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RESEARCH ARTICLE

Loss of *dmrt1* restores zebrafish female fates in the absence of *cyp19a1a* but not *rbpms2a/b*

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ABSTRACT

Sex determination and differentiation is a complex process regulated by multiple factors, including factors from the germline or surrounding somatic tissue. In zebrafish, sex-determination involves establishment of a bipotential ovary that undergoes sex-specific differentiation and maintenance to form the functional adult gonad. However, the relationships among these factors are not fully understood. Here, we identify potential Rbpms2 targets and apply genetic epistasis experiments to decipher the genetic hierarchy of regulators of sex-specific differentiation. We provide genetic evidence that the crucial female factor *rbpms2* is epistatic to the male factor *dmrt1* in terms of adult sex. Moreover, the role of Rbpms2 in promoting female fates extends beyond repression of Dmrt1, as Rbpms2 is essential for female differentiation even in the absence of Dmrt1. In contrast, female fates can be restored in mutants lacking both *cyp19a1a* and *dmrt1*, and prolonged in *bmp15* mutants in the absence of *dmrt1*. Taken together, this work indicates that *cyp19a1a*-mediated suppression of *dmrt1* establishes a bipotential ovary and initiates female fate acquisition. Then, after female fate specification, Cyp19a1a regulates subsequent oocyte maturation and sustains female fates independently of Dmrt1 repression.

KEY WORDS: Rbpms, Sex-determination, Bipotential, Cyp19a1a, Dmrt1, TGFβ

INTRODUCTION

Although sex determination is a common biological process crucial to reproduction and, thus, species survival in sexually reproducing organisms, the mechanisms controlling this process vary extensively among animal species. The decision to develop as one sex or the other is triggered in different species by many environmental factors, including temperature and nutrition status, presence of sex chromosomes and the function of many sex-associated genetic factors that converge upon regulation of downstream sexual differentiation factors, for example, *Sox9* (*SRY-box 9*), *Foxl2* (*forkhead box L2*), *dmrt1* (*doublesex and mab-3 related transcription factor 1*) and *Cyp19a1a* (*aromatase; cytochrome P450, family 19, subfamily a*) (Kossack and Draper, 2019; Matson et al., 2011; Uhlenhaut et al., 2009; Lau et al., 2016). In domesticated laboratory strains of zebrafish, sex determination appears to be polygenic (Anderson et al., 2012; Liew et al., 2012; Moore and Roberts, 2013; Wilson et al., 2014), being influenced by numerous genetic factors. Like most teleost fish, zebrafish are a gonochoristic species, eventually differentiating into one of two sexes, and in general do not switch sex as adults (Avise and Mank, 2009). However, the zebrafish juvenile gonad, which is initially undifferentiated, further develops into a bipotential ovary that contains early-stage oocytes but remains poised to develop as either an ovary or a testis (Takatsu et al., 2013). This plasticity provides an excellent model for studying genetic factors and interactions between essential regulators of sex-specific differentiation among vertebrates.

Factors regulating sex differentiation and maintenance vary in function, from transcription factors regulating sex-specific programs, to regulators of signaling molecules, and even signaling molecules themselves. Doublesex and Mab-3 related transcription factor 1 (Dmrt1) is a transcription factor crucial for male gonad differentiation and maintenance across vertebrates (Matson et al., 2010, 2011; Webster et al., 2017; Kopp, 2012; Kim et al., 2007; Raymond et al., 2000). In mammals, Dmrt1 acts by directly activating male-determining genes and simultaneously suppressing female-determining genes (Matson et al., 2011; Murphy et al., 2010). Recent studies have verified that *dmrt1* is expressed in both the germline and somatic gonad in zebrafish and, as in mammals, is required for male differentiation, as mutants disrupting Dmrt1 cause female-biased sex ratios (Webster et al., 2017; Lin et al., 2017). Aromatase, encoded by *cyp19*, required for the final steps of estrogen synthesis (Bulun et al., 1994; Simpson et al., 1994), is a crucial factor for sex differentiation in fish (Takatsu et al., 2013; Dranow et al., 2016). Zebrafish have two copies of *cyp19* (ovarian *cyp19a1a* and brain *cyp19a1b*) and, in contrast to *dmrt1*, knockout of ovarian *cyp19a1a* results in an all-male phenotype that is characterized by early transition to male gonad features with an apparent absence of a bipotential ovary stage of development (Yin et al., 2017).

Bone morphogenetic protein 15 (Bmp15) is a signaling molecule expressed primarily by the oocyte in mice and zebrafish (Dranow et al., 2016; Clelland et al., 2006; Dube et al., 1998). Bmp15 has recently been shown to be required in zebrafish for maintenance of the female gonad, after sex has been determined, and has been postulated to promote specification of *cyp19a1a*, producing somatic fates that promote further oocyte and follicle development (Dranow et al., 2016). Knockout of *bmp15* in zebrafish causes early switching of mutant sex from female to male, and thus results in recovery of only fertile adult males (Dranow et al., 2016). Although work in mammalian systems indicates that TGF-β signaling, particularly GDF-9 and BMP15, are crucial for granulosa cell fate and development (Peng et al., 2013a; Otsuka et al., 2011; Su et al., 2004), Gdf9 appears to be dispensable in zebrafish because loss of function causes no overt phenotypes and does not worsen phenotypes caused by loss of *bmp15* (Dranow et al., 2016).

Recent work from our laboratory has identified an RNA-binding protein, RNA-binding protein of multiple splice forms 2 (Rbpms2), as a crucial germline-expressed factor for female sex differentiation in

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zebrafish (Kaufman et al., 2018). Rbpms2 is conserved among vertebrates and has been shown to regulate Bmp in the gastrointestinal tract and smooth muscle plasticity in mammalian systems (Nakagaki-Silva et al., 2019; Sagnol et al., 2014; Notarnicola et al., 2012), and to play a significant role in zebrafish cardiac development (Kaufman et al., 2018). More specifically, *RBPMS2* is expressed in chick visceral smooth muscle cell (SMC) precursors and blocks SMC differentiation by promoting expression of the BMP inhibitor noggin RNA (Notarnicola et al., 2012). In addition, based on studies in HEK 293T cells, RBPMS has been postulated to promote nuclear accumulation of Smad2/3 and transcriptional activation of Smad2/3 targets by a mechanism that requires Smad2/3 phosphorylation (Sun et al., 2006). Although *rbpms2* is expressed in many cell types, including primary oocytes in mammals (Bgee version 14.1; Ensembl ID: ENSMUSG00000032387), the function of Rbpms2 in mammalian sex determination has not been investigated. Our laboratory has demonstrated that simultaneous loss of both redundant copies of *rbpms2* [*rbpms2a* and *rbpms2b*, i.e. in *rbpms2* double mutants (DMs), referred to here as *rbpms2DM*s] results in recovery of exclusively fertile males (Kaufman et al., 2018); however, how Rbpms2 promotes female fates is not clear. It has also been shown that both the somatic gonad and meiotic oocytes contribute to establishing and maintaining the female gonad in domesticated zebrafish (reviewed by Kossack and Draper, 2019). Therefore, it is expected that the sex-specific identities of the somatic gonad and germ cells must match to develop a functional gonad successfully.

Although crucial roles for each of the above-mentioned genes in determining sexual fate in zebrafish have been established, and it is known that female and male pathways function antagonistically (Kossack and Draper, 2019; Herpin and Schartl, 2015), precisely where each factor resides in the hierarchy of genes regulating germ cell and somatic gonad sex and their interactions in gonadal differentiation remains incompletely understood. In this work, we surveyed known regulators of sex determination to identify potential Rbpms2 targets. Specifically, we used genetic epistasis experiments and cell biological approaches to tease apart the genetic hierarchy of these crucial factors in sex determination. We provide evidence that TGF-β signaling is activated in early germ cells of the bipotential ovary and in wild type becomes activated in the somatic gonad upon sexual differentiation, and that in *rbpms2DM*s initial activation of TGF-β in the bipotential germline is intact. Because zebrafish first establish a bipotential ovary, based on the presence of histologically evident early oocytes that eventually differentiate as an ovary or a testis, we reasoned that antagonism of male promoting factors such as Dmrt1 would be key to female-specific differentiation.

Surprisingly, we found that loss of *dmrt1* was not sufficient to suppress the male-only differentiation phenotype of the *rbpms2DM*s, thus *rbpms2* is epistatic to *dmrt1* in terms of adult sex. Moreover, Rbpms2 has a role beyond simply repressing or antagonizing Dmrt1, as it is required to promote female fates and for female sex-specific differentiation even in the absence of Dmrt1. Interestingly, unlike either *rbpms2DM*s, which develop as fertile males, or *dmrt1*^{uc27} mutants, which develop as fertile females, the triple mutants (*rbpms2; dmrt1TM*s) were sterile in breeding and immunohistological assays, indicating that germ cells might be lost as a consequence of cell autonomous failure to establish sex-specific identity, later requirement for *dmrt1* in germline maintenance, nonautonomous defects in somatic gonad development, or mismatched germ cell and somatic gonad sex-specific identity. Further demonstrating the distinct contribution of Rbpms2 to promoting female fates, we found that loss of *dmrt1* was sufficient to suppress the early male-differentiating phenotype of *cyp19a1a* mutant gonads, as evidenced by the presence of oocytes containing the female-specific marker Buckyball and morphologically normal Balbiani bodies in *cyp19a1a; dmrt1DM* gonads (Heim et al., 2014). In contrast, early *cyp19a1a* single mutant gonads prematurely take on a male identity and fail to develop any oocytes. Taken together these findings indicate that *cyp19a1a* acts during at least two steps of female-specific differentiation. First, *cyp19a1a*-mediated suppression of *dmrt1* is key to establish a bipotential ovary and initiate female fate acquisition in zebrafish, possibly by promoting *rbpms2*, which is required for female-specific differentiation, even in the absence of Dmrt1. Ultimately, female fates are not maintained in *cyp19a1a; dmrt1DM*s, probably due to the later Bmp15-dependent expression of Cyp19a1a that is required for subsequent follicle differentiation and oocyte maintenance by a mechanism that is independent of inhibition of Dmrt1. In support of this notion, maintenance of female fate can be prolonged in *bmp15* mutants that also lack *dmrt1*; however, these oocytes fail to progress to vitellogenic stages and *bmp15;dmrt1DM*s eventually switch to male fate.

RESULTS AND DISCUSSION

Identification of candidate Rbpms2 target RNAs

Because *rbpms2DM*s can develop as fertile males that complete meiosis and spermatogenesis but fail to complete female differentiation (Kaufman et al., 2018), we reasoned that *rbpms2* is not required for meiosis per se, but instead is required to prevent the germline from adopting a male fate. This could occur by promoting expression of female factors, repressing male factors or a combination of both. Because Rbpms2 is an RNA-binding protein, we surveyed genes required for meiosis, oogenesis and sex determination for presence of the previously identified Rbpms RNA-binding site consensus sequence, *CAC(n3-12)CAC* (Farazi et al., 2014). The presence of a Rbpms RNA-binding site indicates that an RNA might be a potential target for Rbpms2 regulation. Targets involved in gonad development (particularly those with similar phenotypes to *rbpms2*) are compelling targets for investigation. We identified five or more Rbpms RNA-binding sites within the untranslated regions (UTRs) and exons of the male fate regulator *dmrt1*; *cyp19a1a*; the TGF-β signaling molecule, *bmp15*; and bmp receptors, *bmpr1ab*, *bmpr1bb* and *bmpr2b* (Table 1), making them compelling targets for regulation by Rbpms2.

Molecular characterization of *rbpms2DM* juvenile gonads

Because Rbpms2 regulates BMP signaling in other contexts, RNAs encoding Bmp15 ligand and Bmp receptors were identified as potential targets of Rbpms2 regulation based on the presence of Rbpms RNA-binding sites, and because Bmp15 is required in zebrafish for maintenance of the female gonad later in development, we examined *bmpr* expression in *rbpms2* mutants. We previously showed that cellular features of *rbpms2DM* juvenile gonads are comparable to wild-type gonads (Kaufman et al., 2018). To determine whether *rbpms2DM* juvenile gonads resemble the wild type molecularly, with respect to Bmp-mediated signaling, we prepared cDNA from trunks at day 21 post fertilization (d21), prior to sex-determination, and used RT-PCR to examine zebrafish *bmp* receptors known to be expressed in the gonad, *bmpr1ab, bmpr1bb, bmpr2b* (Dranow et al., 2016), and *ef1alpha* (also known as *eef1a*) as control. We observed comparable expression of *bmpr* transcripts between *Rbpms2* mutants and wild-type siblings (Fig. 1A).

TGF-β signaling in the differentiating gonad

In mammalian systems, TGF-β signaling mediated by GDF-9 and BMP15 form an oocyte-granulosa cell feedback loop (Otsuka et al.,

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Table 1. Rbpms2-binding sites in genes involved in meiosis, oogenesis and sex determination

Gene name	RRE	Location	Sequence	Function	Reference
buckyball	Yes	3′UTR	CACTTCACTATAAATGTACACATGGACAC	Balbiani body assembly, polarity	Marlow and Mullins (2008)
		Exon 3	ACACAGACACA		
speedy/ RINGO	Yes	3′UTR	CACAATCACACAC	Meiotic progresssion/maturation	Dinarina et al. (2008)
		3′UTR	CACTCAATACAC		
		3′UTR	CACACTCAAAACAC		
		3′UTR	CACTGATGACAC		
mos	Yes	Exon 1	CACCAATCCCCGTCAC	Release of primary arrest	Sagata et al. (1988)
		Exon 1	CACCTGCAC	Establishment of secondary arrest	Ziegler and Masui (1973)
		Exon 1	CACCACGTGCAC		
		Exon 1	CACCTGCACGCGCACGGCGTAGTGCAC		
		Exon 1	CACAAGTGACAC		
		Exon 1	CACGTTTACGCAC		
nanos1	Yes	3′UTR	CACATGTATTGCAC	RNA-binding protein	Zhou and King (1996)
vasa	No			RNA heliase; RNA-binding protein	Schupbach and Wieschaus (1986)
dmrt1	Yes	5′UTR	CACGAGCTCTGGAATGCAC	Transcription factor	Lin et al. (2017)
		Exon 1	CACGGCTTCGTGTCAC		Webster et al. (2017)
		Exon 1	CACCGCTGAAGGGCCAC		
		3′UTR	CACTATCTAGAAGAACAC		
		3′UTR	CACCAACAC		
bmp15	Yes	Exon 1	CACATATTCAC	TGF-β signaling molecule, folliculogenesis	Dranow et al. (2016)
		Exon 2	CACCTACACA		Otsuka et al. (2011)
		Exon 2	CACAAAGCCGGAGCAC		
		Exon 2	CACCTCAACAC		
		Exon 2	CACCCCAC		
		3′UTR	CACTTTATTAACAC		
cyp19a1a		Exon 1	CACCATCGACCACACAAATCAC	Aromatase, estrogen synthesis	Kishida and Callard (2001)
		Exon 3	CACTTCAC		Townsley et al. (1973)
		Exon 4	CACCACCTCCAC		
		Exon 5	CACAAAAAGCAC		
		3′UTR	CACTCTCTGATCCAC		
bmpr1aa		5′UTR	CACAACTACAC	Bmp type 1 receptor	Smith et al. (2011)
		Exon 4	CACGACTTTGGACAC	Expressed in early oocytes	Pulkki et al. (2011)
		Exon 8	CACCAGACCCTCAC	Nodal activity during left-right axis specification	Dranow et al. (2016)
		3′UTR	ACACAGTTTGGTTTCTCAC		
bmpr1ab		5′UTR	CACTTCCAC	Bmp type 1 receptor	Pulkki et al. (2011)
		Exon 1	CACCTTCAC	Expressed in early oocytes	Dranow et al. (2016)
		Exon 2	CACAAACAACAC	Nodal activity during left-right axis specification	
		Exon 8	CACTACCCTGGACAC		
		Exon 8	CACCTCCAC		
		Exon 11	CACAATCCAGCCTCAC		
bmpr1bb		5′UTR	CACACACACAC	Bmp type 1 receptor	Pulkki et al. (2011)
		Exon 12	CACCACGCTGGACAC	Expressed in early oocytes	Dranow et al. (2016)
		Exon 13	CACGCGTGTGGGCAC		
		3′UTR	CACTGACAATCACCAACACACAC		
		3′UTR	CACAGGAGTCAC		
		3′UTR	CACACACACACACAC		
bmpr2b		Exon 12	CACAAGTTTGTCCACCACAACCACC ACAACAGGCCTCAC	Bmp type 2 receptor	Bauer et al. (2001)
		Exon 12	CACTCGGAGGACAC	Expressed in early oocytes	Monteiro et al. (2008)
		Exon 12	CACTCCCAC	Left-right asymmetry	
		Exon 12	CACTCCGGTCGCAC		
		Exon 13	CACTGATACAC		
foxl2a		Exon 1	CACCACCACCAC	Oocyte maintenance	Yang et al. (2017)
		Exon 1	CACGGCTGCAGCAC		

Continued

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Table 1. Continued

Gene name	RRE	Location	Sequence	Function	Reference
		Exon 1	CACGAGAGCAAACACTCTGCACTACAC		
		3′UTR	ACACATCAACATATTCAC		
gsdf		5′UTR	CACAGACAACATCCAC	TGF-β signaling molecule	Yan et al. (2017)
		Exon 5	CACCGCGCACCAC	Female germ cell specification	
		3′UTR	ACACACACATAACACACTCACACAC ACACACACACACACA	Oocyte maturation	
		3′UTR	CACTGCTGTAGCACACACACA CACACACACACA		
		3′UTR	CACAGAAANACAC		
		3′UTR	CACAACTGTACTACAC		
gdf9		5′UTR	CACCTCCGGCCCAC	Expressed in early oocytes, no known role	Dranow et al. (2016)
		5′UTR	CACTGATGCGCAC		
		Exon 1	CACCTGTACAACAC		
		Exon 2	CACAAGAACAC		
		Exon 2	CACAACCAC		
cdk21		5′UTR	CACAAGTAATTACACCCAA CACTGATTATAACAC	Male-specific proliferation and meiosis	Webster et al. (2018)
		3′UTR	CACAGATCAC		

RRE, Rbpms recognition element. Rbpms binds to tandem CAC motifs (underlined) separated by a spacer of variable length.

2011; Su et al., 2004; Matsumoto et al., 2012; Tsukamoto et al., 2011a,b; Peng et al., 2013b; Wigglesworth et al., 2013). By contrast, in zebrafish, Gdf9 appears to be dispensable because *gdf9* loss neither causes overt phenotypes nor worsens phenotypes caused by loss of *bmp15* (Dranow et al., 2016); thus, the role of TGF-β in the differentiating zebrafish gonad remains unclear. To investigate potential involvement of TGF-β, we examined the localization of p-Smad2, an indicator of active TGF-β signaling (de Sousa Lopes et al., 2003; Nakao et al., 1997; Zhang et al., 1996). In bipotential (d21) wild-type (Fig. 1B) and *rbpms2DM* (Fig. 1C) juvenile gonads, p-Smad2 was detected with DAPI in the nuclei of germ cells marked by *ziwi*-GFP (Leu and Draper, 2010). As differentiation proceeded in wild-type females, p-Smad2 remained strong in the nuclei of early oocytes and became activated in the nuclei of somatic gonad cells (Fig. 1D). Nuclear p-Smad2 was also detected in the somatic gonad of wild-type (Fig. 1E) and *rbpms2DM* (Fig. 1F) males, although its presence appeared weaker than in females and declined in differentiating spermatogonia. Based on these observations, we conclude that TGF-β is active in the early germline and soma of the differentiating gonads of wild-type zebrafish, reminiscent of the patterns observed in mammals (Xu et al., 2002); however, the relevant ligand(s) in zebrafish remain to be identified. Moreover, although initial activation of p-Smad2 in the germline was intact, the strong signals achieved in germline and soma of wild-type females were not detected in *rbpms2DM*s. Because Rbpms2 binds to and has been implicated in potentiating activation of Smad2/3 targets (Sun et al., 2006), it is possible that Rpbms2 promotes female fates by boosting TGF-β signaling. However, based on the expression and immunohistochemical (IHC) data herein and the earlier sex-reversal phenotypes of *rbpms2DM*s compared with *bmp15* and *bmpr1bb* (also known as *alk6b*) mutants (Dranow et al., 2016; Neumann et al., 2011), altered Bmp signaling is not likely to be responsible for the initial failure of oocyte development observed in *rbpms2* mutants.

Epistasis analysis between the female factor *rbpms2* and male factor *dmrt1*

A key and evolutionarily conserved regulator of male-specific gene expression and antagonist of female fates that is necessary for male-specific development is the Doublesex and Mab3 related transcription factor, Dmrt1 (Webster et al., 2017; Ge et al., 2017; Huang et al., 2017; Zhang et al., 2016; Zhao et al., 2015; Lindeman et al., 2015; Koopman, 2009; Raymond et al., 1999; Koopman and Loffler, 2003). Zebrafish mutants lacking *dmrt1* develop mostly as fertile females, the opposite phenotype of *rbpms2DM*s (Webster et al., 2017); *rbpms2* and *dmrt1* are both expressed as RNAs in stage I oocytes (Webster et al., 2017; Kaufman et al., 2018). Because female development initiates but ultimately fails in *rbpms2DM*s, and because we identified Rbpms2 binding sites in the 3′UTR of *dmrt1* transcripts, it is possible that Rbpms2 promotes female differentiation by repressing *dmrt1* directly and/or by antagonizing the male-specific program mediated by Dmrt1. We reasoned that if *rbmps2* mutants develop as fertile males because of failed antagonism of the Dmrt1-mediated male pathway, then loss of *dmrt1* should restore female development in *rbpms2DM*s. However, if *rbpms2* acts downstream of *dmrt1* or acts in a distinct pathway, then *dmrt1* mutants would fail to differentiate as females even in the absence of Rbpms2. To test this hypothesis, we conducted genetic epistasis analysis between mutants lacking *rbpms2* and *dmrt1* functions. We generated adults lacking *rbpms2a/b* and *dmrt1* and screened for sex-specific secondary sex traits and fertility (Fig. S1). To determine the sex-specific identity of germ cells in the early gonads, and to resolve the epistatic relationship between *rbpms2* and *dmrt1*, we performed IHC analysis of d28 gonads. In triple heterozygous (HHH) siblings, gonads had numerous germ cells and early-stage oocytes (Fig. 2A) or were testis-like gonads (Fig. 2B) (Heim et al., 2014; Bontems et al., 2009). The *rbpms2DM* gonads were either intersex with some early oocytes and clusters of spermatogonia-like cells (Fig. 2C), or were testis-like (Fig. 2D) as we previously reported (Kaufman et al., 2018). All *dmrt1* single mutant gonads examined had early oocytes (Fig. 2E), whereas *rbpms2;dmrt1TM*s (Fig. 2F) resembled their *rbpms2DM* siblings. Based on these data, *rbpms2* is required for female-specific differentiation, even in the absence of *dmrt1*, and is therefore epistatic to *dmrt1*.

Like *rbpms2DM*s (Fig. 3A,B; Fig. S1), *rbpms2;dmrt1TM* adults developed exclusively as males, indicating that *rbpms2* is also epistatic to *dmrt1* in terms of adult sex. However, unlike *rbpms2DM*s, which were fertile males, or *dmrt1* mutants with

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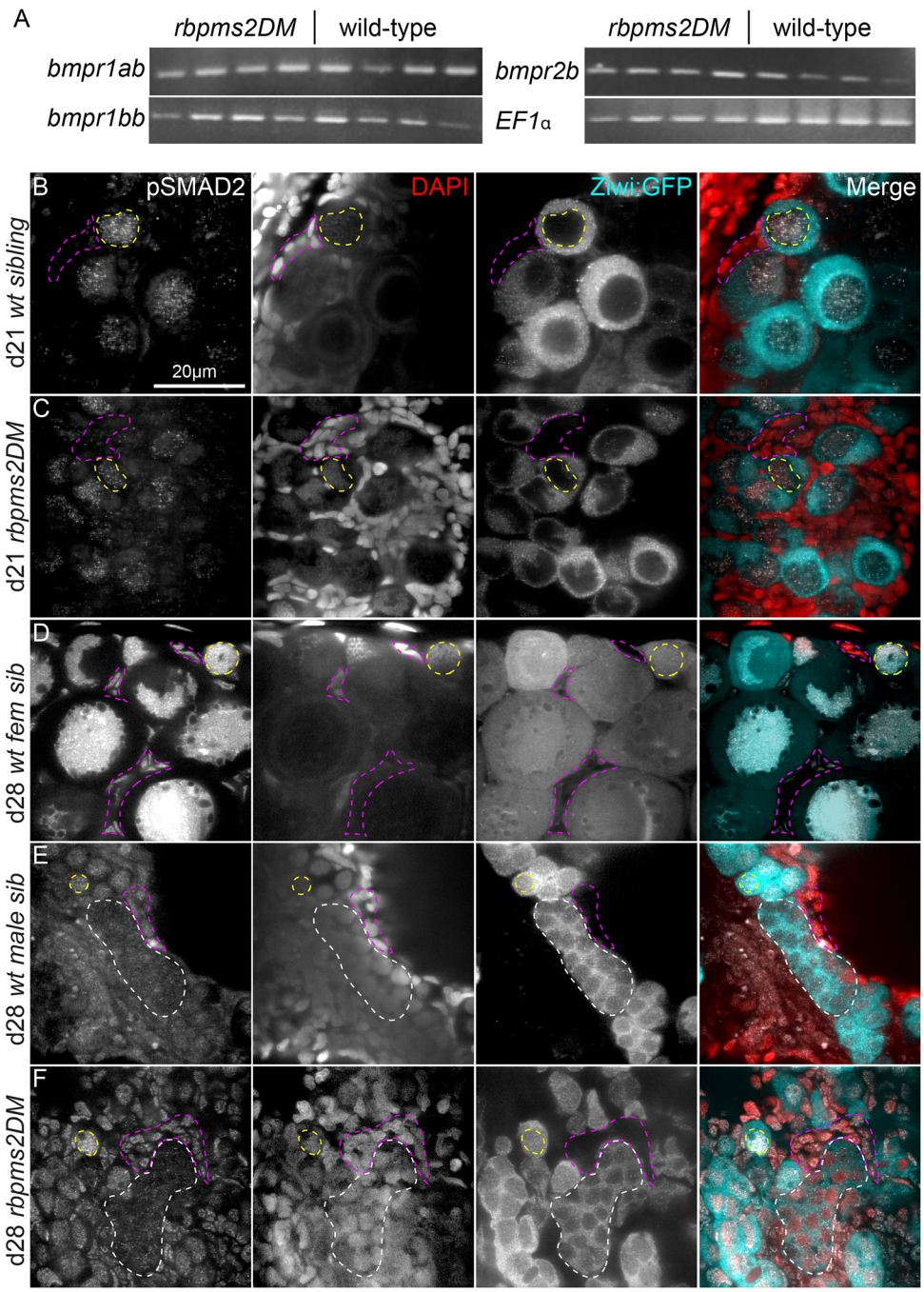

Fig. 1. Molecularly bipotential juvenile gonads of *rbpms2DM*s and activation of p-SMAD in early germ cells and differentiating gonads. (A) RT-PCR on dissected trunks from individual d21 *rbpms2DM*s and wild-type siblings reveals no differences in expression of relevant receptors. (B-F) p-Smad2, DAPI and *ziwi*:GFP staining. Representative slices from Z-series reveal localization of p-Smad2 in bipotential and early differentiating gonads of d21 and d28 juvenile zebrafish, respectively. In undifferentiated bipotential ovaries of both wild-type (*n*=3) (B) and *rbpms2DM* (*n*=3) (C), p-Smad2 is detected in the nuclei of germ cells (example outlined with yellow dashed line) but not the nuclei of the surrounding somatic cells (examples outlined in pink dashed lines). (D) By d28 in early differentiated females (*n*=4), p-Smad2 remained localized to the germ cell nuclei and began to be expressed in somatic nuclei. Similarly, p-Smad2 was expressed at d28 in both germ cell nuclei (examples outlined in white dashed lines) and somatic nuclei of wild-type males (*n*=4) (E) and *rbpms2DM* gonads (*n*=4) (F). 'Wild-type' denotes wild-type or heterozygous animals as well as *rbpms2a* or *rbpms2b* single mutants that are heterozygous or homozygous for the wild-type allele at the other *rbpms2* locus because these animals are phenotypically normal.

some Rbpms2 intact (compound genotypes with various *rbpms2* combinations), the TM males were sterile in fertility assays (failed to induce spawning in mating assays) (Fig. 3B,C; Fig. S1). Interestingly, *dmrt1* mutants that were also *rbpms2b* mutants, with just one copy of *rbpms2a* intact (compound HMM), were sterile males like the TMs (Fig. 3B). Thus, although *rbpms2a* and *rbpms2b* have redundant functions, requiring loss of both to observe the male-only phenotype, it appears that *rbpms2b* is more important for sustaining female fates because, even in the absence of *dmrt1*, one copy of *rbpms2b* but not *rbpms2a* is sufficient to maintain female fates. Identifying the basis of this difference in *rbpms2* activity is an important area of future investigation. Unlike the ovary or testis of wild-type zebrafish (Fig. 3D,E), *rbpms2DM*s or the ovary of *dmrt1* mutants (Fig. 3F), TM adult males were found to lack germ cells upon inspection of the dissected gonad (Fig. 3G). It appears that germ cells of the TM gonad are lost as they attempt to transition to a male fate. In previous studies, roughly 30% of *dmrt1* mutants recovered were sterile males due to failed spermatogenesis and somatic gonad deficits (Webster et al., 2017; Wu et al., 2020), raising the possibility that the sterile testes observed in TMs could simply be a result of loss of *dmrt1*. Because we only recovered *dmrt1* mutant females, we were unable to compare testis development in *dmrt1* single mutants and TMs. Because we did not detect Rbpms2 protein in testis (Fig. S2) or in somatic cells in the ovary (Kaufman et al., 2018), Rbpms2 probably acts cell autonomously to promote female germline fate, which could secondarily impact somatic gonad fate. However, *dmrt1* transcripts have been detected in both germ and somatic cells (Webster et al., 2017),

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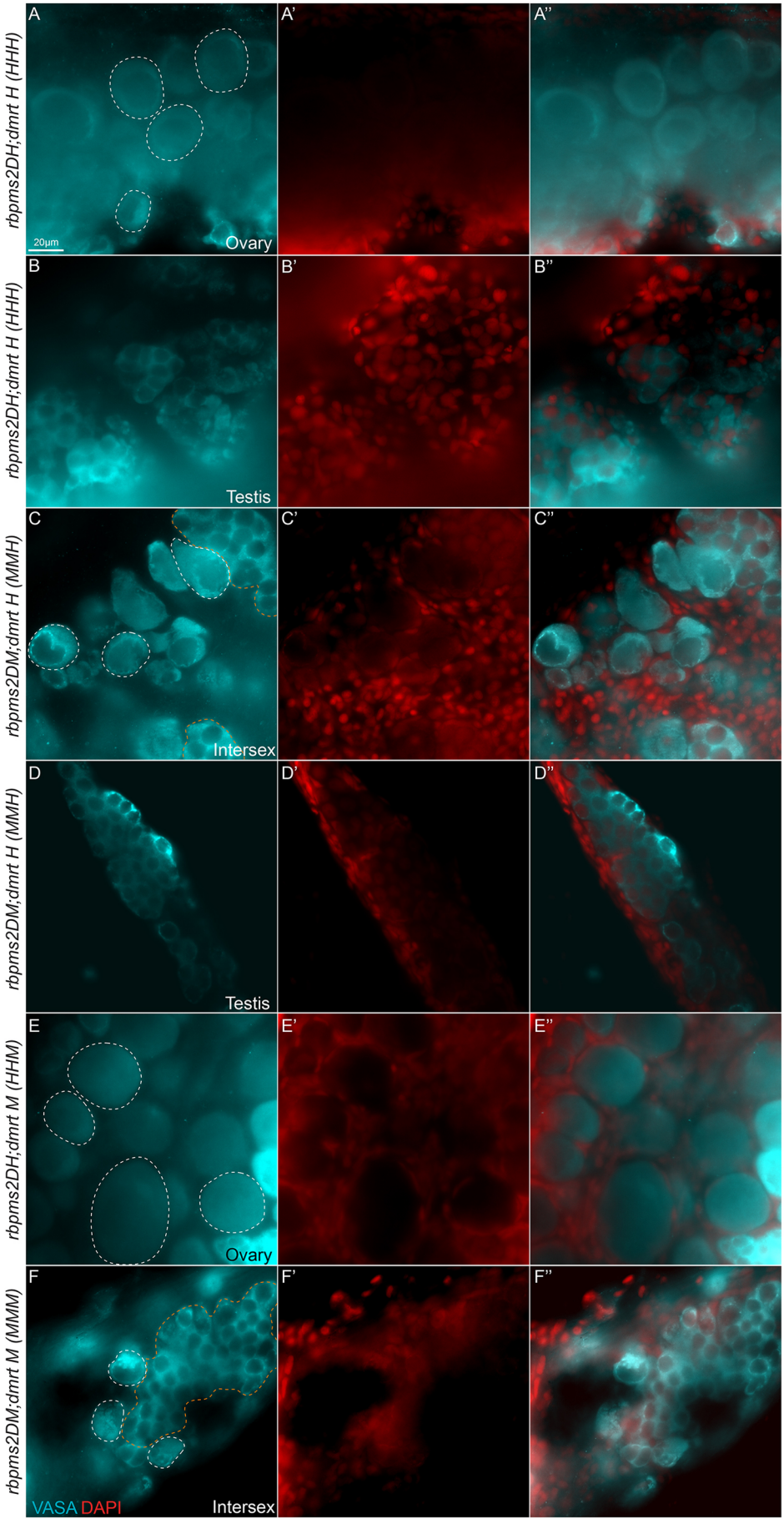

Fig. 2. Rbpms2 is required for female gonad differentiation even in the absence of an essential male fate regulator. (A-F″) Vasa (A-F), DAPI (A′-F′) and merged (A″-F″) staining. Representative slice from a Z-stack reveals gonad development of d28 juvenile zebrafish, including female (*n*=1/3) (A) or male (*n*=2/3) (B) triple heterozygote (HHH) siblings. All *rbpms2DM* (MMH) gonads recovered were either intersex (*n*=2/3) (C) or male (*n*=1/3) (D). The *dmrt1* mutant siblings (HHM) were female (*n*=3/3) (E) and triple mutant siblings (MMM) were intersex (*n*=3/3) (F). Oocyte-like cells are outlined in white dashed lines and testis-like cells in orange dashed line.

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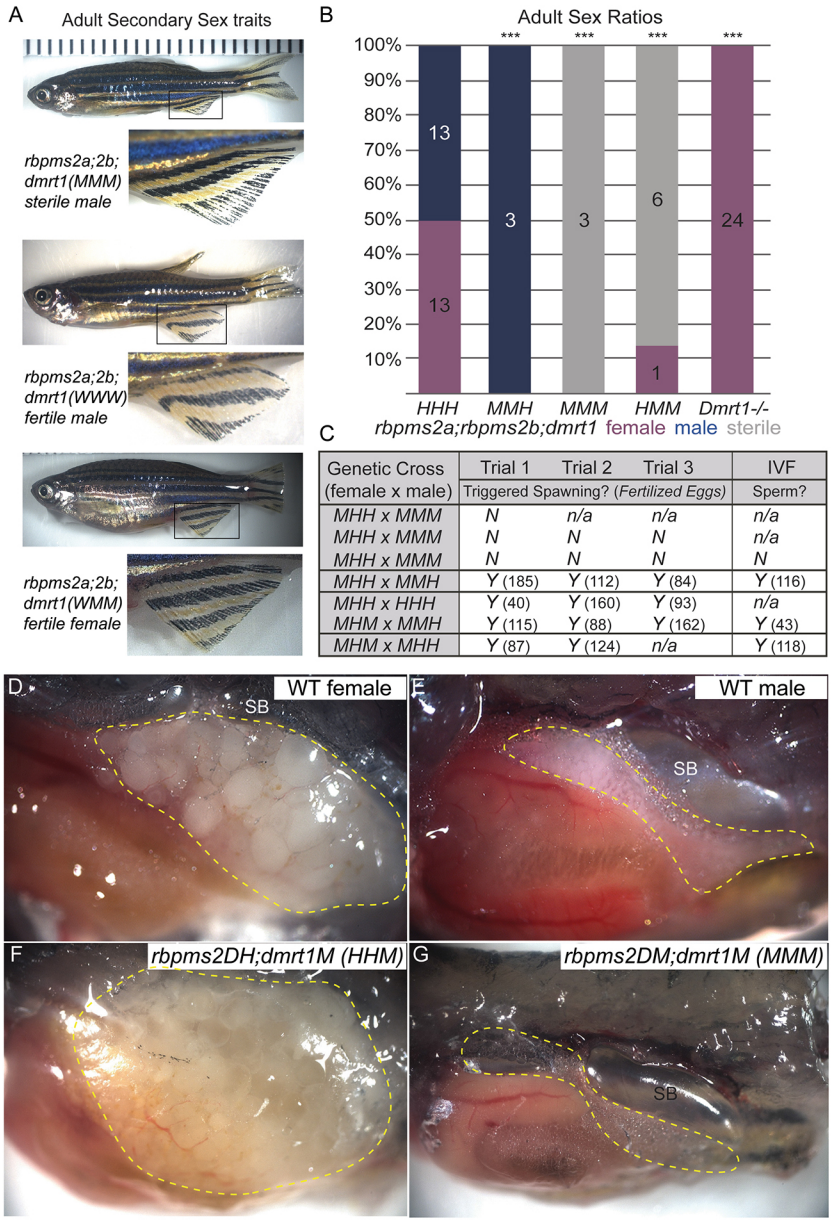

Fig. 3. *rbpms2;dmrt1TM*s are sterile, unlike *rbpms2DM*s. (A) Secondary sex traits were assessed to identify fish from each category. Representative images show body shape and color; insets are magnified views of the anal fin. (B) Adult sex ratios of progeny pooled from three triple heterozygote intercrosses for given genotypes. All double mutants also heterozygote for *dmrt* (MMH) were male as previously observed in *rbpms2DM*s, and all *dmrt1* mutants in any other genetic combination (HHM, MHM, etc.) were female, as previously reported for *dmrt1*$^{uc27/uc27}$. All triple mutants (MMM) were sterile males. Numbers indicate number of animals for each condition. Animals mutant for *dmrt1* and any combination of a wild-type or heterozygous for a *rbpms2* allele (WMM, WHM, HHM, etc.) were combined because they were phenotypically wild type, with the exception of *rbpms2;dmrt1HMM* as it had a different phenotype. The X^2 goodness of fit test with Bonferroni correction was used for multiple comparisons. ***P<0.001 indicates groups that deviated from the expected 50:50 sex ratio. (C) Fertility was assessed in mating trials as indicated in the chart. For each cross, if a male triggered spawning or if sperm was acquired through IVF, the numbers (in parentheses) of fertilized eggs per trial for natural breeding are indicated. n/a indicates fish that were not able to be mated for subsequent mating trials. (D-G) Representative lateral view images of representative adult with tissue resected to reveal gonads (outlined in yellow dashed lines) of wild-type female (D), wild-type male (E), *dmrt1*$^{uc27/uc27}$ (*rbpms2;dmrt1HHM*) female (F) and *rbpms2;dmrt1MMM* (G) fish. TM fish lacked morphologically obvious gonads proximal to the swim bladder (SB) compared with fertile sibling wild type or single mutants.

thus germ cells might be lost in *rbpms2;dmrt1TM*s because of a cell autonomous failure to establish sex-specific identity (e.g. the germ cells are neither truly male or female without Rbpms2 and Dmrt1), nonautonomous defects in somatic gonad development, as a consequence of mismatched germ cells and somatic gonad identity, or as a result of a later germ cell autonomous requirement for Dmrt1 in germline maintenance or development of the somatic gonad (Webster et al., 2017; Matson et al., 2010; Wu et al., 2020; Lei et al., 2007; Masuyama et al., 2012). Nonetheless, these results indicate that Rbpms2 is required to promote expression of factors required for female fates, despite absence of *dmrt1*.

Loss of *dmrt1* restores bipotential and female development in the absence of *cyp19a1a*

Loss of the germ cells in *rbpms2;dmrt1TM* adults could simply be a consequence of lack of sex-specific identity or a mismatch between germ cells and somatic cell sex-specific identities. If so, the gonads lacking two opposing sex-specific differentiation factors should disrupt the sex-specific identity of the gonad, potentially force a mismatch between somatic and germ cell identity, and recapitulate the sterile male phenotype observed in *rbpms2;dmrt1TM*s. Somatic-gonad derived factors such as Dmrt1 and Cyp19a1a are continually expressed in adult gonads and loss of either gene disrupts stochastic gonad differentiation as either male or female (Webster et al., 2017; Dranow et al., 2016), with loss of *dmrt1* resulting in female sex bias (Webster et al., 2017) and loss of *cyp19a1a* resulting in recovery of only male adults (Dranow et al., 2016), indicating that the sexual phenotype of the somatic gonad must be actively maintained by the functions of these proteins. However, the genetic relationship between these two essential factors had not been appreciated in zebrafish. If bipotential ovary formation fails in *cyp19a1a* mutants due to failure to antagonize the Dmrt1-mediated male pathway, then loss of *dmrt1* should restore development of a bipotential ovary and potentially later female fates in *cyp19a1a* mutants. Conversely, if male gonadogenesis requires *dmrt1* antagonism of *cyp19a1a*, then loss of *cyp19a1a* should restore male-specific fates in *dmrt1* mutants. However, if *dmrt1* and *cyp19a1a* independently contribute to sex-specific differentiation, then *cyp19a1a;dmrt1DM*s lacking both

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sex-specific identity factors would fail to confer a sex-specific identity to the somatic gonad or result in a mismatch of soma and germ cell identity, leading to sterile males as observed in *rbpms2; dmrt1TM*s. To test this hypothesis, we conducted genetic epistasis analysis between mutants lacking *cyp19a1a* and *dmrt1* and determined the sex-specific identity of the double mutant germ cells using IHC analysis of d45 gonads (Fig. 4; Fig. S3). In *cyp19a1a*$^{-/+}$*;dmrt1*$^{-/-}$ (referred to as *cyp19a1a;dmrt1HM*) siblings, all gonads examined had numerous early-stage oocytes (Fig. 4A), consistent with the previously reported *dmrt1* mutant female-bias phenotype (Webster et al., 2017; Wu et al., 2020). As expected, all c*yp19a1a*$^{-/-}$*;dmrt1*$^{-/+}$ (*cyp19a1a;dmrt1MH*) sibling gonads examined were testis-like and lacked the oocyte marker, Buc (Fig. 4B) (Heim et al., 2014; Bontems et al., 2009). By contrast, *cyp19a1a;dmrt1DM*s either contained early oocytes with normal Balbiani bodies marked by Buc (Fig. 4C) or were testis-like with clusters of undifferentiated cells that lacked Buc expression (Fig. 4D). Based on these data, it appears that the loss of *dmrt1* is sufficient to restore development of a bipotential ovary and initial female differentiation in the absence of *cyp19a1a*, possibly by preventing *dmrt1* inhibition of *rbpms2*.

To investigate the long-term effects of the absence of *cyp19a1a* and *dmrt1* on differentiation and fertility, we screened adult mutants for sex-specific secondary sex traits and fertility (Fig. 5 and Fig. S4). The *cyp19a1a;dmrt1MH* (Fig. 5A) and *cyp19a1a;dmrt1HM* (Fig. 5B) adults appeared male and female, respectively, based on external secondary sex traits. *cyp19a1a;dmrt1MM*s appeared male, based on inspection of secondary sex traits (Fig. 5C,D) similar to *rbpms2;dmrt1DM*s. Similarly, in an independent study using different alleles, *cyp19a1a;dmrt1* were all masculinized (Wu et al., 2020). In that study, masculinization of *cyp19a1a; dmrt1MM*s was attributed to lower serum estrogen levels, which are required for development of female features (Wu et al., 2020; Brion et al., 2004). As expected, male *cyp19a1a;dmrt1MH* siblings developed testes (Fig. 5E,I) and female *cyp19a1a;dmrt1HM* siblings developed ovaries (Fig. 5F,J) with late stage oocytes, including

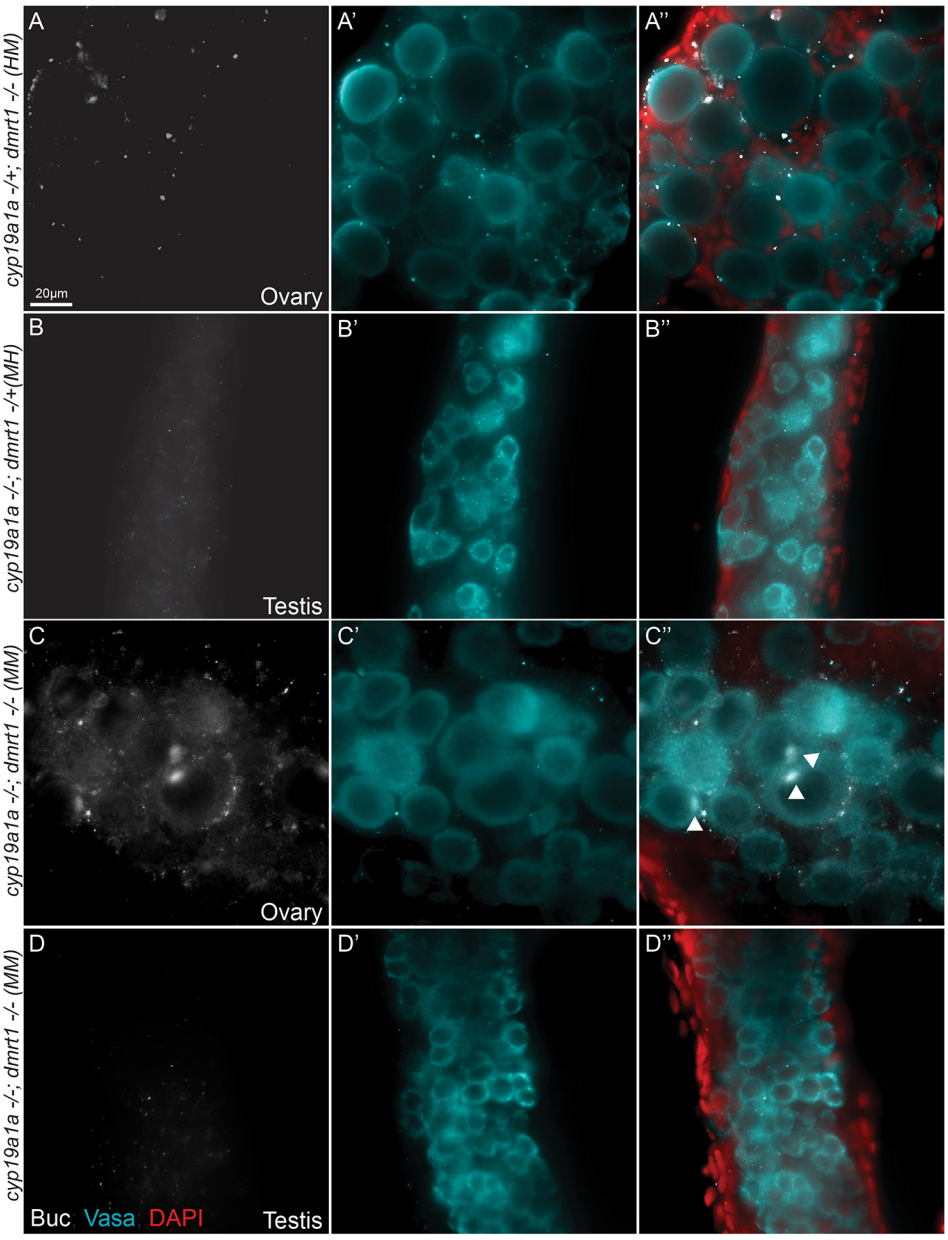

Fig. 4. Cyp19a1a promotes bipotential and female development by inhibition of *dmrt1*. (A-D″) Buc (A-D), Vasa (A′-D′) and Buc/Vasa/DAPI merged (A″-D″) staining in representative slices from individual Z-series allows visualization of gonocyte development and sex-specific differentiation in d45 juvenile zebrafish, including female *cyp19a1a*$^{uc38/+}$*; dmrt1*$^{uc27/uc27}$ (HM) (*n*=3/3) (A), male *cyp19a1a*$^{uc38/uc38}$*;dmrt1*$^{uc27/+}$ (MH) (*n*=5/5) (B) and *cyp19a1a*$^{uc38/uc38}$*;dmrt1*$^{uc27/uc27}$ (MM) gonads that were either male (*n*=1/4) with male germ cells (C) or female (*n*=3/4) with oocytes (D), as shown by expression of the oocyte marker Buc (white) and indicated with white arrowheads.

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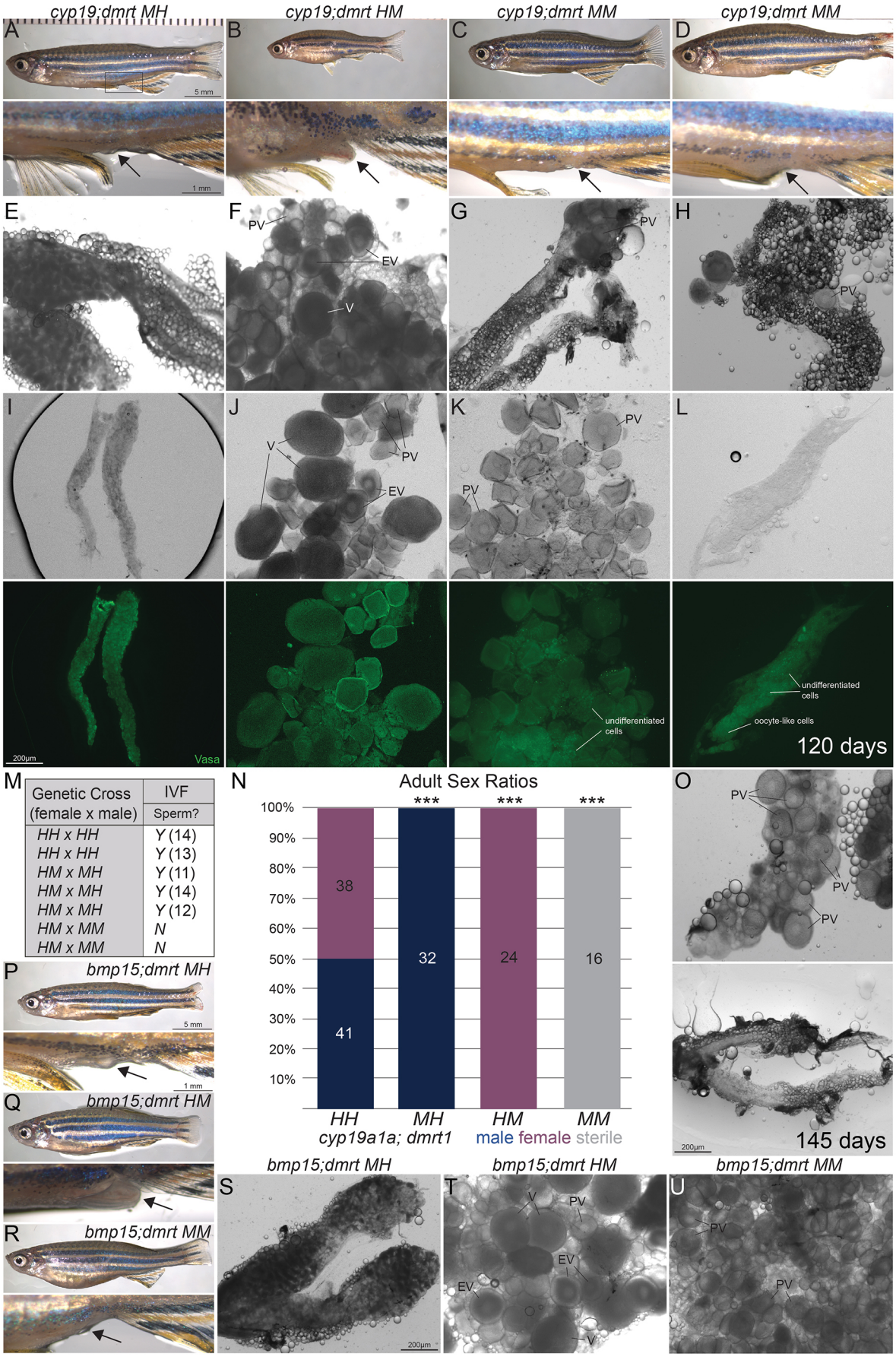

Fig. 5. See next page for legend.

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Fig. 5. Cyp19a1a is required for oocyte maturation and maintenance of female fates, independently of antagonism of *dmrt1*. (A-D) Representative lateral view images show the external secondary sexual characteristics of d120 adults: *cyp19a1a;dmrt1MH* (*n*=7) male (A), *cyp19a1a;dmrt1HM* (*n*=7) female and *cyp19a1a;dmrt1MM* (*n*=5) mutants (C,D). Male MH fish (A) had a slender body shape and lacked a genital papilla (indicated by arrow). Female HM fish (B) had a round body shape with protruding abdomen and extended genital papilla. Similar to MH males, MMs (C,D) exhibited male secondary sex traits. (E-H) However, upon dissection, MH males (E) had testes, HM females (F) had ovaries that contained pre-vitellogenic (PV) early-vitellogenic (EV) and vitellogenic (V) stages of oocytes, whereas MMs (G,H) had gonads that were atypical in overall structure and contained oocytes that only progressed to the PV stage. (I-L) Additional representative brightfield and Vasa (green) overview images of *cyp19a1a;dmrt1MH* (I), *cyp19a1a;dmrt1HM* (J) and *cyp19a1a;dmrt1MM* (K,L) gonads. (M) Fertility was assessed by natural mating or by IVF extraction of sperm followed by fertilization of eggs. For each cross or if sperm was acquired through IVF, the numbers (in parentheses) of fertilized eggs per trial are indicated. (N) Adult sex ratios of progeny pooled from heterozygote intercross and double heterozygote crossed to a mutant;heterozygote (HH×MH) for given genotypes. All *cyp19a1a*^{uc38} mutants that were also heterozygous for *dmrt1* (MH) were males, all *dmrt1*^{uc27} mutants that were also heterozygous for *cyp19a1a*^{uc38} (HM) were female. All *cyp19a1a*$^{uc38/uc38}$;*dmrt1*$^{uc27/uc27}$ double mutants appeared male based on secondary sex traits, but female based on primary gonad sex and were classified as sterile because of the lack of mature gametes. Numbers indicate individual animals for each condition. X^2 goodness of fit test with Bonferroni correction was used for multiple comparisons. ***P<0.001 indicates groups that deviated from the expected 50:50 sex ratio. (O) Representative gonads from two d145 *cyp19a1a;dmrt1MM* fish (total, *n*=4) show an ovary containing PV oocytes and a gonad lacking oocytes. (P-R) Representative lateral view images show the external secondary sex traits of d145 *bmp15;dmrt1* adults. The *bmp15;dmrt1MH* (*n*=7) (P) and *bmp15;dmrt1HM* (*n*=7) (Q) fish display normal secondary sex characteristics of males and females, respectively. MMs (*n*=4) (R) had a female body shape but lacked protruding genital papillae. (S-U) Upon dissection, MH males (S) had testes, HM females (T) had ovaries and MMs (U) had ovaries that contained PV oocytes.

pre-vitellogenic (PV), early vitellogenic (EV) and vitellogenic (V) oocytes. In contrast, despite their male secondary sex traits, D120 *cyp19a1a;dmrt1MM* gonads (*n*=4/5) (Fig. 5G,H,K,L; Fig. S4) were surprisingly ovary-like and contained early oocytes that progressed as far as the PV stage of oogenesis. The absence of vitellogenic growth suggests that, although the bipotential ovary can be established and oogenesis can progress through prophase I without *cyp19a1a* in the absence of *dmrt1*, further oocyte differentiation requires *cyp19a1a*. Failure of *cyp19a1a;dmrt1MM* oocytes to progress beyond PV stages is reminiscent of *bmp* mutant ovary phenotypes and is consistent with a role for *cyp19a1a* in promoting oocyte and follicle development downstream of *bmp15* (Dranow et al., 2016). Accordingly, at d145, half of the recovered *cyp19a1a;dmrt1MM* gonads (Fig. 5O; Fig. S4) were ovaries with oocytes stalled at the PV stage, whereas the other half no longer contained oocytes. Consistent with failed oocyte differentiation in the absence of *cyp19a1a* and a requirement for *dmrt1* in spermatogenesis or somatic gonad development, double mutants were sterile in fertility assays (absence of sperm or eggs in mating assays) (Fig. 5M). Similarly, in the study by Wu and colleagues, histological analysis revealed *cyp19a1a;dmrt1MM*s with well-developed ovaries despite the masculine secondary sex traits and observed failure to maintain oocytes and progressive sex-reversal of double mutants (Wu et al., 2020). Thus, Cyp19a1a function antagonizes Dmrt1 to establish the bipotential ovary, but is also required later in a *dmrt1*-independent manner to maintain female fate and promote oocyte differentiation in adulthood.

Bmp15 is an oocyte-derived protein required to promote oocyte differentiation beyond the PV stage and is required for development of *cyp19a1a*-positive granulosa cells (Dranow et al., 2016). In *bmp15* mutants, initial *cyp19a1a* is intact, but later *cyp19a1a* expression is *bmp15* dependent. Therefore, to explore late *cyp19a1a* function in the context of loss of *dmrt1* and investigate the regulatory relationship between *bmp15* and *dmrt1*, we examined secondary sex characteristics and sex ratios in mutants lacking *bmp15* and *dmrt1* (Fig. 5P-U; Fig. S5). As expected, *bmp15;dmrt1MH* adults (90-145 d) were exclusively male based on secondary sex traits (Fig. 5P) and primary gonad sex (Fig. 5S), and *bmp15;dmrt1HM* adults were female (Fig. 5Q,T). Although *bmp;dmrt1MM*s initially appeared feminized based on their body shape, they had male papillae (Fig. 5R). Like *cyp19;dmrt1MM*s, *bmp;dmrt1MM* gonads were ovaries with early stage oocytes that did not progress beyond PV stages (Fig. 5U), as expected if Bmp15 promotes oocyte development by inducing Cyp19a1a-producing somatic gonad fates. That oocytes persist longer in *bmp15;dmrt1MM* ovaries (Fig. 5U) than in *bmp15;dmrt1MH* single mutants (Fig. 5S), indicates that *dmrt1* contributes to sex reversal of *bmp15* mutants, probably by promoting or reinforcing expression of factors required for spermatogenesis or male-specific differentiation of the somatic gonad.

Previous work showed that *cyp19a1a* is required to establish a bipotential ovary containing early oocytes (Dranow et al., 2016). Within the bipotential ovary, Cdk21 promotes mitotic divisions of gonocytes (Webster et al., 2018) and Amh limits gonial proliferation and promotes maturation (Lin et al., 2017). In contrast to *cyp19a1a* mutants, *rbpms2*, *bmp15* and *amh* mutants develop a bipotential ovary and begin to develop oocytes that ultimately fail prior to prophase I in the case of *rbpms2* (Kaufman et al., 2018) and after diplotene arrest in the case of *bmp15* (Dranow et al., 2016) and *cdk21* (Webster et al., 2018), leading to eventual differentiation of an adult testis in these cases (Fig. 6A-D). This suggests that Cyp19a1a is required earlier in gonad development than Rbpms2, Amh or Bmp15 (Fig. 6B-D). Here, we provide genetic evidence that mutation of *dmrt1* (Fig. 6E) restores bipotential ovary development in the absence of *cyp19a1a* and allows development of a gonad that contains early oocytes (Fig. 6G). That Rbpms2 is not expressed in *cyp19a1a* single mutants (Fig. S6), which develop exclusively as males, and that normal Balbiani bodies were detected in *cyp19a1a;dmrt1DM*s indicate that inhibition of Dmrt1 allows expression of Rbpms2 and female development in the absence of Cyp19a1a. Moreover, these results support a regulatory relationship between *dmrt1* and *cyp19a1a* and suggest that Cyp19a1a is required to establish the bipotential ovary and does so by antagonizing Dmrt1 activity (Fig. 6). That bipotentiality is restored indicates that this relationship is probably mutually antagonistic, with Dmrt1 antagonizing early Cyp19a1a activity under normal conditions, poising the system for male differentiation. However, despite the ability of *cyp19a1a;dmrt1DM*s to initially form a bipotential ovary that can develop as male or female, subsequent oogenesis and ovary maintenance fails, leading to recovery of all male *cyp19a1a;dmrt1DM* adults (Fig. 6G). Because the primary function of Cyp19a1a enzyme is to catalyze the final step of estrogen (estradiol) synthesis (Bulun et al., 1994; Simpson et al., 1994), the antagonistic relationship between Cyp19a1a and *dmrt1* is probably an indirect relationship mediated by the estrogens produced by Cyp19a1a.

Indeed, recently another group independently discovered the same epistatic relationship between *cyp19a1a* and *dmrt1* and provides evidence in support of estrogen involvement (Wu et al., 2020). Additionally, that *cyp19a1a;dmrt1DM*s can establish a bipotential ovary, but eventually resolve as males, indicates that Cyp19a1a is dispensable for establishment of the bipotential ovary and initiation of female development in the absence of Dmrt1. However, this work

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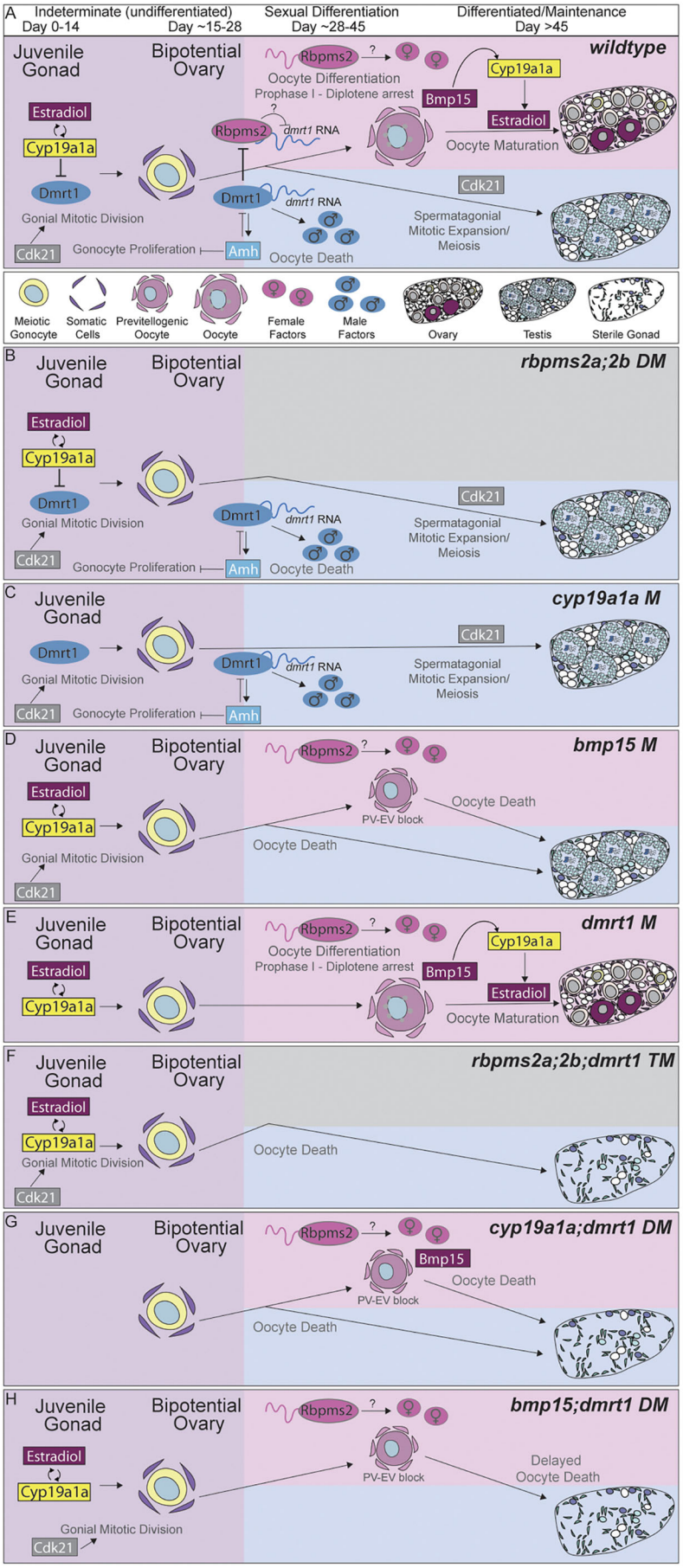

Fig. 6. Model: *Cyp19a1a* inhibits *dmrt1* to promote bipotential ovary formation and acquisition of female fates, which requires *rbpms2* even in the absence of *dmrt1*. (A-F) Timeline (from left to right) of requirement for each essential regulator of sex determination. (A) Cyp19a1a is required for the transition from juvenile gonad to bipotential ovary by antagonizing Dmrt1 activity. Cdk21 promotes mitotic divisions of gonocytes, whereas Amh limits gonial proliferation within the bipotential ovary. Inhibition of Dmrt1 allows expression of Rbpms2, either directly or indirectly, and female development in the absence of Cyp19a1a. Rbpms2 probably promotes female fates temporally upstream of Bmp15 and estrogen (estradiol), which promote follicle maturation beyond pre-vitellogenic phases. Cyp19a1a is an enzyme that functions to catalyze the final step of estrogen synthesis; therefore, the antagonistic relationship between Cyp19a1a and Dmrt1 is probably an indirect relationship mediated by the estrogens produced by Cyp19a1a. Although Cyp19a1a is dispensable to establishment of the bipotential ovary and initiation of female development in the absence of Dmrt1, it appears to be required for subsequent oocyte maturation and maintenance of female fates independently of antagonism of Dmrt1. Bmp15 produced in oocytes is required to promote oocyte differentiation and *cyp19a1a*-positive granulosa cells, indicating that the subsequent failure of *cyp19a1a;dmrt1* double mutant oocytes to mature is a result of loss of the later granulosa cell-derived source of Cyp19a1a, which appears not to require inhibition of Dmrt1. (B-H) Model of mutant scenarios for each essential regulator. (B) In *rbpms2a;rbpms2b* double mutants, Rbpms2 repression of *dmrt1* transcript is alleviated and Dmrt1 is translated, leading to male differentiation. Within the testis, Cdk21 promotes spermatogonial expansion and meiosis. (C) In *cyp19a1a* mutants, Cyp19a1a no longer antagonizes *dmrt1*, probably due to reduced estradiol synthesis, and Dmrt1 is expressed prematurely, leading to precocious male differentiation. (D) In *bmp15* mutants, oocytes do not progress past the PV stage, in part because Bmp15 is required for late Cyp19a1a production. (E) In *dmrt1* mutants, Dmrt1 is not present to antagonize Rbpms2, and female differentiation ensues. (F) In *rbpms2a;rbpms2b;dmrt1* triple mutants, female differentiation cannot occur in the absence of Rbpms2, whereas male differentiation cannot proceed because of loss of Dmrt1, resulting in the formation of a sterile testis. (G) In *cyp19a1a;dmrt1* double mutants, loss of *dmrt1* is sufficient to restore the transition from juvenile gonad to bipotential ovary and to prevent precocious male differentiation as occurs in *cyp19a1a* mutants. However, despite the absence of *dmrt1*, oocytes lacking all Cyp19a1a (*cyp19a1a;dmrt1* double mutants) or (H) Bmp15-dependent Cyp19a1a (*bmp15;dmrt1* double mutants) fail to progress to vitellogenic stages and female development ultimately fails, indicating that Cyp19a1a promotes oocyte progression independently of *dmrt1* antagonism. Probably because of later requirements for *dmrt1* in testis development, loss of both *dmrt1* and *cyp19a1a* or *bmp15* eventually results in sterile male adults.

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and recent work from Wu and colleagues (Wu et al., 2020) indicate that Cyp19a1a is also required for subsequent oocyte maturation and maintenance of female fates by a mechanism that does not depend on antagonism of Dmrt1. Consistent with this notion, earlier work demonstrated that *bmp15* produced in oocytes is required to promote oocyte differentiation and development of *cyp19a1a*-positive granulosa cells (Dranow et al., 2016). Moreover, in human ovaries and granulosa-like cells, activin promotes CYP19 expression via SMAD2 (Mukasa et al., 2003; Nomura et al., 2013); thus, it seems likely that subsequent failure of *cyp19a1a;dmrt1DM* oocytes to mature is due to lack of the later granulosa cell-derived source of *cyp19a1a*, which appears to utilize a Dmrt1 inhibition independent mechanism as maturation fails and cannot be restored in the absence of Dmrt1 whether all Cyp19a1a is lacking (*cyp19a1a;dmrt1DM*) or only the early source is present (*bmp15;dmrt1DM*) (Fig. 6G,H). Finally, Rbpms2 probably promotes female fates temporally upstream of Bmp15 (Dranow et al., 2016) and estrogen (estradiol) (Wu et al., 2020) because Rbpms potentiates transcription mediated by Smad2/3 (Sun et al., 2006) and mutant oocytes lacking Bmp15 or estrogen (Wu et al., 2020) reach the diplotene stage of oogenesis or beyond in the absence of Dmrt1, whereas *rbpms2* mutants do not.

Our study identified potential Rbpms2 targets and used genetic epistasis experiments and cell biological approaches to decipher the genetic hierarchy of crucial factors involved in sex-specific differentiation of the germline and somatic gonad. We show that TGF-β signaling is activated in early germ cells of the bipotential ovary, and in the wild type becomes activated in the somatic gonad by d28, during the early stages of sexual differentiation. In *rbpms2DM*s, initial activation of TGF-β in the germline is intact. We provide genetic evidence that *dmrt1* antagonizes the crucial female factor *rbpms2*, acting either upstream or in a parallel mutually antagonistic pathway. Based on their opposite mutant phenotypes and the presence of Rbpms2 binding sites in the *dmrt1* 3′ UTR, Rbpms2 may promote female fate by preventing translation of Dmrt1. Accordingly, loss of Rbpms2 would lead to production of Dmrt1 and male differentiation (Fig. 6). However, our genetic data indicate that, once produced, Dmrt1 or its targets antagonize or promote elimination of Rbpms2 and other factors required for acquisition of female fates, as Rbpms2 protein is not detected in testis.

Moreover, Rbpms2 function in promoting female fates extends beyond simply repressing or antagonizing *dmrt1*, as it is essential for female sex-specific differentiation, even in the absence of Dmrt1, indicating that additional Rbpms2 targets are required for female fates (Fig. 6F). In contrast to loss of *rbpms2* function, female fates can be restored or prolonged in mutants lacking *cyp19a1a* and *dmrt1* or *bmp15* and *dmrt1*.

Conclusion

Taken together, this work and the work of Wu et al. (2020) indicate that *cyp19a1a* acts during at least two steps of female-specific differentiation. Early *cyp19a1a*-mediated suppression of *dmrt1* is required to establish a bipotential ovary and initiate female fate acquisition in zebrafish, possibly by promoting expression of *rbpms2*, which is required for female-specific differentiation, even in the absence of Dmrt1. Finally, once female fates have been established, Cyp19a1a is required for subsequent oocyte maturation and maintenance of female fates by a mechanism that does not depend on antagonism of Dmrt1.

MATERIALS AND METHODS

Zebrafish

Wild-type zebrafish embryos of the AB strain were obtained from pairwise crosses and reared according to standard procedures (Westerfield, 2000). Embryos were raised in 1× embryo medium at 28.5°C and staged as described (Kimmel et al., 1995). All procedures and experimental protocols were in accordance with NIH guidelines and approved by ISMMS (protocol # 17–0758 INIT) IACUC. The zebrafish *rbpms2a*^{ae30} allele was previously generated in our laboratory using CRISPR-Cas9 mediated mutagenesis (Kaufman et al., 2018) and the *rbpms2b*^{sa9329} allele was obtained from the Sanger Institute's Zebrafish Mutation Project (Kettleborough et al., 2013). The zebrafish *dmrt1*^{uc27}, *bmp15*^{uc31} and *cyp19a1a*^{uc38} alleles were obtained from Bruce W. Draper, University of California, Davis (Webster et al., 2017; Dranow et al., 2016).

Genotyping

Restriction fragment length polymorphism

Genomic DNA was extracted from adult fins, juvenile fins or single embryos using standard procedures (Westerfield, 2000). The genomic region surrounding *rbpms2a*^{ae30} was amplified using the primers 5′-TTTGCTAA-AGCCAACACGAA-3′ and 3′-ATTCACCCTGGCCAGAGTTT-5′, followed by digestion of the wild-type strand with the enzyme BsurI (New England Biolabs, R0581S) (Kaufman et al., 2018). The genomic region surrounding *rbpms2b*^{sa9329} was amplified using the dCAPs primers 5′-CA-CTTATCAAGCTAACTTCAAAGCAGA-3′ and 3′-TGAAAGGGGACA-AATAAGTCA-5′, followed by digestion of the mutant strand with the enzyme MboII (New England Biolabs, R0148S) as described previously (Kaufman et al., 2018). The genomic region surrounding *bmp15*^{uc31} was amplified using the primers 5′-AGCCTTTCAGGTGGCACTCG-3′ and 3′-GGGAGAAAGTGTTTTTCAGTGG-5′, and *dmrt1*^{uc27} using primers 5′-GTTGTAACTGGCAGCTGGAGA-3′ and 3′-GGCGATGAGTCTGCATT-TCT-5′, but these products were resolved without digestion. After 40 cycles of PCR at 60°C annealing, samples were digested for 30 min using specified restriction enzymes. Digested PCR products were resolved using a 1.5% ultrapure agarose (Invitrogen) and 1.5% Metaphor agarose (Lonza) gel.

High resolution melt curve analysis

PCR and melting curve analyses were performed as described (Parant et al., 2009). PCR reactions contained 1 µl of LC Green Plus Melting Dye (BioFire Diagnostics), 1 µl of Taq buffer, 0.8 µl of dNTP mixture (2.5 mM each), 1 µl of each primer (as indicated below) (5 µM), 0.05 µl of Taq (Genscript), 1 µl of genomic DNA and water up to 10 µl. PCR and melt curve analyses were performed in a Bio-Rad CFX96 Real-Time System, using black/white 96-well plates (Bio-Rad HSP9665). PCR reaction protocol was 98°C for 1 min, then 34 cycles of 98°C for 10 s, 60°C for 20 s, and 72°C for 20 s, followed by 72°C for 1 min. After the final step, the plate was heated to 95°C for 20 s and then cooled to 4°C. Melt curve analysis was performed over a 72–92°C range and analyzed with Bio-Rad CFX Manager 3.1 software. All mutations were confirmed by TA cloning and sequencing. Primers used were as follows: *rbpms2a*^{ae30}, 5′-ACACGAAGATGGCGAAGAGT-3′ and 3′-C-AGGGTGCAGGTTGGAAG-5′; *rbpms2b*^{sa9329}, 5′-ATGAGGGTTCAC-TTATCAAGCTA-3′ and 3′-TCCGGTCAGCTGTAATGTCTAA-5′; *dmrt1*^{uc27}, 5′-CTCTCGCTGCAGAAACCAC-3′ and 3′-GGCGATGAGT-CTGCATTTCT-5′; *cyp19a1a*^{uc38}, 5′-CCAACTGACCTGGAATGTGTG-3′ and 3′-AGCTACAATACTGCTGCTGCTA-5′.

RT-PCR

Trunks were dissected at d21 from the specified genotypes and placed into Trizol (Life Technologies). RNA was extracted using standard phenol-chloroform-isoamyl alcohol (PCI) extraction and was used for oligo(dT) cDNA preparation (using Invitrogen SuperScript III reverse transcriptase). RT-PCR was performed using the following primers: *bmpr1ab*, 5′-GATG-CCACAAACAACACCTG-3′ and 3′-GCAACCAAAGTGAAGCAACA-5′; *bmpr1bb*, 5′-GAGGCAGATGGGTAAACTG-3′ and 3′-CTCCTGTGTTC-TGTTGAG-5′; *bmpr2b*, 5′-CGGCCTCTGGGAGAAAACAC-3′ and 3′-T-GGCCTCATCTCTGTGTATAG-5′; *ef1alpha*, 5′-AGCCTGGTATGGT-TGTGACCTTTCG-3′ and 3′-CCAAGTTGTTTTCCTTTCCTGCG-5′. PCR products were resolved using a 1.5% ultrapure agarose (Invitrogen) gel and visualized with a Biorad gel imager.

Immunofluorescence

For whole-mount immunofluorescence (IF) of gonads, trunks or tissue at d28, d45 and d120 were fixed in 4% paraformaldehyde overnight at 4°C, dehydrated

in MeOH and placed at −20°C. In the case of trunks, an incision was made to open the body cavity during staining and gonads were dissected for imaging. Rabbit anti-phospho-Smad2 antibody (Millipore AB3849-I) was used at 1:200. Rabbit anti-Buckyball antibody y1165 was used at 1:500 (Heim et al., 2014). Rabbit anti-Rbpms2 antibody (Abcam A170777) was used at 1:500. Chicken anti-Vasa antibody was a gift from Bruce W. Draper (Blokhina et al., 2019) and used at 1:3000 dilution. AlexaFluor488, CY3 (Molecular Probes) secondary antibodies were diluted at 1:500. Images were acquired using a Zeiss Axio Observer inverted microscope equipped with ApotomeII and a CCD camera. Images were processed in ImageJ/FIJI, Adobe Photoshop and Adobe Illustrator.

Sexing zebrafish and fertility assays

Secondary sex traits

Fish were sexed based on morphologically distinct secondary features of females and males, such as body shape, fin coloration, genital papillae and tubercle characteristics. Females are typically larger than males, have a more rounded abdomen and have a pale body and anal fin stripes, protruding genital papillae and smooth pectoral fins lacking spiky tubercles. In contrast, males appear smaller than females, have a slender body shape, display dark yellow body stripes and anal fins, lack protruding genital papillae and have spiky tubercles on their pectoral fins. Images were acquired using a Zeiss Stemi 508 dissecting scope equipped with a color CCD camera. Images were processed in Zeiss ZenBlue, ImageJ/FIJI, and Adobe Illustrator.

Primary gonad sex

Gonads were dissected from either d90, d120 or d145 adult fish. Gonads were either imaged live in brightfield at 40× magnification and fixed for further analysis, or fixed and stained with Vasa antibody (1:3000) then imaged in brightfield and GFP at 40× magnification. Gonad overview images were acquired using a Zeiss Axio Zoom dissecting scope equipped with an Apotome II and a CCD camera. Images were processed in Zeiss ZenBlue, ImageJ/FIJI, and Adobe Illustrator.

Fertility

Fertility was assessed in mating trials where couples for a given genotype were paired and bred for several repeat mating trials. For each cross, if a male triggered spawning, the number (in parentheses in Fig. 3C) of fertilized eggs per trial was counted. For sperm acquired through *in vitro* fertilization (IVF), the number of eggs successfully fertilized was also counted. n/a indicates that fish were not available for subsequent mating trials.

Statistical analyses

Statistical analyses were performed using Graphpad Prism 8 and Excel; we performed a chi^2 test to compare the observed sex ratios to the expected 50:50 sex ratio. We then compared each group to the heterozygote siblings using a chi^2 goodness of fit test with a Bonferroni correction for multiple comparisons, using an adjusted significance value determined by the number of comparisons to be calculated. Where groups were pooled, expanded data is available in the Supplementary material.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: O.H.K., F.L.M.; Methodology: S.R., O.H.K., F.L.M.; Formal analysis: S.R., O.H.K., F.L.M.; Investigation: S.R., O.H.K., F.L.M.; Resources: F.L.M.; Data curation: S.R., O.H.K., F.L.M.; Writing - original draft: S.R., F.L.M.; Writing - review & editing: S.R., O.H.K., F.L.M.; Visualization: F.L.M.; Supervision: F.L.M.; Project administration: F.L.M.; Funding acquisition: S.R., O.H.K., F.L.M.

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Figure S1. Adult sex ratios in *Rbpms2a;2b;dmrt1* TMs and siblings

Graph of adult sex ratios of progeny from three triple heterozygote intercrosses for given genotypes.

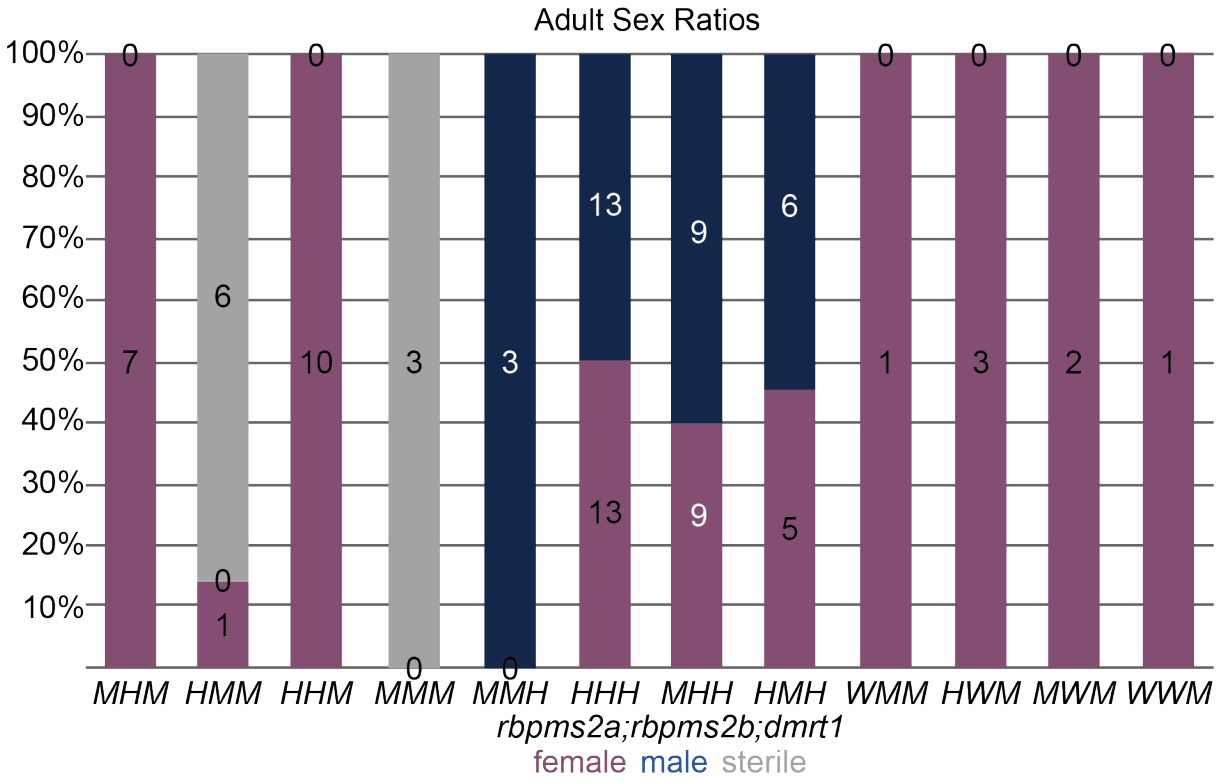

Figure S2. Rbpms2 is expressed in oocytes but not somatic gonad or testis. (A) Wild-type gonad labeled with Rbpms2 antibody and DAPI reveals Rbpms2 expression within oocytes (example outlined with yellow dashed line) but not somatic gonad cells (examples outlined in pink dashed lines) or (B) testis.

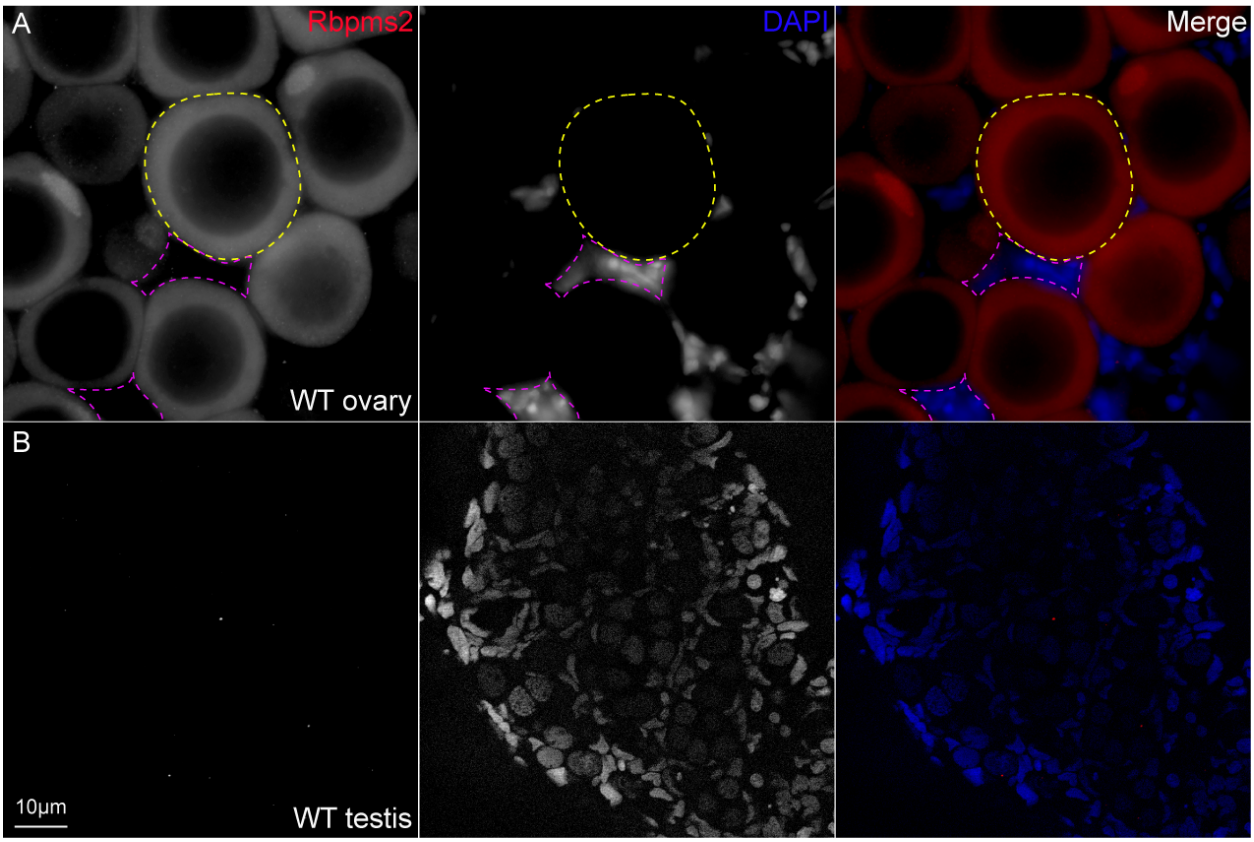

Figure S3. Loss of *dmrt1* restores bipotential female ovary development and early oogenesis in *cyp19a1a* mutants. (A-G) Buc, Vasa (A'-G') and Buc/Vasa/DAPI merged (A''-G'') staining in representative slices from individual Z-series to visualize gonocyte development in gonads of d120 adult zebrafish, including (A) female *cyp19a1a*$^{uc38/+}$;*dmrt1*$^{uc27/uc27}$ *(HMs)* (n=3/3), (B) male *cyp19a1a*$^{uc38/uc38}$;*dmrt1*$^{uc27/+}$ *(MHs)* (n=3/3), and *cyp19a1a*$^{uc38/uc38}$;*dmrt1*$^{uc27/uc27}$ *(DM)* (n=3/3) gonads that were female with (C) pre-vitellogenic (PV) oocytes, or (D,E) had regions with PV oocytes or early stage Ia/Ib oocytes, as indicated by Buc staining (white), or (F, G) contained PV oocytes and regions containing many undifferentiated cells. Representative images from different regions of the same gonad.

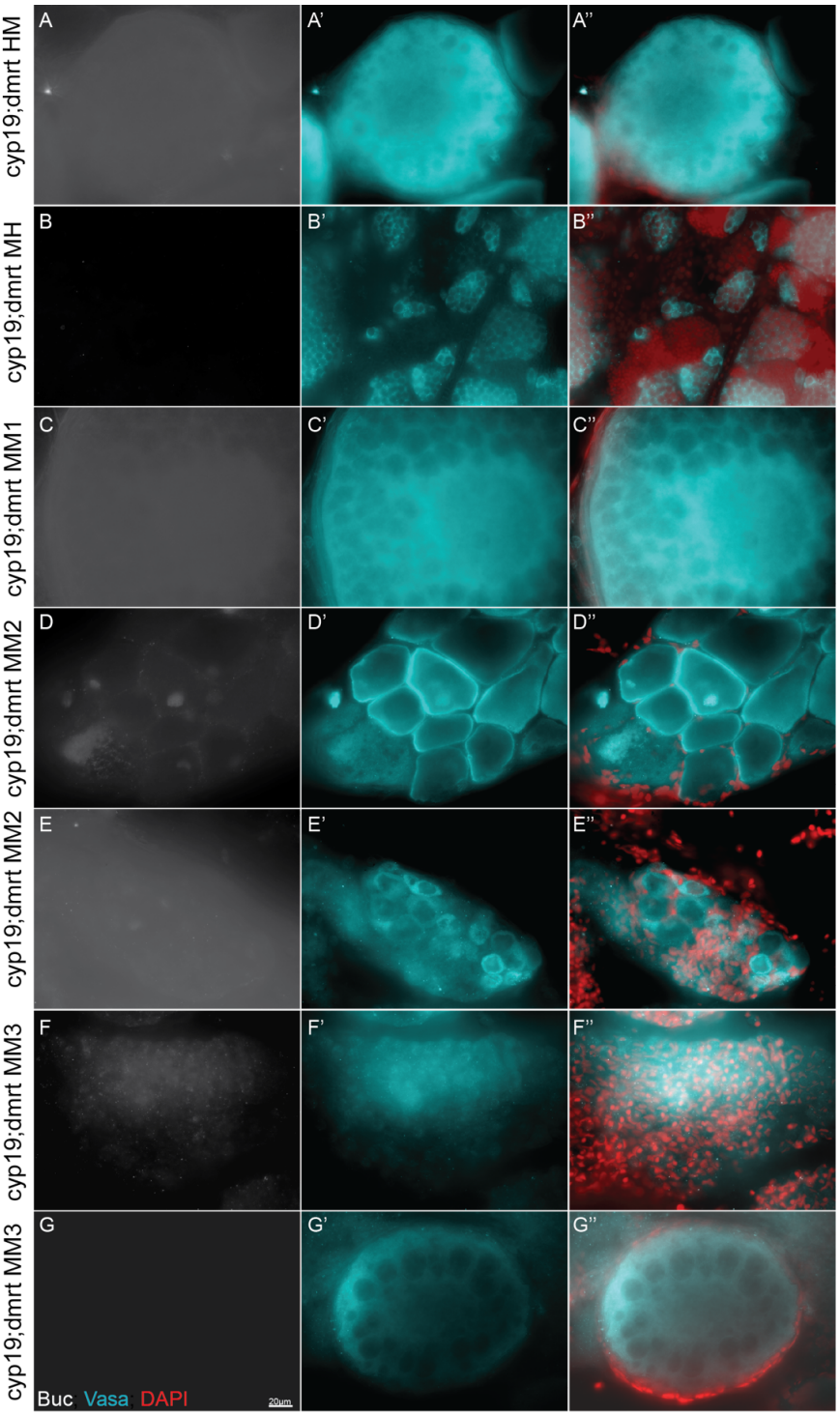

Figure S4. Secondary sex traits and primary gonad sex differentiation in *cyp19a1a;dmrt1DMs.* *cyp19a1a*$^{uc38/uc38}$*; dmrt1*$^{uc27/+}$ (MH) and *cyp19a1a*$^{uc38/+}$*;dmrt1*$^{uc27/uc27}$ (HM) display normal secondary sex characteristics of males and females, respectively at 120 days (A-D) and 145 days (M-R). (A-D and M-R are lateral views with rostral to the left and caudal to the right). (A, M) Male MH fish had a slender body shape, lacked a genital papilla (indicated by arrows), had spiky tubercles (2x zoom in dashed box insets), and a yellow anal fin. (B, N) Female HM fish had a round body shape with protruding abdomen, extended genital papilla, lacked spiky tubercles, and had a lighter anal fin. Similar to *MH* males, (C-D and O-R) *cyp19a1a*$^{uc38/uc38}$*; dmrt1*$^{uc27/uc27}$ (MM) double mutants exhibited male secondary sex traits. Upon dissection, at both 120 and 145 days, (E) *MH* males had testes, (F) *HM* females had ovaries and (G, H, U, X) *MMs* had gonads that contained pre-vitellogenic (PV) oocytes or (V, W) had underdeveloped testes. Adult sex ratios for independent groups (I-L) based on clutch, age, and genotype.

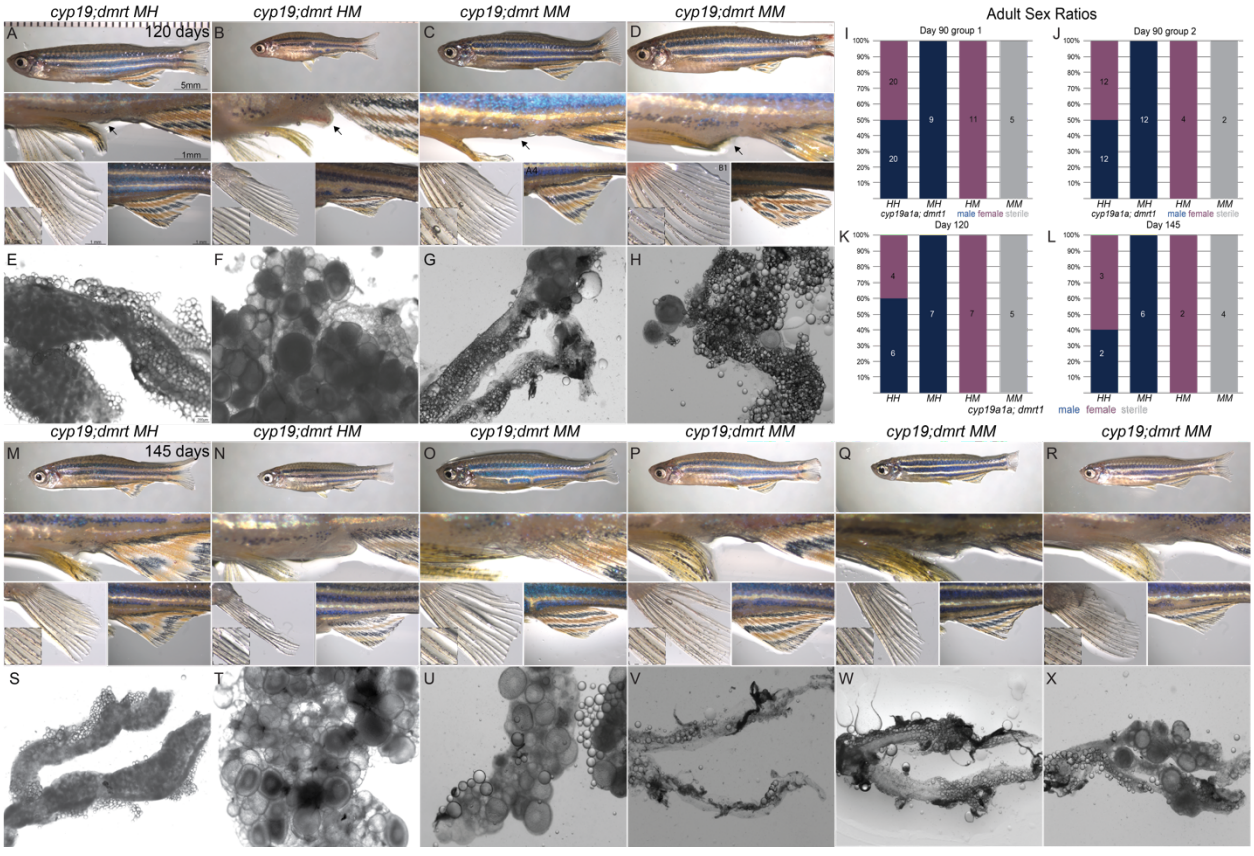

Figure S5. Secondary sex traits and primary gonad sex in *bmp15;dmrt1MMs*. (A-D) *bmp15*$^{uc31/uc31}$*; dmrt1*$^{uc27/+}$ (MH) and *bmp15*$^{uc31/+}$*; dmrt1*$^{uc27/uc27}$ (HM) displayed normal secondary sex characteristics of males and females, respectively at 145 days. (A, B) Male *MHs* (n=7) had a slender body shape, lacked a genital papilla (indicated by arrows), had spiky tubercles (2x zoom in dashed box insets). (C, D) Female *HMs* (n=7) had a round body shape with protruding abdomen, extended genital papilla, lacked spiky tubercles. (I-P) *bmp15*$^{uc31/uc31}$*;dmrt1*$^{uc27/uc27}$ (MM) double mutants (n=4) exhibited a mix of characteristically male and female secondary sex traits. All *MMs* had a round body shape with protruding abdomen and lacked a genital papilla, though half (J,K) had tubercles and half (I,L) did not. Upon dissection, (E,F) *MH* males had testes, (G,H) *HM* females had ovaries and (M-P) *MMs* had ovaries that contained pre-vitellogenic (PV) oocytes. Adult sex ratios of pooled *HHxMH* progeny from d90-d145 were analyzed with X^2 goodness of fit test with Bonferroni correction for multiple comparisons, $p<0.0001$ indicated groups deviated from the expected 50:50 sex ratio. For individual comparisons to HH sex ratios, all groups were significantly different; MM ($p=0.002$), HM ($p<0.0001$) and MH ($p<0.0001$).

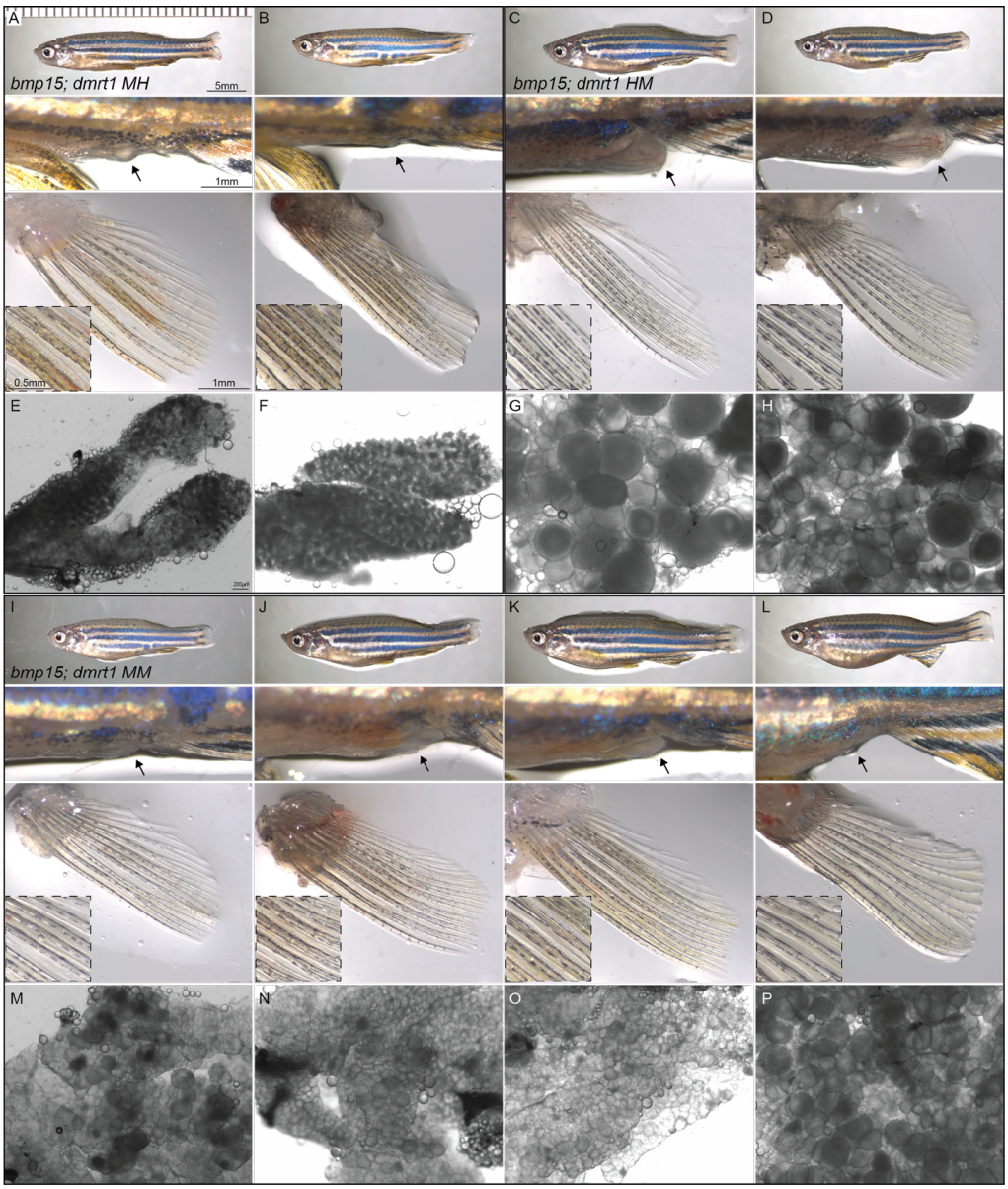

Figure S6. Rbpms2 expression in *cyp19a1a* mutant gonads. (A-D) Rbpms2, Vasa (A'-D') and Rbpms2/Vasa/DAPI merged (A''-D'') staining in representative slices from individual Z-series to visualize gonocyte development in gonads of d50 adult zebrafish, including (A) male *cyp19a1a*$^{+/+}$(n=3/3) with differentiated testis containing male germ cells marked by Vasa (cyan) that do not express Rbpms2 and (B-D) male *cyp19a1a*$^{uc38/uc38}$ mutants (n=6/6), that were either still differentiating as early testis (B), or differentiated testis with male germ cells (C, D), and did not express Rbpms2.