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The P Granules of *C. elegans*: A Genetic Model for the Study of RNA–Protein Condensates

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Abstract

P granules are RNA/protein condensates in the germline of *Caenorhabditis elegans*. Genetic analyses have begun to identify the proteins that regulate P granule assembly in the cytoplasm of zygotes. Among them, the RGG-domain protein PGL-3, the intrinsically disordered protein MEG-3, and the RNA helicase LAF-1 all bind and phase separate with RNA *in vitro*. We discuss how RNA-induced phase separation, competition with other RNA-binding proteins, and reversible phosphorylation contribute to the asymmetric localization of P granules in the cytoplasm of newly fertilized embryos. P granules contain RNA silencing complexes that monitor the germline transcriptome and may provide an RNA memory of germline gene expression across generations.

Keywords

RNA granules; P granules; *C. elegans*; Germline condensates

Cell biologists have long appreciated that cells contain micro-compartments not enclosed by membranes. In his classic study [1], Wilson concluded that the cytoplasm of star fish and sea urchin eggs “is a mixture of liquids in the form of a fine emulsion consisting of a continuous substance in which are suspended drops.” More than 100 years later, the concept of the cytoplasm as an emulsion is enjoying a revival, as several nuclear and cytoplasmic bodies have been reported to exhibit liquid-like characteristics: round shape, permeable surface, dynamic internal components, and ability to fuse [2-5]. The P granules of *Caenorhabditis elegans* are a particularly compelling example of a liquid-like condensate because of the rapid cycles of dissolution and condensation that P granules undergo in embryos [4]. The genetics of *C. elegans* also enabled the identification of molecules that drive condensate dynamics *in vivo*. In this review, we discuss the principles that have emerged from the study of P granules, in particular the central roles that RNA and intrinsically disordered proteins play in the formation of the “suspended drops.”

P Granules Exhibit Liquid-like Properties

P granules were first identified by Susan Strome and Bill Wood, who discovered serendipitously a rabbit IgG serum that recognized “granules” in *C. elegans* embryos [6].

The granules were observed exclusively in the P lineage (hence the name “P granules”), the cell lineage that gives rise to the germline. Strome and Wood recognized that similar structures had been observed by electron microscopy in the germline of other organisms, including the famous polar granules of *Drosophila* [7]. Electron microscopy images revealed a dense, fibrillar internal structure, which led to the “granule” designation [8]. It was not until the advent of GFP-tagging and confocal microscopy that the remarkable liquid-like properties of P granules came to light.

P granules are present in the germline throughout the life cycle of the worm (Fig. 1) [6]. In growing oocytes, P granules are stable, semi-spherical structures that decorate the cytoplasmic face of nuclei. During oocyte maturation, P granules move into the cytoplasm by a poorly understood mechanism that involves remodeling of the nuclear membrane and endoplasmic reticulum [9,10]. After fertilization, in the one-cell zygote, P granules become highly dynamic as they partition to the posterior side of the cell, where the germline will form [4,11]. Asymmetric partitioning of P granules is repeated during four consecutive cell divisions, which eventually give rise to the founder cell of the germline, the P blastomere P₄ [11]. Live imaging of P granules in zygotes was first achieved by injecting a fluorescently labeled antibody against P granules. These studies revealed that some P granules in the anterior cytoplasm are disassembled and others move toward the posterior cytoplasm [12]. Live imaging of the P granule protein PGL-1 tagged with GFP showed that preferential disassembly in the anterior and preferential assembly in the posterior is sufficient to localize P granules [4]. The realization that P granules partition by a process resembling localized dissolution and condensation was a major clue that these structures might have liquid characteristics [4]. Additional observations lent support to this view. Photoactivation experiments confirmed that PGL-1 released from dissolving granules in the anterior cytoplasm is recycled into condensing granules in the posterior cytoplasm [13]. Fluorescent recovery after photo-bleaching experiments showed that PGL-1 is dynamic within the granules and exchanges rapidly with the bulk cytoplasm, as expected for a liquid phase [4]. Estimates based on fluorescence recovery rates suggested a viscosity similar to that of glycerol. P granules were also occasionally observed to fuse with one another, returning to a circular shape after fusion. Applying forces to the perinuclear granules of growing oocytes caused the granules to deform and appear to drip off nuclei. The authors postulated that these liquid properties might derive from a collection of weakly “sticky” molecules that phase separate from the cytoplasm to form condensed droplets [4].

PGL-1 and PGL-3 Form a Core Condensate that Recruits Other P Granule Components

What are the “sticky” molecules that drive P granule assembly? Over 40 proteins have been reported to localize to P granules [14]. Most are predicted RNA-binding proteins. Some remain in P granules throughout development, while others associate with P granules at specific stages. A genome-wide screen identified over 150 genes required for maintenance of PGL-1::GFP condensates in adult gonads [15]. The genes cover a range of a wide range of cellular processes that impact P granule morphology and stability during development. Proteins required specifically for granule assembly were identified by ectopic expression

experiments and by studies in zygotes where it is possible to observe the emergence of new granules in the cytoplasm. These analyses have begun to reveal a hierarchy for granule assembly.

PGL-1 and PGL-3 are two homologous proteins that contain a C-terminal RGG box (Arg-Gly-Gly repeats) predicted to bind RNA [16] and a unique dimerization domain that has RNA endonuclease activity *in vitro* [17] (Fig. 2). In mutant zygotes lacking PGL-1 and PGL-3, P granules do not assemble normally [18]. Other P granule proteins either form smaller condensates or remain diffusively distributed in the cytoplasm. When expressed ectopically in mammalian cells, PGL-1 and PGL-3 form dense, RNA-rich condensates that can recruit other co-expressed P granule proteins [18]. Condensate formation requires the PGL dimerization domain, but not the RGG box. The RGG box is required, however, to recruit other P granule proteins to the PGL condensate. These observations, and the fact that PGL-1 and PGL-3 are present in P granules throughout development, suggest that these proteins form a core condensate that recruits other RNA-protein complexes to P granules by binding to RNA [18].

MEG-3 and MEG-4 Stabilize and Localize PGL Condensates in Zygotes

Proper segregation of PGL condensates to the posterior of zygotes requires MEG-3 and 4, two homologous intrinsically disordered proteins [19]. MEG-3 and MEG-4 contain a long, N-terminal intrinsically disordered region (IDR) and a shorter ordered C-terminal tail that contains a partial high-mobility group motif (Fig. 2). A similar domain structure has been described for the recently identified GCNA family of intrinsically disordered proteins [20]. Members of this family contain a divergent N-terminal disordered domain followed by a C-terminal ordered domain that typically, but not always, contains a zinc finger, a protease domain and a high-mobility group motif. The molecular function of GCNA proteins is not known but may be linked to a unique aspect of germline biology, since GCNA family members are often expressed in reproductive tissues and a mouse GCNA mutant is male sterile [20].

MEG-3 and MEG-4 are only expressed in maturing oocytes and early embryos and only localize to P granules in embryos [19]. In newly fertilized zygotes, MEG-3 and MEG-4 are uniformly distributed in the cytoplasm and co-localize with PGL in small granules. As zygotes approach mitosis, MEG-3 re-localize in a posterior-rich cytoplasmic gradient. This re-localization precedes the change in the distribution of PGL-1 condensates. By mitosis, both co-localize again in strong condensates in the posterior [21]. The temporal shift between MEG-3 and PGL-1 re-localization suggested that the former might drive the latter and genetic analyses confirmed this hypothesis. In embryos lacking MEG-3 and MEG-4, PGL-1 and PGL-3 condensates are fewer, do not become localized to the posterior, and are inherited by both daughter cells [19,22] (Fig. 3). The PGL condensates eventually dissipate and *meg-3meg-4* embryos develop without P granules. In contrast, in embryos lacking PGL-1 and PGL-3, MEG proteins still redistribute to the posterior and form condensates that are segregated asymmetrically to P blastomeres, as would P granules [21] (Fig. 3). These observations have suggested that MEG proteins form a localized scaffold that favors the

growth of PGL condensates in the posterior cytoplasm, creating a diffusive flux that clears PGL condensates from the anterior cytoplasm [21].

A Competition for RNA Localizes P Granules in Zygotes

Experiments with recombinant proteins revealed that MEG-3 and PGL-3 form condensates *in vitro* under physiological salt concentrations (150 mM KCl or 150 mM NaCl) [21,23]. For both MEG-3 and PGL-3, addition of RNA stimulates phase separation. Addition of short (30 nt) poly-U oligonucleotides increased the number and sizes of MEG-3 condensates [21]. Similarly, addition of total RNA purified from *C. elegans* embryos lowered the critical concentration of PGL-3 protein needed to observe condensates *in vitro* [23]. PGL-3 has a C-terminal RGG domain predicted to bind RNA, and this domain was required for phase separation *in vitro* [23]. MEG-3 binds with nanomolar affinity to poly-U *in vitro*, but the domain responsible is not yet known [21]. The MEG-3 IDR binds RNA with lower affinity and requires higher levels of RNA to stimulate phase separation. Together, these results suggest that binding to RNA stimulates the condensation of MEG-3 and PGL-3.

A requirement for RNA offered a possible explanation for why P granules assemble preferentially on the posterior side of zygotes. At the same time that P granules become enriched in the posterior, MEX-5 (and its homolog MEX-6) redistribute in a mirror image, anterior-rich gradient [24] (Fig. 3). MEX-5 and MEX-6 are abundant zinc-finger RNA-binding proteins that recognize short poly-U tracts in RNA [25]. Poly-U tracts are common in *C. elegans* 3' untranslated regions and MEX-5 and MEX-6 have been predicted to bind to most mRNAs present in zygotes [25]. MEX-5 and MEX-6 are therefore excellent candidates for proteins that might suppress P granule assembly in the anterior by competing with P granule proteins for RNA. Consistent with this hypothesis, in zygotes that lack MEX-5/6, P granules assemble throughout the cytoplasm [13,24] (Fig. 3). Conversely, in zygotes that express a mutant version of MEX-5 that remains uniformly distributed, P granule assembly is suppressed throughout the cytoplasm and this suppression requires the MEX-5 RNA-binding domain [21] (Fig. 3). An attractive hypothesis is that MEX-5 and MEX-6 function as a localized RNA sink which suppresses P granule assembly in the anterior cytoplasm by lowering the concentration of RNA available to MEG-3 and PGL-3 for phase separation.

In vitro experiments confirmed that MEX-5 can suppress condensation of MEG-3 and PGL-3 by competing for RNA [21,23]. Pre-incubation of the RNA-binding domain of MEX-5 with poly-U RNA eliminated RNA-stimulation of MEG-3 condensation, and this inhibition could be reversed by adding excess RNA [21]. Similarly, the RNA-binding domain of MEX-5 blocked phase separation of PGL-3 at low RNA concentrations, but not in the presence of excess RNA [23]. Saha *et al.* [23] tested the validity of such an RNA competition model further using mathematical modeling. Their model included parameters defined *in vitro* to describe the interactions between PGL-3, MEX-5, and RNA. MEX-5 binds RNA with 20-fold higher affinity compared to PGL-3, and both appear to bind most mRNAs indiscriminately. The model was also based on concentration estimates for each component derived from RNA and protein mass spectrometry measurements in embryo lysates. Remarkably, the model was sufficient to predict phase separation of PGL-3 into

condensates that could respond within minutes to a local increase in MEX-5. The PGL-3 condensates dissolved in the high MEX-5 domain and grew in the low MEX-5 domain.

In its simplest form, the RNA competition model predicts that MEG-3 and PGL-3 should respond to the MEX-5 gradient independently from each other. Observations *in vivo*, however, have shown that this is not the case. In *meg-3meg-4* mutant zygotes, PGL-3 granules do not localize to the posterior cytoplasm despite a normal MEX-5 gradient. The MEG proteins, in contrast, respond to the MEX-5 gradient even in the absence of PGL-1/3 [21]. These observations suggest that only MEG-3/4 sense the MEX-5 gradient directly. Why? The answer may lie in a recent study that examined how MEX-5 regulates the distribution of another RNA-binding protein POS-1.

A Role for Polo Kinase in the Localization of P Granules?

Like MEG-3 and MEG-4, POS-1 forms a posterior-rich gradient in response to MEX-5 [26]. When MEX-5 becomes enriched in the anterior cytoplasm, POS-1 mobility increases in the region of high MEX-5 causing POS-1 to redistribute from anterior to posterior [27]. A localized change in protein mobility is sufficient to drive the formation of a protein concentration gradient anti-correlated with the diffusion gradient [27,28]. Formation of the POS-1 gradient requires not only MEX-5 but also the Polo-like kinase 1 (PLK-1) [29]. PLK-1 is a kinase that binds to MEX-5 and relocates with it to the anterior cytoplasm [30]. PLK-1 phosphorylates POS-1 *in vitro*, and mutations that block PLK-1 phosphorylation *in vitro* reduce POS-1 diffusivity and block gradient formation *in vivo*. Mutations in MEX-5 or POS-1 that lower RNA binding also prevent formation of the POS-1 gradient [29]. Together these findings suggest the following model. MEX-5 brings PLK-1 to RNA complexes containing POS-1. Phosphorylation of POS-1 by PLK-1 lowers POS-1 affinity for RNA and increases its diffusion, causing POS-1 to relocate from the anterior to the posterior. In this model, MEX-5 does not directly compete with POS-1 for binding to RNA, but rather reduces POS-1 affinity for RNA by promoting POS-1 phosphorylation [29]. This model predicts that only proteins with PLK-1 sites will be sensitive to the MEX-5 gradient. It will be interesting to determine whether MEG-3 is also a PLK-1 substrate, and whether phosphorylation by PLK-1, rather than a direct competition with MEX-5, is what reduces MEG-3's propensity to phase separate with RNA in the anterior cytoplasm.

Phosphorylation and Dephosphorylation of MEG Proteins Promote Granule Disassembly and Reassembly

Phosphorylation by a different kinase has already been implicated in the regulation of P granule dynamics. MEG-3 and MEG-4 were initially identified in a screen for substrates for the DYRK kinase MBK-2 and the phosphatase PP2A^{pptr-1/pptr-2} [19,22]. *In vitro*, recombinant MEG-3 can be phosphorylated by MBK-2 and binds directly to the PP2A substrate adaptor PPTR-1. In *mbk-2* mutants, P granules assemble throughout the cytoplasm. Conversely in *pptr-1* mutants, P granules disassemble during mitosis throughout the cytoplasm. Depletion of *meg-3* partially restores P granules in *pptr-1* mutants, suggesting that MEG-3 destabilizes P granules when hyperphosphorylated [19]. These epistasis experiments suggest that phosphorylation of MEG-3 by MBK-2 promotes granule

disassembly and de-phosphorylation promotes granule reassembly. Unlike MEX-5 and PLK-1, MBK-2 and PPTR-1 do not show obvious asymmetry in the cytoplasm, suggesting that these regulators promote granule dynamics throughout [19]. Reversible phosphorylation of the MEGs could help sensitize the granules to the MEX-5/PLK-1 gradient, by transiently weakening MEG affinity for RNA during each cell cycle. The MEG proteins are very rich in serines (over 100s serines in MEG-3), so in principle phosphorylation could be used as a tunable mechanism to regulate granule assembly [31]. It will be important to determine the effects of phosphorylation on MEG-3 RNA binding and phase separation.

Multiple Phases in P Granules

In vitro and *in silico* models for P granule assembly have assumed so far that P granules are one-phase liquids that derive their physical characteristics directly from the liquid–liquid phase separation properties of key components [23]. Live imaging of PGL-1::GFP initially supported this view by showing that P granules are well mixed, spherical organelles that can fuse with one another and that contain mobile components that rapidly exchange with the cytoplasm [4]. Closer inspection of live P granules using high-resolution lattice light sheet microscopy, however, revealed that P granules are in fact non-homogeneous, imperfect spheres, whose contours and internal domains are constantly rearranging on a millisecond time scale [19]. Most strikingly, MEG-3 and PGL-3 appear to occupy distinct domains in the granules: MEG-3 concentrates at the periphery in a discontinuous, ribbon-like pattern that encircles a central PGL-3 core. Since the MEG phase is required to stabilize the PGL phase in zygotes, it is tempting to speculate that the MEG phase serves as a dynamic “shell” that partially isolates the PGL phase from the cytoplasm.

The lattice-light sheet study only analyzed the distribution of MEG-3 and PGL-3, but dozens of other proteins have been reported to localize to P granules [14]. Whether these form additional phases is not yet known. Besides MEG-3 and PGL-3, only one other P granule protein has been reported so far to form condensates *in vitro*: the DDX3 class RNA helicase LAF-1 [32] (Fig. 2). Unlike PGL-3 and MEG-3, phase separation of LAF-1 is not stimulated by RNA, but RNA changes the properties of LAF-1 condensates. LAF-1 could bind a short RNA oligo (poly-U 50) with nanomolar affinity. Addition of poly-U reduced the viscosity of LAF-1 condensates and increased the mobility of LAF-1 molecules [32]; longer RNAs, however, had the opposite effect perhaps due to entanglement of RNA molecules leading to increased viscosity [33]. Interestingly, single-molecule experiments using FRET sensors on a small 30-mer RNA oligo, revealed that, at low concentrations, LAF-1 induces tight compaction of the RNA molecule. At the higher concentrations required for phase separation, LAF-1 form dimers and RNA dynamics increase. Addition of ATP decreased LAF-1's affinity for RNA and decreased LAF-1-induced RNA dynamics [34]. These observations suggest a possible role for ATP hydrolysis in promoting the disassembly of LAF-1/RNA complexes. A role for helicases in tuning the assembly/disassembly of RNP complexes has also been proposed for stress granules [35]. Whether LAF-1 tunes P granule dynamics *in vivo* is not yet known. An interesting property of LAF-1 condensates is their relatively low concentration and high porosity compared to condensates made with globular proteins. Dextran (10 kD) can penetrate LAF-1 condensates, but larger dextrans are excluded, as also observed for P granules *in vivo* [33]. Embryos depleted of LAF-1 still form

granules, but these lack PGL-1, suggesting a role for LAF-1 in recruiting or stabilizing the PGL-1 phase *in vivo* [32]. It will be interesting to combine LAF-1, PGL-3, and MEG-3 condensates *in vitro* to better understand how these components interact.

Perinuclear P Granules

By the time the germline founder cell is born (P₄), most P granules are perinuclear and few P granules remain free in the cytoplasm. Electron microscopy studies in the gonad of adult hermaphrodites have revealed that perinuclear P granules associate with clusters of nucleopores [9]. Perinuclear localization of P granules requires the FG-repeat protein GLH-1 and FG-containing nucleoporins [36,37]. FG domains are known to form dense condensates or “hydrogels” *in vitro* [38]. Multimerization of the GLH-1 FG domain is sufficient to form perinuclear granules when ectopically expressed in intestinal cells [37]. Wild-type GLH-1, however, cannot form granules on its own unless co-expressed with PGL-1. An intriguing possibility is that the FG domain of GLH-1 (and the related GLH-2,3, and 4) phase separates with FG domain-containing nucleoporins, and this brings the GLH/PGL phase in direct contact with nucleopores. Consistent with this, like nucleopores, perinuclear P granules are sensitive to aliphatic alcohols and exclude macromolecules greater than 70 kD [37]. GLH-1 associates with MEG/PGL condensates from the one-cell stage [39] onward, yet the transition from cytoplasmic P granules to perinuclear P granules happens progressively in the P lineage and is not complete until the P₄ blastomere. What prevents P granules from associating with nuclei in earlier blastomeres is not known.

P Granules as Guardians of the Germline

What is the function of P granules? Embryos that cannot assemble or localize cytoplasmic P granules in the one-cell stage (e.g., *meg-3/meg-4* mutants or *pptr-1* mutants) grow up into larvae that eventually assemble perinuclear P granules *de novo* during postembryonic development [19]. *meg-3meg-4* animals are viable and 70% are fertile. Thus, assembly of P granules in embryos is not a prerequisite to specify the germline or to assemble P granules in later stages [13,19]. These observations suggest that, despite their prominence, P granules only play a supporting, non-essential role in the specification of the embryonic germline.

Although P granules are not essential structures in embryos, they are essential for germ cell differentiation during post-embryonic development and *pgl* and *glh* mutants are sterile [16,40]. To examine the effect of eliminating all P granules in developing germ cells, Updike *et al.* [41] used an RNAi strategy to knock down PGL-1, PGL-3, GLH-1, and GLH-4 at the same time. Remarkably, they found that germlines lacking P granules proliferate normally through the larval stages but fail to initiate oogenesis during the larval-to-adult transition and become sterile adults that lack oocytes [41]. The sterile gonads expressed somatic transcripts and some germ cells were observed to differentiate into neuronal-like cells. Transcriptomic analyses revealed thousands of miss-regulated transcripts, including many transcripts normally expressed in sperm and somatic cells [42,43]. These findings suggest that P granules promote the differentiation of germ cells into functional gametes by preventing ectopic gene expression.

How do P granules silence unwanted gene expression in germ cells? Several lines of evidence suggest that P granules are the cellular compartment where small RNA pathways monitor the germline transcriptome. In *C. elegans*, endogenous small RNAs distinguish authentic germline transcripts from potentially harmful “foreign” transcripts, such as those derived from transposons, repetitive sequences, or transgenes [44]. The latter are recognized and silenced by piRNAs, a class of small RNAs that are complexed with the PIWI-related argonaute PRG-1. Germline protein-coding transcripts are recognized by another class of endogenous small RNAs that associate with a different argonaute called CSR-1 (pronounced CAESAR). CSR-1 modulates the levels of transcripts that code for maternal proteins inherited by zygotes. CSR-1 regulation ensures that embryos inherit the right levels of maternal proteins to support early development before zygotic transcription begins [45]. Both CSR-1 and PRG-1 localize to P granules. In animals depleted of CSR-1, PGL-1 form grossly enlarged condensates that fill the germline [15]. Enzymes required for the biogenesis of small RNAs and their amplification also localize to P granules or to other condensates that flank P granules on the nuclear membrane [44]. The DICER ribonuclease that generates small RNAs exists in a complex with GLH-1 [46], and GLH-1 is required for inheritance of RNAi-triggered gene silencing [47]. RNA condensates analogous to P granules in the *Drosophila* germline have also been linked to small RNA biogenesis [48].

In developing germ cells, P granules cover 70% of nuclear pores, and thus, most germline mRNAs must traverse a P granule before reaching the cytoplasm [9]. *In situ* hybridization experiments confirmed that newly transcribed mRNAs reside transiently in P granules before diffusing into the cytoplasm [49]. The small RNA machinery, therefore, is perfectly positioned in P granules to scan new transcripts as they emerge from the nucleus. P granules may also function as a privileged compartment where argonautes and other mRNA regulators can associate with their target transcripts without competition from ribosomes. Consistent with this idea, localization of the Pumilio homolog FBF-2 to P granules is required for maximal binding of FBF-2 to its mRNA targets [50]. An important function of P granules, therefore, may be to facilitate the formation of silencing RNA–protein complexes before RNAs enter the ribosome-rich cytoplasm. Whether the liquid properties of P granules contribute to this function is not yet known.

Conclusions and Future Prospects

Almost 10 years ago, P granules were proposed to assemble by spontaneous phase separation of protein and RNAs from the cytoplasm [4]. Since then, the identification of proteins that regulate P granule assembly has begun to reveal fundamental principles of P granule assembly.

First, P granules are non-homogeneous, multiphase condensates. The core PGL phase coassembles with dozens of RNA-binding proteins, including the helicase LAF-1 and the FG-containing GLH-1 protein which anchors P granules to nuclear pores. In newly fertilized zygotes, the intrinsically disordered MEG-3 protein forms a dynamic outer shell that stabilizes and segregates the core PGL phase in the cytoplasm. Electron microscopy studies suggest that perinuclear P granules also contain distinct phases, but the molecules responsible for these distinct compartments are not yet known [9]. Like P granules, nucleoli,

stress granules, and the polar granules of *Drosophila* have all also been reported to contain distinct phases or compartments [35,51-53]. Liquid phases assembled *in vitro* can reproduce aspects of nucleolar compartmentalization [53]. A challenge for the future will be to understand how different phases contribute to granule assembly and stability *in vivo*.

Second, RNA promotes P granule assembly. Free RNA stimulates phase separation of P granule proteins, and the RNA-binding protein MEX-5 locally suppresses P granule assembly in the anterior cytoplasm. P granules are rich in polyadenylated mRNAs [54], but whether all mRNAs or a subset are selected by P granules is not yet known. P bodies favor poorly translated mRNAs [63], and non-sequence specific aggregation of polysome-free RNA has been proposed to drive the assembly of stress granules [55]. P bodies and P granules share certain protein components and occasionally dock onto each other, but do not fuse and remain distinct granules [56]. The fungus *Ashbya gossypii* maintains distinct RNA granules in a common cytoplasm by discriminating between different RNAs based on secondary structure [57]. In *Drosophila* polar granules, RNAs are arranged in homotypic clusters that occupy distinct stereotypical territories [51,52,58]. Whether similar RNA specificity and organization exist in P granules is not yet known.

Third, reversible phosphorylation drives cycles of granule dissolution and condensation in dividing cells. In particular, the kinase MBK-2 promotes P granule disassembly in zygotes by phosphorylating MEG-3. Interestingly, the MBK-2 homolog DYRK3 has also been implicated in the dissolution of stress granules [59] and several mitotic condensates [60] in human cells. Phosphorylation, in principle, could interfere with RNA binding by introducing negative charges on IDRs that interact with RNA [31], but this model has not yet been tested directly for P granules. RNA helicase activity could also contribute to granule dynamics, since the ATPase activity of LAF-1 was shown to promote disassembly of LAF-1 condensates *in vitro* [34].

Most RNA-binding proteins that localize to cytoplasmic granules contain IDRs and the phase separation properties of IDRs are often assumed to underlie granule formation [61]. What roles IDRs play in P granule assembly, however, are not yet known. MEG-3 contains a long IDR (>500 amino acids) that readily phase separates *in vitro*, but the MEG-3 IDR is not sufficient for granule formation *in vivo* [21]. Similarly, the RGG domain of PGL-3 is required for phase separation *in vitro* but is dispensable for granule assembly *in vivo* [18]. Defining the role of IDRs *in vivo* and understanding the constraints that the crowded cytoplasmic environment imposes on phase separation is an important goal for future studies.

Perhaps some of the most intriguing questions remaining have to do with P granule function. As was posed over 100 years ago by Wilson [1]: are the condensates “living or lifeless”, “active or passive”? Are P granules factories that synthesize small RNAs and assemble silencing complexes on mRNAs? Or are they passive RNA filters or sponges? Wilson was also intrigued by the possibility that the “suspended drops” might be able to replicate or self-propagate [1]. In *C. elegans*, traits acquired in ancestral generations are transmitted to future generations by small RNA pathways [62]. It is tempting to speculate that small RNAs that shape the transcriptome could be transferred from mother to progeny *via* P granules. How

the liquid-like properties of P granules enable their unique RNA functions promises to be an exciting area of inquiry for years to come.

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Abbreviations used:

IDR	intrinsically disordered region
PLK-1	Polo-like kinase 1

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Fig. 1.

P granules are present throughout the life cycle of *C. elegans*. Schematics showing different stages of the *C. elegans* germline life cycle. In embryos (ovals in figure), P granules segregate asymmetrically with the P lineage that gives rise to the primordial germ cells Z2 and Z3. Z2 and Z3 proliferate during larval stages (not shown) and eventually generate oocytes (square cells, and sperm not shown). P granules (green) are perinuclear during most of germline development. P granules become cytoplasmic in oocytes right before fertilization and return to their perinuclear location in embryos by the 100-cell stage.

Fig. 2.

P granule proteins shown to undergo phase separation *in vitro*. Schematics showing the domain organization of PGL-3, MEG-3 and LAF-1. Intrinsically disordered domains are in green. MEG-3 is rich in serines (14%, yellow lines).

Fig. 3.

Genetic hierarchy for P granule asymmetry in zygotes. Schematics summarizing the key results (A) that have led to our understanding of the genetic hierarchy (B) that regulates P granule asymmetry in zygotes. (A) Schematics showing the distribution of MEX-5/6 (pink), MEG-3/4 (orange), and PGL-1/3 (green) in zygotes of the indicated genotypes. In wild type, MEX-5/6 (pink) forms an anterior-rich gradient opposite the posterior-rich MEG-3/4 gradient and the posterior MEG/PGL condensates (P granules). In zygotes lacking PGL-1/3 [*pgl-1/3(0)*], the MEG-3/4 condensates still localize but appear smaller. In zygotes lacking MEG-3/4 [*meg-3/4(0)*], PGL condensates do not localize despite normal formation of the MEX-5/6 gradient. In zygotes lacking MEX-5/6 [*mex-5/6(0)*], MEG/PGL granules form throughout the cytoplasm. In zygotes where MEX-5 is uniformly distributed, MEG/PGL condensates dissolve throughout the cytoplasm (purple). In zygotes where MEX-5 is uniform but lacks its RNA-binding domain (ZF-), MEG/PGL condensates assemble throughout the cytoplasm. (A) Genetic hierarchy: MEX-5/6 gradient localizes MEG-3/4 in an opposite cytoplasmic gradient. MEG-3/4 condensates form scaffolds that stabilize PGL-1/3 condensates.