



Germ granules and gene regulation in the *Caenorhabditis elegans* germline

Carolyn M. Phillips ^{1,*} and Dustin L. Updike ^{2,*}

¹Department of Biological Sciences, University of Southern California, Los Angeles, CA 90089, USA and

²The Mount Desert Island Biological Laboratory, Bar Harbor, ME 04672, USA

*Corresponding author: cphil@usc.edu (C.M.P.); dupdike@mdibl.org (D.L.U.).

Abstract

The transparency of *Caenorhabditis elegans* provides a unique window to observe and study the function of germ granules. Germ granules are specialized ribonucleoprotein (RNP) assemblies specific to the germline cytoplasm, and they are largely conserved across Metazoa. Within the germline cytoplasm, they are positioned to regulate mRNA abundance, translation, small RNA production, and cytoplasmic inheritance to help specify and maintain germline identity across generations. Here we provide an overview of germ granules and focus on the significance of more recent observations that describe how they further demix into sub-granules, each with unique compositions and functions.

Keywords: *C. elegans*; germline; germ granules; P granules; Mutator foci; Z granules; piRNAs; WormBook

Overview of germ granules

Early studies of animal germline specification noted visibly dense assemblies on the cytoplasmic surface of germline nuclei [reviewed in Eddy (1975)]. These assemblies have been called by various names across species and throughout germline development, but in recent years a consensus has emerged to collectively refer to them as germ granules. In *C. elegans*, the first observed germ granules were called P granules because they segregate with the P cell lineage (germline blastomeres) during embryogenesis [Figure 1, A–D; reviewed in Strome (2005), Hubbard and Greenstein (2005)]. Today, the term “germ granule” defines a collection of dynamic and germline-specific perinuclear assemblies that can be distinguished through high-resolution microscopy. Other cytoplasmic assemblies, such as P bodies, stress granules, and those which form in arrested oocytes will not be covered here. In early embryos, the terms germ granule and P granule are used interchangeably, but as development progresses, germ granules further demix into sub-granules that, along with P granules, include *Mutator* foci, Z granules, SIMR-1 foci, and likely other perinuclear assemblies yet to be defined (Figure 1, C–G). *Caenorhabditis elegans* provide a window to observe the dynamics of these sub-granules, revealing their function in the germline and specific contributions of their individual components. These findings can then be extended to better define the roles of germ-granule proteins in other animals.

Germ granules and their subtypes are heterogeneous ribonucleoproteins. Of the ~90 *C. elegans* proteins currently known to be germ-granule enriched (Table 1), almost all have RNA binding domains. They include RNA (mostly DEAD-box) helicases, mRNA stabilizing and destabilizing proteins, translation initiation

factors, small RNA-binding Argonautes, RNA polymerases, mRNA export and nuclear pore complex components, and many proteins harboring KH, CCCH zinc-finger, Tudor, and LOTUS domains. Together, assemblies of these germline proteins at the nuclear periphery position them to regulate mRNA abundance, translation, small RNA production, and cytoplasmic inheritance through cell divisions and across generations. In the proceeding paragraphs, we explore the function of germ granules, and what the composition and modularity of known germ-granule subtypes reveal about their function.

Organization and function of P granules

Overview of P granules

The discovery of P granules in *C. elegans* (Strome and Wood 1982) followed the discovery of asymmetric segregation of germ granules that had been previously described during early embryonic cleavages in several invertebrates and amphibians (Ritter 1890; Hegner 1911; Penners 1922; Smith 1966; Mahowald et al. 1976). It was recognized that partitioning of P granules in early embryos could be used to understand how asymmetry is established prior to the first cell division [(Kemphues et al. 1988; reviewed in Rose and Gonczy (2014)]. Moreover, the accessibility of P granules provided a way to identify their core components and observe their formation, condensation, and dissolution in early embryogenesis (reviewed in Seydoux 2018).

Structural composition of P granules

The list of P-granule-associated proteins continues to expand (Table 1). Many of these proteins associate transiently with P granules in the early embryo or at other stages of germline

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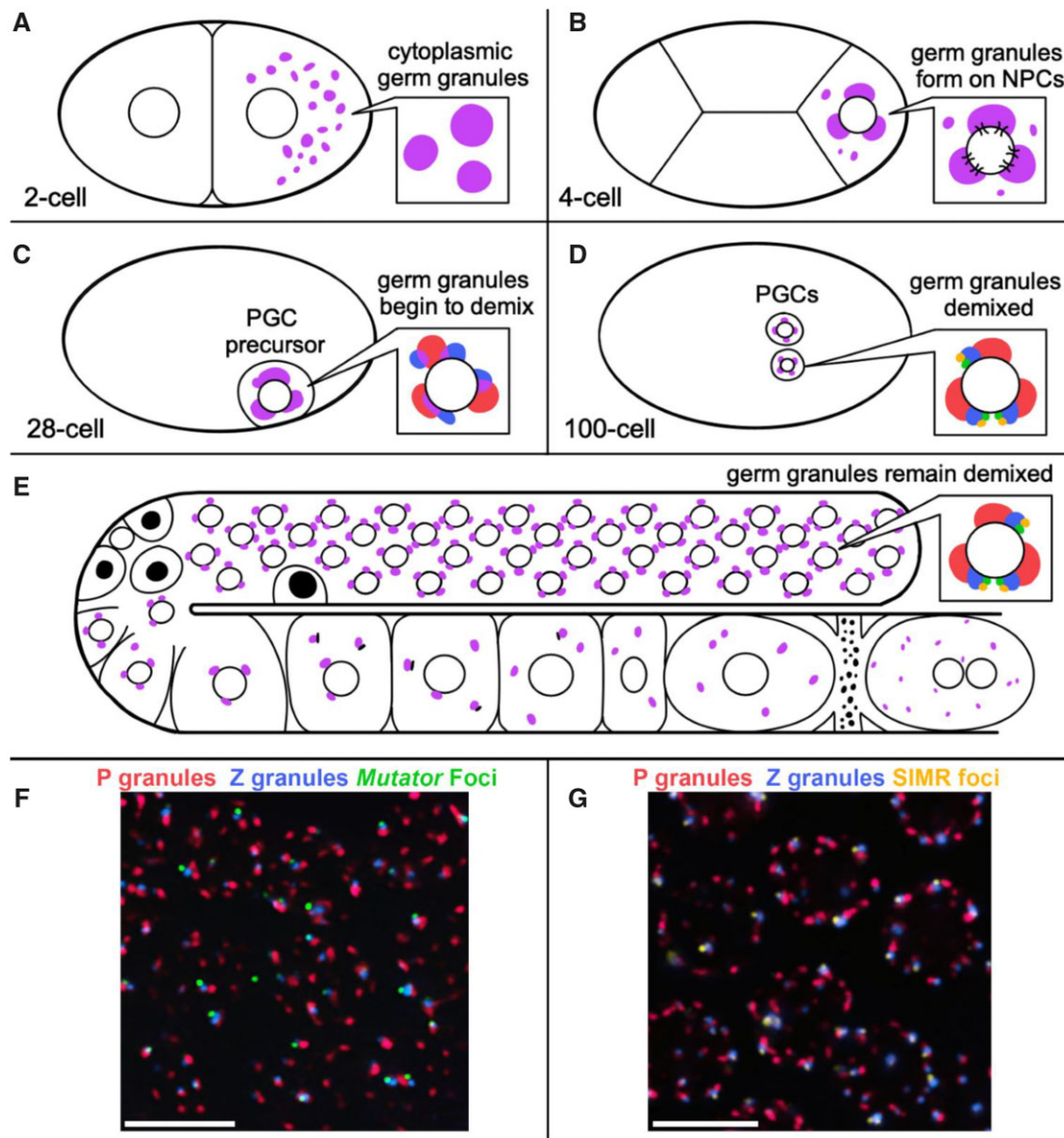


Figure 1 Germ granule distribution and demixing during development. (A) Posteriorly localized germ granules (purple) in the 2-cell embryo are dispersed in the cytoplasm. (B) Germ granules in the 4-cell embryo begin to adhere to the cytoplasmic surface of the nuclear envelope and cluster NPCs. (C) Germ granule demixing begins at the 28-cell stage in primordial germ cell (PGC) precursors. (D) Germ granules in the PGCs of 100-cell embryos have demixed into adjacent P granules, Z granules, SIMR foci and Mutator foci. (E) Germ granules remain demixed in adult germ cells (inset). PGL proteins, but not GLH proteins, are cleared from P granules during physiological apoptosis (black nuclei). P granules disperse into the cytoplasm of oocytes prior to fertilization, initially with part of the nuclear envelope attached. (F, G) Immunofluorescence image of adult germ cells (pachytene) with demixed sub-granules. (images Celja Uebel. scale bars = 5 μm).

development and gametogenesis. A smaller contingent can be described as constitutive proteins that are found in P granules at all stages of the *C. elegans* lifecycle. Among these are a few novel proteins (DEPS-1, PGL-1, and PGL-3), the Vasa DEAD-box germline helicases (GLH-1, GLH-2, GLH-3, and GLH-4), other related DEAD-box helicases (RDE-12, LAF-1, VBH-1), and some of the Argonaute proteins (CSR-1, PRG-1, and worm-specific Argonaute (WAGO)-1). PGL-1 and PGL-3 are scaffolding proteins that interact through two dimerization domains, exhibit RNA endonuclease activity *in vitro*, and have the ability to repress tethered mRNA expression *in vivo* (Aoki et al. 2016, 2021). Ectopic expression of PGL-1 and PGL-3, either in the soma of *C. elegans* or in mammalian cell culture, is sufficient to nucleate P-granule-like condensates (Hanazawa et al. 2011; Updike et al. 2011). Interestingly, different proteins nucleate germ-granule formation in other animals,

supporting the idea that germ-granule nucleators evolved through convergent evolution [reviewed in Kulkarni and Extavour (2017)]. Recombinant PGL-3 can self-assemble under physiological conditions *in vitro* (Saha et al. 2016). *In vivo*, the posterior condensation of PGL-1 and PGL-3 in the zygote is facilitated by a MEG-3 and MEG-4 scaffold to coat PGL assemblies, and LOTUS-domain MEG-3 interacting proteins MIP-1 and MIP-2 (Wang et al. 2014; Chen et al. 2016; Smith et al. 2016; Putnam et al. 2019; Folkmann et al. 2021; Price et al. 2021; Schmidt et al. 2021; Cipriani et al. 2021). During spermatogenesis, PGL-1 and PGL-3 are cleared from P granules while GLH proteins are retained, suggesting that nucleators are not always needed to maintain P granules after they are formed [discussed in Updike and Strome (2010)].

The RNA composition of P granules is better defined in embryos than in adults. Early studies demonstrated that embryonic

Table 1 Germ granule proteins (P granules, Z granules, *Mutator* foci, SIMR foci, and unknown)

Germ granule	Protein	Description	References
P	ALG-3	Argonaute expressed during spermatogenesis	Conine et al. (2010)
P	ALG-4	Argonaute expressed during spermatogenesis	Conine et al. (2010)
P	ALG-5	Argonaute associated with miRNAs	Brown et al. (2017)
P	CAR-1	Cytokinesis, apoptosis, and RNA-binding 1 TRAL/Lsm14	Audhya et al. (2005), Boag et al. (2005), Squirrell et al. (2006)
P	CCF-1	CCR4/NOT deadenylase complex	Gallo et al. (2008)
P	CDE-1	Uracil nucleotidyltransferase	van Wolfswinkel et al. (2009)
P	CGH-1	Dhh1/DDX6 DEAD-box helicase	Navarro et al. (2001)
P	CSR-1	Argonaute required for endo-siRNA	Claycomb et al. (2009)
P	DCAP-1	mRNA decapping enzyme	Squirrell et al. (2006)
P	DCAP-2	mRNA decapping enzyme	Lall et al. (2005)
P	DCR-1	Dicer-related RNase	Beshore et al. (2011)
P	DDX-19	DDX19 DEAD-box helicase	Sheth et al. (2010)
P	DEPS-1	Defective P granules and Sterile	Spike et al. (2008a)
P	DRH-3	Dicer-related DEAD-box helicase	Claycomb et al. (2009)
P	EGO-1	RNA-directed RNA polymerase (RdRP)	Claycomb et al. (2009)
P	ERH-2	21U-RNA maturation	Cordeiro Rodrigues et al. (2019) and Zeng et al. (2019)
P	FBF-2	PUF-domain fem-3 mRNA 3'UTR-binding factor	Voronina (2012)
P	GLD-1	RNA-binding KH domain	Jones et al. (1996)
P	GLD-2	Poly(A) polymerase	Wang et al. (2002)
P	GLD-3	RNA-binding KH domain	Eckmann et al. (2002)
P	GLD-4	Poly(A) polymerase	Schmid et al. (2009)
P	GLH-1	Vasa DEAD-box helicase	Gruidl et al. (1996)
P	GLH-2	Vasa DEAD-box helicase	Gruidl et al. (1996)
P	GLH-3	Vasa DEAD-box helicase	Kuznicki et al. (2000)
P	GLH-4	Vasa DEAD-box helicase	Kuznicki et al. (2000)
P	GLS-1	GLD-3/4 interacting protein	Rybarska et al. (2009)
P	HENN-1	3' RNA methyltransferase	Kammaing et al. (2012)
P	HRDE-2	Heritable RNAi deficient	Spracklin et al. (2017) and Lewis et al. (2020)
P	IFE-1	eIF4E mRNA cap-binding	Amiri et al. (2001)
P	IFE-3	eIF4E mRNA cap-binding	Cordeiro Rodrigues et al. (2019), Zeng et al. (2019), and Huggins et al. (2020)
P	IFET-1	eIF4E transporter	Sengupta et al. (2013)
P	LAF-1	DDX3 DEAD-box helicase	Hubert and Anderson (2009)
P	MBK-2	DYRK3 YAK-related kinase	Stitzel et al. (2007)
P	MEG-1	Maternal effect germ cell defective	Leacock and Reinke (2008)
P	MEG-2	Maternal effect germ cell defective	Leacock and Reinke (2008)
P	MEG-3	Maternal effect germ cell defective GCNA-IDR	Wang et al. (2014)
P	GEI-12		
P	MEG-4	Maternal effect germ cell defective GCNA-IDR	Wang et al. (2014)
P	MEX-1	CCCH-type zinc-finger protein	Guedes and Priess (1997)
P	MEX-3	RNA-binding KH domain	Draper et al. (1996)
P	MIP-1	LOTUS-containing MEG-3 interacting protein	Cipriani et al. (2021)
P	MIP-2	LOTUS-containing MEG-3-interacting protein	Cipriani et al. (2021)
P	NOS-2	Nanos-related protein	Subramaniam and Seydoux (1999)
P	NPP-8	NUP155 NPC protein	Voronina and Seydoux (2010)
P	NPP-10	NUP98 NPC protein	Voronina and Seydoux (2010)
P	NXF-1	NXF1/TAP-like mRNA export factor	Sheth et al. (2010)
P	OMA-1	CCCH-type zinc-finger protein	Shimada et al. (2002)
P	OMA-2	CCCH-type zinc-finger protein	Shimada et al. (2002)
P	PAB-1	Poly(A)-binding protein 1	Gallo et al. (2008)
P	PAN-1	LRRTM4 DEAD-box helicase	Gao et al. (2012)
P	PARN-1	Poly(A)-specific 3'-5'-exoribonuclease	Tang et al. (2016)
P	PATR-1	Pat1 decapping cofactor	Gallo et al. (2008)
P	PGL-1	GGG-containing P granule endoribonuclease	Kawasaki et al. (1998)
P	PGL-2	PGL-1 related	Kawasaki et al. (2004)
P	PGL-3	GGG-containing P granule endoribonuclease	Kawasaki et al. (2004)
P	PID-1	piRNA-induced silencing defective	Cordeiro Rodrigues et al. (2019) and Zeng et al. (2019)
P	PID-3	piRNA-induced silencing defective	Cordeiro Rodrigues et al. (2019) and Zeng et al. (2019)
P	PICS-1		
P	PID-4	piRNA-induced silencing defective	Placentino et al. (2021)
P	PID-5	Aminopeptidase, piRNA-induced silencing defect	Placentino et al. (2021)
P	PIE-1	CCCH-type zinc-finger protein	Mello et al. (1996)
P	PLP-1	Pur alpha-like protein	Witze et al. (2009)
P	POS-1	CCCH-type zinc-finger protein	Tabara et al. (1999a)
P	PRG-1	Argonaute required for piRNA synthesis	Batista et al. (2008)
P	PUF-8	PUF (Pumilio/FBF) domain 3'UTR-binding factor	Ariz et al. (2009)
P	RDE-12	RNAi defective DEAD-box helicase	Sheth et al. (2010)

(continued)

Table 1 (continued)

Germ granule	Protein	Description	References
P	RNP-8	RRM poly(G) RNA binding	Kim <i>et al.</i> (2009)
P	SIR-2.2	Sirtuin 4-like protein deacetylase	Jedrusik-Bode <i>et al.</i> (2013)
P	Sm prot.	Splicing factors	Barbee <i>et al.</i> (2002)
P	SPN-2	eIF4E-binding protein	Li <i>et al.</i> (2009)
P	SPN-4	RNP-type RNA-binding domain	Ogura <i>et al.</i> (2003)
P	TIA-1	TIA-1 RNP-type RNA-binding domain	Gallo <i>et al.</i> (2008)
P	TOFU-6	21U-RNA fouled up	Cordeiro Rodrigues <i>et al.</i> (2019) and Zeng <i>et al.</i> (2019)
P	VBH-1	Vasa Belle-like DEAD-box helicase	Salinas <i>et al.</i> (2007)
P	WAGO-1	Argonaute required for endo-siRNA	Gu <i>et al.</i> (2009)
P	WAGO-3	Argonaute present in sperm	Schareier <i>et al.</i> (2021)
P	Y51F10.2	TRIM32 E3 ubiquitin-protein ligase	Lee <i>et al.</i> (2020)
M	MUT-2 RDE-3	Mutator with predicted nucleotidyltransferase activity	Phillips <i>et al.</i> (2012)
M	MUT-7	Mutator with predicted 3'-5' exoRNase activity for miRNA end processing	Phillips <i>et al.</i> (2012)
M	MUT-8 RDE-2	Mutator RNAi defective	Phillips <i>et al.</i> (2012)
M	MUT-14	Mutator resembling the DDX3 DEAD-box helicase	Phillips <i>et al.</i> (2012)
M	MUT-15 RDE-5	Mutator RNAi defective	Phillips <i>et al.</i> (2012)
M	MUT-16 RDE-6	Mutator RNAi defective	Phillips <i>et al.</i> (2012)
M	NYN-1	NYN domain ribonuclease homolog	Uebel <i>et al.</i> (2018)
M	NYN-2	NYN domain ribonuclease homolog	Uebel <i>et al.</i> (2018)
M	RDE-8	mRNA-binding endo-RNase that positively regulates RdRP activity	Tsai <i>et al.</i> (2015)
M	RRF-1	RNA-directed RNA polymerase (RdRP)	Phillips <i>et al.</i> (2012)
M	SMUT-1	Synthetic mutator DDX3 DEAD-box helicase-like	Phillips <i>et al.</i> (2014)
Z	LOTR-1	LOTUS and Tudor domain protein	Marnik <i>et al.</i> (2021)
Z	PID-2	piRNA-induced silencing defective	Placentino <i>et al.</i> (2021) and Wan <i>et al.</i> (2021)
Z	ZSP-1		Wan <i>et al.</i> (2021)
Z	WAGO-4	Argonaute required for RNAi inheritance	Wan <i>et al.</i> (2018)
Z	ZNFX-1	NFX1-type zinc finger-containing protein	Ishidate <i>et al.</i> (2018) and Wan <i>et al.</i> (2018)
S	HPO-40	SIMR-1-like Tudor-domain protein	Manage <i>et al.</i> (2020)
S	RSD-2	RNAi spreading defective	Manage <i>et al.</i> (2020)
S	SIMR-1	siRNA defective and mortal germline Tudor-domain protein	Manage <i>et al.</i> (2020)
?	FBF-1	PUF-domain fem-3 mRNA 3'UTR-binding factor that docks next to PGL-1	Voronina <i>et al.</i> (2012)
?	MINA-1	RNA-binding KH protein that docks next to PGL-1	Sendoel <i>et al.</i> (2019)

P granules retain both maternally expressed and developmentally regulated mRNAs (Seydoux and Fire 1994; Subramaniam and Seydoux 1999; Schisa *et al.* 2001). More recently, MEG-3 iCLIPs identified approximately 500 specific mRNAs that are enriched in embryonic P granules (Lee *et al.* 2020). *In situ* hybridization studies revealed that nascent mRNAs pass into and through P granules, and that their perinuclear enrichment may be a result of their slowed diffusion during the transit (Sheth *et al.* 2010). In contrast to mRNAs, ribosomal RNAs are not enriched in P granules and even appear excluded (Schisa *et al.* 2001; Marnik *et al.* 2019), suggesting that P granules are devoid of translation. In fact, some P-granule localized transcripts are correlated with low translational status and ribosome coverage, and global translation inhibition directs numerous transcripts to embryonic P granules in a MEG-3 dependent but nonsequence specific manner (Lee *et al.* 2020; Parker *et al.* 2020). These results suggest embryonic P granules are a way to maintain a pool of maternal mRNAs in germline precursors until they resume zygotic transcription.

Association of P granules with the nuclear pore complex

The association of *C. elegans* P granules on the cytoplasmic surface of the nuclear periphery reflects the general distribution of germ granules across species. In the *C. elegans* germline, P granules cluster nuclear pore complexes (NPCs), and 75% of NPCs are covered by P granules (Figure 1B; Pitt *et al.* 2000). NPCs not

covered by P granules are only found where a prominent lobe from the nucleolus contacts the nuclear envelope, suggesting that rRNAs enter the cytoplasm without passing through P granules (Sheth *et al.* 2010). Nuclear export factors, such as NXF-1 and DDX-19, and peripheral nucleoporins, such as NPP-8, NPP-9, and NPP-10, localize to the base of P granules (Sheth *et al.* 2010; Voronina and Seydoux 2010). Upon the cytoplasmic dispersal of P granules during oogenesis, some P granules retain attached NPCs (Pitt *et al.* 2000). In addition, RNAi depletion of a number of nucleoporins cause P granules to detach from the nuclear periphery and disperse into the cytoplasm (Updike and Strome 2009; Voronina and Seydoux 2010). These findings demonstrate the tight association between P granules and NPCs.

P granules and NPC contacts are likely mediated through glycine-rich FG-repeat domains present in several nucleoporins (FG-Nups) and P-granule proteins such as GLH-1, GLH-2, GLH-4, DDX-19, RDE-12 (Sheth *et al.* 2010). Unstructured FG-repeat domains fill up the pore of NPCs to establish the size exclusion barrier between the nucleus and cytoplasm. A current model is that the regularly spaced phenylalanines form weak hydrophobic interactions to create a mesh or smart sieve (Schmidt and Görlich 2016). Proteins under 40 kD can diffuse freely through the sieve, while larger proteins require a karyopherin for import and export. P granules extend the 40 kD size exclusion barrier beyond the pore and into the cytoplasm, and weak concentrations of hexane-diol capable of disrupting the hydrophobic interactions within

the pore also disperse P granules (Updike et al. 2011). P-granule FG-repeat proteins form hydrophobic tethers with FG-Nups to maximize coverage of NPCs, positioning P granules to receive nascent transcripts that exit the nucleus. Deleting FG-repeats from GLH-1 and GLH-2 increases P-granule size and sphericity as they lose contact with the nuclear periphery (Marnik et al. 2019; Chen et al. 2020).

P granules regulate germline apoptosis

Over half of the oogenic germ cells undergo physiological apoptosis (Gartner et al. 2008). Germ cells are connected to a cytoplasmic syncytium, and excess germ cells function as nurse cells and dump their mitochondria and other cytoplasmic contents into the shared cytoplasm as apoptosis is initiated (Raiders et al. 2018). PGL-1 and PGL-3 disappear from these apoptotic cells, suggesting that P granules and their components not only play a role in the formation of germ cells, but also their preservation (Pitt et al. 2000; Sheth et al. 2010). In response to UV damage, *pgl-1* and *pgl-3* mutants show elevated levels of apoptosis; the pro-apoptotic factor Apaf1/CED-4 accumulates, and Sirtuin/SIR-2.1, which functions as an antiapoptotic factor in the nucleus, is translocated into the cytoplasm. These findings suggest that P granules (or the presence of PGL proteins in P granules) suppress these pro-apoptotic activities (Min et al. 2016). Supporting these findings, somatic programmed cell death is suppressed in *synMuvB* mutants that express somatic P granules; similarly, ectopic expression of PGL-1 or PGL-3 from transgenes is sufficient to repress apoptosis in the soma in a SIR-2.1-dependent manner (Al-Amin et al. 2016). PGL proteins are cleared by autophagy in somatic blastomeres during embryogenesis (Zhang et al. 2009, 2018a). Similarly, PGL proteins in the adult germline are cleared by autophagy following UV-induced DNA damage, linking the requirement of autophagy machinery to UV-induced apoptosis in the germline (Min et al. 2019).

P granules promote germline gene expression

Given the complex composition of P granules and the redundancy involved in their nucleation, depleting single P-granule components does not clear P granules from the adult germline. However, core P-granule proteins and electron-dense P-granule assemblies are no longer detected in *mex-3 gld-1* double mutants, and this absence correlates with germ-to-soma transdifferentiation (Ciosk et al. 2006). The simultaneous RNAi depletion of multiple core P-granule proteins inhibits fertility and also causes germ-to-soma transdifferentiation (Updike et al. 2014), and an increase in somatic expression in the germline of older adults (Knutson et al. 2017). These results suggest that P granules safeguard germline development through mRNA surveillance mechanisms that repress the accumulation and translation of somatic transcripts that become stochastically expressed. The analysis of *deps-1*, *glh-1*, *glh-2*, *glh-4*, *pgl-1*, and *pgl-3* single mutants has revealed underproliferated germlines and defects in gametogenesis at restrictive temperatures, while sterility in double mutants can make it difficult to distinguish primary from secondary effects on gene expression (Kawasaki et al. 2004; Spike et al. 2008a, 2008b). Before the onset of these defects in *deps-1* and *glh-1* single mutants, expression profiling reveals only subtle germline expression changes (Spike et al. 2008a, 2008b). The same is the case in healthy germlines of young adults after simultaneous RNAi depletion of multiple transcripts (*pgl-1*, *pgl-3*, *glh-1*, *glh-4*), except for a global increase of spermatogenic transcripts in proximal germ cells slated to undergo oogenesis (Campbell and Updike 2015; Knutson et al. 2017).

Mechanisms used by P granules to recognize and suppress somatic and spermatogenic expression in the adult germline are areas of continuing focus. During spermatogenesis PGL proteins are cleared from secondary spermatocytes (Amiri et al. 2001), while GLH proteins, which are necessary for the completion of spermatogenesis (Kuznicki et al. 2000), are retained in P granules until they are deposited in the residual body near the completion of spermatogenesis (Gruidl et al. 1996). The expression of P-granule associated Argonaute proteins like CSR-1, WAGO-1 and WAGO-3 is accompanied by transient expression of Argonautes ALG-3 and ALG-4 during spermatogenesis; while all of these Argonautes are implicated in paternal inheritance, CSR-1, WAGO-1 and WAGO-3 persist in sperm while ALG-3 and ALG-4 become deposited in residual bodies (Conine et al. 2010, 2013; Schreier et al. 2021). How the temporospatial expression of each these factors impact spermatogenesis still needs to be resolved. Recent studies have shown that CSR-1 has both long (a) and short (b) isoforms, but the long CSR-1a isoform is selectively expressed during spermatogenesis in L4 hermaphrodites, where it primarily targets spermatogenic genes (Nguyen and Phillips 2021; Charlesworth et al. 2021). Dimethylarginine modifications to the CSR-1a isoform are necessary for this target specificity (Nguyen and Phillips 2021). Translational initiation may also be involved; for example, the PGL-associated isoform of the m7G cap-binding eIF4E initiation factor IFE-1 promotes sperm translation—an activity likely repressed with the occurrence of PGLs in the first wave of oogenesis (Amiri et al. 2001; Henderson et al. 2009; Friday et al. 2015). In contrast, a second isoform of the m7G cap eIF4E, IFE-3, associates with eIF4E transporter IFET-1 to drive oocyte translation and the sperm-to-oocyte switch but is not required for spermatogenesis (Sengupta et al. 2013; Huggins et al. 2020).

P granules as sites of mRNA surveillance

The tight association with the NPC positions P granules to survey transcripts for foreign or somatic sequences as they exit the nucleus. This epigenetic memory of germline expression is conferred through the small RNA machinery within P granules. The PIWI-class Argonaute, PRG-1, is a constitutive P-granule component that associates with more than 10,000 distinct Piwi-interacting RNAs (piRNAs), also known as 21U RNAs in *C. elegans* due to their 21-nt length and 5' bias for uracil, to form piRNA-induced silencing complexes (piRISCs). These piRISCs use imperfect complementarity to engage and surveil the entire germline transcriptome, including mRNAs, non-coding RNAs, and transposable elements (Ruby et al. 2006; Wang and Reinke 2008; Batista et al. 2008; Lee et al. 2012; Bagijn et al. 2012; Shen et al. 2018; Zhang et al. 2018b). piRNA targeting can initiate heritable epigenetic silencing that, in many cases, bypasses the need for piRNAs to maintain that silencing in subsequent generations (Shirayama et al. 2012; Ashe et al. 2012; Luteijn et al. 2012). This heritable silencing is mediated by WAGO 22G-RNAs, 22-nt siRNAs with a 5' guanosine bias, that are bound by a class of WAGO proteins. WAGO 22G-RNAs are synthesized by RNA-dependent RNA polymerases (RdRPs) associated with the *mutator* complex (described in more detail below; Gu et al. 2009; Phillips et al. 2012).

The biogenesis of piRNAs requires both 5' and 3' processing. First, the 5' piRNA end maturation is carried out by the PETISCO/PICS complex, made up of PID-1, PID-3/PICS-1, ERH-2, TOFU-6, and IFE-3 (Cordeiro Rodrigues et al. 2019; Zeng et al. 2019; Perez-Borrajero et al. 2021). This maturation involves decapping and removal of two nucleotides from the 5' end of the piRNA precursor (Gu et al. 2012). The PETISCO/PICS complex is also P-granule associated, though the co-localization of this complex with

known P-granule factors may be imperfect (Cordeiro Rodrigues et al. 2019; Zeng et al. 2019; Perez-Borrajero et al. 2021). For example, perinuclear IFE-3 granules appear to dock next to PGL-1 labeled granules (Huggins et al. 2020). 5' end maturation is followed by loading of the piRNA precursor into PRG-1 where the 3' end is trimmed by the P granule-localized exonuclease PARN-1 (Tang et al. 2016). Finally, the 3' end of the piRNA is 2'O-methylated by the methyltransferase HENN-1, which may also localize to P granules (Billi et al. 2012; Montgomery et al. 2012; Kamminga et al. 2012). While initially the mobilization of transposable elements was assumed to cause the transgenerational decline in fertility in *prg-1* mutants, *prg-1* sterility correlates more with perturbed P-granule structure and may be independent of genomic stability (Spichal et al. 2021). Also correlating with the progressive loss of fertility in *prg-1* mutants, is the accumulation of 22G-RNAs antisense to replicative histone genes and ribosomal RNA genes and a corresponding reduced expression of histone mRNAs (Barucci et al. 2020; Montgomery et al. 2020; Reed et al. 2020; Wahba et al. 2021). Whether reduced expression of rRNAs and histones, disruption of P granules, or another unknown factor is underlying cause of transgenerational sterility in *prg-1* mutant animals is a matter that will need further investigation.

Opposing piRNA-mediated silencing, 22G-RNAs bound by the P granule-associated Argonaute CSR-1 (CSR-1 22G-RNAs) license protein-encoding transcripts for germline expression (Claycomb et al. 2009; Gu et al. 2009; Seth et al. 2013; Wedeles et al. 2013; Cecere et al. 2014; Tu et al. 2014). CSR-1 22G-RNAs are produced through the activity of the EGO-1 RdRP (Claycomb et al. 2009), yet the mechanism by which EGO-1 activity is initiated on CSR-1 target transcripts has been a bit of an enigma. While WAGO 22G-RNAs can be initiated by piRNAs or other classes of primary siRNAs, there has been no known primary siRNA class in the CSR-1 pathway. However, recent work has demonstrated that CSR-1 slicer activity is necessary to trigger biogenesis of CSR-1 22G-RNAs within the coding region of CSR-1 target genes. Interestingly, CSR-1 slicer activity is not required for initiation of EGO-1 RdRP activity in CSR-1 target 3'UTRs. Thus, while it is unknown what triggers the recruitment of EGO-1 to target 3'UTRs, these new data indicate that within target gene bodies EGO-1 22G-RNA synthesis may be initiated following cleavage by 22G-RNA-bound CSR-1, independent of any primary siRNAs (Singh et al. 2021). Furthermore, it appears that CSR-1 22G-RNA biogenesis occurs on actively translated mRNAs in the cytoplasm, in contrast to other 22G-RNAs which are mostly synthesized in germ granules (Singh et al. 2021). Lastly, CSR-1 22G-RNAs can be uridylylated by the P granule-localized nucleotidyl transferase CDE-1, to restrict their accumulation (van Wolfswinkel et al. 2009).

Through CSR-1 22G-RNA/WAGO 22G-RNA opposition, P granules have the capacity to retain a memory of germline expression. How CSR-1 promotes the expression of its 22G-RNA targets is unclear; however, one model is that CSR-1 may secure the passage of its targets through P granules and into the cytoplasm where they can ultimately be translated. Compromising CSR-1 and select components upstream of CSR-1 22G-RNA synthesis cause a very distinct enlarged P-granule phenotype (Vought et al. 2005; Claycomb et al. 2009; Urdike and Strome 2009; Campbell and Urdike 2015; Andralojc et al. 2017). This could reflect the pooling of CSR-1 22G-RNA target transcripts that can no longer make their way through and into the cytoplasm. Interestingly, while CSR-1 22G-RNA target mRNA levels change very little in dissected germlines and whole worm lysates, expression profiling in early embryos suggest a P-granule independent role for the slicer activity of CSR-1 in clearing its maternally deposited targets

from somatic blastomeres (Quarato et al. 2021). Determining whether germ granules in other animals confer a similar mRNA surveillance system to retain an epigenetic memory of germline expression will be a critical next step.

Organization and function of Mutator foci

Overview of Mutator foci

The term “mutator” was first used to describe spontaneous mutations caused by insertions of the transposon Tc1 (Eide and Anderson 1985; Collins et al. 1987). Later experiments linked the phenomenon of transposon activation to the disruption of RNA interference pathways via two parallel genetic screens, one for defects in RNAi and the other for germline mobilization of transposons (Ketting et al. 1999; Tabara et al. 1999b). The overlap of these two screens provided some of the first evidence that the RNAi pathway is required for transposon silencing. Since then, nearly a dozen *mutator* genes have been identified, with their protein products found to interact to form the *mutator* complex and function in the production of WAGO 22G-RNAs and ERGO-1 26G-RNAs (see Table 1; Ketting et al. 1999; Tabara et al. 1999b; Tijsterman et al. 2002; Vastenhouw et al. 2003; Tops et al. 2005; Chen et al. 2005; Grishok et al. 2005; Robert et al. 2005; Kim et al. 2005; Phillips et al. 2012; Tsai et al. 2015). Consequently, loss of the *mutator* complex results in defects in the production of WAGO 22G-RNAs and ERGO-1 26G-RNAs but not other classes of small RNAs such as piRNAs, microRNAs (miRNAs), CSR-1 22G-RNAs, and ALG-3/4 26G- and 22G-RNAs (Gu et al. 2009; Zhang et al. 2011; Lee et al. 2012; Phillips et al. 2014; Tsai et al. 2015). Interestingly, the loss of ERGO-1 26G-RNAs in *mutator* mutants can be attributed to the disruption of a homeostatic feedback loop mediated by *mutator*-dependent 22G-RNAs at the *eri-6/7* gene locus rather than the direct involvement of the *mutator* proteins in ERGO-1 26G-RNA biogenesis (Rogers and Phillips 2020b). Therefore, the *mutator* complex is thought to function primarily for the amplification of WAGO 22G-RNAs through the activity of RdRPs.

Mutations in genes from this group not only have active germline transposition and defects in exogenous RNAi, but they also are temperature-sensitive sterile and have more male progeny, suggestive of chromosome segregation defects (Ketting et al. 1999; Tabara et al. 1999b; Zhang et al. 2011; Wallis et al. 2019). The fertility defects at elevated temperature are present in spermatogenic and oogenic cells, though more severe during spermatogenesis, manifesting within a single generation at elevated temperature (Rogers and Phillips 2020a). In contrast, the oogenic defect accumulates over several generations, becoming progressively sterile over 2–3 generations at elevated temperature (Rogers and Phillips 2020a). While the spermatogenic defect has not been fully characterized, the oogenic defect can be attributed to changes in germline chromatin accessibility resulting in the aberrant expression of somatic and spermatogenic genes in oogenic nuclei (Rogers and Phillips 2020a).

Composition and assembly of Mutator foci

Assembly of *mutator* complex and accumulation of the *mutator* complex into visible perinuclear germline foci, referred to as *Mutator* foci, depend on the scaffolding properties of MUT-16 (Figures 1F and 2, A and C; Phillips et al. 2012; Uebel et al. 2018). Both structured and unstructured/disordered regions of MUT-16 are required for *Mutator* foci assembly and for the recruitment of other *mutator* complex members (Uebel et al. 2018). Specifically, the most C-terminal region of MUT-16 (~275 amino acids), is both necessary and sufficient for the germline *Mutator* foci

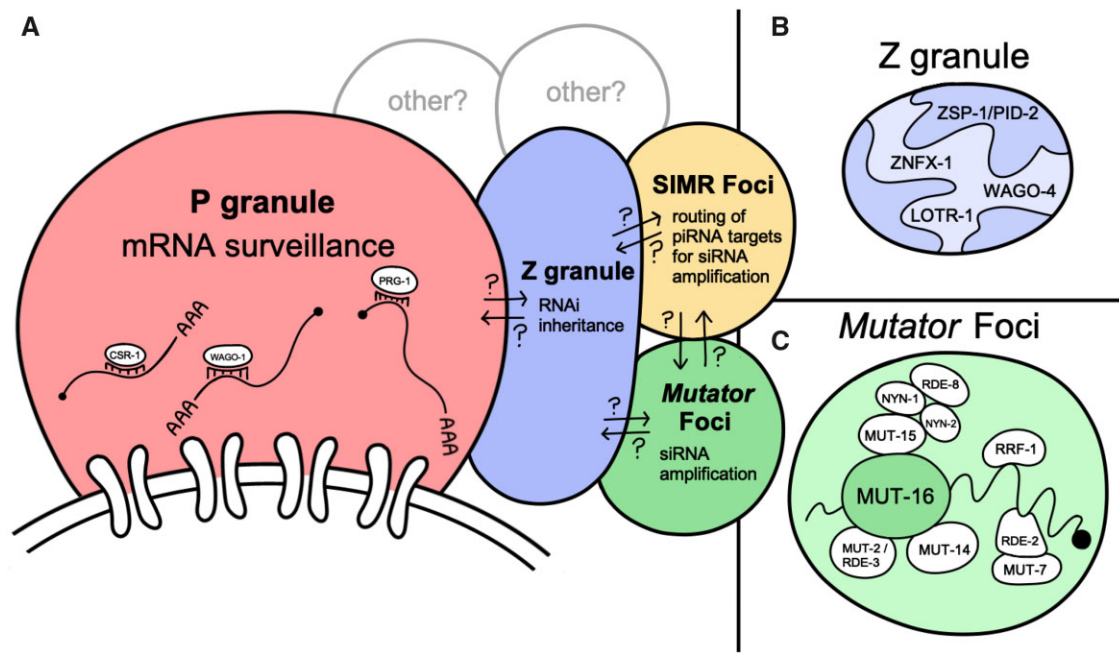


Figure 2 Physical and functional compartmentalization of germ granules. (A) P granules are positioned to receive and scan nascent transcripts as they exit the nucleus. This is accomplished through CSR-1, PRG-1, and WAGO-1 Argonautes and their associated small RNAs, which can distinguish foreign transcripts from those licensed for germline expression. Z granules promote amplification of siRNAs for memory storage and transgenerational silencing. SIMR foci route piRNA targets for siRNA amplification. Mutator foci are sites of siRNA amplification. The trajectory of small RNAs and mRNAs through the granules is still unknown and possible routes are indicated by arrows. (B) Substructure within Z granules is mediated through ZSP-1/PID-2. (C) Mutator foci are nucleated by MUT-16, which contains both structured and disordered regions. Additional mutator complex proteins are recruited to Mutator foci through either direct or indirect interactions with MUT-16.

assembly (Phillips et al. 2012). MUT-2/RDE-3, a nucleotidyltransferase that adds untemplated poly(UG) tails (pUG tails) to the 3' termini of cleaved siRNA-targeted mRNAs, is recruited to the complex through a structured region near the N-terminus of MUT-16 (Chen et al. 2005; Phillips et al. 2012; Uebel et al. 2018; Shukla et al. 2020). The same region also recruits a DEAD-box helicase MUT-14, which acts redundantly with its paralog SMUT-1 in the initiation of WAGO 22G-RNA amplification (Tijsterman et al. 2002; Phillips et al. 2014), and MUT-15, a protein with no known domains that recruits NYN-1 and NYN-2 to Mutator foci (Uebel et al. 2018). NYN-1 and NYN-2, two NYN-domain proteins, in turn recruit to Mutator foci the NYN-domain endoribonuclease RDE-8, which associates with MUT-2/RDE-3 and cleaves siRNA-targeted mRNAs for MUT-2 pUGylation (Tsai et al. 2015; Uebel et al. 2018).

The disordered central region of MUT-16 recruits the RdRP RRF-1 and RDE-2, a protein with no known domains, which then recruit the 3'-5' exonuclease MUT-7 to Mutator foci (Ketting et al. 1999; Tops et al. 2005; Phillips et al. 2012; Uebel et al. 2018). The RNAs targeted by the MUT-7 exonuclease are currently unknown. It is also worth noting that the RdRPs EGO-1 and RRF-1 are redundantly required for mutator complex-dependent small RNA amplification; however, only RRF-1 localizes to Mutator foci and co-IPs with mutator complex proteins (Gu et al. 2009; Phillips et al. 2012; Manage et al. 2020). Perhaps the distinct localization of the two RdRPs can be attributed to their activity in two distinct 22G-RNA pathways: EGO-1 is also required for biogenesis of CSR-1 22G-RNAs (Claycomb et al. 2009), suggesting that EGO-1 may act primarily with CSR-1 in P granules or the cytoplasm for CSR-1 22G-RNA biogenesis, whereas RRF-1 acts with the mutator complex for WAGO 22G-RNA biogenesis. Perhaps then, only in the absence of RRF-1, EGO-1 can compensate for the loss by also

generating WAGO 22G-RNAs with the Mutator complex. Despite the distinction, both RdRPs, EGO-1 and RRF-1, and MUT-16 co-IP with the Dicer-related helicase DRH-3 and the Tudor-domain protein EKL-1 (Gu et al. 2009; Thivierge et al. 2011; Manage et al. 2020). DRH-3 and EKL-1 are required for both CSR-1 22G-RNA and WAGO 22G-RNA biogenesis making them likely members of the mutator complex (Claycomb et al. 2009; Gu et al. 2009), though it has not been demonstrated that they colocalize with mutator complex proteins at Mutator foci.

Regulation of Mutator foci

Several variables have been shown to modulate the presence and intensity of Mutator foci. Early observations of Mutator foci indicated that they are present in germ cells but not somatic cells, which was initially perplexing due to the necessity of the mutator complex to promote RNAi and WAGO 22G-RNA production in somatic cells (Chen et al. 2005; Kim et al. 2005; Gent et al. 2010; Phillips et al. 2012; Uebel et al. 2018). Interestingly, increasing the concentration of MUT-16 in somatic cells can lead to the formation of ectopic Mutator foci that are capable of nucleating other mutator complex proteins (Uebel et al. 2018). This result suggests that somatic cells are not lacking any factors required for Mutator foci formation, but rather that somatic cells do not possess MUT-16 at high enough concentration to nucleate visible Mutator foci. In addition to protein concentration, Mutator foci are also regulated by environmental temperature, as elevated temperatures promote their dissolution (Uebel et al. 2018). Examination of Mutator foci suggested that their intensity is greatest in the mitotic region, transition zone, and late pachytene/diplotene (Phillips et al. 2012; Uebel et al. 2020). This association of foci intensity with germ cell progression can be monitored through the examination of mitotic and meiotic mutants, demonstrating that

extended regions of mitosis but not the transition zone can expand the region of intense *Mutator* foci and establishing that foci intensity is modulated by germline cell cycle stage (Uebel *et al.* 2020). Finally, similar to work demonstrating that RNA is integral to P-granule stability (Sheth *et al.* 2010), injection of the transcriptional inhibitor α -amanitin results in dispersal of *Mutator* foci and indicating that RNA is a critical component of this granule (Uebel *et al.* 2020).

Organization and function of Z granules

Z granules are defined by the localization of ZNFX-1, a superfamily one (SF1) RNA helicase and zinc-finger domain protein, and WAGO-4, a WAGO-clade Argonaute protein (Ishidate *et al.* 2018; Wan *et al.* 2018). Loss of ZNFX-1 and WAGO-4 disrupt RNAi inheritance from one generation to the next but do not disable the response to double-stranded RNA within a single generation (Ishidate *et al.* 2018; Wan *et al.* 2018; Xu *et al.* 2018). Curiously, in a transgene expression assay, mutations in *znfx-1* do not simply disrupt the inheritance of silencing signals but rather alter the stability of transgene expression, with the transgenes switching back and forth between silencing and expressed states across generations (Ishidate *et al.* 2018). Furthermore, ZNFX-1 co-immunoprecipitates with Argonaute proteins in activating and silencing pathways, including PRG-1, WAGO-1, and CSR-1 (Ishidate *et al.* 2018). These data suggest that Z granules may not solely be required for RNAi inheritance of silencing signals but instead for the inheritance of both expressed and silent epigenetic states.

There is some discrepancy regarding the small RNA that associates with WAGO-4, with separate reports indicating that it primarily associates with CSR-1 22G-RNAs or WAGO 22G-RNAs (Xu *et al.* 2018; Wan *et al.* 2021). However, these data, along with data indicating that WAGO-4 binds small RNAs antisense to genes targeted by exogenous RNAi, suggest that, like ZNFX-1, WAGO-4 may act with both activating and silencing small RNA pathways (Xu *et al.* 2018). Yet, precisely how ZNFX-1 and WAGO-4 promote balanced epigenetic inheritance is still unclear. One clue comes from the sequencing of small RNAs in a *znfx-1* mutant. This mutant shows a shift in the distribution of both CSR-1 and WAGO 22G-RNAs across target mRNAs and toward the 5' end, indicating that one role for ZNFX-1 may be the redistribution of RdRPs from the 5' end to the 3' end of target mRNAs (Ishidate *et al.* 2018). Another Z-granule protein called LOTR-1 contributes to this function, as *lotr-1* mutants exhibit a similar loss of small RNAs from the 3' ends of WAGO and *mutator* targets and impacts transgenerational RNAi inheritance (Marnik *et al.* 2021). The Tudor domain of LOTR-1 positions ZNFX-1 and LOTR-1 in perinuclear Z granules, while the LOTUS domain of LOTR-1 appears to associate with cytoskeletal and 3'UTR-binding components. While the LOTUS-domain proteins MIP-1 and MIP-2 interact with the helicase GLH-1 to help nucleate P-granule assembly, LOTR-1 functions similarly by interacting with the helicase ZNFX-1 to help nucleate Z-granule assembly (Marnik *et al.* 2021; Cipriani *et al.* 2021).

More recently, PID-2/ZSP-1, a protein with intrinsically disordered regions (IDRs), has been shown to localize to the surface of Z granules where it regulates Z-granule size, number, and fluidity (Figure 2B; Placentino *et al.* 2021; Wan *et al.* 2021). PID-2/ZSP-1 is required for germline RNAi and heritable silencing downstream of piRNAs, and, similarly to *znfx-1*, mutations in *pid-2/zsp-1* display only modest alterations in overall small RNA levels or levels of small RNAs mapping to previously defined categories of 22G-RNAs (Ishidate *et al.* 2018; Placentino *et al.* 2021; Wan *et al.* 2021).

Furthermore, like *znfx-1*, *pid-2/zsp-1* mutants alter the distribution of both WAGO 22G-RNAs and CSR-1 22G-RNAs along mRNA targets (Ishidate *et al.* 2018; Placentino *et al.* 2021). However, *pid-2/zsp-1* mutants have reduced small RNAs at the 5' end of target mRNA transcripts, which is opposite to the effect of a *znfx-1* mutation and suggests a possible role for PID-2/ZSP-1 in the processivity of the RdRPs (Placentino *et al.* 2021). Nonetheless, the fact that most small RNA target genes still produce small RNAs, including germline genes targeted by RNAi, yet *pid-2/zsp-1* mutants have strong desilencing of a piRNA sensor and fail to silence RNAi-targeted germline mRNAs, suggests that PID-2/ZSP-1 may somehow fail to couple siRNA production to gene silencing (Placentino *et al.* 2021; Wan *et al.* 2021). Altogether, there are clearly still questions to be answered regarding the precise role Z granules play in RNA silencing and RNA inheritance.

Organization and function of SIMR foci

SIMR-1, an extended Tudor domain protein, was initially identified through a MUT-16 immunoprecipitation followed by mass spectrometry of interacting proteins (Manage *et al.* 2020). Loss of *simr-1* has no RNAi-defective (Rde) or Enhanced RNAi (Eri) phenotypes, though the mutation causes a depletion of piRNA-dependent but not piRNA-independent *Mutator* targets. Further, *simr-1* mutants can desilence a piRNA sensor and prevent sterility after reestablishing WAGO-class 22G-RNA production, a phenotype previously only associated with piRNA pathway mutants (de Albuquerque *et al.* 2015; Phillips *et al.* 2015; Manage *et al.* 2020). The piRNAs themselves are unaffected in a *simr-1* mutant, so together, this data suggests that SIMR-1 acts in the piRNA pathway, downstream of piRNA biogenesis but upstream of the *mutator* complex. Interestingly, SIMR-1 also forms germline foci, but these foci are discrete from P granules, Z granules, and *Mutator* foci (Figures 1G and 2A; Manage *et al.* 2020). Rather, SIMR-1 colocalizes with RSD-2, a factor required for exogenous RNAi introduced at low doses and production of 22G-RNAs at ERGO-1 target mRNAs (Tijsterman *et al.* 2004; Han *et al.* 2008; Zhang *et al.* 2012; Sakaguchi *et al.* 2014) and that has no known association with the piRNA pathway. Thus, the current hypothesis is that the SIMR-1 foci function to promote interactions between primary and secondary small RNA pathways, RSD-2 for exogenous RNAi and ERGO-1 target genes and SIMR-1 for piRNA target genes. Also, likely co-localizing with SIMR-1 and RSD-2 is the SIMR-1 paralog, HPO-40. While no function has yet been attributed to HPO-40, it has a similar localization pattern as SIMR-1 and fails to colocalize completely with MUT-16 (Manage *et al.* 2020). Since no other components of the SIMR foci are currently known, further study will be necessary to reveal the molecular details of this compartment.

Interactions between germ-granule compartments

The current model of germ granule organization in the *C. elegans* germline proposes sequential stacking of P granules, Z granules, and *Mutator* foci at the nuclear periphery (Figure 2A; Wan *et al.* 2018). SIMR foci are also found in tripartite structures, adjacent to Z granules and opposite P granules (Manage *et al.* 2020), however the orientation of all four condensates relative to one another is still undetermined. Nonetheless, the consistency of which these granules are found in this stacked organization suggests that the interaction between condensates may promote efficient small RNA-based silencing and inheritance. In the germline progenitor cells of early embryos, Z-granule proteins,

ZNFX-1 and WAGO-4, co-localize to P granules rather than forming discrete structures; however, after the 100-cell stage of embryonic development, the Z granules demix into discrete condensates (Figure 1C; Wan et al. 2018). Similarly, the *Mutator* foci and SIMR foci are first observed as robust granules around the 100-cell stage of embryonic development in the Z2/Z3 progenitor germ cells, but unlike Z granules, the *Mutator* foci and SIMR foci appear to nucleate *de novo* from cytoplasmically localized proteins rather than through demixing of proteins from the P granule (Figure 1D; Uebel et al. 2018, 2021; Wan et al. 2018). These events roughly coincide with initiation of transcription from embryonic germ cells, suggesting that the presence of newly synthesized mRNAs traversing the nuclear pores and their recognition by various small RNA pathway proteins may drive changes in germline condensate morphology (Seydoux and Dunn 1997; Wan et al. 2018).

Based on their protein composition, *Mutator* foci are considered hubs of WAGO 22G-RNA biogenesis, positioned near P granules and nuclear pores to capture recently-transcribed target mRNAs for small RNA amplification. Simultaneous RNAi knock-down of four core components of the P granule is sufficient to disrupt *Mutator* foci formation, while *Mutator* foci are not required for P-granule assembly (Phillips et al. 2012; Singh et al. 2021). SIMR foci also do not require *Mutator* foci for formation and, reciprocally, *Mutator* foci do not require SIMR foci; however, interactions between SIMR foci and other germ-granule compartments have not been thoroughly tested (Manage et al. 2020). Similar to *Mutator* foci, ZNFX-1 appears to require P granules for localization, as *csr-1* and *glh-1* mutants, which disrupt P granules, also disrupt ZNFX-1 localization (Ishidate et al. 2018). Interestingly, loss of the P-granule protein DEPS-1, alters P-granule, Z-granule and *Mutator* foci morphology, while loss of *mutator* complex proteins disrupt DEPS-1 localization, suggesting there may be a more complex interplay between condensates (Wan et al. 2018; Suen et al. 2020).

Similar to P granules, Z granules and *Mutator* foci have properties associated with phase-separated condensates, canonically characterized by rapid internal recovery after photobleaching (Brangwynne et al. 2009; Uebel et al. 2018; Wan et al. 2018). Furthermore, *Mutator* foci assemble after reaching a critical concentration threshold, which may explain their presence in germ cells but not somatic cells. They are disrupted by elevated temperature and, like P granules, they dissolve in low concentrations of an aliphatic alcohol that is thought to only affect weak hydrophobic interactions (Updike et al. 2011; Uebel et al. 2018). These liquid-like properties may allow a continuation of RNA exchange through distinct granule compartments, coordinating RNA silencing between granules.

Proteins and RNAs not yet assigned to specific germ-granule compartments

While the above-described sub-granules constitute the most well-characterized of the germ-granule compartments, several other proteins have been associated with germ granules but whether they form distinct compartments or overlap with a known compartment is unclear. For example, the Tudor domain protein RSD-6 localizes to foci near P granules referred to as R2 bodies (Yang et al. 2014). RSD-6, along with the DEAD box helicase RDE-12, associate with mRNAs targeted by RNAi and are required for production of secondary siRNAs at ERGO-1 and exogenous RNAi targets (Zhang et al. 2012; Shirayama et al. 2014; Yang et al. 2014). Interestingly RDE-12 localizes to both the R2 bodies and to

P granules, possibly shuttling between the two; it has been proposed that RDE-12 may carry primary siRNA-targeted mRNAs from P granules to R2 bodies for *mutator*-dependent siRNA amplification (Yang et al. 2014). Given the overlap in phenotypes and likely function between *rsd-2*, in SIMR foci, with *rsd-6* and *rde-12*, in R2 bodies, a likely conclusion is that SIMR foci and R2 bodies are one in the same; however, this inference has yet to be proven.

Another example of a germ granule-associated protein which has not been precisely localized to one of the known germ-granule compartments is FBF-1. FBF-1, and its paralog FBF-2, are PUF-family RNA binding proteins that are required in germline stem cells to silence the expression of mRNAs required for meiosis (Voronina et al. 2012). While FBF-2 seems to associate with P granules, FBF-1 forms both cytoplasmic and perinuclear foci, the majority of which do not overlap with P granules though many are immediately adjacent (Voronina et al. 2012). Two additional PUF-family proteins, PUF-3 and PUF-11, in combination with FBF-1 and FBF-2, are required for germline proliferation and have been localized to unidentified cytoplasmic and perinuclear foci germline foci (Haupt et al. 2020).

Similarly, the KH protein MINA-1, identified in a screen for apoptosis regulators, binds the 3'UTRs of genes associated with germ cell development and localizes to a germ-granule compartment adjacent to P granules (Sendoel et al. 2019). Curiously, MINA-1 down-regulates the Z-granule Argonaute protein WAGO-4, and loss of *mina-1* leads to enhanced exogenous RNAi (Sendoel et al. 2019). Further work will be necessary to uncover the precise role of MINA-1 in small RNA and gene regulatory pathways.

Finally, in addition to proteins that have yet to be localized to specific germ granules, mRNAs have also been localized to the perinuclear germ-granule regions. Specifically, mRNAs are differentially regulated by miRNAs in germ cells compared to somatic cells, where germline miRNA-targeted mRNAs are stabilized while somatic miRNA-targeted mRNAs are destabilized (Dallaire et al. 2018). These miRNA-targeted mRNAs localize adjacent to P granules dependent on GLH-1, but not to any specific, known compartment (Dallaire et al. 2018). Thus, the localization of RSD-6, FBF-1, PUF-3, PUF-11, MINA-1, and miRNA-targeted mRNAs to undetermined perinuclear germ-granule compartments will necessitate further co-localization studies to determine whether these proteins are components of known germ-granule compartments or whether they are the defining members of a newly identified compartments.

Germ granules likely collaborate in heritable gene silencing

Overview of transgenerational inheritance

In many eukaryotes, including *C. elegans*, endogenous siRNAs can transmit epigenetic information, including responses to environmental stress, from parents to offspring (Rechavi and Lev 2017). When siRNAs carried by either egg or sperm are deposited into the embryo, they are amplified through the activity of RdRPs. mRNA targets of the deposited siRNAs become RdRP templates, leading to further production of abundant secondary siRNAs and enhanced silencing. Loss of many RNAi pathway and germ-granule components lead to a germline mortal (Mrt) phenotype, where the animals become progressively less fertile over generations (Buckley et al. 2012; Simon et al. 2014; Sakaguchi et al. 2014; Spracklin et al. 2017; Ishidate et al. 2018; Wan et al. 2018, 2021; Manage et al. 2020; Placentino et al. 2021). This phenotype hints at the possibility that sterility arises from changes in germ cell gene

expression that become exacerbated by the transgenerational small RNA amplification cycle.

Phenotypic variation can depend on parental or ancestral genotype

It has been observed that mutations in multiple germ-granule components, including *pgl-1*, *glh-1*, and *meg-3*; *meg-4*, are either RNAi defective or RNAi inheritance defective (Robert et al. 2005; Spike et al. 2008b; Wang et al. 2014; Spracklin et al. 2017; Ouyang et al. 2019; Dodson and Kennedy 2019). The implication being that germ granules are essential for RNAi. However, this RNAi-defective phenotype can be uncoupled, or “transgenerationally disconnected,” from the germ granule-defective genotype. For example, wild-type animals with *meg-3*; *meg-4* mutant ancestors can exhibit RNAi defects and *meg-3*; *meg-4* mutants from wild-type ancestors can exhibit a wild-type RNAi response (Lev et al. 2019; Ouyang et al. 2019; Dodson and Kennedy 2019). This phenotypic disconnect can last >8 generations, indicating that loss of germ granules can have transgenerational repercussions. It is worth noting, however, that the expected coupling of phenotype and genotype ultimately returns, suggesting the information needed to coordinate small RNA gene silencing is genome-encoded (Dodson and Kennedy 2019).

P granules and piRNAs coordinate siRNA biogenesis and transgenerational inheritance

If embryonic P granules are not required, per se, for germline RNAi, what role do they play in coordinating siRNA production and RNA silencing (Dodson and Kennedy 2019)? P granules house multiple RNAi pathway proteins, including Argonaute proteins and RdRPs, and in their absence, aberrant siRNAs are generated at some genes while siRNAs are lost at others. Changes in small RNA poly-uridylation have also been observed, which could affect small RNA stability or sorting into distinct Argonaute proteins, ultimately altering RNA silencing efficacy (van Wolfswinkel et al. 2009; Xu et al. 2018; Lev et al. 2019). Some of the aberrantly targeted genes include genes required for RNAi. For example, *rde-4* mRNA expression is reduced when P granules are dispersed in *deps-1* mutants (Spike et al. 2008a). In addition, *sid-1* and *rde-11* have increased small RNAs and reduced mRNA expression when embryonic P granules are dispersed in *meg-3*; *meg-4* double mutants, presumably reducing RNAi's efficacy in this mutant (Ouyang et al. 2019; Dodson and Kennedy 2019). Curiously, the misexpressed small RNAs tend to be very consistent between replicates and experiments, leading to the question of why specific genes are mistargeted and if certain sequences or features drive aberrant small RNA targeting following the loss of germ granules. One clue comes from the fact that mutants in *prg-1*, as well as transgenerational RNA silencing mutants *hrde-1* and *znfx-1*, can restore RNAi competence to *meg-3*; *meg-4* mutants (Ouyang et al. 2019). Both *sid-1* and *rde-11* appear to be direct targets of PRG-1, as their siRNA levels are reduced and their mRNA expression increased in a *prg-1* mutant (McMurchy et al. 2017; Shen et al. 2018; Ouyang et al. 2019). This comes as a surprise, as these genes are expressed in wild-type animals and required for an effective response to exogenous RNAi; however, the findings invoke a “safe harbor” model where P granules can protect some transcripts from transgenerational piRNA-mediated silencing (Ouyang et al. 2019).

While it is generally understood that piRNAs promote siRNA-mediated gene silencing, it may not be that simple. For example, PRG-1 is required maternally to coordinate siRNA biogenesis and prevent siRNA-mediated silencing of essential genes (de

Albuquerque et al. 2015; Phillips et al. 2015). In fact, following the loss of PRG-1 and piRNAs, siRNA-mediated silencing of the rDNA locus directly contributes to transgenerational sterility (Wahba et al. 2021), while siRNA-mediated silencing at histone genes can become permanent (Barucci et al. 2020; Reed et al. 2020; Shukla et al. 2021). While the signal that predisposes some genes to silencing in *prg-1* mutants is unknown, it has been hypothesized that the lack of a poly(A) tail common in histone transcripts and rRNAs (Montgomery et al. 2020; Reed et al. 2020), or the susceptibility of transcripts to perpetual poly-UG (pUG) RNA/siRNA cycling (Shukla et al. 2020, 2021; Wahba et al. 2021), could make specific genes more permissive for siRNA biogenesis. Like the “safe harbor” model above, PRG-1 targeted transcripts may be protected from permanent RDE-3-mediated siRNA silencing of *Mutator* foci if they are sequestered within P granules and away from RDE-3 silencing in *Mutator* foci (Shukla et al. 2021). As these details are worked out, the roles of germ-granule demixing are sure to become more apparent.

Outlook and outstanding questions

The occurrence of germ-granule demixing into distinct sub-granules leaves a number of questions unanswered. To start with, what are the events in germline blastomeres that trigger demixing? Is demixing a passive phenomenon caused by the localized synthesis of particular small RNAs, the clustering of similar RNA modifications and their binding proteins, or the condensation of phases similar to those that partition nucleolar sub-compartments (Feric et al. 2016; Lafontaine et al. 2021)? Instead, is demixing an active process that requires energy metabolism and the cytoskeleton? Is there directionality or progression from the emergence of one sub-granule to the next? Then once formed, how is the separation of these compartments maintained?

Determining what constitutes a P granule, Z granule, *Mutator* focus, or SIMR focus, their upper and lower size limits, and how each are defined is left to an investigator's discretion. This requires us to ask whether distinct compositions of germ-granule compartments exist and whether this indicates functional specialization. At any given time are the RNA and protein constituents of the individual sub-granules the same, and if not, does the association, loss, or exchange of RNAs and proteins constitute a new compartment with distinct functions? If the answer is yes, it will be important to understand the extent that proteins are shared across sub-granules, and if proteins moving between compartments are trafficking RNAs or other substrates from one location to the next. If so, what then is the trajectory of RNAs between sub-granules? Does this trajectory reflect changing functions for these RNAs, or an assembly line for their progressive modifications? And then, finally, how are these activities coordinated to ensure germline integrity? The answer to these and other questions should be attainable with further study and the increasing availability of new tools and improved imaging resolution.

Data availability

The authors state that all data necessary for confirming the conclusions presented in the manuscript are represented fully within the manuscript.

Conflicts of interest

The authors declare that there is no conflict of interest. Conclusions, implications and opinions expressed within the content are solely the authors.

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