# Mammalian germ cells are determined after PGC colonization of the nascent gonad

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**Mammalian primordial germ cells (PGCs) are induced in the embryonic epiblast, before migrating to the nascent gonads. In fish, frogs, and birds, the germline segregates even earlier, through the action of maternally inherited germ plasm. Across vertebrates, migrating PGCs retain a broad developmental potential, regardless of whether they were induced or maternally segregated. In mammals, this potential is indicated by expression of pluripotency factors, and the ability to generate teratomas and pluripotent cell lines. How the germline loses this developmental potential remains unknown. Our genome-wide analyses of embryonic human and mouse germlines reveal a conserved transcriptional program, initiated in PGCs after gonadal colonization, that differentiates germ cells from their germline precursors and from somatic lineages. Through genetic studies in mice and pigs, we demonstrate that one such gonad-induced factor, the RNA-binding protein DAZL, is necessary in vivo to restrict the developmental potential of the germline; DAZL's absence prolongs expression of a *Nanog* pluripotency reporter, facilitates derivation of pluripotent cell lines, and causes spontaneous gonadal teratomas. Based on these observations in humans, mice, and pigs, we propose that germ cells are determined after gonadal colonization in mammals. We suggest that germ cell determination was induced late in embryogenesis—after organogenesis has begun—in the common ancestor of all vertebrates, as in modern mammals, where this transition is induced by somatic cells of the gonad. We suggest that failure of this process of germ cell determination likely accounts for the origin of human testis cancer.**

germ cell | commitment | teratoma | pluripotency | *Dazl*

During embryogenesis, cells segregate into germline and somatic lineages. In mammals, this split is first evident around the time of gastrulation, when intercellular signaling induces the formation of primordial germ cells (PGCs) (1, 2). Comparative studies reveal that an inductive method of germline segregation likely existed in the common ancestor of all vertebrates (3). However, some vertebrates, such as fish, frogs, and birds, have acquired a different approach to germline segregation. It occurs much earlier in these species—during the first cell divisions of the zygote—through the action of maternally supplied RNAs known as germ plasm (4).

Despite these different strategies for PGC formation, emerging evidence suggests that migratory PGCs of nonmammalian vertebrates remain developmentally uncommitted to gametogenesis, retaining the capacity for somatic differentiation. In frogs, PGCs arising via germ plasm readily differentiate into somatic cells when transplanted into host embryos (5). Similarly, in fish, mismigrated PGCs readily adopt somatic fates if depleted of *Dnd1* (6). In salamanders, where PGCs arise through inductive processes, irreversible commitment of the germline occurs late in development, long after gastrulation is complete and somatic lineages are established (7). In mammals, migratory PGCs can form teratomas if transplanted to ectopic sites (8) and give rise to pluripotent cell lines in culture (9–11). It has also been suggested that presumptive PGCs (labeled genetically by *Prdm1-Cre*) in the posterior region of the embryo during allantoic elongation may contribute to nongametogenic lineages (12, 13). Taken together, these observations suggest that migratory PGCs of vertebrates retain a broad developmental potential, regardless of their mode of segregation. That is, migratory PGCs, while clearly cells of the germline (the entire lineage from zygote to gamete), may not yet be germ cells, which, by definition, are committed to producing gametes and no other cell types (14).

To better understand germline commitment in mammals, we examine the transition that occurs as PGCs invade the nascent gonads. We find a transcriptional program, initiated in human and mouse PGCs after colonization of the gonad, that distinguishes germ cells from their migratory germline precursors, and from soma. Through genetic studies, we demonstrate that this program is necessary for germ cell commitment in mammals. In embryonic mice deficient in one factor induced at PGC colonization—the

## Significance

In mammals, the germline is set aside early in development for the later production of the gametes, either eggs or sperm. It remains unknown when, and how, the precursor cells (termed primordial germ cells [PGCs]) become committed to produce only gametes, and no other cell type. We identify an embryonic transition occurring late in development, after PGCs colonize the nascent gonad, that serves to commit these cells to produce only gametes. Our findings have broad implications for the origin of germ cell tumors in humans, and for the stepwise commitment of the germline in mammals and other vertebrates.

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Data deposition: Data generated using the Mouse Diversity Genotyping Array (Thermo Fisher Scientific) for the SNP genotyping of 129S2 and 129S4 substrains have been deposited at the Gene Expression Omnibus under accession no. GSE87771. Data generated from control and *Dazl*-deficient germ line cells at E10.3 and E11.5 have been deposited under the Sequence Read Archive BioProject accession no. PRJNA434733.

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RNA-binding protein *Dazl*—the germline remains developmentally uncommitted, retaining expression of a network of pluripotency factors, the capacity for pluripotent cell line derivation, and the potential to form gonadal teratomas in mice and pigs.

## Results

**Germ Cell Determinants of Nonmammalian Vertebrates Are Expressed in Mice and Humans upon PGC Colonization of the Nascent Gonads.** We searched for changes in gene expression that accompany PGC colonization of gonads in mammals. To this end, we reanalyzed published transcriptomes of migratory and gonadal germline cells from mouse (15) and human (16) embryos. Our analyses in mice identified 74 genes whose expression increased robustly after PGCs colonized the gonads (fold change of >4 [presented as $\log_2$ transformed] from E9.5 to E11.5, false discovery rate (FDR) < 0.05; Fig. 1*A* and Datasets S1 and S2). To determine whether this program of gene expression is similarly induced in human embryos, we reanalyzed the transcriptomes of single cells within a comparable developmental window (XY: weeks 4 to 9; XX: weeks 5 to 7 and 8; Dataset S3 and *SI Appendix*, Fig. S1). Of the 74 genes induced in mice, 44 have one-to-one orthologs in humans. As a set, these 44 genes are up-regulated in both XY and XX human germline cells after gonadal colonization, indicating that the program induced in the mouse germline at colonization is conserved in humans (Fig. 1*B*). Of particular importance, 13 genes up-regulated in mice were also significantly up-regulated in both the XY and XX human germlines (Fig. 1*C*).

Might this conserved program of gene expression, initiated after PGCs colonize the nascent gonads, serve to distinguish germ cells from other cell types in vivo? To address this, we examined, across diverse tetrapods, the expression breadth of these 13 genes. By reanalyzing RNA sequencing (RNA-seq) datasets of nine tissues from five mammals, as well as chicken and frog, we found that 10 of the 13 genes were predominantly or exclusively expressed in the adult testis, regardless of whether the germline is segregated by induction (as occurs in the mammalian epiblast) or via germ plasm (as occurs in the frog and chicken; Fig. 1*D*). In both mouse and human, each of these 10 gonad-specific genes is expressed predominantly in germ cells of embryonic gonads (Fig. 1 *E* and *F*). Notably, several factors activated on PGC colonization are components of germ plasm in nonmammalian animals, including *DAZL*, *DDX4* (the mammalian ortholog of *Vasa*), *MAEL*, and *TDRD12*.

By comparison, the set of genes expressed in migratory PGCs of mouse and human embryos, immediately prior to gonadal colonization, does not display such gonad-specific expression in tetrapods; instead, these genes are expressed across adult tissues (*SI Appendix*, Fig. S2 *A–D*). The same is true for a curated set of PGC-defining factors gleaned from the literature, and also for a set of genes activated on PGC-like cell derivation (*SI Appendix*, Fig. S2 *A–D*). Further, analysis of 500,000 random-sampled gene sets found none that displayed gonad specificity comparable to that of the 13 genes up-regulated as PGCs colonize the gonads (*SI Appendix*, Fig. S2*E*).

In sum, a set of genes whose expression defines germ cells of vertebrates is first activated in the mouse and human germlines after embryonic PGCs colonize the gonads. These genes include orthologs of germ plasm components critical to germline commitment in diverse nonmammalian metazoa (Dataset S2), raising the possibility that one or more of these genes direct germ cell commitment in mammals, and that this occurs following gonadal colonization.

If this is true, then the transcriptional profiles of the germline should provide evidence of its uncommitted nature prior to gonadal entry. Indeed, we find that, in both humans and mice, migratory and newly colonized germline cells express naïve and general pluripotency factors, which identify developmentally uncommitted cells in vivo (in the inner cell mass) and in vitro (in embryonic stem [ES] cells) (17–19) (*SI Appendix*, Fig. S3). [Pluripotency factors are similarly expressed in migratory PGCs of fish and birds (20, 21).] Further, in humans and mice of both sexes, these pluripotency factors are markedly down-regulated after PGC colonization and induction of the germ cell-defining program of gene expression (*SI Appendix*, Fig. S3). Might activation of this program be necessary to down-regulate pluripotency factors and restrict the developmental potential of the germline?

**Expression of Pluripotency Factors, and the Capacity for Deriving Pluripotent Cell Lines, Are Prolonged in *Dazl*-Deficient Mice.** We considered whether the program of gene expression induced after PGCs colonize the gonads functions in germ cell commitment. Evidence from a range of vertebrates suggests that *DAZL*, encoding an RNA-binding protein, might contribute to this function. For example, *DAZL* orthologs function in the germ plasm of fish (22, 23), frogs (24, 25), and birds (26). In C57BL/6 mice (B6), *Dazl* is necessary for licensing—the acquisition of meiotic and gametogenic competence—after PGCs colonize the gonads (27), and studies of mouse and human ES cells have suggested that *DAZL* limits the expression of pluripotency factors in vitro (28, 29). An opposing view—that *Dazl* serves to maintain germline pluripotency—emerges from reports that pluripotent embryonic germ (EG) cell lines could not be derived from *Dazl*-deficient embryos (30), and that the *Dazl*-deficient germline is unable to form gonadal teratomas (28), which arise when pluripotent cells differentiate to generate tissues of all three germ layers.

To reexamine the relationship between DAZL expression and germline commitment, we generated a reporter allele of DAZL expression (where both *Dazl* and *tdTomato* are translated from a single nascent RNA, referred to as *Dazl-tdTomato*; *SI Appendix*, Fig. S4*A*), and intercrossed this with a second fluorescent reporter allele, *Nanog:GFP* (a reporter of uncommitted cells). Flow cytometry revealed an abundance of *Nanog:GFP*-positive cells in E11.0 embryos (~12 tail somites [ts]) carrying both reporters, with few if any of these cells also expressing the DAZL reporter (Fig. 2*A* and *SI Appendix*, Fig. S4*B*). By 15 ts, however, we began to detect DAZL reporter expression in a small population of *Nanog:GFP*-positive cells. With increasing embryonic age, we continued to find a small group of cells expressing both reporters, while an increasing proportion expressed the DAZL reporter alone (no longer positive for *Nanog:GFP*). By 27 ts (~E12.5), very few cells were *Nanog:GFP*-positive, irrespective of the chromosomal sex of the embryo. These findings demonstrate, at cellular resolution, that the onset of DAZL expression is tightly correlated with the subsequent restriction of *Nanog:GFP* expression, across the entire population, within 36 h.

Might *Dazl* be necessary for this restriction? To test this, we crossed the *Nanog:GFP* reporter to both B6 and 129S4/SvJae (129S4) backgrounds, and monitored expression in each strain (*SI Appendix*, *SI Discussion* and Fig. S5). At E15.5, germline expression of the *Nanog:GFP* reporter was absent from the gonads of control embryos, but maintained in *Dazl*-deficient gonads, regardless of the embryo's genetic background or sex (Fig. 2*B* and *SI Appendix*, Fig. S4*C*). In support, reanalysis of published RNA-seq data from *Dazl*-deficient mouse ovaries (31) shows that a set of "general" and "naïve" pluripotency factors (19) remain expressed at E14.5 (*SI Appendix*, Fig. S4*D*). These observations indicate that *Dazl* is necessary, in vivo, to extinguish the expression of key markers of uncommitted cells.

To test whether *Dazl* is necessary to restrict the developmental potential of the germline, we attempted to derive pluripotent cell lines from control and *Dazl*-deficient B6 embryos. Germline cells isolated from control E10.5 embryos readily gave rise to EG cell colonies, with a mean derivation efficiency of 10.0 ± 3.4 colonies per 100 EGFP-positive cells plated (mean ± SD, $n = 8$ embryos; Fig. 2*C* and *SI Appendix*, Table S1). Colony formation declined precipitously with increasing embryonic age, irrespective of the

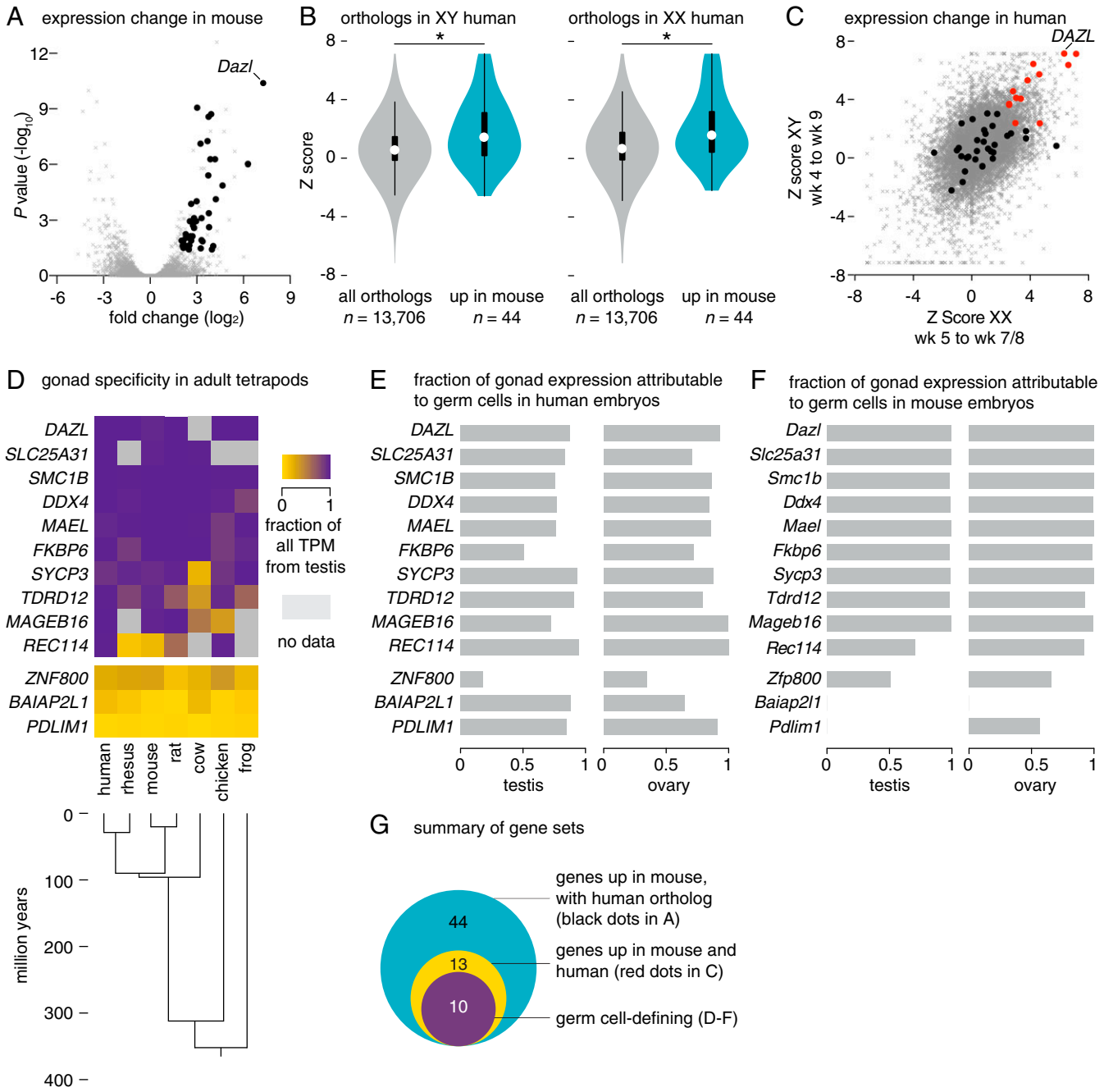


**Fig. 1.** A conserved program of germ cell transcription is induced upon PGC colonization of nascent gonads in mice and humans. (*A*) Gene expression changes in mouse germline between E9.5 and E11.5, as measured by RNA-seq. Black dots denote 44 genes that are up-regulated and have single human orthologs (fold change > 4, FDR value < 0.05); gray dots denote all other expressed genes ($n$ = 11,282). (*B* and *C*) Gene expression changes in XY and XX human embryonic germlines between weeks 4 and 9, as measured by single cell RNA-seq. (*B*) Violin plots; as a set, genes induced in mouse germline (from *A*, $n$ = 44) show greater expression increases in XY (*Left*) and XX (*Right*) human germline after PGCs colonize the gonads than do the set of all expressed orthologs ($n$ = 13,706; **P* value < 0.0007 by Wilcoxon rank sum test; black bar, interquartile range; circle, median value). (*C*) Scatter plot; black and red dots denote genes robustly up-regulated in mice, and possessing a single human ortholog (from *A*, $n$ = 44); red dot genes are also significantly up-regulated in both XY and XX human germlines ($n$ = 13). Gray dots denote all other expressed genes. (*D*) Heatmap; summary of gonad specificity of commonly up-regulated genes (red dots in *C*, $n$ = 13), by RNA-seq, in 9 adult tissues from 7 tetrapods. Specificity fraction is determined by dividing testis expression (in TPM, transcripts per million) by sum of expression in all analyzed adult tissues, for each species. Genes with no annotated ortholog are shown in gray. (*E* and *F*) Germ cell expression of commonly up-regulated factors (red dots in *C*, $n$ = 13) in (*E*) human embryonic testis and ovary and (*F*) mouse E14.5 testis and ovary by RNA-seq (*SI Appendix, SI Materials and Methods*). Ratio of 1 indicates germ cell-specific expression; 0 indicates somatic cell expression. (*G*) Euler diagram of gene sets identified through analyses in *A*–*F*.

donor embryo's sex (E11.5: 1.2 ± 0.72 colonies, $n$ = 15 embryos). At E12.5, only 5 of 18 control embryos gave rise to any EG colonies (0.03 ± 0.05 colonies), and no EG colonies were derived from E13.5 onward, irrespective of sex ($n$ = 43 embryos). These observations were as expected, given prior reports that EG colony formation declines sharply after PGCs arrive at the gonads in mice (32), and the similarly transient capacity to derive pluripotent-like cells from human gonads (33).

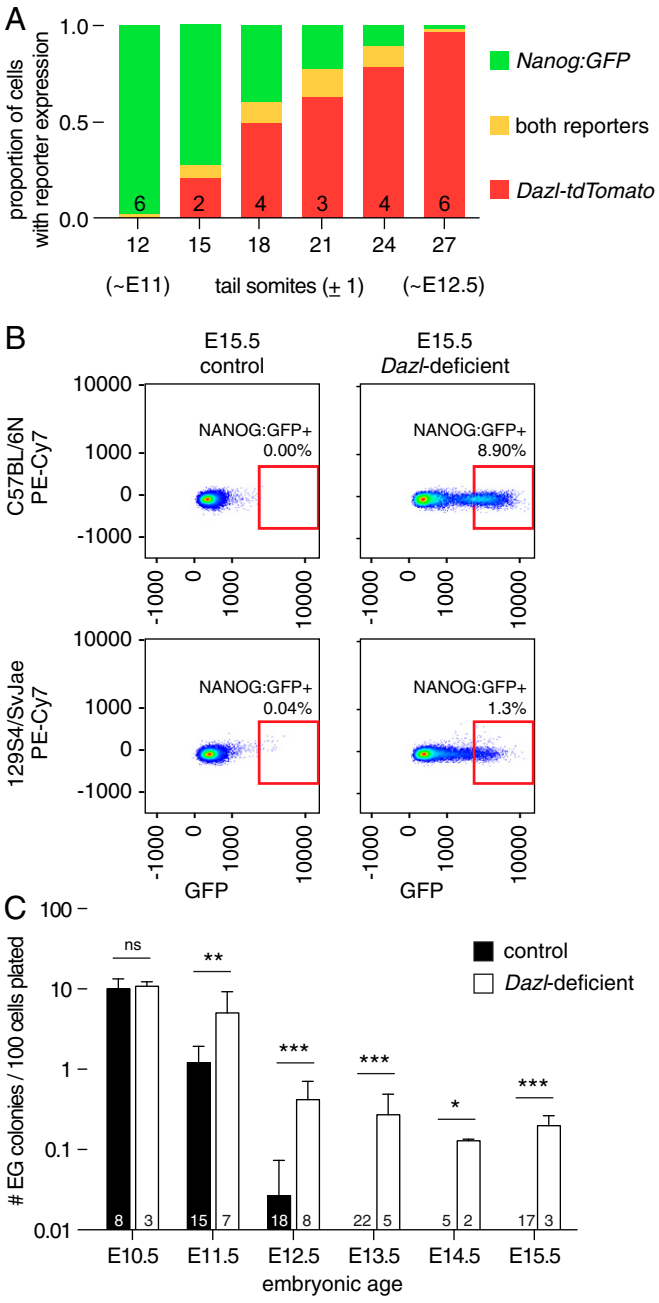


**Fig. 2.** *Dazl* is required for restricting developmental potential of the germline in diverse strains of mice. (*A*) Developmental time course of germline using *Dazl-tdTomato* and *Nanog:GFP* reporters, detected by flow cytometry. At ~12 ts (~E11), most cells expressed only the *Nanog:GFP* reporter (green). As development proceeds, the proportions of cells expressing both fluorescent reporters (orange), or only the *Dazl-tdTomato* reporter (red), change. Numbers of embryos tested are listed in each column, and the fraction of cells expressing each reporter is shown as an average. (*B*) Flow cytometry for Nanog:GFP-positive cells of E15.5 control and *Dazl*-deficient ovaries of indicated strains. Autofluorescence in PE-Cy7 channel is shown on *y* axis. Red box indicates area in which Nanog:GFP-positive cells were counted. (*C*) Derivation of EG cell lines from control and B6.*Dazl*-deficient embryos. Cells were collected by fluorescence-activated cell sorting (FACS) at embryonic age indicated on *x* axis, and cultured under defined conditions. After 10 d, EG cell colonies were counted, and rate of EG cell derivation (per 100 EGFP-positive cells plated) was calculated. Number of embryos tested is listed in each column, mean + SD, **P* value < 0.05, ** <0.01, *** <0.001, ns = not significant, using *t* test or Fisher's exact test as appropriate.

Our experimental observations with *Dazl*-deficient embryos differed strikingly from littermate controls. At E10.5 (before *Dazl* is expressed), *Dazl*-deficient embryos gave rise to EG colonies at a frequency comparable to controls (10.8 ± 1.5 colonies per 100 EGFP-positive cells, $n = 3$ embryos; Fig. 2*C*). However, all *Dazl*-deficient embryos retained the capacity to generate EG colonies beyond E12.5 ($n = 10$ embryos, combined from E13.5 to E15.5), regardless of the embryo's sex. Importantly, we first observed a significant difference between control and *Dazl*-deficient embryos at E11.5 (5.0 ± 4.2 colonies per 100 EGFP-positive cells, $n = 7$ embryos), concomitant with initiation of *Dazl* expression in the germline (Fig. 2*A*). Likewise, *Dazl*-deficient embryos isolated from an F1 cross between 129S4 and B6 mice retained the capacity to give rise to EG cell colonies until at least E15.5; the pluripotency of these cell lines was confirmed by injection into recipient blastocysts and resultant chimerism (*SI Appendix*, Fig. S4 *E–H*).

Taken together, these data establish that the *Dazl*-deficient germline continues to express pluripotency factors, and retains a PGC-like capacity for the derivation of pluripotent cell lines, even several days after colonization of the genital ridges in mice. We conclude that *Dazl* is necessary to restrict the developmental potential of the mouse germline after colonization of the gonads, regardless of genetic background, and prior to sexual differentiation of the germline.

Are other germ cell-defining factors (Fig. 1*D*) also required for this restriction? To address this question, we collected germline cells immediately prior to and shortly after gonadal entry, from control and *Dazl*-deficient embryos (34). RNA-seq analysis of these cells revealed that, apart from *Dazl*, expression of each of the other germ cell-defining factors is initiated upon gonadal entry in both control and *Dazl*-deficient embryos, indicating that DAZL is not required for their expression (*SI Appendix*, Fig. S4*I*). Further, this demonstrates that their expression is not sufficient to restrict *Nanog:GFP* expression in the *Dazl*-deficient germline—nor does their expression preclude derivation of EG cell lines (Fig. 2 *B* and *C*).

**Spontaneous Gonadal Teratomas in *Dazl*-Deficient Mice of both Sexes.** We next considered the fate of the *Dazl*-deficient germline in mice. In wild-type 129 males (but not females), teratomas arise spontaneously, at low but significant frequency, from germline cells (35). We hypothesized that these tumors arise from PGCs that enter the gonads but nonetheless remain uncommitted, retaining their teratoma-forming potential.

We collected 129S4.*Dazl*-deficient males and examined the testes for spontaneous teratomas. In control 129S4 mice at 4 mo of age, we found two teratomas among 127 males (Fig. 3 *A–C*), consistent with the low incidence reported in this strain (35). By contrast, among *Dazl*-deficient males, 19 of 69 (28%) exhibited testicular teratomas by 4 mo of age. To determine whether teratomas were present even earlier in postnatal life, we collected 129S4 mice at 4 wk of age. In controls, we found one teratoma among 188 males (Fig. 3*B*). By contrast, among *Dazl*-deficient males, 20 of 65 (31%) displayed testicular teratomas. Clearly, teratomas form at a markedly elevated rate in 129S4.*Dazl*-deficient males early in postnatal life, if not before.

Our earlier studies of EG cell derivation and *Nanog* reporter expression indicated that germline cells become restricted in their developmental potential independent of and prior to their sexual differentiation. Accordingly, we asked whether the germline in 129S4.*Dazl*-deficient ovaries can give rise to spontaneous teratomas. At 2 mo of age, we found no ovarian teratomas in 131 control females (Fig. 3*B*). By contrast, 11 of 108 (10%) *Dazl*-deficient females displayed teratomas (Fig. 3 *A–C*).

Do these tumors arise from pluripotent mitotic cells (such as PGCs), or from cells that have completed meiosis I and have undertaken parthenogenetic activation of pluripotency? In humans, ovarian teratomas may arise from either mitotic or meiotic

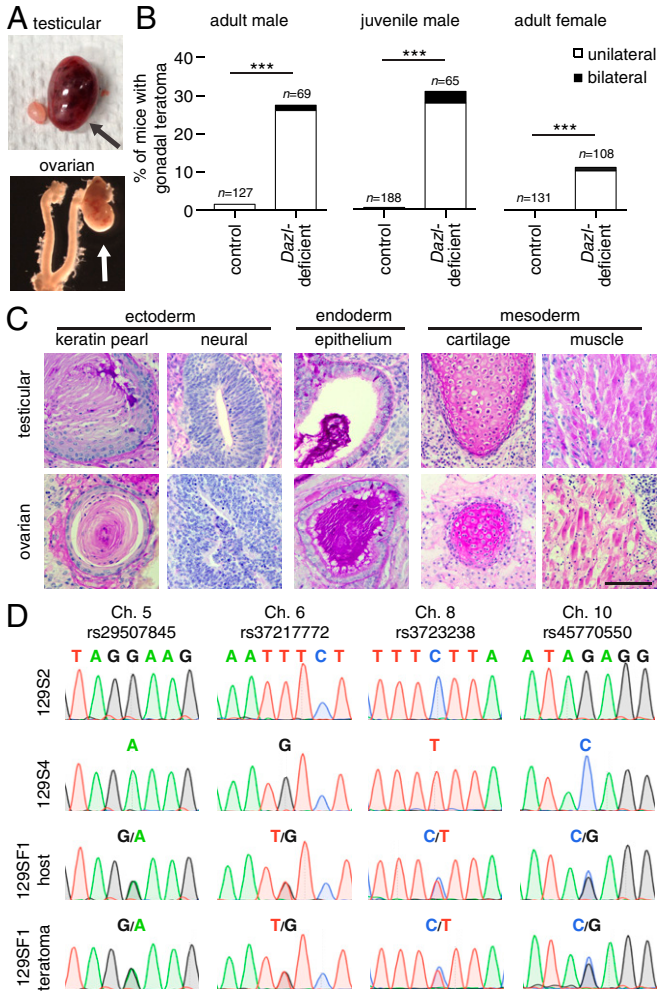


**Fig. 3.** Spontaneous gonadal teratomas occur in *Dazl*-deficient mice, and arise from mitotic cells. (*A*) Appearance of unilateral gonadal teratomas (arrows) in 129S4.*Dazl*-deficient mice. (*B*) Incidence of gonadal teratomas in mice. Males were dissected by 4 mo (adult male) or at 4 wk of age (juvenile male), and adult females were dissected at 2 mo of age; $n$ = number of animals examined, ***$P$ value $\leq 0.0001$ using Fisher's exact test. (*C*) Representative histology of teratomas from testis (*Upper*) and ovary (*Lower*) stained with periodic acid-Schiff reagent (PAS). (Scale bar, 100 μm.) (*D*) Representative Sanger sequencing at 4 SNP loci from 129S2 and 129S4 mice (*Upper*), and from 129SF1 host and teratoma (*Lower*). Teratomas are heterozygous at each SNP locus.

cells. In mice, there are no published reports of ovarian teratomas arising from mitotic cells; ovarian teratomas have been observed in LTXBJ mice, but these arise from meiotic germ cells, via parthenogenesis (36). To ascertain the cellular origin of teratomas in *Dazl*-deficient mice, we tested tumors for loss of heterozygosity (LOH)—a hallmark of cells that have completed meiosis I, and of parthenogenesis. To assay LOH, we first crossed the 129S4.*Dazl* mice with a genetically distinguishable substrain—129S2/SvPasCrl (129S2)—in which *Dazl* deficiency produces teratomas (*SI Appendix*, Fig. S6*A*). Importantly, the 129S4 and 129S2 substrains differ at 11 single-nucleotide polymorphisms (SNPs), each on a different chromosome (Dataset S4 and *SI Appendix*, Table S2; ref. 37). By intercrossing these mice, we generated offspring that are heterozygous at each SNP. We genotyped the resultant gonadal teratomas, along with normal host tissue, and detected no LOH in 10 ovarian and 10 testicular teratomas (Fig. 3*D* and *SI Appendix*, Fig. S6*B*), implying that these teratomas arose from mitotic cells.

Collectively, these studies demonstrate that *Dazl* is necessary for the commitment of germ cells, in the gonads, from their uncommitted precursors, independent of sex. *Dazl*-deficient gonadal teratomas do not reflect the parthenogenetic reactivation of pluripotency in meiotic cells, but instead arise from mitotic germline cells that have retained broad developmental potential.

Are other germ cell-defining factors also required for this commitment? Like *Dazl*, *Ddx4* and *Gcna* are germ plasm constituents, first expressed after PGC colonization of the gonads in humans and mice (Fig. 1 and refs. 38 and 39). To determine whether *Ddx4* or *Gcna* is similarly necessary for germ cell commitment, we crossed null alleles for *Ddx4* [*Ddx4-Cre* (40)] and *Gcna* (38) to a 129S4 background and measured teratoma incidence. We found no gonadal teratomas in 129S4.*Ddx4*-deficient mice, and a single tumor in a 129S4.*Gcna*-deficient male (*Ddx4*: $n = 25$ males, 23 females; *Gcna*: $n = 50$ males; *SI Appendix*, Fig. S6 *C* and *D*), indicating that, while necessary for the completion of spermatogenesis (38, 41), both *Ddx4* and *Gcna* are dispensable for germ cell commitment.

**Sex Reversal Shows That the Testis Is a Favorable Site for Teratoma Formation.** Despite the common developmental origin of teratomas in *Dazl*-deficient males and females, their incidence is higher in males (Fig. 3*B*). To test whether this male bias reflected an intrinsic difference in the developmental potency of XY germline cells, or the testicular environment to which XY PGCs migrate, we reversed the gonadal sex of both XX and XY animals. In mammals, gonadal sex is determined by expression of a Y-linked gene, *Sry*. An *Sry* transgene (*TgSry*) can induce male development in XX embryos (42), and disruption of *Sry* results in female development of XY embryos.

We generated sex-reversed *Dazl*-deficient mice and assessed teratoma incidence. Teratomas were observed much less frequently in *Dazl*-deficient 129S4 females (either XX or XY) than in *Dazl*-deficient XY males (Fig. 4*A*). Strikingly, we observed testicular teratomas in all XX male mice (XX *TgSry*, $n = 34$ animals, 33 with bilateral teratomas). Thus, the mouse testis is a more favorable environment than the ovary for teratomas to arise from either the XX or XY *Dazl*-deficient germline. We conclude that XX germline cells are as susceptible to teratoma formation as their XY counterparts, if not more so, given an equivalent gonadal environment. Our findings establish that, in the absence of *Dazl*, both XX and XY germline cells can form spontaneous teratomas, overturning the view that a Y-linked gene is essential for teratoma formation in male mice (43).

**Ablating *Bax*-Mediated Cell Death Increases Teratoma Formation in *Dazl*-Deficient Male Mice.** Our findings led us to hypothesize that many (or most) gonadal germline cells either die or form spontaneous teratomas in the absence of *Dazl* function. Indeed, these two fates—cell death or teratoma—might represent alternative outcomes, in vivo, of germline cells whose developmental potential has not been restricted. If this were the case, then curtailing cell death pathways in *Dazl*-deficient mice should increase the incidence of spontaneous gonadal teratomas. To attenuate apoptotic cell death, we intercrossed mice carrying the *Dazl* null allele with mice carrying a *Bax* null allele (44, 45). Among *Dazl*-deficient;*Bax*-heterozygous males, we observed a dramatically increased incidence of teratomas (68%; including 23 with bilateral teratomas, from 50 mice; Fig. 4*B* and *SI Appendix*, Fig. S6*E*) compared with *Dazl*-deficient;*Bax* wild-type male littermates (42%; including one with bilateral teratomas, from 26 males). Strikingly, we observed bilateral teratomas in all 17 double-knockout males examined (compared with none in 51 *Bax*-deficient males, where at least one copy of *Dazl* remained intact). We conclude that, in the testes of 129S4.*Dazl*-deficient males, the failure to restrict the developmental potential of the germline usually leads to *Bax*-mediated cell death. By genetically

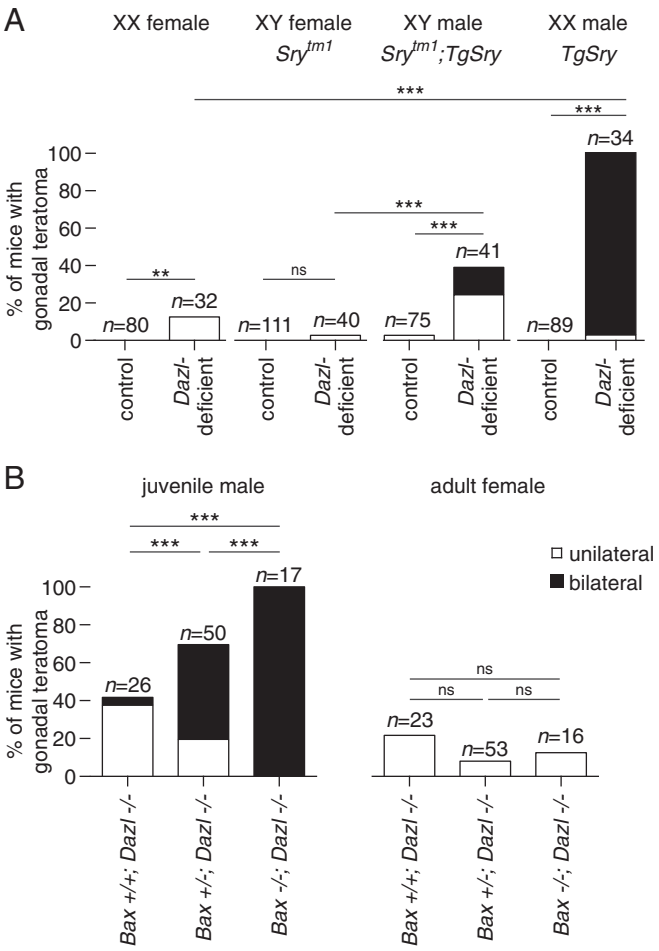


**Fig. 4.** Teratoma formation in *Dazl*-deficient mice is affected by sex reversal, and by ablation of *Bax*-mediated cell death. (*A*) Incidence of gonadal teratomas in sex-reversed mice. Mutation of *Sry* (*Sry*$^{tm1}$) causes XY embryos to develop as anatomic females. Expression of *Sry* transgene (*TgSry*) causes XX embryos to develop as anatomic males. (*B*) Incidence of testicular teratomas (*Left*) and ovarian teratomas (*Right*) in *Dazl*-deficient mice (–/–) who were either homozygous wild-type (+/+), heterozygous (+/–), or deficient (–/–) for *Bax*; *n* = number of animals examined, ***P* value < 0.01, *** <0.0001, ns = not significant using Fisher's exact test.

attenuating or eliminating *Bax*-mediated cell death, the *Dazl*-deficient germline's broad developmental potential—and, thereby, its capacity for teratoma formation—is revealed more fully in vivo. In females, however, we observed no significant effect of *Bax* deficiency on the incidence of ovarian teratomas in *Dazl*-deficient mice (Fig. 4*B* and *SI Appendix*, Fig. S6*F*). This finding parallels our observations in sex-reversed mice, demonstrating again that the sexual identity of the somatic gonad strongly influences the likelihood of teratoma formation (Fig. 4*A*).

***DAZL*-Deficient Pigs Develop Spontaneous Teratomas.** Given that *Dazl* is necessary for germ cell commitment in mice, we assessed whether this holds true in other mammals. We tested this in pigs, an outgroup to primates and rodents (Fig. 5*A*). Using a transcription activator-like effector nucleases (TALEN)-mediated gene editing strategy, we generated female pigs with targeted disruptions of *DAZL* (*SI Appendix*, Table S3). We examined the ovaries of 20 *DAZL*-deficient females that were at least 6 mo old: 13 had large ovarian tumors, and 3 of the 13 had bilateral tumors (Fig. 5 *B* and *C*). Histological examination revealed that these tumors were teratomas, containing disorganized mixtures of tissues derived from all three germ layers (Fig. 5*D*), similar to our findings in *Dazl*-deficient mice. We did not observe evidence of ovarian teratomas in 151 female controls of similar age [nor did we observe testicular teratomas in any of three previously generated (46) *DAZL*-deficient male pigs analyzed at 11 wk or 9 mo of age].

To assay whether *DAZL* is necessary for survival of germline cells in the testes of pigs, as it is in diverse strains of mice (*SI Appendix*, Fig. S5), we examined testes of *DAZL*-deficient pigs at 11 wk and at 9 mo of age. In all gonads analyzed, we found no evidence of germ cells in either ovaries or testes (Fig. 5 *D* and *E*). Consistent with this, immunohistological analysis revealed that all cells within the seminiferous epithelium expressed the Sertoli cell factor SOX9 (Fig. 5*F*). These data indicate that *DAZL* is required for the survival of germline cells in the swine testis.

Combined with our data in humans and mice, these observations in pigs demonstrate, across eutherian mammals, that *DAZL* is necessary to restrict the developmental potential of PGCs after their arrival at the gonads.

**Embryonic *Dazl* Expression Is Sufficient for Germline Survival and Oogenesis.** Finally, we assessed whether a brief period of *Dazl* expression is sufficient to initiate germ cell commitment, after which *Dazl* might be dispensable for gametogenesis in mice. We constructed a conditional *Dazl* allele on a B6 background (referred to as B6.*Dazl-2L*), and then generated a null allele (*Dazl-1L*) using a germline-specific Cre recombinase (40) (*Ddx4-Cre*; *SI Appendix*, Fig. S7 *A* and *B*). Next, we temporally ablated *Dazl* from ~E14.5 onward, using *Ddx4-Cre* (Fig. 6*A*). This ablation occurred after PGCs extinguished *Nanog:GFP* and their potential to give rise to EG cells (Fig. 2 *A* and *C*), and after sexual differentiation had commenced in both sexes and meiosis had been initiated in females. In conditional knockout mice (B6.*Dazl-1L/2L*;*Cre*, referred to as *Dazl* cKO), we observed germ cells in ovaries and testes at all ages tested, through 8 mo of age (Fig. 6 *B–D*). In *Dazl* cKO ovaries, we confirmed DAZL's absence in germ cells at birth (marked by the *LSL-tdTomato* reporter, recombined in germ cells by *Ddx4-Cre*; Fig. 6*E*). In *Dazl* cKO testes, we confirmed the loss of DAZL expression in spermatogonia (marked by the Oct4:EGFP transgene and the germ cell marker GCNA; some tubules retained DAZL expression and exhibited variable spermatogenesis). These data suggest that, once germ cell commitment has occurred, *Dazl* is no longer necessary for germ cell viability in mice on a B6 background (*SI Appendix*, *SI Discussion*).

To test whether *Dazl* is dispensable during the remainder of gametogenesis, we assessed the fertility of *Dazl* cKO mice. *Dazl* cKO females were fertile through at least 8 mo of age ($n = 5$ females, with the recombined allele transmitted to all progeny; Fig. 6*F*). In contrast, *Dazl* cKO males were sterile, with no spermatozoa observed in the epididymis of adults ($n = 5$ males; Fig. 6*G*). Thus, *Dazl* has additional functions in postnatal spermatogenesis, consistent with previous descriptions on a mixed genetic background (47–49).

We conclude that a brief period of *Dazl* expression, after PGC colonization of the gonads, is sufficient for germline commitment and the initiation of gametogenesis in mice, as shown by the survival of germ cells in the gonads of both sexes of *Dazl* cKO mice, and by the completion of oogenesis and fertility in females.

## Discussion

Our studies sought to answer a fundamental question: When, where, and how does the mammalian germline restrict its developmental potential and irreversibly commit to gametogenesis? One view has been that mammalian PGCs are unipotent germ cells—only capable of giving rise to gametes (50–54). This view is challenged by several lines of evidence that reveal the broad developmental potential of migratory PGCs, including the expression of a network of pluripotency factors, the ability to

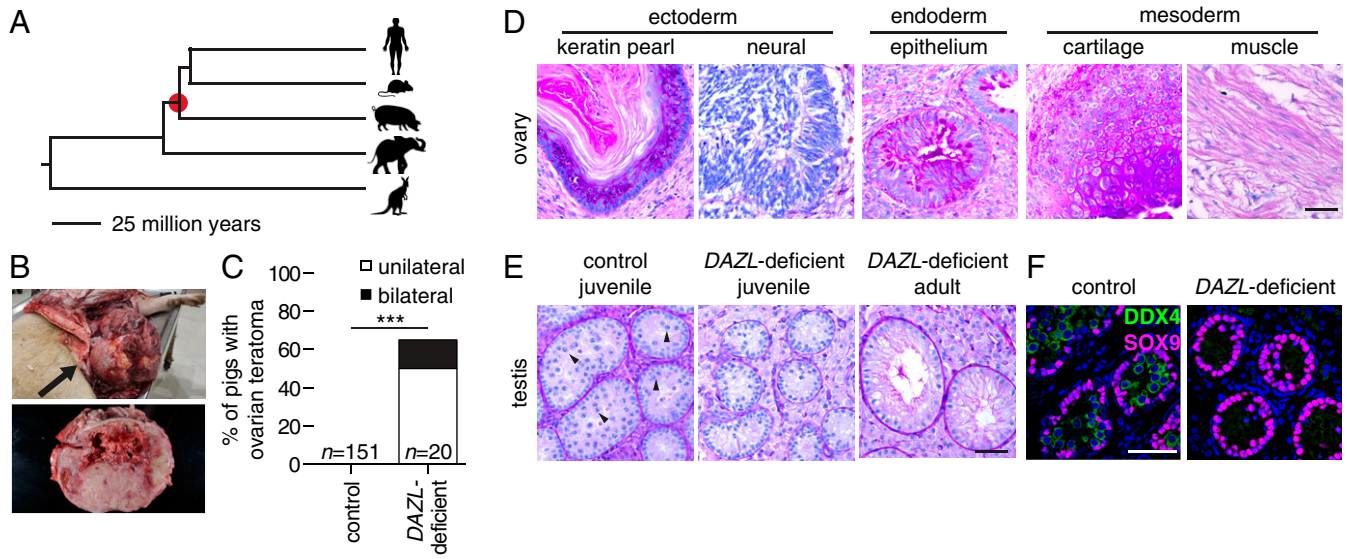


**Fig. 5.** Spontaneous ovarian teratomas in *DAZL*-deficient pigs. (*A*) The pig is an outgroup to rodents and primates, sharing a common ancestor 95 million years ago (red dot). (*B*) Ovarian teratoma (arrow) in *DAZL*-deficient pig. The tumor measured 46 × 23 × 28 cm and weighed 15.4 kg. (*C*) Incidence of ovarian teratomas in control and *DAZL*-deficient pigs. ***$P$ value < 0.0001 using Fisher's exact test. (*D*) Representative histology of teratoma from ovary, stained with PAS. (*E*) Histology of control (*Left*, 18 wk, spermatocytes marked with arrow head) and *DAZL*-deficient testis at 11 wk (*Center*) and 9 mo of age (*Right*), stained with PAS. (*F*) Immunofluorescence of control (*Left*, 18 *wk*) and *DAZL*-deficient pig testis (*Right*, 11 wk). Germ cells were stained with DDX4 (green), and somatic cells were stained with SOX9 (magenta). DNA was stained with DAPI (blue). (Scale bars, 50 μm.)

produce pluripotent cell lines in culture, the occurrence of spontaneous gonadal teratomas, and, most recently, evidence that presumptive PGCs may contribute to the allantois (12, 13). Comparable observations in fish (6), frogs (5), and salamanders (7) corroborate the view that, among vertebrates, migratory-stage PGCs are not yet irreversibly committed, but instead retain the capacity for somatic differentiation, regardless of whether germline segregation occurs via induction, or by germ plasm.

Our present studies in mammals provide definitive genetic evidence that the germline's broad developmental potential is restricted after PGC colonization of the gonads, and that *Dazl* is necessary for this to occur. We find that, in mouse embryos lacking *Dazl* function, PGCs migrate to the gonads but maintain expression of a network of pluripotency factors, and retain the ability to give rise to pluripotent cell lines until at least E15.5 in both sexes (Fig. 2 and *SI Appendix*, Fig. S4). We further corroborated this by following the fate of the *Dazl*-deficient germline in adult 129S mice, which formed gonadal teratomas at a remarkable frequency. We found testicular teratomas in 87 of 324 *Dazl*-deficient males, compared to 6 of 747 control mice (*SI Appendix*, Table S4). The incidence was even higher in certain circumstances. For example, 33 of 34 *Dazl*-deficient, sex-reversed (XX) males developed bilateral gonadal teratomas, as did 17 of 17 *Dazl*-deficient;*Bax*-deficient XY males (Fig. 4). Strikingly, we also discovered ovarian teratomas in 35 of 300 *Dazl*-deficient females, compared to none in 426 control mice, demonstrating that *Dazl* is necessary for germ cell commitment in both sexes.

Taken together with published reports, our findings suggest a sequence of commitment steps during mammalian germline development. In developmental biology, a cell is "specified" when it will develop autonomously after isolation from the embryo; specified cells are not yet irreversibly committed, and may adopt other fates if transplanted to a new position (55). For example, the fate of specified trophectoderm or of the inner cell mass may be altered upon relocation within the preimplantation embryo (56). Similarly, mammalian PGCs appear to be specified shortly after their induction (1, 2), without being irreversibly committed to gametogenesis.

In contrast, a cell is "determined" (fully committed) when its fate cannot be reversed by grafting (55). By these definitions, cells are determined when their potential is restricted, regardless of environment. It is informative here to revisit the work of Leroy Stevens, who discovered that PGCs can give rise to teratomas in mice (35, 57). [Likewise, migratory-stage PGCs from amphibians can give rise to somatic cell lineages if transplanted (5) or in cell culture (7).] Stevens demonstrated, by grafting PGCs, that they lose the capacity to form teratomas after colonizing the gonads (8, 58). Combining Stevens' observations with our own, we conclude that germ cells are determined in mice after PGCs colonize the gonads, and that the induction of *Dazl* is necessary for this commitment (Fig. 7). Utilizing a conditional allele, we show that a brief period of *Dazl* expression is sufficient for this commitment to gametogenesis.

This model is further corroborated by our findings in *DAZL*-deficient pigs, where the germline similarly retains the capacity for teratoma formation after gonadal colonization. These findings—in two species whose most recent common ancestor lived about 95 million years ago—strongly suggest that *DAZL*-dependent commitment, occurring after gonadal colonization, operated in the last common ancestor of all eutherian mammals. Moreover, in fish (22, 23), frogs (24, 25), and birds (26), orthologs of *DAZL* are essential constituents of germ plasm, which functions in segregating germline from soma. Thus, *DAZL* is a key factor in germline commitment, regardless of whether germline segregation occurs by induction or germ plasm.

**Distinguishing Gonadal Germ Cells from Their Migratory Germline Precursors.** If the germline's broad developmental potential is extinguished only after PGC colonization of the gonads, then the roles of key regulators expressed in PGCs prior to gonadal entry must also be considered. Here we will incorporate findings from both mammals and nonmammalian vertebrates to suggest that these critical regulators of migratory PGCs repress the cells' capacity for somatic differentiation, insulating them from inductive cues.

Consider *Nanos* and *Dnd1*, which are expressed in migratory PGCs of many vertebrates and encode repressors of cellular differentiation. When *dnd1* is knocked down in fish, PGCs adopt

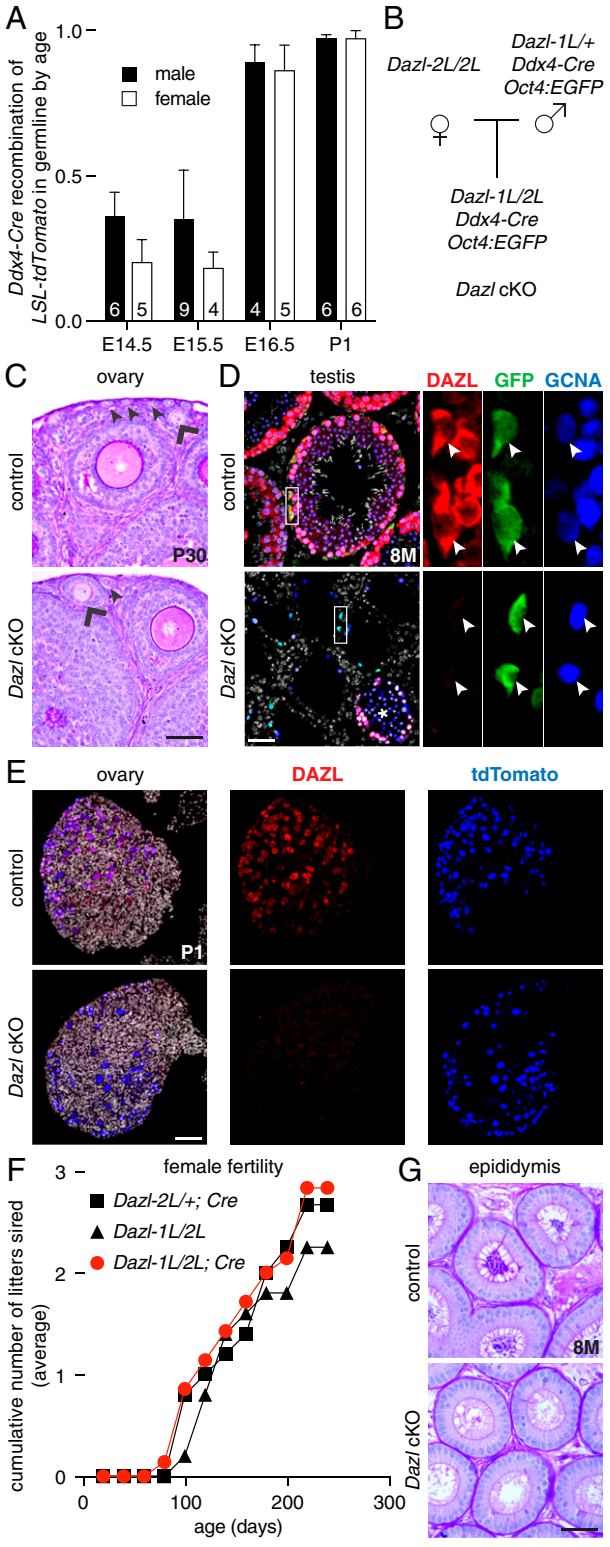


**Fig. 6.** A brief period of *Dazl* expression is sufficient for germ cell commitment, and for completion of oogenesis. (*A*) Time course of *Ddx4-Cre* (*Mvh*$^{Cre\text{-}mOrange}$) recombinase activity in embryonic germline using a fluorescent Cre reporter mouse line, *LSL-tdTomato*. Cre-mediated recombination (resulting in tdTomato expression) in Oct4:EGFP-positive cells is assayed by flow cytometry. Numbers of embryos tested are listed in each column, mean + SD. (*B*) Breeding scheme for *Dazl* conditional knockout (*Dazl* cKO) mice. (*C*) Histology of control (*Upper*) and B6.*Dazl* cKO ovary (*Lower*) stained with PAS at 20 d of age, with primary (arrow head) and secondary (chevron) follicles marked. (*D*) Immunofluorescence of control (*Upper*) and B6.*Dazl* cKO testis (*Lower*) at 8 mo of age. Germ cells are immunostained by GCNA (blue), and undifferentiated spermatogonia are immunostained by GFP (green) expressed from the *Oct4:EGFP* reporter. Cre recombination is confirmed by immunostaining for DAZL (red). DNA is stained with DAPI (gray). *Insets* show each marker in Oct4:EGFP-positive spermatogonia (arrow head); * denotes tubule with incomplete *Dazl* recombination in cKO testis. (*E*) Immunofluorescence of control (*Upper*) and B6.*Dazl* cKO ovary (*Lower*) at postnatal day 1. Germ cells are immunostained by tdTomato protein (expressed following recombination of *tdTomato-LSL* allele by *Ddx4-Cre*; blue). Cre recombination is confirmed by immunostaining for DAZL (red). DNA is stained with DAPI (gray). (*F*) B6.*Dazl* cKO females remained fertile for at least 8 mo. (*G*) B6.*Dazl* cKO males were sterile, with no spermatozoa in epididymal ducts. All data are mean + SD. (Scale bars, 50 μm.)

somatic cell fates in response to morphogenic signals that they would normally resist (6). Similarly, in frogs, *nanos1*-deficient PGCs migrating through the endoderm inappropriately activate a set of endoderm-defining factors (59). When either of these factors, *Dnd1* (60, 61) or *Nanos3* (62), is deleted in rodents, PGCs give rise to teratomas at high frequency. These comparative observations support the view that both *Nanos* and *Dnd1* repress the expression of somatic factors, thereby insulating PGCs from inductive cues encountered during migration.

Similarly, nascent mammalian PGCs express a repertoire of factors (including *Prdm1* [*Blimp1*] and *Tfap2c*, and also *SOX17* in primates and pigs) that serve to repress cell differentiation in other contexts (63–66). These factors have also been observed to repress somatic gene expression during the derivation and culture of human and mouse PGC-like cells (67–70). Taken together, these observations suggest that PGC-expressed regulators function to insulate the migratory germline from ectopic gene expression, thereby preventing uncommitted PGCs from adopting somatic cell fates. Expression of these factors, however, is insufficient to irreversibly restrict germline potential; PGCs express a network of pluripotency factors, and will produce teratomas when transplanted (8). By contrast, once mammalian PGCs colonize the gonads, a definitive program of germ cells is induced—a program marked by the expression of deeply conserved, germ cell-exclusive factors (and a reciprocal down-regulation of pluripotency factors), and, functionally, by extinguishing the capacity for pluripotent cell line derivation and teratoma formation (Fig. 7).

Unlike other genes whose ablation yields teratomas, *Dazl* is expressed only in the germline (71, 72), and only after PGC colonization of the gonad (60, 61). Thus, *Dazl* deficiency provides unambiguous evidence of germ cell commitment occurring on PGC arrival at the nascent gonad.

That mechanisms exist to insulate PGCs from somatic inductive cues could also help explain why mouse PGCs do not contribute to somatic lineages when injected into blastocysts (73). PGC-expressed factors may prevent these cells from contributing to chimerism upon injection into blastocysts, without necessarily being instructive for germ cell determination. Thus, PGCs have been said to exhibit a "latent" pluripotency (74) whose unmasking results in teratomas and EG cell lines. The failure of mouse PGCs to contribute to chimerism could also reflect a developmental incompatibility between mouse PGCs and the environment of the blastocyst, as observed elsewhere when donor cells are not developmentally matched to their host (75). In any case, the negative results in mice contrast with a study in pigs, where freshly isolated PGCs, following injection into blastocysts, were reported to contribute to somatic lineages, as assayed by transgene expression (76).

**Implications for Gametogenesis In Vitro.** Since our model states that migratory PGCs remain uncommitted, in vitro gametogenesis from these immature cells should not be possible. In seeming contradiction, complete gametogenesis from mouse ES and induced pluripotent stem cells has been reported; however, this

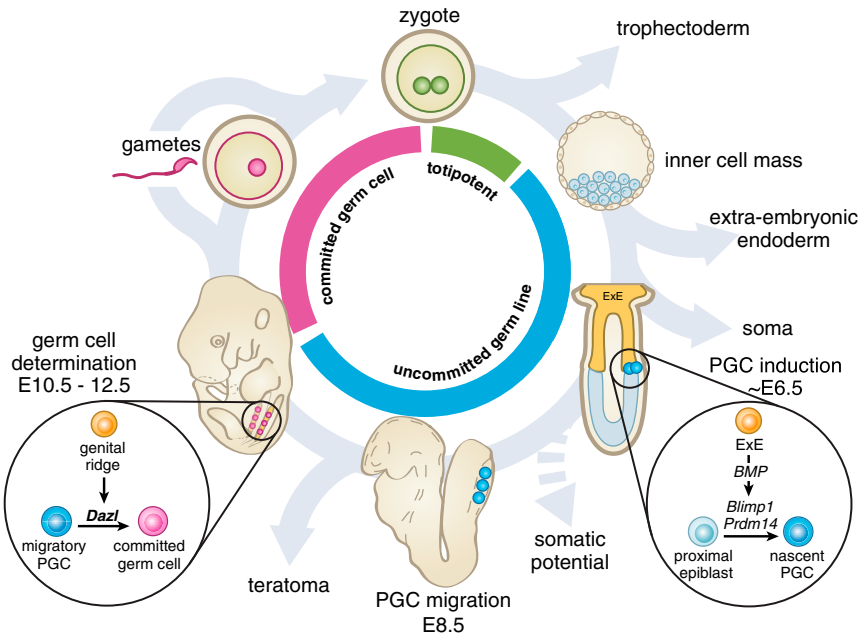


**Fig. 7.** A proposed model for germ cell commitment in mammals. The germline comprises all cells whose descendants include gametes. At fertilization, the totipotent zygote has the capacity to give rise to all cell lineages. As development proceeds, extraembryonic and somatic lineages differentiate away from the germline. PGCs (blue cells) are induced from the epiblast, preserving the germline, but also maintaining a broad developmental potential. At PGC colonization of the gonads, expression of a germ cell program—marked by *DAZL*—is induced in germline cells by the genital ridge. Our studies show that *DAZL* is necessary for the restriction of developmental potential in the germline, resulting in the determination of germ cells (pink cells). Determined germ cells then undertake gametogenesis and must cycle through fertilization to reestablish totipotency and continue the germline cycle in a new diploid individual. ExE, extraembryonic ectoderm.

occurred only after PGC-like cells were combined with somatic gonadal tissue, which led to *Dazl* expression (77, 78). We suggest that this soma-directed induction of *Dazl*, together with DNA demethylation to facilitate *Dazl* induction (79, 80), likely accounts for the ability of PGC-like cells to differentiate into functional gametes. Identifying the gonad-derived factors that induce *Dazl* expression in vivo will help investigators to recapitulate the entirety of gametogenesis in vitro (81, 82).

**Implications for the Pathogenesis of Germ Cell Tumors.** Our insights into germ cell commitment in the embryo have ramifications for our understanding of germ cell tumors (GCTs), the most common cancer in young men (83). Specifically, our present studies in embryos converge in striking fashion with recent epidemiological and genome-wide association (GWA) studies of GCTs. For example, GWA studies implicate pluripotency factors (e.g., *PRDM14*, *SALL4*, *TFCP2L1*, and *ZFP42*), as well as genomic sites of binding for transcription factors of pluripotency (e.g., *KLF4*, *NANOG*, *POU5F1*, and *SOX2*), in the pathogenesis of testis cancer (84, 85). Most importantly, *DAZL* has been identified as a susceptibility locus, implicating *DAZL* as a key factor in both the pathogenesis of GCTs in humans (86) and germ cell commitment. Consistent with a critical role for apoptosis, GWAS has implicated *BAK1* in the heritability of GCTs (87), akin to our observations in *Bax*-deficient mice. Along similar lines, the receptor:ligand pair *KIT* and *KIT ligand* (*KITLG*) function in several cellular contexts to protect cells from apoptosis (88). Like *BAK1*, polymorphism at *KITLG* is implicated in the heritability of human GCTs, and teratoma incidence in mice (87, 89, 90). Human GWA studies also implicate *GATA4* (84), which is required in mice for gonad development and induction of *Dazl* (81). Reinforcing the view that somatic gonadal development is central to germ cell determination, young children with disorders of gonadal development are at markedly increased risk of germline neoplasia (91). These many connections lead us to suggest that germline neoplasms arise from embryonic cells that, having failed to complete germ cell determination on their arrival at the gonad, remain uncommitted and susceptible to tumor formation. Accordingly, our revised understanding of germ cell commitment will help clarify the developmental origin of GCTs, and inform efforts to account for their dramatically increased incidence in recent decades (92).

In conclusion, we demonstrate that germ cell determination in mammals occurs late in embryonic development—after the body plan has been established, and organogenesis begun—through an ancient germ cell program induced as PGCs colonize the nascent gonads (Fig. 7). This model has deep implications for the genesis of germline neoplasms in humans, and for the stepwise commitment and determination of germ cells in mammals and across the vertebrata.

## Materials and Methods

Further details can be found in *SI Appendix, SI Materials and Methods*.

**Animals.** All experiments involving mice or pigs conformed to principles and guidelines approved by the Committee on Animal Care at the Massachusetts Institute of Technology, Cincinnati Children's Hospital Medical Center, or by International Center for Biotechnology, respectively. Further details can be found in *SI Appendix, SI Materials and Methods*.

**Cell Isolation.** Embryos carrying fluorescent reporter alleles were dissected, and gonadal cells were subjected to flow cytometry, followed by pluripotent cell line derivation, or to RNA isolation for transcriptional analysis, as described in *SI Appendix, SI Materials and Methods*.

**Histology.** Gonads were removed and fixed in 4% paraformaldehyde, or Bouins' solution, embedded in paraffin, sectioned, and stained for immunohistology, or stained with hematoxylin and periodic acid-Schiff. Teratoma formation was confirmed by the presence of cells from each somatic germ layer. Full details are available in *SI Appendix, SI Materials and Methods*.

**Transcriptional Analyses.** Sequence data were aligned to the appropriate reference genome, and differential expression was calculated as outlined in *SI Appendix, SI Materials and Methods*.

**Data Availability.** Data generated by array and RNA-seq have been deposited at Gene Expression Omnibus (accession no. GSE87771) and Sequence Read Archive (accession no. PRJNA434733), respectively.

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