DMRT1: An Ancient Sexual Regulator Required for Human Gonadogenesis

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Abstract

Transcriptional regulators related to the invertebrate sexual regulators *doublesex* and *mab-3* occur throughout metazoans and control sex in most animal groups. Seven of these *DMRT* genes are found in mammals, and mouse genetics has shown that one, *Dmrt1*, plays a crucial role in testis differentiation, both in germ cells and somatic cells. Deletions and, more recently, point mutations affecting human *DMRT1* have demonstrated that its heterozygosity is associated with 46,XY complete gonadal dysgenesis. Most of our detailed knowledge of DMRT1 function in the testis, the focus of this review, derives from mouse studies, which have revealed that DMRT1 is essential for male somatic and germ cell differentiation and maintenance of male somatic cell fate after differentiation. Moreover, ectopic DMRT1 can reprogram differentiated female granulosa cells into male Sertoli-like cells. The ability of DMRT1 to control sexual cell fate likely derives from at least 3 properties. First, DMRT1 functionally collaborates with another key male sex regulator, SOX9, and possibly other proteins to maintain and reprogram sexual cell fate. Second, and related, DMRT1 appears to function as a pioneer transcription factor, binding “closed” inaccessible

chromatin and promoting its opening to allow binding by other regulators including SOX9. Third, DMRT1 binds DNA by a highly unusual form of interaction and can bind with different stoichiometries.

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Prologue: A Brief Overview of *DMRT* Genes

The *DMRT* gene family was initially discovered in the late 1990s based on sequence similarity between products of the fruitfly sex-determining gene *dsx* and the nematode male regulator *mab-3* [Baker and Ridge, 1980; Shen and Hodgkin, 1988; Raymond et al., 1998]. These invertebrate sexual regulators encode transcription factors sharing a motif consisting of an intertwined zinc-binding module followed by an alpha-helical recognition domain that confers sequence-specific DNA binding [Erdman and Burtis, 1993; Erdman et al., 1996; Yi and Zarkower, 1999; Zhu et al., 2000; Murphy et al., 2007, 2015] (Fig. 1a, b). This bipartite motif was named the DM domain after *dsx* and *mab-3*, and the gene family containing the motif were christened *DMRT* genes, for “Doublesex- and mab-3-related transcription factors”. Since *dsx* and *mab-3* perform some analogous functions in sexual development and *dsx* can partially substitute for *mab-3* in vivo, it appeared like-

ly that the sequence similarity indeed represented descent from a common ancestral sexual regulator [Raymond et al., 1998]. Subsequent molecular cloning and nucleotide sequence searches have revealed that nearly all metazoan animals have *DMRT* genes, including some from basal clades [Chong et al., 2013; Wexler et al., 2014]. However, *DMRT* genes are not found outside metazoans, suggesting that they arose close to the origin of multicellularity and thus potentially played an ancient role in specifying differential cell fates, sexual or otherwise.

Most animals have multiple *DMRT* genes, ranging from 4 in insects and 7 in mammals [Raymond et al., 1998, 1999b; Ottolenghi et al., 2002; Kim et al., 2003; Volff et al., 2003] to 11 in ctenophores such as the starlet sea anemone *Nematostella*, which diverged early in metazoan evolution [Wexler et al., 2014]. In addition, a number of genes with some similarity to *DMRT* genes but lacking a functional DNA-binding domain have been identified, particularly in mammals. Some of these *DMRT*-like genes show sex-specific expression [Veith et al., 2006], but no functional analysis of these genes has been reported. *DMRT* genes can play varied roles in different tissues, but in species where *DMRT* gene function has been investigated most have at least one *DMRT* gene involved in sexual development, acting at the level of sex determination, sex differentiation, or, in some cases, both [Matson and Zarkower, 2012]. The functions of *DMRT* genes can be limited to one tissue or cell type or can be more general. For example, in mice *Dmrt6* and *Dmrt7* (also called *Dmrtb1* and *Dmrtd2*, respectively) regulate germ cell differentiation but appear not to be required in any other cell types [Kim et al., 2007b; Zhang et al., 2014]. At the other end of the spectrum, *dsx* controls most sexually dimorphic aspects of development in insects, regulating a wide variety of distinct functions in different cell types [Baker and Ridge, 1980; Robinett et al., 2010]. Moreover, *dsx* is alternatively spliced to generate sex-specific *Dsx* protein isoforms that direct male- or female-specific differentiation throughout the body [Burtis and Baker, 1989; Robinett et al., 2010]. This distributed and diverse regulatory repertoire allows *dsx* to act as a primary sex-determining gene in a system that, unlike sex development in vertebrates, does not involve gonadal sex hormones. Because *DMRT* genes regulate sexual differentiation in species as divergent as nematodes [Shen and Hodgkin, 1988; Mason et al., 2008], flatworms [Chong et al., 2013], insects [Geuverink and Beukeboom, 2014], crustaceans [Kato et al., 2011], and vertebrates [Raymond et al., 2000; Matsuda et al., 2002; Smith et al., 2009; Ge et al., 2017; Webster et al., 2017], it seems likely that that sexual regulation is an an-

cestral function of this gene family [Matson and Zarkower, 2012]. As such, *DMRT* genes are by far the most deeply conserved regulators of sexual development found to date, and it seems likely that they have characteristic functional properties that have helped them maintain a crucial role in this process for hundreds of millions of years in organisms with diverse strategies and mechanisms of sexual development.

***DMRT* Genes in Mammals and *DMRT1* as a Conserved Sexual Regulator**

Mammals have 7 *DMRT* genes [Volff et al., 2003], as well as up to 3 X-linked *DMRT*-like genes encoding proteins that appear to lack a functional DM domain [Veith et al., 2006]. Mouse gene targeting has probed the functions of all 7 bona fide autosomal *Dmrt* genes. Mutations in 4 genes, *Dmrt1*, *Dmrt4* (*Dmrta1*), *Dmrt6* (*Dmrtb1*), and *Dmrt7* (*Dmrtd2*), disrupt gonadal development in one or both sexes [Raymond et al., 2000; Balciuniene et al., 2006; Kawamata and Nishimori, 2006; Kim et al., 2007b; Zhang et al., 2014], confirming roles in sexual differentiation, while loss of *Dmrt2* disrupts embryonic patterning [Seo et al., 2006]. Mutations in *Dmrt3* and *Dmrt5* (*Dmrta2*) mainly affect the nervous system, disrupting patterning and development of the hippocampus and cerebral cortex [Konno et al., 2012; Saulnier et al., 2013; De Clercq et al., 2018]. *Dmrt3* also is important for spinal interneuron function in locomotion in mice, and variants of *DMRT3* determine gait patterns in horses [Andersson et al., 2012; Vallstedt and Kullander, 2013].

Dmrt1 is the only *DMRT* gene shown to play a conserved role in mammalian sexual differentiation. *Dmrt1* is expressed specifically in the gonads of mice and humans, in both somatic and germline cells. In mice, *Dmrt1* mRNA is first detected in the genital ridges around E10.5 in both somatic cells and primordial germ cells (PGCs) of both sexes, and it becomes highly male-enriched by about E14.5 as testis differentiation initiates [Raymond et al., 1999a]. Similarly, *DMRT1* protein is expressed from E10.5 to E11.5 in somatic and germ cells of both sexes. It becomes male-specific in somatic cells from E12.5 to E13.5, and expression continues in Sertoli cells thereafter [Lei et al., 2007]. *DMRT1* is expressed in germ cells of both sexes through E13.5, disappearing by about E15.5 [Lei et al., 2007]. In males, *DMRT1* is re-expressed in spermatogonia just before birth and maintained in spermatogonia subsequently [Lei et al., 2007; Matson et al., 2010]. During steady state adult spermatogenesis, *DMRT1*

is expressed in mitotic cells, from *Id4-gfp*-expressing spermatogonial stem cells (SSCs) through differentiated B spermatogonia, disappearing in preleptotene spermatocytes that are entering meiosis [Matson et al., 2010].

Human DMRT1 expression is less fully characterized but appears similar to that of mice. *DMRT1* mRNA was detected only in the testis, among 50 tissues surveyed by RNA blotting [Raymond et al., 1998]. During fetal development, *DMRT1* mRNA is detectable by gestational week 11 (GW11) in both sexes. DMRT1 protein is most abundant in pre-Sertoli cells between GW10 and GW20, before becoming strong in spermatogonia from about GW24 onward [Jorgensen et al., 2012]. DMRT1 also is expressed in oogonia and oocytes prior to GW20 and downregulated upon meiotic entry, as in mice [Jorgensen et al., 2012]. In prepubertal testes, DMRT1 protein is highly expressed in spermatogonia and in pre-Sertoli cells, followed by lower expression in Sertoli cells [Jorgensen et al., 2012]. In adult testes, DMRT1 is expressed both in Sertoli cells and in spermatogonia, except in OCT2-positive A-dark cells, which are thought to represent reserve SSCs [Jorgensen et al., 2012]. Similarly, single cell RNA sequencing (scRNA-seq) has found that *Dmrt1* mRNA is expressed postnatally in Sertoli cells and in SSCs, differentiating spermatogonia, and preleptotene spermatocytes, but not in meiotic and post-meiotic germ cells [Guo et al., 2018; Wang et al., 2018].

Ancient Involvement of *DMRT1* in Vertebrate Sex Determination and Its Evolution

Not only is *DMRT1* conserved in mammalian sex regulation but it also is a universal or near-universal sex regulator in other vertebrates. Studies in non-mammalian vertebrates have uncovered *DMRT1* or a close paralog controlling gonadal sex determination in species with diverse sex determination mechanisms, ranging from fish to birds to reptiles and amphibians [Matson and Zarkower, 2012; Ge et al., 2017]. Moreover, in several vertebrate clades, changes in *DMRT1* appear to be associated with the acquisition of new sex determination mechanisms. In the fish Medaka, for example, sex is dominantly determined by a *DMRT1* paralog called *Dmy* or *Dmrt1bY* that is located in the non-recombining region of the Y chromosome and acts analogously to *SRY* in mammals [Matsuda et al., 2002; Nanda et al., 2002]. *Dmy* and the X/Y sex chromosome pair containing it are found only in a handful of Medaka species, suggesting a comparatively recent origin for this gene variant and these sex chromosomes

[Matsuda et al., 2003; Kondo et al., 2004]. A plausible model is that *Dmy* formed by a duplication of *Dmrt1* that caused constitutive expression of the new gene, rendering individuals carrying it male, regardless of their genotype otherwise, and thus created a neo-Y chromosome. In birds, sex is determined by a ZZ/ZW chromosome system, with ZZ eggs developing as males and ZW eggs as females. Avian *DMRT1* is located on the Z and missing from the W chromosome [Nanda et al., 1999]. Knock-down and overexpression experiments in chickens support the model that *DMRT1* is required in 2 copies for male gonadal fate [Smith et al., 2009; Lambeth et al., 2014]. Here, a likely model is that deletion of *DMRT1* in an avian progenitor was associated with formation of a new sex chromosome pair. In the African clawed frog *Xenopus laevis*, a *DMRT1* paralog, *DM-W*, is found on the W chromosome of a different ZW sex chromosome system [Yoshimoto et al., 2008]. In ZW individuals, *DM-W* dominantly interferes with function of the autosomal *DMRT1* gene, preventing testis differentiation [Yoshimoto et al., 2008]. *DM-W* has an N-terminal DM domain but lacks some of the C-terminal sequences found in *DMRT1*, suggesting that it formed via a partial duplication of *DMRT1*, resulting in a neo-W chromosome [Bewick et al., 2011]. In all of these cases it is impossible to distinguish whether a mutation affecting *DMRT1* triggered the formation of new genetic sex-determining mechanisms or instead co-occurred with those events, but it does appear that *DMRT1* and its close paralogs determine sex in a wide range of vertebrates and have done so for hundreds of millions of years. Function of *DMRT1* in vertebrate sex determination is not limited to genetic mechanisms: in the red-eared slider turtle, *DMRT1* is required for temperature-dependent sex determination, possibly via temperature-dependent methylation of the *DMRT1* promoter [Ge et al., 2017]. The involvement of *DMRT1* in such a breadth of sex determination systems suggests that it plays a central role in the process and has properties that make it hard to displace.

DMRT1 and Disorders of Sexual Development: Distal 9p Deletions Define a Critical Region

The discovery of human *DMRT1* and its mapping to 9p24.3 [Raymond et al., 1998] was of immediate relevance for disorders of sexual development (DSD) genetics. The association between monosomy of the distal short arm of chromosome 9 (9p), where *DMRT1* is located, and partial or complete 46,XY gonadal dysgenesis

Table 1. *DMRT1* microdeletions and missense mutations associated with human DSD

Lesion	Phenotype	Notes	Citation
103-kb deletion removing <i>DMRT1</i> exons 1 and 2	46,XY complete DSD	Hemizygous	Ledig et al. [2010]
35-kb deletion removing <i>DMRT1</i> exons 3 and 4	46,XY ovotesticular DSD	Hemizygous	Ledig et al. [2012]
700-kb deletion extending distally from 30 kb 5' of <i>DMRT1</i> TSS	46,XY complete DSD 46,XY partial DSD	Hemizygous; probands are siblings	Calvari et al. [2000]
<i>DMRT1</i> R111G missense mutation in DM domain	46,XY complete DSD	Heterozygous; reduced DNA affinity, altered stoichiometry	Murphy et al. [2015]
<i>DMRT1</i> Y84C missense mutation in DM domain	46,XY complete DSD	Heterozygous; maternally inherited	Fan et al. [2017]
<i>DMRT1</i> R80S missense mutation in DM domain	46,XY complete DSD	Heterozygous; reduced DNA affinity	Buonocore et al. [2019]; this paper
<i>DMRT1</i> L139Q missense mutation adjacent to DM domain	46,XY complete DSD	Heterozygous	Chauhan et al., [2017]
<i>DMRT1</i> P74L missense mutation in DM domain	46,XY Sertoli-only syndrome	Heterozygous; reduced DNA affinity	T. Maric and M. Murphy, in preparation

has been clear for several decades [Jotterand and Juillard, 1976; Fryns et al., 1986; Crocker et al., 1988; Hoo et al., 1988; Huret et al., 1988; Bennett et al., 1993; Ogata et al., 1997; Veitia et al., 1997, 1998]. Distal 9p monosomy also can be associated with cognitive deficits, developmental delay, and a characteristic array of dysmorphic craniofacial features [Alfi et al., 1976]. An early question was whether these effects are genetically linked, much as heterozygosity of *SOX9* causes both campomelic dysplasia and DSD [Foster et al., 1994; Wagner et al., 1994]. Identification of shorter 9p deletions narrowed the critical region for DSD to an interval on 9p24.3, near the telomere, again containing *DMRT1* [McDonald et al., 1997; Flejter et al., 1998; Guioli et al., 1998; Veitia et al., 1998; Calvari et al., 2000; Ottolenghi and McElreavey, 2000]. Because these shorter deletions are found in patients with 46,XY gonadal dysgenesis but lacking the non-urogenital features of monosomy 9p, it is clear that the gonadal and extragonadal aspects of 9p deletion syndrome are genetically separable.

9p deletions consistently affect *DMRT1* but most also remove other genes, including the adjacent paralog *DMRT3*, which is immediately proximal to *DMRT1* on 9p [Ottolenghi et al., 2000]. The co-involvement of *DMRT3* in so many DSD-associated 9p deletions raised the possibility that gonadal dysgenesis might result from combined hemizygosity of *DMRT1* with this gene. This possibility cannot be definitively excluded but appears unlikely on several grounds. First, although both genes are expressed in the fetal gonad, targeted deletion of *Dmrt3* (and the nearby *Dmrt2*) in the mouse has not revealed a

function in gonadal development [Seo et al., 2006; De Clercq et al., 2018; Desmaris et al., 2018]. Moreover, deletion of the interval containing *Dmrt1* and *Dmrt3* in mice, either using CRISPR/Cas9 [Inui et al., 2017] or by meiosis-mediated recombination between *loxP* sites (D.Z., unpubl. data), does not cause a more severe phenotype than mutation of *Dmrt1* alone. The most compelling line of evidence is the identification of DSD cases involving 9p microdeletions and point mutations that affect only *DMRT1*. At least 3 hemizygous microdeletions have been reported that likely affect just *DMRT1* (Table 1). One, a deletion of 103 kb in a patient with 46,XY complete gonadal dysgenesis, removed exons 1 and 2 of *DMRT1* [Ledig et al., 2010], while a second deletion of 35 kb, associated with 46,XY ovotesticular DSD, removed exons 3 and 4 of *DMRT1* [Ledig et al., 2012]. A 3rd deletion was found in two 46,XY sisters, one with complete gonadal dysgenesis and female external genitalia and the other with partially virilized DSD (ovotestes and virilized female external genitalia) [Calvari et al., 2000]. This deletion removed an interval of 700 kb located distal to *DMRT1* but extending within about 30 kb of the *DMRT1* transcriptional start site, and thus may have disrupted *DMRT1* upstream regulatory elements. The difference in DSD severity between these 2 sisters helps address the long-standing question of why 9p deletion-associated DSD is variable. The degree of DSD can range from male genitalia with small dysgenic testes to complete gonadal DSD with sex reversal and does not correlate in an obvious way with the extent of the 9p deletion. In addition, there has been no indication of loss of *DMRT1* heterozy-

Fig. 1. The DM domain and DMRT1/DNA interaction. **a** X-ray crystal structure of the human DMRT1 DM domain from Murphy et al. [2015] with major structural domains indicated. **b** Conservation of metazoan DM domains. Positions of structural domains and sites of human mutations associated with DSD are indicated. **c** X-ray crystal structure of human DMRT1 DM domains bound to DNA from Murphy et al. [2015], with DMRT1 protomers in distinct colors. Two protomers (pink and blue) insert in the DNA major groove on one side of the binding site, while a single pro-

tomers (green) inserts in the major groove on the other side. Red oval indicates central basepair of the punctuated pseudopalindromic DNA site. **d** DM domain amino acids affected by human DMRT1 point mutations associated with DSD. Affected amino acid side-chains are shown as space-filling models and labeled. R80 makes contact with the DNA phosphate backbone, Y84 is in the zinc module, and R111 makes contacts with conserved DNA bases in the recognition site.

gosity in 9p-associated DSD. Given the range of severity and the finding that siblings with the same deletion can have distinct DSD phenotypes, it is more likely that variable penetrance of distal 9p deletions reflects interaction between *DMRT1* hemizygosity and genetic background, rather than indicating a contiguous gene syndrome or a difference in functional *DMRT1* copy number.

While distal 9p clearly is required in 2 copies for normal male gonadogenesis, it is less clear whether it plays a role in human ovarian development or function. On the one hand, primary hypogonadism and premature ovarian failure have been reported in 46,XX individuals with terminal 9p deletions [Muroya et al., 2000; Ogata et al., 2001; Fujimoto et al., 2004; Bartels et al., 2013], suggesting a significant role for this region in female gonadal development and function. On the other hand, the maternal inheritance of 9p deletions [Calvari et al., 2000; Quinonez et al., 2013] as well as reports of 46,XX fetuses and adults with distal 9p deletions but apparently normal ovaries [Vialard et al., 2002; Fujimoto et al., 2004; Ounap et al., 2004] suggest that distal 9p hemizygosity does not preclude ovarian differentiation and oogenesis. In sum, it appears that one or more dose-sensitive loci on distal 9p, potentially including *DMRT1*, play a significant but not essential role in ovarian differentiation.

***DMRT1* Point Mutations Confirm Its Role in Male Gonadogenesis**

Despite DNA sequencing efforts by a number of laboratories, *DMRT1* point mutations associated with DSD or male infertility that convincingly cause loss of gene function have been surprisingly rare [Raymond et al., 1999b; Calvari et al., 2000; Ottolenghi et al., 2000; Tewes et al., 2014; Eggers et al., 2016]. Nonetheless, exome sequencing has so far detected 4 heterozygous point mutations in 46,XY individuals with complete gonadal dysgenesis that are likely to reduce *DMRT1* function (Table 1). All 4 mutations affect amino acids in or adjacent to the DM domain (Fig. 1b). One de novo mutation, R111G [Murphy et al., 2015], alters a highly conserved amino acid that makes specific hydrogen-bond contacts with bases in the DNA binding consensus site and also coordinates G118 to help stabilize the DM domain tertiary structure. Substitution with alanine at this position nearly eliminates in vitro DNA binding, confirming the importance of this residue [Murphy et al., 2015]. Substitution of R111 to G severely reduces DNA binding affinity of the mutant protein but also allows it to alter the

Fig. 2. Sex-reversing R111G and R80S mutations reduce DNA binding by *DMRT1* in vitro. Left panel: *DMRT1* protein translated in vitro was incubated with radiolabeled double-stranded DNAs previously shown to bind dimers, trimers, or tetramers of *DMRT1* as previously described [Murphy et al., 2015]. Reactions contained unprogrammed reticulocyte lysate (–), lysate programmed with wild-type synthetic human *Dmrt1* mRNA (WT), or synthetic mRNA encoding the R111G or R80S mutant *DMRT1* proteins, as indicated. After incubation, reactions were subjected to native gene electrophoresis and autoradiography. Mobility of dimers, trimers, and tetramers are indicated by arrowheads. The R111G mutation reduced trimer and dimer binding more severely than tetramer binding, while the R80S mutation reduced dimer and tetramer binding more severely than trimer binding. Right panel: *DMRT1* proteins were incubated with DNA containing a *DMRT1* binding site from the *Foxl2* locus. Both mutations severely reduced binding to this site.

stoichiometry of binding by wild-type protein in vitro, suggesting that R111G may function as a dominant negative mutation [Murphy et al., 2015]. A second mutation, Y84C, alters a position in the zinc-binding module of the DM domain [Fan et al., 2017]. Mutations at this position have not been observed in model organisms or tested in vitro, but the introduction of a cysteine is likely to disrupt folding of the zinc-binding module and consequently affect DNA affinity. The Y84C mutation was maternally inherited, further indicating that *DMRT1* heterozygosity can be compatible with female fertility. The 3rd mutation, a de novo R80S change, alters a conserved residue in the DM domain that makes hydrogen-bond contacts with the DNA backbone [Murphy et al., 2015; Buonocore et al., 2019]. Molecular dynamics simulation predicted that this change should reduce DNA affinity [Buonocore

et al., 2019]. To directly test that prediction, we have compared in vitro DNA binding affinity of full-length human wild type, R80S-, and R111G-substituted DMRT1 proteins to labeled synthetic DNA oligonucleotides by electrophoretic mobility shift analysis (EMSA) (Fig. 2). The R80S mutant protein bound less efficiently than the wild type. As discussed below, DMRT1 can bind DNA as a dimer, trimer, or tetramer. R80S mutant DMRT1 binding was reduced less severely than that of R111G DMRT1 on DNAs that bind DMRT1 trimers and dimers but more severely on a site that binds DMRT1 tetramers. Both mutant proteins showed severely reduced binding on a DMRT1 tetramer site from the *Foxl2* locus. Collectively, the data were consistent with the R80S mutation being responsible for DSD in the affected patient. The 4th mutation, L139Q, is a de novo change affecting a conserved residue adjacent to the DM domain [Chauhan et al., 2017]. This residue is not included in the published X-ray structure, it has not been altered in model organism genetic screens, and its role in DNA binding has not yet been tested; thus, how the L139Q mutation affects DMRT1 function remains to be established. In addition, a heterozygous P74L mutation has been found in 2 brothers with Sertoli-only syndrome. It is predicted to affect minor groove recognition and reduces DMRT1 DNA binding affinity, suggesting that reduced DMRT1 function also can cause infertility without DSD (T. Maric and M. Murphy, in preparation).

A handful of other *DMRT1* mutations or rare variants outside the DM domain have been reported in DSD [Raymond et al., 1999b] and male infertility cases [Tewes et al., 2014]. It is possible that these variants represent loss-of-function mutations, but given the lower sequence conservation of the affected regions and the lack of a simple and robust functional assay for DMRT1, it cannot yet be excluded that these are merely uncommon neutral variants. The low discovery rate for *DMRT1* mutations in DSD and the absence of homozygous *DMRT1* mutations is notable. One suggested explanation is that *DMRT1* deletions also are associated with male infertility, allowing the load of *DMRT1* mutations to be purged by spermatogenic failure [Lopes et al., 2013].

Insights into *DMRT1* from Mouse Conditional Genetics: *DMRT1* and Spermatogenesis

Conditional gene targeting technology in mice has allowed a detailed dissection of *Dmrt1* function in a mammal with similar reproductive biology to that of humans.

Conditional genetics has confirmed the importance of *Dmrt1* in gonadal development has found similarities and differences to its function in humans and has uncovered some unexpected roles for DMRT1. In the mouse, DMRT1 plays a major role in male but not female gonadal development, as appears to be the case in humans [Raymond et al., 2000]. However, in mice the *Dmrt1* gene is fully recessive rather than haploinsufficient. This difference in dose-sensitivity is not unusual for genes involved in mouse and human sex regulation. For example, *SOX9*, *WT1*, and *SF1* are haploinsufficient for human male gonadogenesis but recessive for testis differentiation in the mouse [Parker et al., 1999; Barrionuevo et al., 2006]. Homozygous deletion of *Dmrt1* in XX mice causes a deficit in primordial follicles but does not significantly reduce female fertility [Krentz et al., 2011]. By contrast, XY *Dmrt1* homozygous mutants have severely dysgenic gonads [Raymond et al., 2000]. However, XY homozygous *Dmrt1* mutant mice still undergo male nongonadal development, in contrast to 46,XY humans with heterozygous *DMRT1* point mutations or deletions, who can have complete gonadal dysgenesis at an early enough stage to cause complete male-to-female phenotypic sex reversal. Indeed, testicular development is superficially normal in *Dmrt1*^{-/-} mice of mixed genetic background until birth, with gonadal dysgenesis becoming apparent mainly between 1 and 4 weeks postnatally [Raymond et al., 2000; Fahrioglu et al., 2007; Kim et al., 2007a]. It therefore appears that there are fundamental differences, at least in timing, between the requirements for *DMRT1* in human and *Dmrt1* in mouse. While these differences must be kept in mind, the mouse has nonetheless been useful for deeper exploration of DMRT1 function than is possible in humans.

Conditional gene targeting has allowed the dissection of requirements for *Dmrt1* in germ cells and Sertoli cells and has revealed distinct functions in different cell types and at different stages of development. The role of *Dmrt1* in the germ line has been reviewed in detail elsewhere [Zarkower, 2013; Zhang and Zarkower, 2017] and will be summarized briefly here. Consistent with its expression in fetal PGCs and postnatal spermatogonia, *Dmrt1* is required both for the establishment of postnatal spermatogenesis and subsequently for the maintenance of the progenitor pool that supplies adult steady state spermatogenesis [Fahrioglu et al., 2007; Kim et al., 2007a]. Fetal loss of *Dmrt1* blocks perinatal mitotic reactivation, migration, and viability of prospermatogonia [Kim et al., 2007a]. Later, in steady-state adult spermatogenesis, *Dmrt1* has multiple distinct roles affecting spermatogonial stem

cells: it activates the stem cell regulator *Plzf* to help maintain the SSC pool and it is required also in *Ngn3*-positive committed progenitor cells to let them replenish the SSC pool when they are artificially depleted [Zhang et al., 2016]. In steady state spermatogenesis, *Dmrt1* also has a key role in differentiating spermatogonia. In these cells, rather than promoting self-renewal, *Dmrt1* promotes differentiation and proliferation by activating genes including *Sohlh1* and inhibits premature meiotic initiation by inhibiting retinoic acid signaling and repressing *Stra8* [Matson et al., 2010]. Normally meiotic initiation is triggered in part by degradation of DMRT1 via a β -TrCP ubiquitin ligase complex [Nakagawa et al., 2017]. The importance of this regulation is revealed when β -TrCP is depleted, which stabilizes DMRT1 in preleptotene cells, reduces STRA8 expression in these cells, and prevents meiosis. The suite of germline DMRT1 functions collectively allows spermatogonia to proliferate and differentiate, supported by a stable stem cell pool, and promotes long-term fertility. How DMRT1 activity is modified to perform different roles at different stages of spermatogonial development is important but just beginning to be understood, as discussed below.

Insights from Mouse Conditional Genetics: *DMRT1* and Cancer

DMRT1 not only controls normal spermatogenesis but also is linked to germ cell cancer in both mice and humans. In mice of mixed or C57BL/6J genetic background, the *Dmrt1* mutant testis appears normal until shortly after birth, but in male mice of the 129 Sv/J inbred genetic background, mutant fetal germ cells fail to undergo cell cycle arrest and downregulation of pluripotency regulators, and this results in a very high incidence of testicular teratomas [Krentz et al., 2009]. These tumors differ in a number of respects from the testicular germ cell tumors most common in humans [Oosterhuis and Looijenga, 2005]. However, genome-wide association studies have shown strong association of *DMRT1* with human testicular germ cell cancer [Turnbull et al., 2010; Kanetsky et al., 2011; Kratz et al., 2011]. Gain of distal 9p, involving *DMRT1*, also is found in all cases of the rare germ cell cancer spermatocytic seminoma, which is found exclusively in older men [Looijenga et al., 2006]. These tumors are thought to arise from a different stage of germ cell development and may result from ectopic *DMRT1* activity [Oosterhuis and Looijenga, 2005].

Insights from Mouse Conditional Genetics: *DMRT1* and Somatic Sexual Cell Fate

The role of DMRT1 in Sertoli cells is most germane to DSD. *Dmrt1* null mutants or conditional mutants with *Dmrt1* deleted in fetal bipotential progenitor cells have apparently normal testes at birth, but mutant Sertoli cells fail to complete differentiation and are unable to support spermatogenesis beyond meiotic prophase [Raymond et al., 2000; Kim et al., 2007a]. Unexpectedly, however, deletion of *Dmrt1* in pre-Sertoli or Sertoli cells not only prevents the completion of differentiation but also causes their transdifferentiation to granulosa-like cells, the female supporting cell counterpart [Matson et al., 2011]. This sexual transdifferentiation occurs even when *Dmrt1* is deleted in fully differentiated post-mitotic adult Sertoli cells, demonstrating that male sexual cell fate must be continuously maintained from at least puberty onward. Loss of *Dmrt1* in Sertoli cells causes ectopic expression of key female sex regulators including *Foxl2*, *Wnt4*, *Rspo1*, *Esr1*, and *Esr2*, and genetic analysis indicates that these genes drive the male-to-female transdifferentiation that ensues [Minkina et al., 2014]. Whether the transdifferentiated granulosa-like cells are able to support oogenesis is unknown, because germ cells in the mutant XY gonad undergo apoptosis and efforts to co-transplant mutant XY somatic cells together with wild type XX oocytes have not been successful (D.Z., unpubl. data).

Why do Sertoli cells need a sex maintenance system? When *Dmrt1* is deleted, the feminizing genes are activated by inappropriate retinoic acid (RA) signaling. Blocking RA signaling or deleting the RA receptor *Rara* suppresses this activation and largely blocks transdifferentiation [Minkina et al., 2014]. A likely model is that DMRT1 not only limits RA signaling in spermatogonia but also restricts the activity of RA in somatic cells, thereby allowing RA to promote meiosis in spermatocytes without causing premature meiotic initiation in spermatogonia or sexual cell fate transdifferentiation in Sertoli cells.

Granulosa cells also need sex maintenance: deletion of *Foxl2* in the ovary causes a reciprocal, female-to-male, transdifferentiation. These results suggest that DMRT1 and FOXL2 anchor antagonistic regulatory networks that ensure the stability of somatic sex throughout postnatal life [Uhlenhaut et al., 2009]. Consistent with this view, deletion of *Foxl2* suppresses transdifferentiation of XY *Dmrt1* mutants [Minkina et al., 2014] and the transcriptome of XX gonads expressing ectopic *Dmrt1* is very similar to that of XX gonads lacking *Foxl2* [Lindeman et al., 2015]. Whether *DMRT1* is required in the human gonad

for the testis determination decision or subsequently for testis differentiation, and whether it maintains male sexual fate in the human gonad are unknown. This uncertainty is in part because early failure of human testicular development, for example in *SRY* or *SOX9* mutants, results in highly dysgenic “streak” gonads and female internal and external genitalia, a condition known as Swyer Syndrome (MIM 400044).

DMRT1 not only is required to maintain male fate in the XY gonad but also can impose male fate on somatic cells of the XX gonad. Ectopic expression of DMRT1 in the ovary from a single copy transgene, starting either around E11.5 in the fetal gonad, driven by *Sf1-Cre*, or in the adult gonad, driven by *UBC-CreERT2* or *Hsd17b1-Cre*, can reprogram granulosa cells into Sertoli-like cells organized into tubule-like structures [Lindeman et al., 2015] and can sexually transform the ovarian transcriptome (Lindeman and Zarkower, unpubl. data). These results indicate that DMRT1 can act instructively to determine male cell fate. However, transdifferentiation did not start until about 1–2 weeks after birth, similar to the timing of transdifferentiation in XY *Dmrt1* mutants. Even expressing DMRT1 from about E9.5 using *wt1-Cre* did not cause fetal masculinization. This is perhaps unsurprising, given that DMRT1 normally is expressed at similar levels in bipotential Sertoli/granulosa progenitor cells of the 2 sexes prior to sex determination. One study has found that a multicopy *Dmrt1* cDNA driven by the *wt1* promoter can cause partial female-to-male sex reversal [Zhao et al., 2015]. However this observation involved progeny of a single transgenic founder with extremely high expression of *Dmrt1* and some of its targets, in contrast to the approximately physiological DMRT1 expression level in the previous study. Also, a different multicopy transgenic study also expressing *Dmrt1* from the *wt1* promoter found no effect on sex determination [Agbor et al., 2013]. Possible explanations for the discrepancy are that highly overexpressed DMRT1 and/or its targets competes for regulatory cofactors shared with transcription factors involved in female sex determination, or that the *wt1-Dmrt1* transgene in the single affected founder line was inserted in or near a locus involved in female gonadogenesis.

The DM Domain and Gene Regulation

DMRT proteins are site-specific DNA binding proteins, and genetic experiments have confirmed that they regulate transcription of target genes by activation or re-

pression. The DMRT1 DNA-binding consensus sequence in vitro and in vivo is a 13-bp pseudopalindromic DNA motif, which is also bound by other DMRT proteins [Murphy et al., 2007]. DMRT proteins can form heteromers on DNA both in vitro and in vivo, raising the possibility that they may interact functionally by forming mixed complexes with different activities [Murphy et al., 2007; Zhang et al., 2014; Zhang and Zarkower, 2017]. X-ray crystallography has revealed that the DM domain inserts its recognition helix into the DNA major groove and makes sequence-specific hydrogen bond contacts with key DNA bases in the manner of many other site-specific DNA binding proteins (Fig. 1c, d) [Murphy et al., 2015]. The DM domain can bind DNA as a trimer, with an antiparallel dimer of protomers on one side of the binding site and a single protomer on the other side. Unexpectedly, however, the antiparallel dimerized protomers jointly insert their recognition helices into the same segment of an inherently wide major groove. This binding mode, involving 2 helices contacting the same major groove segment, may be unique to the DM domain, as it has not been observed among the hundreds of other protein/DNA complexes structurally analyzed to date. Both in vitro and in vivo DMRT1 is able to bind DNA not only as a trimer but also as a dimer or tetramer, based on EMSA (see Fig. 2) and high resolution ChIP-exo analysis [Murphy et al., 2015]. The stoichiometry of binding depends both on the specific sequence and the inherent shape of the particular binding site.

Understanding how DMRT1 functions requires an understanding of how it can impose a new fate on cells even after differentiation is complete and how it can perform very different functions in different contexts, for example in somatic versus germ cells or in SSCs versus differentiating spermatogonia. There are several properties that could help account for these abilities. These properties include the differences in stoichiometry of DNA binding, interactions with cell type-specific transcriptional regulators, the ability to bind closed chromatin, effects on genome organization, or a combination of these. The significance of the alternative DNA binding stoichiometries remains unclear, as individual stoichiometries do not correspond in an obvious way with targets that are, for example, activated or repressed by DMRT1. R111 plays a key role in DNA base recognition (Fig. 1d), and the dominant change in DNA binding stoichiometry caused by the R111G mutation hints that context-dependent binding modes may be important. The other DSD-associated DMRT1 mutations have not yet been tested for such dominance.

Fig. 3. Model of DMRT1 regulation of sexual cell fate maintenance and reprogramming. Top: In sexual cell fate maintenance, DMRT1 (purple) occupies distal regulatory elements, in many cases with SOX9 (blue) and other regulators (brown). This binding helps keep chromatin accessible (“open”) and promotes expression of genes involved in Sertoli cell fate and function. Bottom: In reprogramming, DMRT1 binds inaccessible (“closed”) chromatin and promotes its conversion to a form accessible to SOX9 and other regulators. Occupancy by DMRT1 and other regulators relieves repression and promotes activation of genes involved in Sertoli cell fate and function. How DMRT1 affects chromatin accessibility and potentially chromatin interactions is unknown, as are whether DMRT1 binding stoichiometry affects these functions and whether DMRT1 binding can promote formation of repressive chromatin at granulosa-expressed genes.

There is stronger evidence that some of the other properties are significant. Genetic and molecular analysis suggest that DMRT1 functionally interacts with SOX9 both in the fetal and adult testis. Deletion of *Sox9* causes more severe sexual transdifferentiation of *Dmrt1* mutant testes [Minkina et al., 2014], and deletion of *Sox9* with its close paralog *Sox8* also limits reprogramming of granulosa cells by DMRT1 in the ovary [Lindeman et al., 2015, 2021]. ChIP-seq experiments show that DMRT1 and SOX9 often bind near one another on target genes in the fetal testis [Rahmoun et al., 2017], in juvenile Sertoli cells, and when expressed ectopically in the ovary [Lindeman et al., 2021], suggesting that they have joint effects on target gene expression. DMRT1 also functionally interacts with DMRT6 in spermatogonia, but here the relationship may be antagonistic [Zhang et al., 2014]. DMRT1 promotes spermatogonial differentiation and activates spermatogonial regulators such as *Sohlh1*, whereas DMRT6 is expressed late in spermatogonial development, in intermediate and B spermatogonia, and helps repress the mitotic and differentiation programs prior to meiosis by turning off spermatogonial regulators like *Sohlh1* and *Sohlh2*, as well as *Dmrt1*. DMRT1 and DMRT6 bind to an overlapping set of genomic sites, and ChIP-reChIP shows that they can bind the same site at the same time on several key target genes, suggesting that they form heteromers in which the presence of DMRT6 may switch regulation from activation to repression.

DMRT1 appears to act as a pioneer transcription factor, binding “closed” low-modification chromatin and opening it to binding by other transcriptional regulators [Lindeman et al., 2021]. In granulosa cells, ectopic DMRT1 can bind differentially accessible regions (DARs) that normally are open in Sertoli cells but closed in granulosa cells. Many of these sites are not efficiently bound by SOX9 when it is expressed alone, but expression of DMRT1 with SOX9 allows binding by SOX9, consistent with DMRT1 pioneering for SOX9 and presumably other regulatory factors [Lindeman et al., 2021]. Pioneer function requires the ability to bind nucleosomal DNA and, while this has not yet been demonstrated for DMRT1 in vitro, structural modeling suggests that DMRT1 should be able to interact with its site in a nucleosomal context. Finally, DNA binding by DMRT1 as a multimer suggests the possibility that it could mediate chromatin contacts that could help form functional domains. Preliminary HiC data indicate that ectopic DMRT1 does result in formation of new chromatin loops (M.W.M and D.Z., unpubl. data), but further work will be required to determine their significance for gene expression and how directly DMRT1 participates in the formation of these structures. A model for how DMRT1 controls maintenance and reprogramming of sexual cell fate is shown in Figure 3.

Epilogue: Open Questions and Future Directions

The past 2 decades have revealed much about *DMRT* genes in general and *DMRT1* in particular. We now have convincing evidence that *DMRT1* is an important regulator of testicular differentiation in most or all vertebrates, is involved in the evolution of new vertebrate sex determination mechanisms, has a number of distinct functions in the mouse gonad both during and after development, and is a key locus involved in 9p deletion-associated DSD. We also are gaining a more detailed view of how *DMRT1* engages with target genes, know the identities of many of these genes, and are beginning to identify other regulators that cooperate with *DMRT1* to control gonadal gene expression.

Much remains to be learned, however. Among the open questions relating to the role of *DMRT1* in DSD are whether *DMRT1* controls sex determination or sex differentiation in the human gonad (or both), how genetic background intersects with *DMRT1* loss in DSD, whether male sex maintenance exists in the human testis, and whether other genes on distal 9p contribute to testicular differentiation or male fertility. Most of these are questions not easily resolved using mice or other model organisms, but advances in organoid technology may soon accelerate progress [Sakib et al., 2019].

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Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

D.Z. wrote the manuscript and M.W.M. performed EMSA experiments and edited the manuscript.

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