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REVIEW

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Germ granules in *Drosophila*

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Abstract

Germ granules are hallmarks of all germ cells. Early ultrastructural studies in *Drosophila* first described these membraneless granules in the oocyte and early embryo as filled with amorphous to fibrillar material mixed with RNA. Genetic studies identified key protein components and specific mRNAs that regulate germ cell-specific functions. More recently these ultrastructural studies have been complemented by biophysical analysis describing germ granules as phase-transitioned condensates. In this review, we provide an overview that connects the composition of germ granules with their function in controlling germ cell specification, formation and migration, and illuminate these mysterious condensates as the gatekeepers of the next generation.

KEYWORDS
germ granules, localized translation, mRNA clusters, Oskar, phase separation, RNA granules, RNA localization, vasa, *nanos*

1 | GERM PLASM AND GERM GRANULES OF *DROSOPHILA*—FIRST DESCRIPTIONS

In his search for a "heritable substance" that is transmitted from generation to generation, German biologist August Weismann proposed in 1893 that the offspring owes its origin to a "peculiar substance of extremely complicated structure" called the germ plasm.[1] It is this structure, he explained, that distinguishes cells that give rise to the next generation from those that produce the "perishable body." Experimental support for such a germ cell determinant was later provided by Robert Hegner's pricking experiments where he removed the germ plasm from the beetle *Calligrapha punctate* and found that the resulting embryos lacked morphologically discernable germ cells.[2] With microscopy techniques of the time, Hegner described the germ plasm as containing "special bodies," which were dense and stained like "chromatin."[3] However, it was not until the early 1960s, when the detailed electron microscopic (EM) studies of *Drosophila melanogaster* and other Drosophilid embryos by Tony Mahowald revealed the existence of morphologically unique structures found within the germ plasm, called the germ granules (Figure 1A). Mahowald described these granules as round, membraneless bodies about 0.2 to 0.5 μm in diameter, which contained fibrous material and ribosomes and stained with nucleic acid markers.[8] These studies also revealed that granules could have a defined structure, with the periphery more electron dense than the core, and could often closely associate with mitochondria[9] (Figure 1A, B). While the morphology of granules changed during *Drosophila* development, and could even vary among *Drosophila* species, Mahowald noted that the electron-dense, fibrous nature of germ granules was a hallmark of the germline lineage throughout the germline life cycle and shared among species.

Functionally, Mahowald and his postdoctoral fellow Karl Illmensee demonstrated the deterministic potential of the *Drosophila* germ plasm by transplanting it from the posterior pole, where germ cells form, to an ectopic anterior location in the embryo.[10,11] Nuclei located in the transplanted region formed cells with the morphology of "pole cells," as the primordial germ cells (PGCs) in *Drosophila* are called. Moreover, these ectopic pole cells gave rise to functional germ cells after being transferred into a host embryo.[10,11] Some of the key proteins and

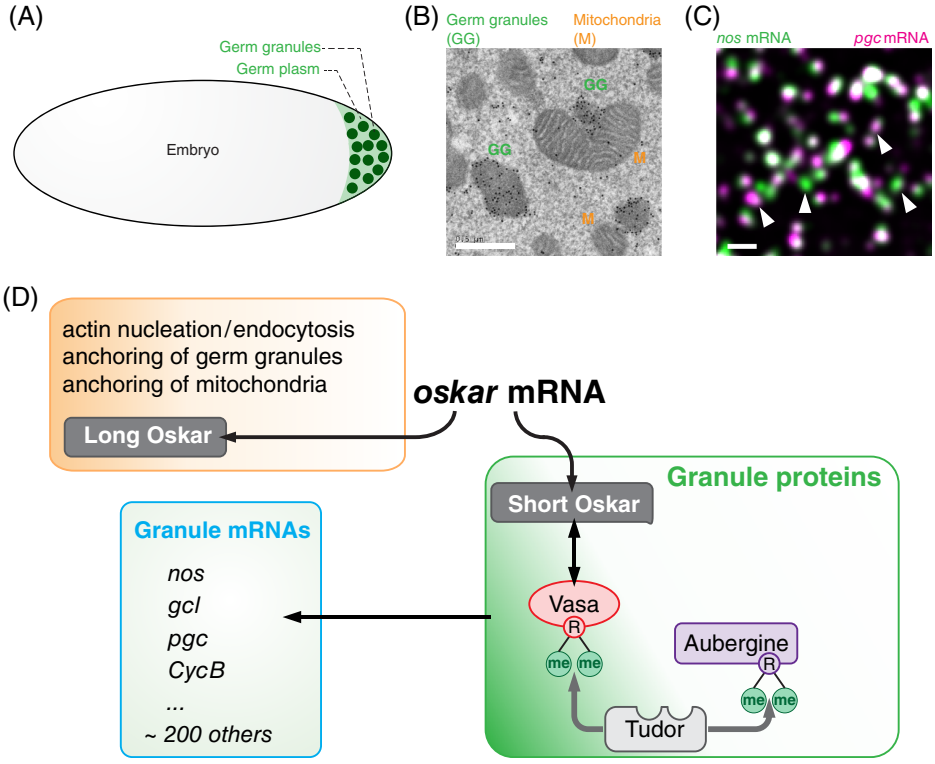

FIGURE 1 Formation of germ granules in *Drosophila* embryo (figure adapted from Reference 4. A, Germ granules form in the specialized cytoplasm called germ plasm at the embryo's posterior pole. B, An EM image showing that germ granules (labeled by immunogold particles staining Vasa protein; marked with green "GG") are more electron dense than surrounding germ plasm and are closely associated with mitochondria (marked with orange "M"). C, Germ granules accumulate *nos* (green) and *pgc* (magenta) homotypic mRNA clusters,[5-7] that are often colocalized within the same granule but that do not mix with each other.[7] White arrows point at granules that are populated by only *nos* or *pgc* demonstrating that germ granules are heterogeneous in mRNA composition. D, *oskar* mRNA translates into Long and Short Osk isoforms that regulate distinct aspects of germ plasm and germ granules formation. R-me indicates a methylated arginine. Scale bar in B is 500 nm and in C it is 1000 nm

mRNAs contained in germ plasm were later genetically identified as the products of so-called maternal effect genes required maternally to regulate the assembly of germ plasm during oogenesis and the function of germ cells in the resulting embryo. Mutations in genes required for germ plasm assembly lead to a "grand-childless" phenotype due to the loss of germ cells in the progeny of mutant mothers.[12-14] Among these genes, *oskar* (*osk*) plays a central role in the organization of germ plasm and the formation of germ cells in the early embryo. An instructive role for Oskar protein akin to the germ plasm transplantation experiments of Mahowald and Illmensee was demonstrated genetically by expressing a transgene that encoded the open reading frame (ORF) of *oskar* with an RNA localization signal that anchored this transgene to the anterior pole of the embryo.[14,15] Thus, the anterior localization of *osk's* ORF and the resulting local production of Osk protein at the anterior pole was sufficient to attract other germ plasm components, leading to assembly of germ granules and specification of functional germ cells at the ectopic location.[15] Interestingly, Oskar is not conserved beyond dipterans[16] and is not a marker for all stages of germ line development (reviewed in Reference 4). However, orthologs of other germ plasm components are found throughout the animal kingdom and present in germ cells throughout their life cycle.[4] Indeed, the core germ granule components Vasa, an ATP-dependent RNA helicase, the translation factors Nanos, Pumilio and Dazl, Tudor (Tud), the founder of the Tudor domain family of proteins and Aubergine (Aub), a Piwi family Pi RNA-binding protein,[4] all have critical, evolutionary-conserved roles in the germline across species. Therefore, deciphering the principles of germ granule formation and function in *Drosophila* allows us to understand the roles of these droplets in shaping the biology of germ cells in other organisms, including humans.

Early EM studies revealed that germ granules first appear as small and dense bodies at the posterior pole of the developing oocyte, are later inherited by the fertilized embryo and finally become engulfed by the newly formed pole cells. Modern molecular biology, microscopy and genetic tools revealed that these granules accumulate maternally provided messenger ribonucleic acids (mRNAs) and proteins critical for the establishment of PGCs and the germline; in their absence, germ cells do not form and the resultant embryo is sterile (reviewed in Reference 4). Thus, while Weismann was looking for the heritable substance, the DNA, he instead identified germ granules as the hallmark substance that provides continuity of the species, the subject discussed in this review.

2 | ONTOGENY AND ORGANIZATION OF GERMPLASM

2.1 | Localization of Oskar and other core germ plasm components

Germ granule formation in *Drosophila* is intricately linked to oocyte polarity and relies on the coordinated transport of a single mRNA, *oskar*, toward the posterior pole of the oocyte, where this mRNA remains localized throughout late oogenesis and early embryogenesis. To ensure the continuity of the species, *Drosophila* evolved an intricate mechanism by which *osk* reaches the posterior pole (described in detail[4]). Briefly, *osk* mRNA is synthesized by the nurse cells, which are

sister, germline cells of the oocyte connected with the oocyte by large inter-cellular bridges called ring canals. The nurse cells have large, polyploid nuclei that synthesize transcripts and proteins for the transcriptionally silent oocyte. Dynein motors transport *osk* and many other mRNAs into the growing oocyte along the minus-end microtubule-directed transport from nurse cells into the oocyte.[17] Afterward and concurrent with repolarization of the oocyte microtubule network, *osk* transport particles shift preference toward the kinesin-mediated, plus end-directed transport, which leads to accumulation of *osk* mRNA at the oocyte's posterior pole. Concerted action of F-actin and cortical microtubules establishes a sustained anchoring of *osk* transcripts at the posterior cortex.[18,19] However, this anchoring is not static. Instead, the granules display corralled movement or move directionally on the cytoskeletal network spanning several micrometers across the posterior cortex.[18]

Enroute from nurse cells into the oocyte, several *osk* molecules become packaged into large ribonucleoprotein particles (RNPs) containing the double-stranded RNA-binding protein Staufen and the exon junction complex components Mago nashi, Y14, eIF4AIII and Barentsz.[20-24] Transport and mRNA localization to the posterior pole rely on the ability of *osk* mRNA to dimerize and are coordinated by *cis*-acting sequences positioned within the *osk* 3′UTR and the first exon junction in *osk*.[25] Additional sequences in the *osk* 3′UTR are specifically recognized by translational regulatory proteins such as the RNA-binding protein Bruno, which represses *oskar* translation during transport.[26-28] The switch from minus- to plus-end–directed microtubule transport depends on the germline-specific isoform of tropomyosin.[29-31] In addition to this early, motor-dependent enrichment, *osk* accumulation also relies on a motor-independent enrichment process during later stages of oogenesis and is mediated by the RNA-binding proteins Rump and Lost.[32] This accumulation of *osk* together with the continuous production of Oskar protein amplifies the amount of germ plasm available to form germ granules.[5,18,32,33]

Upon localization, the repressive activity of Bruno is inhibited, allowing *osk* translation. Together these regulatory mechanisms ensure that *oskar* mRNA reaches the posterior pole with high efficiency and that Oskar protein is specifically synthesized there. Perhaps given the complex and possibly energy-consuming nature of *osk* transport to the posterior pole, it is not surprising that only few mRNAs aside from *osk* use directed microtubule-mediated transport to localize within the oocyte or are as tightly regulated as *osk* in the oocyte. For example, approximately 200 maternally provided mRNAs that enrich in germ plasm nucleated by Oskar[34,35] localize passively, through a diffusion and entrapment mechanisms (see below[36]). While this entrapment mechanism seems similar to the late stage localization of *osk* mRNA, *osk* accumulates in RNP particles that are distinct from germ granules, which contain the majority of localized mRNAs at the posterior pole.[6,7,19]

2.2 | Organization of proteins in germ granules

Germ plasm is a specialized cytoplasm that forms at the embryo's posterior pole and is populated by core granule proteins Oskar, Vasa, Tud and Aub, a variety of RNA-binding proteins involved in various aspects of RNA biology, maternally deposited mRNAs, piRNAs and mitochondria (Figure 1A-D) (reviewed in Reference 37). While most of the RNA-binding proteins enriched in germ plasm are also found elsewhere in the embryo, the core granule proteins are almost exclusively found only at the posterior pole.[38,39] The exact composition of germ plasm is not known, however, recent quantitative imaging data demonstrated that the vast majority of core germ plasm proteins (>94%) are condensed into granules, with very little of these proteins diffusing in the intergranular germ plasm space.[38] Indeed, their concentration in the intergranular space is similar to their concentration in somatic regions.[38] For germ plasm mRNAs, the majority of transcripts that enrich at the posterior remain dispersed in somatic regions of the embryo with only up to 4% of a particular mRNA enriched in germ plasm.[7,40] Despite this small fraction, however, these mRNAs become 8- to 10-fold more concentrated upon localization.[7] Some mRNAs that enrich in germ plasm also tend to preferentially accumulate in granules. For instance, whole-mount in situ labeling of 59 mRNAs revealed that these transcripts interact with germ granules; they arrange as crescents surrounding the dividing PGC nuclei in older embryos, a spatial organization driven entirely by germ granule proteins (Figure 2A, see granule organization around nuclei of pole buds).[41,42] Interestingly, transcriptome analysis with microarrays of purified fluorescently labeled PGCs revealed that over 1700 different transcripts enrich in PGCs compared to the surrounding soma,[46] suggesting that the diversity of germ plasm transcripts could be much higher than originally believed. It is unknown whether all these various transcripts associate with granules or instead with other unknown germ plasm components. Regardless, PGCs that remain transcriptionally silent longer than the surrounding soma (see below) could benefit from this enrichment as the diversity of localized mRNAs could provide quiescent PGCs with the necessary maternally provided material for PGCs to reach activation of their zygotic genome upon maternal to zygotic transition (MZT).

At the posterior pole, *osk* mRNA translates into two protein isoforms: a longer 606 amino acid (aa) isoform called Long Oskar (Long Osk), which is translated from the first start codon, and a shorter, 467 aa isoform called Short Oskar (Short Osk) translated from the second start codon (Figure 1D).[47] Interestingly, the two isoforms have different subcellular localizations and functions. Long Osk is found in close association with the egg cortex and is required for the sustained association of *osk* mRNA with the posterior pole and localization of mitochondria (see also below), while Short Osk is both necessary and sufficient for germ plasm assembly and germ cell formation (Figure 1D).[19,39,47-49] Short Osk forms granules in the absence of other germ granule components in cultured *Drosophila* cells and human cells,[38] indicating that condensation into a granule is an innate property of Oskar protein. Additionally, early *Drosophila* embryos form two types of germ granules: the cytoplasmic granules also called "polar granules" that associate with maternally deposited mRNAs and promote formation of PGCs, and nuclear germ granules that promote mitotic divisions of PGCs (Figure 2A-C).[38] Both granules are mostly round and nonmembrane bound, nucleated by Short Osk and recruit Vasa,[8,38,49-51] while only cytoplasmic granules also recruit Aub, Tud and known granule-enriched mRNAs.[38] Nuclear germ granules are bigger

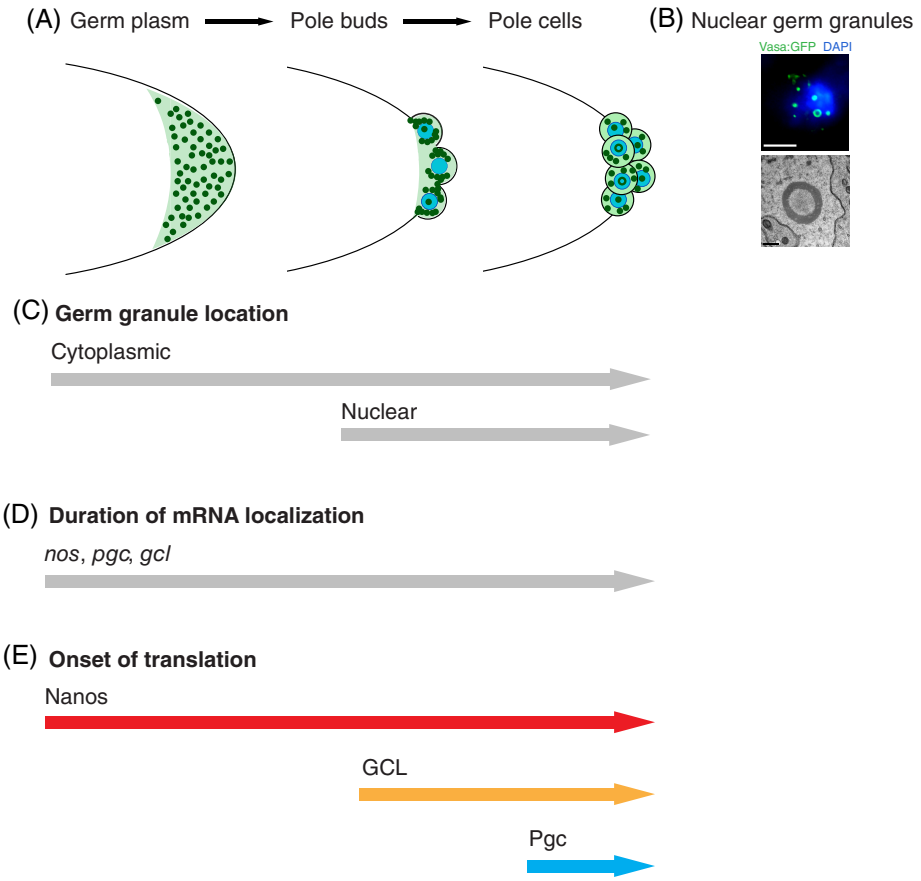

FIGURE 2 Spatial organization of germ granules and of granule-associated posttranscriptional regulation through early embryo development. (A-C) Initially, germ granules are uniformly distributed within germ plasm (0-1.5 hours old fertilized eggs). Once the pole buds form at the posterior pole, germ granules then transport via dynein motors coupled to astral microtubules toward the centrosomes associated with pole bud nuclei.[41] As such they become organized into crescents surrounding dividing nuclei in pole buds and pole cells (1.5-3 hours old fertilized eggs) (A).[41,42] During this time, nuclear germ granules form that are also often hollow (B, C).[38] Image in B shows a nucleus of a pole cell stained with DAPI (blue) that accumulates hollow nuclear germ granules stained with Vasa:GFP protein (green). EM image in B shows nuclear germ granules formed by Short Osk in *Drosophila*-cultured cells lines. (D, E) *nos*, *pgc* and *CycB* mRNAs persistently localize in germ granules throughout early embryogenesis (D) but they nevertheless display distinct onsets of translation (E) to allow the body patterning of the early embryo (Nanos), cellularization of pole buds into primordial germ cells (Gcl) and transcriptional silencing of newly formed primordial germ cells (Pgc)[42-45] (figure adapted from Reference 42). Scale bar in B is 5 μm (fluorescent image) and 1 μm (EM image)

and often appear hollow in EM and fluorescence images (Figure 2B),[38,50] and some cytoplasmic germ granules seem to have similar protein lucid cores. In both types of granules, this core appears less electron dense in EM images than the outside granule rim (Figure 2B).[8,38,50,51] A short nuclear localization sequence controls nuclear import of Osk and cotransport of Vasa into PGC nuclei where the two proteins condense into the same granules. As with cytoplasmic granules, the majority of Osk and Vasa is found in granules rather than freely diffusing in the nucleoplasm.[38] How these nuclear granules regulate of PCG number is currently unknown.

Short Osk contains two, structurally discernable protein domains. The N-terminus forms the LOTUS domain named after the Limkain (a human autoantigen whose function and binding partners are unknown), Oskar, and Tudor-containing proteins 5 and 7 (TDRD5 and TDRD7 proteins, respectively), two mammalian members of the germline Tudor group.[52] The C-terminus is a novel RNA-binding domain that shares similarity to SGNH hydrolases but lacks its enzymatic activity.[53] Recent structural studies revealed that the LOTUS domain folds into a winged-helix-turn-helix fold motif. The beta-sheets of the LOTUS domain facilitate Osk dimerization while the extended helices interact with the C-terminal RecA-like helicase domain of Vasa (Figure 1D).[54-56] Short Osk-Vasa interaction stimulates Vasa's ATPase activity in the presence of single-stranded and double-stranded RNA and is required for posterior localization of Vasa.[49,55] It is unclear whether and how Osk and Vasa regulate RNA biology of granule mRNAs. For example, Osk could help recruit mRNAs to granules via its RNA-binding domain. Indeed, in vitro and in vivo experiments demonstrated that Oskar interacts with germ granule-enriched mRNAs *nanos* (*nos*), *germ-cell-less* (*gcl*), *polar granule component* (*pgc*) as well as its own mRNAs, albeit with low affinity and in a sequence unspecific manner.[54,57] Vasa on the other hand could unwind secondary RNA structures and RNA duplexes of localized

mRNAs, possibly to facilitate their localization and translation in granules. The Osk-Vasa complex may therefore play an instructive role in attracting mRNAs to the posterior pole.

In addition to mRNAs, germ granules recruit Tudor[58] and Aubergine[59] proteins (Figure 1D). Aub is a member of the Argonaute/Piwi family of proteins and binds to small piRNAs that regulate transposable elements but has also been implicated in mRNA binding (see below[60]). Tud consists of 11 Tudor domains, each able to bind symmetrically methylated arginines found in Aub. Tud tethers Aub via its Tudor domain to the germ plasm, as mutations that affect Tudor's ability to bind dimethylated arginines strongly reduce the localization of Aub to the posterior pole (Figure 1D).[61,62] Interestingly, not all Tudor domains bind Aubergine equally well and identical mutations in distinct Tudor domains have different effects on Aubergine localization.[62] This finding suggests that the Tudor domains have additional, yet unknown specificities and can possibly organize multiple proteins containing dimethylated arginines. One such protein could be Vasa, also a methylated protein,[63] suggesting that Vasa's persistent anchoring to granules could depend on both Osk and Tud. This possibility further suggests that Tudor's role in germ granule formation could be as a scaffolding protein. Indeed, EM studies of embryos expressing mutated versions of Tud protein revealed that in the absence of Tud protein, embryos form fewer granules that are also far less electron dense than their wild-type counterparts.[51,58] Additionally, identical mutations in different Tudor domains also result in distinct appearance of germ granules,[51] indicating that Tud plays a central role in maintaining the integrity and the morphology of germ granules in *Drosophila* (Figure 1D).

Oskar/Vasa and Tudor/Aubergine form specific complexes of high affinity interactions. However, these proteins also associate into large membraneless droplets that by other criteria such as high concentration, mobility of components and variable stochiometry resemble RNA droplets such as stress granules, processing bodies and germ granules called P-granules of the round worm *Caenorhabditis elegans* (*C. elegans*). These RNA granules, including germ granules in *Drosophila*, are composed of diverse RNA-binding proteins and mRNAs and form by phase separation, a process best described as oil-and-water demixing.[37,38,64] Proteins that phase separate often contain intrinsically disordered regions (IDRs) (regions that do not adopt a particular protein fold) or low complexity sequences (LCs) (regions within a protein containing little diversity in amino acid composition).[64-67] Indeed, a 160 aa long IDR resides between the Osk's LOTUS and its RNA-binding domain, while the first 47 aa of the LOTUS domain also harbors a LC sequence.[38] When truncated versions of Oskar are expressed in *Drosophila* cultured cells, deletion of either the LOTUS domain, Osk domain, its IDR or LC fails to abolish condensation of Short Osk indicating that the four Short Osk regions act redundantly to form a granule. However, these protein truncations condense less efficiently indicating that these regions synergize to augment Short Osk condensation.[38] Interestingly, Long Osk never forms granules be it in embryos or in cultured cells despite sharing all structural features with Short Osk.[38,39,47] Therefore, the N-terminal region of Long Osk interferes with the ability of the protein to condense. The biological relevance of this interference in embryos is not understood.

Biophysical studies further demonstrated that *Drosophila* germ granules display both liquid-like and hydrogel-like properties and are thus best described as phase-transitioned condensates. Specifically, a fraction of core granule proteins readily exchanges with the granule environment while a fraction appears highly immobile and is retained within granules.[38] Such properties are likely relevant as they can have profound functional consequences for the development of the germline. The liquid properties could enhance biochemical reactions occurring within granules while the more stable conformation could ensure that granule regulatory proteins persist throughout early embryonic development. Indeed, functional germ granules form during late oogenesis[68] and persist through early embryogenesis, a process that lasts many hours when fertilization is delayed.[69,70] The persistence of granules and its components is fundamentally important for the formation of germ cells in *Drosophila*. For example, embryos of certain Osk and Tud alleles that fail to form granules or form only small granules are largely defective in RNA localization and do not form germ cells.[14,51,58] Thus, robust mRNA localization within a liquid granule environment that affords biochemical reactions would support dynamic and prolonged posttranscriptional regulation locally at the posterior pole and enable synthesis of effector proteins necessary for germ cell fate and function (Figure 2A-E).

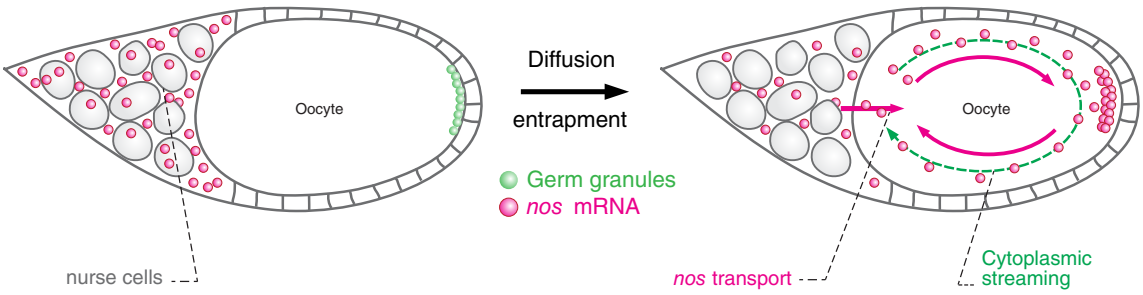

FIGURE 3 Mechanisms of mRNA enrichment to germ granules. mRNAs such as *nos* (pink) are transcribed in nurse cells during oogenesis and afterward dumped into a transcriptionally silent oocyte. Cytoplasmic streaming swirls these mRNA through the oocyte, which brush along the germ granules (green) formed at the posterior pole. mRNAs enrich in germ granules passively in a diffusion/entrapment-dependent mechanism. Figure adapted from Reference 36

2.3 | Organization of mRNAs in granules

An estimated 200 mRNAs are specifically enriched in the germ plasm (Figure 1D).[34] Most enriched mRNAs reach the posterior and anchor into germ granules during mid-oogenesis by passive diffusion-entrapment mechanisms (Figure 3).[36] Here, transcriptionally active nurse cells dump their content into a quiescent and growing oocyte. The oocyte forms microtubule bundles, which cause cytoplasmic streaming in the oocyte that swirls the cytoplasm through the oocyte enabling mRNAs to entrap as they brush along the germ granules formed at the posterior pole (Figure 3).[36] Deep-sequencing studies have revealed that the initial entrapment of mRNAs into granules could be mediated by RNA:RNA interactions via short RNAs called piRNAs loaded into the Aubergine protein. In this model, partial complementarity is established between piRNAs and target mRNAs, which is sufficient to initially recruit and anchor a variety of transcripts in germ granules.[60] For several mRNAs, the sequences proposed to mediate complementarity between piRNAs and target transcripts reside within the mRNAs' 3′ untranslated regions (UTRs).[60] This result agrees well with previous findings using transgenically expressed reporter mRNAs, which demonstrated that sequences necessary and sufficient for posterior localization of *nos*, *gcl* and *pgc* reside within their 3′UTR.[42,43,71] Additionally, recent high-resolution microscopy studies have shed new light on the mechanism by which mRNAs such as *nos*, *CycB*, *gcl* and *pgc* may become highly enriched and organized within germ granules. While individual mRNAs localize as single transcripts, upon enrichment, however, between 2 to more than 40 mRNA molecules derived from the same gene organize into homotypic mRNA clusters within germ granules (Figure 1C).[5-7] Importantly, these homotypic clusters occupy defined positions from the center to the periphery of granules, while the core granule proteins Osk, Vasa, Tud and Aub, that make the granules and recruit other granule components including mRNAs are homogeneously distributed within the same granule.[7] These results suggest that the assembly of homotypic clusters is driven at least in part, by the granule mRNAs themselves, possibly through direct RNA-RNA interactions. In support of a process mediated by RNA-RNA interactions, mRNAs seem more likely to associate with each other than equally distributing across all granules, leaving some granules devoid of specific mRNA species (Figure 1C).[5-7] Additionally, granule mRNAs display an ability to self-recruit to granules,[5] further supporting the possibility that the RNA-RNA interactions could play a central role in mRNA enrichment and organization of mRNAs in *Drosophila* germ granules. Thus, a unified and fascinating theme is emerging from studies of RNA droplets in the fly: that RNA:RNA interactions could play a central role in the enrichment and organization of mRNAs in *Drosophila* germ granules.

mRNA enrichment at the posterior pole within germ granules has important consequences for germ cell development and function. (a) Increased concentration. While only 3% to 4% of the total amount of a given mRNA is localized at the posterior pole, the final concentration in the granule is 8- to 10-fold of that found elsewhere in the embryo.[7,40] (b) mRNA stability. Maternally synthesized mRNA and proteins deposited in the developing egg during oogenesis are degraded in the somatic regions of the embryo during the MZT, when the embryonic genome is activated. Germ plasm mRNAs are stabilized and protected from this degradation and MZT occurs at a later developmental stage in germ cells.[46,72,73] (c) Translational regulation. A common feature of posteriorly localized mRNAs is that they are translated while the 96% of unlocalized counterparts distributed throughout the embryo that will give rise to future soma, are translationally repressed.[42,71] (d) mRNAs stored in the germ granules are translated temporally in the order of need (Figure 2E).[42]

For many mRNAs it has been shown that translational repression outside of germ granules depends on the RNA-binding protein Smaug, which recruits the CCR4/NOT deadenylases to the respective mRNAs.[74,75] For *nanos* mRNA, translational activation has been linked to germ plasm localization via Oskar, which displaces Smaug from *nos* thereby preventing the deadenylation of *nos* CCR4/NOT complex and increasing the stability of the mRNA while simultaneously also relieving translational repression imposed by Smaug.[76] It is unlikely that this is a general regulatory mechanism for germ plasm-localized mRNAs as not all posteriorly localized mRNAs are Smaug targets.[46] Additionally, the developmental time when posteriorly localized mRNAs are translated varies from immediately upon localization (*nos*), to later stages of development when nuclei enter the germ plasm (*gcl*), when germ cells form (*pgc*) or when germ cells reach the embryonic gonad (*CycB*) (Figure 2A,E).[42] Curiously, the spatial organization of homotypic mRNA clusters within granules does not predict when an mRNA becomes translated nor how effectively it will be protected from somatic mRNA decay.[7] Whether this mRNA self-organization may influence other aspects of posttranscriptional control of localized transcripts is presently unclear.

2.4 | Functions of germ plasm and germ granules

Components enriched in the germ plasm and germ granules control critical germ cell functions. Several of these functions can be directly linked to specific mRNAs, including those required for germ cell fate specification (*nos*), germ cell formation (*gcl*), transcriptional silencing (*pgc*), germ cell migration (*tre1*) and germ cell survival (*wun2*). Other germ cell functions, such as mitochondrial inheritance and germ cell genome integrity also rely on maternally synthesized factors. The latter, however, are not linked to a single gene but to the deposition of small RNAs, called piRNAs, which protect germ cells against transposable elements.

2.4.1 | Nanos suppresses somatic fate in germ cells

Germ cell specification requires the function of the *nanos* gene. *nanos* translation is initiated specifically at the posterior pole as soon as the mRNA becomes localized (Figure 2A,D,E) and the resulting Nanos protein forms a posterior-to-anterior gradient. Germ granule localization of *nos* followed by local translation and formation of the gradient plays a critical role in establishing the anterior-posterior segmental patterning of the embryo.[43] Nanos together with its cofactor Pumilio inhibits the translation of maternal *hunchback* (*hb*) mRNA[77] and the absence of Hb protein in the posterior region allows the expression of

gap genes required for abdomen formation.[78,79] Thus, in the absence of maternally provided Nos, embryos fail to form an abdomen.[13] Additionally, failure to localize *nos* and defects in posterior Nos protein translation account for the abdominal phenotype of the maternal effect genes required for germ plasm assembly (so called posterior group phenotype).[13,79] Nanos is not required for germ cell formation per se, but it is required to preserve a germ cell-specific cell cycle program. As a consequence, in *nanos* mutant embryos, pole cells express somatic genes, undergo apoptosis, migrate aberrantly and lose their ability to give rise to functional germ cells.[80,81] How Nanos promotes germ cell specification remains unclear, however, recent experiments have provided some insight into this question. The tumor suppressor L(3)mbt was shown to secure somatic cellular identity in *Drosophila* ovaries and larval brains by repressing germline-specific genes, including *nanos*.[82] Given its well-documented role in translational regulation, it is likely that the primary role of Nanos is to repress factors that promote somatic development rather than to actively promote germ cell development, akin to its recently reported role in *C. elegans*.[83] Whether Nanos also has an independent role in actively promoting germ cell fate is less clear.

2.4.2 | The GCL protein regulates germ cell formation

Germ cells are the first cells formed in the *Drosophila* embryo. The early embryo begins its development without cell membranes. Instead, zygotic nuclei undergo rapid, synchronous divisions. Subsequently, nuclei line up along the egg cortex where they are transformed into "bona fide" cells. This occurs in two stages. Nuclei that reach the posterior pole cellularize first and give rise to pole cells, the PGCs, while several divisions later, the remaining nuclei are enveloped by cell membranes and generate all somatic tissues.[44,70,84,85] Pole cells form during the 10th nuclear division cycle during the time when GCL translates at the posterior pole (Figure 2A,D,E).[42] At this stage, apical, actin-filled membrane caps form around each nucleus where they basally constrict to separate the future germ cells from the rest of the embryo. GCL protein controls this basal constriction.[44] Afterward, cellularized nuclei divide via the canonical anaphase constriction.[44] *Drosophila* therefore exemplifies an extreme case of how germ line-soma dichotomy is achieved. Not only are the two cell populations specified at different times during development, but also the cellular events leading to germ cell and somatic cell formation are strikingly different and are controlled by separate sets of genes. For example, germ cell formation and specification rely on maternally synthesized factors and occur even when zygotic mRNA transcription is blocked.[44]

A rate-limiting component specific to pole cell formation is the Germ-cell-less protein. GCL is a BTB/BACK domain protein with homologs found from nematodes to humans and distinguished by the conserved GCL motif in the C-terminal region.[45] GCL is not directly involved in transcriptional silencing or centrosome segregation during germ cell formation as previously suggested.[44,86,87] Instead, GCL functions as a substrate-specific adaptor for the Cullin 3-Ubiquitin-ligase complex.[45] GCL targets the receptor tyrosine kinase (RTK) Torso for degradation via the conserved GCL substrate-binding motif. During pole cell formation, GCL translocates from the inner nuclear membrane to the cell membrane where it leads to the degradation of Torso. Amazingly, pole cell formation is fully restored in *gcl*, *torso* double mutants, indicating that Torso is the single critical target of GCL in the early embryo and involved in the formation of basal constriction promoted by GCL.[44,45] How Torso activation inhibits this constriction is not fully understood. Preliminary studies suggest that this process is independent of the conventional downstream components of the Torso RTK including MAP kinase-mediated transcriptional activation of target genes.[45] These studies have also suggested that additional, yet unknown germ plasm-localized factors can promote basal furrow constrictions in the absence of *gcl* and *torso*.

2.4.3 | Pgc represses transcription in germ cells

Major transcriptional activity in germ cells is not observed until they reach the gonad, although PGCs become transcriptionally competent shortly after they form.[88] *Polar granule component* (Pgc) protein, which translates once PGCs cellularize (Figure 2A,D,E),[42] is required for transcriptional silencing of primordial germ cells. *Pgc* mRNA was first suggested to encode a noncoding RNA, however, later studies demonstrated that instead it encodes a 71 amino-acid peptide[89,90] This peptide blocks transcriptional elongation by inhibiting transcription elongation factor b (P-TEFb).[90] P-TEFb promotes transcriptional elongation by phosphorylating the carboxy-terminal repeat domain (CTD) of RNA polymerase II (RNAPII) at the Serine 2 position. In embryos derived from *pgc* mutant mothers, pole cells form but they now transcribe genes that are otherwise expressed only in somatic cells.[91] As a result, maternally deposited, germ cell-specific components are lost due to precocious activation of the MZT that at this developmental stage normally only takes place in the soma.[92] Consequently, germ cells in *pgc* mutant embryos are unable to migrate during later developmental stages and instead undergo apoptosis.[91] Thus, transcriptional silencing in germ cells maintains the germ line-soma dichotomy by preventing the somatic program unfolding in primordial germ cells.

2.4.4 | Tre1 and Wunen regulate germ cell survival and migration

Germ cells form at the posterior pole, and subsequently move toward the somatic tissues of the gonad, which are specified in the mesoderm of the abdomen. Successful survival and migration of germ cells is linked to reproductive success of the offspring. The number of germ cells formed depends on the overall amount of germ plasm. For example, reduction in the germ plasm organizer Osk reduces germ cell number while an increase in Oskar has the opposite effect.[15,93] The amount of germ plasm inherited is graded from the center of the posterior tip to the periphery: germ cells in the center inherit more germ plasm than those at the periphery. Not all germ cells reach the gonad and about 35% to 45% of germ cells are eliminated during migration. The ability to survive and reach the gonad is directly correlated with

the amount of germ plasm inherited.[94] Indeed, the levels of a single germ plasm-localized mRNA, Wunen 2, which encodes a protein that is a homolog of mammalian lipid-phosphate phosphatase 3, was shown to be a quantitative regulator of germ cell survival.[94] Another regulator of germ cell migration, the G-protein-coupled receptor Tre1 is also synthesized maternally and deposited as a localized mRNA to the germ plasm and translated there. Tre1 receptor activation orients germ cells as they exit the gut and directs their migration toward the somatic gonad.[95]Thus, heterogeneity of germ plasm, inherited at the time primordial germ cells form, predetermines the success of these cells for future generations.

2.4.5 | Mitochondria and germ plasm

The transmission of mitochondria through the germ line is an essential component of maternal inheritance. Mitochondria are passed from the mother to the progeny in most organisms and specific mechanisms exist to eliminate paternal mitochondria.[96] Consequently, the mitochondria of PGCs will constitute the pool from which all mitochondria of the next generation originate. The original EM images suggested that germ granules and mitochondria could closely associate in the germ plasm.[68] Subsequently, it was proposed that mitochondrial large and small ribosomal RNAs (mtrRNA), which are transcribed by the mitochondrial genome, were extra-mitochondrial in the germ plasm and associated with germ granules.[97] It was hypothesized that these extra-mitochondrial mtrRNAs induced the assembly of "mitochondrial-like" ribosomes within the germ plasm and directed translation of germ plasm mRNAs with mitochondrial codon usage.[98,99] While intriguing, recent high-resolution imaging showed that mtrRNA localizes separate from germ granule markers such as *nanos* mRNA and is strictly confined to the mitochondria at the posterior pole as well as elsewhere in the embryo.[48] This study shows that mitochondria are enriched at the posterior pole, consistent with the previous EM observations. Closer examination of the mechanisms by which mitochondria become enriched at the posterior pole showed that this localization requires the Long Osk (Figure 1D) and is independent of the germ plasm inducing function of Short Osk.[48] Both Long Osk as well as just the 138 aa N-terminal extension of Oskar, which distinguishes Long Osk from Short Osk, are sufficient to localize mitochondria to an ectopic location.[48] Previous studies have suggested that Long Osk enhances the cortical recruitment and maintenance of *osk* mRNA at the posterior pole by mediating the formation of actin filaments and recruitment of endosomes.[19,100] The new studies further show that Long Osk also directly associates with actin cytoskeleton and that this function is critical for mitochondrial enrichment at the posterior pole.[48] The functional significance of this mitochondrial enrichment in PGCs is unclear. Germ cells form in the absence of Long Osk on condition that Short Osk is provided separately, however, their number is reduced and they contain fewer mitochondria. This reduction in PGC number could be an indirect consequence of loss of Long Osk diminishing the ability of *osk* mRNA to anchor at the cortex, thereby translating less Short Osk and forming fewer germ granules. Alternatively, this finding may suggest a more direct requirement for increased mitochondrial activity during PGC formation. The later idea is supported by experiments that interfered with prokaryotic (and mitochondrial) translation specifically at the posterior pole and resulted in a reduction in germ cell number.[99]

Beyond a potential functional role during the formation of germ cells, enrichment of mitochondria could impact germ cell development in other ways. Germ granules are closely associated with mitochondria (Figure 1B) throughout their lifecycle and this association may be important for the success of germ cells. Recent experiments using heteroplasmic flies that simultaneously harbor wild-type mitochondrial DNA and mutant DNA defective in mitochondrial oxidative phosphorylation showed that functional mitochondria are actively selected during early oogenesis.[101] Thus, enrichment of mitochondria in germ plasm at the PGC stage may widen the bottle neck and increase the pool size from which good mitochondria can be selected for inheritance at a later developmental stage.

2.4.6 | Germ granules and the control of transposable elements through germline the life cycle

Transposable elements (TE) account for about one-third of the *Drosophila* genome. Their activity is regulated in the germline by a gonad-specific subclass of Argonaute proteins, called Piwi, Argonaute 3 and Aubergine, and the Piwi-interacting (Pi) small RNAs (piRNAs). Immunity to TEs is transmitted maternally through the deposition of piRNAs in the germ plasm. Together with Piwi, maternally inherited piRNAs repress the transcription of TEs by recruiting heterochromatin to TE loci.[102-104] With Aubergine, piRNAs slice TE transcripts in the cytoplasm and initiate an amplification loop, which generates new piRNAs in the sense and antisense orientation. According to the "ping-pong" model, sense-piRNAs and TE transcripts fuel the production of new TE-complementary piRNAs, thereby providing continued and adaptable immunity against TE activity.[105] Recent studies using immunoprecipitation followed by RNA sequencing suggested that in addition to targeting TEs, maternally deposited piRNAs associated with Aubergine and Tudor proteins also enriched for germ plasm localized mRNA.[60] In this scenario, piRNA-mRNA recognition would trap mRNAs in the germ plasm. In these experiments, piRNA-mRNA pairs did not show any sequence specificity for germ plasm localized RNAs and it was therefore proposed that enrichment of localized mRNAs is achieved by special mRNA features such as longer 3′UTRs.[60] While intriguing, other studies have suggested that Aubergine may bind directly and preferentially to germ plasm-enriched mRNAs to stabilize them. In contrast, in the soma Aubergine/mRNA interactions lead to mRNA degradation.[106,107]

3 | CONCLUSION

Germ granules in *Drosophila* are the hubs for RNA biology: they enrich and posttranscriptionally regulate a subset of mRNAs crucial for the development of the germline to ensure fecundity of the offspring. A century of experimentation described *Drosophila* germ granules as

electron dense, amorphous and nonmembrane bound organelles. Genetic analysis identified critical proteins and mRNAs enriched in germ granules, revealed their activity and regulatory mechanisms. Most recent biophysical studies have shown that germ granules form by phase separation. Yet, fundamental questions remain unaddressed. Is germ plasm functional or is the germline-inducing activity restricted to germ granules? If so, what is its function? Is the composition of germ plasm different from the composition of germ granules? Aside from mRNA enrichment and storage, what additional functions, if any, do germ granules have, perhaps in regulating posttranscriptional reactions? How do homotypic mRNA clusters form in germ granules and how does their organization contribute to their biological function? What are the conserved and unique principles of composition, structure and function of germ granules compared to other droplets formed by phase separation? New quantitative, super-resolution imaging studies combined with biochemistry and genetics are providing a framework to begin addressing some of these questions. These methods allow us to dissect the function of germ granules and explore how concentration of protein and mRNA components in granules establishes a highly specialized program that controls posttranscriptional regulation of mRNAs required for many aspects of early germ cell biology. As for the past century, with modern technology and approaches these mysterious condensates will continue to fascinate us.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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