**ABSTRACT**

Fertility and gamete reserves are maintained by asymmetric divisions of the germline stem cells to produce new stem cells or daughters that differentiate as gametes. Before entering meiosis, differentiating germ cells (GCs) of sexual animals typically undergo cystogenesis. This evolutionarily conserved process involves synchronous and incomplete mitotic divisions of a GC daughter (cystoblast) to generate sister cells connected by intercellular bridges that facilitate the exchange of materials to support rapid expansion of the gamete progenitor population. Here, we investigated cystogenesis in zebrafish and found that early GCs are connected by ring canals, and show that Deleted in azoospermia-like (Dazl), a conserved vertebrate RNA-binding protein (Rbp), is a regulator of this process. Analysis of dazl mutants revealed the essential role of Dazl in regulating incomplete cytokinesis, germline cyst formation and germline stem cell specification before the meiotic transition. Accordingly, dazl mutant GCs form defective ring canals, and ultimately remain as individual cells that fail to differentiate as meioocytes. In addition to promoting cystoblast divisions and meiotic differentiation programs and migrate to the site in which the gonad differentiates, these cells can be regarded as germ line stem cell (GSC) progenitors. Here, we investigated cystogenesis in zebrafish and found that early GCs are connected by ring canals, and show that Deleted in azoospermia-like (Dazl), a conserved vertebrate RNA-binding protein (Rbp), is a regulator of this process. Analysis of dazl mutants revealed the essential role of Dazl in regulating incomplete cytokinesis, germline cyst formation and germline stem cell specification before the meiotic transition. Accordingly, dazl mutant GCs form defective ring canals, and ultimately remain as individual cells that fail to differentiate as meioocytes. In addition to promoting cystoblast divisions and meiotic entry, dazl is required for germline stem cell establishment and fertility.

**KEY WORDS:** Dazl, Germline cyst, Fertility, Incomplete cytokinesis, Germline stem cells, Ring canals

**INTRODUCTION**

In many organisms, the germline is among the first cell types to be set aside (Ginsburg, 1994; Illmensee and Mahowald, 1976; Illmensee et al., 1976; Wolf et al., 1983). Early germ cells, called primordial germ cells (PGCs), are specified by maternal factors or by inductive signals (Farrell et al., 2018; Lawson et al., 1999; Lawson and Hage, 1994). Once specified, PGCs ignore somatic differentiation programs and migrate to the site in which the gonad forms (Braut et al., 1999; Gross-Thebing et al., 2017; Exhaust and Akam, 2003; Marlow, 2015; Nieuwkoop and Sutasurya, 1979; Strome and Updike, 2015). There, the PGCs proliferate, enter meiosis and differentiate to produce the gametes: sperm in males and oocytes in females. Although the earliest stages of PGC development in zebrafish have been studied (Barton et al., 2016; Marlow, 2015; Paksa and Raz, 2015; Raz, 2003), the cellular and molecular mechanisms mediating the transition from PGC to germline stem cell (GSC) specification are less well understood.

**Germline stem cells**

Nanos, a conserved marker of GSCs in zebrafish, medaka and mice (Nakamura et al., 2010; Sada et al., 2009; Aoki et al., 2009; Beer and Draper, 2013; Cao et al., 2019), is required for the maintenance of GSCs in vertebrates and invertebrates (Forbes and Lehmann, 1998; Sada et al., 2009). The mechanisms that establish the GSCs are unknown, but blocking Wnt diminishes nanos2 and impedes ovary regeneration (Cao et al., 2019). In mouse testis and zebrafish germ cells (GCs), nanos2 is required for the maintenance of GSCs (Sada et al., 2009; Cao et al., 2019). In zebrafish, the related nanos3 contributes to the maintenance of GSCs and may act redundantly to nanos2 in females but not males (Beer and Draper, 2013; Draper et al., 2007). Although zebrafish nanos2 is expressed in a subset of premeiotic gonocytes, neither it nor nanos3 are required for GSC specification (Beer and Draper, 2013; Cao et al., 2019). Instead, Nanos family members in zebrafish may prevent premature differentiation of GSCs, in part by translational repression of meiosis factors like their counterparts in flies (Wang and Lin, 2004) and mice (Barrios et al., 2010; Suzuki et al., 2010). Thus, the cellular and molecular mechanisms that establish zebrafish GSCs have yet to be discovered.

**Germline cyst formation**

A common and evolutionarily conserved feature of GCs is the asymmetric division of GSCs to produce a stem cell and a premeiotic daughter. These differentiating divisions have been classified as type I or type II divisions (Saito et al., 2007; Saito and Tanaka, 2009). Cells resulting from type-I divisions directly differentiate as meiotic cells and are observed in juvenile and adult teleosts (Marlow and Mullins, 2008; Saito et al., 2007). Type II divisions generate cystoblast cells that divide mitotically with incomplete cytokinesis to generate interconnected sisters in Drosophila (Cox and Spradling, 2003), Xenopus (Kloc et al., 2004) and medaka (Saito et al., 2007). The interconnections generated from incomplete cytokinesis are known as ring canals, or ring channels (Brown and King, 1964; de Cuevas et al., 1997; Fawcett et al., 1959; Greenbaum et al., 2011; Haglund et al., 2011; Koch and King, 1966, 1969; Koch et al., 1967; Lei and Spradling, 2016; Lin and Spradling, 1993; Mahowald, 1971; Marlow and Mullins, 2008; Pepling and Spradling, 1998; Robinson and Cooley, 1996; Seidel et al., 2018; Spradling, 1993; Wolke et al., 2007). In Drosophila, ring canal formation involves the regulation of actin to maintain midbody structures (Tilney et al., 1996). During normal
cell division, sister cells are separated by the cytokinetic furrow which severs the midbody, a transient connection between cells. For incomplete cytokinesis, the midbody actomyosin meshwork is stabilized to block abscission and maintain the contractile ring such that ring canals form between connected cells (Greenbaum et al., 2011; Haglund et al., 2011; Hime et al., 1996; Robinson and Cooley, 1996). In mice, the inactive serine-threonine kinases TEx14 and CEP55 regulate intercellular bridge stability in part by blocking abscission factors (Greenbaum et al., 2011, 2006; Kim et al., 2015; Morita et al., 2007). Intercellular bridge and germline cyst formation is a conserved feature of germ cell biology and is crucial for fertility (e.g. Greenbaum et al., 2006). Intercellular bridges of GCs could fulfill several functions, including facilitating intercellular communication to yield synchronous cells (Lei and Spradling, 2013; Pepling and Spradling, 1998), coordinating crucial stages, such as meiotic entry (Stanley et al., 1972), maintaining gamete equivalence and potentially acting to detect and remove abnormal cells (Braun et al., 1989; LeGrande, 2001).

**Dazl and fertility**

The RNA-binding protein (Rbp) Deleted in azoospermia-like (Dazl) is a member of the Deleted in azoospermia (Daz) family, which is composed of Daz, Daz-like (Dazl) and BOULE (Fu et al., 2015). Daz family members are germline specific and contribute to various aspects of GC development in invertebrates and vertebrates (Fu et al., 2015). Although dazl is required for GC development, loss of daz family members disrupts distinct aspects of GC development in different species (Alphey et al., 1992; Courtot et al., 1992; Eberhart et al., 1996; Fukuda et al., 2018; Gill et al., 2011; Iyer et al., 2016; Karashima et al., 2000; Maines and Wasserman, 1992; Ruggiu et al., 2007; Saunders et al., 2003; Schrans-Stassen et al., 2001). Depletion of Xenopus dazl disrupts PGC migration and causes PGC deficiency (Houston and King, 2000). In mammals, Dazl also regulates meiotic RNAs (Haston et al., 2009; Kim et al., 2012; Li et al., 2019; Medrano et al., 2012; Zagore et al., 2018) and commitment to germline fate (Nicholls et al., 2019). These studies identify diverse and opposing activities for Dazl, including regulation of RNA stability, acting both as translational activator and repressor of its target RNAs in different contexts (Chen et al., 2014; Li et al., 2019; Mikedis et al., 2020; Yang et al., 2020; Zagore et al., 2018). In zebrafish PGCs, Dazl overexpression antagonizes miR-430 and promotes polyadenylation of maternally provided germline RNAs (Maegawa et al., 2002; Takeda et al., 2009); however, the role of Dazl in the zebrafish germ line remains unknown.

We examined the earliest stages of gonadogenesis and provide evidence that Dazl is required for germline cyst formation and is crucial for GC amplification establishment of GSCs and fertility. We describe zebrafish cystogenesis: the process by which PGCs transition from spatially separated individual cells to closely positioned GC clusters that undergo complex cytoplasmic and nuclear morphological changes to form germline cysts. Additionally, we show that GC numbers increase concomitantly with cyst formation. Analyzing F-actin distribution revealed that premeiotic cyst cells are interconnected by intercellular actin rings that resemble ring canals based on confocal and ultrastructural analyses, and contain aggregated or branched actin-rich structures reminiscent of spectrosomes and fusomes (Cooley and Theurkauf, 1994; Deng and Lin, 1997; Lighthouse et al., 2008; Lin et al., 1994; Robinson and Cooley, 1997; Tilney et al., 1996; Xue and Cooley, 1993; Yue and Spradling, 1992). Analysis of zinc finger nuclease (ZFN) and CRISPR-induced dazl mutant alleles revealed that zygotic dazl is essential for incomplete cytokinesis and germline cyst formation. In contrast to wild type, dazl mutant GCs form defective ring structures, and ultimately remain as individual cells that fail to differentiate as GSCs or meiocytes. Our findings support a novel requirement for dazl in GCs for cystogenesis and GSC establishment, linking cyst formation to PGC specification and fertility in zebrafish.

**RESULTS**

**Germline cyst formation**

After arriving at the gonad, PGCs proliferate to form a bipotential gonad (Leu and Draper, 2010; Tong et al., 2010; Tzung et al., 2015; Wang et al., 2007). Sex determination is influenced by the timing of meiotic entry and oocyte abundance, such that more GCs promote female fate (Ye et al., 2019). Conversely, low numbers of GCs result in male development (Dai et al., 2015; Orban et al., 2009; Tzung et al., 2015). Yet, despite these correlations, little is known about GC specification in zebrafish. Type II divisions of GSCs are thought to be amplifying divisions and have been observed in juvenile and adult teleosts (medaka and in zebrafish) (Beer and Draper, 2013; Marlow and Mullins, 2008; Saito et al., 2007). In zebrafish, GCs proliferate between 5 and 14 days (Leerberg et al., 2017; Leu and Draper, 2010; Tong et al., 2010; Tzung et al., 2015; Wang et al., 2007); however, how cysts form is unknown.

To investigate cystogenesis, we labeled wild-type GCs with anti-Vasa antibody between 7 and 14 days (Fig. S1). At 7-10 days, Vasa+ cells were detected in wild type as individual GCs or clustered groups of individuals (Fig. S1A,B). At this stage, GC nuclei were condensed DNA, as revealed by DAPI, with no visible nucleolus, and the nuclear cytoplasm interface was highly folded with a high nucleus to cytoplasm ratio (Fig. S1A). Following clustering, GCs underwent a morphological transition; individual GCs became difficult to discern within a Vasa+ mass (Fig. S1C). Previous studies indicate that GC proliferation occurs during this stage (Leerberg et al., 2017); thus, these morphological changes may represent mitotic divisions (Tong et al., 2010). The simultaneous obscuring of individual cell boundaries within clusters suggests the phenomena is synchronous (Fig. S1D). As this phenomenon likely corresponds to the amplification of GC numbers preceding germline cyst emergence, hereafter, we refer to this process as cystogenesis. Subsequently, GCs within forming cysts adopted a compact round nucleus with a prominent actin ring structures (Fig. 1E-E″); Fig. S3). These changes were associated with the formation of ring canals between connected cells (Fig. 1F); however, it was unclear whether cyst cells were interconnected by cytoplasmic bridges like those observed in juvenile ovaries (Marlow and Mullins, 2008).

To examine cyst architecture, cyst size and to investigate whether cysts were interconnected by cytoplasmic bridges, we labeled GCs with Vasa antibody and F-actin with phalloidin to visualize cortical actin and the cytoskeleton. At 10 days, cysts of various stages were observed in wild-type gonads (Fig. 1A,B-B″; Fig. S2A-F″), including GCs in two-cell (Fig. 1A,C-C″; Fig. S2A-F″) and four-cell (Fig. 1A,D-D″) configurations. Cyst cells were connected by prominent actin ring structures (Fig. 1E-E″; Fig. S3). These structures appeared before the reported onset of meiotic marker expression in zebrafish (Beer and Draper, 2013; Rodriguez-Mari et al., 2013; Tzung et al., 2015); therefore, we conclude that these are premeiotic GC cysts.

**dazl mutants**

Although Dazl has not been previously implicated in cyst formation, Dazl was a compelling candidate regulator of this conserved...
Crispr/cas9 mutagenesis (Gagnon et al., 2014). We recovered a dazl allele (Δ7ΔΔ) that causes a premature stop codon within exon 6, revealing a frameshift mutation that caused a premature stop codon within dazl, truncating all functional domains (Fig. 2B,C). Using CRISPR/Cas9 methods targeting exon 6, we recovered two additional alleles: dazlae57 and dazlae58. Sequencing of genomic DNA (gDNA) and mutant cDNA revealed two distinct insertion-deletion mutations (Fig. 2B,C; Fig. S4). The dazlae57 allele harbors a 2 bp deletion, a 9 bp insertion and 2 bp substitution that result in a premature stop codon predicted to truncate Dazl and eliminate the Daz motif (Fig. 2B,C). The dazlae58 allele, a 15 bp insertion with a 12 bp deletion, results in a five amino acid in-frame deletion generating a truncated protein of 225 amino acids with intact RNA recognition motif (RRM) and Daz motifs. Genotyping assays were developed for each dazl mutant allele (Fig. 2D; Fig. S5).

Dazl protein is first detected in PGCs just before cystogenesis

Although dazl transcripts are maternally provided in zebrafish (Kosaka et al., 2007), the distribution of Dazl protein was not known. To examine Dazl protein, we generated C and N-terminal anti-Dazl antibodies (Yenzym) and found that although endogenous Dazl was not detectable in PGCs at 30 hours post fertilization (hpf) (Fig. 3A), it was first detectable just before cyst formation. In 12-14 day wild-type gonads, Dazl protein was detected as puncta in Vasa+ GCs, but not somatic gonad cells, which lack Vasa (Fig. 3B,D; Fig. S6), indicating that Dazl is germline specific. Dazl protein appeared reduced in dazlae57 mutants (Fig. 3C) and was not detectable in dazlae58 (Fig. 3E,F). At 29 days, in wild type, Dazl protein was diffusely localized in the cytoplasm of the GCs (Fig. 3G). Thus, the N-terminal antibody is specific for Dazl and detects the truncated dazlae57 protein, revealing that zygotic Dazl is produced before cystogenesis. Consistent with lack of detectable Dazl protein in PGCs at 30 hpf, we observed no deficits in PGC specification, PGC number, germ granule formation or GC migration in dazlae57, dazlae58 or dazlae59 mutants (Fig. S7). Therefore, if dazl is required for GC specification, PGC granule formation or GC viability, maternal dazl must fulfill these functions.

Dazl regulates cystogenesis

To determine whether dazl was required for cystogenesis, we examined Vasa and actin, labeled with phalloidin, in dazl mutants. Between 7 and 10 days, Vasa persisted in dazl mutants, indicating that zygotic Dazl is not required for Vasa expression (Fig. 4; Figs S8, S9). As in wild type (Fig. 4A; Figs S8-S11), Vasa+ cells (GCs) of dazl mutants were single and clustered individuals (Fig. 4B; Figs S8-S11), adjacent to somatic gonadal cells (Fig. 4; Fig. S8). Like wild type at 10 days (Fig. 4C; Figs S8, S10), dazl mutant cells transitioned to a cystogenic state and underwent synchronous nuclear/cytoplasmic morphological changes (Fig. 4D; Figs S8, S11). Some dazl mutant GCs showed evidence of amplification, including a compact nucleus (DAPI), but Vasa was diffusely cytoplasmic, and Vasa perinuclear aggregates were less apparent at this time (Figs S8, S10, S11). During the transition-amplification step, somatic gonadal cells enclosed GCs in both wild type and mutants. These results indicate that initiation of the transition and early amplification phases does not require dazl. Next, we examined dazl mutants at 12 days, when cysts emerge in wild type. In contrast to the interconnected Vasa+ cyst cells in heterozygotes (Fig. 4E), no organized cysts formed in dazl mutants; instead, dazl mutant cells resembled the earlier individual GC morphology (Fig. 4F; Fig. S8). The cytoplasm of the mutant cells appeared convoluted, and perinuclear enrichment of Vasa granules appears reduced in dazl mutants (Fig. S6), indicating that Dazl is germline specific. Dazl protein appeared reduced in dazlae57 mutants (Fig. 3C) and was not detectable in dazlae58 (Fig. 3E,F). At 29 days, in wild type, Dazl protein was diffusely localized in the cytoplasm of the GCs (Fig. 3G). Thus, the N-terminal antibody is specific for Dazl and detects the truncated dazlae57 protein, revealing that zygotic Dazl is produced before cystogenesis. Consistent with lack of detectable Dazl protein in PGCs at 30 hpf, we observed no deficits in PGC specification, PGC number, germ granule formation or GC migration in dazlae57, dazlae58 or dazlae59 mutants (Fig. S7). Therefore, if dazl is required for GC specification, PGC granule formation or GC viability, maternal dazl must fulfill these functions.

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was not evident (Fig. S8). At this stage, cortical actin accumulated at cell membranes, making the membrane appear thicker. In addition, at 8 days and 10 days, dazl mutant GCs became larger than wild type. In contrast, although wild-type gonads were full of cysts at 14 days (Fig. 4G-I; Figs S8, S10, S11), dazl mutant GCs remained as individuals, indicating failed or severely delayed cystogenesis (Fig. 4H,J-L; Figs S8, S10, S11). Because somatic gonadal cells were intact, and because dazl mutant cell area and volume did not decrease, suggesting failed division (Fig. S12). In contrast, although wild-type gonads were full of cysts at 14 days (Fig. 5C-C″) as they initiated amplification. After this stage, postmitotic GC nuclei had smooth nuclear membranes and large nucleoli (Fig. 5E-E″). Although dazlΔ7/+ mutants initiated amplification (Fig. 5D-D″, Fig. 4D; Figs S8, S10), mutant GCs did not maintain a smooth nuclear morphology at 10 days or thereafter, and instead of forming cysts were individual cells with folded raisin-like nuclear membranes resembling wild-type GCs at 10 days (Fig. 5F-F″).

Formation of nuclear indentations has been reported in other cell types and is associated with cell state or cell cycle progression in cultured cells (Aureille et al., 2019), and transcriptional modulation during mouse oogenesis (Almonacid et al., 2019). Whether the change in nuclear morphology from the smooth appearance of early PGCs (Knaut et al., 2000; Strasser et al., 2008) to the raisin-like morphology that precedes the amplification step reflects activation of transcriptional programs necessary for the PGC to GSC transition is unknown. To determine whether germline-specific zygotic programs are activated without Dazl, we examined ziwi, a zygotically expressed GC marker, using the established ziwi:GFP reporter (Leu and Draper, 2010). At 8 days, zygotic activation of the ziwi reporter persists in dazlΔ7/+ or dazlΔ7/Δ7 mutants (Fig. 6A) undergoing amplification. At 10 days, the reporter persists in dazlΔ7/+ while dazlΔ7/Δ7 mutants could be due to abnormal cyst architecture or cytokinesis, such as failure to arrest the
cytokinetic furrow, particularly given that Dazl interacts with cytokinesis and ring canal factors (Reynolds et al., 2005; Rosario et al., 2019, 2017; Zagore et al., 2018). Actin is a prominent marker of intercellular connections and subcellular structures of GC cysts in other species, including the spectrosome of GSCs, and the branched fusome that connects germline cyst cells (de Cuevas et al., 1997; Djagaeva et al., 2005; Hime et al., 1996; Kloc et al., 2004; Lin and Spradling, 1995; Snapp et al., 2004; Spradling et al., 1997) and the ring channels of *Caenorhabditis elegans* (Seidel et al., 2018). Close inspection of actin (labeled with phalloidin) in wild-type gonads at 8 days and 10 days revealed an actin-rich density within GCs (Fig. 7A,A′,C,C′). These actin aggregates were also present in *dazl* mutant GCs (Fig. 7B,B′,D,D′). By 12 days, when cysts are abundant in wild-type gonads, actin was present in branched structures (Fig. 7E,E′). In contrast, at this stage in *dazl* mutants, the actin-rich aggregates persisted in Vasa+ cells (Fig. 7F,F′). By 14 days, these actin structures were no longer detected in wild-type cysts (Fig. 7G,G′,I). However, the actin densities persisted with up to two actin aggregates in *dazl* mutant GCs (*n*=3/6 individuals), as opposed to the single aggregate observed earlier in *dazl* mutants (compare Fig. 7H,H′ and Fig. 7B,B′,J), possibly reflecting failed division or cell fusion.

The main structures connecting cells within cysts are ring canals or ring channels, thought to be the products of arrested cytokinesis (Haglund et al., 2011; Hime et al., 1996; Robinson and Cooley, 1996; Seidel et al., 2018). Although full ring canal structures were not apparent within a single plane of a z-stack, projections of ten slices spanning the structures combined with supplemental three-dimensional projections revealed circular actin-rich structures between cells (Fig. 8; Movies 1-6).

At 10 days, actin rings were present between wild-type and *dazl* mutant cyst cells (Fig. 8A-A″, Movie 1; Fig. 8B-B″, Movie 2), consistent with normal onset of cystogenesis without *dazl*. In wild-type cysts, conspicuous actin rings remained at 12-14 days (Fig. 8C-C″,E-E″; Movies 3, 5). In contrast, the actin rings appeared compromised, possibly collapsed, in *dazl*−/−, with membranes closely abutting one another as cells individualized (Fig. 8D-D″,F-F″; Movies 4, 6). Altogether, we conclude that *dazl* is essential for incomplete cellularization and germline cyst formation.

To further examine the architecture of the actin rings, we examined gonad ultrastructure using transmission electron microscopy (TEM). Consistent with the confocal data, at 14 days electron dense intracellular bridges were detected between wild-type GCs (Fig. 8G-I; Fig. S13). In contrast, no ring canals or
cysts were detected in the mutant GCs, which were all individual cells (Fig. 8J). Based on the confocal and ultrastructural analysis, we conclude that GC progenitors are connected by intercellular ring canals, resembling those previously observed in zebrafish ovaries (Marlow and Mullins, 2008), and that Dazl is required for cyst structure and GC amplification.

**Type II/cystogenic divisions are required for fertility**

As discussed, two modes of GSC divisions, type I/direct differentiating and type II/cystogenic, have been described (Marlow and Mullins, 2008; Nakamura et al., 2010; Saito et al., 2007). In mouse, ring canals are dispensable for fertility in females but are required in males (Greenbaum et al., 2006). Conversely,
defects in ring canal formation in Drosophila disrupt the fertility of both sexes (Hime et al., 1996; Yue and Spradling, 1992). Likewise, type II divisions fail in dazl mutants. To determine whether cystogenic divisions are required for fertility in zebrafish, dazl mutants were raised to adulthood. As expected, based on the germline specific expression of dazl (Kosaka et al., 2007; Maegawa et al., 1999), dazl−/− had no overt morphological defects; however, dazlΔ7/Δ7 (n=11) and dazlΔ57Δ57 (n=6) adults were exclusively sterile males, whereas dazlΔ34Δ34 adults were fertile (n=23 males; n=6 females).

To confirm that infertility of dazl mutants was specific to the dazl mutation, and to assess mutant allele strengths, we performed complementation tests. Although heterozygosity for each allele caused no phenotypes or fertility deficits (Fig. 9A,B,D,D′,E,E′; n=9), dazlΔ57Δ57 mutants were exclusively sterile males (Fig. 9C,F,F′; n=2). Examination of Vasa-labeled GCs (Leerberg et al., 2017) and DNA (DAPI) at 2 months of age revealed oocytes (Fig. 9G) or normal testis morphology (Fig. 9H) in wild-type siblings, and lack of Vasa+ GCs in dazlΔ57Δ57 mutants (Fig. 9I; n=2). Like wild type, dazlΔ57Δ57 mutants were fertile males or females (n=5 females, n=10 males). Based on these observations, we conclude that dazl-mediated cystogenesis/type II division is essential for GSC establishment and fertility in zebrafish. Moreover, dazlΔ57 and dazlΔ34 are strong loss-of-function alleles, but dazlΔ34 retains all functional domains and sufficient activity to support normal germline development.

**Checkpoint inactivation cannot suppress germ cell loss in dazl mutants**

Loss of germline can occur by cell death, failure to maintain GC fate and differentiation, or an absence of supporting somatic cell types (Gross-Thebing et al., 2017; Kossack and Draper, 2019; Leerberg et al., 2017; Rodriguez-Mari et al., 2010; Slanchev et al., 2005; Uchida et al., 2002; Wang et al., 2007). During normal development, apoptosis of GCs is associated with male-specific differentiation (Pradhan et al., 2012; Rodriguez-Mari et al., 2010; Siegfried and Nusslein-Volhard, 2008; Slanchev et al., 2005; Tzung et al., 2015; Uchida et al., 2002; Wang et al., 2007). Mutation of the tumor suppressor tp53 can block cell death and oocyte loss in some zebrafish mutants (Miao et al., 2017; Rodriguez-Mari et al., 2010; Shive et al., 2010). Similarly, in mouse, infertility phenotypes associated with germline cell death are suppressed by chek2 mutation, which acts via Tp53 and Tp63 (Bolcun-Filas et al., 2014). Zebrafish have a single chek2 gene on chromosome 5. The zebrafish chek2Δ4aΔ350 mutant allele was recovered in the Sanger screen (Kettleborough et al., 2013). By sequencing gDNA and cDNA from mutants, we confirmed that chek2Δ4aΔ350 is a nonsense allele harboring a C to A mutation that creates a premature stop codon (Q-stop), yielding a truncated Chek2 protein lacking the kinase domain (Fig. S14). As in Drosophila (Abdu et al., 2002) or mouse (Bolcun-Filas et al., 2014), mutation of zebrafish chek2 caused no overt phenotypes and did not interfere with fertility (n=9 females and n=13 males). Therefore, chek2, on its own, is dispensable for germline development and fertility in zebrafish.

To determine whether eliminating tp53 or chek2 could suppress germline loss in dazl mutants, we generated double mutants lacking dazl and tp53 or chek2. Analysis of dissected 20 dpf, 40 dpf and adult (>6 months) gonads revealed ovary or testis in tp53 or chek2 mutants that were heterozygous for dazlΔ34Δ34 (n=9). In contrast, all dazlΔ34Δ34 mutants were sterile because dazlΔ34Δ34 were either fertile males or females (n=11) (Fig. S16). These results indicate that dazl is required for cyst development and germline maintenance independently of chek2 and Tp53.
Dazl promotes germline stem cell fate

Although migration of PGCs has been extensively studied (reviewed by Barton et al., 2016; Marlow, 2015; Paksa and Raz, 2015), the cellular and molecular mechanisms by which PGCs transition to GSCs, in zebrafish, remain unknown. The only established marker of zebrafish GSCs is nanos2 (Beer and Draper, 2013; Cao et al., 2019), but neither it nor nanos3 are required to specify GSCs in zebrafish (Beer and Draper, 2013; Cao et al., 2019).

To determine whether germline loss in dazl mutants was a result of impaired GSC specification or maintenance, we used RNAscope to analyze nanos2 expression in wild type and dazl mutants at 7-14 days, earlier than previously analyzed. In addition, we examined dazl mRNA expression, which also had not been analyzed during this period, using Vasa to mark all GCs. At 7 days and 14 days, dazl transcripts were detected as cytoplasmic puncta in Vasa+ wild-type GCs (Fig. 10A,C,E,G), and were less abundant in dazlae57/ae57 mutants at this stage (Fig. 10D,F,H,I). Therefore, we conclude that all GCs initiate nanos2 expression, which also had not been analyzed during this period, using Vasa to mark all GCs. At 7 days and 14 days, dazl transcripts were detected as cytoplasmic puncta in Vasa+ wild-type GCs (Fig. 10A,C,E,G), and were less abundant in dazlae57/ae57 mutants at this stage (Fig. 10D,F,H,I). Therefore, we conclude that all GCs initiate
expression of nanos2, but only a few become the nanos2 high GSCs. Because induction of nanos2 is intact, but high nanos2 is not achieved in dazl mutants, we conclude that dazl is required for GSC specification, either by directly promoting stabilization of nanos2 transcripts or by promoting the PGC to GSC transition required for their specification.

**DISCUSSION**

Our study examines the earliest stages of gonadogenesis and provides evidence that the conserved Rbp Dazl is required for germline cyst formation and plays crucial roles in GC amplification and establishment of GSCs to promote fertility. We provide genetic evidence that Dazl is required to form germline cysts interconnected by actin rings that resemble ring canals, based on confocal and ultrastructural analysis. Specifically, dazl mutant GCs are able to initiate cyst formation, but ring canals are not maintained. It is unclear whether failed cysts return to the individual state or are quickly eliminated, such that only pre-cystogenic cells remain (Fig. 11). In either case, mutant cells ultimately remain as individuals that fail to differentiate as meiocytes (Fig. 11).

Consistent with our findings, regulators of incomplete cytokinesis were among the Dazl targets identified in immunoaffinity screens (Kim et al., 2015; Reynolds et al., 2005; Rosario et al., 2019, 2017; Zagore et al., 2018). In addition to promoting type II cystoblast divisions, we show dazl is required for GSC establishment. Finally, we show that Dazl and type II cystogenic divisions are essential for...
fertility, as GCs are lost from dazl mutants by a mechanism independent of checkpoint regulators.

Conserved dazl functions in gametogenesis and the PGC to GSC transition

In cultured cells, dazl promotes meiotic entry (Chen et al., 2014; Haston et al., 2009; Jung et al., 2017; Yu et al., 2009). Similarly, in mouse, dazl is required for ‘licensing’, or acquisition, of meiotic competence and to generate gametes; dazl mutant GCs remain PGC-like and fail to develop as male or female (Gill et al., 2011; Hu et al., 2015). In medaka, dazl also regulates gametogenesis and GC development (Li et al., 2016; Xu et al., 2007), but is not required for sexually dimorphic gonad development (Nishimura et al., 2018). We show that zygotic dazl is required for gametogenesis in zebrafish. However, in contrast to mouse dazl mutant gonads, which fail to sexually differentiate, and medaka dazl mutants, which develop as either sex (Nishimura et al., 2018), zebrafish dazl mutants develop exclusively as males. In medaka, recovery of male or female dazl mutants with only early PGCs indicated that PGC-like cells could support development of both sexes (Nishimura et al., 2018). Although PGCs form in zebrafish dazl mutants, no females were recovered. Thus, zebrafish PGCs devoid of dazl cannot support cystogenesis, establishment of GSCs or fertility. Similarly, work in mouse and pig indicates that commitment to germline fate only occurs after the PGCs reach the gonad and requires dazl (Nicholls et al., 2019).

A role for dazl in germline cyst formation

In many organisms, proliferation of GCs involves synchronous incomplete divisions that form germline cysts. During cyst formation, GSCs form cytoplasmic divisions (Leu and Draper, 2010; Pepling and Spradling, 1998). We characterized cystogenesis in juvenile zebrafish gonads and demonstrate a requirement for Dazl in germline cyst formation. This process...
begins with the congregation of individual migratory GCs, which then undergo synchronous division to generate cysts. Notably, as in zebrafish \textit{dazl} mutants, \textit{dazl}−/− GCs of mouse (Chen et al., 2014; Gill et al., 2011; Hu et al., 2015; Lin et al., 2008) and medaka (Nishimura et al., 2018) remain as individual cells, which were described as PGC-like (Chen et al., 2014; Gill et al., 2011; Nishimura et al., 2018). We found that PGCs lacking \textit{dazl} initiate cystogenesis, including changes in nuclear morphology and gene expression, but fail to maintain intercellular bridges.

Nuclear indentations have been associated with cell state, behavioral changes or transcriptional modulation (Hulspas et al., 1994; Seirin-Lee et al., 2019; Aureille et al., 2019; Almonacid et al., 2019). Whether the transformation of nuclear morphology from smooth in PGCs (Knaut et al., 2000; Strasser et al., 2008) to the raisin-like morphology that precedes amplification represents the activation of transcriptional programs necessary for PGC differentiation remains to be determined. Initiation of this step independently of Dazl, combined with \textit{de novo} expression from the \textit{ziwi} promoter and persistent Vasa expression, indicate that zygotic transcriptional programs are initiated; however, this transition subsequently fails in mutants.

Although GCs without Dazl initiated cystogenesis, ring canal integrity was compromised, possibly due to a failure to mature bridges or arrest cytokinesis. Although Dazl was not previously implicated in cyst formation, it interacts with Tex14 RNA, a known ring canal regulator formation (Greenbaum et al., 2006; Reynolds et al., 2005; Rosario et al., 2017; Zagore et al., 2018). Thus, Dazl likely promotes ring canal formation or maintenance via Tex14 or a related protein. In wild type, actin aggregates progress to branched structures that ultimately resolve in late cysts. It is unclear why these structures seem to disappear after cysts form. Labeling and lineage tracing will be required to determine whether cyst breakdown occurs, as in mice, or if instead cysts are stable and there is selection, like in \textit{Drosophila} (de Cuevas et al., 1997; Lei and Spradling, 2013; Pepling and Spradling, 1998). In contrast, actin remains in aggregates, which are subsequently duplicated in \textit{dazl} mutants. These duplicated structures potentially indicate cell fusion or, more likely, failed cytokinesis because although wild-type GCs become smaller, cell size does not change in \textit{dazl} mutant GCs.

Interestingly, during cystogenesis, the characteristic perinuclear Vasa granules of PGCs (Knaut et al., 2000) are transiently lost and are later re-established in premeiotic cystocytes. Significantly, zygotic Dazl is not required for Vasa translation, but Dazl protein or successful cyst formation is required to re-establish perinuclear Vasa aggregates. In zebrafish, all premeiotic GCs express Vasa, but a subset also express \textit{nanos2}; those expressing both are the presumed GSCs (Beer and Draper, 2013; Cao et al., 2019; Draper, 2017). Because all GCs seem to enter a cyst state in wild type from which Vasa+ premeiotic cells and a few \textit{nanos2}+ GSCs emerge, and because GCs of both populations are lost in \textit{dazl} mutants, it is tempting to speculate that the germline cyst not only serves to amplify premeiotic GC numbers but also plays a role in GSC specification. Work in mouse and pig similarly implicates \textit{dazl} in commitment to germline fates (Nicholls et al., 2019), suggesting that whether PGCs are specified by maternal inheritance or are induced later, \textit{dazl} plays an evolutionarily conserved role in commitment or specification of the germline after PGCs reach the gonad anlage.
A Dazl-mediated PGC to GSC transition

The PGCs in zebrafish are specified by inheritance of maternal factors and are maintained by maternal and zygotic programs. As the precursors for the GSC, PGCs are essential for germline fate. Nanos is a conserved marker of GSCs in zebrafish, medaka and mice (Nakamura et al., 2010; Sada et al., 2009; Aoki et al., 2009; Beer and Draper, 2013; Cao et al., 2019), and is required for the maintenance of GSCs in vertebrates and invertebrates (Forbes and...
In mice, it is known that Dazl is required to promote GSC fate. Future analysis is required to determine whether Dazl directly regulates nanos2 stability or promotes GSC fate by another mechanism, potentially via allocation of GSC versus cystoblast fate during the cystogenic divisions.

**MATERIALS AND METHODS**

**Fish strains**

dazl<sup>Δ7</sup> and nanos2<sup>Δ13</sup> mutant fish strains were generated using CRISPR/Cas9 mutagenesis as previously described (Gagnon et al., 2014). dazl<sup>Δ7</sup> was generated using ZFNs (Ekker, 2008; Foley et al., 2009b) as detailed below. Complementation tests were performed by intercrossing carriers of each dazl mutant allele. To generate double mutants, dazl<sup>Δ7</sup> was crossed with tpa<sup>2.5</sup> or the chk2<sup>20128003</sup> allele, generated in The Sanger Institute’s Zebrafish Mutation Project (Kettleborough et al., 2013) and obtained through the Zebrafish International Resource Center (ZIRC). Genomic DNA and cDNA from chk2<sup>20128003</sup> mutant tissues were sequenced to verify the genomic mutation and determine the transcript produced from the mutant allele (primers in Table S1). To visualize the GCs, mutations were crossed into the zp<sup>1326</sup> GSC reporter line (Leu and Draper, 2010). All procedures and experimental protocols were performed in accordance with National Institutes of Health guidelines and were approved by the Einstein (protocol 20140502) and Icahn School of Medicine at Mount Sinai Institutional (ISMMS) Animal Care and Use Committees (IACUC, 2017-0114).

**Dazl genotyping**

Genomic DNA was obtained either from dissected adult trunk, fin clip or whole larvae. Samples were lysed in an alkaline lysis buffer ([25 mM NaOH and 0.2 mM EDTA (pH 12)], heated at 95°C for 20 min, then cooled to 4°C before the addition of neutralizing buffer ([20 mM Tris-HCl and 0.1 mM EDTA (pH 8.1)]) (Truet et al., 2000). gDNA from dazl<sup>Δ7</sup> or dazl<sup>Δ13</sup> was PCR-amplified for 40 cycles with an annealing temperature of 59°C, followed by an EcoI47I restriction enzyme digestion for 1 h (fast digest, EcoI47I, Thermo Scientific). Undigested and digested products were resolved in a 3% MetaPhor 1:1 (Lonza)/agarose gel. DNA and cDNA from isolated GCs, GSCs and gonocytes were amplified and digested as described above.
Eco147I cuts the wild-type fragment. A PCR amplified fragment annealed at 57°C flanking the dazl<sup>57</sup> region was digested with AflIII enzyme and then resolved on a 3% MetaPhor/agarose gel. AflIII cuts the mutant fragment.

tp3<sup>52114K</sup> was identified as described previously (Berghmans et al., 2005). High resolution melt analysis assays were also developed for dazl<sup>57</sup>, dazl<sup>134</sup>, tp3<sup>52114K</sup>, ckh<sup>2063050</sup> and the dazl<sup>667a7</sup> (described below) alleles (primers listed Table S1).

**Mutagenesis**

dazl<sup>134</sup> and dazl<sup>57</sup> alleles were generated by CRISPR/Cas9 mutagenesis as previously described (Gagnon et al., 2014). dazl single-guide RNAs (sgRNA) targeting exon 6 were designed using the CHOPCHOP web tool (Montague et al., 2014) (Table S1). Following annealing of the gene-specific target and the constant oligonucleotides, the fragment was filled using T4 DNA polymerase. The resulting fragment was transcribed and purified to yield sgRNA using the MEGAScript SP6 kit (Life Technologies, Ambion) (Jinek et al., 2012). The sgRNA [1 nl of 12.5 ng/μl of sgRNA and 1 nl of Cas9 protein (300 ng/μl)] was co-injected with phenol red (Sigma-Aldrich) at the one-cell stage. At 24 hpf, uninjected and injected embryos (n=8 each) were assayed by PCR amplification (primers listed in Table S1), followed by T7 endonuclease digest (Hwang et al., 2013), and those with new banding patterns were sequenced to confirm mutagenesis. Injected embryos were raised to adulthood, and individuals carrying mutations were compared with the wild-type allele, indicative of dazl<sup>134</sup> knock-in founders carrying mutations in the germline. Recovered alleles were identified by genotyping their progeny, as described above. Smaller bands compared with the wild-type allele, indicative of de novo mutations, were extracted from the gel, cloned into a PCR4 TOPO vector (Sigma-Aldrich) and sequenced in both directions to determine the mutated sequence. Fish harboring dazl<sup>134</sup> or dazl<sup>57</sup> mutations were outcrossed to AB fish. All mutations were verified by sequencing both gDNA and cDNA from mutant animals. Total RNA was extracted from pooled embryos from heterozygote intercrosses or AB strain wild type (n=20-30) using Trizol (Life Technologies, 15596). cDNA was prepared using a SuperScript III/IV Reverse Transcription Kit (Life Technologies, 18080-051). RT-PCR was performed to amplify the dazl coding region using Easy-A High Fidelity Taq polymerase (Agilent, 601400) (primers listed in Table S1). The PCR fragments were TOPO cloned into pCR4/GW/TOPO (Invitrogen, K250020) and sequenced (MacroSeq). Sequences were analyzed using Sequencer or MacVector software.

The dazl<sup>57</sup> allele, a 7 bp deletion resulting in a frame shift and subsequent premature stop codon at amino acid 54, was generated using ZFNs (Foley et al., 2009a). The dazl genomic region was PCR amplified and sequenced. ZFNs targeting the region in exon 2 just upstream of the RRM were purchased from Sigma-Aldrich and injected (500 pg) at the one-cell-stage into wild-type embryos. The dazl ZFN set (spacers underlined) was as follows:

```plaintext
5'-GATCTGGTATCCCGGTGGCTGATAT-3'
3'-ATTAACACATAT-AGTCCAAATTTACTCGATCA-5'
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**Dissections**

Fish were anesthetized with a lethal dose of tricaine (MS-222) (400 mg/l) and dissected. Fish were positioned laterally, and anteroposterior body length was measured before dissection. Images were acquired using an Olympus SZ61 dissecting microscope equipped with a high-resolution digital camera (model S97809) and Picture Frame 2.0 software.

**Dazl antibodies**

The YenZym custom antibody service was used to design, produce and raise chicken polyclonal antibodies against the following Dazl epitope: (residues 16-35) CYSGDIQKHRQGFPSLKLNS-amide (YZ6741 and YZ6742).

**Immunostaining**

For whole-mount immunofluorescence-stained embryos or ovaries, tissues were fixed in 3.7% paraformaldehyde overnight at 4°C. The next day, washes were performed in PBS then dehydrated in MeOH and stored at −20°C. Chicken anti-Vasa antibody (Blokhina et al., 2019) was used at a 1:5000 dilution or rabbit anti-Vasa (SAB2702444, MilliporeSigma, 1:5000) anti-β-catenin (C7202, Sigma-Aldrich, 1:500), anti-LaminB1 (Ab16048, abcam, 1:1000), Dazl (YZ6741, 1:500). Alexa Fluor 488, Alexa Fluor 568, CY3 and C5 (Molecular Probes) secondary antibodies were diluted 1:500. For F-actin labeling, samples were fixed overnight at 4°C in 3.7% paraformaldehyde in actin stabilization buffer (Becker and Hart, 1999) and were then permeabilized in 2% Triton X-100. Following the primary antibody solution, Alexa Fluor 568 phallolidin was added. Samples were mounted in Vectashield with DAPI and images were acquired using a Zeiss Axio Observer inverted microscope equipped with Apotome and a charged-coupled device (CCD) camera, a Zeiss Zoom dissecting scope equipped with Apotome1, or a Leica SP5 DMi or SP8 DMi at the microscopy CoRE at the Icahn School of Medicine Mount Sinai, NY, USA. Image processing was performed using Zenpro (Zeiss), Leica Application Suite, ImageJ/FIJI, Adobe Photoshop, Adobe Illustrator and Imaris (Oxford Instruments). Confocal images were acquired using a Leica SP5 DMi with a 40x or 63x objective. The acquisition setting was set between sample and experiments to XY resolution 1024×1024 (or 512×512 as indicated), zoom 2.5x, pinhole was adjusted to 1.1 μm of the z thickness and increments between images in stacks were 0.2 μm. Laser power and gain were set for each antibody or fluorescent compound to 2-10% below saturation condition.

**Fluorescent in situ hybridization**

Fluorescent in situ hybridization (RNAscope) was performed using the Multiplex Fluorescent Reagent Kit v2 (ACD Bio, 323100), according to the whole-mount RNAscope protocol described previously (Campbell et al., 2015). Animal trunks were fixed overnight in 4% paraformaldehyde at 4°C, washed with PBT (0.1% Tween), dehydrated in MeOH and placed at −20°C overnight. RNAscope Blank C1 probe, Dr-nanos2-C2 probe (ACD Bio, 843461-C2) and Dr-dazl-C3 probe (ACD Bio, 469261-C3) were used. The signal was amplified using Opal 570 (Aloya Biosciences, FP1488001KT) for Dr-nanos2-C2 probe and Opal 690 (FP1497001KT) for Dr-dazl-C3 probe, respectively. Fluorescent in situ hybridization was followed by immunostaining for Vasa as described above and DNA was labeled by DAPI.

**Electron microscopy**

Fish were euthanized in tricaine and trunks were immediately fixed in Karnovsky’s solution (2% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate) for at least 24 h. The samples were then washed with cacodylate buffer, osmicated with 1% osmium tetroxide for 1 h, and en bloc stained with 2% uranyl acetate for 1 h. After a quick rinse in water and seven 10-min incremental ethanol dehydration steps (25%–100%), samples were infiltrated with a mixture of propylene oxide and an epoxy resin (Epon, Electron Microscopy Sciences). Samples were polymerized in pure resin in a vacuum oven at 60°C for 72 h. Semi-thin sections (2 μm) were cut until the gonadal zone was reached. Five consecutive ultrathin sections were cut at 90 nm using a diamond knife (Diatome) on an ultramicrotome (Leica EM UC7) and mounted onto a formvar-supported slot grid (Electron Microscopy Sciences). Serial sections were imaged using a Hitachi H7500 TEM at 75 kV and 2048X2048 pixel, and 16 bit images were taken using a CCD camera (AMT Imaging).

**Germ cell, cyst and early oocyte quantification**

Vasa protein was used as a marker to identify and count individual GCs. Z-series stacks of gonads at each stage were obtained using a Zeiss Axio Observer inverted microscope equipped with Apotome and a CCD camera, a Zeiss Zoom dissecting scope equipped with Apotome1, Leica SP5 DMi or Leica SP8 DMi at the Microscopy CoRE at the Icahn School of Medicine Mount Sinai. Cells were manually counted by analyzing each slice within each z-stack for Vasa positivity and nuclear morphology (DAPI), defining each category in the cystogenesis process and the number of cells per cyst. Quantification of actin-rich structures was performed by counting each actin-rich structure through the z-stack. Cell area and volume was measured after manual segmentation of the cell in each plane through the z-stack. Quantification of nanos2 or dazl foci was performed manually by counting each...
corresponding dot in each Vasa+ cell in the gonad for the specified stages. In the case of the transition/amplification stage, only the foci surrounding the nucleus were counted because cell boundaries between cells were not well defined. Image analysis for all experiments above were performed using ZEN pro (Zeiss), Leica Application Suite, ImageJ/FIJI or Imaris (Oxford instruments).

Statistical analysis
Statistical differences were assessed using GraphPad Prism software and paired two-tailed Student’s t-test. Significant differences are indicated in figures by asterisks (* P<0.01; ** P<0.05; *** P<0.001; n.s., not significant).

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Competing interests
The authors declare no competing or financial interests.

Author contributions

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