Perspective

Germ Granules Allow Transmission of Small RNA-Based Parental Responses in the ''Germ Plasm''

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SUMMARY

In the recent decade small RNA-based inheritance has been implicated in a variety of transmitted physiological responses to the environment. In *Caenorhabditis elegans*, heritable small RNAs rely on RNA-dependent RNA polymerases, RNA-processing machinery, chromatin modifiers, and argonauts for their biogenesis and gene-regulatory effects. Importantly, many of these factors reside in evolutionary conserved germ granules that are required for maintaining germ cell identity and gene expression. Recent literature demonstrated that transient disturbance to the stability of the germ granules leads to changes in the pools of heritable small RNAs and the physiology of the progeny. In this piece, we discuss the heritable consequences of transient destabilization of germ granules and elaborate on the various small RNA-related processes that act in the germ granules. We further propose that germ granules may serve as environment sensors that translate environmental changes to inheritable small RNA-based responses.

INTRODUCTION

More than a century ago August Weismann postulated that acquired traits are not inherited, and that "germ plasm," a *nuclear* substance, is passed from parental germ cells to the progeny's germ cells to ensure the heredity of the species qualities (Weismann, 1893). Weismann's followers "evolved" the "germ plasm" term and instead used it to describe maternally contributed *cytoplasmic* substance, consisting of germ granules, that is required for the formation of germ cells in the next generation (Eddy, 1976; Hegner, 1914; Ritter, 1890). Ironically, recent studies demonstrate that these germ granules play a crucial role in the inheritance of regulatory small RNAs that can defy Weismann's law and allow acquired traits to be inherited. In the recent decade regulatory small RNAs were implicated in the transmission of various phenotypes in worms (Ewe et al., 2020; Gammon et al., 2017; Moore et al., 2019; Rechavi et al, 2011, 2014), flies (Casier et al., 2019; Hermant et al., 2015), plants (Ito et al, 2011, 2016; Liu et al., 2019; Zhong et al., 2013), and mice (Chen et al., 2016; Conine et al., 2018; Gapp et al., 2014; Grandjean et al., 2015; Rodgers et al., 2015; Zhang et al., 2018b). Notably, transgenerationally heritable small RNAs seem to differ from DNA-based genetics: their inheritance obeys a different set of rules (Houri-Ze'evi et al., 2016; Houri-Zeevi et al., 2020), their biogenesis can be induced in response to the environment (Ni et al., 2016; Rechavi et al, 2011, 2014), and their cellular pools change across generations in a continuous (rather than discrete) manner that seems to dilute (Vastenhouw et al., 2003; Alcazar et al., 2008; Houri-Ze'evi et al., 2016) or concentrate (Dodson and Kennedy, 2019; Houri-Ze'evi et al., 2016; Lev et al., 2017; 2019b; Ouyang et al., 2019; Simon et al., 2014). This review covers the role of the germ granules in regulating transgenerational small RNA responses and focuses on knowledge gained from studies in the nematode *Caenorhabditis elegans,* which plays an instrumental role (Perez and Lehner, 2019) in uncovering the principles and rules of transgenerational epigenetic inheritance.

In *C. elegans* exposure to synthetic exogenous double-stranded RNA leads to the production of small RNAs (Aoki et al., 2007; Pak and Fire, 2007; Vasale et al., 2010). These small RNAs silence the complementary gene and are inherited to the progeny (Buckley et al., 2012; Burton et al., 2011; Fire et al., 1998; Vastenhouw et al., 2006). Likewise propagation of RNA viruses leads to the production of antiviral small RNAs that silence the viral genes across multiple generations and protects the progeny (Gammon et al., 2017; Rechavi et al., 2011). Beyond the two examples above, heritable small RNAs were implicated in a variety of inherited responses to different environmental conditions. Inheritance of small RNAs results, for example, in enhanced longevity in the progeny of starved worms (Rechavi et al., 2014), altered endoderm

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development following parental diapause (Ewe et al., 2020), competent chemotactic behavior (Posner and Toker, 2019), inherited bacterial avoidance (Kaletsky et al., 2020; Moore et al., 2019; Palominos et al., 2017; Pereira et al., 2020), transgenerational loss of fertility in specific mutants (Mortal germline phenotype) (Bar-ucci et al., 2020; Buckley et al., 2012; Lev et al., 2017; Simon et al., 2014), and heritable gene-expression re-sponses to temperature changes (Ni et al., 2016; Schott et al., 2014).

The transgenerational inheritance of small RNAs in the germline relies on small RNA-binding argonauts (Buckley et al., 2012; Luteijn et al., 2012; Shirayama et al., 2012), RNA-dependent RNA polymerases (RdRPs) (Rechavi et al., 2011), additional biogenesis factors (Grishok et al., 2000), and in some cases (but not always) also chromatin mod-ifiers (Ashe et al., 2012; Kalinava et al, 2017, 2018; Lev et al, 2017, 2019a; Mao et al., 2015; Shirayama et al., 2012). The argonaute proteins act as effectors guided by the bound small RNAs to regulate the expression of the tar-geted gene. The RdRPs use the gene's mRNA as template to produce additional "secondary" small interfering RNAs (siRNAs) in every generation to maintain the response. This inheritance requires nuclear RNAi factors (*nrde* genes) (Buckley et al., 2012; Burton et al., 2011; Mao et al., 2015). However, many of the factors involved, including multiple argonauts and the RdRPs, reside in cytoplasmic germ granules (see Table 1).

Germ granules are cytoplasmic RNA-rich membrane-less condensates found in the germline (Eddy, 1976; Krieg et al., 1978; Wolf et al., 1983). Germ granules are evolutionary conserved and have been described in dozens of organisms, including worms, flies, and mice (Eddy, 1976). In *C. elegans,* the best studied class of germ granules are the P granules. The P granules are transmitted from the mother's germline to the embryo and segregate together with the P lineage, which ultimately forms the adult germline in the progeny (Hird et al., 1996). In contrast to the germ granules of *Drosophila melanogaster,* the P granules in *C. elegans* are not required for *specification* of germ cell identity (Gallo et al., 2010). Instead, the P granules are required for *maintaining* germline identity and proper germline gene-expression patterns. The proteins PGL-1 and PGL-3 are required for germ granule nucleation (Hanazawa et al., 2011; Kawasaki et al., 2004; Updike et al., 2011) while the GLH1 and GLH-4 proteins are promoting the anchoring of germ granules to nuclear periph-ery (Updike et al., 2011). Simultaneous knockdown of these four key germ granule components leads to ectopic expression of sperm genes in the germline (Campbell and Updike, 2015), and in later adulthood, to additional ectopic expression of somatic transcripts (Knutson et al., 2017; Updike et al., 2014). How germ granules maintain germline gene expression is an ongoing field of research; however, recent studies shed new light on these mechanisms (see Box 1 for outstanding questions).

GERM GRANULES SURVEY MRNAS AS THEY LEAVE THE NUCLEAR PORES

In the cytoplasm, the germ granules are strategically located adjacent to the nuclear pores, where they survey mRNAs as they are released into the cytoplasm. In agreement with this model, studies using fluorescence *in situ* hybridization (FISH) demonstrated that mRNAs transiently move through the germ granules when exiting the nuclear pore (Schisa et al., 2001; Sheth et al., 2010). Although the germ granules are largely perinuclear, they transiently lose their perinuclear localization during specific developmental stages. For example, in mature oocytes the germ granules are released into the cytosol and after fertilization migrate to the posterior part of the zygote to segregate with the germline blastomeres (Strome and Wood, 1983). Later during embryonic develop-ment, toward the 24-cell stage, the germ granules regain their perinuclear localization (Strome and Wood, 1983).

Germ granules can be subclassified into more specific entities of physically adjacent condensates, some of which stereotypically divide into separate entities at defined developmental stages (Wan et al., 2018). So far three classes of germ granules have been identified: the P (Hird et al., 1996), Z (Wan et al., 2018), and M (or Mutator) (Phillips et al., 2012) granules. Each subclass harbors different small RNA-related proteins. For example, the RdRP EGO-1 is localized in the P granules, whereas the argonaute WAGO-4 is localized in the Z granules (Claycomb et al., 2009; Wan et al., 2018) (for a comprehensive list see Table 1). The P and the Z granules separate to form two distinct condensates specifically in the primordial germ cells in the em-bryo and also later during the pachytene stage of the adult germline (Wan et al., 2018). This separation leads to the formation of a tri-condensate a "PZM" structure, where the Z granule is hypothesized to bridge between the P and M granules. Accordingly, the PZM assemblage's structure was hypothesized to play a role in organizing and coordinating small RNA pathways (Wan et al., 2018).

GERM GRANULES REGULATE GENE EXPRESSION VIA THE SMALL RNA MACHINERY

Initial genetic studies showed that mutants in structural germ granule proteins such as *pgl-1* (Robert et al., 2005; Spike et al., 2008b) and *deps*-1 (Houri-Ze'evi et al., 2016; Spike et al., 2008b) and later also *glh-1* (Spracklin et al.,

Protein Name(s)	Function	Germ Granule Localization	Temperature-dependent Fertility Defects
WAGO-1	Argonaute	P granules (Gu et al., 2009)	Unknown
CSR-1	Argonaute	P granules (Claycomb et al., 2009) and Z granules (Charlesworth et al., 2020)	General sterility in hermaphrodites (Yigit et al., 2006), temperature-sensitive sterility in males (Conine et al., 2013)
WAGO-4	Argonaute	Z granules (Wan et al., 2018)	Yes (Wan et al., 2018; Xu et al., 2018)
PRG-1	Argonaute	P granules (Batista et al., 2008; Wang and Reinke, 2008)	Yes (Batista et al., 2008; Simon et al., 2014; Yigit et al., 2006)
ALG-3/4	Argonautes	P granules (Conine et al., 2010)	Yes (Conine et al., 2010)
ALG-5	Argonaute (microRNAs)	P granules (Brown et al., 2017)	General (not temperature-dependent) defective fertility (Brown et al., 2017)
EGO-1	RdRP, small RNA biogenesis	P granules (Claycomb et al., 2009)	General sterility (Qiao et al., 1995; Smardon et al., 2000)
DRH-3	Helicase, small RNA biogenesis	P granules (Claycomb et al., 2009)	Yes (Claycomb et al., 2009; Nakamura et al., 2007)
RRF-1	RdRP, small RNA biogenesis	M granules (Phillips et al., 2012)	Unknown
DCR-1	Ribonuclease, small RNA biogenesis	Interacting with P granules (Barucci et al., 2020; Beshore et al., 2011)	Yes (Pavelec et al., 2009; Welker et al., 2010)
RDE-3(MUT-2)	Poly-UG polymerase, small RNA biogenesis	M granules (Phillips et al., 2012)	Yes (Chen et al., 2005)
RDE-12	Small RNA biogenesis	P granules (Shirayama et al., 2014)	Unknown
CDE-1(CID-1 or PUP-1)	Poly(A) polymerase, implicated in sorting of small RNAs	P granules (van Wolfswinkel et al., 2009)	Yes (Spracklin et al., 2017)
ZNFX-1	Small RNA biogenesis	Z granules (Ishidate et al., 2018; Wan et al., 2018)	Yes (Wan et al., 2018)
RDE-8	Small RNA biogenesis, mRNA cleavage.	M granules (Tsai et al., 2015)	Unknown
MUT-16, MUT-15, MUT-8 (RDE-2)	Small RNA biogenesis.	M granules (Phillips et al., 2012)	Yes (Vastenhouw et al., 2003)
MUT-14	RNA helicase, small RNA biogenesis	M granules (Phillips et al., 2012)	Yes (Vastenhouw et al., 2003)
MUT-7	Exonuclease, small RNA biogenesis	M granules (Phillips et al., 2012)	Yes (Ketting et al., 1999)

Table 1. Germ Granule-Localized Small RNA Factors

> **Box 1. Outstanding Questions:**
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> 1. What is the role of the organization of small RNA factors in the different sub-classes of germ granule?
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> 2. How the developmental changes in germ granule composition affect small RNA biology?
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> 3. What is the role of germ granules in sorting of amplified siRNAs to their partner argonauts?
>
> 4. Which environmental cues impact germ granule structure and composition *in vivo*, and how?

2017) and *meg-3;4* (Wan et al., 2018) have defective small RNA-mediated RNAi responses. Conversely, mutating or knocking down small RNA-related factors such as the argonaute CSR-1, the RdRP EGO-1, and the helicase DRH-3 disrupts germ granule structures (Claycomb et al., 2009; Updike and Strome, 2009; Vought et al., 2005). In addition, mutants in many small RNA-related factors exhibit fertility defects that are commonly temperature sensitive (see Table 1), similar to mutants in germ granule proteins. For example, the M granule protein MUT-16 is required for biogenesis of endogenous (Mutator-class) small RNAs (Phillips et al., 2012). When grown at elevated temperatures, *mut-16* mutants exhibit reduced fertility and ectopic expression of somatic and sperm genes in the germline, thus phenocopying P granule mutants and the outcome of P granule knockdown (Campbell and Updike, 2015; Kawasaki et al., 1998, 2004; Knutson et al., 2017; Rogers and Phillips, 2020a; Spike et al., 2008a; Updike et al., 2014). Similarly, knockdown of the argonaute CSR-1 results in ectopic expression of sperm genes (Campbell and Updike, 2015). Overall, evidence from co-localization and genetic analyses strongly suggest a mechanistic link between small RNAs and germ granules. The presence of the ribonuclease RDE-8 in the M granules also supports this idea. This ribonuclease was shown to be recruited by the small RNA machinery and to be required for the formation of cleaved RNA fragments and small RNAs from the targeted mRNA (Tsai et al., 2015). A recent study provided further evidence for germ granule-mediated regulation of gene expression, which is mediated by the recruitment of the small RNA machinery. In this study, artificial tethering of a germline expressed transcript to the P granules resulted in robust silencing of the transcript (Aoki et al., 2018). Importantly, silencing was dependent on both the integrity of the P granules and the P granule argonaute WAGO-1. Altogether, the mechanism of germ granules' regulation of gene expression seems to be, at least in part, mediated by the small RNA machinery (summarized in Figure 1).

AMPLIFICATION OF SMALL RNAS IN GERM GRANULES

Different primary small RNA pathways such as dsRNA-derived small RNAs, ERGO-1-associated small RNAs, and piRNAs converge by inducing the production of similar secondary siRNAs (reviewed by Houri-Zeevi and Rechavi, 2016). These secondary siRNAs are ~22 base pair long with a strong 5' guanine (22G) bias (Gu et al., 2009; Pak and Fire, 2007; Aoki et al., 2007). The secondary siRNAs are antisense to the mature mRNA, covering mainly the exons. The amplification step, which is central to siRNA-mediated silencing and siRNA inheritance seems to take place in the germ granules. The RdRPs that produce the amplified siRNAs, EGO-1 and RRF-1, are localized in the germ granules (Claycomb et al., 2009; Phillips et al., 2012). In addition, mutants lacking core germ granule components misexpress different populations of amplified siRNAs (Dodson and Kennedy, 2019; Lev et al., 2019b; Ouyang et al., 2019; Suen et al., 2020) (discussed further below). Additionally, a Z granule component, the helicase-domain protein ZNFX-1, contributes to the alignment pattern of amplified small RNAs aligning along the targeted gene. In its absence, siRNAs are produced preferentially from the 5' of the transcript (Ishidate et al., 2018). A recent study showed that the amplification step requires the presence of germ granule-localized RNA fragments with untemplated UG tails (19–75 bp long) (Shukla et al., 2020). This study demonstrated that targeting of mRNAs by silencing siRNAs leads to the production of poly-UG-tagged RNA fragments that derive from the targeted mRNAs. The poly-UG fragments then facilitate the production of additional amplified siRNAs. Together the poly-UG RNAs and amplified siRNAs form a feedforward loop that maintains the production of siRNAs and the silencing of the targeted gene. Both RDE-3, a poly-UG ribo-nucleotidyl-transferase, and the poly-UG RNAs are localized in the germ granules (specifically the M granules) (Shukla et al., 2020). In conclusion, accumulating evidence shows that germ granules serve as centers of small RNA amplification (summarized in Figure 1).

SORTING OF SMALL RNAS IN GERM GRANULES

Another vital step in small RNA-mediated regulation of gene expression is their assignment to different argonauts. It is generally thought that the identity of the binding argonaute determines, to some extent,
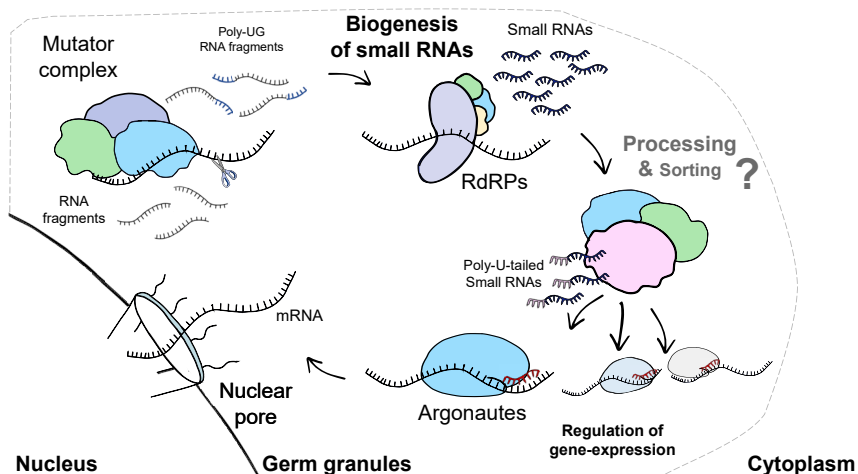
Figure 1. Conceptual Scheme Depicting the Different Small RNA-Related Processes that Occur Inside the Germ Granules

When exiting the nucleus, the mRNA molecule passes through the germ granule. Guided by small RNA molecules different argonauts regulate the expression of the mRNA. RNA-dependent-RNA polymerases (RdRPs) amplify and maintain the small RNA response across generations by creating additional small RNAs. The amplification process involves a feedback between poly-UG RNA fragments derived from the targeted mRNA and small RNA production. The small RNAs are then processed and sorted to the different argonaute proteins. The sorting process is hypothesized to involve untemplated poly-urydilation of the small RNAs, which direct the small RNAs to specific argonauts.

the effect of the amplified small RNA on its targeted mRNA. Despite their structural similarity (same length and 5' nucleotide bias), the different amplified 22G siRNAs are bound by different argonauts (such as WAGO-1, WAGO-4, CSR-1, and HRDE-1). How the amplified siRNAs find their correct partner argonaute is still unclear. Untemplated poly-urydilation of siRNAs is thought to be involved in this process. The siRNAs that bind with the CSR-1 argonaute (specifically the CSR-1b isoform, Charlesworth et al., 2020; van Wolfswinkel et al., 2009), tend to have longer untemplated poly-U tails when compared with the siRNAs that bind with the WAGO-1 and HRDE-1 argonauts (de Albuquerque et al., 2015; Houri-Ze'evi et al., 2016; Lev et al., 2019b; Phillips et al., 2015; van Wolfswinkel et al., 2009). Poly-uridylation of siRNAs is partially dependent on CDE-1, a germ granule-localized poly uracil polymerase (van Wolfswinkel et al., 2009; Xu et al., 2018). Accordingly, it was hypothesized that CDE-1-dependent uridylation targets the modified siRNAs for loading to specific argonauts such as CSR-1 (van Wolfswinkel et al., 2009). WAGO-4-bound siRNAs are also poly-urydilated (Xu et al., 2018). In addition, the binding of WAGO-4 to these siRNAs is defective in *cde-1* mutants (Xu et al., 2018). RNAi inheritance is defective in both *cde-1* and *wago-4* mutants (Lev et al., 2019b; Spracklin et al., 2017; Wan et al., 2018; Xu et al., 2018). Altogether, these results lead the authors to suggest that CDE-1-dependent uridylation of small RNAs promotes the transition of siRNAs from the CSR-1 argonaute to WAGO-4 to facilitate their inheritance (Xu et al., 2018). The recent finding that like WAGO-4, the CSR-1b argonaute localizes to the Z germ granule is in accordance with this view (Charlesworth et al., 2020; Wan et al., 2018). The argonaute HRDE-1 is crucial for RNAi inheritance (Ashe et al., 2012; Buckley et al., 2012; Kalinava et al., 2017; Lev et al., 2017; Luteijn et al., 2012; Pak et al., 2012; Shirayama et al., 2012). A recent study showed that mutants of core germ granule factors MEG-3/4 exhibit ectopic uridylation of HRDE-1-class siRNAs and unexpected *HRDE-1-independent* inheritance of RNAi (Lev et al., 2019b). These findings further support the idea that germ granules play a role in sorting of small RNAs to specific pathways. Although poly-uridylation of siRNAs seem to distinguish between siRNA associated with different argonauts, it is still unclear whether it plays a role in their sorting. For example, it is possible that the poly-uridylation occurs only after the loading of the siRNA to the argonaute. Future studies will clarify the roles of poly-uridylation in sorting of siRNAs, and how this process is regulated by the germ granules (see Box 1 for outstanding questions).

GERM GRANULES REGULATE THE INHERITANCE OF SMALL RNAS

Initial studies reported that worms with disrupted germ granule structures (defective in their organization or morphology) cannot initiate RNAi-derived siRNA responses (Robert et al., 2005; Spike et al., 2008b). The presence of key siRNA inheritance factors in the germ granules and their intricate organization further

suggested that the structure of the germ granule structures will be required for inheritance of small RNAs. Recent studies on fertile mutants defective in germ granule structures revealed a more complicated picture. The proteins PGL-1, MEG-3 and MEG-4 are core constituents of germ granules (Kawasaki et al., 1998; Wang et al., 2014). The MEG-3 and MEG-4 proteins function redundantly to scaffold the germ granules (Wang et al., 2014). The stability of the germ granules is further regulated by the PP2A phosphatase and its regulatory subunit PPTR-1 (Gallo et al., 2010) (reviewed in detail by Seydoux, 2018). Accordingly *meg-3;meg-4* and *pptr-1* mutants have small germ granules that do not segregate well with the germ cell lineage in the embryo (Gallo et al., 2010; Wang et al., 2014). Post embryogenesis, however, the germ granules are normally formed in *meg-3;meg-4* mutants (Wang et al., 2014). Despite having largely disrupted embryonic germ granules, these mutants were recently found to display potent small RNA inheritance (Lev et al., 2019b). Moreover, *pptr-1* and *meg-3;meg-4* exhibit enhanced transgenerational RNAi-mediated silencing that lasts for tens of generations (Lev et al., 2019b). Therefore, the integrity of the germ granules in the embryo is not essential for the inheritance of RNAi-derived small RNAs. Instead, the structure of the germ granules seems to play a role in limiting the duration of the heritable response.

Homozygous mutants lacking functional *meg-3;meg-4* or *pgl-1* genes gradually lose their ability to initiate RNAi responses across generations (Dodson and Kennedy, 2019; Lev et al., 2019b; Ouyang et al., 2019). This explains why previous studies found *pgl-1* and *meg-3;meg-4* mutants to have defective RNAi responses (Robert et al., 2005; Spike et al., 2008b; Wan et al., 2018). Further investigations showed that in the absence of *meg-3;meg-4*, ectopic siRNAs are transmitted to the progeny (Dodson and Kennedy, 2019; Lev et al., 2019b). These siRNAs accumulate across generations and ultimately render the mutants refractive to RNAi (Dodson and Kennedy, 2019; Lev et al., 2019b; Ouyang et al., 2019). Removal and subsequent rescue of the HRDE-1 argonaute "resets" the ability of the *meg-3;4* mutants to initiate an RNAi response (Lev et al., 2019b), showing that HRDE-1-dependent siRNAs are the heritable agents that mediate this transgenerational phenomenon. Among the accumulating ectopic siRNAs in the *meg-3;meg-4* mutants are siRNAs that target the *sid-1* and *rde-11* genes (Dodson and Kennedy, 2019; Lev et al., 2019b; Ouyang et al., 2019). These genes encode for proteins required for the RNAi responses, and specifically for the inter-tissue spreading and amplification steps of RNAi (Feinberg and Hunter, 2003; Yang et al., 2012; Zhang et al., 2012). These siRNAs silence the *sid-1* and *rde-11* mRNAs in *meg-3;meg-4* mutants, and their inheritance is dependent on HRDE-1 (Dodson and Kennedy, 2019; Ouyang et al., 2019). Elegant RNA-FISH experiments revealed that the germ granules encapsulate the *sid-1* and *rde-11* mRNAs and that in *meg-3;meg-4* mutants this compartmentalization is impaired (Ouyang et al., 2019). The *sid-1* and *rde-11* genes are regularly targeted by piRNAs, but in the wild type this targeting does not lead to the production of amplified siRNAs and silencing of these genes. The authors suggested that the germ granules protect the mRNAs of *sid-1* and *rde-11* from piRNA targeting (Ouyang et al., 2019). Altogether, heritable siRNA-mediated silencing of the *sid-1* and *rde-11* accounts for the observed heritable RNAi defects found in the *meg-3;meg-4* mutants. Notably, this mechanism cannot explain the enhanced transgenerational inheritance of RNAi responses of *pptr-1* mutants and early generation (following homozygosity) of *meg-3;meg-4* mutants. Instead, the genome-wide disruption of competing heritable small RNA species found in these mutants seems to contribute to these inheritance dynamics (Lev et al., 2019b). For example, *pptr-1* mutants exhibit a reduction in the total amounts of endogenous siRNAs (endo-siRNAs, Lev et al., 2019b). The endo-siRNAs compete over shared machinery (such as RdRPs) with exogenous dsRNA-derived siRNAs (exo-siRNAs). Therefore, in the absence of endo-siRNAs, exogenous siRNA production is potentiated. Indeed, *pptr-1* mutants were shown to synthesize higher levels of exo-siRNAs (Lev et al., 2019b). Similar mechanism was described for other conditions (or mutants) where worms exhibit an enhanced RNAi phenotype (Duchaine et al., 2006; Houri-Ze'evi et al., 2016; Lev et al., 2017). Recently an *eri-6/7*-based feedback between MUT-16 and ERGO-1 small RNA pathways (Rogers and Phillips, 2020b) was described. It is possible that similar feedback mechanism between different small RNA pathways may also be involved in these inheritance patterns. In summary, recent studies revealed that germ granules maintain proper endogenous small RNA production and regulate the animal's capacity to produce and inherit RNAi-derived siRNAs.

HERITABLE SMALL RNAS MAINTAIN THE EFFECTS OF GERM GRANULE DISRUPTION ACROSS GENERATIONS

As germ granules regulate heritable small RNAs, even transient changes to the germ granule assembly or composition can have transgenerational consequences. In accordance with this idea, wild-type progenies that are isolated from a cross with *meg-3;meg-4* mutants exhibit defects in both initiation and inheritance of RNAi responses (Dodson and Kennedy, 2019; Lev et al., 2019b). These defects are maintained in the wild-type

lineage for multiple generations, and their inheritance depends on the argonaute HRDE-1 (Dodson and Kennedy, 2019; Lev et al., 2019b). This heritable phenotype was accompanied by inheritance of many HRDE-1-dependent endogenous siRNAs to the wild-type progeny (Dodson and Kennedy, 2019; Lev et al., 2019b). Recently, heritable phenotypes have also been described for mutants of the germ granule-residing argonauts ERGO-1 and PRG-1. In both cases, the heritable phenotypes are mediated by small RNAs. The F1 wild-type progeny of mutants of the P granule argonaute ERGO-1 are more sensitive to RNAi (Almeida et al., 2019). Similarly, wild-type progeny of mutants in the P granules-residing argonaute PRG-1 have reduced fertility for multiple generations (Barucci et al., 2020). Importantly, other examined germ granule components were unaltered in *prg-1* mutants. Nevertheless, both studies highlight that transient changes to germ granule-residing small RNA factors can lead to heritable phenotypes. In conclusion, changes to germ granule integrity or composition can affect the physiology of ensuing generations via their effect on regulatory small RNAs.

Interestingly, the *meg-3;meg-4*-defective RNAi initiation phenotype is transmitted more efficiently through the maternal lineage, as wild-type progeny of *meg-3;meg-4* males have less severe RNAi defects than progeny of *meg-3;meg-4* hermaphrodites (Lev et al., 2019b). This maternal effect is not due to inheritance of the defective granules (Lev et al., 2019b). Intriguingly, the earliest report on the M granule protein MUT-7, which plays a key role in small RNA biogenesis, described a similar phenomenon (Ketting et al., 1999). Heterozygotus *mut-7* (+/−) mutants had less severe RNAi defects if they were segregated from a cross with a mutant male (Ketting et al., 1999). Recently, a similar maternal parent-of-origin effect was reported for small RNA-based transgenerational inheritance of an endoderm development phenotype (Ewe et al., 2020). Further research would reveal if this is a general theme for small RNA-based heritable responses.

DO GERM GRANULES MAKE HERITABLE SMALL RNAS SENSITIVE TO THE ENVIRONMENT?

As discussed above, various environmental challenges induce heritable physiological responses. Heritable small RNAs have been implicated in the inheritance of some of these effects. It is, however, still unclear how abiotic environmental changes induce the production of specific small RNA species. The ability of germ granules to affect heritable small RNA populations mark them as a likely candidate mediator (Dodson and Kennedy, 2019; Lev et al., 2019b; Ouyang et al., 2019). Germ granules possess liquid-like properties such as fusion, dripping, and wetting (Brangwynne et al., 2009). As biophysical entities, the P and M germ granules are sensitive to temperature and tend to disperse as temperatures rise (Putnam et al., 2019; Uebel et al., 2018). Transient growth at higher temperatures elevates the expression of transgenes and repetitive elements (Klosin et al., 2017). These gene-expression changes are inherited for multiple generations. Growth at high temperatures also interferes with piRNA production (Belicard et al., 2018) and temperature shifts impact heritable small RNAs and the expression of some of their endogenous targets (Manage et al., 2020; Ni et al., 2016; Schott et al., 2014). As discussed above, mutants in structural germ granule components and germ granule-residing small RNA factors exhibit temperature-dependent sterility. It is therefore tempting to speculate that the intrinsic biophysical sensitivity of germ granules to temperature allows them to translate transient temperature shifts into changes in the pools of heritable small RNAs. As germ granules are highly conserved, it is possible that they will play a similar role in other organisms. Future studies will uncover the involvement of germ granules in rendering small RNAs responsive to the environment.

AUTHOR CONTRIBUTIONS

I.L and O.R. conceptualized and wrote the paper.

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