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Primary sex determination in birds depends on DMRT1 dosage, but gonadal sex does not determine adult secondary sex characteristics

Jason Ioannidis1,2, Gunes Taylorb,1, Debiao Zhaoa, Long Liuc, Aileen Idoko-Akohb, Daqing Gongc, Robin Lovell-Badgeb, Silvana Guiolib,3, Mike J. McGrewa,2,3, and Michael Clintoana,3

1Division of Functional Genomics and Development, The Roslin Institute, Royal (Dick) School of Veterinary Studies, EH25 9RG Midlothian, United Kingdom; 2Laboratory of Stem Cell Biology and Developmental Genetics, The Francis Crick Institute, NW1 1AT London, United Kingdom; and 3College of Animal Science and Technology, Yangzhou University, 225009 Yangzhou, People’s Republic of China

In birds, males are the homogametic sex (ZZ) and females the heterogametic sex (ZW). Primary sex determination is thought to depend on a sex chromosome gene dosage mechanism, and the most likely sex determinant is the Z chromosome gene Doublesex and Mab-3–Related Transcription factor 1 (DMRT1). To clarify this issue, we used a CRISPR-Cas9–based monoleucine targeting approach and sterile surrogate hosts to generate birds with targeted mutations in the DMRT1 gene. The resulting chromosomally male (ZZ) chicken with a single functional copy of DMRT1 developed ovaries in place of testes, demonstrating the avian sex-determining mechanism is based on DMRT1 dosage. These ZZ ovaries expressed typical female markers and showed clear evidence of follicular development. However, these ZZ adult birds with an ovary in place of testes were indistinguishable in appearance to wild-type adult males, supporting the concept of cell-autonomous sex identity (CASI) in birds. In experiments where estrogen synthesis was blocked in control ZW embryos, the resulting gonads developed as testes. In contrast, if estrogen synthesis was blocked in ZZ embryos that lacked DMRT1, the gonads invariably adopted an ovarian fate. Our analysis shows that DMRT1 is the key sex determination switch in birds and that it is essential for testis development, but that production of estrogen is also a key factor in primary sex determination in chickens, and that this production is linked to DMRT1 expression.

Significance

Here, we show that DMRT1 dosage is the key sex determination factor in birds and is essential for testis development. Furthermore, we provide additional evidence that birds, in contrast to mammals, have acquired cell-autonomous sex identity (CASI) and that the sex hormone environment does not significantly influence avian secondary sexual characteristics. This finding highlights an evolutionary divide between mammals and nonmammalian vertebrates. In mammals, the sex chromosomes determine the type of gonad formed, and sex hormones largely define the secondary sexual phenotype. In birds, the sexual phenotype is directly determined by the sex chromosome content of individual cells in different tissues. Our findings help advance our understanding of the evolution of sex determination systems and the nature of sex identity.

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The authors declare no competing interest.

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J.I. and G.T. contributed equally to this work.
2To whom correspondence may be addressed. Email: jason.ioannidis@roslin.ed.ac.uk or mike.mcgrew@roslin.ed.ac.uk.
3S.G., M.J.M., and M.C. contributed equally to this work.

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To elucidate the role of DMRT1 dosage in chicken sex determination, we used an efficient CRISPR-Cas9 targeting approach and surrogate germ cell hosts to generate chickens with targeted mutations in DMRT1 and analyzed the effects on gonadal development. Here, we clearly demonstrate that avian gonadal sex fate is dependent on DMRT1 dosage and that the mechanism involves moderation of E2 production. The presence of DMRT1 is essential for testicular differentiation, but not for the early stages of ovarian differentiation. Our analysis further supports the concept of cell-autonomous sex identity (CASI) (18) as our results show the development of secondary sexual characteristics of nonreproductive tissues in birds is independent of gonadal sex.

Results

Generation of DMRT1-Mutant Birds Using Surrogate Hosts. To generate DMRT1 knockout chickens, we used CRISPR-Cas9 to target the DMRT1 gene in cultured chicken primordial germ cells (PGCs). As DMRT1 is essential for meiosis and gametogenesis in mammals (19, 20), we targeted a loss-of-function mutation into a single DMRT1 allele in ZZ PGCs (21). ZZ germ cells heterozygous for loss-of-function mutations in essential meiotic genes will successfully navigate meiosis and produce functional gametes (22). We simultaneously delivered a high fidelity CRISPR/Cas9 vector and two single-stranded oligodeoxynucleotides (ssODNs) into in vitro propagated male tdTomato+/− heterozygote PGCs: one oligonucleotide to create a premature stop codon and a protospacer adjacent motif (PAM) mutation, and a second oligonucleotide, which contained a PAM mutation encoding a synonymous amino acid change in DMRT1 (SI Appendix, Table S1). We isolated clonal male PGC populations and identified clones containing the correct (ZZ DMRT1+/−; formatted as ZD+ZD− for simplicity hereafter) mutations in the DMRT1 locus (n = 10 of 25 clones) (Fig. 1A, SI Appendix, Fig. S1, and Methods).

Targeted (ZD+ZD−) PGCs were injected into transgenic surrogate host chicken embryos containing an inducible Caspase9 (iCaspase) targeted to the germ cell-specific DAZL locus (deleted in azoospermia) (23). Treatment of iCaspase9 host embryos with the dimerization drug AP20187 (B/B) ablates the endogenous...
germ cells, such that the only gametes that develop are derived from donor PGCs. The surrogate host chicks were hatched and raised to sexual maturity, and then surrogate males (ZD+ZD+ G0 founders carrying ZD+ZD- PGCs) were naturally mated to ZD+W wild-type hens (Fig. 1B). This mating produced chromosomally male and female G1 offspring that were wild type for DMRT1 (ZD+ZD+ and ZD+W), chromosomally male birds that were heterozygous for functional DMRT1 (ZD+ZD-), and chromosomally female birds that lacked functional DMRT1 (ZD-W). PCR and red fluorescent protein (RFP) fluorescence expression indicated that 51.6% of DMRT1 embryos were RFP-positive, suggesting that all offspring derived from exogenous PGCs (see Methods and SI Appendix, Fig. S1 and Table S3 for DMRT1-allele transmission data).

**ZZ DMRT1 Heterozygote Embryos Show Gonadal Sex Reversal.** Fertile G1 eggs from G0 founder males mated to wild-type females were incubated and examined for gonadal development. Our initial characterizations were performed on embryos at day 13.5 of development (E13.5) as clear morphological differences between male and female gonads are apparent by this stage. As expected, in E13.5 ZZ chicken embryos, the testes appeared as two similar sized, cylindrical structures lying on either side of the midline while ZW embryos contained a left ovary, which acquired an elongated flattened appearance, and a small right ovary, which subsequently regressed. The E13.5 testis comprised a core medulla containing germ cell-filled sex cords while the left ovary contained a relatively unstructured medulla surrounded by a thickened cortex containing germ cells (Fig. 2A).

Examination of the gross morphology of the gonads in ZD+ZD- embryos, however, showed that the targeted mutation of DMRT1 had a significant effect on gonadal development, with clear morphological signs of sex reversal (Fig. 2A). Unlike the typical paired structures seen in the wild-type ZZ embryo, the ZD+ZD- clearly contained an ovary-sized structure on the left side and a much smaller structure on the right side, like the ZD+W control (n = 5 of 5). In ZD-W embryos, the left gonad also appeared to be an ovary although smaller in size than the wild-type counterpart (n = 3 of 3) (Fig. 2A).

**Fig. 2.** Gonadal development in DMRT1-mutant embryos. Gross morphology of gonads (A) and Müllerian ducts (B) in ZD+ZD+ and ZD+W embryos and ZD+ZD- and ZD-W DMRT1-mutant embryos (n = 3–7 embryos per genotype). Immuno-sections from right (R) and left (L) gonads from E13.5 wild-type and DMRT1-mutant embryos (C–F). Expression of DMRT1, aromatase (AROM) and AMH (C, E) and SOX9, FOXL2, and of PGC-specific marker (VASA) (D, F). A minimum of three embryos of each genotype were examined. Arrows indicate gonads in A and Müllerian ducts in B. Asterisks indicate Wolffian ducts in B. c, cortex; m, medulla. (G) Relative gene expression of DMRT1 and of testis and ovary markers in gonads of E8.5 wild-type and DMRT1-mutant embryos. Individual expression levels were calculated relative to levels in ZD+ZD+. Five replicates on pools of two gonads per genotype. Bars represent mean ± SD. Different letters specify statistically significant groups, P < 0.05.
It is interesting to note that, by E13.5, both Müllerian ducts had regressed in the ZD+W male while both Müllerian ducts were retained in ZD-W embryos, similar to ZD+W embryos (Fig. 2B). This result is unexpected, as it was previously published that down-regulation of DMRT1 blocks Müllerian duct formation (24). We also observed that the right Müllerian ducts of both ZD+W and to ZD+W embryos showed early signs of regression while, in contrast, the right Müllerian duct of ZD-ZD- embryos showed no sign of regression (Fig. 2B).

Sections of E13.5 gonads were examined by immunohistochemistry (IHC) to reveal spatial expression patterns of DMRT1 and of established tests (AMH, SRY-box 9 (SOX9)) and ovary (FOXL2, aromatase [Cytochrome P450 Family 19 Subfamily A member 1 (CYP19A1)]) marker proteins, and PGC-specific markers (Fig. 2 C–F). Sections from both right and left ZD+ZD+ gonads showed a typical male medulla with obvious sex cords comprised of PGCs and somatic cells that expressed DMRT1, SOX9, and AMH, overlaid by a thin epithelial layer. In contrast, the right and left ZD-W gonads were structurally distinct. As expected, the medulla of both right and left gonads expressed FOXL2 and aromatase; however, the right gonad was markedly smaller in size. In addition, the left gonad was enclosed within an obvious thickened cortex on the ventral surface, which contained the PGCs. Analyses of sections of gonads from ZD+ZD+ embryos revealed that they were indistinguishable from ZD+W ovaries in terms of structure and molecular profiles. The medullary regions expressed FOXL2 and aromatase and did not contain sex cords or express SOX9 or AMH. DMRT1 was expressed at low levels, reduced in size, compared to ZD+W gonads, but otherwise appeared to be typical ovaries; left and right medullas were FOXL2- and aromatase-positive, and SOX9- and AMH-negative, and the left gonad included a PGC-containing cortex. It is clear from these analyses that the loss of a single functional copy of DMRT1 from this analysis that the loss of a single functional copy of DMRT1 blocks Müllerian duct regression while, in contrast, the right Müllerian duct of ZD+W ovaries showed an obvious thickened cortex on the ventral surface, which contained the PGCs. Analyses of sections of gonads from ZD-W embryos revealed that they were indistinguishable from ZD+W ovaries in terms of structure and molecular profiles. The medullary regions expressed FOXL2 and aromatase and did not contain sex cords or express SOX9 or AMH. DMRT1 was expressed at low levels, compared to ZD+W controls, indicating a potential delay in meiotic entry during embryonic development. As expected, the ZD-W gonads completely lacked DMRT1 expression while, in contrast, there was significant expression of the “female” marker FOXL2 in ZD-ZD+ gonads. Although FOXL2 transcript levels in the latter were lower than those in wild-type ovaries, IHC analyses suggested that FOXL2 protein levels were similar (Fig. 2C). Expression levels of aromatase in ZD-ZD- gonads were similar to those found in control ZD+W ovaries. Expression patterns typical of ovaries were also evident in gonads from ZD-W embryos completely lacking DMRT1 although the levels of ovary-specific markers were reduced compared to both ZD+W and ZD-ZD- gonads.

It is clear from these analyses that gonadal development in ZD-ZD- embryos is similar to that seen in control ovaries of ZW female embryos.

**Meiosis in DMRT1-Mutant Embryos.** DMRT1 is also highly expressed in germ cells and has been implicated in the control of meiotic entry and progression in different vertebrate species (19, 25). To assess the effects of DMRT1 loss on germ cell development, we monitored expression of a selected meiotic marker at E13.5 and E17.5, after the initiation of meiosis in the chicken (Fig. 3A). Meiotic progression was assessed by monitoring gamma H2A variant (HMV) variant (HMV) and histone family member X (H2AX), an indicator of double-stranded DNA breaks (22, 26). As expected, γH2AX was not expressed in germ cells of ZD-ZD+ gonads at either developmental stage, while germ cells in ZD+W gonads expressed γH2AX at both stages with a reduction in expression at E17.5. In the germ cells of gonads from ZD-ZD+ embryos, γH2AX was present at both stages and, at E17.5, γH2AX expression was more abundant, compared to ZD+W controls, indicating a potential delay in meiotic entry in ZD+ZD- gonads. In the gonads of ZD+W embryos, there was no evidence of γH2AX expression at either developmental stage, suggesting a delay or failure of meiosis.

**Follicular Development in DMRT1-Mutant Chicken.** To determine whether the gonadal sex reversal observed during embryonic development was permanent, we examined gonads of birds at 5 wk posthatch. Histological sections of gonads were stained with hematoxylin and eosin (H&E) or processed for IHC to examine expression of male and female markers (Fig. 3B). The gonads of ZD-ZD+ birds exhibited typical testicular structures, with seminiferous tubules showing strong expression of SOX9 and DMRT1. The gonads of ZD-W birds displayed a clear cortex with oocyte-containing follicles of different sizes. FOXL2 was highly expressed in the granulosa cells encapsulating the oocyte, and aromatase was expressed in the thecal tissue surrounding the follicles. The structure and the expression patterns of FOXL2 and aromatase were evident in the gonads of ZD-ZD- birds, similar to the ZD+W birds, and small follicles were clearly present. However, no larger follicles were observed in ZD-ZD- birds. The gonads of ZD+W birds contained no oocytes/follicles, and FOXL2 and aromatase were observed in cells dispersed throughout the cortex. It is clear from this analysis that the testis-to-ovary sex reversal in ZD-ZD- was permanent and complete. It is well established that DMRT1 is highly expressed in both male and female germ cells, and the absence of oocytes/follicles in the gonads of ZD+ZD- birds is likely a direct result of this, leading to an ovo-failure of the germ cells to progress into meiosis. As expected, neither the ZD-ZD+ nor the ZD+W birds produced eggs (Fig. 3D).

**Gonadal Sex Reversal Does Not Affect Secondary Sex Characteristics.** We have previously established that chickens possess a degree of CASE: i.e., the secondary sexual phenotype depends, at least partly, on the sex-chromosome content of the somatic cells and not simply on gonadal hormones (18). The generation of ZD-ZD- birds that possess an ovary instead of testes enabled us to investigate
the extent of CASI in chickens. In terms of secondary characteristics, male birds are heavier (possess greater muscle mass and bone density), they have larger combs and wattles, they possess hackle feathers (hood), and they develop leg spurs (Fig. 4A). We assessed sexually mature adult birds at 24 wk of age. It is clear from these images that the chromosomally male bird with an ovary (ZD+ZD-) was identical in appearance to the wild-type ZD+ZD+ bird: with large comb and wattles, hackle feathers, and obvious leg spurs. ZD-W birds were similar in appearance to ZD+W birds. Given that the ZD+ZD- bird possesses an ovary rather than testes (SI Appendix, Fig. S4D), this suggests that these typical male secondary sexual characteristics are due to CASI and independent of gonadal hormones.

We monitored the body weight of wild-type and DMRT1-mutant birds over a 28-wk period (Fig. 4B). In this line of layer chickens, weights of wild-type male and female birds diverge at 10 wk (70 d), resulting in adult males that were ~20% heavier than adult females. The ZD+W birds followed an almost identical growth pattern to ZD+ZD+ birds. ZD+ZD- birds showed an identical weight increase to ZD+ZD+ birds up to 120 d but then showed an even greater weight gain until 150 d of age. Postmortem examination suggested that this additional weight accrues from abdominal fat deposits: a phenomenon also associated with capons (27) (castrated cockerels). These results suggest that the weight difference between the ZD+ZD+ birds and ZD+ZD- was due to the loss of testes rather than the acquisition of an ovary. This further suggests that secondary sex characteristics of nonreproductive tissues in chickens are primarily due to the sex chromosome content of cells/tissues and independent of gonadal hormones.

Surprisingly, we observed that the ZD+ZD- birds contained mature oviducts derived from both Müllerian ducts; in wild-type male birds, both Müllerian ducts regress while, in wild-type female birds, only the left Müllerian duct is retained, becoming the mature oviduct (SI Appendix, Fig. S4 B and C). In the adult ZD+ZD- birds, two mature oviducts were present and connected to the cloaca. Examination of the reproductive ducts of E17.5 embryos showed that, while the right Müllerian ducts of both ZD+W and ZD+ZD- embryos had fully regressed, the right Müllerian ducts of ZD+ZD- embryos exhibited only a slight shortening (SI Appendix, Fig. S4A). It is well established that wild-type female birds with one oviduct generate low levels of AMH during gonadal development so the retention of both Müllerian ducts in ZD+ZD- birds is consistent with a complete loss of AMH expression at embryonic stages (Fig. 2G and SI Appendix, Fig. S3B).

Female Sex Reversal by E2 Blockade Requires DMRT1. Multiple reports have established that E2 plays a key role in ovarian differentiation in chickens (10, 28). Studies with mixed-sex gonadal chimeras have shown that the presence of a small portion of aromatase-expressing ZW (ovarian) tissue is sufficient to induce cortex formation in the left gonad of wild-type ZZ embryos (10) while it is also well established that blockade of the synthesis of E2 in ZD+W embryos results in a sex reversal and the gonads develop as testes. Here, we assessed the effects of blocking E2 synthesis on gonadal development in DMRT1 mutants, by injecting E25 eggs with an inhibitor of aromatase activity (fadrozole). Following reincubation of eggs, gonads were collected at E13.5 of development and processed for IHC with antibodies against DMRT1 and other gonadal markers (Fig. 5).

The ZD+ZD+ gonad displayed obvious PGC-containing medullary sex cords with strong DMRT1 and SOX9 expression. The ZD+W gonad had a clear PGC-containing outer cortex and displayed medullary expression of FOXL2 and aromatase. The gonads of fadrozole-treated ZD+W embryos were clearly affected and showed clear evidence of female-to-male sex reversal;
the medulla contained sex cords with germ cells, aromatase expression was reduced and SOX9 expression was evident, and no cortex was present. $Z^{D^+}Z^{D^+}$-treated embryos displayed a similar pattern, demonstrating a rescue of the male-to-female sex reversal phenotype. This indicates that embryos with a single copy of DMRT1 will develop testes in the absence of estrogen. In contrast, fadrozole treatment of $Z^D$-$W$ embryos did not result in female-to-male medullary sex reversal: medullary sex cords did not form, and the expression of FOXL2 and aromatase was maintained; however, a thickened cortex is absent. These findings show that blocking E2 synthesis allows testis formation in $Z^{D^+}Z^{D^+}$, but not in $Z^D$-$W$, embryos (Fig. 5). Therefore, although a lack of E2 prevents the development of an obvious cortex in fadrozole-treated $Z^D$-$W$ embryos, DMRT1 is essential for testis development.

Discussion

To clarify the role of DMRT1 in sex determination and gonadal development in chickens, we used a CRISPR-Cas9-based approach to generate male offspring carrying disrupting mutations in DMRT1. $Z^{D^+}Z^{D^+}$ genome edited PGCs were transmitted through a novel sterile surrogate host, leading to 100% germline transmission. The G1 offspring presented the four chromosomal genotypes in a 1:1:1:1 ratio: $Z^{D^+}Z^{D^+}$, $Z^D$-$W$, $Z^{D+}Z^D$, and $Z^D$-$W$. The equal transmission of all four possible genotypes demonstrates the $Z^{D^+}$ and $Z^D$ spermatozoa formed in the surrogate host gonad (the somatic tissue of which has a $Z^{D^+}Z^{D^+}$ genotype) were all viable.

The gonads of $Z^{D^+}Z^D$ embryos resembled the gonads of wild-type female embryos at the equivalent stage at all developmental time points examined (E5.5 to E 17.5). These findings clearly demonstrate that the loss of a single copy of DMRT1 in male birds results in ovarian rather than testicular development and represent definitive proof of a DMRT1-dependent dosage-based mechanism of sex determination in birds. To determine whether this switch in gonadal fate persisted posthatch, we examined the gonads of these birds at 5 wk of age and again found that these resembled the gonads found in wild-type females. The tissue is

![Fig. 4. Phenotyping of adult DMRT1 mutants. (A) Physical appearance of wild-type and of DMRT1-mutant birds at 24 wk. (B) Body weight of wild-type and DMRT1-mutant birds. Asterisks indicate a statistically significant difference in body weight between each of the ZZ genotypes ($Z^{D^+}Z^{D^+}$, $Z^D$-$Z^D$) and each of the ZW genotypes ($Z^D$-$W$, $Z^D$-$W$), on days 120 and 192 (n = 5–8 per genotype). P < .05.](https://doi.org/10.1073/pnas.2020909118)
clearly ovarian, with a thickened cortex containing follicles, with oocytes surrounded by granulosa and theca layers. Although these ovaries contained significant numbers of small and medium-sized follicles, there was a lack of large follicles, and these birds did not ovulate/lay eggs at sexual maturity. In the wild-type female (ZD+W), follicular maturation and ovulation are stimulated by signals from the hypothalamic-pituitary axis (HPA), and the lack of a female HPA in sex-reversed males (ZD+ZD-) may indicate a requirement for DMRT1 in the cellular allocation of the right Müllerian duct in ZD+ZD- embryos is also shorter than its left counterpart. The mechanism underlying this persistence of the right Müllerian duct in ZD+ZD- embryos is unclear although regression in ZD+W embryos is thought to involve AMH or AMHR2 signaling. In any event, it appears that the retained Mullerian duct tissue is able to respond to the same factors that compensate for this loss and enable Mullerian duct formation.

We also analyzed gonads of ZD+W embryos and found that loss of DMRT1 had little effect on gonadal sex identity, in that female embryos clearly had a left ovary with a thickened cortex containing germ cells. However, when we examined these ovaries at 5 wk posthatch, there were no obvious follicles and no evidence of oocytes although the cortex did contain granulosa cells and theca cells. This suggests that the absence of functional DMRT1 leads to a loss of germ cells in posthatch female birds. Given that DMRT1 is highly expressed in germ cells and implicated in meiosis in other species, we analyzed meiotic progression in late stage embryos (E13.5 and E17.5) by monitoring the expression of γH2AX, which indicates DNA double-strand breaks typical of meiotic recombination (26). For ZD+ZD- embryos, the pattern of marker expression and DNA fragmentation in cortical PGCs was similar, although delayed, to that seen in wild-type female embryos. In contrast, no γH2AX expression was detected in cortical PGCs of chromosomally female embryos lacking DMRT1 (ZD-W); a similar PGC phenotype to that observed in DDX4-mutant (DEAD-Box Helicase 4) chickens where the germ cells are lost (22). Taken together, these findings suggest that, in these birds, the loss of DMRT1 either prevented or delayed meiosis and resulted in the loss of germ cells.

It is clear from our studies that the loss of one copy of DMRT1 in chromosomally male embryos results in the induction of the gene network underlying ovary development: the spatial and temporal expression of first FOXL2 and then aromatase is identical to that seen in wild-type female embryos. This suggests that the presence of two functional copies of DMRT1 in wild-type male embryos suppresses, either directly or indirectly, the expression of FOXL2. In goats, FOXL2 is a primary ovarian factor that stimulates gonad formation. In chickens, FOXL2 stimulates medulla formation, which, in turn, leads to a reduction in aromatase expression, which catalyzes the conversion of androgens to estrogens (30–32). It is well established that E2 also plays a major role in sex determination in birds. Estrogen treatment of chromosomally male embryos leads to ovary formation and inhibition of E2 synthesis in chromosomally female embryos results in ovary-to-testes sex reversal (8, 10). In this study, we have investigated the effects of blocking E2 synthesis in embryos with targeted mutations in DMRT1. We have demonstrated that the left gonad in ZD+W embryos develops as an ovary; however, if E2 synthesis is blocked in these embryos, both gonads develop as testes. Interestingly, when E2 synthesis is blocked in chromosomally female embryos that lack DMRT1, the gonads do not develop as testes, suggesting that DMRT1 is essential for testis formation. The gonad medulla of these embryos continues to express FOXL2 and aromatase, but, because E2 synthesis is blocked, cortex formation is not induced. It is noteworthy that the early gonads of ZD+W embryos are smaller than those of ZD+W embryos, perhaps reflecting a requirement for DMRT1 in the cellular allocation and/or proliferation of the early gonad. Fig. 6D summarizes the fate of the gonadal medulla and cortex under the influence of different combinations of DMRT1 and E2. We hypothesize that primary sex determination in chickens depends on whether or not the gonadal medulla expresses E2. In ZD+W embryos, high levels of the Z chromosome DMRT1 suppress FOXL2 expression, which, in turn, leads to a reduction in aromatase expression and to low levels of E2 synthesis and allows sex cord formation to be induced. In ZD+W embryos, levels of DMRT1 are not sufficient...
Methods

highlights the unique feature of CASI in birds. While providing evidence for an important role of DMRT1 in the center of the avian gonadal sex-determining mechanism, characteristic.

Previously, it was considered that the male and female secondary sexual characteristics of vertebrates were largely dependent on the outcome of primary sex determination and that gonadal hormones played a major role in defining the sexual phenotype. More recently, it has become generally accepted that male:female differences are due to the combined effects of gonadal hormone differences and differences in the sex-chromosome constitution of individual cells and tissues, a classic example being that of marsupial body dimorphism (reviewed in ref. 33). We and others have established that birds possess a CASI and that this plays a major role in defining secondary sexual characteristics (18, 34, 35). Analysis of the adult birds in this study suggest that CASI may be the dominant factor in establishing secondary sexual characteristics of vertebrates. The male sexual phenotype and that gonadal hormones have little or no effect on external secondary sexual characteristics. The male birds with ovary in place of testes are virtually identical in growth rate and appearance to wild-type males and display no female characteristics.

Taken together, our findings clearly place DMRT1 dosage in the center of the avian gonadal sex-determining mechanism, while providing evidence for an important role of DMRT1 in germ cell and Müllerian ducts fate. Finally, this work further highlights the unique feature of CASI in birds.

Genome Editing and Generation of DMRT1 Mutant Birds. Germ cells were isolated from Hy-line Brown layer embryos heterozygote for an RFP reporter gene (36) at Hamburger–Hamilton (HH) stage 16° and cultured in vitro (37). Briefly, 1 μL of embryonic blood was aspirated from the dorsal aorta of embryos and placed in FAOT (FGF, Activin, ovotransferrin) culture medium (37). Expanded germ cell populations (3 wk) were cotransfected with 1.5 μg of high fidelity CRISPR-Cas9 vector (HF-PX459 V2.0) which included a targeting guide (single guide RNA [sgRNA]) for the DMRT1 locus and two single-stranded donor oligonucleotides (ssODNs) (5 pmol of each) (SI Appendix, Table S1) using Lipofectamine 2000 (Thermo Fisher Scientific) (21).

Twenty-four hours after transfection, PGCs were treated with puromycin (at 400 ng/mL) for 48 h to select for edited cells. Following puromycin treatment, PGCs were sorted into single wells of 96-well plates using a FACSaria III (BD Biosciences) at one PGC per well in 110 μL of FAOT to produce clonal populations. PGCs were expanded in culture, DNA was extracted for analysis, and then clonal PGCs were cryopreserved in STEM-CELLBANKER (AMSBio).

Generating Surrogate Host Chicken. Clonal PGCs were thawed, and 1 μL of cells from an individual PGC clone carrying the desired edits for DMRT1 was injected via the dorsal aorta into stage 16 HH+ transgenic surrogate host embryos containing an iCapsase9 targeted to the germ cell-specific DAZL locus (23, 38). Then, 1.0 μL of 25 mM B/B (in dimethyl sulfoxide [DMSO]) (AP20187; Takara) was added to 50 μL of PGCs (3,000 PGCs per microliter) before injection, and, subsequently, 100 μL of Penicillin/Streptomycin containing 3 μL of 0.5 mM B/B drug (in ETOH) was pipetted on top of the embryo. Treatment of the transgenic surrogate hosts with B/B drug ablates the endogenous germ cells, such that the only gametes that can form are from the donor PGCs. Fourteen surrogate host chicks were hatched from two injection experiments. Four surrogate host chicks carried the iCapsase9 transgene. Two male iCapsase9 surrogate hosts (with somatic genotype ZD+ZD+), carrying germ cells heterozygous for DMRT1 (ZD+ZD-), were crossed with wild-type hens (ZD+W) to produce G1 embryos for analysis and hatched to create G2 offspring. All animal experiments were conducted under United Kingdom Home Office license. Experimental protocols and studies were approved by the Roslin Institute Animal Welfare and Ethical Review Board Committee.

Genetic Screening. DNA was extracted from cells and embryonic tissues using the PureLink Genomic DNA Mini Kit (Thermo Fisher Scientific) according to the manufacturer’s instructions. To amplify the DMRT1 locus, PCR reactions included 100 ng of genomic DNA (gDNA) and QS high-fidelity polymerase

Fig. 6. Overview of sex determination in chickens. (A) Outcomes resulting from different combinations of DMRT1 and E2. (B and C) Schematics illustrating regulation of gene networks that define male and female reproductive systems (DMRT1: ++/−/− = 2/1/0 copies; E2 and Cortex: ++/−/− = present/absent).
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Primary sex determination in birds depends on Dmrt1 dosage, but gonadal sex does not determine adult secondary sex characteristics

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II. IHC. IHC was carried out according to the protocol described by Stern (43). Gonads were fixed in 4% paraformaldehyde for 2 h at 4 °C. Tissues were equilibrated in 15% sucrose/0.1 M phosphate buffer overnight, embedded in 15% sucrose plus 7.5% gelatin/0.1 M phosphate buffer (pH 7.2), and snap frozen using isopentane. Ten-micrometer-thick sections were cut on a cryostat (OFS 5000; Bright Instruments) and mounted on Super Frost Plus slides (Thermo Fisher Scientific). Slides were dehydrated for 30 min in PBS at 37 °C and blocked in PBS containing 10% donkey serum, 1% bovine serum albumin (BSA), and 0.3% Triton X-100 for 2 h at room temperature. Incubation with primary antibodies (SI Appendix, Table S2) was carried out overnight at 4 °C, followed by washing twice in PBS containing 0.3% Triton X-100, and incubation with secondary antibodies for 2 h at room temperature. After washing four times in PBS containing 0.3% Triton X-100, the sections were treated with 5 μg/ml Hoechst nuclear stain solution (10 μg/ml) for 5 min. Imaging was carried out using a Leica DMLB Upright Fluorescent microscope (Leica Camera AG).

Data Analysis. All summary data values are expressed as mean ± SD. GraphPad Prism (Graphpad) was used to produce graphs and for statistical analyses. Statistical analysis of qPCR data included a one-way ANOVA analysis followed by Tukey’s multiple comparison test for post hoc comparisons. P < 0.05 was set as the statistical significance threshold.

Data Availability. All study data are included in the article and/or SI Appendix.

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