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Structural and functional organization of germ plasm condensates

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Abstract

Reproductive success of metazoans relies on germ cells. These cells develop early during embryogenesis, divide and undergo meiosis in the adult to make sperm and oocytes. Unlike somatic cells, germ cells are immortal and transfer their genetic material to new generations. They are also totipotent, as they differentiate into different somatic cell types. The maintenance of immortality and totipotency of germ cells depends on extensive post-transcriptional and post-translational regulation coupled with epigenetic remodeling, processes that begin with the onset of embryogenesis [1,2]. At the heart of this regulation lie germ granules, membraneless ribonucleoprotein condensates that are specific to the germline cytoplasm called the germ plasm. They are a hallmark of all germ cells and contain several proteins and RNAs that are conserved across species. Interestingly, germ granules are often structured and tend to change through development. In this review, we describe how the structure of germ granules becomes established and discuss possible functional outcomes these structures have during development.

Germ granules: a hallmark of the animal germ cell lineage

Germ granules is a term that refers to the RNA-rich condensates that mostly form in the cytosol of germ cells. Depending on the organism and its developmental stage, several types of germ granules have been described. These include nuage, which in immature and differentiating germ cells, sponge body, Balbiani body and chromatoid body, which form in gametes, and germinal granules, polar granules and P granules, which accumulate in the oocytes and embryos [3]. Germ granules control mRNA abundance, translation, mRNA decay, and small RNA production to specify the germ cell fate, control the formation and

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Tatjana Trcek: Conceptualization, Resources, Supervision, Funding acquisition, Writing — original draft, Project administration, Writing — review and editing. **Austin Chiappetta:** Writing — original draft, Writing — review and editing. **Jeffrey Liao:** Writing — original draft, Writing — review and editing. **Siran Tian:** Writing — original draft, Writing — review and editing.

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number of germ cells as well as protect the germline genome from transposon-induced DNA damage.

In different organisms, germ granules change their name, form, composition, and association with other cellular structures, often within the same organism as it develops. They can be cytosolic and in contact with the nuclear pore complex (NPC), mitochondria and endoplasmic reticulum (ER) or form in the nucleus. They often interact with other RNA granules or 'de-mix' to form distinct condensates that remain attached to each other. Despite this diversity, germ granules share several proteins and messenger ribonucleic acids (mRNAs), including Vasa, the DEAD-box RNA helicase, PIWI and Aubergine, two Argonaute family of proteins involved in piRNA biosynthesis, Tudor-domain proteins required for protein:protein interactions, splicing, translational and RNA decay regulators, *nanos* mRNA, which codes for a translational regulator and small RNAs called piRNAs required for repression of transposon jumping (reviewed in [3]).

As a whole, germ granules largely appear as small and round puncta with a diameter no larger than a few micrometers. However, their internal structure is rarely symmetric and instead displays a heterogeneous composition. In this review we focus on the germ granules of *Caenorhabditis elegans* and *Drosophila melanogaster*, whose formation and function are best understood and summarize the properties and structures of germ granules in these species in Table 1. Interestingly, several structural features reported for the *C. elegans* and *Drosophila* germ granules have also been observed for the germ granules in zebrafish (Table 1), suggesting that structural features are conserved among germ granules across species. We end our review with *oskar*, the gene required for the formation of polar granules in *Drosophila* and whose products form condensates with complex structures and distinct developmental roles. Many of the principles that govern the formation and function of the *C. elegans* and *Drosophila* germ granules also apply to germ granules that form in other organisms, indicating that the fundamental properties of germ granules are conserved. Studying the germ granules that form in these model organisms will ultimately allow us to understand how germ granules help establish the next generation in humans.

Germ granules in *Caenorhabditis elegans*

In *C. elegans*, the germ granules and the surrounding germ plasm are maternally loaded into the oocyte. After fertilization and through four asymmetric divisions (P_1, P_2, P_3, P_4), the germ granules progressively partition to the posterior P lineage and eventually exclusively enrich in germ cells (Figure 1A) [7]. Because of this, germ granules of the early *C. elegans* embryo are also called P granules and the two terms are used interchangeably. Until this point, P granules are largely cytoplasmic without an apparent association with a particular subcellular structure. However, once P granules segregate to the P_4 germline blastomere, they arrange around the nuclear envelope to form nuage, the germ granules that also abut nurse cell nuclei in *Drosophila* oocytes (Figure 1B) [8–11]. There, they remain associated with the nuclear envelope throughout the rest of germline development [7]. Once perinuclear, the germ granule material begins to separate into at least four distinct germ granule condensates, namely P granules, Mutator foci, Z granules and SIMR-1 foci (Figure 1B). These condensates interact with each other and remain associated with the nuclear

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envelope (reviewed in [12]). Through early development, the germ granules in *C. elegans* undergo dramatic subcellular, structural and compositional changes, with some of them likely having functions in the establishment of the *C. elegans* germline.

Germ granules of the *C. elegans* early embryo

Initially discovered by immunostaining in 1982 [9], P granules were the first cytoplasmic RNA granules reported to display liquid-like behaviors [13]. They are mostly round, can fuse with each other, exchange components with the cytosol and form by a controlled condensation and dissolution [13]. During the first few embryonic divisions, P granules disassemble and re-assemble in the germ plasm concentrated at the posterior of the developing zygote [13–15]. Similar to *Drosophila* germ granules, electron microscopy imaging revealed these granules as 'numerous cytoplasmic and electron dense spherical bodies of an apparent fibrous nature' that mature into larger granules throughout divisions of the P_1 to P_4 blastomeres [11].

P granules contain a myriad of proteins required for their assembly and structural properties. Specifically, P granules form through condensation of the PGL-1 and PGL-3 paralogs, which contain a self-association domain and an RNA-binding RGG domain [16]. Crystal structures revealed that PGL-1 and PGL-3 form homodimers and provide a scaffold to P granules [17]. They are recruited to P granules by two RNA helicases, LAF-1 and GLH-1 (the Vasa helicase in *C. elegans*), and an intrinsically disordered protein DEPS-1 [16,18–21].

However, PGL-1/3, LAF-1, GLH-1 and DEPS-1 form labile condensates that dissolve quickly when isolated [22] and require two additional proteins to stabilize them, namely the intrinsically disordered RNA-binding proteins (RBPs) MEG-3 and its paralog MEG-4, with MEG-3 providing a greater contribution [23]. Lattice light-sheet microscopy and fluorescence recovery after photobleaching (FRAP) analysis demonstrated that MEG-3 forms stable, gel-like clusters that are resistant to dilution and which adsorb to the surface to reinforce the more labile, amorphous PGL-1/3 phase (Figure 1A) [22,23]. Without affecting the viscosity of the PGL condensate, MEG-3 clusters regulate the structural integrity of P granules by lowering the surface tension of the PGL phase, slowing down its coarsening and forming a physical barrier to coalescence, thus acting as Pickering agents [24]. Furthermore, post-translational modifications control the formation of P granules. MEG-3 is a substrate of the MBK-2/DYRK kinase and the $PP2A^{PPTR-½}$ phosphatase, and phosphorylation of MEG-3 promotes granule disassembly while its dephosphorylation promotes granule assembly [23]. MEG-3 also recruits MBK-2 to P granules where the kinase reduces the viscosity of PGL-3 droplets, increases its dynamics and tunes the cytoplasm-granule exchange of PGL-3 [24]. This tuning accelerates P granule dissolution and growth in the polarizing zygote to ensure their segregation to the P1 blastomere [24]. Lastly, MEG-3 is an RBP and co-precipitates with roughly 500 mRNAs in embryonic lysates, some of which are required for fertility [25].

In vitro and *in vivo* experiments demonstrated that RNA promotes phase separation of diverse proteins and that it is required for the formation and maintenance of RNA granules [8,26–37]. In agreement with these observations, RNA also enhances condensation of MEG-3 and hence the formation of P granules [38]. However, the distinct multi-phase

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appearance of the MEG-3/PGL-1 condensates depends on the HMG-like motif found at the C-terminus of MEG-3 rather than the RNA [39], indicating that the structural organization of P granules is primarily driven by protein:protein interactions.

Aside from the capacity of PGL and MEG proteins to condense, the asymmetric accumulation of P granules at the posterior cortex also requires the PAR and MEX polarity regulators. Here, the kinase activity of the PAR-1 protein, which partitions with P granules [40], generates an anterior-posterior gradient of two redundant RBPs, MEX-5 and MEX-6, by modulating their diffusion, with a slower fraction accumulating at the anterior [41,42]. This gradient is critical, as MEX-5/6 compete for RNA binding with MEG-3/4 and thus prevent the formation of P granules at the anterior pole, where the concentration of MEX-5 protein is high [38]. Once the asymmetry of MEG-3/4 concentration is established, PGL-1/3 can nucleate P granules through their self-association domain and recruit other P granule proteins [16].

Given the complex nature of the accumulation and structural organization of P granules, it is surprising that the asymmetric distribution of P granules is not required for the fertility of *C. elegans* [14]. Indeed, in the absence of expression of the *pptr-1* gene, which encodes a regulatory subunit of the PP2A phosphatase, P granules distribute equally between the germline and somatic blastomeres. And yet, the *pptr-1* mutants correctly specify the germline and have normal fertility. In addition, animals mutant for MEG-1, MEG-3 and MEG-4 proteins, all germ plasm components, are sterile, indicating that MEG proteins provide an essential activity that is required for the fertility of *C. elegans* independently of P granules [23]. In support of this notion, a new type of germ plasm condensate nucleated by MEG-1 and MEG-2 termed the germline P-body has recently been identified [43]. The germline P-body is distinct from P granules and forms independently from them but tends to form in close association with P granules. It enriches mRNA decapping and deadenylation enzymes and its components are required for the regulation of translation and stability of P granule mRNAs. MEG-1 and MEG-2 mutant embryos do not form germline P-bodies and do not develop the germline, supporting the observations that MEG-1, MEG-3 and MEG-4 mutant animals are sterile [23].

Experiments that examined the effect of MEG mutations on the formation of the germline also revealed that P granules as well as their complex structure are not required for the specification of germ cells in *C. elegans*. Importantly, as phase separation depends critically on the concentration of its components [44], it is possible that the formation of P granules could simply result from a highly concentrated germ plasm at the posterior pole without necessarily imparting an important regulatory function required for the germline in *C. elegans*. Alternatively, P granules could instead protect the germline from stress, as the fertility in *pptr-1* mutants becomes reduced when animals are reared at higher temperatures [14]. Further studies analyzing the germ plasm components of the P cell blastomeres are needed to identify the factors that specify the germ cell lineage in early *C. elegans* embryos.

Germ granules of the adult *C. elegans* germline

Once germ granules segregate to the P4 blastomere, they arrange around the nuclei to form the nuage (Figure 1B), where they remain for the remainder of worm development [7]. Like

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the cytoplasmic P granules, the nuage is membraneless and composed of diverse proteins and RNAs.

The nuage undergoes characteristic structural changes (reviewed in [12]). Its constituents de-mix into at least four distinct condensates, namely P granules, Z granules, SIMR foci and Mutator foci, which remain attached to each other in adult germ cells (Figure 1B) [45,46]. The consistency with which these four condensates interact with each other and the fact that several proteins that are required for the synthesis and function of silencing small RNAs (sRNAs) enrich in them suggest that nuage could be involved in RNA-mediated interference. This subject has been extensively covered in other excellent reviews and will only be summarized here (reviewed in [12,47,48]).

Briefly, *C. elegans* produces a variety of small regulatory RNAs (sRNAs) that are between 20 to 30 nucleotides (nts) in length. They are bound by different Argonaute proteins, which, along with the RNA sequences they pair with, endow the sRNAs with their specific activity. sRNAs silence gene expression by targeting cytoplasmic mRNAs for degradation [49,50], confer chromatin-based gene silencing by targeting nascent mRNAs as they are being transcribed in the nucleus [51–55] and silence expression of transposons, pseudogenes and repetitive elements [50,56].

Among the sRNAs involved in the RNA surveillance and gene expression silencing are the 21 nt long sRNAs with a strong bias for the 5′ uracil (21U-RNAs) called piRNAs. They represent a large group of genomically encoded regulatory RNAs mostly expressed in the germline and associated with the PIWI-class Argonaute protein PRG-1 [48,57,58]. In response to piRNA activity, a second type of sRNAs, called the 22G-RNAs, with a characteristic 22 nt length and a strong bias for guanosine at the 5′ position is formed. They are bound by worm-specific Argonaute proteins (WAGOs) and amplify the piRNA-triggered gene silencing response [49,59]. Remarkably, the silencing by sRNAs can be carried from a parent onto an offspring thus conferring a multigenerational sRNA-mediated silencing even after the initial exposure to the target RNA is removed (reviewed in [12,47,48]).

However, piRNAs bind to their target RNAs with high mismatch tolerance for base pairing. While this tolerance enables piRNAs to exhibit a broad targeting capacity, it also creates a problem as it renders most germline transcripts susceptible to silencing [60–63]. To circumvent this problem, a protective mechanism is set in place in which the Argonatue protein CSR-1 binds to abundant 22G-RNAs that target many germline-expressed transcripts thereby opposing the engagement of the PRG-1-bound piRNAs [61,64,65]. CSR-1 thus licenses germline mRNAs for expression through a process called self/non-self discrimination [64].

How the balance between silencing and licensing is achieved is not clear. However, the spatial organization of germ granules suggests a possible mechanism. PRG-1, CSR-1 and several WAGO proteins enrich in P granules [12,47,57,58] indicating that the decision to silence or license mRNAs likely occurs within the same cellular compartment. In addition, P granules interact with the nuclear pore complex (NPC) of the germ cell nuclei [9,66]. This association is driven by the interactions mediated between the phenylalanine-glycine

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(FG) repeats of the GLH-1/2/4 RNA helicases and the FG repeats of the nucleoporins of the NPC [67]. These interactions are thought to create an exclusion barrier reminiscent of the one formed within the central channel of NPCs [67]. In addition, P granules enrich nascent mRNAs in transcriptionally active germ cells [66], which suggests that P granules could be required for the surveillance of newly synthesized transcripts and distinguish foreign mRNAs from those licensed for germline expression as they exit the nucleus [66].

In addition, Z granules, Mutator foci and SIMR foci that form adjacent to P granules each accumulate distinct proteins that are active in different parts of the sRNA pathway, suggesting that these condensates might also be the sites where those reactions occur. Specifically, Mutator foci harbor the RNA-dependent RNA polymerases (RdRPs), which are required for the synthesis of WAGO 22G-RNAs. Therefore, the mutator complex is thought to function primarily for the amplification of WAGO 22G-RNAs and transgenerational silencing through the activity of RdRPs [50,68]. Meanwhile, Z granules contain ZNFX1 and WAGO-4 proteins, indicating that Z granules might be involved in transgenerational epigenetic inheritance. Finally, SIMR foci, which are found adjacent to Z granules and opposite P granules [46], are believed to traffic target RNAs for sRNA amplification (reviewed in [12,47]).

Interestingly, similarly to P granules of early embryos and adult animals, RNA is integral for the formation of Mutator foci, as injection of the transcriptional inhibitor α-amanitin triggers their dissolution [66,69]. The formation of Mutator foci is also disrupted at higher temperatures and in the presence of aliphatic alcohols that affect weak hydrophobic interactions [38,67,70], suggesting that like P granules, Mutator foci also exhibit liquid-like properties. This liquid behavior could be important as it may enable exchange of RNAs and other components with P granules, Z granules and SIMR foci and coordinate RNA silencing among these condensates.

While the molecular mechanisms that separate P granules, Mutator foci, Z granules and SIMR foci remain unknown, it is likely that the structured domains of these proteins regulate the interactions between their respective protein components similar to distinct heterotypic assemblies observed for MEG-3 from PGL-1 P granules in the early embryo [71]. Domain analyses of these proteins and the interactions of these condensates are needed to elucidate how these protein assemblies form and function.

Germ granules in *Drosophila*

As in *C. elegans*, *Drosophila* also forms nuage, a concatenated ring of germ granules that surrounds the nuclear envelope of the oocyte nurse cells in the adult fly. Due to enrichment of proteins involved in piRNA biogenesis, the nuage has been hypothesized as the site of piRNA synthesis. As the oocytes mature, a second type of germ granule begins to form at the posterior within the germ plasm. These granules, termed the polar granules, are inherited by the developing embryo and remain enriched at the posterior where they instruct the formation of primordial germ cells (PGCs). In contrast with nuage in *C. elegans*, the nuage in *Drosophila* remains relatively poorly understood. Meanwhile, research spanning 60 years

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has taught us a great deal about the biology of polar granules. We therefore dedicate the sections below to discussing the structure and function of these condensates.

Early structural studies on *Drosophila* polar granules

Counce and Mahowald have systematically characterized the morphology and fine structure of *Drosophila* polar granules with light and electron microscopy. They observed that the pole plasm, the specialized cytoplasm composed of proteins and mRNAs required for germ cell formation, also contains 'cytoplasmic granular inclusions' termed polar granules, which first appear in the posterior ooplasm during mid-oogenesis [72–74]. Mahowald described these granules as electron-dense, membraneless, round and composed of an interwoven meshwork of fibrils that undergo morphological changes as the oocytes and embryos develop [72–74]. During late oogenesis, they form a narrow band and tend to associate with each other and with mitochondria (Figure 2A) (reviewed in [75]). After fertilization, they then disperse and become more discrete with an average size of 200 to 500 nanometers [73] (Figure 2A). Once the PGCs bud off from the posterior of the embryo, polar granules once again change their behavior. They become associated with dynein motors, which transport polar granules on astral microtubules towards the centrosomes associated with the nuclei of newly forming PGCs [76]. Interestingly, at this point, a new type of germ granule begins to form in the nuclei of PGCs (Figure 2A) [74,77,78]. These nuclear granules, Mahowald noted, are morphologically distinct from the cytoplasmic ones and adopt a donut shape with a hollow center [74].

While polar granules in different *Drosophila* species vary in size and morphology, they nevertheless share the same fundamental structure, in which the electron-dense cytoplasmic fibrils and ribosomes are associated with the periphery of polar granules [74] while RNA appears inside the granules [79]. With successive passages in sucrose gradients to purify the polar granules, Mahowald noted that ribosomes remain tightly associated with polar granules, suggesting that these ribosomes could be directly attached to granules, possibly via the mRNA [80]. In addition, polar granules often form in close association with other cellular organelles including the nuclei, mitochondria and the ER [74,75,81,82]. These observations suggest that the association of polar granules with these organelles could be important for the formation of PGCs.

Formation of polar granules

Extensive genetic, biochemical and microscopy analysis demonstrated that pole plasm and polar granules have a critical role in the specification of germ cells in the fly (reviewed in [10,83,84]). They are necessary and sufficient for the formation of PGCs [85–88] and their removal disrupts the patterning of the body axis and is lethal for the development of the fly [89]. Since the early 1960s, several studies have characterized the function and ultrastructure of polar granules using electron microscopy, while recent advancements in quantitative, super-resolution microscopy have furthered our understanding of the organization of proteins and mRNAs inside polar granules [77,90–93].

Polar granules form by condensation of germ plasm proteins concentrated at the posterior end of the oocyte [77]. An immunoprecipitation followed by mass spectroscopy

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identified 117 different proteins as polar granule constituents [94]. These include the well-characterized core granule proteins Oskar (Osk), Vasa, Tudor (Tud) and Aubergine (Aub), which function specifically in germ plasm and are required for the formation of PGCs, as well as other proteins that function elsewhere in the embryo. Many are RBPs [94] with known roles in post-transcriptional regulation. This is an anticipated result given that polar granules enrich and regulate mRNAs required for the formation of PGCs.

Polar granules become fully functionalized during oogenesis [87] and persist anchored at the posterior end for many hours and even days when fertilization of the oocyte is delayed [95,96]. Biophysical studies revealed that polar granules display liquid and hydrogel-like properties [77]. This dual nature might be functionally relevant as it could both enhance reactions that occur in polar granules as well as increase the stability and persistence of polar granules. Indeed, the concentration of Osk and Vasa in polar granules is 21 to 15-fold higher in granules compared with their surroundings, respectively [77,97]. In addition, the fact that purification does not dissolve polar granules [77] further supports the observations that polar granules are resistant to even harsher environmental perturbations.

Two proteins, Osk and Tud, seem to provide this incredible robustness. Osk, the nucleator of polar granules, contains a self-dimerization motif, which also enables interactions with Vasa, and an RNA-binding domain, which binds polar granule mRNAs including *nanos* (*nos*), *germ-cell-less* (*gcl*) and *polar granule component* (*pgc*) [98,99]. These two domains are connected by a long intrinsically disordered region (IDR) [77,98,99]. Importantly, the two domains and the IDR allow Osk to nucleate polar granules by engaging in many protein: protein and protein:RNA interactions (see section 5.2).

In addition, Osk recruits Tud via its IDR [100,101]. Tud, the founding member of the Tudor family of proteins, consists of 11 Tudor domains that promote protein:protein interactions and the formation of higher-order protein complexes (reviewed in [102]). It binds symmetrically methylated arginines found in other polar granule proteins including Aub and Vasa [103–105]. Aub is a member of the Argonaute/Piwi family of proteins involved in piRNA biogenesis and regulation of transposable elements, as well as in localization and decay of polar granule mRNAs [106,107], while Vasa is a conserved DEAD-box RNA helicase [108]. With its many domains and interacting partners, Tud forms a scaffold required for the higher-order assembly of polar granules. Indeed, without Tud, embryos form fewer granules that are also less electron-dense [101,109]. In addition, different Tudor mutations can change the morphology of polar granules [109], further supporting the role of Tud in the assembly of polar granules.

Polar granules enrich and post-transcriptionally regulate a subset of maternally provided mRNAs, which code for proteins that control specification (*nos*, *pgc* mRNAs), migration (*nos* mRNA), cellularization (*gcl* mRNA), division (*CycB*, *nos* mRNAs) and transcription (*pgc* mRNA) of PGCs (reviewed in [10,83,110]). Of the nearly 6300 different mRNAs transcribed and provided by the mother to the oocyte during oogenesis [111], only 59 show a clear association with germ granules [112], with an additional 140 displaying a tendency for enrichment at the posterior pole [113]. These latter mRNAs most likely do not partition with polar granules and might therefore preferentially associate with germ plasm proteins.

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Genetic and microscopy analysis suggest that polar granule mRNAs translate while associated with polar granules and that their translation relies critically on germ plasm components [114]. Some of these transcripts contain sequences that spatiotemporally regulate their translation. For instance, the 3′ untranslated region (UTR) of *nos* forms a conserved two-stem RNA structure, termed the translational control element (TCE), which regulates the translation of *nos* [115,116]. The TCE is bound by Smaug which prevents the translation of *nos* [39]. Upon enrichment into polar granules, Osk, which is mostly condensed in polar granules [77], displaces Smaug from *nos* and prevents its deadenylation [117]. Translation of *nos* thus ensues, which generates a posterior-anterior gradient of Nanos protein, required for the patterning of the early embryo [118–120]. In mutations that prevent the formation of polar granules, *nos* mRNA remains translationally repressed, which causes embryonic lethality [114,119].

While only up to 4% of a particular mRNA enriches in polar granules [93,121], the system is remarkably buffered, as even a strong reduction in the enrichment of *nos* still provides enough Nanos protein to enable embryonic development [116]. Of the 59 mRNAs predicted to enrich in polar granules [112], only *nos*, *pgc*, *gcl* and *CycB* have well defined roles in germline development (reviewed in [10]), with the role of other mRNAs remaining unexplored. When the embryo begins transcription upon the maternal-to-zygotic transition (MZT), the PGCs remain transcriptionally silent until they too reach the PGC-specific MZT [122]. These additional uncharacterized transcripts and those that associate with the germ plasm could increase the diversity of transcripts within the germ plasm and provide PGCs the required transcriptome to promote their development until PGCs activate their genome.

Structural organization of polar granule components

RNA granules often form spatially distinct sub-compartments. This heterogeneous organization has been observed in RNA condensates in different species including nucleolus [123], P granules in *C. elegans* [22], germ granules in zebrafish [5], and polar granules in *Drosophila* [90,93].

In *Drosophila* polar granules, mRNAs display a distinct spatial organization. Here, mRNAs derived from different genes enrich within the same granule. However, rather than mixing with each other, transcripts derived from the same gene instead form homotypic multi-transcript assemblies [90,93] that de-mix from each other and occupy distinct granular territories (Figure 2B) [93]. Importantly, the mRNA itself actively participates in the formation of these assemblies. As the mRNAs enrich in polar granules, they are initially recruited by polar granule proteins using specific RNA sequences located in their 3′UTR called ‘RNA localization elements’ [91,116,121]. After this initial ‘seeding’ step, the mRNAs can then self-recruit into the granules [91]. This self-recruitment mechanism is poorly understood, however it could engage indirect RNA:RNA interactions, which involve RBPs as intermediates, or direct intermolecular RNA:RNA base pairing.

Once localized in granules, mRNAs then self-sort into homotypic assemblies through a de-mixing process that is independent of the polar granule proteins and of RNA localization elements [92]. Interestingly, unlike the homotypic mRNA assemblies, the core granule proteins Osk, Vasa, Tud and Aub, that form the polar granules and recruit polar

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granule mRNAs, appear co-organized within the same granules (Figure 2B) [93]. This co-organization was observed using laser scanning confocal microscopy [93] and hence, Osk, Vasa, Tud and Aub could appear de-mixed if examined with super-resolution microscopy. Polar granules therefore resemble a ‘bag of marbles’ with the mRNA and protein components assuming distinct polar granule locations. This distinct polar granule structure further supports the observation that core granule proteins are dispensable for mRNA sorting within polar granules [92]. Importantly, similar homotypic mRNA organization has been observed in germ granules in zebrafish and *C. elegans* [5,124], suggesting that homotypic mRNA assemblies could be a universal feature of germ granules. It is unclear what is the functional relevance of homotypic mRNA assemblies (Table 1). One model suggests that they could concentrate regulators required for post-transcriptional regulation and augment the efficiency of these reactions. For instance, within the assemblies, the ribosomes could become efficiently recycled, which could increase the likelihood of additional rounds of translation [84].

The exact mechanisms of mRNA assembly in polar granules are also unclear. One of the models that describe polymer assembly is the ‘sticker and spacer’ model [125]. This model defines how polymers with interaction domains (stickers) that are connected by separating sequences (linkers) interact with each other within an assembly. Specifically, the model proposes that multivalent sticker-sticker interactions could be the main driving force for the formation of the assembly, with the spacers modulating the dynamics of assembly [126]. For instance, in IDRs of proteins, polar and aromatic amino acids serve as stickers to drive phase separation of proteins by weak polar, electrostatic and π–π stacking interactions [127]. Interestingly, an *in vitro* mixture of RNA homopolymers composed of adenines (poly(A)) and cytosine (poly(C)), or of cytosine (poly(C)) and uracil (poly(U)), separates into homotypic assemblies containing only poly(A) and poly(C), or poly(C) and poly(U) in the absence of other cellular components [128]. These data suggest that in addition to the Watson–Crick base pairing, which requires complementary sequences, other RNA:RNA interactions such as base stacking, could serve as RNA stickers and drive RNA assembly. However, the identity and distribution of stickers and spacers in the RNA are entirely unexplored. In addition, it is unclear what RNA feature determines the specificity for homotypic RNA assembly. Differences in molecular charges and surface tension have been proposed to drive the formation of sub-granule compartments [126,129,130] and a similar mechanism could drive homotypic RNA assembly in polar granules. A systematic investigation of RNA properties including RNA sequences, structure and length is required to understand the mechanisms of RNA assembly.

Oskar, a gene whose products form distinct cellular structures with unique functions

During *Drosophila* development, the profound interplay of structure and function is demonstrated by *oskar* (*osk*), the gene responsible for the formation of germ plasm condensates. The three *osk* gene products, namely *osk* mRNA, Long Oskar (Long Osk) and Short Oskar (Short Osk), the two protein isoforms coded by *oskar* mRNA, engage in strong structure-function relationships. They have unique roles in supporting oogenesis

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and early embryogenesis and in specifying the germline and each occupy distinct cellular compartments. Interestingly, *oskar* messenger ribonucleoproteins (mRNPs) and Short *Osk* each form condensates, while Long *Osk* does not, despite sharing most of its protein sequence with Short *Osk*. The three gene products functionally inter-depend on one another, yet must be spatially excluded from each other for their proper functioning. In the sections below, we discuss how this dichotomy becomes established and how it functions to promote fly development.

The formation of *oskar* mRNP condensates

osk mRNA is transcribed during oogenesis by the nurse cells and an intricate series of events results in *osk* becoming localized to the oocyte posterior. The regulation of *osk* localization begins with the splicing of its pre-mRNA. Here, the removal of the first intron located in the coding region of the gene creates the Spliced Oskar Localization Element (SOLE), which, together with the components of the exon junction complex, is required for the kinesin-1 mediated transport and localization of *osk* to the posterior end of the oocyte [131,132]. In addition, *osk* contains a second RNA localization element located in the *osk* 3′UTR. This element contains a GC-rich palindrome presented in a well-defined stem loop. The palindrome triggers dimerization between neighboring *osk* mRNPs allowing one *osk* mRNP to piggy-back on the other while being transported to the posterior end [133,134].

Such localization-primed *osk* is transported into the nurse cell cytosol, where it becomes remodeled by several RBPs including the translational repressor Bruno [135,136]. Bruno binds to multiple sites in the *oskar* 3′UTR and prevents formation of the 48S initiation complex [135]. Despite being ribosome-free, polysome gradients revealed that the translationally silenced *osk* purifies as a ‘heavy particle’ that contains multiple *osk* mRNPs. The formation of these particles is driven by Bruno with the help of polypyrimidine tract-binding (PTB) protein [135,137]. In addition, an unconventional RNA-binding isoform of the actin filament-binding protein tropomyosin associates with kinesin-1 and the SOLE element in the nurse cell cytosol thereby establishing transport-ready *osk* mRNPs [138]. Dynein motors then transport *osk* transport particles along minus end-directed microtubules from nurse cells into the adjacent oocyte through the ring canals [139], which connect adjacent nurse cells and the oocyte, allowing transport among them [140,141]. Once the oocyte microtubule network repolarizes, plus end-directed transport along microtubules by kinesin-1 helps enrich *osk* transport particles at the posterior. There, the *osk* transport particles group into even larger assemblies that can contain hundreds of copies of *osk* mRNA [90,93] referred to as founder granules [142]. Repression of *osk* in founder granules is released and translation of the two Osk isoforms ensues. This transition to translational activation is poorly understood, though it is known to require de-repression of *osk* imposed by Bruno as well as activation of translation by the IGF-II mRNA-binding protein (Imp) protein [143].

Recent *in vitro* work by the Ephrussi lab demonstrated that the founder granules undergo a liquid-to-solid phase transition required for the localization and translation of *osk* mRNP [144]. In the liquid phase, *osk* mRNA transport granules efficiently enrich *osk* mRNAs and grow while their solid phase allows partitioning of Bruno, PTB and other translational

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regulators to control translation of Oskar protein and promote fly development [144]. Thus, the solid state of *osk* granules appears necessary for the localization and translational of *osk* mRNA at the posterior. This result is surprising, as granules with a solid-like state are generally thought to be less biochemically active and repressive than those with a more liquid-like state [144–146]. Furthermore, the solid state of *osk* founder granules was also found to be important for mediating their selective permeability of regulators *in vitro* [144], suggesting that the activating effect of the solid state could be explained by the exclusion of factors which inhibit *osk* translation. Here, RNA chaperones and helicases could prove critical for the maintenance of the functional solid state of *osk* mRNP condensates. For instance, these proteins could limit the growth of *osk* condensates and promote the switch from liquid to solid state as well as prevent potential mis-regulation of *osk* translation induced by promiscuous RNA:RNA interactions, which could be promoted within the highly concentrated, solid state of the *osk* founder granule [128,147].

Importantly, Vasa could be one such regulator. With the help of the eukaryotic initiation factor 5B (eIF5B), Vasa stimulates isoform-specific translation of *osk* mRNA, as lack of Vasa results in reduced Short Osk protein while Long Osk expression is unaffected [148,149]. Interestingly, Vasa and Bruno physically interact with one another [150], suggesting that Vasa may promote translation of *osk* by releasing its repression by Bruno. However, Vasa might unwind a complex secondary RNA structure in *osk* to promote translational switching between the two Oskar isoforms. Indeed, Vasa unwinds double-stranded RNA [151], which could facilitate identification of a start codon embedded within the RNA structure during scanning by the small ribosomal subunit. Alternatively, Vasa could control the material properties of *osk* founder granules by regulating the magnitude of base-pairing in the condensate and thus regulate translation of *osk*.

Formation and function of Oskar protein condensates

There are two Osk protein isoforms, generated by alternative translation initiation. Long Osk is produced via initiation at an upstream start codon and Short Osk by a downstream in-frame start codon [149]. Both isoforms share the LOTUS domain required for dimerization of Osk and the C-terminal OSK RNA-binding domain that binds and recruits mRNAs such as *nos*, *pgc* and *gcl* to germ granules [98,99]. The two domains are separated by a long IDR [77]. Immunoprecipitation of Short Osk identified 117 protein interactors [94], of which only a few have well established roles in development of the fly germline. Specifically, Tudor is required for the formation of germ cells [101], while Vasa is also required for the patterning of the embryos [152]. The two proteins interact with Oskar differently. Vasa interacts with its LOTUS domain [153], while Tudor is recruited through Valois, which interacts with Oskar's IDR [77,100,154].

The structural organization of the Oskar protein enables multiple protein:protein and protein:RNA interactions, which act synergistically to promote efficient condensation of Oskar protein [77]. The general structure of Oskar (oligomerization and substrate-binding domains separated by a long IDR [77]) is typically found in proteins with a strong tendency to phase separate and form RNA granules including G3BP1/2, which form stress granules [35], PGL1/3, which form P granules in *C. elegans* [16,17,19,155], NPM1, required for the

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formation of the nucleolus [156–159], and coilin, which forms the Cajal body [160–162]. However, despite sharing most of the protein sequence with Short Osk, Long Osk does not phase separate [77]. Long Osk has an additional 138 amino acids N-terminal extension (NTE) [149], indicating that the NTE inhibits condensation of Long Osk.

The NTE also determines the cellular location and function of the two isoforms. Long Osk accumulates underneath the posterior cortex where it associates with the endocytic membranes (Figure 3A) [163]. Short Osk, on the other hand, accumulates in germ granules and serves as their nucleator (Figure 3A) [149,164]. Long Osk stimulates endocytosis and the formation of filamentous (F) actin which are required for anchoring and accumulation of the germ granules to the posterior [97,149,163,165–167]. Finally, Long Osk also anchors mitochondria at the posterior pole [94] where they are thought to supply energy for the formation of the germline and enable mitochondrial inheritance of germ cells.

The NTE inhibits interaction of Vasa with the LOTUS domain in Long Osk [164] indicating that the NTE somehow controls the ability of Long Osk to interact with Short Osk binding partners, possibly by controlling the conformation of Long Osk. Interestingly, despite relying on Oskar protein for the establishment of the *Drosophila* germline, the wasp *Nasonia* has only Short Osk, which is required for germ granule assembly and pole cell formation and lacks Long Osk [168]. Rather than forming many small, well-separated germ granules as observed in the embryos of *Drosophila melanogaster* [93], a single, large germ granule called the oosome nucleated by a protein with high sequence homology to Short Osk forms instead [168]. These data suggest that Long Osk or its NTE could also control the structural organization of germ granules. In support of this hypothesis, a second type of germ granule forms in the nuclei of the PGCs in *Drosophila* once these cells begin to emerge (Figure 3B). These granules, termed nuclear germ granules, contain Short Osk and Vasa but lack Long Osk, and control the synchrony and frequency of divisions of PGCs [77]. They are 2.5-times bigger than the cytoplasmic polar granules normally present at the posterior and are also morphologically distinct. They are large and hollow, and appear closely associated with the nuclear envelope [77]. It is unclear whether the hollow structure has a functional role or whether it is a manifestation of an underlying biophysical process. For instance, negatively charged molecules trigger a hollow appearance of condensates made of positively charged proteins [26]. A similar principle could give rise to a hollow appearance of nuclear germ granules without necessarily generating a specific biological function.

Furthermore, unlike their cytoplasmic counterparts, nuclear germ granules do not enrich polar granule mRNAs indicating that they do not regulate a particular post-transcriptional process. Finally, the nuclear localization sequence located in the Osk IDR mediate transport of Short Osk and Vasa into the PGC nuclei. Despite sharing the NLS with Short Osk, Long Osk does not localize into the nuclei (Figure 3B), indicating that the NTE also interferes with the nucleocytoplasmic transport of this protein [77].

How does a single protein achieve the specificity to mediate these different functions? It is likely that different chaperone proteins recruited by the NTE modulate the activity of the two protein isoforms at the posterior pole. In addition, the NTE might change the conformation

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of Long Osk and prevent interactions with proteins typically observed for Short Osk thereby changing the biophysical properties, localization and activities between the two isoforms.

The long noncoding function of *osk* mRNA

Beyond simply encoding its protein products, *osk* mRNA is involved in several noncoding functions. These functions were first uncovered in flies that did not express *osk* mRNA. These *osk* RNA null phenotypes, which manifested during oogenesis, were much more severe than those triggered by mutations that allow localization of *osk* mRNA but prevented expression of the Oskar protein, which merely prevent embryonic patterning and formation of germ cells. During oogenesis, the meiotic chromosomes of the oocyte form a single compact cluster within the nucleus called the karyosome. In the absence of *osk* mRNA, the karyosome does not form properly, and the chromosomes disperse within the nucleus of the oocyte [169]. The resultant oocytes cannot mature and females carrying *oskar* RNA null mutations fail to lay eggs. Surprisingly, the expression of the *osk* 3′UTR alone is sufficient to suppress this phenotype, indicating that the *osk* transcript acts as a regulatory element of *Drosophila* oogenesis [169]. This regulation is manifested by the ability of *osk* 3′UTR to bind and sequester protein regulators including Bruno, thereby titrating the abundance and activity of this translational repressor during oogenesis [170]. Through this negative feedback, *osk* and Bruno regulate each other's activities required for the soma/germline distinction in the fly.

This ability of *osk* RNA to sequester translational repressors and regulate chromosomal integrity is reminiscent of the one observed for the long noncoding RNA *NORAD* in mammalian cells. *NORAD* accumulates in the cytosol where, similar to *osk*, it forms assemblies containing several *NORAD* transcripts [171]. *NORAD* contains multiple binding sites for the translational repressor Pumilio and, during stress, *NORAD* assemblies sequester Pumilio and titrate it away from the cytosol. In the absence of *NORAD*, the cytosolic concentration of Pumilio increases, leading to Pumilio hyperactivity and genome instability in mammalian cells. These phenotypes can be rescued by a synthetic RNA that sequesters Pumilio, demonstrating that RNA-driven phase separation can regulate RBP activity and identifying an essential role for this process in genome maintenance [171].

Lastly, while founder granules contain *osk* mRNA and polar granules contain Short Osk, the two types of condensates never mix (Figure 3A) [90,93]. This strict spatial segregation is critical for germ cell development as enrichment of *osk* mRNA in polar granules inhibits the formation of PGCs [90,142]. The mechanism of this inhibition is not clear. However, one could speculate that as with Bruno, *osk* mRNA might sequester translational regulators within polar granules, making them unavailable for translation to mRNAs enriched in polar granules. Distinct localization mechanisms must anchor the founder granules and polar granules proximal to each other yet keep them separated throughout germline development. In addition, site-specific degradation maintains this spatial separation, as the *osk* mRNA becomes quickly degraded even as germ granule proteins remain stable and persist throughout early embryogenesis [142].

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Outlook

Quantitative, super-resolution microscopy of germ granules coupled with purification and reconstitution experiments provided new insights into the biology of these RNA condensates. We now understand that these granules are rarely homogeneous, can compositionally and structurally change during the development and present different, intricate structures. These discoveries, however, raise new, critical questions. How is the structure of germ granules spatiotemporally regulated? How are proteins and mRNAs regulated in granules and what is the role of granule structure in this regulation? How do the biophysical properties of germ granules instruct the structure and function of germ granules?

To answer these questions, the structure of germ granules must be perturbed to examine whether structure provides a particular function to germ granules. However, this task is challenging, as perturbations that disrupt the structure of germ granules could also alter other germ granule functions. For instance, deletion of Oskar's OSK RNA-binding domain markedly changes the morphology of nuclear germ granules [77]. However, this mutation also diminished Oskar's ability to bind RNA and interact with Vasa [77], which makes separating the role of germ granule morphology from germ granule composition on the function of these condensates difficult. Similarly, the location of homotypic mRNA clusters in *Drosophila* polar granules is determined by the mRNA abundance. Here, mRNA clusters with fewer mRNAs reside at the periphery of germ granules, while those that contain more mRNAs reside in the center of the germ granules. By changing the abundance of mRNA clusters, mRNA clusters can move in and out of germ granules [92]. However, since the concentration of an mRNA and its associated regulators within the cluster might affect how efficiently a particular mRNA is regulated, it is challenging to ascertain whether structural changes, caused by the movement of mRNA clusters, affect mRNA regulation and the function of germ granules.

In most cases, it is still unclear how a particular germ granule structure becomes established, however *in vitro* phase separation experiments provide some clues. These assays revealed that molecular charges, modulated by cellular ions, are important regulators of granule formation and morphology [26,172]. Thus, without changing their composition, germ granules could exhibit different structures in different cellular environments. This mechanism could explain how the *C. elegans* P granules adopt different structures depending on the cellular location and developmental stage of the worm.

Furthermore, a single amino acid change can trigger accelerated gelation and de-mixing of otherwise identical proteins, as was recently observed for the WT Fused in Sarcoma (FUS) protein and its mutant counterpart [173]. Thus, striking structural changes of germ granules could be caused by even small alterations of the protein sequence, including post-translational modifications. Studies that carefully investigate the composition, behavior and properties of germ granules *in vivo* will have to be applied to decipher how structure of germ granules might influence their function.

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Data Availability

Data sharing plan is not applicable to this work.

Abbreviations

ER	endoplasmic reticulum
IDR	intrinsically disordered region
MZT	maternal-to-zygotic transition
NPC	nuclear pore complex
NTE	N-terminal extension
PGCs	primordial germ cells
PTB	polypyrimidine tract-binding
RBPs	RNA binding proteins
RdRPs	RNA-dependent RNA polymerases
SOLE	Spliced Oskar Localization Element
TCE	translational control element

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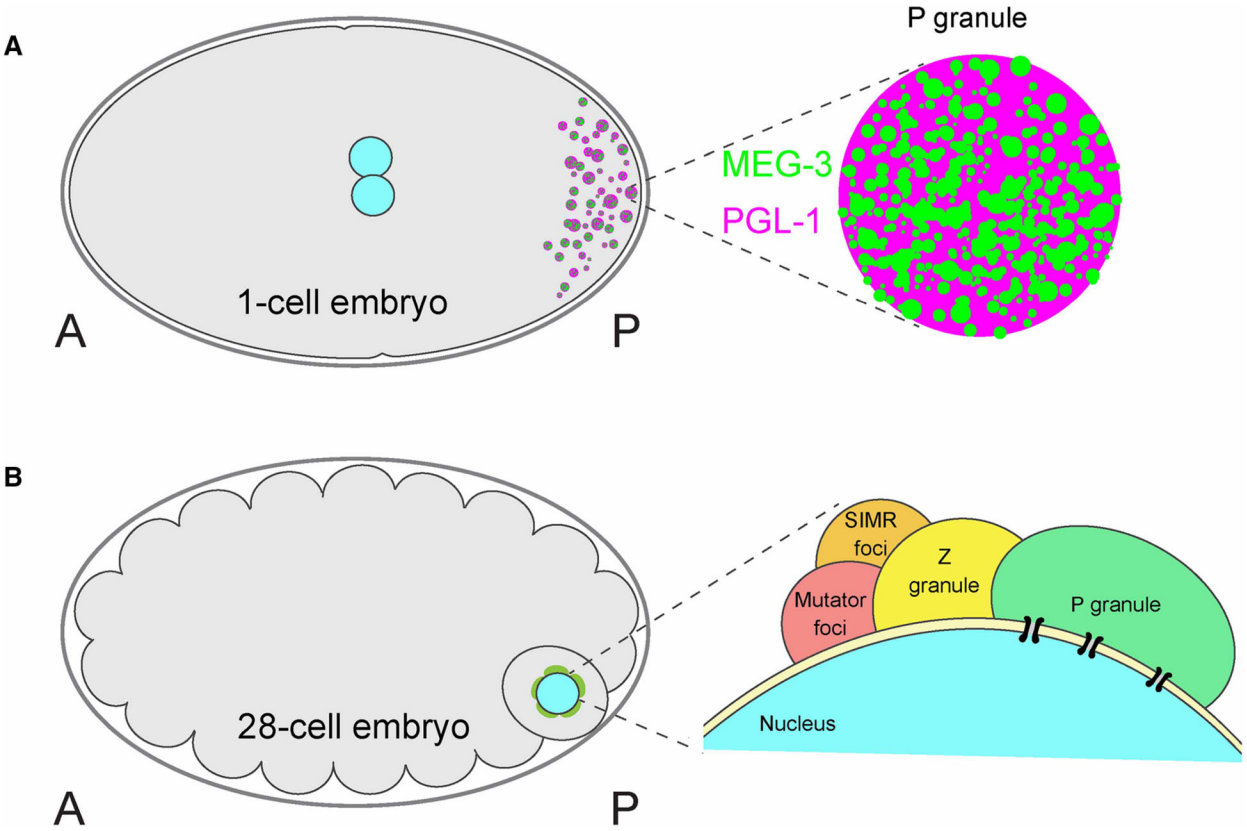

Figure 1. Morphology and structural organization of *C. elegans* germ granules.

(**A**) In the early embryo, germ granules known as P granules accumulate at the posterior pole (P) of the embryo. MEG-3 clusters (green), which assemble on the surface of the PGL-3 droplets, stabilize the PGL-3 (magenta) phase. Prior to P4 blastomere, P granules also associate with germline P-bodies, that represent the outermost layer surrounding P granules, but which can also exist in the cytoplasm independently of P granules **(43)**. A: anterior. Blue circles: nuclei. (**B**) In the older embryo, germ granules arrange into nuage that surround the nuclei of early germ cells. The constituents of nuage demix into P granules, Z granules, SIMR foci and Mutator foci, which remain attached to each other. P granules also interact with the nuclear pore complexes of the germ cells. Figure adapted from **(47)**. The figure was created with BioRender.com.

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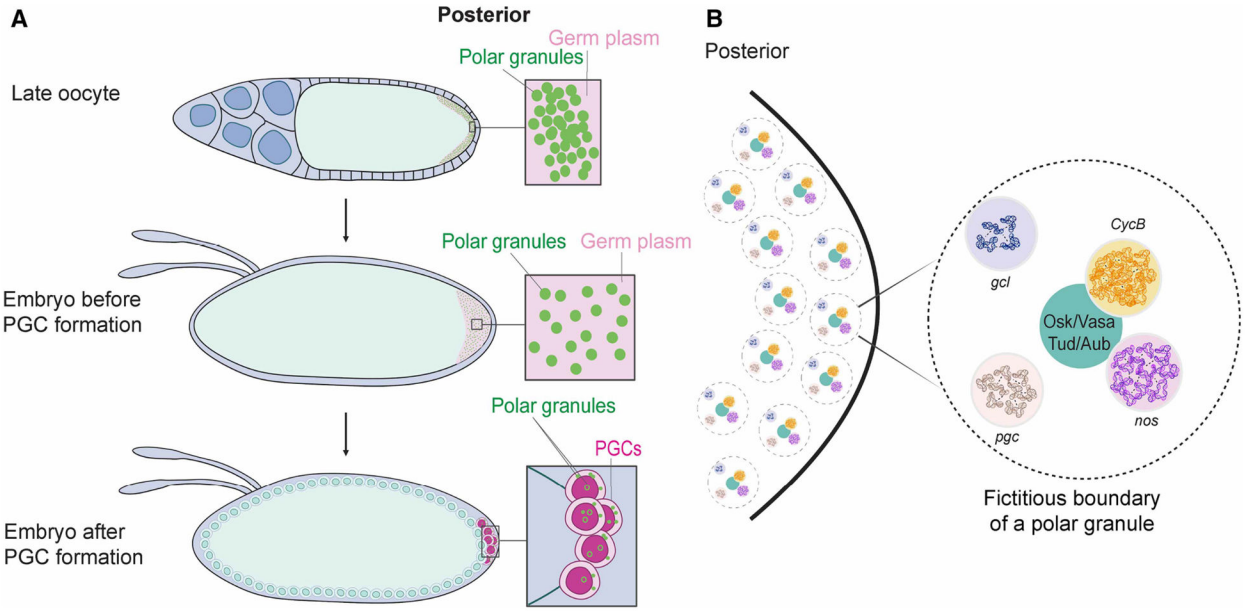

Figure 2. Morphology and structural organization of *Drosophila* polar granules.

Polar granules (green) initially form a narrow band within the germ plasm (light pink) at the posterior of the late oocyte. Afterwards they become more discrete and disperse in the embryo before PGC formation. Once PGCs (dark pink) are formed, hollow polar granules also form in the PGC nuclei. (**B**) Polar granule mRNAs *nos* (purple), *CycB* (yellow), *pgc* (brown), and *gcl* (dark blue) de-mix into homotypic clusters within the same granule. In contrast, core polar granule proteins (green) Osk, Vasa, Tud and Aub, co-assemble within the same granule. Such structural organization of the granule is reminiscent of ‘a bag of marbles’ in which each transcript and granule proteins occupy spatially distinct territories within a fictitious boundary of a germ granule (dashed line). The figure was created with BioRender.com

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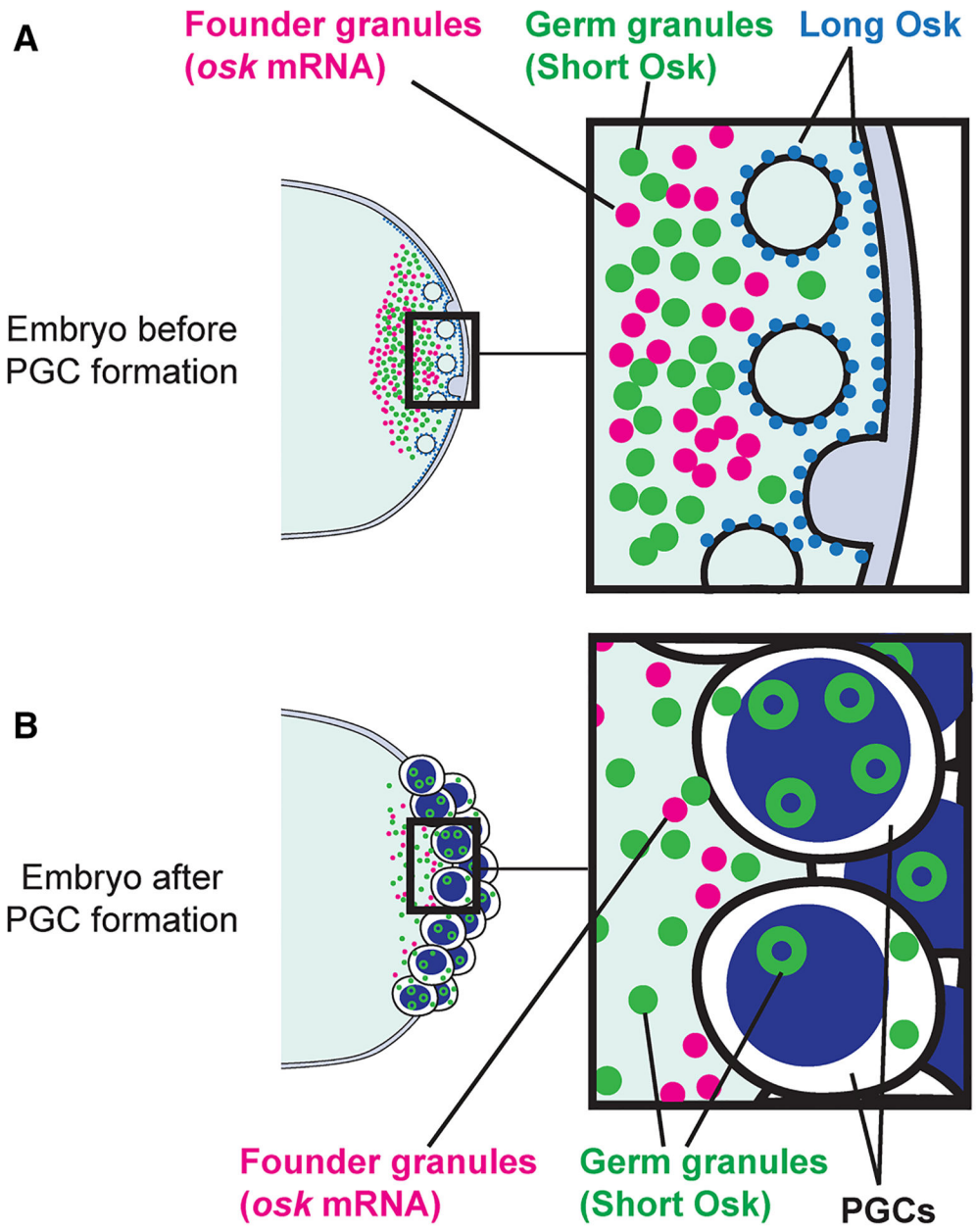

Figure 3. *osk* gene products form distinct compartments at the posterior.

(**A**) Before PGC formation, osk mRNA is found in founder granules (magenta), while Short Osk is found in polar granules (green), which do not overlap with founder granules. Meanwhile, Long Osk does not form granules, and instead associates with endocytic membranes. (**B**) Polar granules, but not founder granules become part of PGCs. In addition, nuclear polar granules form within the nuclei of PGCs. These germ granules are often hollow and larger than their cytoplasmic counterparts. The remaining founder granules and polar granules in the embryo cytoplasm get degraded. The localization of Long Osk after PGC formation is unknown.

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Table 1

Comparison of germ granule features among species

	P granules (*C. elegans*)	**Nuage (*C. elegans*)**	**Polar granules (*D. melanogaster*)**	**Germ granules (zebrafish)**
Proposed function	Regulation of mRNA localization, translation, decay	mRNA licensing, sRNA generation	Regulation of mRNAs localization, translation, decay	Regulation of mRNA localization, translation, decay
Nucleator	PGL1/3	?	Oskar	Bucky Ball [4]
Localization	Zygote posterior	Perinuclear	Posterior pole	Balbiani Body in oocytes
Morphology	Mostly round	Concatenated/ Amorphous	Mostly round	Concatenated/Amorphous
Display structure	✓	✓	✓	✓ [4–6]
RNA required for germ granule assembly	✓	?	?	?
Homotypic mRNA assemblies	✓	?	✓	✓ [5]

This review focuses on germ granules in *C. elegans* and *Drosophila*, a similar heterogeneous organization of proteins and mRNAs observed in germ granules in these two species has also been observed for the germ granules in zebrafish *Danio rerio*.