Germ Cell Lineage Homeostasis in *Drosophila* Requires the Vasa RNA Helicase

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**ABSTRACT** The conserved RNA helicase Vasa is required for germ cell development in many organisms. In *Drosophila melanogaster* loss of PIWI-interacting RNA pathway components, including Vasa, causes Chk2-dependent oogenesis arrest. However, whether the arrest is due to Chk2 signaling at a specific stage and whether continuous Chk2 signaling is required for the arrest is unknown. Here, we show that absence of Vasa during the germarial stages causes Chk2-dependent oogenesis arrest. Additionally, we report the age-dependent decline of the ovariole number both in flies lacking Vasa expression only in the germarium and in loss-of-function *vasa* mutant flies. We show that Chk2 activation exclusively in the germarium is sufficient to interrupt oogenesis and to reduce ovariole number in aging flies. Once induced in the germarium, Chk2-mediated arrest of germ cell development cannot be overcome by restoration of Vasa or by downregulation of Chk2 in the arrested egg chambers. These findings, together with the identity of Vasa-associated proteins identified in this study, demonstrate an essential role of the helicase in the germ cell lineage maintenance and indicate a function of Vasa in germline stem cell homeostasis.

**KEYWORDS** vasa; Vasa helicase; Chk2; piRNA pathway; germline; oogenesis

**DEVELOPMENT** of the *Drosophila* female gonad begins during the third larval instar with the formation of 16–25 somatic niches that will give rise to the future germaria (Panchal et al. 2017). Each germarium hosts germline stem cells (GSCs) that produce the germ cell lineage (Wieschaus and Szabad 1979). In adult females, germ cell development begins with the division of a GSC into a self-renewing stem cell and a differentiating daughter cell, the cystoblast (CB). The CB undergoes four rounds of mitosis with incomplete cytokinesis, such that a stage 1 egg chamber is ultimately composed of an oocyte and 15 nurse cells, surrounded by a layer of follicular epithelial cells [reviewed in Gilboa and Lehmann (2004)]. A newly formed egg chamber buds off from the germarium and joins a linear array of developing egg chambers to form an ovariole. Each *Drosophila* ovary consists of 16–25 ovarioles, corresponding to the number of germaria formed in the third-instar larva.

*Drosophila* oogenesis has been intensively studied and many genes found to regulate development of the germ cell lineage. Among the germline proteins essential for oogenesis is the conserved RNA helicase Vasa (Vas). Vas is expressed throughout oogenesis and localizes to the posterior pole of the oocyte and early embryo. In situations leading to absence of Vas from the oocyte posterior pole, germline and posterior patterning determinants fail to localize, germ (or pole) plasm does not form, and the resulting embryos lack posterior structures and primordial germ cells (Lasko and Ashburner 1988, 1990; Hay et al. 1990). In contrast to late oogenesis and embryos, little is known about the role of Vas during early oogenic stages. In early oogenesis *vas* has been implicated in the translational control of *mei-p26* and in regulation of GSC mitotic chromosome condensation (Liu et al. 2009; Pek and Kai 2011b). Complete absence of *vas* triggers oogenesis arrest induced by checkpoint kinase 2 (Chk2) (Lasko and Ashburner 1990; Durdevic et al. 2018). Whether Chk2 is activated at a specific stage and whether continuous Chk2 signaling is required to arrest oogenesis has been unknown.

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Using GFP-fused wild-type and trapping mutant (E400Q) Vas, we identified new Vas-associated proteins, several of which have a function in early germ cell development. To address the importance of Vas activity in early germ cell lineage development, we took a genetic approach. We found that, in addition to the previously described oogenesis arrest (Lasko and Ashburner 1988, 1990), loss-of-function vas mutation causes an age-dependent reduction of the number of egg chamber–producing ovarioles. Our study reveals that sole exclusion of Vas from the germarium causes Chk2-dependent arrest of oogenesis and a reduction of ovariole number in aging flies. Importantly, once induced in the gerarium, Chk2-mediated oogenesis arrest and germline proliferation decline are not overcome by downregulation of Chk2 at later oogenic stages. Our study shows that Chk2 activity exclusively in the gerarium is sufficient to interrupt germ cell development. Activity of Vas RNA helicase early in oogenesis is essential to prevent activation of Chk2 signaling and license the germline component of the Drosophila ovary for further development.

Materials and Methods

Fly stocks and husbandry

The following Drosophila stocks were used: w^{1118}, vas^{cn1} b^{l}/CyO (vas^{P0}, Schüpbach and Wieschaus 1986), b^{l} vas^{jd}/CyO (vas^{D1}, Tearel and Nusslein-Volhard 1987; Lasko and Ashburner 1990), P(w^{+}mC = vas-GAL4.2.6)/TM6B (Kyoto Drosophila Genomics Resource Center: 109997), y^{1} w*; P(mata4-GAL-VP16)67; P(mata4-GAL-VP16)15 (matTub-Gal4, FBST0080361), P(w^{+}mC = UASp-GFP.vas^{WT})/TM2 and P(w^{+}mC = UASp-GFP.vas^{DQAD})/VK00033/TM2 (GFP-vas^{WT} and GFP-vas^{DQAD}, Xiol et al. 2014), P(Trip.GL00020)attP2/TM3 (TripSm, FBST0035573), P(Trip.GL00094)attP2 (TripPw, FBST0035573), and P{Ubi-GFP.S65T}PAD1 (GFP, FBST004888). All flies were kept at 25°C on standard Drosophila medium.

Generation of transgenic flies and expression of the transgenes

The vas transgene carrying the K295N substitution (vas^{GNT}) was created by introducing the point mutation in wild-type vas complementary DNA sequence by site-directed mutagenesis using QuikChange II XL Site-Directed Mutagenesis Kit (Agilent). The transgene is subsequently cloned downstream of GFP into the pUASP-K10attB vector (GenBank: EU729723.1, Koch et al. 2009), from which the K10 sequence was removed. Transgenes were integrated into the attP transposable element insertion site of the landing site line P{nos-phiC31/intNLs}; P{Bac[y+]attP3B/VK00033} (FBST0024871).

All transgenes are expressed using the GAL4/UAS-system. Gal4-drivers were under control of two promoters with distinct expression patterns: vas-Gal4 is expressed throughout oogenesis and matTub-Gal4 is excluded from the gerarium (Supplemental Material, Figure S1B).

Fecundity and hatching assays

Virgin females of w^{1118} control and vas^{PD/D1} and vas^{D1/D1} genetic backgrounds with or without expressed transgenes were mated with w^{1118} males for 24 hr at 25°C. The crosses were then transferred to apple-juice agar plates, which were used to collect eggs at 24 hr intervals over 3 or 3–20 days. The number of laid eggs on each plate was counted and the plates were kept at 25°C for 24 hr and the number of hatched larvae was also counted (Table S1 and Table S2). Experiments were performed in five independent replicates.

Ovarian morphology and quantification of ovariole number

Ovaries were dissected from 3- to 20-day-old flies in PBS. To assess ovarian morphology, ovaries were directly imaged on Olympus SZX16 stereomicroscope. The length of the ovaries was measured using Fiji (Table S3, Table S4, and Table S5). For determination of egg chamber–producing ovariole number females were frozen and held at ~20°C before dissection. Ovaries were manually dissected under magnification in a drop of PBS. The ovarioles were gently separated from each other using wolfram needles. The ovariole number of each female was defined as a summary of the number of egg chamber–containing ovarioles in the right and left ovary (Table S6 and Table S7).

Vas localization analysis

For Vas localization, ovaries from wild-type flies (w^{1118}) and vas mutants (vas^{PD/D1} and vas^{D1/D1}) were fixed by incubation at 92°C for 5 min in preheated fixation buffer (0.4% NaCl, 0.3% Triton X-100 in PBS), followed by extraction in 1% Triton X-100 for 1 hr at room temperature (RT). Fixed ovaries were incubated with primary antibodies Vas (rat; 1:500; Tomancak et al. 1998) and subsequently with secondary antibodies Alexa Fluor 647 conjugated donkey anti-rat IgG (1:1000; Jackson ImmunoResearch). Ovaries from flies expressing fusion proteins were fixed in 2% paraformaldehyde and 0.01% Triton X-100 for 15 min at RT. Fixed ovaries were mounted on glass slides for examination of GFP fluorescence for wild-type Vas using a Zeiss LSM 780 confocal microscope. Nuclei were visualized with DAPI.

Cuticle preparation

To examine larval cuticles, eggs were allowed to develop fully for 24 hr at 25°C, dechorionated in bleach, and then transferred to a microscope slide bearing a drop of Hoyer’s medium mixed 1:1 with lactic acid. Cuticle preparations were heated at 65°C overnight before examination using Zeiss Axioshot microscope. The number of counted larvae with or without abdomen is represented in Table S8.

Immunohistochemical staining of ovaries and embryos

Freshly hatched females were mated with wild-type males and kept for 2–3 days on yeast at 25°C before dissection. Ovaries
were dissected in PBS and immediately fixed by incubation at 92° for 5 min in preheated fixation buffer (0.4% NaCl, 0.3% Triton X-100 in PBS), followed by extraction in 1% Triton X-100 for 1 hr at RT. Fixed ovaries were incubated with primary antibodies Aub (rabbit; 1:500; Homolka et al. 2015), Ago3 (mouse; 1:250; Gunawardane et al. 2007), and Vas (rat; 1:500; Tomancak et al. 1998). The following secondary antibodies were used: Alexa Fluor 488 conjugated goat anti-rabbit (1:1000; Invitrogen, Carlsbad, CA), anti-mouse IgG (1:500; Invitrogen), and Alexa Fluor 647 conjugated donkey anti-rat IgG (1:1000; Jackson ImmuNoResearch). Nuclei were stained with DAPI.

For embryo staining, freshly hatched females were mated with wild-type males and fed with yeast for 2–3 days at 25° before egg collection. Embryos (0–1 or 1–3 hr) were collected and dechorionated in 50% bleach, then fixed by incubation at 92° for 30 sec in preheated fixation buffer (0.4% NaCl, 0.3% Triton X-100 in PBS), followed by devitellinization by rigorous shaking in a 1:1 mix of heptane and methanol. After washing in 0.1% Tween-20, embryos were either immediately incubated with primary antibodies against Aub (rabbit; 1:500; Homolka et al. 2015), Ago3 (mouse; 1:250; Gunawardane et al. 2007), and Vas (rat; 1:500; Tomancak et al. 1998), or stored in methanol at −20° for staining later on. The following secondary antibodies were used: Alexa Fluor 488 conjugated goat anti-rabbit (1:1000; Invitrogen), anti-mouse IgG (1:500; Invitrogen), and Alexa Fluor 647 conjugated donkey anti-rat IgG (1:1000; Jackson ImmuNoResearch). Nuclei were stained with DAPI.

The samples were observed using a Zeiss LSM 780 or Leica SP8 confocal microscope. Oocytes and embryos with Aub- and Ago3-positive pole plasm were counted in three independent replicates (Table S9 and Table S10).

**Protein extraction and Western blotting**

For whole protein lysates of ovaries, ~20 pairs of ovaries from 3- to 5-day-old flies were homogenized in protein extraction buffer [25 mM Tris, pH 8.0, 27.5 mM NaCl, 20 mM KCl, 25 mM sucrose, 10 mM EDTA, 10 mM EGTA, 1 mM DTT, 10% (v/v) glycerol, 0.5% NP40, 1% Triton X-100, 1× Protease Inhibitor Cocktail; Roche]. For whole protein lysates of ovaries, 3- to 5-day-old \( \text{vas}^{PD/D1}; \text{vas-Gal4} > \text{GFP-vas}^{WT} \), \( \text{vas}^{PD/D1}; \text{vas-Gal4} > \text{GFP-vas}^{UQ10} \) and control Act5C-Gal4/CyO; Ubi-GFP flies were dissected in PBS and homogenized in protein extraction buffer [25 mM Tris, pH 8.0, 27.5 mM NaCl, 20 mM KCl, 25 mM sucrose, 10 mM EDTA, 10 mM EGTA, 1 mM DTT, 10% (v/v) glycerol, 0.5% NP40, 1% Triton X-100, 1× Protease Inhibitor Cocktail; Roche]. For whole protein lysates of ovaries, 3- to 5-day-old vasPD/D1; vas-Gal4 > GFP-vasWT, \( \text{vas}^{PD/D1}; \text{vas-Gal4} > \text{GFP-vas}^{UQ10} \) and control Act5C-Gal4/CyO; Ubi-GFP flies were dissected in PBS and homogenized in protein extraction buffer [25 mM Tris, pH 8.0, 27.5 mM NaCl, 20 mM KCl, 25 mM sucrose, 10 mM EDTA, 10 mM EGTA, 1 mM DTT, 10% (v/v) glycerol, 0.5% NP40, 1% Triton X-100, 1× Protease Inhibitor Cocktail; Roche]. After 10 min incubation on ice an equal volume of protein extraction buffer without detergents was added to the samples, and the samples were centrifuged twice for 15 min at 16,000 × g. Immunoprecipitations were performed using GFP-trap magnetic agarose beads (ChromoTek) at 4° for 1 hr on 20 mg of protein lysates. The beads were washed five times for 5 min at 4° in 1× in protein extraction buffer, then 1× in high-salt buffer (25 mM Tris, pH 8.0, 1 M NaCl, 0.5% NP40, 1% Triton X-100, 1× Protease Inhibitor Cocktail; Roche), then in 1× medium salt buffer (25 mM Tris, pH 8.0, 0.5 M NaCl, 0.5% NP40, 1% Triton X-100, 1× Protease Inhibitor Cocktail; Roche), then in 1× low-salt buffer (25 mM Tris, pH 8.0, 150 mM NaCl, 0.5% NP40, 1% Triton X-100, 1× Protease Inhibitor Cocktail; Roche), and finally, in 1× low-salt buffer without detergents (25 mM Tris, pH 8.0, 150 mM NaCl, 1× Protease Inhibitor Cocktail; Roche). After washing, precipitated proteins were eluted from the magnetic beads in elution buffer (200 mM glycine, pH 2.5) and neutralized with 1/10 volume of 1 M Tris base (pH 10.4).

Proteomic experiments were performed as described in Casabona et al. (2013). In brief, three samples were stacked in the SDS polyacrylamide gel and after Coomassie staining each lane was cut into three blocks, which were processed separately. After digestion with trypsin (sequencing grade; Promega, Madison, WI), the resulting peptides were analyzed by liquid chromatography with tandem mass spectrometry (LTQ-Orbitrap Velos pro; Thermo Fisher Scientific). Peptides and proteins from each mass spectrometry run were identified using Scaffold software and results for each lane were displayed. The selection criteria for the displayed proteins were as follows: a minimum of two peptides per protein should be identified and the peptide Mascot score should be at least 20. The experiment was performed in two biological replicates and specific interaction partners were determined.
by statistical analysis of control and positive samples using extracted spectral counts. A protein was considered as a high-confidence binding partner if its enrichment was $\geq 2$ and the $P$-value was $\leq 0.05$ in positive immunoprecipitations compared to controls. All proteins that showed enrichment $\geq 2$ but had a higher $P$-value were considered low-confidence hits. $P$-values were computed using the web-based Quantitative Proteomics $P$-value Calculator (Chen et al. 2014), which applies a distribution-free permutation method based on simulation of the log(ratio). A pseudocount of 1 was used in all samples for proteins with no spectral counts. Unweighted spectrum counts for both replicates and the results of the statistical analysis are provided in Table S11. Analyses of gene ontology enrichment of biological processes and cellular components (Table S11) are based on Bioconductor package clusterProfiler (https://bioconductor.org/packages/release/bioc/html/clusterProfiler.html).

To validate the association of Vas with proteins identified in our proteomic analysis, immunoprecipitation of GFP and GFP-VasDQAD was performed as described above, using 30 mg of protein lysates. Co-immunoprecipitated proteins were detected by Western blotting using antibodies directed against Arm (goat; 1:500; Santa Cruz Biotechnology), Aub (rabbit; 1:1000; Homolka et al. 2015), Bel (rabbit; 1:500; Pek and Kai 2011a), GFP (rabbit; 1:5000; Chemokine), Nop60B (rabbit; 1:500; Riccardo et al. 2007), PABP (rabbit; 1:5000; kind gift from Matthias Hentze), Rm62 (rabbit; 1:250; Lei and Corces 2006), and Tub (mouse; 1:10,000, TS168; Sigma). The following secondary antibodies were used: HRP-conjugated donkey anti-rabbit (1:10,000; GE Healthcare), HRP-conjugated sheep anti-mouse IgG (1:10,000; GE Healthcare), and HRP-conjugated rabbit anti-goat IgG (1:10,000; Sigma). To reprobe Western blot membranes with another antibody, we removed bound primary and secondary antibodies from membranes using Restore PLUS Western reagent (Thermo Scientific) according to the manufacturer’s recommendations.

Fluorescence in situ RNA hybridization

Fluorescence in situ RNA hybridization experiments were performed as described in Gaspar et al. (2017). In brief, ovaries were dissected in PBS and immediately fixed in 2% paraformaldehyde and 0.05% Triton X-100 in PBS for 20 min at RT. After washing in PBT (PBS plus 0.1% Triton X-100) samples were treated with 2 $\mu$g/ml proteinase K in PBT for 5 min and then were subjected to 95°C in PBS plus 0.05% SDS for 5 min. Samples were prehybridized in 200 $\mu$l hybridization buffer (300 mM NaCl, 30 mM sodium citrate, pH 7.0, 15% ethylene carbonate, 1 mM EDTA, 50 $\mu$g/ml heparin, 100 $\mu$g/ml salmon sperm DNA, 1% Triton X-100) for 10 min at 42°C. Fluorescence-labeled oligonucleotides (12.5–25 nM) were prewarmed in hybridization buffer and added to the samples. Hybridization was allowed to proceed for 2 hr at 42°C. Samples were washed three times for 10 min at 42°C in prewarmed buffers (1× hybridization buffer, then 1× hybridization buffer:PBT in a 1:1 mixture, followed by 1× PBT). The final washing step was performed in prewarmed PBT at RT for 10 min. The samples were mounted in 80% 2,2-thiodiethanol in PBS and analyzed on a Leica SP8 confocal microscope.

Labeling of DNA oligonucleotides for fluorescence in situ RNA hybridization

Labeling of the oligonucleotides was performed as described in Gaspar et al. (2017). Briefly, nonoverlapping arrays of 18–22 nt long DNA oligonucleotides complementary to mnk (Table S12) were selected using the smFISHprobe_finder.R script (Gaspar et al. 2017). An equimolar mixture of oligos for a given RNA was fluorescence-labeled with Alexa Fluor 565– or Alexa Fluor 633–labeled ddUTP using terminal deoxynucleotidyl transferase. After ethanol precipitation and washing with 80% ethanol, fluorescence-labeled oligonucleotides were reconstituted with nuclelease-free water.

RNA extraction and quantitative PCR analysis

Total RNA was extracted from ovaries of 3-day-old flies using TRIzol reagent (Thermo Fisher Scientific). For first-strand complementary DNA synthesis, RNA was reverse-transcribed using the QuantiTect Reverse Transcription Kit (QIAGEN, Valencia, CA). Quantitative PCR was performed on a StepOne Real-Time PCR System (Thermo Fisher Scientific) using SYBR Green PCR Master Mix (Thermo Fisher Scientific). Experiments were performed in biological triplicates with technical triplicates. Relative RNA levels were calculated by the 2$^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001), normalized to rp49 mRNA levels and normalized to respective RNA levels from w$^{118}$ flies. Sequences of primers used for quantitative PCR reaction are presented in Table S12.

Data availability

The authors declare that all data supporting the findings of this study are available within the manuscript and its supplemental files. Supplemental material available at FigShare: https://doi.org/10.25386/genetics.9026579.

Results

Vas helicase activity is required for Drosophila oogenesis

To investigate effects of Vas helicase activity on germ cell lineage development, we used the UAS/GAL4 system to manipulate the expression of wild-type Vas and of two Vas helicase mutants. The Vas mutant proteins contain amino acid substitutions that affect helicase activity at different points in the enzymatic process: the K295N (GKT$^\text{DDQAD}$) substitution prevents release of the ATP-hydrolysis products from the enzyme, whereas the E400Q (DEAD$^\text{DDQAD}$) mutation prevents release of the ATP-hydrolysis products and locks the helicase in an open conformation, whereas the E400Q (DEAD$^\text{DDQAD}$) mutation prevents release of the ATP-hydrolysis products and locks the helicase in a closed conformation (Xiol et al. 2014) (Figure S1A). To monitor expression and localization of the different Vas proteins, we fused them with GFP (GFP-Vas$^{\text{DDQAD}}$, GFP-VasGNT, and GFP-VasWT).

We tested the ability of the proteins to provide Vas function and suppress the oogenesis arrest displayed by vas$^{D1/D1}$
mutants and rescue the abdominal defects (posterior group phenotype) of embryos produced by the hypomorphic vas<sup>PD/D1</sup> females. We assessed ovarian morphology and quantified ovary length as a measure of oogenesis rescue (Figure S1C). The GFP-Vas<sup>WT</sup> transgene fully restored oogenesis to the loss of function vas<sup>PD/D1</sup> flies, whereas the helicase mutants GFP-Vas<sup>DQAD</sup> and GFP-Vas<sup>GNT</sup> did not (Figure 1A and Figure S1D). Furthermore, Vas function provided by the GFP-Vas<sup>WT</sup> transgene promoted abdomen formation in ~50% of embryos produced by both vas<sup>PD/D1</sup> and vas<sup>PD/D1</sup> females (Figure 1, B and C). In contrast, the embryos produced by helicase inactive GFP-Vas<sup>DQAD</sup> and GFP-Vas<sup>GNT</sup> expressing vas<sup>PD/D1</sup> females had a strong posterior group phenotype and did not hatch (Figure 1, B and C). These results suggest that the helicase activity of Vas is required for oogenesis and embryogenesis.

**Localization of PIWI proteins is affected by Vas’s helicase activity**

Localization of Vas in the egg chamber is independent of RNA-binding and helicase activity (Liang et al. 1994; Dehghani and Lasko 2016). We analyzed whether the E40Q and K295N mutations (Figure S1A), which impair Vas helicase activity differently, locking the enzyme in a closed or in an open conformation, respectively, affect localization of the protein. Additionally, as Aub and Ago3 colocalize with Vas (Liang et al. 1994; Malone et al. 2009), we tested if the localization of these two PIWI proteins in the egg chamber and embryo is affected by Vas helicase mutations.

Localization to nurse cell nuage was impaired in the case of GFP-Vas<sup>DQAD</sup> (closed conformation) (Xiol et al. 2014), whereas GFP-Vas<sup>WT</sup> and GFP-Vas<sup>GNT</sup> (open conformation) showed wild-type localization (Figure 2, A and B). This was true for the localization of Aub and Ago3 (Figure S2, A and B and Figure S3A). These findings indicate that an open helicase conformation of the Vas is required for its correct localization, as well as for the localization of Aub and Ago3, whereas helicase activity per se is not.

In oocytes and embryos, GFP-Vas<sup>WT</sup> showed a wild-type localization at the posterior pole (Figure 2, A and B), whereas GFP-Vas<sup>DQAD</sup> was not detected. Further, although we could detect GFP-Vas<sup>GNT</sup> at the posterior pole of the oocyte and the protein was transmitted to the embryo, it was not detected at the posterior pole (Figure 2B and Figure S1E). In the presence of GFP-Vas<sup>WT</sup> Aub and Ago3 showed wild-type localization in oocytes and embryos, whereas GFP-Vas<sup>GNT</sup> only partially restored localization of the two PIWI proteins (Figure 3, A and B, Figure S2, C and D, and Figure S3, B–D). These observations indicate that helicase activity of Vas is necessary for stable localization of the protein itself and of Aub and Ago3 at the embryo posterior pole.

**Transposons are deregulated in flies expressing Vas helicase mutants**

Interaction of Vas with PIWI proteins in the perinuclear nuage of nurse cells is required for PIWI-interacting RNA (piRNA) biogenesis and transposon control (Xiol et al. 2014; Nishida et al. 2015). To investigate the effects of Vas mutations on transposon control, we analyzed RNA levels of several transposons. Quantitative PCR analysis showed that expression of GFP-Vas<sup>WT</sup> and GFP-Vas<sup>DQAD</sup> but not GFP-Vas<sup>GNT</sup> caused resciling of transposons in vas<sup>PD/D1</sup> female germline (Figure

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**Figure 1** Vasa helicase activity is essential for germline and embryo development. (A) Box plot representing length of ovaries of wild-type (w<sup>1118</sup>, vas<sup>D1/D1</sup>, vas<sup>D1/D1</sup>, vas-Gal4 > GFP-Vas<sup>WT</sup>, vas<sup>D1/D1</sup>; vas-Gal4 > GFP-Vas<sup>DQAD</sup>, vas<sup>D1/D1</sup>; vas-Gal4 > GFP-Vas<sup>GNT</sup>, vas<sup>D1/D1</sup>, vas<sup>GNT</sup>; vas-Gal4 > GFP-Vas<sup>WT</sup>, vas<sup>D1/D1</sup>; vas-Gal4 > GFP-Vas<sup>GNT</sup>, vas<sup>D1/D1</sup>, vas<sup>GNT</sup>; and vas-Gal4 > GFP-Vas<sup>DQAD</sup>, vas<sup>D1/D1</sup>; vas-Gal4 > GFP-Vas<sup>GNT</sup>, vas<sup>D1/D1</sup>, vas<sup>GNT</sup> flies. The measurements were performed on 10 flies (n = 10) of each genotype. Dots in the box plot represent values that are 1.5 times greater than the upper limit or 1.5 time smaller than the lower limit of the interquartile range. (B) Box plot representing the number of eggs laid and hatched from wild-type (w<sup>1118</sup>, vas<sup>D1/D1</sup>, vas<sup>D1/D1</sup>; vas-Gal4 > GFP-Vas<sup>WT</sup>, vas<sup>D1/D1</sup>; vas-Gal4 > GFP-Vas<sup>DQAD</sup>, vas<sup>D1/D1</sup>; vas-Gal4 > GFP-Vas<sup>GNT</sup>, vas<sup>D1/D1</sup>, vas<sup>GNT</sup>; vas-Gal4 > GFP-Vas<sup>WT</sup>, vas<sup>D1/D1</sup>; vas-Gal4 > GFP-Vas<sup>GNT</sup>, vas<sup>D1/D1</sup>, vas<sup>GNT</sup>; and vas-Gal4 > GFP-Vas<sup>DQAD</sup>, vas<sup>D1/D1</sup>; vas-Gal4 > GFP-Vas<sup>GNT</sup>, vas<sup>D1/D1</sup>, vas<sup>GNT</sup> flies. Five independent replicates of the experiment were performed. Dots in the box plot represent values that are 1.5 times greater than the upper limit or 1.5 time smaller than the lower limit of the interquartile range. (C) Larval cuticle phenotypes observed in wild-type (w<sup>1118</sup>, vas<sup>D1/D1</sup>; vas-Gal4 > GFP-Vas<sup>WT</sup>, in vas<sup>D1/D1</sup>; vas-Gal4 > GFP-Vas<sup>DQAD</sup>, in vas<sup>D1/D1</sup>; vas-Gal4 > GFP-Vas<sup>GNT</sup>, in vas<sup>D1/D1</sup>; and vas-Gal4 > GFP-Vas<sup>DQAD</sup>, and vas-Gal4 > GFP-Vas<sup>GNT</sup> flies. Bars, 500 μm (larva) and 100 μm (unhatched larva).
3C). GFP-VasDQAD, previously described as a product-release-trap mutant (Xiol et al. 2014), entrap transposon RNAs within the piRNA amplifier complex, from which the cleaved transposon mRNAs cleaved cannot be released, as this requires ATP hydrolysis product release (Nishida et al. 2015). The entrapment of transposon RNAs can lead to transposon downregulation, as residual levels of endogenous Vas in vasD1/D1 flies (Figure S1D) are sufficient to ensure that the piRNA amplification loop never collapses entirely. Our finding that only GFP-VasWT, and not GFP-VasDQAD and GFP-VasGNT, downregulated transposons in loss-of-function vasD1/D1 flies (Figure 3C), suggests that the helicase activity of Vas is essential for transposon control.

**Vas-associated proteins in the Drosophila ovary**

The dynamic association of DEAD-box RNA helicases with multiprotein complexes (Linder and Jankowsky 2011) renders challenging the biochemical detection of their interaction partners. The E400Q mutation, which locks Vas-containing protein complexes, is an ideal biochemical tool for identifying Vas’s interaction partners in vivo (Xiol et al. 2014). We performed co-immunoprecipitation experiments from ovaries expressing GFP-VasWT and GFP-VasDQAD, and identified their associated proteins by mass spectrometry. Flies expressing GFP ubiquitously served as a negative control. We identified 57 proteins associated with GFP-VasWT and 71 associated with GFP-VasDQAD (Figure 4A, Figure S4, A–C, and Table S11). In the case of the GFP-VasWT co-immunoprecipitation, the stringent conditions and absence of a cross-linking reagent (see Materials and Methods) restricted detection to stable complexes. For instance, the Oskar protein, which interacts with Vas at the posterior pole (Breitwieser et al. 1996; Wang et al. 2015; Jeske et al. 2017), was not detected in the co-immunoprecipitations, whether in the case of GFP-VasWT or the “locked” GFP-VasDQAD (Figure 2B, top panels). We validated the specificity of co-immunoprecipitations by Western blot detection of several identified proteins. Our analysis confirmed Armi, Bel, PABP, Nop60B, and Rm62 as new Vas-associated proteins, while Aub served as a positive control (Figure 4B). Among the Vas interactors we identified were Aub, Piwi, Fragile X Mental Retardation 1 (FMR1), and eIF4A (Figure 4A), which have been shown to also be in complex with Vas in early embryos (Megosh et al. 2006; Thomson et al. 2008). Ago3 was not among the interactors, in agreement with previous findings that Bombyx Vas directly associates with Siwi (Bombyx mori Aub homolog) but not with Ago3 (Nishida et al. 2015). Curiously, we found CCHC-type zinc finger nucleic acid binding protein (CNBP) (Figure 4A), previously described to regulate wing development (Antonucci et al. 2014), to co-immunoprecipitate with Vas, suggesting that CNBP might also be involved in germline development. We also detected small ribonucleoprotein particle (Sm) proteins SmB, SmD2, SmD3, SmE, SNRPG, and snRNP-U1-70K to associate with Vas, leading to detection of splicing and spliceosomal complex as enriched gene ontology terms (Figure S4, D and E and Table S11). However, previous reports showed that Sm proteins localize at the posterior pole of the oocyte and that their localization is affected by Vas (Anne 2010; Gonsalvez et al. 2010), indicating involvement of Sm proteins in Vas-related cytoplasmic processes, rather than association of Vas with the nuclear process of splicing.

Figure 2  Helicase activity is required for Vasa localization at the posterior pole of the embryo. (A) Localization of Vasa in egg chambers (top panels) and embryos (bottom panels) of wild-type (w1118), vasD1/D1, vasD1/D1; vas-Gal4 > GAP-VasWT, vasD1/D1; vas-Gal4 > GFP-VasDQAD, and vasD1/D1; vas-Gal4 > GFP-VasGNT flies. Bars, 50 μm (egg chamber), 10 μm (nuage and pole plasm), and 100 μm (embryo). (B) Localization of Vasa in egg chambers (top panels) and embryos (bottom panels) of vasD1/D1, vasD1/D1; vas-Gal4 > GAP-VasWT, vasD1/D1; vas-Gal4 > GFP-VasDQAD, and vasD1/D1; vas-Gal4 > GFP-VasGNT flies. Bars, 50 μm (egg chamber), 10 μm (nuage and pole plasm), and 100 μm (embryo).
Interestingly, the enrichment of proteins known to be involved in the female GSC divisions (Figure 4, A and B, Figure S4D, and Table S11), such as Aub, Rm62, PABP, FMR1, eIF4A, and Piwi, indicates a function of Vas early in oogenesis.

**Vas activity in the gerarium is essential for oogenesis**

Absence of Vas in *Drosophila* females causes oogenesis arrest (Lasko and Ashburner 1988, 1990). To determine at which stage of oogenesis Vas is required, we used either the vas-Gal4 or the matTub-Gal4 driver to express GFP-VasWT at distinct stages of oogenesis; the vas promoter is active throughout oogenesis, whereas the matTub promoter is inactive in the gerarium, but active during the subsequent stages (Figure S1B). matTub-Gal4 driven expression of GFP-VasWT in vasD1/D1 females fully rescued oogenesis in 3-day-old flies, but as these progressed in age, oogenesis was arrested (Figure 5A and Figure S5A). In contrast, vas-Gal4 driven expression of GFP-VasWT in the same vasD1/D1 background restored oogenesis independently of the age of the flies (Figure 5A and Figure S5A). Of note, expression of helicase inactive GFP-VasDQAD and GFP-VasGNT proteins did not rescue oogenesis, regardless of the Gal4-driver used. In addition, analysis of egg chamber development showed that ovarian atrophy takes place between oogenesis stages six and eight and is a result of pyknosis (Figure S5B).

To test whether absence of Vas in the gerarium interferes with germ cell development, we determined the number of egg chamber–producing ovarioles per female. Strikingly, in the case of matTub-Gal4 > GFP-VasWT expressing vasD1/D1 and vasD1/D1 flies, the number of ovarioles decreased with the age of the females, whereas it did not in vas-Gal4 > GFP-VasWT expressing vasD1/D1 flies or in wild-type flies (Figure 5B). Furthermore, egg-laying analysis showed that the number of eggs produced by vasD1/D1; matTub-Gal4 > GFP-VasWT females decreased with the age of the females and eventually stopped altogether (Figure 5C, left diagram). However, the hatching rate of eggs produced by vasD1/D1 females as a result of matTub-Gal4 or of vas-Gal4 GFP-VasWT driven expression of GFP-VasWT did not differ significantly (Figure 5C, right diagram). Taken together, these results indicate that progression and completion of oogenesis depends on the activity of Vas in the gerarium.

**Absence of Vas in the gerarium deregulates transposons in aged flies**

As removal of Vas causes transposon upregulation (Liang et al. 1994; Malone et al. 2009), we tested whether oogenesis arrest in vasD1/D1; matTub-Gal4 > GFP-VasWT females coincides with transposon deregulation. Expression of GFP-VasWT using the matTub-Gal4 or vas-Gal4 drivers silenced transposons to similar levels in vasD1/D1 3-day-old flies (Figure 6A). In aged 20-day-old females, however, we could observe small but significant increase of transposon mRNA levels in vasD1/D1 flies expressing matTub-Gal4 > GFP-VasWT compared to...
vas-Gal4 > GFP-VasWT (Figure 6A). These results suggest that absence of Vas in the germarium leads to a delayed transposon deregulation that coincides with the arrest of germline development.

**Chk2 signaling in the germarium induces oogenesis arrest in vas mutant Drosophila**

We recently showed that mnk (Chk2) and vas interact genetically, and that depletion of Chk2 signaling in loss-of-function vasD1/D1 flies rescues oogenesis, but that the embryos die due to severe DNA damage (Durdevic et al. 2018). Moreover, previous studies determined that Vas is phosphorylated in a Chk2-dependent manner (Abdu et al. 2002; Klattenhoff et al. 2007). To investigate whether the age-dependent oogenesis arrest observed in vasD1/D1; matTub-Gal4 > GFP-VasWT flies is due to the Chk2 signaling, we used RNA interference (RNAi) to knockdown mnk mRNA by either matTub-Gal4 or vas-Gal4 driven expression of mnk double-strand RNA (vasD1/D1; matTub-Gal4 > TRIpmnk and vasD1/D1; vas-Gal4 > TRIpmnk, respectively). Knockdown efficiency tests using quantitative PCR showed mnk mRNA levels to be between 30 and 40% of the wild-type mnk level (Figure S6A). However, fluorescence in situ RNA hybridization analysis showed that upon matTub-Gal4 driven knockdown of mnk, the mRNA was detectable in the gerarium and not in the later stages of oogenesis, whereas vas-Gal4 driven mnk-RNAi downregulated mnk throughout oogenesis (Figure S6B). Furthermore, although vas-Gal4 driven silencing of mnk in vasD1/D1 females restored oogenesis, matTub-Gal4 driven knockdown of mnk did not (Figure 6B). This indicates Chk2-mediated signaling activity in the germarium determines the fate of developing egg chambers. Although the efficiency of vas-Gal4 RNAi-driven downregulation of mnk decreased over time, finally resulting in ovarian atrophy, we observed a more severe age-dependent decrease in the number of egg chamber–producing ovarioles when mnk knockdown was driven by matTub-Gal4 (Figure 6C). These results suggest that in vas mutants Chk2 signaling specifically in the germarium induces arrest of germ cell development.

**Discussion**

We have demonstrated that development of the Drosophila female germline depends on Vas activity in early oogenesis. Our data indicate that progression and completion of oogenesis require helicase active Vas. However, as our fusion proteins show low-expression levels, we cannot rule out that when expressed at higher levels Vas might support oogenesis independently of helicase activity (Dehghani and Lasko 2015). Independent of helicase activity is Vas's subcellular localization, which requires an open conformation of the protein. Helicase mutant GFP-VasDQAD, which is unable to release the ATP-hydrolysis products (Xiol et al. 2014), shows strong association with piRNA pathway components and colocalizes with Aub and Ago3 in large foci in the nurse cells. The granular accumulation of GFP-VasDQAD presumably hinders localization of the helicase to the posterior pole of the oocyte. In contrast, helicase inactive mutant GFP-VasGNT, which remains in an open conformation, displays both wild-type localization and correct subcellular distribution. However, Vas blocked in an open conformation does not maintain its posterior accumulation in the embryos. Thus, we show that localization of Vas in the egg chambers, but not in the embryos, is helicase activity independent and requires open conformation of the protein.
Vas interaction with different factors implicated in promoting the GSC division indicates an intricate network of Vas-associated processes involved in sustaining the germ cell lineage. Moreover, the previously overlooked fact that loss-of-function vas mutant flies undergo an age-dependent reduction of the ovariole number indicates Vas function in early germ cell development. Using stage-specific promoters, we manipulated the expression of Vas and determined that activity of Vas in the germarium is crucial for sustaining germ cell lineage. Our conclusion that oogenesis depends on an early helicase activity of Vas is consistent with the finding that Vas directly interacts with meiotic P26 (mei-P26) mRNA and activates its translation (Liu et al. 2009). Mei-P26 itself has been found to cooperate with proteins such as Bag of marbles and Sex lethal to promote both GSC self-renewal and germline differentiation (Li et al. 2012, 2013). In addition, we identified Vas interaction partners Lingerer, Rasputin, FMR1, and Caprin, which have been shown to cooperate in restricting tissue growth in a nongermline tissue, the Drosophila eye (Baumgartner et al. 2013). Interestingly, these proteins were found to interact in Drosophila ovaries as well (Costa et al. 2005, 2013), suggesting a complex that could act in conjunction with Vas to control growth of Drosophila germ-line tissue. FMR1, Piwi, and Aub were previously shown to interact with Vas in embryos and to be important for primordial germ cell formation (Deshpande et al. 2006; Megosh et al. 2006). In Drosophila ovaries, FMR1 was proposed to participate in the regulation of germline proliferation and GSC maintenance (Yang et al. 2007; Epstein et al. 2009), while Piwi and Aub have been shown to control GSC self-renewal and lineage differentiation (Cox et al. 1998; Ma et al. 2014, 2017). Vas association with Rm62, PABP, and eIF4A suggests that Vas might be a part of a previously described interaction network of Aub, eIF4, PABP, and Rm62 that regulates germ cell lineage development (Ma et al. 2017). We have also shown that Vas associates with Bel and Nop60B, proteins that play a role in male GSC maintenance (Kauffman et al. 2003; Kotov et al. 2016). Our study thus reveals association of Vas with different factors involved in the control of stem cell proliferation and maintenance. Further studies will be required to determine how these proteins collaborate to regulate early germ cell development.
In Drosophila, Chk2 signaling triggered by DNA damage, replication stress, or nuclear lamina dysfunction induces GSC loss (Molla-Herman et al. 2015; Ma et al. 2016; Barton et al. 2018). Our previous study showed that removal of Chk2 in vas mutant flies fully restores oogenesis, while progeny embryos succumb to transposon upregulation and DNA damage (Durdevic et al. 2018). Here, we went further and genetically determined that, in vas mutants, Chk2 signaling exclusively in the germarium is sufficient to cause oogenesis arrest. Furthermore, the decline of ovariole number is Chk2-dependent, indicating that in vas mutants Chk2 signaling compromises germ cell lineage proliferation. An earlier study suggested that Vas interacts with Aub to regulate mitotic chromosome condensation in Drosophila GSCs and that consequently, vas mutants display aberrant chromosome segregation during GSC mitosis (Pek and Kai 2011b). Defects in mitotic chromosome segregation can be the cause as well as the consequence of Chk2 activation in the respective daughter cells (Janssen et al. 2011; Bakhoun et al. 2014). As vas mutant GSCs do not display DNA damage (Pek and Kai 2011b), we speculate that mitotic chromosome segregation defects trigger Chk2 signaling in the daughter GSC and the CB, disrupting GSC self-renewal and development of the germ cell lineage. We conclude that the activity of Vas RNA helicase in germarium is critical to ensure sustained development of the germ cell lineage in Drosophila.

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Author contributions: Z.D. and A.E. conceived and designed the experiments. Z.D. carried out the experiments and analyzed the data. Z.D. and A.E. wrote the manuscript.

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