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Zebrafish *vasa* is required for germ-cell differentiation and maintenance

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Abstract

Vasa is a universal marker of the germ line in animals, yet mutations disrupting *vasa* cause sexually dimorphic infertility, with impaired development of the ovary in some animals and the testis in others. The basis for this sexually dimorphic requirement for Vasa is not clear; in most animals examined, both the male and female gonad express *vasa* throughout the life of the germ line. Here we characterized a loss-of-function mutation disrupting zebrafish *vasa*. We show that maternally provided Vasa is stable through the first ten days of development in zebrafish, and thus likely fulfill any early roles for Vasa during germ-line specification, migration, survival, and maintenance. Although zygotic Vasa is not essential for the development of juvenile gonads, *vasa* mutants develop exclusively as sterile males. Furthermore, phenotypes of *vasa;p53* compound mutants are indistinguishable from those of *vasa* mutants, therefore the failure of *vasa* mutants to differentiate as females and to support germ-cell development in the testis is not due to p53-mediated apoptosis. Instead, we found that failure to progress beyond the pachytene stage of meiosis causes the loss of germ-line stem cells, leaving empty somatic tubules. Our studies provide insight into the function of zebrafish *vasa* during female meiosis, differentiation, and maintenance of germ-line stem cells.

Keywords

Juvenile gonad; p53; Ddx4; germ line; maternal; meiosis

Introduction

Sexual reproduction in multicellular organisms requires the proper segregation and maintenance of germ cells. Germ cells are segregated from other cell types early in embryogenesis by two common mechanisms: In invertebrates and some vertebrates, such as

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Conflict of Interest

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zebrafish and *Xenopus*, germ-cell specification depends on the inheritance of maternal factors present in the egg. On the other hand, in mammals such as mice, germ-cell fate is induced through cell-to-cell contacts. Despite the apparently significant differences in specification mechanism, the molecular factors driving germ-line development are well conserved throughout the animal kingdom. Among these factors is the gene *vasa*, which is expressed in the germ cells of nearly every sexually reproducing animal, including humans (Castrillon et al., 2000; reviewed in Gustafson and Wessel, 2010; Hartung and Marlow, 2014).

Vasa is a RNA-helicase of the DEAD-box helicase family that shares structural and functional similarity with the prototypical DEAD-box helicase eukaryotic translation initiation factor 4A (eIF4A) (Lasko and Ashburner, 1988). Vasa, also known as DDX4, contains 9 conserved motifs at its C-terminal end that are responsible for RNA binding, ATP binding, and ATP hydrolysis (Liang et al., 1994; reviewed in Parsyan et al., 2011). Vasa is also functionally similar to eIF4A in that both of these helicases promote translation. This was demonstrated in *Drosophila* studies wherein Vasa was shown to physically interact with eukaryotic translation initiation factor 5B (eIF5B) promoting the recruitment of the 60S ribosomal subunit and subsequent translation of interacting RNAs (Johnstone and Lasko, 2004; reviewed in Parsyan et al., 2011).

Identification of the *vasa* homolog in zebrafish allowed for the first description of zebrafish primordial-germ-cell development (Yoon et al., 1997). *vasa* RNA is enriched in the cleavage furrows of zebrafish zygotes, and is a specific marker of germ cells in gastrula-stage and later embryos; thus *vasa* has been used as a probe to identify the temporal and spatial origins of the zebrafish germ line (Braat et al., 1999; Yoon et al., 1997). Like its RNA, Vasa protein appears to be continuously present in the germ line from the beginning of germ-cell specification throughout germ-cell maturation (Braat et al., 2000; Knaut et al., 2000). Vasa is expressed in the juvenile bipotential gonad, which is composed of early oocyte-like cells that will either promote female gonadogenesis or apoptose and instead give rise to a male gonad (Rodriguez-Mari et al., 2010; Rodriguez-Mari and Postlethwait, 2011; Siegfried and Nusslein-Volhard, 2008; Takahashi, 1977; Uchida et al., 2002). Following sexual differentiation of this bipotential gonad, Vasa persists in the mature ovary and testis (Beer and Draper, 2013; Braat et al., 2000; Knaut et al., 2000; Leu and Draper, 2010).

The absence of functional Vasa leads to sterility, but the penetrance of this phenotype can vary by sex. In *Drosophila*, *vasa* is expressed in the germ cells of both the ovary and testis, yet *vasa* deficiency results in female-specific sterility (Schupbach and Wieschaus, 1986; reviewed in Raz, 2000). *Vasa* expression persists in the gonads of both sexes in mice, but deficiency in mouse *vasa* homolog (MVH) have male-specific sterility (Tanaka et al., 2000). This variance in the affected gender between *Vasa*-null organisms has led to different hypotheses regarding the possible species-specific and sex-specific roles of *vasa* (reviewed in Gustafson and Wessel, 2010). For example, closely related helicases with functions that are redundant to Vasa may account for the differential requirement for Vasa in the testis or ovary (Johnstone et al., 2005), or that sexual dimorphism in the expression of *Vasa* RNA targets in male and female germ cells of different species influences the phenotype associated with losing *vasa* function (reviewed in Gustafson and Wessel, 2010).

Comprehensive investigation of *vasa*-null phenotypes in other animals will likely elucidate the conserved and species-specific roles of Vasa protein.

In this study we characterized a loss-of-function mutation disrupting zebrafish *vasa*. We show that maternally provided Vasa is stable through the first ten days of zebrafish development, and thus could fulfill any roles for Vasa during germ-line specification, migration, survival, and maintenance. Although Vasa is not required to form the juvenile gonad, *vasa* mutants develop exclusively as sterile males. Our data indicate that failure to support germ-cell development in *vasa* mutants is not due to p53-mediated apoptotic loss of the germ cells as *vasa;p53* compound-mutant phenotypes are indistinguishable from those of *vasa* mutants. Instead, failure to progress beyond the pachytene stage of meiosis and loss of the germ-line stem cells causes all *vasa*-mutant fish to form an immature testis. Thus, zebrafish *vasa* appears to be required for female meiosis, differentiation, and maintenance of germ-line stem cells.

Results

Characterization of a null mutation in zebrafish *vasa*

To investigate the role of *vasa* in the germ-line development of zebrafish, we obtained zebrafish harboring a mutant *vasa* allele from the Sanger Institute's Zebrafish Mutation Project (Kettleborough et al., 2013). We sequenced the *vasa* (*sa6158*) allele and confirmed that it contains a T-to-A point mutation, resulting in a premature stop codon that is predicted to encode a truncated Vasa protein lacking critical functional domains –including the Q-motif, ATP-binding and C-terminal RNA helicase domains; this likely represents a *vasa*-null allele (Fig. 1A) (Liang et al., 1994; Sengoku et al., 2006).

The Zebrafish Ensembl genome browser has annotated seven *vasa* transcripts, formed by inclusion or exclusion of 3 alternative exons from a total of 28 exons, as well as alternative splice sites within conserved exons (Fig. 1B) (Flicek et al., 2014). We identified zebrafish *vasa* mutants using a PCR-based genotyping assay, and confirmed that the mutation in the *vasa*^{*sa6158*} allele is present in a conserved exon affecting all annotated *vasa* transcripts using sequencing analysis; thus, the point mutation eliminates all annotated Vasa protein isoforms (Fig. 1C–D).

Maternal Vasa is stable and zygotic *vasa* is not essential for early germ-line development

Zebrafish germ cells are specified by the inheritance of maternal germ plasm, a mixture of RNAs and proteins present in the egg that direct germ line development. Among these maternal germ plasm components are several RNAs, including *vasa*, *nanos3*, *dazl*, and *dead end* (reviewed in Hartung and Marlow, 2014). Additionally, the Vasa protein itself is maternally inherited and present in zebrafish germ cells of all stages examined (Baat et al., 2000). To our knowledge, the stability of maternal Vasa protein has not been previously described in zebrafish; we therefore investigated maternally provided Vasa protein stability in zygotic *vasa*^{*sa6158/sa6158*}-homozygous mutants (hereafter referred to as *vasa*^{*sa6158*} mutants). We examined larvae of *vasa*^{*sa6158*} heterozygous in-crosses for expression of Vasa protein, finding that maternal Vasa was consistently present in germ cells of *vasa*^{*sa6158*}

mutants examined at days 1, 3, 6, 8, and 10 days post fertilization (d.p.f.) (Fig. 2A, B, and G). At 12 d.p.f., however, only one *vasa*^{sa6158} mutant expressed Vasa in the gonad in a manner comparable to wild-type and heterozygous siblings; the other mutants examined at this stage lacked Vasa staining (n=4) (Fig. 2C, D). By 14 d.p.f., all *vasa*^{sa6158} mutants examined lacked maternal Vasa (n=7) (Fig. 2F). Consistent with the presence of maternal Vasa protein, primordial-germ-cell specification and migration were normal in *vasa*^{sa6158} mutants, as assessed by in situ hybridization for the germ-plasm RNA *nanos3* in shield-stage and 30-hours-post-fertilization embryos (n=8) (Fig. 2H–I). Thus, maternal Vasa is present, and likely fulfills any requirement for Vasa activity in germ cells of *vasa*^{sa6158} mutants from fertilization until 10–12 d.p.f.

***vasa* is required for fertility in adult zebrafish**

Consistent with the germ-line-specific expression of *vasa* in zebrafish (Baat et al., 2000; Knaut et al., 2000; Yoon et al., 1997), zygotic *vasa*^{sa6158} mutants were viable to adulthood. Adult fish heterozygous for *vasa*^{sa6158} were identified in sex ratios comparable to wild-type fish (Fig. 3A–B'' and D–E''). In contrast, all recovered adult *vasa*^{sa6158} mutants were phenotypically male, based on pigmentation hue, abdomen shape, and the presence of male-specific epidermal tubercles on the pectoral fins (n=12) (Fig. 3C–C''). When mated to females, the *vasa*^{sa6158} mutants exhibited male mating behavior and induced female egg-laying, but eggs collected from these matings were unfertilized (n=12 males, cumulative of 1,380 eggs).

The “sterile male” phenotype of *vasa*^{sa6158} mutants is reminiscent of phenotypes displayed by zebrafish mutants in genes required for germ-cell survival, including zebrafish *Piwi* (*ziwi*), *breast cancer antigen 2* (*brca2*), and *fanconi anemia complementation group L* (*fancl*), and also occurs when germ cells are ablated by treatment with *dead end* morpholino or cytotoxins (Dranow et al., 2013; Houwing et al., 2007; Rodriguez-Mari et al., 2010; Rodriguez-Mari and Postlethwait, 2011; Shive et al., 2010; Weidinger et al., 2003). These and other studies have demonstrated that zebrafish lacking germ cells undergo masculine gonadogenesis, forming a testis containing somatic cells but no sperm (Dranow et al., 2013; Rodriguez-Mari et al., 2010; Rodriguez-Mari and Postlethwait, 2011; Siegfried and Nusslein-Volhard, 2008). Thus, to determine if the sterile male phenotype of our *vasa*^{sa6158} mutants was also caused by a lack of germ cells, we examined the gross and histological morphology of the adult *vasa*^{sa6158}-mutant gonads. In contrast to wild-type males, the gonad was not immediately apparent during the gross dissection of *vasa*^{sa6158}-mutant animals. Upon closer examination, however, a thin thread-like organ was visible adjacent to the swim bladder in the normal position of the gonad. When compared side-by-side, wild-type gonads were substantially larger than *vasa*^{sa6158}-mutant gonads (n=7) (Fig. 3F).

Examination of histological sections stained with Haematoxylin and Eosin (H&E) revealed that the adult *vasa*^{sa6158}-mutant gonad lacked germ cells and contained empty testis tubules composed of somatic cells, whereas heterozygous and wild-type testes contained both somatic cells and numerous spermatocytes and spermatozoa (Fig. 3A'', B'' and C''). Based on reverse-transcriptase-PCR (RT-PCR) analysis of transcripts extracted from gonads of *vasa*^{sa6158} mutants, the residual testis tissue expressed the Leydig marker *cyp11c1* and the

Sertoli cell marker *anti-mullerian hormone (amh)*, whereas germ-cell markers including *vasa* and *dazl* were absent (Fig. 3G) (Siegfried and Nusslein-Volhard, 2008; Wang and Urban, 2007). In contrast, male and female fish heterozygous for the *vasa^{sa6158}* allele appeared grossly and histologically normal, and displayed normal fertility. Combined with the histological analysis, these results indicate that the sterile male phenotype of *vasa^{sa6158}* mutants is caused by the absence of germ cells in the adult gonad.

Bipotential gonad formation in *vasa^{sa6158}* mutants

To determine the developmental time point during which germ cells are lost in *vasa^{sa6158}* mutants, we utilized a transgenic germ-cell reporter line expressing green fluorescent protein (GFP) under the *ziwi* promoter, *Tg[ziwi:GFP]* (Leu and Draper, 2010). This transgenic reporter drives GFP expression in germ cells as early as 12 d.p.f., continues to be expressed through adulthood, and marks the germ cells of both sexes, spermatogonia and oocytes (Leu and Draper, 2010). We crossed the *Tg[ziwi:GFP]* fish into the *vasa^{sa6158}* background and examined GFP expression in germ cells of *vasa^{sa6158}* mutants at critical time points in germ-cell development.

Since we had already determined that germ cells expressing Vasa protein were present in our mutants as late as 12 d.p.f., we focused on whether or not *vasa* was required for developing the bipotential gonad of day-21 juveniles. In zebrafish, the bipotential gonad represents an important stage of germ-line development as a transition from purely mitotic proliferation of germ cells to the initiation of meiosis and development of early-stage oocytes occurs during this period (Rodriguez-Mari et al., 2010; Rodriguez-Mari and Postlethwait, 2011; Siegfried and Nusslein-Volhard, 2008; Takahashi, 1977; Uchida et al., 2002).

We examined gonads of *vasa^{sa6158}*-mutant, heterozygous, and wild-type *Tg[ziwi:GFP]* juveniles by observing GFP expression through the body wall of live, anesthetized fish, as previously reported (Leu and Draper, 2010). At day 21 (d21, 0.7–0.8 cm in length), *vasa^{sa6158}* mutants had GFP expression in the gonad region comparable to their wild-type and heterozygous siblings (Fig. 4A and B). To examine the state of the gonads more closely, we euthanized the fish and fixed the tissue to examine dissected gonads from four heterozygous and four *vasa^{sa6158}*-mutant fish. Using DAPI to label DNA content, we observed that d21 *vasa^{sa6158}* heterozygous and mutant fish both contained meiotic nuclei that had developed as far as the pachytene stage of meiotic prophase I (Fig. 4C and D). Moreover, RT-PCR analysis of individual fish showed expression of the early meiotic marker *sycp3* in zygotic *vasa^{sa6158}* mutants (Fig. 4E). Therefore, initiation of meiosis does not appear to be impaired in the mutants.

We also analyzed several germ cell markers by RT-PCR, and detected comparable levels of the early oocyte marker *pou5f3* between *vasa^{sa6158}* mutants and their heterozygous and wild-type siblings (Rodriguez-Mari et al., 2013). *vasa* transcript was measurable in the gonads of one d21 mutant, suggesting that the mutant RNA is not subject to nonsense-mediated decay. Although *vasa^{sa6158}* mutant and heterozygous gonads appeared otherwise identical, heterozygous but not mutant fish expressed the oogenesis markers *nanos3* and *zona pellucida 3 (zp3)*, which likely represent stage-Ib or later-stage oocytes (Fig. 4E).

(Draper et al., 2007; Liu et al., 2006). Taken together, these data indicate that initiation of meiosis and formation of early oocytes in the bipotential gonad of *vasa^{sa6158}* mutants is likely normal; however, differentiation to stage-Ib oocytes (and thereby the diplotene stage of meiosis) is impaired.

Next, we examined the mitotic population of cells in the bipotential gonad. As in other organisms, proliferating zebrafish germ cells remain attached to each other through intercellular bridges, forming a syncytium that is thought to promote synchronous development (Marlow and Mullins, 2008; reviewed in Marlow, 2010). Consequently, mitotic daughter cells share their cytoplasmic contents, including cytoplasmic GFP in the *Tg[ziwi:GFP]* line; further, mitotic and post-mitotic germ-cell cysts in this reporter line can be visualized as groups of nuclei within a continuous GFP-positive mass (Fig. 4F' and G') (Dranow et al., 2013; Leu and Draper, 2010). The mitotic capacity of germ cells in *vasa^{sa6158}* heterozygotes and mutants was examined by counting the number of nuclei in GFP-positive germ-cell clusters of the bipotential gonad. We determined that adjacent cells represented a clonal germ-line cyst by several criteria: (1) continuity of GFP signal within cytoplasm; (2) cell-cycle synchronicity of cyst cells, as judged by the size and condensation of DAPI-stained nuclei; and (3) the presence of a surrounding follicle-cell layer, as visualized by DAPI-stained follicle cell nuclei. Z-sectioning through the tissue was also performed to distinguish between two clusters. We found no difference in average germ-cell-cluster size or overall distribution of cluster size between *vasa^{sa6158}* mutants and heterozygotes at this stage (Fig. 4F', G', and H). Moreover, the germ-line stem-cell marker *nanos2* was comparably expressed in *vasa^{sa6158}* mutants and heterozygotes (Fig. 4E). Therefore, we conclude that at this stage the mitotic capacity of *vasa^{sa6158}*-mutant germ cells and maintenance of the germ-line stem-cell population are intact.

Caspase-mediated apoptosis does not account for the loss of *vasa^{sa6158}*-mutant germ cells

The presence of germ cells in d21 juvenile mutants and their absence in adult *vasa^{sa6158}* mutants suggests that germ cells must be lost in the interim. Apoptosis is the most common mechanism by which germ cells are known to be lost in zebrafish mutants with sterility phenotypes. For example, mutations of the piRNA-processing component *ziwi* or the DNA-damage repair proteins *brca2* and *fancl* result in increased germ-cell apoptosis during the establishment and differentiation of the juvenile gonad (Houwing et al., 2007; Rodriguez-Mari et al., 2010; Shive et al., 2010). Moreover, previous observations in *Drosophila* *vasa*-mutant ovaries or testes from mouse MVH mutants indicate that the loss of *vasa* is associated with germ-cell apoptosis (Styhler et al., 1998; Tanaka et al., 2000).

To pinpoint when the germ cells were being lost, we examined the next developmental time point following formation of the bipotential gonad, at day-28 (d28, 0.9–1.0cm in length), the gonadal tissue has begun definitive differentiation into an ovary or testis (Siegfried and Nusslein-Volhard, 2008; Uchida et al., 2002). Detection of GFP expression through the body wall of live, anesthetized *Tg[ziwi:GFP]; vasa^{sa6158}*-mutant fish at d28 revealed patches of GFP in the gonad region, compared to the solid GFP-positive gonadal mass observed in wild-type and heterozygous fish (Fig. 5A and B). The observation that d28

vasa^{sa6158}-mutant gonads appeared to be losing GFP expression indicated that germ cells were being lost through apoptosis or other mechanisms.

To determine if germ cell loss was associated with apoptosis, we examined *Tg[ziwi:GFP]; vasa*^{sa6158/sa6158} gonads for activation of the apoptotic mediator Caspase-3 at d28. We found no evidence of active Caspase-3 staining in either d28 *vasa*^{sa6158} heterozygous or mutant gonads (n=5 for each) by immunofluorescence staining for cleaved Caspase 3 (Fig. 5C–E), whereas a segment of intestine stained in parallel clearly showed cells positive for Caspase-3 staining (Fig. 5F). Thus, while it is evident that gonad mass is reduced in d28 *vasa*^{sa6158} mutants (compare Fig. 5A to B and C to E), the loss of germ cells is likely not occurring through Caspase-mediated apoptosis.

Germ-cell differentiation and survival requires zygotic *vasa*

In mouse *mvh* mutants, spermatogonial differentiation and meiosis are arrested at the zygotene stage of meiotic prophase I (Tanaka et al., 2000). To determine if zebrafish *vasa* is also required for meiotic progression, we examined d28 gonads for meiotic nuclear content using DAPI. We observed pachytene-stage meiotic nuclei in testis of both *vasa*^{sa6158} mutants and heterozygotes. Although diplotene-stage (or dictyate) meiotic nuclei were clearly apparent in oocytes of *vasa*^{sa6158}-heterozygous animals (Fig. 6A), this stage was not apparent in males of either genotype (Fig. 6B and C) — even in wild-type adult testis tissue (data not shown), likely because rapid progression of spermatogonia through diplotene stage impedes reliable detection of this stage. Despite this technical limitation, we noticed that pachytene-stage germ cells in *vasa*^{sa6158} mutants had intracellular vacuolization that was not visible in their heterozygous siblings (Fig. 6C', red arrows). This striking phenotype may indicate dying cells, despite their Caspase-3-negative status and the absence of nuclear fragmentation, which are both characteristic of apoptotic cells. These findings therefore suggest that zebrafish *vasa* is required for the survival of germ cells and progression beyond the pachytene stage of meiotic prophase I.

In addition to the overall reduced size and patchy appearance of *vasa*^{sa6158}-mutant gonads undergoing differentiation (Fig. 5B), we observed that germ-cell clusters in d28 *vasa*^{sa6158} mutants appeared to be smaller than those of heterozygous siblings (Fig. 6B', C', and D). As we did for d21 bipotential gonads, we quantified the number of nuclei in GFP-positive germ-cell clusters of d28 *Tg[ziwi:GFP]; vasa*^{sa6158/sa6158} and *Tg[ziwi:GFP]; vasa*^{sa6158/+} gonads (n=5 and n=3, respectively). While *vasa*^{sa6158} heterozygotes tended to have a wide range in the number of cells associated with a single germ-cell cluster (from 1 to 50), *vasa*^{sa6158} mutants produced germ-cell clusters no larger than 12-cells. When we charted this data, it was apparent that germ-cell-cluster size between testes of *vasa*^{sa6158} heterozygotes and mutants was significantly different ($P=0.0001$) (Fig. 6D). Additionally, the germ-line stem-cell marker *nanos2* was markedly reduced in d28 *vasa*^{sa6158}-mutant gonads compared to heterozygous siblings (Fig. 6E), suggesting that *Vasa* may play a role in the maintenance of *nanos2* expression or of germ-line stem cells. These data point to a requirement for *vasa* in germ-cell differentiation as well as a possible requirement for *vasa* to support and maintain germ cell proliferation and mitosis, as has been reported for *Drosophila* and sea urchin (Pek and Kai, 2011; Yajima and Wessel, 2011).

Loss of *tp53* does not suppress germ-cell deficits of *vasa*^{sa6158} mutants

Tumor suppressor protein p53 (*tp53*) is a major activator of apoptosis (Berghmans et al., 2005; Fridman and Lowe, 2003), and elimination of *tp53* activity has been demonstrated to suppress germ cell-death in zebrafish *brca2* and *fancl* mutants (Rodriguez-Mari et al., 2010; Shive et al., 2010). Although we did not observe classical markers of apoptosis, such as activated Caspase-3 or nuclear fragmentation, in *vasa*-mutant gonads, we reasoned that if mutant germ cells were undergoing *tp53*-mediated apoptosis, then genetically eliminating *tp53* should suppress the loss of germ cells. Therefore, we generated and analyzed compound mutants with *vasa*^{sa6158} and *tp53*^{M214K} mutations (Berghmans et al., 2005).

Based on fertilization assays, adult compound *vasa*^{sa6158/sa6158};*tp53*^{M214K/M214K} zebrafish mutants had a sterile-male phenotype resembling that of single *vasa*^{sa6158} mutants. Histological analysis was performed to determine whether the basis of the fertility defect in *tp53*;*vasa*-compound mutants arose from an absence of germ cells, as occurs in *vasa* mutants, or a later defect in spermatozoa differentiation. We compared H&E-stained histological sections of *vasa*^{sa6158/sa6158};*tp53*^{M214K/M214K} mutants to their *vasa*^{sa6158}-mutant siblings (n=3 for each genotype), and found no difference in the appearance of the testis tissue (Fig. 7B' and C').

To investigate if the loss of *tp53* can temporarily delay germ-cell apoptosis without permanently rescuing germ-cell loss, we examined the gonads of *vasa*^{sa6158/sa6158} and *vasa*^{sa6158/sa6158};*tp53*^{M214K/M214K} fish at 42 d.p.f., a time-point directly following definitive differentiation of the gonad into testis or ovary. We also did not detect any differences in the gonads of *vasa*^{sa6158/sa6158} and *vasa*^{sa6158/sa6158};*tp53*^{M214K/M214K} fish at this stage (Fig. 6E and F). In half of the gonads examined, however, we noticed what appeared to be lymphocytic infiltration surrounding the gonad tissue, indicating possible resorption of dead or dying cells (Fig. 6H and I). This lymphocytic infiltration was observed in *vasa*^{sa6158}-mutant gonads independent of the presence or absence of the *tp53* mutation. Based on this analysis, we conclude that the *vasa*^{sa6158}-mutant germ line is lost due to failed differentiation and maintenance by a mechanism that is distinct from the p53-mediated apoptotic pathway.

Discussion

The molecular mechanisms that govern germ-line development in a popular genetic model, the zebrafish *Danio rerio*, are in the nascent stages of discovery. In this work, we provide the first description of a zebrafish mutant with a disrupted *vasa* gene. Despite its extensive conservation across animal (reviewed in Gustafson and Wessel, 2010; Hartung and Marlow, 2014), the function of *vasa* in germ cells differs between mice and fruit flies; it is required for spermatogenesis but dispensable for oogenesis in mice, and has precisely the opposite function in *Drosophila* (Schupbach and Wieschaus, 1986; Tanaka et al., 2000). In this study, we undertook a comprehensive analysis of the function of *vasa* in zebrafish, the results of which may shed light on its critical role in gametogenesis.

Zebrafish *vasa* is expressed in the germ line from the beginning of germ-cell specification in cleavage-stage embryos throughout gonadogenesis, and continually throughout

differentiation and establishment of adult gonads of both sexes (Baat et al., 2000; Knaut et al., 2000; Leu and Draper, 2010). In this study, we used zygotic *vasa*^{sa6158} mutants to show that maternal Vasa protein is stable until 10–12 d.p.f.. During this period of development, maternal Vasa is likely able to fulfill any potential roles for *vasa* in germ-cell determination, migration, proliferation, or maintenance. Previous loss-of-function studies using antisense oligonucleotides (morpholinos) targeted to zebrafish *vasa* determined that *vasa* morpholino-treated embryos had normal germ cells and germ-line establishment (Baat et al., 2001). Interestingly, the *vasa* morpholino effectively eliminated Vasa protein in embryos from 1 to 4 d.p.f., implying that Vasa protein is constantly turned over, and that translation is effectively blocked by the *vasa* morpholino (Baat et al., 2001). Thus, the maternal Vasa protein we are detecting in zygotic *vasa*^{sa6158} mutants is not itself stably inherited; rather, translation of maternally inherited *vasa* RNA is likely responsible for the wild-type abundance of Vasa protein until day 10–12 in *vasa*^{sa6158} mutants. Taken together, it appears that functional Vasa is not required for primitive gonad development from 1–4 and 10–21 d.p.f.. The results from Baat et al. (2001) and our current study cannot, however, exclude a function for maternal Vasa in zebrafish germ-cell specification; this can only be addressed by generating a conditional-mutant allele to disrupt maternal *vasa* expression.

The bipotential gonad is primarily composed of undifferentiated germ cells that resemble stage-Ia oocytes, which are morphologically defined by the presence of multiple oocytes in a shared follicle-cell layer, and with meiotic nuclei in the first three stages of meiotic prophase I: leptotene, zygotene, and pachytene (Draper, 2012; Selman, 1993; Siegfried and Nusslein-Volhard, 2008). Our findings indicate that zygotic *vasa* is not required for the formation of a bipotential gonad, as assessed by germ-cell morphology, germ-cell cluster size, and expression of germ-cell and meiotic markers. On the other hand, the bipotential gonad also contains rare, differentiated stage-Ib oocytes, which are characteristically enveloped in a private follicle cell layer (Selman, 1993) and contain a subcellular aggregate now known to be the Balbiani body (Fig. 8) (Draper et al., 2007). During stage Ib of oogenesis, the oocyte undergoes prophase-I arrest at the diplotene (or dictyate) stage. We found that d21 *vasa*^{sa6158} mutants express the primitive oocyte marker *pou5f3* (*oct4*) at levels comparable to their wild-type and heterozygous siblings, an indication that stage-Ia oocytes form normally in the bipotential gonad (Rodriguez-Mari et al., 2013). This is supported by the presence of zygotene- and pachytene-stage meiotic nuclei in *vasa*^{sa6158} mutants. Yet, *vasa*^{sa6158} mutants do not express *nanos3*, whose expression begins in stage-Ib oocytes (Draper et al., 2007). This is the first indication that *vasa* may be required for oocyte differentiation, but also hints at a possible role for *vasa* in the progression of meiosis to the diplotene stage in zebrafish.

During sexual determination, around d28, *vasa*^{sa6158} mutants seem to be transitioning to the male differentiation pathway, as judged by gonad morphology and the absence of oocyte markers *nanos3* and *zp3*. In zebrafish, gonad masculinization does not require the complete loss of germ cells for testis differentiation to proceed, but rather that germ-cell number fall below a threshold (Dranow et al., 2013; Houwing et al., 2007; Saito et al., 2008; Siegfried and Nusslein-Volhard, 2008). We note that *vasa*^{sa6158}-mutant germ cells at the pachytene stage have a peculiar vacuolar appearance, a possible indication of ensuing cell death.

Similar vacuolar appearance has been observed in oocytes of *zili* mutants, which also develop as sterile males (Houwing et al., 2008). Combined with the absence of stage-Ib oocytes in the bipotential gonad, these results indicate a role for *vasa* in the differentiation of germ cells past the pachytene stage of meiotic prophase I. A study using human germ cells differentiated from embryonic and induced pluripotent stem cells found that overexpression of *Vasa* increased germ-cell progression through meiosis at a low frequency (Medrano et al., 2012). Thus, in human cells, VASA may be sufficient for meiotic progression – which is consistent with observations reports for mice and fruit flies (Schupbach and Wieschaus, 1986; Tanaka et al., 2000). Zebrafish *vasa* also seems to be required for germ cell differentiation, and is likely required for progression past pachytene of meiotic prophase I. While zebrafish *vasa* is clearly required for progression of oogenesis, its requirement in spermatogenesis cannot be directly assessed since spermatogenic meiosis typically does not initiate until 30 to 35 d.p.f. (Rodriguez-Mari et al., 2005; Siegfried and Nusslein-Volhard, 2008).

Apoptosis of germ cells is the most-common, known mechanism whereby the germ line is lost in zebrafish (Houwing et al., 2007; Rodriguez-Mari et al., 2010; Shive et al., 2010; Weidinger et al., 2003). Loss of *Vasa* function in mice and fruit flies also point to apoptosis as the mechanism responsible for germ-cell loss (Schupbach and Wieschaus, 1986; Tanaka et al., 2000). We were therefore surprised to find that germ-cell loss in our *vasa^{sa6158}*-mutant gonads was not accompanied by hallmarks of apoptosis, such as activation of Caspase-3 or nuclear fragmentation. Even more compelling is that loss of *tp53* function does not rescue germ-cell loss, as has been demonstrated for zebrafish *brca2* and *fanc1* mutants (Rodriguez-Mari and Postlethwait, 2011; Shive et al., 2010). Although we noticed that pachytene-stage germ cells of d28 *vasa^{sa6158}* mutants have a peculiar vacuolar appearance, this phenotype does not seem to be associated with canonical apoptosis phenotypes based on the above analysis. On the other hand, the p53 homolog, p63, has recently been implicated as an important mediator of germ-cell death in failed meiotic progression in mice (Bolcun-Filas et al., 2014); therefore, it is possible that *vasa^{sa6158}*-mutant germ cells are lost through p63- or other non-p53-mediated apoptotic pathways.

Vasa is also integral to the piRNA biogenesis pathway, acting as a scaffold for the transposon transcripts being processed by Piwi proteins in the piRNA ping-pong amplification loop (Brennecke et al., 2007; Xiol et al., 2014). Studies in mice have shown that MVH mutants have phenotypes closely resembling those of murine knockouts for the piRNA processing proteins MILI and MIWI2 (mouse homologs of zebrafish *zili* and *ziwi*) (Deng and Lin, 2002; Kuramochi-Miyagawa et al., 2004; Kuramochi-Miyagawa et al., 2010; Tanaka et al., 2000). In zebrafish, however, the phenotype of the *ziwi* mutant centers on apoptosis in the bipotential gonad, as judged by Caspase-3 staining, and our analysis demonstrates that *vasa^{sa6158}*-mutant gonads do not undergo Caspase-3-mediated apoptosis at the bipotential stage or later (Houwing et al., 2007). On the other hand, mutants of the other *piwi* homolog, *zili*, fail to undergo meiosis and differentiation and lose their germ cells by 7 weeks of age (Houwing et al., 2008). The *zili*-mutant phenotype resembles the *vasa*-mutant phenotype in that germ-cell differentiation is the main defect and germ cells are ultimately lost through Caspase-3 independent mechanisms (Houwing et al., 2008). The

similarity between the *vasa*- and *zili*-mutant phenotypes suggests a shared mechanism for germ-cell differentiation involving the piRNA pathway, which may be conserved in mice (Kuramochi-Miyagawa et al., 2010). Although the similarity in zebrafish *vasa* and *zili* phenotypes supports a potential role for *vasa* in the piRNA pathway, the difference in timing of germ-cell loss between these two mutants and *ziwi* mutants suggest that other essential roles for *vasa* –perhaps in translational control of target RNAs– manifest prior to any potential requirement for *vasa* in piRNA biogenesis. Identification of the relevant RNA targets of Vasa in various species may help to explain these differences.

Our results indicate that zebrafish *vasa* is required for germ-cell differentiation in the d28 gonad (Fig. 8); however, this leaves a key aspect of the adult phenotype unresolved: the complete absence of germ cells, including undifferentiated stem cells in the gonad. There are two possible explanations for this observation: 1) differentiated germ cells may be required for maintenance of spermatogonial stem cells in zebrafish, or 2) zebrafish *vasa* has an additional function in stem-cell maintenance in adults. Based on reports that adult zebrafish mutants for *brca2* and the hypomorphic allele of *ziwi* contain undifferentiated spermatogonia and spermatocytes, while lacking fully differentiated spermatozoa (Houwing et al., 2007; Shive et al., 2010), it seems unlikely that zebrafish testes require fully differentiated cells to maintain the stem-cell population. The second model proposes that *vasa* itself is required to maintain the stem-cell population in differentiated gonads. Vasa function is required in *Drosophila* and sea urchin for chromosomal condensation and mitotic progression of germ cells (Pek and Kai, 2011; Yajima and Wessel, 2011). Our observation of reduced mitotic capacity in d28 *vasa*^{sa6158}-mutant germ cells and reduced expression of the germ-line stem-cell marker *nanos2* are consistent with these data, and suggest a possible role for zebrafish *vasa* in mitosis and proliferation of germ cells. Interestingly, the existence of an oogonial stem-cell population in the ovaries of mice and humans, while controversial, hinges on the isolation of a rare population of Vasa-positive cells (White et al., 2012; Woods and Tilly, 2013; Woods et al., 2013). In addition to roles in the germ line, Vasa has been implicated in somatic-stem-cell functions in flatworms (Pfister et al., 2008). Thus, in light of our findings and in the context of these studies, it seems plausible that zebrafish *vasa* may be required for germ-line stem-cell maintenance in differentiated adult gonads.

Materials and Methods

Fish strains

The zebrafish *vasa*^{sa6158} allele was obtained from The Sanger Institute's Zebrafish Mutation Project (Kettleborough et al., 2013). *tp53*^{M214K} (Berghmans et al., 2005) and *ziwi:GFP* transgenic reporter lines (Leu and Draper, 2010) were used in this study. Genotyping for the *tp53* allele was performed as previously described (Berghmans et al., 2005). All procedures and experimental protocols performed are in accordance with National Institutes of Health guidelines, and have been approved by the Einstein Institutional Animal Care and Use Committee.

***vasa*^{sa6158} genotyping**

Adult fin or whole larval samples were lysed in PCR buffer with the addition of 0.3% Tween20 (Fisher) and 0.3% Nonidet P40 (Roche), and 0.2 mg/mL Proteinase K (Roche). Samples were lysed overnight at 55°C, and the lysis reaction was heat-killed at 95°C for 10 minutes. To identify fish harboring the *sa6158* allele, we designed a derived Cleaved Amplified Polymorphic Sequence (dCAPS) genotyping assay (Neff et al., 1998) that introduced a cytosine 3' to the point mutation, creating a RsaI restriction site within the PCR-amplified mutant allele. Lysate was used as 10% of the total PCR reaction volume using the following *vasa*^{sa6158} dCAPS primers: 5'-GCCACCTGCATTTTCACTTCCTTCTTGATCG and 5'-GGCCGGAATGAGGAAGTATT. After 40 cycles of PCR at 60°C annealing, samples were digested for one hour using Fastdigest RsaI (Thermoscientific) or conventional RsaI (New England Biolabs). Digested PCR products were resolved using a 1.5% Ultrapure agarose (Invitrogen) and 1.5% Metaphor agarose (Lonza) gel.

Immunofluorescence

Larvae or juvenile fish were euthanized, fixed overnight in 4% paraformaldehyde at 4°C, washed in phosphate-buffered saline (PBS), then fixed for at least one day in 100% methanol. Samples were rehydrated, permeabilized in acetone at -20°C for 12 minutes, washed, and incubated with antibodies overnight at 4°C. Rabbit anti-Vasa antibody (courtesy of Dr. Knaut) was used at 1:5000 dilution; chicken anti-GFP antibody (Invitrogen) was used at 1:500 dilution; and rabbit anti-cleaved Caspase 3 (Sigma) was used at 1:500 dilution. Vectashield (Vector Labs) containing DAPI was used to mount samples and label nuclei. Images were acquired using a Zeiss Axio Observer inverted microscope equipped with Apotome and a CCD camera. Images were processed in ImageJ (National Institutes of Health), Adobe Photoshop, and Adobe Illustrator.

Histology

H&E staining was performed as previously described (Heim et al., 2014). Briefly, fish were euthanized, their trunks were dissected, fixed overnight in 4% paraformaldehyde at 4°C, washed in PBS, and fixed in 100% methanol for at least 1 day. Trunks were decalcified in Immunocal solution (Decal Chemical Corp) overnight at room temperature. After paraffin embedding and sectioning onto slides, tissue was deparaffinized, stained with H&E, coated with Permount solution (Fisher Scientific), coverslipped, and imaged using an AxioSkop2 microscope and AxioCam CCD camera.

Reverse-transcriptase polymerase chain reaction (RT-PCR)

For RT-PCR from adult fish, RNA was isolated from dissected gonads. For RT-PCR from juvenile (d21 and 28 d.p.f.) fish, RNA was isolated from trunks. Tissue was stored in RNAlater (Ambion) at -20°C for 1 day up to 1 month. RNA was extracted using Trizol (Life Technologies), followed by oligo(dT) cDNA synthesis (Invitrogen SuperScript III RT kit). RT-PCR was performed using the following primers: *dazl* forward 5'-TCCGTCCTCCCTGAAGTTGTCT and reverse 5'-TCGTCCTTTGCTCTCCACACCA; *nanos3* forward 5'-AGGACTACATGGGTTTGGCGGA and reverse 5'-

CAATCCGCCGCGTATCAACCAA; *nanos2* forward 5'-TGATGCAGTCCGAGAGTCAG; *nanos2* reverse 5'-CGTCCCTTGCCTTTAGTCTG; *pou5f3* forward 5'-TCATCCCCATCTCTGTCTCC and reverse 5'-CAGAGTGATGCGCTTGTGTT. Primers for *vasa* and *efl1a* (Heim et al., 2014), *sycp3* (Chen et al., 2010), *zp3* (Liu et al., 2006), *cyp19a1a* and *amh* (Siegfried and Nusslein-Volhard, 2008), and *cyp11c1* (Wang and Orban, 2007) were as previously described. Forty PCR cycles were run at an annealing temperature of 57°C.

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Abbreviations

d#	day #
d.p.f	days post fertilization
GFP	green fluorescent protein
H&E	hematoxylin and eosin
RT-PCR	reverse-transcriptase PCR
Vasa/DDX4/MVH	DEAD-box helicase 4. Genes
brca2	breast cancer antigen 2
cyp11c1	cytochrome P450 family 11 subfamily C1
dazl	deleted in azoospermia-like
fancl	fanconi anemia complementation group L
pou5f3	Pou domain class 5 transcription factor 3
sycp3	synaptonemal complex protein 3
tp53	tumor suppressor protein p53
zp3	zona pellucida 3

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Figure 1. The *vasa*^{sa6158} allele results in a truncated protein lacking helicase domains
 (A) Diagram of zebrafish Vasa protein structure, with the *vasa*^{sa6158} allele resulting in a truncated protein lacking annotated Q-motif, ATP-binding motif, and helicase domains. (B) Seven annotated zebrafish *vasa* transcript variants are illustrated. Alternatively spliced exons are indicated in yellow, and the conserved mutated exon in the *vasa*^{sa6158} allele is indicated in red. (C) Chromatography data from wild-type and *vasa*^{sa6158}-mutant genomic DNA. The arrow indicates the T-to-A *vasa*^{sa6158} mutation, whereas the arrowhead points to the nucleotide mutation (G-to-C) inserted by the dCAPs primer to create a RsaI site. (D) The dCAPs PCR-based genotyping assay utilizes the RsaI restriction site in the *vasa*^{sa6158} allele. Heterozygotes have both wild-type (upper) and mutant (lower) bands.

Figure 2. Maternal Vasa is stable throughout early germ-line and gonad development

(A, B) Lateral view (rostral to the left) of Vasa staining in germ cells in 10 d.p.f. larvae. The swim bladder (SB) is outlined in white. (C, D) Vasa staining in 12 d.p.f. larvae. Yellow outline marks the gut. (E, F) Vasa protein in 14 d.p.f. larvae. The blue arrowheads point to germ cells. (G) Table of embryonic and larval stages examined for Vasa staining in germ cells. (H, I) In situ hybridization for the germ-cell marker *nanos3* expressed in germ cells of the shield-stage (H) and 30-hours-post-fertilization (I) embryos from *vasa^{sa6158/+}* in-crosses. Dorsal is oriented toward the top in both panels. Scale bars, 100 μ m (A–F, H–I).

Figure 3. *vasa*^{sa6158}-mutant testes lack germ cells

(A–E) Lateral views (rostral to the left) show overall morphology of wild-type (A, D), *vasa*^{sa6158} heterozygote (B, E), and mutant fish (C). (A'–E') Lateral views (rostral to the left) of dissections reveal visible gonads in situ, outlined in white, in wild-type (A', D') and *vasa*^{sa6158} heterozygote (B', E') fish, but not *vasa*^{sa6158} mutants (C'). SB, swim bladder; L, liver. (A'', B'') H&E-stained sections of wild-type and *vasa*^{sa6158}-heterozygous testes with numerous visible spermatocytes (SC) and spermatozoa (SZ) within tubules (outlined in yellow). (C'') *vasa*^{sa6158}-mutant testis tubules have no detectable germ cells. (D'', E'') Oogenesis is normal in wild-type (D'') and *vasa*^{sa6158}-heterozygous females (E''). (F) Side-by-side comparison of dissected wild-type (top) and *vasa*^{sa6158}-mutant (bottom) testis. (G) RT-PCR of dissected gonads from adult wild-type, *vasa*^{sa6158}-heterozygote, and mutant fish. Scale bars, 0.1 cm (A'–E'); 10 μ m (A''–E'', F).

Figure 4. Zygotic *vasa* is not required for the formation of the bipotential gonad

(A, B) Lateral view (rostral to the left) of d21 *Tg[ziwi:GFP]*-positive juveniles taken through the body wall. (C, D) Meiotic nuclei stained with DAPI. P, pachytene. (E) RT-PCR on samples from individual d21 juveniles. (F–F'', G–G'') Germ-cell (GC) clusters of d21 *Tg[ziwi:GFP];vasa^{sa6158/+}* (F–F'') and *Tg[ziwi:GFP];vasa^{sa6158/sa6158}* (G–G'') gonads. (H) Quantification of germ-cell cluster size in d21 juveniles. Scale bars, 200 μ m (A, B); 20 μ m (C, D, F–F'' and G–G'').

Figure 5. Caspase-3 staining in d28 *vasa*^{sa6158} mutants and siblings

(A, B) Lateral view (rostral to the left) of d28 *Tg[ziwi:GFP]*-positive juveniles taken through the body wall. (C–E) *Tg[ziwi:GFP];vasa*^{sa6158/+} and *Tg[ziwi:GFP];vasa*^{sa6158/sa6158} gonads stained for GFP and active (cleaved) Caspase-3. (F) Intestinal tissue stained for GFP and active Caspase-3 served as a positive control for Caspase-3. Scale bars, 250 μ m (A, B); 100 μ m (C–F). Lateral views of organs (C–E) are oriented with the rostral-caudal axis aligned from top to bottom.

Figure 6. Zygotic *vasa* is required for meiotic progression, germ-cell differentiation, and germ-cell cluster development

(A–A'') Meiotic nuclei and of *Tg[ziwi:GFP];vasa^{sa6158/+}* d28 female. P, pachytene; D, diplotene. (B–B'') Meiotic nuclei and germ-cell (GC) clusters of a *Tg[ziwi:GFP];vasa^{sa6158/+}* d28 male. (C–C'') Meiotic nuclei and GC clusters of a *Tg[ziwi:GFP];vasa^{sa6158/sa6158}* d28 male. Red arrowheads point to germ cells with a vacuolar phenotype. (D) Quantification of germ-cell cluster size in d28 juveniles. Error bars indicate the 95% confidence interval. (E) RT-PCR on samples from individual d28 juveniles. Scale bars, 20 μ m (A–C).

Figure 7. Concurrent loss of *tp53* does not rescue germ-cell loss in *vasa*^{sa6158} mutants
 (A–C) Transverse sections through the whole trunk of wild-type (A), *vasa*^{sa6158/sa6158} (B), and *vasa*^{sa6158/sa6158;tp53^{M214K/M214K}} (C) fish identify the testis in situ (indicated by blue arrowheads). Dorsal is to the top. (A', B', C') Higher magnification images of testes reveal that both *vasa*^{sa6158/sa6158} (B') and *vasa*^{sa6158/sa6158;tp53^{M214K/M214K}} (C') contain no germ cells within the somatic tubules. (D, G) Wild-type differentiated gonads of d42 female (D) and male (G) fish. (E) d42 *vasa*^{sa6158/sa6158} gonad. (F) d42 *vasa*^{sa6158/sa6158;tp53^{M214K/M214K}} gonad. (H, I) D42 *vasa*^{sa6158/sa6158} (H) and *vasa*^{sa6158/sa6158;tp53^{M214K/M214K}} (I) gonads both show evidence of lymphocytic infiltration (indicated by black arrowheads). Scale bars, 0.1 cm (A–C); 10 μ m (A'–I),

A. (wild type) **B.** (mutant) **Figure 8. Proposed model for *vasa* function during gonad differentiation**

(A) In normal zebrafish gonadogenesis, the bipotential gonad forms with numerous stage-Ia oocytes in meiotic prophase I (zygotene and pachytene) and rare stage-Ib oocytes (meiosis I arrested at the diplotene stage). As sexual differentiation takes place, oogenesis can either be sustained, resulting in female sexual development, or oocytes will undergo apoptosis and be replaced by spermatogonia, resulting in male sexual development. (B) We propose that in *vasa*^{sa6158} mutants, stage-Ia oocytes are unable to progress to stage Ib of oogenesis. As differentiation proceeds, pachytene-stage oocytes display a vacuolated phenotype and are lost by p53-independent mechanisms. Although they are specified in the bipotential gonad, germ-line stem cells (GSCs) are not maintained in *vasa* mutants, ultimately resulting in an empty testis devoid of germ cells and sterility.