dmrt1* is dispensable for ovary development in the mouse. Thus, as in invertebrates, a DM domain gene regulates vertebrate male development.

[Key Words: DM domain; *Dmrt1*; sex determination; *doublesex*; *mab-3*]

Received July 15, 2000; revised version accepted September 6, 2000.

In contrast to many developmental processes, for example, body axis establishment or segmentation, genetic and molecular analyses of sex determination have revealed virtually no clear evolutionary conservation between phyla. A potential exception to this lack of conservation is the similarity of the *Caenorhabditis elegans mab-3* gene (Shen and Hodgkin 1988) and the *Drosophila dsx* gene (Baker and Ridge 1980). These genes share a number of properties, suggesting that some mechanisms controlling sexual development may be conserved between nematodes and arthropods (Raymond et al. 1998). Both genes contain the DM domain, a zinc finger-like DNA-binding motif (Erdman and Burtis 1993; Raymond et al. 1998). Functionally, the two genes are also related in several respects: Both directly regulate yolk protein gene transcription (Coschigano and Wensink 1993; Yi and Zarkower 1999), both are required for differentiation of male-specific sense organs (Baker and Ridge 1980; Shen and Hodgkin 1988; Yi et al. 2000), and both mediate male mating behavior (Villegla and Hall 1996; Yi et al. 2000). Furthermore, the male-specific isoform of *dsx*, but not the female-specific isoform, can replace *mab-3* in vivo (Raymond et al. 1998).

DM domain genes have been implicated in vertebrate sexual development by chromosomal location and by embryonic expression. Human *DMRT1* maps to an autosomal locus (9p24.3) that, when hemizygous, is associated with defective testicular development and consequent 46,XY feminization (Crocker et al. 1988; Hoo et al. 1989; Bennett et al. 1993; Veitia et al. 1997, 1998; Flejter et al. 1998; Guioli et al. 1998; Raymond et al. 1998). In birds, which have ZZ/ZW sex determination, *Dmrt1* is found on the Z chromosome (Nanda et al. 1999), again suggesting that two doses of *Dmrt1* (ZZ=male) might be necessary for testis development. In all vertebrates examined, *Dmrt1* is expressed in the differentiating male genital ridges and adult testis, including mammals, birds, and reptiles with temperature-dependent sex determination (Raymond et al. 1999a; Smith et al. 1999; De Grandi et al. 2000; Kettlewell et al. 2000; Moniot et al. 2000). In most vertebrates, including humans, *Dmrt1* expression is higher in genital ridges destined for testis differentiation, starting very early in development, suggesting a likely role in male sex determination or in later testis differentiation. In mouse, *Dmrt1* mRNA is expressed in the genital ridges of both sexes and then becomes testis specific at the end of the sex-determining period. This expression is consistent with a sex-determining role in either sex, or with a later role in testis differentiation, or both.

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Article and publication are at www.genesdev.org/cgi/doi/10.1101/gad.834100.

Taken together, the expression profiles and chromosomal locations of *Dmrt1* in different species strongly suggest that it plays a conserved role(s) in vertebrate male gonad development. However, no functional data have been reported from any vertebrate. To test the role of *Dmrt1* in a mammal, we have generated a deletion allele of the murine *Dmrt1* gene. We find that *Dmrt1* is required for multiple aspects of testis differentiation and is not required for ovary development.

Results and Discussion

Targeted deletion of *Dmrt1*

We generated a targeted deletion in the murine *Dmrt1* gene using the strategy diagrammed in Figure 1A. Because the DM domain is essential for function of MAB-3 and DSX (Shen and Hodgkin 1988; Erdman and Burtis 1993; Raymond et al. 1998), we removed the exon encoding this domain of *Dmrt1*, plus the predicted transcriptional start site and part of the first intron (Fig. 1B). Transcription from the mutant allele is greatly reduced, although a low level can be detected by RT-PCR (Fig. 1C). On the basis of cDNA sequencing and analysis of 5' RACE products (data not shown), the deletion removes the major transcriptional start site and ~300 bp of promoter sequence, so the remaining RNAs presumably originate from a minor upstream start site. Because these transcripts lack the DM domain, they should not be able to encode a functional *Dmrt1* protein. In addition, immunohistochemistry (described below) demonstrates that the mutant allele does not express detectable *Dmrt1* protein. This deletion mutation, *Dmrt1*⁻, therefore appears to be a null allele.

Dmrt1 is required for testis but not ovary differentiation

The *Dmrt1*⁻ mutation causes severe defects in the adult testis (Fig. 2). Although heterozygous *Dmrt1*^{+/-} males

have normal testes (Fig. 2A,B) and are fertile, homozygous *Dmrt1*^{-/-} mutants have severely hypoplastic testes (Fig. 2A). Microscopic examination reveals that *Dmrt1*^{-/-} seminiferous tubules are disorganized and germ cells are missing (Fig. 2C,D; see legend for details). There is evidence of fatty degeneration of interstitial (Leydig) cells with infiltration by macrophages. No ectopic ovarian tissue or Mullerian duct-derived structures are apparent in *Dmrt1*^{-/-} males. Thus, the murine *Dmrt1* null phenotype, like that of human 9p monosomy (Ogata et al. 1997; Guioli et al. 1998; Ion et al. 1998; Veitia et al. 1998), is a failure of testis differentiation, accompanied by germ cell death, rather than a transformation of testis to ovary. Although *Dmrt1* mRNA is expressed in the XX genital ridge during early gonadal development, XX *Dmrt1*^{+/-} and *Dmrt1*^{-/-} mutant mice, like XX humans with 9p deletions, have normal ovaries and are fertile (data not shown). This indicates either that *Dmrt1* does not function in ovary development in the mouse or that any role it plays is functionally redundant.

Pre-meiotic germ cell death in *Dmrt1*^{-/-} mutant testes

Testis development can be divided into three stages as follows: formation of the undifferentiated or indifferent gonad; commitment to testis rather than ovary development; and differentiation into a functional testis. Clearly, testis differentiation is severely defective in *Dmrt1*^{-/-} mutants. To determine when defective testis development begins, we examined earlier stages. Prior to postnatal day 7 (P7), *Dmrt1*^{-/-} testes appear grossly normal relative to *Dmrt1*^{+/-} littermates (Fig. 3A,B; data not shown). However, by P10, just after meiosis normally begins, germ cell numbers in *Dmrt1*^{-/-} testes are greatly reduced (Fig. 3C,D). Levels of apoptosis appear normal in *Dmrt1*^{-/-} testes (not shown), so this may represent re-

Figure 1. Strategy for producing the *Dmrt1* deletion mutant. (A) Diagram of *Dmrt1* wild-type and mutant alleles. Homologous recombination of the targeting vector with the wild-type *Dmrt1* allele (*Dmrt1*⁺) resulted in the loxP-flanked (floxed) allele *Dmrt1*^{neo}. This allele also contains within the first intron a neomycin-resistance cassette (*Pgk-neo*) flanked by FLP recombinase recognition sites (*frt* sites). Mice heterozygous for the *Dmrt1*^{neo} allele were mated with transgenic mice expressing Cre recombinase under the control of a β -actin promoter (Lewandoski et al. 1997), resulting in deletion of the sequences between the two loxP sites, including the major transcriptional start, the first exon (containing the DM domain), *Pgk-neo*, and part of the first intron. The resulting deletion allele is called *Dmrt1*⁻. (B) Genotyping of *Dmrt1* alleles. PCR of genomic DNA from wild-type *Dmrt1*^{+/+}, heterozygous *Dmrt1*^{+/-} and homozygous *Dmrt1*^{-/-} animals is shown. For each animal, PCR reactions were run to test for each allele (CR92/CR99 to detect *Dmrt1*⁺, and KOS1/KOS3 to detect *Dmrt1*⁻), and the products were mixed and resolved by gel electrophoresis. (C) RT-PCR of *Dmrt1* mRNA. RT-PCR of E13.5 testis mRNA was performed using primers from exons 3 and 4, 3' to the region deleted by the *Dmrt1*⁻ mutation (CR132/CR133; see Materials and Methods). *Hprt* mRNA is a positive control.

Figure 2. *Dmrt1* mutant testes are severely dysmorphic. (A) Adult testis size is reduced in *Dmrt1*^{-/-} testes (two large testes at left) relative to *Dmrt1*^{+/-} (four small testes at right). Testes are from 10-month-old adults. (B–D) Sections of *Dmrt1*^{+/-} and *Dmrt1*^{-/-} testes stained with hematoxylin and eosin. (B) Adult (16 weeks postpartum) *Dmrt1*^{+/-} testis, showing normal morphology. (C) *Dmrt1*^{-/-} testis at 6 wk post partum. Seminiferous tubules are present and contain immature Sertoli cells, but are devoid of germ cells. There are numerous Leydig cells. (D) Example of adult (16 weeks postpartum) *Dmrt1*^{-/-} testis. Small numbers of seminiferous tubules are separated by moderate numbers of interstitial cells, some undergoing vacuolation and fatty degeneration. There is extensive infiltration by cells that often contain light brown pigment (ceroid/lipofuscin). These are considered to be of macrophage and/or interstitial cell origin. Degenerating seminiferous tubules are attenuated and contain variable numbers of immature Sertoli cells, foamy macrophages, and as yet unidentified large eosinophilic cells, but are devoid of identifiable spermatogonia and spermatids.

duced germ cell proliferation. The germ cells still present at P10 remain in the center of the seminiferous tubules, indicating that *Dmrt1* is required for the germ cell movements that occur around P7 (see also below). In contrast to *Dmrt1*^{+/-} (Fig. 3C), no meiotic germ cells are evident in the *Dmrt1*^{-/-} testis (Fig. 3D).

By P14, germ cells are absent from the *Dmrt1*^{-/-} mutant testis and the seminiferous tubules, which are filled with abundant immature Sertoli cells, lack a luminal space (Fig. 3E,F). This morphology is quite distinct from the hollow tubules containing normal numbers of differentiated Sertoli cells that are typical of other mutants lacking germ cells, such as *c-kit* (Fig. 3G) or *Dhh* (Bitgood et al. 1996). In addition, Sertoli cells appear to overproliferate in *Dmrt1*^{-/-} mutants. At this stage, the testes of *Dmrt1*^{-/-} and *c-kit* mutants are approximately the same size and contain similar densities of seminiferous tubules (data not shown). However, the number of Sertoli cells in each cross section of seminiferous tubule is elevated in *Dmrt1*^{-/-} relative to either *Dmrt1*^{+/-} or *c-kit* (Fig. 3E–G).

The seminiferous tubule morphology we observe in *Dmrt1*^{-/-} mice resembles phenotypes reported in human infants with deletions of 9p24.3 in several respects. Although detailed histology has only been reported for a small number of human 9p deletion cases, several features have been noted that are similar to the *Dmrt1*^{-/-}

phenotype. These features include reduced testis size (Ogata et al. 1997; Guioli et al. 1998), immature Sertoli cell morphology (Ion et al. 1998), germ cells absent or greatly reduced in number (Hoo et al. 1989; Veitia et al. 1997; Ion et al. 1998) and seminiferous tubules disorganized or lacking a central lumen (Veitia et al. 1997; Ion et al. 1998). These phenotypic similarities to *Dmrt1*^{-/-} mutants suggest that reduced *DMRT1* expression may be at least partly responsible for the defective testis differentiation caused by distal 9p deletions in humans. A direct comparison of human and mouse phenotypes is not possible currently, as extensive searches have failed to identify point mutations in human *DMRT1* (Raymond et al. 1999b; Calvari et al. 2000; Ottolenghi et al. 2000). In addition to *DMRT1*, 9p deletions may remove other genes involved in testis development. However, no correlation is apparent between the size of 9p deletions and the severity of the resulting phenotype. Thus, if testis defects resulting from 9p hemizyosity are caused by combined loss of *Dmrt1* and another gene on 9p, the two genes must be tightly linked. The 9p23–9p24 region contains several other DM domain genes (Ottolenghi et al. 2000; C.S. Raymond, J.R. Kettlewell, and D. Zarkower, unpubl.). It will be important to determine whether these or other genes affected by 9p deletions also are required for testis development.

Figure 3. Germ cell death and Sertoli cell over-proliferation in *Dmrt1* mutant testes. Sections of *Dmrt1*^{+/-} and *Dmrt1*^{-/-} testes of littermates stained with hematoxylin and eosin. (A) P1 *Dmrt1*^{+/-} testis, showing seminiferous tubules with Sertoli cells (small dark blue nuclei) around periphery, and germ cells (larger round red nuclei; arrowheads) in center. (B) Section of P1 *Dmrt1*^{-/-} testis, with normal morphology. Germ cells are present in normal numbers (arrowheads). (C) P10 *Dmrt1*^{+/-} testis. Germ cells have migrated to the periphery (arrowheads). Meiosis has begun and differentiating germ cells (round dark blue nuclei) are visible in some tubules. (D) P10 *Dmrt1*^{-/-} testis. Sertoli cells have begun to over-proliferate. Germ cells are nearly absent, and the few remaining germ cells have failed to migrate to the periphery of the seminiferous tubules (a few germ cells are visible in this section; two are indicated by arrowheads). No meiosis is detectable. (E) P14 *Dmrt1*^{+/-} testis, showing meiotic germ cells (round dark blue nuclei) within seminiferous tubules. (F) P14 *Dmrt1*^{-/-} testis. Immature Sertoli cells are present and have overproliferated, whereas germ cells are absent. (G) P14 *c-kit*^{W/W-v} mutant testis. Germ cells are absent, as in *Dmrt1*^{-/-} testis, but differentiated Sertoli cells are present in normal numbers.

Dynamic expression of *Dmrt1* protein

Dmrt1 mRNA is expressed both in germ cells and in Sertoli cells (Raymond et al. 1999a). Thus, the germ cell

death caused by mutation of *Dmrt1* could reflect either a defect in the germ cells themselves or a defect in Sertoli cells, which promote germ cell survival and differentiation (Rassoulzadegan et al. 1993; Bitgood et al. 1996). To

compare Dmrt1 protein expression with *Dmrt1* mRNA expression, we generated an antibody against the C-terminal portion of the protein. Immunohistochemistry reveals that in the embryonic testis Dmrt1 protein accumulates primarily in Sertoli cell nuclei, with little or no expression detectable in germ cells (Fig. 4A). Starting at P1, Dmrt1 levels rise in germ cells and reach high levels by P7, just before meiosis begins (Fig. 4B–D). The similar timing of increased Dmrt1 expression in germ cells and the onset of germ cell death in the *Dmrt1*^{−/−} mutant testis suggests a possible cell-autonomous function for Dmrt1 in maintaining the germ line. From P7 through adult stage, Dmrt1 protein is present in Sertoli cells and undifferentiated germ cells, but not in differentiating germ cells (Fig. 4E; data not shown). In adult testis, Dmrt1 is expressed in Sertoli cells in all regions of the seminiferous tubules, but is expressed dynamically in premeiotic germ cells (spermatogonia), with high expression only in regions of the seminiferous tubule that are early in the spermatogenic cycle (Fig. 4G). This further suggests that *Dmrt1* may play a role in pre-meiotic germ cells, for example, regulating entry to meiosis or controlling the mitotic cell cycle. The antibody also confirms that no Dmrt1 protein is detectable in *Dmrt1*^{−/−} testes, demonstrating that this is a null allele (Fig. 4F).

Failure of Sertoli cell differentiation in *Dmrt1*^{−/−} testes

Dmrt1^{−/−} Sertoli cells overproliferate, fail to adopt a differentiated morphology, and then die postnatally. To characterize these phenotypes further, we examined expression of several Sertoli cell markers. Prior to P7, no defects were apparent. For example, the early marker Gata-4 is expressed normally in *Dmrt1*^{−/−} Sertoli cells during embryonic and early postnatal development (Fig. 5), as are other early testis markers, including *Dhh*, *Ptch2*, and *Mis* (data not shown). The failure of germ cell

migration in *Dmrt1*^{−/−} at P7 is clearly visible in sections stained for Gata-4, with most *Dmrt1*^{−/−} germ cells failing to move from the center of the tubule to the margin (Gata-4 negative cells; Fig. 5B). This may result from defects in the germ cells, the Sertoli cells, or both, and may reflect a failure of Sertoli/germ cell interaction. At P14, Gata-4 levels have decreased in *Dmrt1*^{+/−}, but remain high in *Dmrt1*^{−/−} Sertoli cells. The persistence of Gata-4 expression in *Dmrt1*^{−/−} Sertoli cells may reflect a cell-autonomous defect. Alternatively, it may result from absence of germ cell-dependent regulation, as Gata-4 expression also persists in the Sertoli cells of *c-kit* mutant testes, which also lack germ cells (Fig. 5).

Expression of the later Sertoli cell marker Gata-1 also is abnormal in *Dmrt1*^{−/−} testes (Fig. 6). Gata-1 normally is expressed in Sertoli cells from about P10, but expression is delayed and reduced, although not absent, in *Dmrt1*^{−/−} mutants (Fig. 6), which further confirms the failure of Sertoli cells to complete differentiation in the *Dmrt1*^{−/−} testis. Gata-1 expression in adult Sertoli cells is dependent on the stage of the spermatogenic cycle (Yomogida et al. 1994; data not shown). It will be of interest to determine whether the cyclical expression of Dmrt1 in spermatogonia and of Gata-1 in the adjacent Sertoli cells are functionally related.

Here we have genetically tested the role of *Dmrt1* in mammalian sexual development. We find that murine *Dmrt1* is necessary in the male gonad for survival and differentiation of both somatic and germ-line cells. Sertoli cell morphology and gene expression are abnormal in *Dmrt1*^{−/−} and the phenotype of the mutant testis differs from that of other mutants simply lacking germ cells. Thus, the defects we observe must be caused at least in part by a failure of Sertoli cell differentiation. The loss of germ cells in *Dmrt1*^{−/−} may be an indirect effect of Sertoli cell inadequacy or a cell-autonomous defect, possibilities that are currently being tested by cell-specific targeting.

Figure 4. *Dmrt1* protein expression. Immunofluorescence of testis sections stained with anti-Dmrt1 antibody. (A) E18.5 testis. Expression is detectable in Sertoli cells and not in germ cells. (B) P1 testis. Expression is detectable Sertoli cells (S) and in some germ cells (g). (C) P4 testis. Expression is detectable in Sertoli cells and becoming strong in most germ cells (g). (D) P7 testis. Expression continues in Sertoli and has become strong in all germ cells. (E) P10 testis. Meiosis has begun. Expression is detectable in Sertoli cells and undifferentiated germ cells (spermatogonia) only. (F) P14 testis. All Sertoli cells and all undifferentiated germ cells express Dmrt1. (G) Sixteen-week-old adult testis. All Sertoli cells (S) express Dmrt1, but spermatogonia (g) express Dmrt1 only in regions early in the spermatogenic cycle (bottom tubule but not the top tubule, which is later in the cycle). (H) P14 *Dmrt1*^{−/−} testis. No Dmrt1 expression is detectable. Brightly autofluorescent interstitial cells in this and other panels are blood cells.

Figure 5. Gata-4 expression persists in *Dmrt1*^{-/-} mutant Sertoli cells. Immunofluorescence of testis sections stained with Gata-4 antibody. Genotype and developmental stage are indicated. (A) P7 *Dmrt1*^{+/-} testis. Sertoli cells strongly express Gata-4 and germ cells do not. Many germ cells have migrated to periphery of seminiferous tubules (arrowheads). (B) P7 *Dmrt1*^{-/-} testis. Gata-4 expression is normal in Sertoli cells. Most germ cells have failed to migrate to the periphery of the seminiferous tubules and remain in the center (arrowheads). (C) P10 *Dmrt1*^{+/-} testis. Sertoli cells continue to express high levels of Gata-4. All germ cells are at periphery of seminiferous tubules (arrowheads). (D) P10 *Dmrt1*^{-/-} testis. Sertoli cells express high levels of Gata-4 and have begun to overproliferate, and germ cells are nearly absent. (E) P14 *Dmrt1*^{+/-} testis. Gata-4 expression has decreased in Sertoli cells. (F) P14 *Dmrt1*^{-/-} testis. Sertoli cells continue to express high levels of Gata-4 and to proliferate. Germ cells are absent. (G) P14 *c-kit*^{W/W-v} mutant testis. Sertoli cells have normal morphology (see Fig. 2G) but express high levels of Gata-4, indicating that germ cells are required for down-regulation of Gata-4 in Sertoli cells.

How similar are mouse and human?

The defects we have observed in *Dmrt1*^{-/-} mutant mice resemble those in humans with distal 9p deletions (Crocker et al. 1988; Ogata et al. 1997; Ion et al. 1998), with two important differences. First, in mouse, *Dmrt1* is recessive, whereas human 9p deletions in some cases are haploinsufficient for testis differentiation. There are several possible explanations for this difference, which

are not mutually exclusive. First, there may be an inherent difference in dose sensitivity in the pathway(s) responsible, because other genes required for testis development are more dose sensitive in human than in mouse (Kreidberg et al. 1993; Swain et al. 1998). Second, genetic background may be important. Human 9p hemizygosity causes testis defects of variable severity and is incompletely penetrant, even when the critical region for testis development is removed by large deletions (Veitia et al. 1998, and references therein), suggesting that background effects may be significant. (In addition, the degree

Figure 6. Gata-1 expression is delayed and reduced in *Dmrt1* mutant Sertoli cells. Immunofluorescence of testis sections stained with Gata-1 antibody. (A) P10 *Dmrt1*^{+/-} testis. Most Sertoli cells have begun to express Gata-1. (B) P10 *Dmrt1*^{-/-} testis. Gata-1 expression is lower than in heterozygous littermate shown in A. (C) P14 *Dmrt1*^{+/-} testis. Most Sertoli cells express high levels of Gata-1. (D) P14 *Dmrt1*^{-/-} testis. Most Sertoli cells express some Gata-1, but generally at lower levels than in heterozygous littermate shown in C. (E) Adult (16 weeks post partum) *Dmrt1*^{+/-} testis. Gata-1 expression is strong in Sertoli cells (brightly staining cells spaced around periphery of seminiferous tubule) and also is present in meiotic germ cells. (F) Six-week postnatal *Dmrt1*^{-/-} testis. Gata-1 expression has not been maintained, and Sertoli cells express low levels. (Most staining is nonspecific background.) (G) Adult *c-kit*^{W/W-v} mutant testis. Sertoli cells express high levels of Gata-1, demonstrating that the germ line is not necessary for high-level Gata-1 expression.

of haploinsufficiency of 9p24.3 in humans may be overestimated, as small deletions with no testis phenotype would go undetected.) Experiments are under way to test whether heterozygous murine *Dmrt1* mutants have a phenotype on different genetic backgrounds. Finally, it is possible that 9p deletions remove an additional gene(s) involved in testis development and thus, although *DMRT1* is recessive, combined hemizygoty with another gene(s) can cause defective testis development in XY individuals retaining a copy of *DMRT1*.

Another important difference between mouse and human is that some 9p-deleted XY patients have Mullerian duct remnants and feminized external genitalia, whereas, perhaps surprisingly, no defects outside of the gonads have been observed in *Dmrt1*^{-/-} mice. This finding indicates earlier defects in Sertoli cell function in human than those observed in the *Dmrt1*^{-/-} mouse. As discussed above, we cannot yet eliminate the possibility that genetic background effects are obscuring earlier functions in the mouse. However, male-specific *DMRT1* mRNA expression occurs at an earlier developmental stage in the human gonad than in mouse (Moniot et al. 2000), and thus *DMRT1* might play an earlier role in human testis differentiation than *Dmrt1* does in mouse. Birds and reptiles also have male-specific *Dmrt1* expression prior to gonad differentiation (Raymond et al. 1999; Smith et al. 1999; Kettlewell et al. 2000), and so *Dmrt1* may be required earlier in gonad development in these species as well.

What is the relationship between Dmrt1, dsx, and mab-3?

Of what significance is the similarity of *Dmrt1* to the invertebrate sexual regulators *dsx* and *mab-3*? The data presented here demonstrate that in mammals, as in nematodes and insects, a DM domain transcription factor controls male sexual development. Does this functional similarity imply a close evolutionary relationship between *Dmrt1* and the invertebrate sexual regulators? Currently, this question cannot be satisfactorily answered for several reasons. First, comparison of protein sequence alone is not very helpful in this case. Even *dsx* and *mab-3*, which perform a number of related biological functions and can be functionally interchangeable, show quite limited sequence similarity, restricted primarily to the DM domain. Of the 12 nematode DM domain genes, the one most similar in sequence to *dsx* is not *mab-3* (C.S. Raymond, W. Yi, and D. Zarkower, unpubl.). We and others have identified several vertebrate DM domain genes, but it is not yet clear whether *Dmrt1* is the one most closely related to *dsx* and *mab-3*. Not all vertebrate DM domain genes are involved in testis development (Meng et al. 1999; J.R. Kettlewell and D. Zarkower, unpubl.). However, other DM domain genes are expressed in the embryonic mouse gonad (J.R. Kettlewell, C.S. Raymond, and D. Zarkower, unpubl.), so it is possible that multiple members of this gene family are involved in vertebrate gonad development. Thus, on the basis of se-

quence comparison, one cannot yet conclude that *Dmrt1* is the mammalian ortholog of *dsx* and *mab-3*.

A second issue confounding the evolutionary question is the fundamentally different biology of mammalian sex determination as compared with that of invertebrates. In worms and flies, sex determination occurs throughout the body, so if *dsx* and *mab-3* are orthologs, they can reasonably be expected to control a number of similar aspects of sexual dimorphism. However, in mammals, sex determination occurs in the embryonic gonad and secreted sex hormones induce sexual dimorphism elsewhere. Thus, there is no a priori expectation that *Dmrt1* should regulate yolk protein transcription or control nervous system sexual dimorphism, as *dsx* and *mab-3* do, or that *Dmrt1* should function anywhere outside of the gonad.

Confounding the situation further, sex determination evolves rapidly and the role of sex-determining genes can change rapidly. An extreme example is *Sry*, which plays a pivotal role in mammalian sex determination but does not exist in birds and reptiles. Similarly, *Sox9* appears to play an early sex-determining role in mammals but a later role in testis differentiation in other vertebrates (Kent et al. 1996; Morais da Silva et al. 1996; Oreal et al. 1998; Spotila et al. 1998; Moreno-Mendoza et al. 1999; Western et al. 1999). Nevertheless, given the evolutionary lability of sex-determining genes, it is particularly striking that *Dmrt1* has been found in all vertebrates in which it has been sought. Furthermore, *Dmrt1* is unique in being expressed very early and sex specifically in the gonad of all classes of vertebrates so far examined, regardless of the sex-determining mechanism used, whether chromosomal or environmental. Determining the evolutionary relationships of *dsx*, *mab-3*, and *Dmrt1* will require the examination of a greater range of species. What is now clear, however, is that DM domain genes play an essential role in sexual development in at least three phyla and probably in others.

Materials and methods

Production of Dmrt1^{-/-} mice

A mouse *Dmrt1* cDNA fragment containing sequences from exon 1 was used to screen a mouse genomic λ phage library from the 129/SvJ strain (Stratagene), and clones containing promoter sequences were isolated and sequenced to obtain *Dmrt1* genomic sequences spanning the first intron. The targeting vector pDZ161 (diagrammed in Fig. 1A) was constructed by the following scheme. A neomycin resistance cassette (*Pgk-neo*) flanked with Flp recombinase recognition sites (*frt* sites), with a *loxP* site 3' to *neo* (derived from pK-11; gift of E. Meyers, M. Lewandoski, and G. Martin; Meyers et al. 1998), was inserted between the *SacI* and *KpnI* sites of pSP73 (Stratagene) to generate pDZ157. 3' to *Pgk-neo* and the *loxP* site we inserted, as a *Apal*/*XhoI* DNA fragment, sequences from the first intron of *Dmrt1* (from 342 bp to 2797 bp downstream of exon 1), generated by PCR. 5' to *Pgk-neo*, we inserted a *NotI*/*EcoRI* PCR fragment extending from 3192 bp 5' of the *Dmrt1* translational start to 342 bp downstream of exon 1. Finally, we inserted a *loxP* site and *NotI* site 481 bp 5' of the *Dmrt1* translational start (~300 bp 5' of the transcriptional start as determined by 5' RACE and

cDNA sequences; data not shown). In the resulting vector, the first exon of *Dmrt1* is flanked by *loxP* sites (floxed). The *Dmrt1*-containing portions of pDZ161 were completely sequenced.

pDZ161 was linearized with *XhoI* and electroporated into ES cells. Two homologous recombinants were identified from 192 G418-resistant colonies by Southern hybridization by use of a DNA probe from intron 1 to screen genomic DNA digested with *HindIII*. Homologous recombination was confirmed on both ends of the targeted region by PCR with primers from outside of the targeted region paired with primers specific to pDZ161 (data not shown). A targeted ES cell clone containing the floxed allele *Dmrt1^{neo}* was injected into C57/Bl6 blastocysts to generate chimeras. Chimeric males were bred with C57/Bl6 females to generate heterozygotes carrying *Dmrt1^{neo}*. *Dmrt1⁺/Dmrt1^{neo}* females were bred with male β -actin-Cre transgenic mice (gift of M. Lewandoski, Lewandoski et al. 1997) to generate heterozygous *Dmrt1* deletion mutants (*Dmrt1⁺/Dmrt1⁻*). Mating of *Dmrt1⁺/Dmrt1⁻* males and females generated homozygous *Dmrt1⁻* mutants. In each litter, all animals were genotyped and assessed for phenotypic sex. To test whether *Dmrt1^{+/-}* might have testis defects, we tested fertility of ~25 adult males and performed histological analysis of ~12 males at different postnatal stages. We observed no defects in either fertility or testis morphology.

Genotyping

For genotyping, tailclip DNA was amplified for 40 cycles. Chromosomal sex was determined by PCR with primers to the Y chromosome gene *Zfy* (below). The wild-type *Dmrt1* allele *Dmrt1⁺* was detected by PCR with CR92/CR99, with an annealing temperature of 55°C. The floxed allele *Dmrt1^{neo}* was detected by PCR with KOS2/CR127, with an annealing temperature of 65°C. The deleted *Dmrt1* allele *Dmrt1⁻* was detected with KOS1/KOS3 with an annealing temperature of 55°C. PCR with CR92/CR99 contained 10% DMSO.

Primers

KOS1 5'-TTCTTGGATGTTCAATCTGTCTC-3'; KOS2 5'-TG CACACGTGCACCCTCGCCATCG-3'; KOS3 5'-TTCTGATT CAGGGAATCTCGCGAC-3'; CR92 5'-CAGTCCATGGC GAACGACGACACATTCCGG-3'; CR99 5'-CTGCAGCGAGC GCATTTGGGCAGC-3'; CR127 5'-CTGCTAAAGCGCATG CTCCAGACTG-3'; CR132 5'-TCAGAGGGACGCATGGTC ATCCAG-3'; CR133 5'-GTGCATCCGGTACTGGGAGCTC AC-3'; ZFYF 5'-CCTATTGCATGGACAGCAGTCTTATG-3'; ZFYR 5'-GACTAGACATGTCTTAACATCTGTCC-3'.

Immunohistochemistry

Rabbit polyclonal antibodies to Dmrt1 were raised against a purified Dmrt1 fusion protein containing glutathione-S-transferase (GST) fused to the C-terminal 108 amino acids of Dmrt1. Antiserum was purified and concentrated by a 33% saturation ammonium sulphate cut, and antibodies to GST were removed by GST-afigel 10 chromatography. The antibody was used at 1 : 2000 dilution with a Cy3-conjugated goat anti-rabbit secondary antibody (Jackson Immunochemicals) at 1 : 1000 dilution.

Gata-1 was detected with a rat monoclonal antibody (Santa Cruz Biotechnology, sc-265) and Gata-4 was detected with a goat monoclonal antibody (Santa Cruz Biotechnology, sc-1237), each at 1 : 200 dilution. Secondary antibodies were Cy3-conjugated rabbit anti-rat or anti-goat (Jackson Immunochemicals) at 1 : 1000 dilution.

Acknowledgments

We thank Robert Anderson, Electra Coucouvanis, David Largaespada, Carlos Manivel, William Shawlot, and members of the Zarkower and Bardwell laboratories for much valuable advice and assistance, and Electra Coucouvanis and William Shawlot for critical reading of the manuscript. We thank David Largaespada, Mark Lewandoski, Andrew McMahon, and Matthew Scott for providing clones and libraries, Mark Lewandoski for providing Cre transgenic mice, and Sandra Horn, David Largaespada, and the University of Minnesota Mouse Genetics Laboratory for expert technical assistance. This work was supported by the NIH (D.Z., V.J.B., C.S.R., and M.W.M.), the Minnesota Medical Foundation (D.Z. and V.J.B.), the University of Minnesota Graduate School (D.Z. and V.J.B.), and the University of Minnesota Center for Developmental Biology (D.Z.).

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Genes Dev. 2000, **14**:

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