Licensing of gametogenesis, dependent on RNA binding protein DAZL, as a gateway to sexual differentiation of fetal germ cells

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Mammalian oocytes and spermatozoa derive from fetal cells shared by the sexes. These primordial germ cells (PGCs) migrate to the developing somatic gonad, giving rise to oocytes or spermatozoa. These opposing sexual fates are determined not by the PGCs' own sex chromosome constitution (XX or XY), but by the sexual identity of the fetal gonad that they enter. We asked whether PGCs undergo a developmental transition that enables them to respond to feminizing or masculinizing cues from fetal ovary or testis. We conducted in vivo genetic studies of DAZL, an RNA-binding protein expressed in both ovarian and testicular germ cells. We found that germ cells in C57BL/6 *Dazl*-deficient fetuses—whether XX or XY—migrate to the gonad but do not develop either male or female features. Instead, they remain in a sexually undifferentiated state similar to that of migrating PGCs. Thus, germ cells in C57BL/6 *Dazl*-deficient fetuses do not respond to sexual cues from ovary or testis, whereas the earlier processes of germ cell specification and migration are unaffected. We propose that PGCs of both XX and XY fetuses undergo licensing, an active developmental transition that enables the resultant gametogenesis-competent cells to respond to feminizing or masculinizing cues produced by the fetal ovary or testis and hence to embark on oogenesis or spermatogenesis. In C57BL/6 mice, *Dazl* is required for licensing. Licensing serves as a gateway from the embryonic processes shared between the sexes—germ cell specification and migration—to the sex-specific pathways of oogenesis and spermatogenesis.

In both XX and XY mouse embryos, sex cells called germ cells arise from a small cluster of pluripotent epiblast cells (1, 2). The resultant primordial germ cells (PGCs) migrate to the developing somatic gonad, where they in due course give rise to either oocytes or spermatozoa. These opposing sexual fates are not determined by the PGCs' own sex chromosome constitution (XX or XY). Instead, the sexual fate of the PGCs is determined by the sexual identity of the fetal gonad to which the PGCs migrate (3–6). Little is known about how PGCs prepare for this binary sexual decision (7–10).

The first morphological difference between germ cells in XX and XY gonads is the chromosome condensation associated with meiosis, which is observed in ovarian (XX) but not testicular (XY) germ cells at about embryonic day (E)13.5 (7, 8). These and other observations have led to the model that whether germ cells develop as oocytes or spermatozoa depends on the time when they initiate meiosis: if meiosis begins during fetal development, the germ cell is committed to the female path, oogenesis; if meiosis does not begin during fetal development, the germ cell is committed to the male path, spermatogenesis (7, 8, 10–12). Accordingly, preventing meiotic initiation in fetal ovarian germ cells should direct them to a male fate.

Recent observations provided an opportunity to test this prediction. *Dazl*, a gene encoding a germ-cell–specific RNA binding protein, is expressed in postmigratory XX and XY germ cells beginning at about E11.5, before sexual differentiation in these cells (13). We have shown previously that germ cells in C57BL/6 XX *Dazl*-deficient fetuses do not initiate meiosis (14).

In other mutants known to disrupt meiosis, one or more aspects of the meiotic program are detectable at the microscopic or molecular levels. By contrast, C57BL/6 XX *Dazl*-deficient germ cells exhibit none of the hallmarks of meiotic initiation or of the early meiotic program, providing a unique opportunity to study nonmeiotic XX germ cells. Evidence of male differentiation in germ cells of C57BL/6 XX *Dazl*-deficient fetuses would validate the current model.

Results and Discussion

We began by examining expression of the *Nanos2* gene, an early marker and driver of male germ cell differentiation (15, 16). In wild-type fetuses, *Nanos2* is expressed in testicular but not ovarian germ cells beginning at E13.5 (15). Using RT-PCR, we tested for *Nanos2* mRNA in gonads of *Dazl*-deficient fetuses (Fig. 1). Contrary to the current model of germ cell sex determination, *Dazl*-deficient ovaries did not express *Nanos2*, suggesting that their germ cells were not masculinizing despite the absence of meiotic initiation.

To substantiate this finding, we tested whether other processes characteristic of male germ cell differentiation occur in *Dazl*-deficient fetal ovaries. In wild-type testes, germ cells exit the mitotic cell cycle and arrest in G1/G0; this arrest begins between E12.5 and E14.5 (17). We determined whether germ cells in *Dazl*-deficient fetal ovaries were cycling or arrested by examining DNA replication. Specifically, we injected pregnant mice with bromodeoxyuridine (BrdU) 18.5 d after mating and harvested fetal ovaries 3 h later. We immunostained sections of these ovaries with antibodies against BrdU and mouse vasa homolog (MVH, also known as DDX4), a germ cell marker. In wild-type ovaries, where germ cells had progressed into meiotic prophase, BrdU was incorporated by somatic but not germ cells, as expected (Fig. 2A). In contrast, in *Dazl*-deficient ovaries, many germ cells incorporated BrdU (Fig. 2B), indicating that they had continued to cycle rather than undergo the G1/G0 arrest characteristic of male fetal germ cells. To confirm this finding, we examined expression of Ki-67, a nuclear protein whose absence marks quiescent (G1/G0 arrested) cells; cycling cells express Ki-67 (18). We found, as expected, that most germ cells in wild-type testes were negative for Ki-67 at E15.5 (Fig. 2C), consistent with the cells having arrested in G1/G0. [Germ cells in wild-type ovaries displayed weak staining, consistent with previous reports (19) (Fig. 2D)]. Most germ cells in *Dazl*-deficient ovaries were positive for Ki-67 (Fig. 2E), reinforcing the conclusion that these
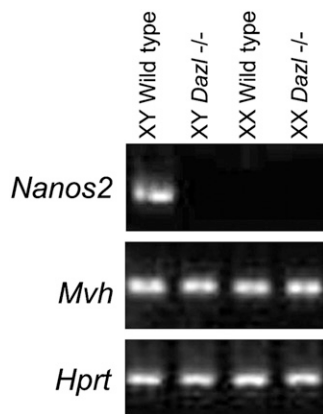
DEVELOPMENTAL BIOLOGY

Fig. 1. *Dazl* is required for expression of *Nanos2* in C57BL/6 fetal germ cells. RT-PCR (reverse transcription–PCR) analysis of *Nanos2, Mvh*, and *Hprt* (ubiquitously expressed control) mRNA in wild-type and *Dazl*-deficient E13.5 gonads.
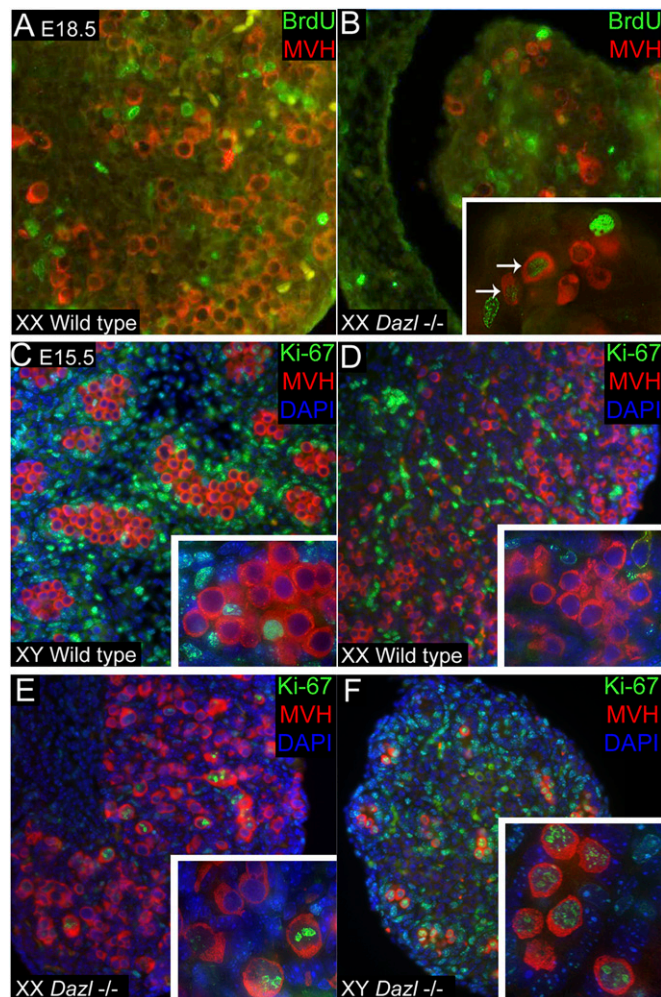

Fig. 2. *Dazl*-deficient germ cells do not undergo cell cycle arrest. (*A* and *B*) Immunohistochemical staining for BrdU (green) and MVH protein (red) in sections of wild-type (*A*) and *Dazl*-deficient (*B*) E18.5 ovaries; BrdU injected 3 h before killing. *Inset* in *Dazl*$^{-/-}$ panel shows higher magnification, deconvolved image. Arrows indicate BrdU-positive germ cells. (*C–F*) Immunohistochemical staining for Ki-67 (green) and MVH (red) proteins in sections of wild-type (*C* and *D*) and *Dazl*-deficient (*E* and *F*) E15.5 gonads. Nuclei stained with DAPI (blue). *Insets* show higher magnification, deconvolved images.

cells had continued to cycle and had not experienced the G1/G0 arrest characteristic of male fetal germ cells.

We then examined a third feature of male differentiation in fetal germ cells: remethylation of the genome. In both sexes, PGCs undergo global DNA demethylation at the end of their migratory phase (20). In fetal testes, germ cells initiate genomewide remethylation by E15.5, whereas germ cells in ovaries do not do so until after birth (20). We examined global levels of DNA methylation in E15.5 germ cells by immunostaining gonadal sections for 5-methylcytosine. As expected, germ cells in wild-type testes displayed readily detectable levels of genome methylation, whereas germ cells in wild-type ovaries did not (Fig. 3 *A* and *B*). Consistent with our earlier findings, germ cells in *Dazl*-deficient ovaries did not stain for 5-methylcytosine (Fig. 3*C*). Thus, germ cells in C57BL/6 XX *Dazl*-deficient fetuses exhibit none of the molecular features of male differentiation. We conclude that the absence of meiotic initiation in fetal germ cells is not sufficient to cause them to embark on the path to spermatogenesis.

Having found that C57BL/6 *Dazl*-deficient ovarian germ cells initiate neither male differentiation (Figs. 1–3) nor meiosis (14), we wondered whether they might engage in nonmeiotic aspects of oogenesis. Specifically, we asked whether *Dazl*-deficient ovarian germ cells express two transcription factors that are early markers and drivers of oogenesis. FIGLA (factor in the germline alpha) and NOBOX (newborn ovary homeobox gene) are expressed in fetal or neonatal ovarian germ cells, where they are required for recruitment of granulosa (somatic) cells to form primordial and primary follicles (21–23). FIGLA is additionally required for expression of zona pellucida genes in oocytes (21, 22). We tested wild-type and *Dazl*-deficient ovaries for *Figla* mRNA by RT-PCR and for NOBOX protein by immunohistochemistry. As previously reported (22), *Figla* was expressed in wild-type ovaries at E14.5 and at birth (Fig. 4*A*). By contrast, we detected no *Figla* mRNA in XX *Dazl*-deficient ovaries (Fig. 4*A*), indicating that their germ cells had not initiated the *Figla*-dependent gene expression programs required for oogenesis. Similarly, whereas NOBOX protein was present in most germ cells of wild-type ovaries at birth (Fig. 4*B*), and in all germ cells of wild-type ovaries at 2 d of age (Fig. 4*C*), this oogenesis factor was absent in germ cells of *Dazl*-deficient ovaries at both time points. In sum, we find no molecular evidence of either
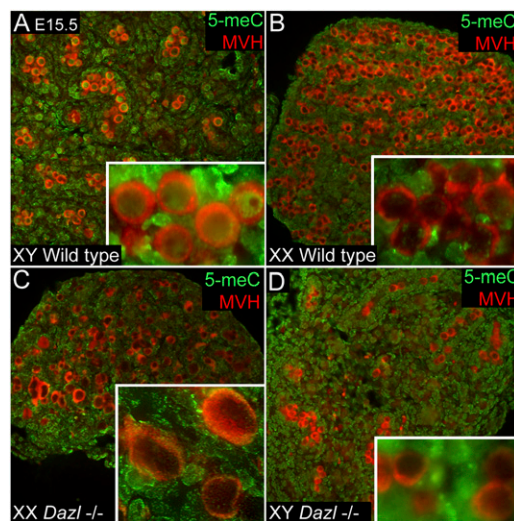
Fig. 3. *Dazl* is required for genome remethylation. Immunohistochemical staining for 5-methylcytosine (green) and MVH (red) in sections of wild-type (*A* and *B*) and *Dazl*-deficient (*C* and *D*) E15.5 gonads.

Gill et al.
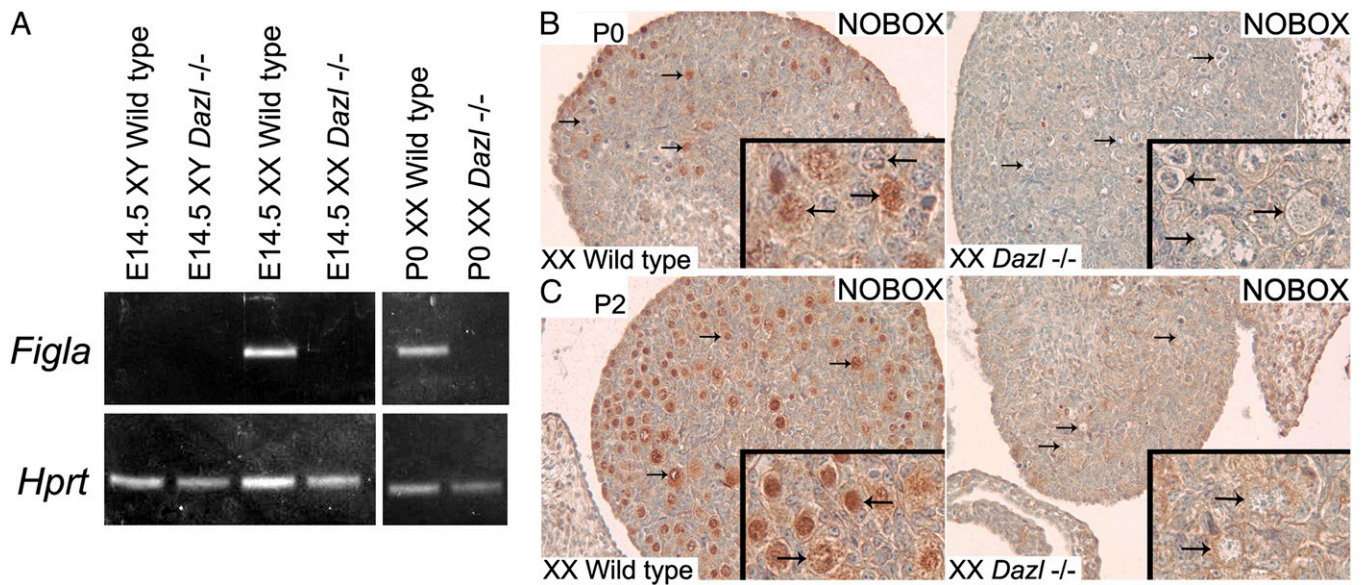
Fig. 4. *Dazl* is required for expression of regulators of oogenesis. (*A*) RT-PCR analysis of *Figla* and *Hprt* mRNA in wild-type and *Dazl*-deficient E14.5 and P0 (day of birth) gonads. (*B* and *C*) Immunohistochemical staining for NOBOX protein in sections of wild-type and *Dazl*-deficient XX gonads at P0 (*B*) and P2 (*C*). Insets show higher magnification images. Arrows indicate representative germ cells.

feminization or masculinization in germ cells of C57BL/6 *Dazl*-deficient ovaries.

These findings in XX gonads led us to closely examine the fate of germ cells in C57BL/6 *Dazl*-deficient XY gonads (24). Given that *Dazl* is expressed in germ cells of both XX and XY gonads before sexual differentiation (13), we considered the possibility that germ cells in *Dazl*-deficient fetal testes might share some phenotypic features of their ovarian counterparts. To explore this possibility, we assayed expression of *Nanos2* and *Figla* in *Dazl*-deficient fetal testes. We found that germ cells of *Dazl*-deficient testes express neither the male marker *Nanos2* nor the female marker *Figla* (Figs. 1 and 4*A*). Thus, in the absence of *Dazl* function, both testicular and ovarian germ cells fail to activate genes required for male-specific and female-specific differentiation.

Might germ cells in *Dazl*-deficient fetal testes nonetheless undergo the G1/G0 arrest observed in wild-type testes or the genomewide remethylation observed there? To address these questions, we immunostained gonadal sections for either Ki-67 or 5-methylcytosine. We found that germ cells in *Dazl*-deficient E15.5 testes were positive for Ki-67 (Fig. 2*F*) and did not display detectable levels of 5-methylcytosine (Fig. 3*D*). Thus, in the absence of *Dazl* function, germ cells in fetal testes do not embark on the pathway to spermatogenesis: they fail to express *Nanos2*, to arrest in G1/G0, and to remethylate their genomes.

In sum, germ cells in C57BL/6 *Dazl*-deficient fetal gonads—both XX and XY—do not appear to undergo sexual differentiation. These findings suggest that PGCs, before expressing *Dazl*, are not primed and poised for sex determination. Instead, they must be readied for this binary decision via an active, *Dazl*-dependent process. We propose a model of in vivo germ cell development (Fig. 5) based on these findings in combination with previous observations. The model posits the existence in vivo of the "gametogenesis competent cell," (GCC), which, like its precursor, the PGC, is sexually undifferentiated and present in both XX and XY embryos. Unlike the PGC, the GCC is capable of embarking on oogenesis or spermatogenesis in response to molecular signals from, respectively, ovary or testis. The transition from PGC to GCC, which we refer to as licensing for gametogenesis, requires *Dazl*. Licensing is thereby distinguished from two earlier processes—PGC specification and migration—that occur normally in the absence of *Dazl*.

This model predicts that, in *Dazl*-deficient fetuses, gonadal germ cells should retain gene expression patterns characteristic of migrating PGCs. We found this to be the case. In wild-type embryos, migrating PGCs express several genes that are subsequently down-regulated in gonadal germ cells. These genes include *Oct4* (also known as *Pou5f1*), *Nanog*, *Sox2*, and *Dppa3* (also known as *stella*). In wild-type fetal ovaries, expression of these genes is extinguished coordinately, in an anterior to pos-
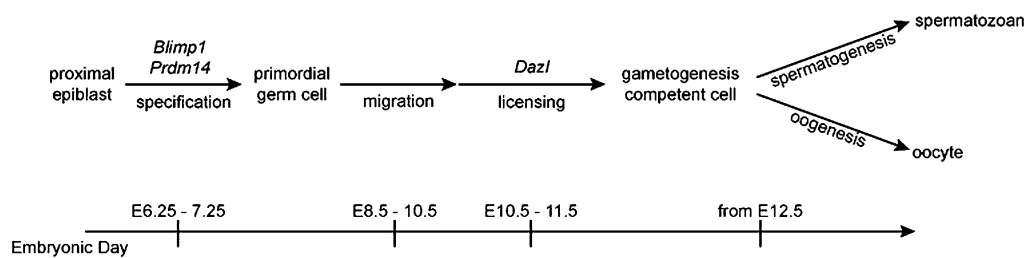
Fig. 5. A proposed pathway of germ cell development in vivo. Primordial germ cells (PGCs) are specified from posterior proximal epiblast cells via extracellular signaling pathways and the activity of germ-cell–intrinsic factors including *Blimp1* and *Prdm14* (30, 32, 37, 38). PGCs then migrate to the site of the developing gonad. At this time, PGCs express *Dazl* (13) and undergo licensing. The resulting gametogenesis-competent cells are sexually undifferentiated but are capable of initiating either spermatogenesis or oogenesis in response to appropriate gonadal cues.
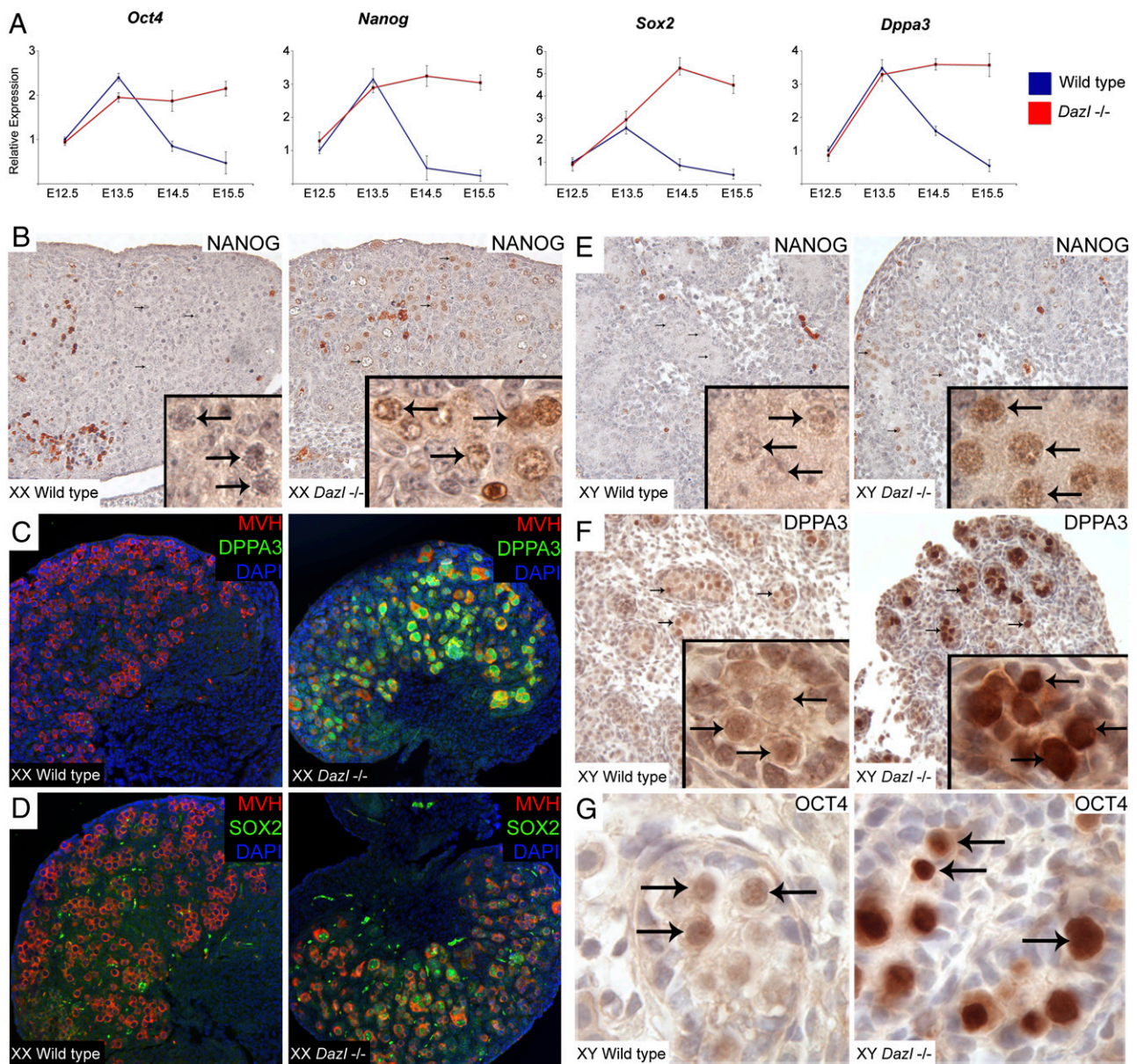
Fig. 6. *Dazl*-deficient gonadal germ cells retain gene expression characteristic of migrating PGCs. (*A*) Quantitative analysis of *Oct4*, *Nanog*, *Sox2*, and *Dppa3* mRNA levels in wild-type and *Dazl*-deficient ovaries at E12.5–E15.5. Plotted here are average fold changes (relative to wild-type E12.5 ovary; all values normalized to *Hprt*) of three independent biological replicates at each time point. Error bars show SD among biological replicates. (*B*) Immunohistochemical staining for NANOG protein in sections of wild-type and *Dazl*-deficient E15.5 XX gonads. *Insets* show higher magnification images. Arrows indicate germ cells. (*C*) Immunohistochemical staining for DPPA3 (green) and MVH (red) protein in sections of wild-type and *Dazl*-deficient E15.5 XX gonads. Nuclei stained with DAPI (blue). (*D*) Immunohistochemical staining for MVH (green) and SOX2 (red) protein in sections of wild-type and *Dazl*-deficient E15.5 XX gonads. Nuclei stained with DAPI (blue). (*E*) Immunohistochemical staining for NANOG protein in sections of wild-type and *Dazl*-deficient E15.5 XY gonads. *Insets* show higher magnification images. Arrows indicate germ cells. (*F*) Immunohistochemical staining for DPPA3 protein in sections of wild-type and *Dazl*-deficient E15.5 XY gonads. *Insets* show higher magnification images. Arrows indicate germ cells. (*G*) Immunohistochemical staining for OCT4 protein in sections of wild-type and *Dazl*-deficient E15.5 XY gonads. Arrows indicate germ cells.

terior wave, beginning at ∼E14.5 (25–27). We measured expression of these genes in fetal ovaries at 1-d intervals, from E12.5 through E15.5, by quantitative RT-PCR. In wild-type ovaries, as expected, mRNA levels for these PGC markers declined sharply after E13.5 (Fig. 6*A*). In *Dazl*-deficient ovaries, by contrast, germ cells continued to express all four genes at high levels through E15.5 (Fig. 6*A*). To confirm these mRNA findings, we assayed the protein products of three of the genes by immunohistochemistry in E15.5 ovaries. For all three factors, we observed little or no protein expression in germ cells of wild-type ovaries, but strong expression in germ cells of *Dazl*-deficient

ovaries (Fig. 6 *B–D*). This retention of PGC-like gene expression in germ cells of C57BL/6 *Dazl*-deficient ovaries corroborates our model.

To extend these studies of ovarian germ cells to their testicular counterparts, we also assayed the NANOG, DPPA3, and OCT4 proteins in E15.5 testes by immunohistochemistry. In wild-type fetal testes, germ cells down-regulate these PGC markers, but in a less ordered manner than in fetal ovaries (28, 29). At E15.5, we detected no NANOG protein in germ cells of wild-type testes, but robust staining in germ cells of *Dazl*-deficient testes (Fig. 6*E*). Similarly, germ cells of *Dazl*-deficient

testes stained much more intensely for DPPA3 and OCT4 than did germ cells in identically treated wild-type testes (Fig. 6 F and G). Thus, in both fetal testes and ovaries, C57BL/6 *Dazl*-deficient germ cells retain PGC-like gene expression, as predicted by our model. A question for the future is whether germ cells must extinguish PGC-like gene expression before initiating sexual differentiation and gametogenesis or whether these processes are governed in parallel.

The C57BL/6 *Dazl*-deficient phenotype reported here differs in an important respect from those of other mutants affecting embryonic or fetal germ cells. Mutations in *Blimp1*, *Prdm14*, and *c-kit*, for example, have revealed critical roles in germ line maintenance or germ cell survival (30–32). Whereas C57BL/6 *Dazl*-deficient germ cells eventually undergo apoptosis (24), this does not occur until well after a primary defect, in cellular differentiation, is observable. This postdefect survival enabled us to appreciate a critical role for *Dazl* in germ cell differentiation and thereby to recognize a previously unknown transition (licensing) and state (the GCC) in fetal germ cell development in vivo (Fig. 2). In C57BL/6 mice, *Dazl* is a competence factor not just for meiotic initiation (14) but for the larger processes of gametogenesis that encompass meiosis and also include the sex-specific cellular differentiation events of oogenesis and spermatogenesis. *Dazl*-dependent licensing serves as a gateway from the embryonic germ cell processes shared between the sexes—specification and migration—to the sex-specific gametogenic processes of fetal, postnatal, and adult life.

Future studies may identify additional factors that are involved in licensing. For example, in mice of some genetic backgrounds, this developmental transition can occur in the absence of *Dazl* (33–35), suggesting the existence of redundant factors or pathways.

Materials and Methods

Mice. Mice carrying the *Dazl*^TM1Hgu^ allele (33) were generously provided by Howard Cooke, MRC Human Genetics Unit, Western General Hospital, Edinburgh, UK. As described previously (14, 24), we crossed *Dazl*^TM1Hgu^/+ mice to C57BL/6 mice (Taconic Farms). All experiments were conducted using mice backcrossed to C57BL/6 between 10 and 22 generations, when >99.9% of the genome is expected to be of C57BL/6 origin; all Y chromosomes and mitochondria are of C57BL/6 origin. *Dazl*-deficient embryos were generated by mating heterozygotes. *Dazl* genotypes were assayed by PCR as previously described (33). The committee on animal care at the Massachusetts Institute of Technology approved all experiments involving mice.

Embryo Collection and Sexing. To establish timed matings, female mice were housed with male mice overnight. Noon of the day when a vaginal plug was evident was considered E0.5. Embryonic gonads and mesonephroi were dissected into PBS. Sex of the tissues was determined by scoring the presence or absence of testicular cords.

BrdU Incorporation Assay. Pregnant mice derived from *Dazl* heterozygous intercrosses were injected intraperitoneally with 50 mg/kg of BrdU. Three hours later, mice were killed and embryonic gonads removed and processed for immunohistochemistry.

Immunohistochemistry. Embryonic gonads were fixed at 4 °C overnight in either 4% paraformaldehyde in PBS or Bouin's solution, embedded in paraffin, and sectioned. Slides were dewaxed, rehydrated, heated in 10 mM sodium citrate buffer, pH 6.0 for 10 min, and blocked for 30 min or 1 h (Table S1). Slides were then incubated with primary antibody overnight at 4 °C. Antibody concentrations, fixatives, and blocking solutions are listed in Table S1. For colorimetric detection, samples were incubated with rabbit or mouse ImmPress reagent (Vector Labs) and developed using DAB substrate kit, 3,3′-diaminobenzidine (Vector Labs). Samples were then counterstained with hematoxylin, dehydrated, and mounted with Permount (Fisher Scientific). For fluorescence detection, samples were incubated with FITC-conjugated antirat or antirabbit and rhodamine-conjugated antigoat secondary antibodies (Jackson ImmunoResearch Laboratories) at a concentration of 1:200 and then mounted with VectaShield mounting media with DAPI (Vector Labs).

Quantitative RT-PCR. Embryonic gonads were dissected away from mesonephroi, submerged in TRIzol (Invitrogen), and then stored at −80 °C. Following genotyping, total RNA was prepared according to the manufacturer's protocol. Total RNA was then DNase treated using DNA Free Turbo (Ambion). A total of 200 ng of total RNA was reverse transcribed using a RETROScript kit (Ambion). Quantitative PCR was performed using SYBR Green Core PCR reagents (Applied Biosystems) on an ABI9700 fast real-time PCR machine (Applied Biosystems). Results were analyzed using the delta-delta Ct method using *Hprt* (hypoxanthine-guanine phosphoribosyltransferase) as a normalization control. RT-PCR primers were picked from the PrimerBank Web site (36); their sequences are listed in Table S2. RT-PCR primers for *Nanos2* were from ref. 15.

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DEVELOPMENTAL BIOLOGY

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