Chapter 6
Multiple Functions of the DEAD-Box Helicase Vasa in *Drosophila* Oogenesis

Mehrnoush Dehghani and Paul Lasko

Abstract The DEAD-box helicase Vasa (Vas) has been most extensively studied in the fruit fly, *Drosophila melanogaster*, and numerous roles for it in germline development have been discovered. Here, we summarize the present state of knowledge about processes during oogenesis that involve Vas, as well as functions of Vas as a maternal determinant of embryonic spatial patterning and germ cell specification. We review literature that implicates Vas in Piwi-interacting RNA (piRNA) biogenesis in germline cells and in regulating mitosis in germline stem cells (GSCs). We describe the functions of Vas in translational activation of two mRNAs, *gurken* (*grk*) and *mei-P26*, which encode proteins that are important regulators of developmental processes, as Grk specifies both the dorsal-ventral and the anterior-posterior axis of the embryo and Mei-P26 promotes GSC differentiation. The role of Vas in assembly of polar granules, ribonucleoprotein particles that accumulate in the posterior pole plasm of the oocyte and are essential for germ cell specification and posterior embryonic patterning, is also described.

6.1 A Brief Summary of *Drosophila* Oogenesis

The DEAD-box helicase Vas functions at many steps during oogenesis. In order to discuss its roles, it is first necessary to outline in overall terms how oogenesis proceeds in *Drosophila*. More detailed descriptions of this developmental process are available (King 1970; Bastock and St Johnston 2008; McLaughlin and Bratu 2015).

Each ovary is composed of approximately 12–14 ovarioles, which can be considered as assembly lines for egg production. At the anterior tip of each ovariole, there is a morphologically distinct region called the germarium, and 2–4 germline stem cells (GSCs) are located near its anterior end. These GSCs are maintained by contact with cap cells, somatic cells that are immediately adjacent at the anterior of the GSCs. Asymmetric division of GSCs results in two daughter cells, one of which is another GSC and the other, which no longer contacts the cap cells, differentiates into a

M. Dehghani • P. Lasko (✉)
Department of Biology, McGill University, 3649 Promenade Sir William Osler, Montréal, Québec, Canada, H3G 0B1
e-mail: paul.lasko@mcgill.ca

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cystoblast (CB). Each CB undergoes four mitotic divisions to produce a cyst of 16 interconnected germ cells, in which one cell at the posterior differentiates into the oocyte and the others become nurse cells. Until late oogenesis, nurse cells remain interconnected among themselves and with the oocyte through cytoplasmic bridges called ring canals. Germline cysts that leave the germarium at this stage are called egg chambers. Meiosis in ovaries starts in the germline cysts, where 2 cells among 16 start meiosis but only 1 differentiates into an oocyte and remains in prophase I until late oogenesis, while the other follows the nurse cell fate. The oocyte then proceeds to metaphase I, where it is arrested again until fertilization (Ables 2015; Lehmann 2012). Egg chamber polarity, which includes posterior position of oocyte and differentiation of epithelial follicle cells, is established via reciprocal interactions between germline and somatic follicle cells (Assa-Kunik et al. 2007; Roth and Lynch 2009; Torres et al. 2003). The oocyte itself is also polarized through a process that originally depends on the cyst structure and becomes more pronounced as the egg chambers develop through a series of 14 defined stages (King 1970) that ultimately produce the mature egg. Oocyte polarity is internally linked to repositioning of the microtubule-organizing center (Grieder et al. 2000; Steinhauer and Kalderon 2006; Theurkauf et al. 1992). Anterior posterior (AP) axis establishment in the oocyte is associated with asymmetric localization of key mRNAs, such as *bicoid* (*bcd*) and *oskar* (*osk*) to the anterior and posterior poles, respectively (Berleth et al. 1988; Ephrussi et al. 1991). The dorsal-ventral (DV) axis is determined by a TGF-α-like protein, gurken (Grk), that accumulates along with its mRNA in the future dorsal-anterior corner of the oocyte, tightly associated with the oocyte nucleus (Neuman-Silberberg and Schüpbach 1993). Oocyte patterning determines polarization of the overlaying epithelial follicle cells, which is critical for proper development of egg shell structures such as respiratory appendages and the micropyle in the anterior-dorsal side of the egg (Berg 2005). During stages 10B and 11, nurse cells transport their cytoplasmic content to the oocyte and undergo apoptosis (Cavaliere et al. 1998). The mature oocyte (stage 14) becomes activated and completes meiosis as it passes through the oviduct (Horner and Wolfner 2008; Mahowald et al. 1983).

6.2 DEAD-Box Family of RNA Helicases

Vasa (Vas) is among the founding members of the DEAD-box protein family, which represents the largest group of nucleic acid helicases (Linder et al. 1989; Linder and Fuller-Pace 2013). DEAD-box helicases unwind double-stranded RNAs in a non-processive manner, an activity requiring ATP binding and hydrolysis.

All DEAD-box helicases contain a highly conserved core of approximately 400 amino acids that is composed of two recombinase A (RecA)-like domains and contains 12 characteristic motifs, which are involved in RNA binding, ATP binding, and inter-domain interactions (Fig. 6.1a). The name of this family originates from motif II, the Asp-Glu-Ala-Asp (D-E-A-D) motif. A similar mechanism of duplex separation is utilized by all DEAD-box proteins, whereby concomitant with ATP binding the

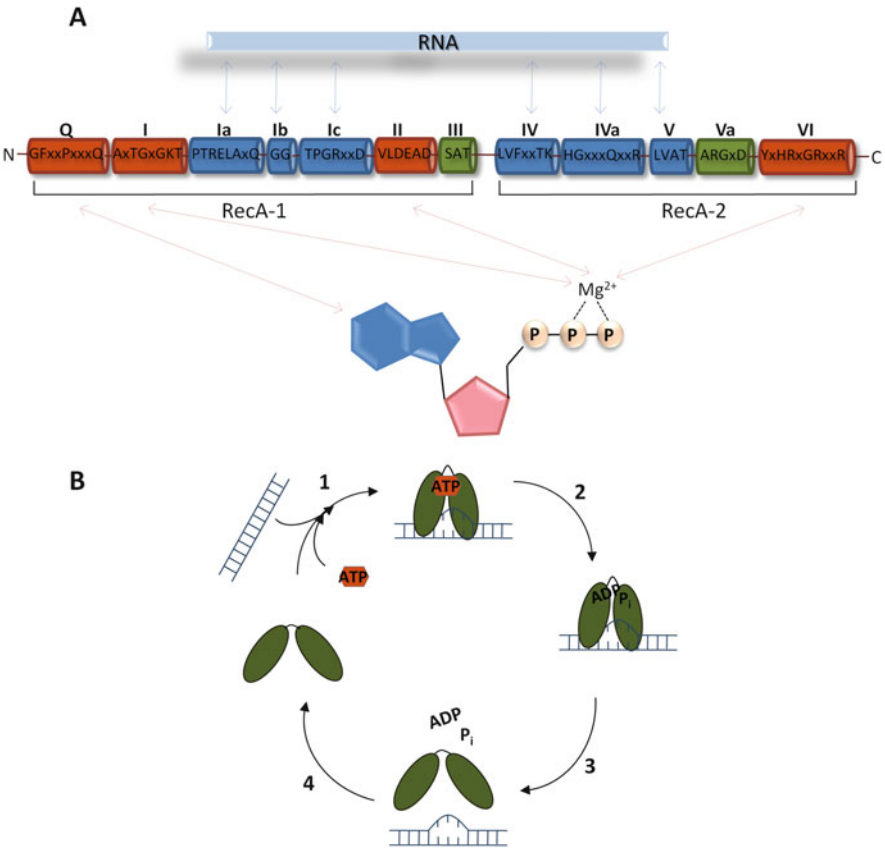

Fig. 6.1 Conserved structure and mechanism of function of DEAD-box proteins. (**a**) DEAD-box proteins share 12 conserved motifs in their core region, which are involved in RNA binding (*blue*), ATP binding (*red*), and inter-domain communications (*green*). These motifs are arranged within two RecA domains (Linder and Jankowsky 2011). (**b**) In DEAD-box proteins, ATP binding, which occurs concomitant with RNA binding, induces a closed conformation in the protein and results in RNA duplex separation (step 1). ATP hydrolysis and product (ADP and P_i) release (steps 2, 3) are subsequently required for dissociation of unwound RNA and to reset the enzyme for further catalytic cycles (step 4) (Chen et al. 2008)

helicase core undergoes a conformation change, closing the cleft between the two RecA domains (Chen et al. 2008; Linder and Fuller-Pace 2013; Rudolph and Klostermeier 2015). The closed state imposes a sharp bend in the RNA strand, bound opposite to the ATP-binding site, and causes local duplex destabilization and RNA unwinding. ATP hydrolysis occurs subsequently to restore open conformation and release RNA before the helicase can start another catalytic cycle (Fig. 6.1b).

Motifs Q, I (also known as the Walker A motif), II (also known as the Walker B motif), and VI are involved in ATP binding and hydrolysis; motifs Ia, Ib, Ic, IV, IVa, and V mainly contain residues which interact with the sugar phosphate

backbone of RNA; and motifs III and Va mediate inter-domain interactions which couple ATP binding with RNA unwinding (Cordin et al. 2006; Linder and Jankowsky 2011; Sengoku et al. 2006). These conserved motifs, however, are often not restricted to one function and contain residues participating in different interactions; for example, motif Ia, in addition to its well-characterized role in RNA binding, is involved in structural rearrangements upon ATP binding (Schwer and Meszaros 2000; Sengoku et al. 2006).

The crystal structure of the helicase core region of Vas (residues 200–623) bound to a single-stranded RNA and AMP-PNP, a nonhydrolyzable analog of ATP, has been solved (Sengoku et al. 2006). This work shows that the N-terminal domain (NTD, residues 233–454) and the C-terminal domain (CTD, residues 463–621) of Vas fold in essentially the same way as for the other DEAD-box proteins, although these two domains in Vas have a much more closed relative orientation possibly due to the specific sequence of the 8-residue linker region (residues 455–462). Interactions between the NTD and CTD of Vas ensure that the bound RNA is sharply bent, and thus duplex formation is disrupted. The interactions of the residues in 12 conserved motifs of Vas with RNA, ATP, and other parts of the protein are essentially the same as those in other DEAD-box proteins. This study also indicates that the core region only interacts with the phosphate-ribose backbone of the RNA and not the base moieties, suggesting that this region does not contribute to any potential sequence specificity of Vas toward its RNA targets.

The conserved helicase core of Vas is flanked by N- and C-terminal sequences, as is the case for most DEAD-box proteins (Hilbert et al. 2009; Rudolph and Klostermeier 2015). The structures and lengths of these flanking domains differ extensively among DEAD-box helicases. In Vas the N-terminal flanking domain of 200 amino acids contains numerous Arg-Gly-Gly (RGG) motifs and a short sequence that is implicated in interactions with two Cullin-RING E3 ubiquitin ligase specificity receptors (Kugler et al. 2010; Dehghani and Lasko 2015). The C-terminal flanking domain of Vas composed of 40 amino acids ends with a series of acidic residues punctuated by a single Trp in the penultimate position. The flanking regions of Vas, with the exception of the stretch of acidic residues and the Trp, evolve very rapidly; for example, in the closely related species, *D. simulans*, the predicted Vas protein (GD23992; Hu et al. 2013) is 51 amino acids longer and only 80% identical to its counterpart in *D. melanogaster*, with most of the divergence in the N-terminal flanking domain.

6.3 Vas and the Biogenesis of piRNAs

Maintaining genome integrity is particularly important in the germline, because deleterious mutations in these cells could be transmitted to the next generation. Active transposable elements (TEs) impose a constant threat to the host cells through their mobility and insertion into essential genes. Therefore, the germline has evolved defense mechanisms to protect its genome against these internal

enemies. Most notably the Piwi-interacting RNA (piRNA) pathway, which is highly conserved among animals, targets TE-derived transcripts for degradation and thus blocks TE replication (Grimson et al. 2008). Increasing evidence shows that Vas is a critical component of piRNA biogenesis.

6.3.1 *piRNA Pathway Protects Genome Integrity in the Germline*

Transposable elements, which were first identified over 50 years ago, are DNA sequences that can move from one location in the genome to another (Pray 2008). TEs comprise a significant portion of all eukaryotic genomes; about 4% of the *Drosophila* genome (and 50% of the human genome) is occupied by transposons and transposon-like repetitive elements (Kaminker et al. 2002; Mills et al. 2007). Transposons are classified based on their mode of propagation. Retrotransposons employ a "copy and paste" mechanism by which the original insertion remains in the genome and a new copy is generated by reverse transcription, resulting in amplification of the element. In contrast DNA transposons move through a "cut and paste" mechanism, which includes excision of the original copy and subsequent repair of the resulting gap in the DNA (Pray 2008). Transposon mobility often has deleterious effects by causing mutations or altering expression of neighboring genes, although it can be also evolutionarily beneficial. The germline is a battlefield for the race between transposons and the host, as TE-mediated changes in the genome could be transmitted to the new generation when they affect germ cells. TEs have evolved mechanisms to ensure their vertical propagation across generations. For example, expression of the active P element transposase in *Drosophila* is restricted to the germline (Seleme et al. 1999). In parallel germ cells have evolved special strategies to tame transposons and protect their genome integrity. One highly conserved mechanism to do this is mediated by piRNAs, a large class of small non-coding RNAs, which target and cleave TE-derived RNA molecules (reviewed by Haase 2016).

piRNAs derive from single-stranded RNA precursors and associate with members of the Piwi clade, which is named after *Drosophila* Piwi (P-element induced wimpy testis) and includes Piwi, Aub, and Ago3 (Meister 2013). Over 90% of piRNAs derive from discrete genomic loci, called piRNA clusters (Fig. 6.2, Brennecke et al. 2007). These loci are mainly found in pericentromeric and telomeric heterochromatin and act as a registry, which contains inactive copies or truncated fragments of all transposons hosted in the genome. Numerous genetic studies have provided a functional link between piRNA clusters and transposon silencing. For example, mutations in the *flamenco*/*COM* locus lead to upregulation of the retrotransposons *gypsy*, *Idefix*, and *ZAM* (Desset et al. 2003; Pelisson et al. 1994). Little is known about piRNA cluster transcription from these, otherwise silenced, heterochromatic regions. The heterochromatin protein 1 (HP1) variant,

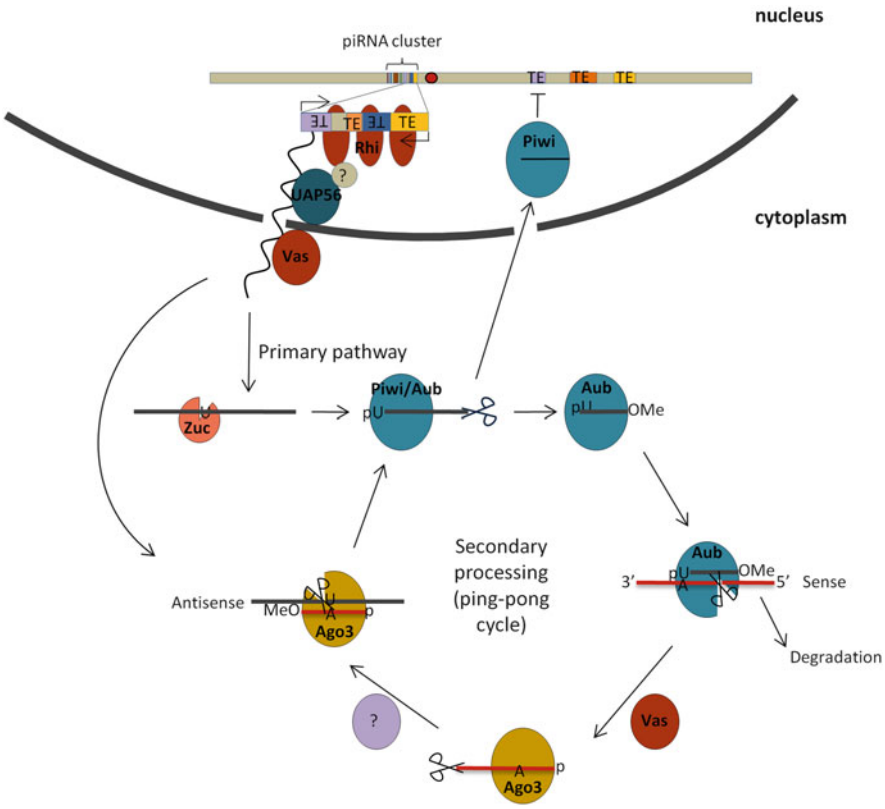

Fig. 6.2 piRNA transcripts are derived from transposon-rich heterochromatin, called piRNA clusters, in the pericentromeric or subtelomeric regions (Brennecke et al. 2007). The *Drosophila* HP1 variant, Rhino (Rhi), binds to piRNA cluster sequences and colocalizes with the DEAD-box protein UAP56 adjacent to the nuclear pore (Klattenhoff et al. 2009; Zhang et al. 2012). The other DEAD-box protein, Vas, is localized to the cytoplasmic face of the nuclear envelope and functions with UAP56 to transfer cluster transcripts to the cytoplasm. During primary processing, the long piRNA transcripts (antisense) are cleaved upstream of the 5′ uridine (U) bias by the nuclease Zucchini (Zuc) and loaded on aubergine (Aub) or Piwi (Nishimasu et al. 2012). The 3′ end of primary piRNA is trimmed by an unknown nuclease and is 2′-O-methylated (Ome). The Piwi-piRNA complex is imported to the nucleus to exert transposon silencing (Huang et al. 2013), whereas Aub-piRNA complex targets transposon transcripts (sense) for cleavage. The 3′ cleavage product is transferred to Ago3 with the help of Vas and undergoes further 3′ end trimming (Xiol et al. 2014). Ago3-piRNA complex then targets antisense transcripts to produce piRNA intermediate fragments, which are delivered to Aub for starting a new cycle (Hirakata and Siomi 2016). TE, transposable element

Rhino (Rhi), is required for production of piRNA precursors from the 42AB dual-strand cluster (Klattenhoff et al. 2009). A recent study shows that an interaction between Rhi and the transcription termination factor Cutoff (Cuff), via the adaptor protein Deadlock, is required for piRNA production, through noncanonical transcription that involves preventing RNA polymerase II from termination and

protecting the 5′ end of the nascent transcripts (Mohn et al. 2014). The long single-stranded transcripts that result are exported to the cytoplasm for processing into small RNAs, through a mechanism that involves the DEAD-box protein UAP56 (Zhang et al. 2012). On piRNA clusters, close to the inner surface of the nuclear membrane, UAP56 colocalizes with Rhino (Fig. 6.2). UAP56 foci are located directly opposite Vas-containing foci on cytoplasmic side of nuclear pores. This together with biochemical and genetic evidence suggests that UAP56 and Vas interact to transfer piRNA transcripts across the nuclear envelope.

Once transcripts reach the perinuclear region of the cytoplasm, they undergo primary processing, which requires activity of the ssRNA-specific endonuclease, Zucchini (Zuc), to generate the monophosphorylated 5′ end (Nishimasu et al. 2012). Subsequently, the pre-piRNA is loaded on Aub or Piwi and the 3′ end is trimmed, by an unknown mechanism, to produce mature piRNAs (Haase 2016; Meister 2013). Piwi-piRNA complexes are translocated to the nucleus to bind complementary sequences in the genome and, by recruiting epigenetic factors such as HP1, silence gene expression (Huang et al. 2013). piRNA-guided epigenetic silencing by Piwi is also an alternative mode for transposon regulation (Saito 2013).

The primary piRNA loaded on Aub targets complementary sequences in transposon transcripts, for their cleavage by Aub slicer activity. Simultaneously collaboration between Aub and Ago3 produces secondary piRNAs through an amplification cycle referred to as the "ping-pong loop."

6.3.2 Vas Function in piRNA Pathway

The role of Vas in piRNA biogenesis was first discovered by studying Mvh (mouse Vasa homolog)-deficient mice (Kuramochi-Miyagawa et al. 2010). Defective spermatogenesis in these mutants was associated with elevated levels of several retrotransposons and impaired de novo DNA methylation of TE regulatory regions, resembling phenotypes observed in Mili- or Miwi2 (mouse homologs of Piwi)-deficient mice (Deng and Lin 2002; Kuramochi-Miyagawa et al. 2004). Further analyses indicated that Miwi2-bound piRNAs are significantly decreased in Mvh mutants and suggested a crucial role for Mvh in the ping-pong cycle (Kuramochi-Miyagawa et al. 2010).

In *Drosophila vas*-null females have aberrant ovarioles with the majority of oocytes not reaching vitellogenic stages of development (Styhler et al. 1998; Tomancak et al. 1998). The phenotype of *vas*-null mutants resembles those of mutants affecting the piRNA pathway, and indeed the activity of the ping-pong pathway is greatly reduced in *vas*-null ovaries (Zhang et al. 2012). Vas is localized to, and is required for, the assembly of the nuage, a specialized perinuclear structure that is most prominent in the large polyploid nurse cells (Hay et al. 1990; Lasko and Ashburner 1990; Liang et al. 1994). Many piRNA components including Aub and Ago3 and the Tud domain proteins Tej and Kumo also localize to the nuage, supporting the idea that nuage functions as a processing site for the amplification cycle of the piRNA pathway (Anand and Kai 2012; Harris and Macdonald 2001;

Patil and Kai 2010; Webster et al. 2015). Vas-containing nuage structures are often localized at or near the cytoplasmic face of nuclear pores, adjacent to and dependent on localization of Rhi and UAP56 foci on the nuclear side (Zhang et al. 2012). As for *rhi* and *uap56* mutants, several transposon families are overexpressed in *vas*-null ovaries. These observations, together with co-immunoprecipitation of piRNA cluster transcripts with Vas and the reduced levels of germline-specific piRNAs in *vas*-null ovaries, suggest that Vas and UAP56 cooperate on opposite sides of nuclear pores for the export of cluster transcripts to cytoplasm.

The role of Vas in the piRNA amplification cycle remained obscure for a long time partly due to the dynamic and transient nature of Vas interactions with RNA, as is the case for other RNA helicases (Linder and Jankowsky 2011). ATP hydrolysis by DEAD-box proteins and product dissociation are required for progression of repeated RNA-unwinding events; thus, mutations in residues, such as those in motif II, that are specifically involved in ATPase activity might be expected to create an RNA clamp and stabilize transient complexes. Therefore, such a mutant allele of *vas* (vas^{DQAD}) was tested in the BmN4 cell line, derived from the silkworm *Bombyx mori*, which is the only cell culture model with active piRNA biogenesis pathways (Kawaoka et al. 2009; Xiol et al. 2014). This study provided several important results that clarified the mechanism of Vas function in the ping-pong cycle. Fluorescence recovery after photobleaching (FRAP) experiments indicated that in contrast to eGFP-BmVas (*B. mori* Vas) in nuage granules, which is rapidly replaced from surrounding cytoplasm, eGFP-$BmVas^{DQAD}$ fails to recover after photobleaching (Xiol et al. 2014). Furthermore, the mutation stabilized Vas in a complex that contains a number of proteins involved in the piRNA amplification cycle such as Siwi (the *Bombyx* ortholog of Aub), Ago3, and Qin/Kumo. Deep sequencing of small RNA associated with Vas^{DQAD} shows that these have characteristics of piRNAs and, in addition, a majority of them corresponded to the Siwi-bound antisense piRNAs. The Vas-Siwi-Ago3-Qin amplifier complex also contained poly(A) piRNA precursors corresponding to transposon transcripts, suggesting that Vas cooperates with Siwi for processing sense strands either by regulating the slicer activity of Siwi or by transferring the sense piRNA intermediates from Siwi to Ago3. Further experiments, using a construct expressing an artificial secondary piRNA precursor (sense), supported the latter function by indicating that 100% of reporter-derived fragments associated with Vas^{DQAD} have 5′ monophosphate ends already generated by Siwi slicer cleavage activity. Together these observations support a model, whereby Vas in its RNA-bound closed conformation provides a platform for the assembly of a piRNA amplifier complex and promotes transfer of the sliced precursor fragments from Siwi/Aub to Ago3 (Xiol et al. 2014).

Co-immunoprecipitation experiments in a subsequent study isolated Siwi from a wild-type BmVas complex, albeit at much lower levels than from the $BmVas^{DQAD}$ complex, which ruled out the possibility that the mutation led to an artifactual association (Nishida et al. 2015). This study also shows that FLAG-BmVas purified from BmN4 cells could liberate RNA from Siwi-piRISC in vitro. The Siwi/BmVas complex, which is required for production of BmAgo3-secondary piRNAs, is found

distinct from a Siwi/BmSpn-E/BmQin complex, involved in primary piRNA production, thus excluding Vas from the primary phase of piRNA processing.

6.4 The Posterior Pole Plasm of the *Drosophila* Oocyte Specifies the Germline in the Developing Embryo

In many organisms, including *Drosophila*, *C. elegans*, *Xenopus*, and zebrafish, there is a physical continuity of germline across generations, meaning that a specific cytoplasmic region in the oocyte, termed germ plasm, contains maternally inherited mRNA-protein complexes that segregate into subsequently developing primordial germ cells (PGCs, Lesch and Page 2012). In the *Drosophila* embryo, the maternally derived germ plasm (called pole plasm), which is located at the posterior pole, is sufficient to induce germ cell fate. This was proven by demonstrating that transplantation of posterior cytoplasm from an early embryo or unfertilized egg to an ectopic site of another embryo induces germ cells at this site, and further these germ cells are functional if transplanted to the posterior pole of a host embryo (Illmensee and Mahowald 1974).

Since *Drosophila* relies so heavily on maternally supplied proteins and mRNA for early development, there is elaborate posttranscriptional regulation of maternal mRNAs mainly at the levels of mRNA localization and translation (Becalska and Gavis 2009; Lasko 2012). Nurse cell-produced materials including germ cell determinants are unidirectionally transported to the oocyte via polarized microtubules (MTs), which in stages 2–6 oocytes emanate from a microtubule-organizing center (MTOC) at the posterior region and extend their plus ends through the ring canals into the nurse cells (Harrison and Huebner 1997; Roth and Lynch 2009; Weil 2014; Goldman and Gonsalvez 2017). In stages 7–10a (mid-oogenesis), MTs reorganize, presumably in response to signals from the posterior follicle cells, and emanate from the lateral and anterior cortex with a slightly biased polarity toward the posterior (Parton et al. 2011; Steinhauer and Kalderon 2006; Zimyanin et al. 2008). mRNAs and proteins involved in germ cell specification and embryonic patterning then become localized to the pole plasm at the posterior region of the oocyte. Different mechanisms have been described for localization of germ cell determinants (Meignin and Davis 2010): (1) degradation in bulk cytoplasm and stabilization at the posterior pole, as observed for *nanos* (*nos*) mRNA (Zaessinger et al. 2006); (2) diffusion and anchoring, for example, reported for *cyclin B*, *germ cell-less* (*gcl*), and *nos* (Forrest and Gavis 2003); (3) active transport mediated by motor proteins and *cis* elements in 3′ UTR of mRNAs such as *oskar* (*osk*) (Clark et al. 2007; Zimyanin et al. 2008); and (4) localized translation in the pole plasm and repression elsewhere, as for Osk (Kim-Ha et al. 1995). Following fertilization and during syncytial divisions, pole plasm complexes are recruited by posterior nuclei through dynein-mediated transport on astral microtubules that emanate from centrosomes (Lerit and Gavis 2011). By this mechanism germ plasm becomes

segregated into the pole buds, which pinch off from the posterior somatic region at mitotic cycle 9 (stage 3, Campos-Ortega and Hartenstein 1985) and undergo two more divisions before the embryo undergoes cellularization. These nuclear divisions are asynchronous and lag behind the synchronous somatic divisions. Germ cell precursors then enter a prolonged quiescence until arriving in the presumptive gonads in late embryogenesis (Su et al. 1998). The mitotic quiescence is partly due to translational repression of *cyclin B* by RNA-binding proteins Nos and Pumilio (Pum) (Asaoka-Taguchi et al. 1999).

6.4.1 *Vas Is a Component of the Polar Granules, RNPs that Assemble in the* Drosophila *Pole Plasm*

The critical role of pole plasm in *Drosophila* germ cell formation was first demonstrated by UV-irradiating the posterior pole of fertilized eggs, which resulted in sterile flies (Geigy 1931). Germ cell formation can be restored to irradiated embryos by transplantation of a new pole plasm (Okada et al. 1974). As mentioned in the introduction, transplantation of pole plasm to an ectopic site results in ectopic formation of pole cells, suggesting that posterior cytoplasm contains all the necessary components for germ cell formation (Illmensee and Mahowald 1974). Ultrastructural analyses show that the main components of germ plasm, the polar granules, which first appear in the posterior region at stage 9, are composed of a non-membrane-bound fibrous meshwork. Polar granules appear as non-membrane-bound fibrous material and become associated with the surface of mitochondria in later-stage oocytes and with the ribosomes in the activated egg (Mahowald 1968; Mahowald et al. 1983). The full composition of polar granules is unknown, but several mRNAs and proteins, including Vas, associate with them.

Vas and other polar granule components expressed during oogenesis are essential for germ cell specification and posterior patterning in the embryos that develop after fertilization of the eggs. In fact, *vas* was first identified in screens for maternal-effect lethal mutations that impair embryonic patterning (Schüpbach and Wieschaus 1986a, b). Homozygous females for *vas* hypomorphic alleles produce embryos which lack polar granules, fail to form germ cells, and exhibit defects in abdominal segmentation (Schüpbach and Wieschaus 1986b). The *vas* gene was subsequently cloned and found to encode a DEAD-box helicase (Hay et al. 1988b; Lasko and Ashburner 1988; Linder et al. 1989). Further immunostaining experiments showed that Vas localizes to the pole plasm and is present in the pole cells and their germ cell derivatives in all stages of development (Hay et al. 1990; Lasko and Ashburner 1990).

Vas localization to the posterior pole plasm depends upon two genes that also encode pole plasm components, *osk* and *stau*, as females mutant for either of these genes do not display posterior localization of Vas. However, Vas localization is initially unaffected in mutants of most other pole plasm components, including *nos*, *tud*, *valois* (*vls*), *gcl*, and *pgc* (Jongens et al. 1992; Lasko and Ashburner 1990;

Nakamura et al. 1996). Yeast two-hybrid experiments indicate direct interaction between Vas and Osk, which is considered an essential step for initiating pole plasm assembly (Breitwieser et al. 1996). This interaction persists upon deleting residues 321–661 of Vas. Recent work further demonstrates that deletion of amino acids 3–200 of Vas, but not 3–139, severely reduces its localization to the pole plasm and to the nuage (Dehghani and Lasko 2015). As the latter occurs independently of Osk, the interval 140–200 must be involved in additional interactions between Vas and its protein partners that promote Vas localization. Furthermore, this work shows that a non-conservative point mutation in T546, an RNA-binding residue, abolishes Vas localization. Since mutations in other RNA-binding motifs do not have similar effects, T546 most likely plays a role in protein-protein interactions required for Vas localization (Dehghani and Lasko 2015; Sengoku et al. 2006). Precise localization of Vas at the posterior region, followed by other components of pole plasm, depends on the C-terminal motif, 636–646, which is conserved among closely related *Drosophila* species. When only a form of Vas deleted for this motif is present, the concentration of pole plasm at the posterior region is insufficient for its function, resulting in a phenotype where most pole buds fail to develop into germ cells and instead adopt a somatic fate (Dehghani and Lasko 2015).

Posterior localization of Vas becomes detectable at stage 10a (Lasko and Ashburner 1990). Live imaging of GFP-tagged Vas and Aub indicates that nuage particles become displaced to the cytoplasm of nurse cells and then enter the oocyte through ring canals (Snee and Macdonald 2004). This study argues against the previous model suggesting that pole plasm is directly formed from the nuage fragments, by providing evidence that Vas becomes dissociated from these precursors and is later entrapped in the pole plasm, after its de novo nucleation by Osk. The direct interaction between Vas and Osk is essential for pole plasm assembly, as long Osk, which has significantly lower affinity to Vas compared to short Osk, is not able to induce ectopic Vas localization and germ cell formation in the anterior region (Breitwieser et al. 1996). Furthermore, persistent trafficking of pole plasm components, such as Vas, on cortical microtubules, mediated by dynein, is required for their retention at the posterior cortex (Sinsimer et al. 2013).

There is evidence that regulation of Vas stability through mechanisms that affect its ubiquitination state contributes to maintenance of its posterior localization. Vas can be co-purified from *Drosophila* ovaries and embryos with the ubiquitin-specific protease Fat facets (Faf), which also localizes to the pole plasm (Huang et al. 1995; Liu et al. 2003). Embryos from *faf* mutant females form fewer pole cells than wild type, consistent with decreased levels of Vas and its posterior localization in these mutants. In addition, Vas purified from *faf* ovaries is highly ubiquitinated, suggesting that Vas is stabilized in pole plasm through its Faf-dependent deubiquitination. Vas ubiquitination in pole plasm is also modulated by the two ubiquitin Cullin-RING E3 ligase specificity receptors, Fsn and Gustavus (Gus), which both bind to the same motif (DINNN) on Vas (Kugler et al. 2010; Styhler et al. 2002). *fsn* mutants show a precocious accumulation of Vas in pole plasm, whereas *gus* mutants additionally lacking one copy of *cullin-5* exhibit delay in posterior localization of Vas. While Fsn clearly acts as a negative regulator of Vas

stability, the function of Gus is more complex, as its overexpression also reduces levels of Vas in the ovaries.

In *vas* mutants, posterior localization of *nos* mRNA is abolished, consistent with defects in abdominal segmentation in these mutants (Gavis and Lehmann 1992). Nos, together with another RNA-binding protein Pum, blocks translation of the maternal mRNA, *hunchback* (*hb*), thereby creating a gradient of Hb protein across the egg, which opposes the steep posterior-anterior gradient of Nos (Murata and Wharton 1995; Tautz 1988). Hb acts as a transcriptional activator of the genes essential for development of anterior structures; thus, its repression in the posterior region is critical for abdominal segmentation (Lehmann and Nüsslein-Volhard 1987).

6.5 Vas Is a Translational Activator of *gurken* and *mei-P26* mRNAs

The EGFR ligand, gurken (Grk), is secreted by the oocyte and induces dorsal fate in the overlying epithelial follicle cells, through its interaction with the EGFR receptor, Torpedo (Top) (González-Reyes et al. 1995; Roth et al. 1995). During oogenesis *grk* mRNA is expressed by the nurse cells and transported to the oocyte via a mechanism depending on dynein and its cofactors, BicD and Egl (Mach and Lehmann 1997; Neuman-Silberberg and Schüpbach 1996). Throughout oogenesis *grk* mRNA remains tightly associated with the oocyte nucleus, which in early stages is positioned at the posterior. Starting at stage 8, growing microtubules push the nucleus to the anterodorsal corner of the oocyte, and accordingly *grk* mRNA forms a crescent between nucleus and cell cortex in this region (Steinhauer and Kalderon 2006; Zhao et al. 2012). The exact mechanisms of *grk* localization are unknown, but this process is mediated at different stages by dispersed elements in the 5′ UTR, coding region, and 3′ UTR of the *grk* transcript (Thio et al. 2000).

Genetic interactions suggest that translation of unlocalized *grk* mRNA is inhibited through a complex including Cup and Squid (Sqd) and that another component of this complex, PABP55B, through its interaction with Encore (Enc), facilitates translation of localized *grk* by a yet unknown mechanism (Clouse et al. 2008). *grk* mRNA is not efficiently translated in *orb* mutant ovaries, suggesting that polyadenylation mediates translation of localized *grk*, a mechanism through which PABP55B is also proposed to exert its regulatory effect on Grk translation (Chang and Matzuk 2001; Clouse et al. 2008). Furthermore, Grk protein level, but not the level of its mRNA, is severely reduced in *vas*-null mutants, whereas mRNA localization remains normal (Styhler et al. 1998). A direct interaction between Vas and the translation factor, eIF5B, required for 60S ribosomal subunit joining at the initiation step (Pestova et al. 2000), was found through a yeast two-hybrid screen against *Drosophila* ovarian cDNAs (Carrera et al. 2000). A mutant form of Vas, $Vas^{\Delta 617}$, does not interact with eIF5B and once expressed in the ovaries does

not support Grk translation, suggesting that Vas directly activates Grk expression by recruiting eIF5B to *grk* transcripts (Johnstone and Lasko 2004).

Grk translation is blocked as a response to meiotic checkpoints that in turn are activated by unrepaired dsDNA breaks (DSBs, Ghabrial and Schupbach 1999). Thus, mutations in genes such as *okra* and *spindle-B*, which are involved in DSB repair, cause defects in dorsoventral patterning. *vas* mutants produce similar nuclear morphology phenotypes as spindle-class mutants, suggesting that meiotic checkpoints act through downregulation of Vas (Styhler et al. 1998; Tomancak et al. 1998). Vas expression is not affected in spindle-class mutants; however, band shift assays indicate that the mobility of Vas protein from ovarian lysates prepared from these mutants is decreased compared to wild type and is restored by an additional mutation in *mei-41*, a gene involved in checkpoint pathway. These observations support a model, whereby meiotic checkpoints suppress Vas activity in Grk translation through posttranslation modifications (Abdu et al. 2002; Ghabrial and Schupbach 1999). Surprisingly, reducing the genetic dosage of the translation factor eIF1A suppresses this effect in *spnB* mutants, increasing both Grk level and the amount of grk mRNA associated with polysomes (Li et al. 2014). eIF1A facilitates recruitment of Met-tRNAi-eIF2-GTP ternary complex (TC) to the 40S ribosomal subunit and thus promotes translation initiation (Hinnebusch 2014). The unexpected inhibitory effect of eIF1A on Grk translation is thought to be associated with three upstream open reading frames (uORFs) in the *grk* 5′ UTR (Li et al. 2014). uORFs often reduce translation from the main ORF, by attracting pre-initiation complex and creating a block on the mRNA as the ribosome stalls on premature stop codons (Barbosa et al. 2013). eIF1A mutations could result in a larger fraction of ribosomes passing the uORFs before TC recruitment, enabling more efficient translation from the main ORF (Fekete et al. 2005; Li et al. 2014).

In addition to its likely role in Grk translation, Vas has been implicated in translation of *mei-P26*, which promotes GSC differentiation through its function in the microRNA (miRNA) pathway (Liu et al. 2009; Neumuller et al. 2008; Page et al. 2000). Vas-mRNA complexes were purified from early embryos through a tandem immunoprecipitation approach with anti-Vas antibody (Liu et al. 2009). In this manner 221 mRNAs co-purified with Vas, 13.3% of which were enriched in the pole cells. One of the recovered mRNAs was *mei-P26*, and subsequent work showed that Vas directly interacts with 3′ UTR of *mei-P26*, depending on a stretch of ten consecutive U residues, and promotes Mei-P26 expression (Liu et al. 2009). Polyuridine tracts are not overrepresented among the mRNAs that co-purified with Vas; however, germ cell-enriched mRNAs appear more frequently when the Vas-bound mRNAs are filtered for the presence of a (U)-rich motif, an approach which may identify other direct targets of Vas. Preferential binding of Vas to the polyuridine motif compared to the neighboring motifs depends on sequences in its N- or C-terminal regions, as the Vas core protein (VCP, residues 200–623) does not exhibit such discriminatory affinity. Mei-P26 protein level is significantly decreased in $vas^{\Delta 617}$ ovaries, suggesting that Vas interaction with eIF5B is required for translational activation of Mei-P26 in GSCs.

A direct role for Vas in activating translation of one or more pole plasm RNAs has long been assumed but has never been convincingly demonstrated. There is evidence that the translational repressor Bruno (Bru), which represses translation of *osk* outside of the pole plasm, interacts with Vas. This raises the possibility of Vas being involved in translational activation of *osk* in the pole plasm by removing Bru (Webster et al. 1997).

6.6 Vas Function in Regulating Mitosis

In *vas*-null (vas^{PH165}) ovaries, germarium atrophy is associated with a decrease in GSCs, CBs and dividing cystocyte clusters, which are recognized based on their specific structures, spectrosomes, and fusomes (Styhler et al. 1998). This phenotype resembles, although is less severe than, that observed in null *nos* mutants. Nos is required for GSC proliferation (Forbes and Lehmann 1998); however, evidence to support that Vas function in regulating GSC divisions is mediated by *nos* is still lacking. More recently it was shown that concomitant with a delay in mitotic progression, a higher percentage of GSCs and CBs in *vas* null, compared to wild type, do not progress to metaphase and remain in prometaphase (Pek and Kai 2011a). Consistent with this Vas becomes localized at pericentromeric piRNA clusters in GSCs and CBs during prometaphase/metaphase, dependent on its interaction with two piRNA pathway components Aub and Spn-E. Co-IP from GSC-/CB-enriched *bag-of-marbles* (*bam*) mutant ovaries indicates that Vas directly interacts with the condensin I components, Barren (Barr) and CAP-D2. Localization of Barr to chromosomes, which is required for chromosome condensation and segregation, is abolished in *vas*-null GSCs and CBs and is restored by transgenically expressed Vas (Bhat et al. 1996; Pek and Kai 2011a; Somma et al. 2003). Interestingly $vas^{\Delta 617}$, which does not interact with eIF5B, could still rescue Barr recruitment to the chromosomes in *vas* null, suggesting that *vas* role in mitotic progression of germline stem cells does not require its function in translation regulation. Recent work also shows that the fecundity of *vas*-null females is surprisingly restored by expressing Vas proteins that carry mutations in the conserved core domains and are very likely to be enzymatically inactive. This suggests that an early function of Vas, possibly associated with mitotic division of GSCs, is independent of its catalytic activity and perhaps requires Vas only as a scaffolding protein (Dehghani and Lasko 2015).

Bel, the *Drosophila* ortholog of mammalian DDX3, has the closest sequence homology to Vas and is expressed both in somatic cells and in germ cells (Johnstone et al. 2005). Bel, which is required for larval growth and adult fertility, can functionally substitute for yeast Ded1p, a protein implicated in translation (Chuang et al. 1997), suggesting that the essential role of Bel in development is through regulation of translation. In addition, Bel has been shown to function in gene silencing through RNA interference (RNAi) (Ulvila et al. 2006; Zhou et al. 2008). Similar to Vas, which regulates mitotic progression in GSCs via its interaction with piRNA components, Bel functions, through endogenous (endo)-siRNA pathway, to promote

chromosome segregation in somatic cells (Pek and Kai 2011a, b). This function is through a physical interaction between Bel and the condensin I components, Barr and CAP-D2, while localization of Bel to the pericentric region of chromosomes is mediated by endo-siRNA pathway components, Ago2 and Dicer-2. The C-terminal end of Vas is distinct from Bel and other DEAD-box proteins and contains several acidic residues. Replacement of the seven C-terminal residues of Vas with those of Bel ($Vas^{c.\ bel}$) most profoundly affects early progression of oogenesis and piRNA biogenesis. Oocytes that escape this early block, when $Vas^{c.\ bel}$ is examined in a *vas* hypomorphic background, are however less severely affected than for most *vas* mutants, and some give rise to viable, fertile embryos (Dehghani and Lasko 2015, 2016). This suggests that the specific interactions of Vas with components of piRNA pathway are dependent on its unique acidic C-terminus. The C-terminal motif also contains a tryptophan residue (Trp660), which is very highly conserved among Vas orthologues. Mutating this residue to glutamic acid abolishes Vas function in germ cell formation and embryonic viability and dramatically decreases its function in piRNA pathway and activating Grk translation; yet it does not significantly affect female fecundity. Thus, these two elements in the Vas C-terminal end, Trp660 and the acidic residues, are both required for the full activity of Vas in *Drosophila* oogenesis and early embryogenesis (Dehghani and Lasko 2016).

6.7 Conclusion

Nearly 30 years of intensive study has revealed a large number of roles for Vas in germline development in *Drosophila*. This work has implicated Vas in a variety of RNA-dependent processes that underlie key developmental events in oogenesis and early embryogenesis. A great deal has been learned about exactly how Vas helps facilitate piRNA biogenesis and translational activation, but mechanistic detail is lacking about some of its other functions. Future work aimed at understanding the composition of Vas-containing ribonucleoprotein complexes will lead to further insights into how Vas functions during transit-amplifying divisions of germ cells and in the pole plasm.

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