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Research

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Ap-Vas1 distribution unveils new insights into germline development in the parthenogenetic and viviparous pea aphid: from germ-plasm assembly to germ-cell clustering

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Targeting the distribution of germ-cell markers is a widely used strategy for investigating germline development in animals. Among these markers, the *vasa* (*vas*) orthologues, which encode ATP-dependent RNA helicases, are highly conserved. Previous studies have examined asexual (parthenogenetic) and viviparous embryos of the pea aphid *Acyrthosiphon pisum* using a cross-reacting Vas antibody. This study utilized a specific antibody against Ap-Vas1, a Vas orthologue in the pea aphid, to gain new insights into germline development. The Ap-Vas1-specific antibody facilitates earlier detection of germ-plasm assembly at the oocyte posterior, challenging the previous assumption that germ-plasm assembly begins only at the onset of embryogenesis. Treatment of oocytes and early embryos with cytoskeleton inhibitors suggests that germ-plasm assembly primarily depends on actin, in contrast to the fly *Drosophila melanogaster*, where both actin and microtubules are essential. Since pea aphids lack an orthologue of *osk*, which encodes the protein Osk responsible for anchoring Vas to the germ plasm in *Drosophila*, this suggests that pea aphids employ distinct mechanisms for *osk*- and microtubule-independent formation of the germ plasm. Moreover, the clustering of germ cells into germarium-like structures in the extraembryonic region before entering the embryos suggests a gonad formation process different from that in *Drosophila*, where germ cells begin to cluster into germaria after settling within the embryonic gonads. Therefore, the analysis of the Ap-Vas1 distribution provides a deeper understanding of germline development in asexual pea aphids, uncovering novel aspects of parthenogenetic and viviparous reproduction in insects.

Keywords: asexual development, cytoskeleton inhibitors, germline specification, germline genes, insect ovaries

Introduction

Germ cells are unique among animal cell types due to their exclusive role in transmitting genetic information to future generations. The differentiation of germ cells from somatic cells, a process known as germ-cell specification, typically occurs during embryonic development (Saffman and Lasko 1999). In certain species, such as the fly *Drosophila melanogaster*, this process initiates at the onset of development (Santos and Lehmann 2004). Two principal modes of germ cell specification are recognized in animals, both substantiated by histological and molecular evidence: the germ plasm-driven mode (GPM) and the signal-induction mode (SIM) (Extavour and Akam 2003, Lynch et al. 2011). In GPM, germ cells are specified by the

incorporation of germline determinants from the maternal germ plasm—a specialized subcellular cytoplasm in developing oocytes or uncellularized embryos. Conversely, in SIM, germ cells arise from somatic cells that respond to signals from neighboring cells within a cellularized embryo. The factors dictating the choice between GPM and SIM across different species remain a topic of ongoing research.

Irrespective of the specification strategy, the maintenance of germline development hinges on several conserved genes (Ewen-Campen et al. 2010). Notably, the *vasa* (*vas*) gene, which encodes an ATP-dependent RNA helicase, is among the most conserved germline markers (Raz, 2000). Initially identified in *Drosophila*, *vas* null mutations in females result in embryos devoid of germ cells and exhibiting truncated abdomens, underscoring the critical role of *vas* in germ-cell formation and posterior development (Hay et al. 1988b, Lasko and Ashburner 1988). During early embryogenesis, the distribution of *Drosophila vas* mRNA and Vas protein is asynchronous. Vas protein localizes to the germ plasm at the posterior pole of the multinucleated and uncellularized embryos (syncytia), whereas *vas* mRNA is ubiquitously distributed. This suggests that Vas protein, rather than *vas* mRNA, is integral to the assembly of germ plasm in *Drosophila* (Hay et al. 1988a, 1988b).

In the parthenogenetic and viviparous pea aphid (*Acyrthosiphon pisum*), commonly known as the asexual pea aphid, a cross-reacting Vas antibody detects Vas signals posteriorly localized to the presumptive germ plasm in uncellularized embryos. After cellularization, Vas-positive signals are predominantly observed in morphologically distinct germ cells throughout embryogenesis (Chang et al. 2006). These findings suggest that (i) Vas signals in the posterior germ plasm are incorporated into the primordial germ cells located at the embryonic posterior in the pea aphid and (2) the Vas protein serves as a conserved germline marker and is essential for germline specification and development. Consequently, the asexual pea aphid, like *Drosophila*, may utilize a germ plasm-driven mode (GPM) to determine germ-cell fate, with the Ap-Vas1 protein likely playing a role in germ-plasm assembly (Lin et al. 2022).

Nevertheless, in *A. pisum*, similar patterns of asynchronous distribution of *vas*/Vas are observed prior to their co-localization in the germ cells. For instance, transcripts of the *Ap-vas1* gene, a *vas* ortholog in *A. pisum*, do not accumulate in the germ plasm before cellularization (Chang et al. 2007). However, a cross-reacting Vas antibody can detect Vas signals in the presumptive germ plasm of uncellularized embryos (Chang et al. 2006). In contrast to *Drosophila*, Vas-positive germ plasm is not detected in the oocyte of the asexual pea aphid and only emerges at the egg posterior after the first mitotic nuclear division within the syncytia (Chang et al. 2006). This delayed localization may reflect fundamental differences in germ-plasm formation between aphids and flies. Alternatively, cross-reactivity of the Vas antibody with the Ap-Vas proteins encoded by the four *Ap-vas* paralogues (*Ap-vas1* to *Ap-vas4*) could obscure the clarity of germ plasm-specific signals. This hypothesis is supported by the observation that only *Ap-vas1* is germline-specific, unlike its paralogues *Ap-vas2*, *Ap-vas3*, and *Ap-vas4* in asexual pea aphids (Shigenobu et al. 2010, Lin et al. 2014).

To investigate the hypotheses, the development of an antibody specific to Ap-Vas1 was prioritized. The enhanced specificity of the Ap-Vas1 antibody has unveiled new insights into germ-plasm assembly, germ-cell proliferation, and the maternal-embryonic transition of Ap-Vas1 expression during early embryogenesis. Additionally, the discovery of germ-cell clusters near embryos prior to katatrepsis challenges conventional views on germarium formation, thereby enriching our understanding of germline development in asexual pea aphids.

Materials and Methods

Maintenance and Staging of Pea Aphids

Pea aphids, known as the NTU clone, were reared on garden pea plants (*Pisum sativum*) in growth chambers set at 20 °C without humidity control. We assume they were exposed to the local humidity, which ranged from 65% to 85% relative humidity (RH). Parthenogenetic reproduction was sustained under a long-day photoperiod of 16 h light and 8 h darkness (16L:8D) (Lin and Chang 2016). Embryos were staged according to Miura et al. (2003).

Ovarian Culture, Drug Treatment, and Immunostaining

The ovaries were dissected from adult asexual pea aphids and transferred into a culture medium. This medium comprised Grace's Insect Medium (G9771; Sigma-Aldrich, St. Louis, MO, USA), 4 mg/ml Glucose (GLU501; Bioman, New Taipei City, TW), 100 µg/ml Penicillin-Streptomycin (P4333; Sigma-Aldrich), 150 µg/ml 20-Hydroxyecdysone (H5142; Sigma-Aldrich), and 8 ug/ml Insulin (KSK0105; Funakoshi, Tokyo, JP). Three pairs of ovaries, comprising 42 ovarioles (each pair of ovaries containing 14 ovarioles, with 7 ovarioles per ovary), were dissected, pooled, and equally divided into three groups: group 1 was supplemented with 2 µg/ml of Cytochalasin D (C8273; Sigma-Aldrich), group 2 with 20 µg/ml of Colchicine (C9754; Sigma-Aldrich), and group 3 with 0.1% methanol as a negative control. Each group of ovarioles was cultured simultaneously in 2.5 ml of the medium described above for 20 min at 25 °C. Following incubation, the ovaries were fixed in 4% paraformaldehyde for 20 min at room temperature and then prepared for immunostaining.

The Ap-Vas1 antibody used in this study is from the same batch that was applied in previous studies to detect Ap-Vas1 (Lin et al. 2014, Lin and Chang, 2016). Detailed information about the production and purification of the anti-rabbit primary antibody against Ap-Vas1 in this study has been published by Lin et al. (2014). This antibody is polyclonal and affinity-purified. In total, 451 amino acids in the N-terminal region of Ap-Vas1 (amino acids 4–454) were selected as the antigen because its sequences appear divergent compared with the other three Ap-Vas proteins (Ap-Vas2, Ap-Vas3, and Ap-Vas4) (see Fig. S1 in Lin et al. (2014)). The specificity of the Ap-Vas1 antibody has been examined, showing that it does not cross with Ap-Vas2, Ap-Vas3, and Ap-Vas4 (see Fig. 1A in Lin et al. (2014)). The dilution ratio of Ap-Vas1 antibody for chromogenic staining is 1:500 (Supplementary Fig. S2), for immunofluorescence staining is 1:50 (Figs. 1–3).

Immunostaining was performed following the protocol described by Lin and Chang (2016). As described in the drug treatment experiments, 42 ovarioles were stained per experiment. With each ovariole contains seven egg chambers and each experiment was repeated at least five times, approximately 1,400 embryos were examined in total. For embryos at developmental stages 1 to 6 (Figs. 1–3), a minimum of 30 samples per stage were analyzed, consistently revealing uniform staining patterns across all stained embryos.

Imaging

Fluorescent images were captured using a Leica TCS SP5 confocal laser scanning microscope (Leica Microsystems, Exton, PA, USA), and z-stack projections were constructed using LCS Lite confocal microscope acquisition and analysis software (Leica Microsystems). For chromogenic staining, biotinylated goat anti-rabbit IgG (1:200) (Vector Laboratories, Newark, CA, USA) was used as a secondary antibody. DIC (differential interference contrast) images of whole-mount embryos were captured using a Leica Digital-Modul-R

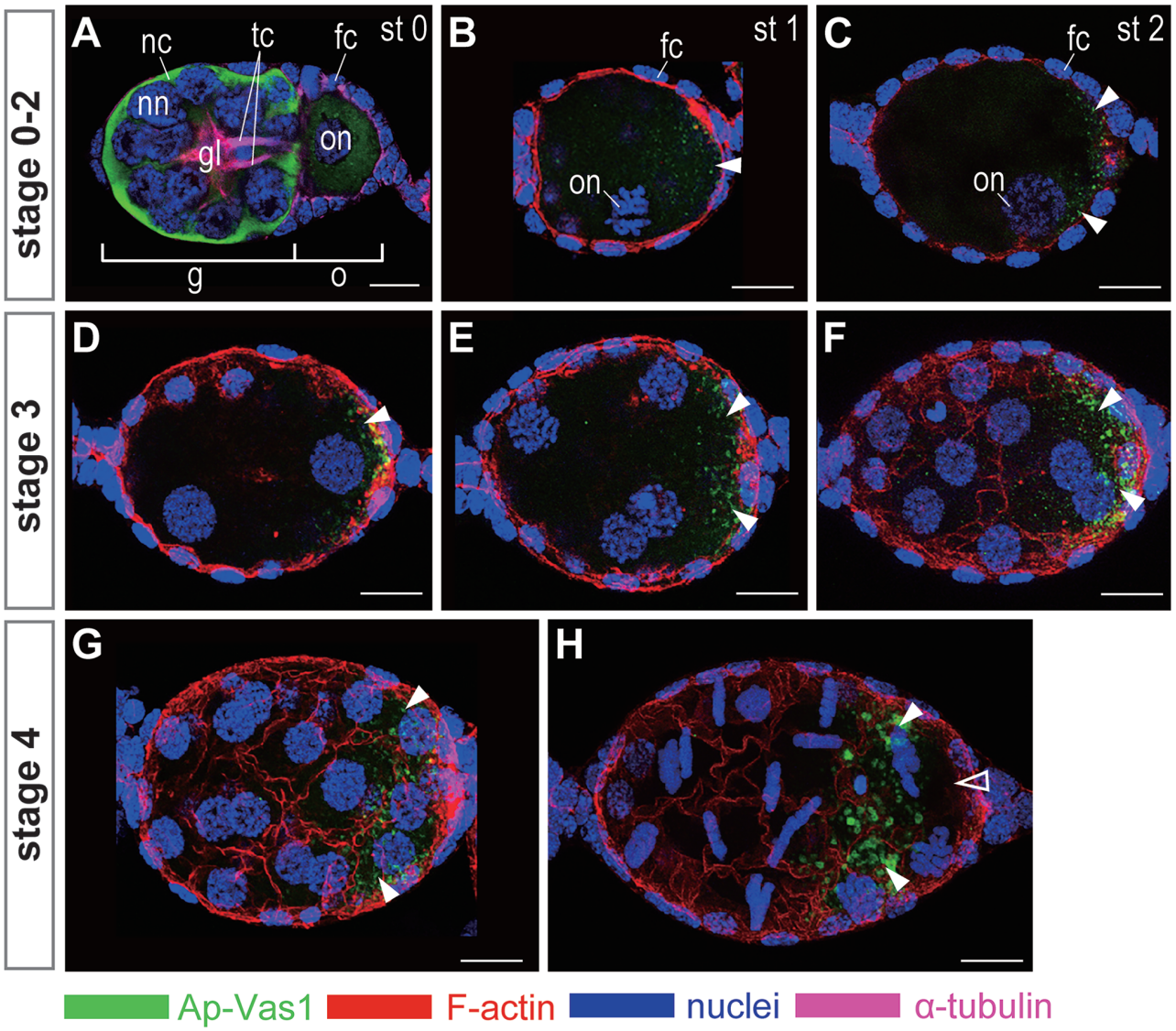

Fig. 1. Posterior localization of Ap-Vas1 in oocytes and syncytia of the asexual pea aphid. Dissected ovarioles, composed of germaria, oocytes, and embryos, were subjected to staining with the Ap-Vas1 antibody, anti-α tubulin antibody, phalloidin-TRITC (targeting F-actin), and DAPI (targeting nuclei). The anterior of germaria and egg chambers is positioned to the left. The color keys are provided below the images. Images shown in panels (B)–(H) were merged from 4 to 6 focal planes to maximize the detection of Ap-Vas1 signals. To emphasize tubulin signals concentrated in the trophic cords, the clearest focal plane was selected for display in (A). **(A)** Germaria and unsegregated oocytes (stage 0). Ap-Vas1 was randomly distributed in the cytoplasm of nurse cells (trophocytes) and protruding oocytes. The trophic cords, prominently stained by the anti-α tubulin antibody, connected the germarial lumen to the oocyte. **(B)** Segregated oocyte (stage 1). Initial Ap-Vas1 aggregation was observed in the posterior region of the egg chamber (arrowhead). **(C)** Oocytes maturation division (stage 2). Compared to the stage-1 (see panel B), Ap-Vas1 localization in the posterior (arrowheads) became more prominent. Nuclear staining highlighting the polar body, a characteristic of stage-2 oocytes (Miura et al. 2003), is shown in Supplementary Fig. S1A. **(D–F)** Synchronous nuclear divisions of the uncellularized embryos (syncytia; stage 3). Embryos contained 2 (D), 4 (E), or 8 (F) dividing nuclei, respectively. Ap-Vas1 signals (arrowheads) remained localized at the egg posterior. As nuclear numbers increased, the Ap-Vas1 signals became more distinct. **(G, H)** Syncytial blastoderm (stage 4). Before blastoderm formation, syncytia contained 16 nuclei (G) and 32 nuclei (H), respectively. Ap-Vas1 localization (arrowheads) detached from the posterior inner periphery of the egg chambers. In panel (H), a noticeable gap (hollow arrowhead) was visible between the Ap-Vas1 signals and the posterior boundary. Abbreviations: fc, follicle cells; g, germarium; gl, germarial lumen; nc, nurse cells; nn, nuclei of nurse cells; o, oocyte; on, oocyte nucleus; pb, polar body; st, stage; tc, trophic cord; -Ctrl, negative control. Scale bars: 10 μm.

(DMR) (Leica, Wetzlar, Germany) connected to a Cannon EOS 5D Mark II digital camera (Canon, Tokyo, Japan).

Results

Initial Identification of Ap-Vas1 Localized to the Oocyte Posterior

Expression of Ap-Vas1 was detected in nurse cells and unsegregated oocytes of the asexual pea aphid (Fig. 1A). In these cells, staining signals were not restricted to any subcellular region in the cytoplasm. In contrast, posterior aggregation of Ap-Vas1 was initially identified in segregated oocytes (stage 1) (Fig. 1B). This finding surpasses our previous discovery, where posterior localization of Vas signals was first detected in embryos undergoing the second nuclear division, using a cross-reacting antibody against grasshopper and fly Vasa proteins (stage 3) (Chang et al. 2006). The use of the Ap-Vas1-specific antibody likely enables the earlier detection of signals.

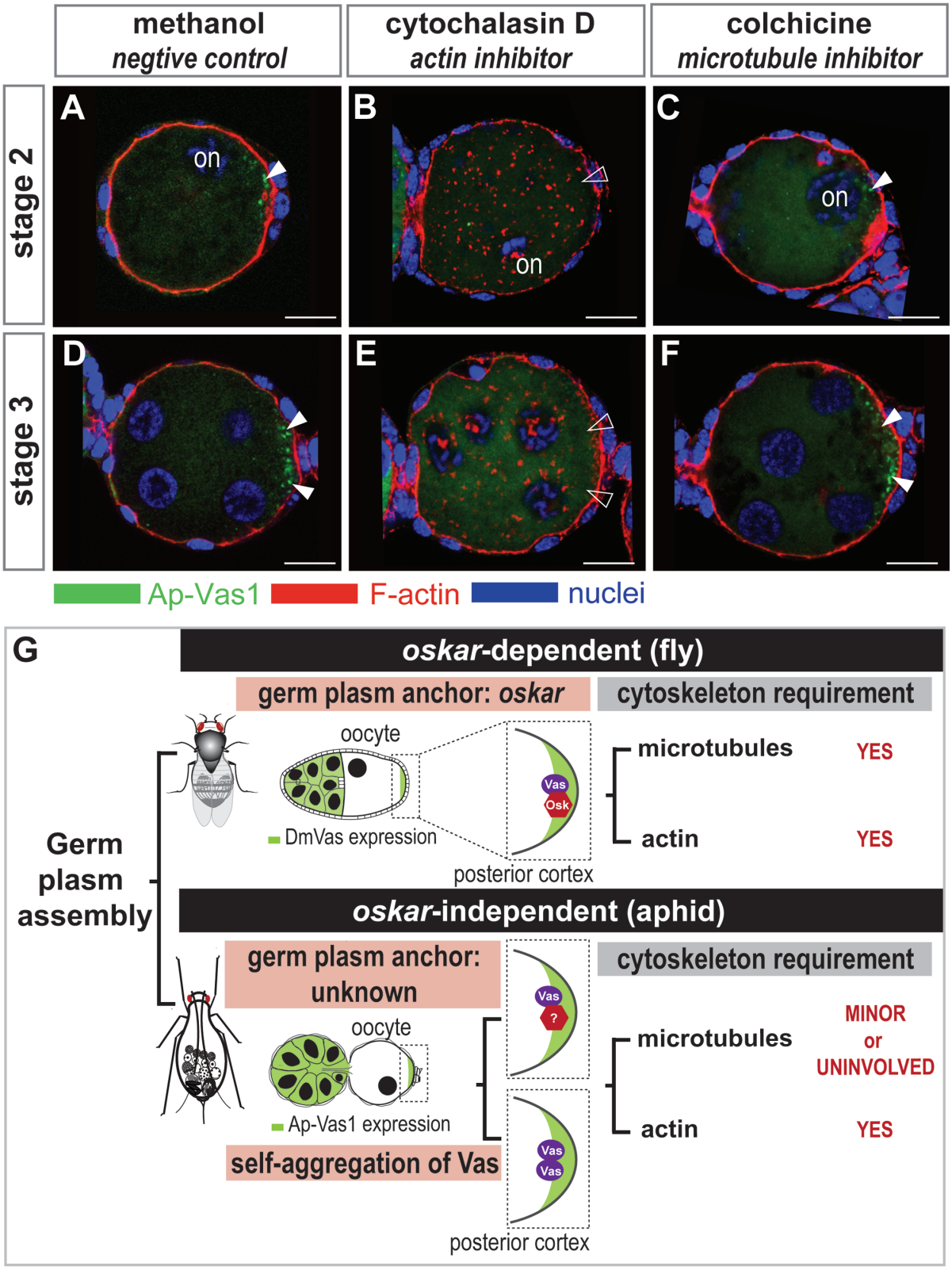

Fig. 2. Actin-dependent localization of Ap-Vas1. Dissected ovaries were cultured with 0.1% methanol with cytochalasin D or colchicine. Subsequently, the ovaries were stained with the Ap-Vas1 antibody, phalloidin-TRITC (targeting F-actin), and DAPI (targeting nuclei). The color keys are provided below the images. The anterior of the egg chambers is positioned to the left. **(A, D)** Embryos cultured in 0.1% methanol, serving as the negative control for cytochalasin D and colchicine. Posterior localization of Ap-Vas1 (arrowheads) was observed in stage-2 oocyte and stage-3 embryo. **(B, E)** Embryos cultured with cytochalasin D, an inhibitor of actin polymerization. Posterior localization of Ap-Vas1 was not observed, and F-actin signals became randomly distributed. **(C, F)** Ovaries cultured with colchicine, a compound that blocks the polymerization of microtubules. Posterior localization of Ap-Vas1 (indicated by arrowheads) remained detectable. **(G)** Comparison of germ plasm assembly between flies (*D. melanogaster*; *osk*-dependent) and aphids (*A. pisum*; *osk*-independent). Scale bars: 10 μm.

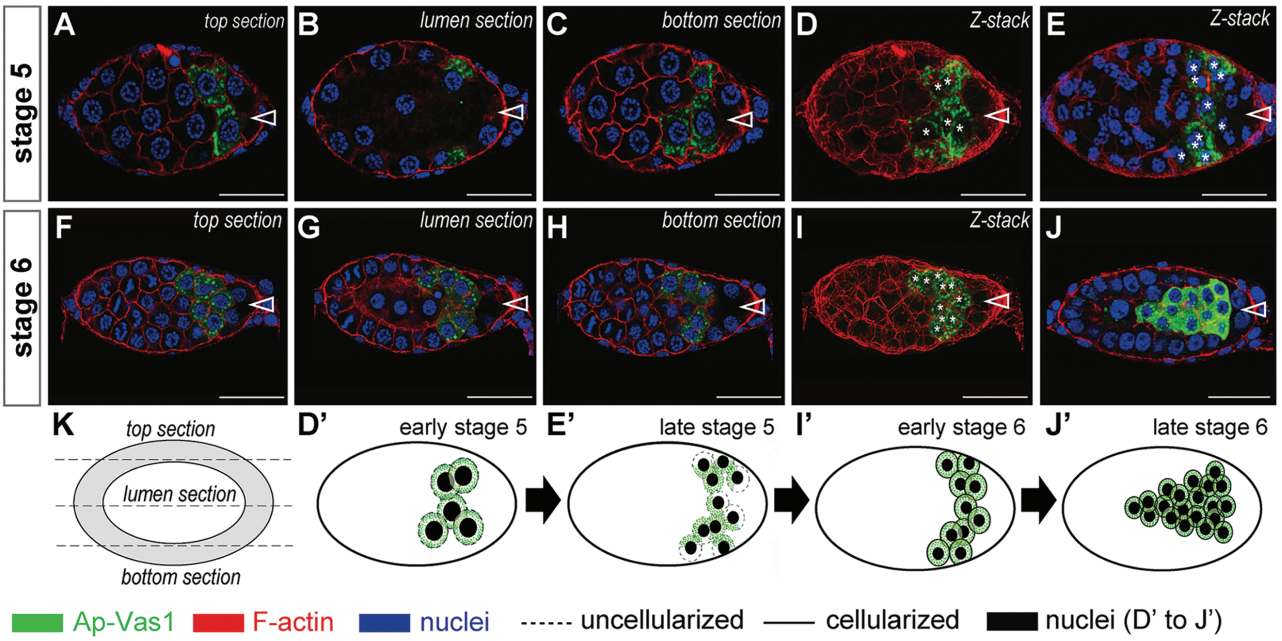

Fig. 3. Formation of the primordial germ cells (PGCs) and proliferation of PGCs during blastoderm development. Embryos were stained with the Ap-Vas1 antibody, phalloidin-TRITC (targeting F-actin), and DAPI (targeting nuclei). Color keys are indicated below the panels. The anterior of the egg chambers is positioned to the left. **(A–E)** Formation of PGCs (stage 5). (A–C) Three images were taken from a stage-5 embryo, from the outer surface to the opposite side. (D) A stacked image of pictures shown from (A) to (C). To solely display the accumulated Ap-Vas1 signals without interference from nuclear staining, DAPI signals were not included. The locations of PGC nuclei, derived from DAPI staining, are indicated by asterisks. (E) A mature stage-5 embryo contains twice the number of duplicated PGC nuclei, totaling 10, compared to (D). Cartoon illustrations of PGCs in (D) and (E) are displayed in (D′) and (E′), respectively. **(F–J)** Cellularization of PGCs (stage 6). (F–H) Three images were taken from a stage-6 embryo, from the outer surface to the opposite side. (I) A stacked image of pictures shown from (F) to (H). Similar to panel (D), DAPI signals were not included and nuclei were labeled with asterisks. In panel (I), the displayed embryo highlights 10 PGCs. However, in a mature stage-6 embryo shown in (J), the count of duplicated PGCs doubles 20. Cartoon illustrations of PGCs in (I) and (J) are shown in (I′) and (J′), respectively. PGCs were not cellularized (D′, E′) until stage 6 (I′, J′). Relative positions of focal sections are shown in panel (K). The hollow arrowheads signify the locations of posterior syncytia (Miura et al. 2003). Scale bars: 20 μm.

Posterior enrichment of Ap-Vas1 persisted in stage-2 egg chambers, where the presence of the polar body (Supplementary Fig. S1A)—a stage-2-specific characteristic—was also identified (Fig. 1C). Moreover, we observed that the posterior localization of Ap-Vas1 became increasingly pronounced as nuclei divided within the syncytial embryos prior to cellularization (Fig. 1D–F). Signals of background staining in the negative controls, where the Ap-Vas1 antibody was not applied, were nearly undetectable (Supplementary Fig. S1B, C), further confirming the specificity of Ap-Vas1 localization in uncellularized egg chambers (Fig. 1B–H).

As the dividing nuclei approached the inner periphery of the egg chambers, Ap-Vas1, which had initially aggregated at the posterior pole, became disassociated from the inner periphery (Fig. 1G, H). This redistribution led to the formation of posterior syncytium (PS) between the posterior pole and the Ap-Vas1-positive germ plasm (Fig. 1H). Given that Ap-Vas1 specifically labels morphologically identifiable germ cells in middle and late embryos (Supplementary Fig. S2), its posterior localization starting from stage 1 of development reveals the initial assembly of the germ plasm.

Cytoskeleton-dependent Localization of Ap-Vas1 to the Germ Plasm

The localization of RNA or protein in insect oocytes is known to depend on cytoskeletal networks (Steinhauer and Kalderon 2006, Kugler and Lasko 2009). To determine which type of cytoskeleton is required for anchoring Ap-Vas1 to the germ plasm in asexual pea aphids, dissected ovaries containing viviparous embryos were treated with chemical inhibitors, and the distribution of Ap-Vas1 was then examined (Fig. 2). In the negative control, where chemical inhibitors were replaced with an equivalent volume of methanol solvent, the posterior localization of Ap-Vas1 was maintained (Fig. 2A, D). This indicates that the culture of dissected ovarioles containing oocytes and embryos supported oogenesis and embryogenesis.

In ovaries treated with cytochalasin D, a compound known to disrupt the polymerization of actin filaments, Ap-Vas1 localization was not detected at the posterior region of the egg (Fig. 2B, E). Conversely, in ovaries cultured in a solution containing colchicine, which depolymerizes microtubules, posterior localization of Ap-Vas1 was still observed (Fig. 2C, F). The Ap-Vas1 signals observed in the cytoplasm of colchicine-treated eggs at stage 2 of development (Fig. 2C) may either diffuse from posteriorly localized Ap-Vas1 or be transported from the germarium. Given that the Ap-Vas1 signals localized at the posterior of stage-3 embryos (Fig. 2F) are more prominent than those in stage-2 eggs (Fig. 2C), it is likely that the Ap-Vas1 randomly distributed in the cytoplasm at stage 2 is in transition, awaiting posterior localization. If colchicine disrupts the posterior localization of Ap-Vas1 in stage-2 eggs, it is unlikely that this localization would remain intact in stage-3 embryos (Fig. 2F). Therefore, most of the Ap-Vas1 present in the cytoplasm of stage-2 eggs is likely further localized to the posterior during stage 3. Consequently, we infer that microtubules may either play a minor role or be uninvolved in the assembly of germ plasm in the pea aphid. A summary of cytoskeleton-dependent assembly of germ plasm in aphids, compared to that in *Drosophila*, is illustrated in Fig. 2G.

Proliferation and Clustering of Embryonic Germ Cells

Using the Ap-Vas1 antibody combined with confocal microscopy, two previously unreported features of germline development in the asexual pea aphid were uncovered. First, two waves of germ-cell proliferation were detected. During the syncytial blastoderm stage, which was about to undergo cellularization, Ap-Vas1 enveloped five nuclei at the inner periphery of the cell membrane (Fig. 3A–D, D') (Chang et al. 2006). As cellularization neared completion, a wave of nuclear duplication occurred, with Ap-Vas1 surrounding 10 nuclei (Fig. 3E, E'). Subsequent examination of egg chambers after cellularization revealed Ap-Vas1 signals in ten posterior cells (Fig. 3F–I, K, I'). Remarkably, embryos approaching gastrulation exhibited a second wave of nuclear duplication, resulting in an increase to 20 Ap-Vas1-positive cells (Fig. 3J, J').

Second, "germarium-like" clusters containing Ap-Vas1 germ cells were first identified in the dorsal region near embryos before katatrepsis (embryo flip) (Fig. 4). The morphology of these germ-cell clusters was not discernible by chromogenic *in situ* hybridization, where germ cells were identified by the antisense riboprobe of *Ap-vas1*, previously known as *Apvas* (Chang et al. 2007). Following katatrepsis, seven germaria clustered to form an ovary on the dorsal side of the abdomen (Supplementary Fig. S2F–H). Consequently, each pea aphid embryo possesses a pair of ovaries, totaling fourteen germaria (Supplementary Fig. S2F'–H'). Notably, germ cells were located extraembryonically before the completion of katatrepsis (Fig. 4A; Supplementary Fig. S2 A–C, E); however, soon after katatrepsis, they were settled within the embryos (Supplementary Fig. S2 F–H). The Ap-Vas1-positive germaria identified under the extended abdomen, for example, provide evidence of this (Supplementary Fig. S2F).

Discussion

Germline Specification in the Asexual Pea Aphid: Temporal and Spatial Assembly of the Germ Plasm

Germline development in the asexual pea aphid, as in other animals, begins with the specification of germ cells (Grimaldi and Raz 2020). The presence of Ap-Vas1-positive germ plasm (Fig. 1) suggests that germline specification in the asexual pea aphid is analogous to that in the fly *Drosophila melanogaster*. In *Drosophila*, germ-cell formation is directed by germ-plasm components asymmetrically localized within a subcellular region of the syncytial egg (Santos and Lehmann 2004). This mechanism in *Drosophila*, a representative model of holometabolous insects, contrasts with that observed in hemimetabolous insects. In several hemimetabolous species, including grasshoppers (Chang et al. 2002), crickets (Mito et al. 2008), and milkweed bugs (Ewen-Campen et al. 2013), germ cells originate from somatic cells in cellularized embryos through the signal induction mode (SIM). Notably, a maternal germ plasm has not been identified in these hemimetabolous species.

At the molecular level, the germ plasm-driven mode (GPM) observed in pea aphids is a rare exception among hemimetabolous insects. Conversely, GPM has been identified in several holometabolous insects, including flies, mosquitoes, and wasps (Quan and Lynch 2016, Kemph and Lynch 2022). These observations suggest that the evolution of maternal germ plasm in aphids occurred independently within Hemimetabola. Moreover, GPM is not unique to asexual aphids; maternal germ plasm has also been observed in sexual and oviparous pea aphid embryos (Lin et al. 2014). Given that parthenogenetic viviparity in aphids is an evolutionary innovation derived from sexual oviparity (Davis 2012), this suggests that GPM originated in sexual morphs and was subsequently inherited by asexual morphs.

The preformation of germ cells in aphids (Fig. 1), similar to *Drosophila* in forming germ cells before embryonic cellularization, may help pea aphids preserve the integrity of their germ plasm. This deduction is based upon the fact that invasion of the endosymbiont *Buchnera aphidicola* occurs after germ-cell formation (Miura et al. 2003). If endosymbiont invasion were to occur before germ-cell formation, the germ plasm located in the posterior of the egg would likely be disrupted by the invading endosymbionts. Conversely, if germ cells were derived from somatic cells in aphids, their locations would differ from current observations. Evidence shows that germ cells appear extraembryonic before katatrepsis in the pea-aphid embryos (Supplementary Fig. S2 A–C, E). Hence, if germ cells were induced by their neighboring cells within the embryo, the observed extraembryonic locations of germ cells would not be consistent with this mechanism. This would contradict the existing evidence. Therefore, the specification of germ cells in the asexual pea aphid is strongly suggested to rely on the maternal germ plasm, which expresses Ap-Vas1 (Fig. 1).

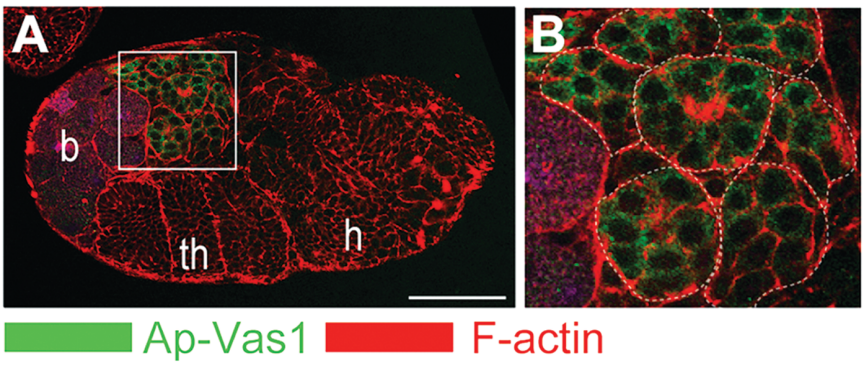

Fig. 4. Cluster of germ cells. Embryos shown in this figure are at stage 14 of development, preceding katatrepsis. The embryonic head is positioned to the right, with dorsal orientation at the top. **(A)** A stage-14 embryo stained with Ap-Vas1 antibody and phalloidin-TRITC (targeting F-actin). Germ cell expressing Ap-Vas1 are clustered at the dorsal region. **(B)** Magnification of the clustered germ cells. F-actin signals highlight the morphology the germarium-like structures (dashed line). Color keys are indicated below the images. Abbreviations: b, bacteria; h, head; th, thorax. The embryonic abdomen is not clearly visible in the shown focal plane. Scale bars: 100 μm.

Comparison of Germ-plasm Assembly between Flies and Aphids: *osk*-dependent versus *osk*-independent Mechanisms

Both flies and aphids possess the Vas protein as a conserved component in the germ plasm, but the assembly of germ plasm manifests differently in these two creatures. Notably, a homolog of *Drosophila osk*, which acts as an upstream anchor for Vas in *Drosophila* (Ephrussi and Lehmann 1992), is absent in the pea aphid (The International Aphid Genomics Consortium, 2010). This suggests that either (1) the posterior localization of Ap-Vas1 relies on either a non-*osk* anchor, or (2) Ap-Vas1 self-interacts to group within the germ plasm (Fig. 2G) (Lin et al. 2022). If a non-*osk* upstream anchor exists in the pea aphid, the results of cytoskeleton-inhibitor treatments suggest that the posterior localization of Ap-Vas1 primarily depends on actin (Fig. 2B, E) rather than microtubules (Fig. 2C, F). This differs from the localization of *osk* mRNA to the oocyte posterior in *Drosophila*, where both microtubules and actin are required—microtubules direct the transport of *osk* mRNA to the egg posterior, while actin facilitates its accumulation (Kugler and Lasko 2009). If the microtubule-dependent transport mechanism is not conserved or is not involved in the pea aphid, the transport of non-*osk* anchors to the posterior region, as proposed in the first hypothesis, may depend on alternative mechanisms relying on actin. If the second hypothesis proves correct, the self-interaction of Ap-Vas1 may depend on actin-based motor proteins, such as Myosin-V, as demonstrated in *Drosophila* (Krauss et al. 2009). Nevertheless, it remains possible that actin regulates the aggregation of Ap-Vas1 through a mechanism independent of Myosin-V.

Maternal-embryonic Transition of Ap-Vas1 Expression: A Comparison of the Stability of *Ap-vas1* mRNA and Ap-Vas1 Protein

The telotrophic ovarian tubules (ovarioles) in aphids are characterized by a germarium located in the anterior region. Nutrients synthesized within the germarium are transported via trophic cords to support the development of oocytes and uncellularized embryos (Büning 1985). It is suggested that the Ap-Vas1 protein detected in uncellularized eggs originates from the nurse cells within the germarium (Fig. 1A–F, G, H; Fig. 3A–E). Previously, a downregulation of *Ap-vas1* mRNA during blastoderm formation was observed. From gastrulation onward, the intensity of *Ap-vas1* in the germ cells significantly increased (Chang et al. 2007), indicating a transition from maternal inheritance to embryonic expression of *Ap-vas1* between blastulation and gastrulation. Unlike *Ap-vas1*, there was no significant degradation of Ap-Vas1 during blastoderm formation (Fig. 3D, E), suggesting that Ap-Vas1 is more stable than *Ap-vas1*, despite both being maternally supplied from the germarium. However, after blastoderm formation, the intensity of Ap-Vas1 observed in duplicated germ cells remains consistent (Fig. 3J, J') compared to that in newly-formed germ cells (Fig. 3I, I'), indicating that the signal intensity is not diluted by germ-cell multiplication. We infer that embryos synthesize Ap-Vas1 to compensate for any intensity reduction. This suggests that embryonic expression of Ap-Vas1 begins in the duplicating germ cells after blastoderm formation (Fig. 3J, J').

Formation of Gonads in Viviparous Embryos of Aphids: Germ-cell Clustering Occurs before Coalescence with the Gonadal Mesoderm

Using the antisense riboprobe of *Ap-vas1*, germ cells within the germaria were initially labeled in the dorsal region of embryos after katatrepsis, suggesting that migrating germ cells coalesce with the gonadal mesoderm and subsequently form gonads consisting of germaria within the flipped embryos. Due to limitation of signal sensitivity, the chromogenic *in situ* hybridization of *Ap-vas1* did not label germarium-like structures before katatrepsis (Chang et al. 2007). Nonetheless, the germarium-like clusters of germ cells revealed by signals from Ap-Vas1 and F-actin may prompt a revision of the previous understanding of gonad formation in pea aphid embryos. The germ-cell clusters identified in the dorsal region, adjacent to the embryos before katatrepsis (Fig. 4A; Supplementary Fig. S2E, E'), indicate that the germ cells initially formed subclusters with germarial morphology. These subclusters likely later amalgamated with the somatic gonadal mesoderm to form germaria after katatrepsis.

When considering the temporal and spatial formation of gonads, *Drosophila* and the asexual pea aphid exhibit notable differences. In *Drosophila*, newly formed germ cells enter the embryo during early gastrulation through germ band folding, and subclusters of germ cells form after the germline-soma coalescence (Moore et al. 1998). In contrast, the asexual pea aphid may utilize katatrepsis, a phenomenon absent in *Drosophila*, to facilitate the delayed entry of germ cells into the embryos after gastrulation. Furthermore, posterior syncytium (PS) between the posterior pole and germ plasm in aphids is not observed in *Drosophila* before cellularization (Santos and Lehmann 2004). PS formation in the uncellularized embryos of pea aphids creates a space for the later invasion of the endosymbiont *Buchnera aphidicola*, which is known to occur from gastrulation onward (Miura et al. 2003). Additionally, the migration of germ cells in the pea aphid might be guided by signals emanating from the somatic gonads, as observed in *Drosophila* (Deshpande et al. 2017, Kim et al. 2021). Additionally, the pea aphid, like *Drosophila* and other animal models (Barton et al. 2024), may utilize a conserved mechanism to guide germ cells into the somatic gonads through signals emanating from the somatic gonads. Observations of migrating germ cells closely associated with *Buchnera* and its surrounding bacteriocytes suggest that *Buchnera*, the bacteriocytes, or both may play a role in mediating these attracting signals. Notably, the close association between germ-cell migration and *Buchnera* movement has also been observed in other aphid species, such as *Myzus persicae* (green peach aphid) (Lin et al. 2022). If *Buchnera* and bacteriocytes contribute to guiding germline migration, this may represent a conserved mechanism among aphids.

In conclusion, the specific antibody targeting Ap-Vas1, a recognized germline marker in the pea aphid, has uncovered key aspects of germline development, including the assembly of germ plasm in the oocyte posterior, the maternal-embryonic transition of Ap-Vas1 expression, and the clustering of germ cells outside the embryos before katatrepsis. These findings, combined with our former discoveries about germ-cell migration (Chang et al. 2007), provide a detailed scenario of germline development in the asexual and viviparous embryos of pea aphids. However, the current data do not offer direct clues to explain how the pea aphid assembles the germ plasm without using a *Drosophila osk* orthologue or the extent to which these two species share common mechanisms for germline development (Fig. 2G). Addressing these questions is crucial for gaining a comprehensive understanding of germline evolution and development in insects.

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Author contributions

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Supplementary material

Supplementary material is available at *Annals of the Entomological Society of America* online.

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