**Mutator Foci Are Regulated by Developmental Stage, RNA, and the Germline Cell Cycle in Caenorhabditis elegans**

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**ABSTRACT**

RNA interference (RNAi) is a crucial gene regulatory mechanism in *Caenorhabditis elegans*. Phase-separated perinuclear germline compartments called Mutator foci are a key element of RNAi, ensuring robust gene silencing and transgenerational epigenetic inheritance. Despite their importance, Mutator foci regulation is not well understood, and observations of Mutator foci have been largely limited to adult hermaphrodite germlines. Here we reveal that punctate Mutator foci arise in the progenitor germ cells of early embryos and persist throughout all larval stages. They are additionally present throughout the male germline and in the cytoplasm of post-meiotic spermatids, suggestive of a role in paternal epigenetic inheritance. In the adult germline, transcriptional inhibition results in a pachytene-specific loss of Mutator foci, indicating that Mutator foci are partially reliant on RNA for their stability. Finally, we demonstrate that Mutator foci intensity is modulated by the stage of the germline cell cycle and specifically, that Mutator foci are brightest and most robust in the mitotic cells, transition zone, and late pachytene of adult germlines. Thus, our data defines several new factors that modulate Mutator foci morphology which may ultimately have implications for efficacy of RNAi in certain cell stages or environments.

**KEYWORDS**

germ granules
siRNAs
Mutator foci
germline
*C. elegans*

RNA interference (RNAi) is an evolutionarily conserved strategy to ensure proper gene expression across a wide range of eukaryotes (Fire et al. 1998; Shabalina and Koonin 2008). The effectors of RNAi are members of the Argonaute protein family, which bind small regulatory RNAs ranging from 18-30 nucleotides in length. Together these components form the RNA Induced Silencing Complex (RISC), which targets fully or partially complementary transcripts of exogenous or endogenous origin through direct cleavage, recruitment of exonucleases, or repression of translational complexes (Hutvagner and Simard 2008; Wu and Belasco 2008). By these means, small RNAs play key roles in development, fertility, chromosome segregation, and defense against foreign genetic elements such as transposons and viruses.

In *C. elegans*, RNA silencing is mediated by a network of ~27 Argonaute proteins associated with three distinct classes of small RNAs: micro-RNAs (miRNAs), Piwi interacting-RNAs (piRNAs), and small interfering RNAs (siRNAs). *C. elegans* siRNAs are categorized in two distinct groups: primary siRNAs and secondary siRNAs. Primary siRNAs are cleaved by Dicer from double-stranded RNA and are bound by primary Argonaute proteins like RDE-1 and ERGO-1, whereas secondary siRNAs are produced from primary siRNA-targeted or piRNA-targeted templates by the RNA-dependent RNA polymerases (RdRPs), RRF-1 and EGO-1, and are bound by the worm-specific Argonaute (WAGO) clade of Argonaute proteins (Yigit et al. 2006; Aoki et al. 2007; Pak and Fire 2007; Sijen et al. 2007; Gu et al. 2009; Vasale et al. 2010). Secondary siRNAs are crucial for signal amplification and transgenerational silencing; mutant animals that fail to produce secondary siRNAs display multiple defects including failure to respond to RNAi, temperature-sensitive sterility, and transposon mobilization (Ketting et al. 1999; Tabara et al. 1999; Vastenhouw et al. 2003; Zhang et al. 2011).

Secondary siRNA amplification primarily occurs in the mutator complex, which forms perinuclear foci, known as Mutator foci, in...
C. elegans germ cells (Phillips et al. 2012). These Mutator foci are nucleated by MUT-16, which directly interacts with the RdRP RRF-1, and other key siRNA biogenesis proteins to assemble mutator complexes at these sites (Phillips et al. 2012; Uebel et al. 2018). The C-terminal region of MUT-16, which contains regions of intrinsic disorder, is both necessary and sufficient for Mutator foci formation. Furthermore, Mutator foci behave as phase-separated condensates, with liquid-like properties such as rapid recovery after photobleaching, temperature and concentration dependent condensation, and disruption upon perturbation of weak hydrophobic interactions (Uebel et al. 2018). Additionally, Mutator foci are adjacent to P granules, which are phase-separated mRNA surveillance centers important for maintenance of the germ cell fate and fertility (Pitt et al. 2000; Brangwynne et al. 2009; Sheth et al. 2010; Updike et al. 2014; Campbell and Updike 2015; Knutson et al. 2017). Because P granules also contain proteins associated with the small RNA pathways, including both primary and secondary Argonaute proteins (Wang and Reinke 2008; Claycomb et al. 2009; Gu et al. 2009; Conine et al. 2010), we hypothesize that P granules and Mutator foci interact at the nuclear periphery to coordinate small RNA silencing of nascent germline transcripts.

Though P granule regulation and morphology has been extensively studied in all stages of C. elegans development (Strome and Wood 1982; Updike and Strome 2009), investigation of Mutator foci is primarily limited to observations within the adult hermaphrodite germline (Phillips et al. 2012; Uebel et al. 2018). Here, we examine Mutator foci throughout embryonic, larval, male, and hermaphrodite germline development and begin to assess factors that regulate or influence Mutator foci morphology. We determine that Mutator foci first appear as bright, distinct puncta in the Z2/Z3 progenitor germ cells (PGCs), and that these foci persist throughout all subsequent developmental stages. We then demonstrate that Mutator foci are present in the male germline throughout spermatogenesis, and that MUT-16 is sequestered into the cytoplasm of post-meiotic spermatids. While probing regulatory mechanisms for Mutator foci, we discover that these phase-separated compartments are at least partially dependent on RNA for their stability in pachytene region of the gonad. Finally, we find that Mutator foci are largest and most robust in the mitotic cells, the transition zone, and the late pachytene of adult germlines. By RNAi of key germline development proteins, we discover that the mitotic cell stage, but not the transition zone, is a determinant of robust Mutator foci. Thus, through these observations, we better define Mutator foci in all developmental stages and probe the regulatory factors that influence morphology of this secondary siRNA amplification center.

MATERIALS AND METHODS

C. elegans Strains

Worms were grown at 20°C according to standard conditions (S. Brenner 1974). Strains used in this study include:

**USC1266** mut-16(cmp41[mut-16::mCherry::2xHA + loxP]) I; pgl-1(sam33[pgl-1::gfp::3xFLAG]) I V

**USC717** mut-16(cmp3[mut-16::gfp::3xFLAG + loxP]) I (Uebel et al. 2018)

**USC1385** mut-16(cmp3[mut-16::gfp::3xFLAG + loxP]) I; him-8(tm611) IV

USC1266 was created by crossing DUP75 (pgl-1(sam33[pgl-1::gfp::3xFLAG])) (Andralojc et al. 2017) and USC896 (mut-16(cmp41 [mut-16::mCherry::2xHA + loxP])] (Uebel et al. 2018; Nguyen and Phillips 2020). USC1385 was created by crossing USC217 (mut-16(cmp3[mut-16::gfp::3xFLAG + loxP])]) (Uebel et al. 2018) and CA257 (him-8(tm611)) (Phillips et al. 2005).

**Antibody staining and microscopy**

For fixed embryo imaging, gravid adult mut-16::mCherry::2xHA; pgl-1::gfp::3xFLAG animals were placed in 4 μL water on SuperFrost slides and burst to release embryos by application of a glass coverslip. Slides were placed on a dry-ice cooled aluminum block for freeze-crack permeabilization. After coverslip removal, slides were fixed in 100% ice-cold Methanol for 15 min and washed three times in 1xPBST for 5 min each. DAPI was added to the first wash. Embryos were mounted in 10 μL NPG-Glycerol mounting medium and imaged (Phillips et al. 2009). Embryos were staged by approximate cell counts and position of the Z2/Z3 PGCs. To minimize artifacts, no antibody staining was used, as the proteins of interest are fluorescently tagged at their endogenous loci. To avoid FRET activation and channel bleed-through, 20 Z-stacks were captured first with 542 nm (red), followed by 475 nm (green) and 390 nm (blue) excitation.

For live imaging, undissected larva, hermaphrodites, or adult males were mounted in M9 containing <1% sodium azide to inhibit movement or in M9 with no sodium azide, and images were collected first with 542 nm excitation followed by 475 nm laser excitation. Larval images were compiled from 10 Z-stacks and staged by gonad morphology. Male germline live images were compiled from 20 Z-stacks. Adult hermaphrodite germline live images were compiled from 5 Z-stacks.

For immunostained germlines, adult males or hermaphrodites were dissected in egg buffer containing 0.1% Tween-20 and fixed in 1% formaldehyde as described (Phillips et al. 2009). mut-16::GFP::3xFLAG; him-8(tm611) germlines were immunostained with 1:500 rabbit anti-GFP (Thermo Fisher A-11122), and all other germlines were stained with 1:2000 mouse anti-FLAG (Sigma F1804) and 1:250 rabbit anti-SYP-1 (MacQueen et al. 2002). Fluorescent Alexa-Fluor secondary antibodies were used at 1:1000 (Thermo Fisher). Immunostained germlines were compiled from 25 Z-stacks.

All imaging was performed on a DeltaVision Elite (GE Healthcare) microscope using a 60x N.A. 1.42 oil-immersion objective. For all images, 0.2 μm Z-stacks were compiled as maximum intensity projections and pseudo-colored using Adobe Photoshop. Image brightness and contrast were adjusted for clarity.

**Transcriptional Inhibition**

Anterior gonads of young adult (~24 hr post L4) hermaphrodites were microinjected with 200 μg/mL α-amanitin until the solution flowed around the germline bend, as previously described (Sheth et al. 2010; Uebel and Phillips 2019). A vehicle control injection of RNAse-free water did not disrupt foci, nor were foci disrupted in the uninjected posterior gonad. The observed results were reproducible despite slight variation in microinjected volume and gonad integrity. Due to injection and imaging time requirements, animals were imaged at three, five, and seven hours ±15 min post-injection. At least three animals were imaged for each time point.

**RNA interference**

For all RNAi experiments, sequence-confirmed RNAi clones gld-1, gld-2, clk-2, atx-2, ama-1 and L4440 (control) in HT115 (DE3) bacteria were grown overnight at 37°C to maximum density. Cultures were concentrated 10-fold and 100 μL was plated on NGM plates with 5 mM IPTG and 100 μg/mL Ampicillin. For gld-2 gld-1 double
RNAi, the concentrated cell cultures were combined at equal volume before plating. Plates were stored at room temperature for at least 24 hr to allow for IPTG induction. Synchronized L1 worms were then plated and grown at 20° for 70 hr before dissection and imaging, unless otherwise stated. A minimum of four gonads for each RNAi treatment were analyzed. RNAi experiments were performed twice to ensure consistent phenotypes.

**Quantification of Mutator foci intensity**

 Gonads were divided by length into 10 equal regions ending at the first single-file diplotene/ diakinesis nucleus. Raw TIFs were loaded into FIJI and backgrounds were subtracted with a 50-pixel rolling ball radius. Each image was then thresholded identically, creating a binary image displaying only above-threshold foci. Each region was processed using the “analyze particles” function and the resulting particle quantification data were graphed and analyzed in Excel.

**Data Availability**

All strains are available either at the Caenorhabditis Genetics Center (CGC) or upon request from the Phillips lab. The authors affirm that all data necessary for confirming the conclusions of the article are present within the article, figures, and tables. Supplemental material available at figshare: https://doi.org/10.25387/g3.12749888.

**RESULTS**

**Robust Mutator foci first appear in the Z2/Z3 progenitor germ cells in C. elegans embryos**

Current characterization of Mutator foci is largely restricted to observations within the adult germline, without consideration for earlier stages of development. *C. elegans* embryos undergo invariant cell divisions, the first of which gives rise to the asymmetric AB and P1 blastomeres. The P1 cell subsequently produces the P2, P3, and P4 cells, the latter of which divides around the 100-cell stage to form the Z2 and Z3 PGCs, which undergo no further divisions until hatching and feeding. After hatching, the PGCs eventually give rise to the ~2,000 cells that comprise the adult germline. Recent imaging by Ouyang et al. (2019) observes punctate Mutator foci in the PGCs of later comma-stage embryos (>550 cells), which describes the onset of elongation around 400 min post fertilization. To determine the earliest formation of Mutator foci, defined here as distinctly punctate MUT-16 fluorescence, we imaged endogenous MUT-16::mCherry and PGL-1::GFP in fixed embryos at representative developmental stages. Since MUT-16 is required for the localization of all known mutator complex proteins, we use MUT-16 foci as a proxy for Mutator foci, and use the terms interchangeably. At the 2-cell stage, P granules segregate to the cytoplasm of the P1 blastomere, while MUT-16 predominantly appears as diffuse cytoplasmic signal in both AB and P1 cells (Figure 1A and A'). At the 16-cell stage, P granules begin to associate with the nuclear periphery of the P4 cell (Updike and Strome 2010), yet MUT-16 remains diffusely cytoplasmic in all cells of the embryo (Figure 1B and B'). As the embryos reach the 30-cell stage, coinciding with the beginning of gastrulation (Sulston et al. 1983), MUT-16 continues to be expressed throughout the cytoplasm of all embryonic cells (Figure 1C), though we sometimes observe faint punctate Mutator foci adjacent to the large, perinuclear P granules (Figure 1C' and Figure S1). At this stage, any PGL-1 remaining in the somatic blastomeres is eliminated via autophagy, temporarily creating small PGL-1 foci throughout the embryo (Hird et al. 1996; Zhang et al. 2009), yet diffuse MUT-16 expression in the cytoplasm is unchanged. Interestingly, in addition to the cytoplasmic MUT-16 signal in the 100-cell stage, we consistently observe punctate Mutator foci surrounding the newly formed Z2 and Z3 PGCs (Figure 1D and D'). The distinct foci are adjacent to P granules and at the nuclear periphery, comparable to Mutator foci localization in adult germ cells. Since we observe foci in the 300-cell stage, and Ouyang et al. (2019) observes foci in the later comma stage, Mutator foci appear to persist throughout later stages of embryonic development (Figure 1E and E'). Thus, our imaging reveals that diffuse cytoplasmic MUT-16 is present in all embryonic cells at all stages of embryonic development, and punctate Mutator foci can form as early as the 30-cell stage, but are most consistent and robust in the Z2/Z3 PGCs.

**Mutator foci persist in all larval stages of C. elegans**

To expand on our characterization of Mutator foci throughout development, we examined MUT-16 and PGL-1 expression during all larval stages, including the “survival-state” dauer stage induced by starvation (Cassada and Russell 1975). In fed L1 larva, the PGCs are easily identified by perinuclear P granules and, despite intestinal autofluorescence, MUT-16::mCherry is clearly visible in punctate Mutator foci (Figure 2A). Mutator foci are present in both early and late L2 stages (Figure 2B-C). Additionally, Mutator foci are visible in the L2d dauer larva, whose germ lines are developmentally quiescent (Figure 2D). Finally, Mutator foci are present in the early L3 developmental stage, at which point two “arms” of the gonad begin to form and branch from the central somatic gonad primordium (Figure 2E). Similar to MUT-16 expression in adults, larval germ cells also show faint cytoplasmic expression and MUT-16 exclusion from the nucleus. Since Mutator foci have been observed previously in L4 and adult stages (Phillips et al. 2012), we can conclude that Mutator foci are present across all stages of larval development.

**Mutator foci localizes throughout spermatogenesis and MUT-16 is deposited in the cytoplasm of spermatids**

In addition to an emphasis on adult germlines, current studies of Mutator foci rely almost exclusively on hermaphrodite germlines for characterization. To fully characterize Mutator foci in male germ-lines, we live imaged mut-16::mCherry, pgl-1::gfp males. Mutator foci appear similar to hermaphrodite germlines throughout the mitotic tip, the transition zone, and the pachytene region (Figure 3A). However, we observe a divergence in the localization patterns of P granules and Mutator foci in spermatogenesis. Previous literature reports that PGL-1 and PGL-3 disassemble in late spermatogenesis, though the P granule component GLH-1 persists until eventual segregation into residual bodies of budding spermatids (Gruidl et al. 1996; Amiri et al. 2001; Updike and Strome 2010). While we observe PGL-1 disassembly in late spermatogenesis, we continue to see punctate Mutator foci around nuclei with no PGL-1 signal (Figure 3A). Mutator foci were previously observed to localize to the nuclear periphery despite lack of detectable PGL-1 via a glh-1/glh-4 RNAi knockdown (Phillips et al. 2012), and our findings support the independent localization of Mutator foci.

To visualize Mutator foci and corresponding nuclei more clearly throughout spermatogenesis, we dissected and fixed mut-16::gfp male germ-lines. Using DAPI-stained DNA as a guide for morphological identification of chromatin state, we observe punctate Mutator foci throughout the condensation zone and into the division zone (Figure 3B). Mutator foci are present around nuclei in the karyosome stage, a poorly understood state of nuclear compaction thought to promote chromosome organization prior to meiotic division (Shakes et al. 2009).
Because chromosomes are highly condensed in the karyosome stage and the nuclear envelope size remains unchanged, Mutator foci appear farther away from the DAPI-stained bodies. Additionally, during late diakinesis and metaphase, the nuclear envelope begins to break down, which may explain why some punctate Mutator foci in the division zone are no longer associated with DAPI-stained nuclei (Figure 3B).

Unexpectedly, we discovered MUT-16::GFP signal in the cytoplasm of post-meiotic spermatids in a unique granular pattern reminiscent of WAGO-1 localization in spermatids (Conine et al. 2010) (Figure 3C).

Figure 1  Distinct Mutator foci appear in the Z2/Z3 progenitor germ cells. (A-E) Distribution of PGL-1::GFP (green) and MUT-16::mCherry (red) in methanol-fixed embryos of representative stages. DNA is stained with DAPI (blue) for visualization. Scale bars, 15 μm. (A'-E') Enlarged insets of boxed areas from merged embryos in A-E. Small MUT-16 puncta are visible in the 30-cell stage (yellow arrowhead). Distinct MUT-16 foci are consistently visible in Z2/Z3 cells of both 100- and 300-cell embryos (white arrowheads). Scale bars, 1 μm.

Figure 2  Mutator foci are present in L1-L3 larval stages. (A-E) Live imaging of mut-16::mCherry; pgl-1::gfp expression in representative larval stages. PGL-1::GFP granules (green) associate with germ cells in the developing gonad in all larval stages. MUT-16::mCherry foci (red) are also present. Merged panels and enlarged insets (dashed region) show PGL-1 granules and MUT-16 foci interacting at the nuclear periphery. Merge scale bars, 5 μm. Inset scale bars, 1 μm.
Distinguishes diplotene nuclei, karyosome formation, the division zone, or Chromosome morphology is visualized by DAPI (blue) and distinct within the cytoplasm of post-meiotic spermatids. All scale bars, 5 μm.

The presence of MUT-16 in spermatids carries implications for paternal inheritance of Mutator components and paternal deposition of WAGO class 22G-RNAs, which have been shown to rescue fertility of piRNA-depleted RNAi defective hermaphrodites (Phillips et al. 2015).

**RNA influences Mutator foci stability and morphology**

Many phase-separated condensates are comprised of multivalent interactions between RNA and proteins, often referred to as ribonucleoprotein (RNP) granules. Concentration, secondary structure, and RNA length have been shown to regulate size, interaction, and formation of RNP granules both in vivo and in vitro (Langdon et al. 2018; Garcia-Jove Navarro et al. 2019). Previous work demonstrated that the presence of RNA is integral to sustaining P granule integrity in vivo (Sheth et al. 2010). Five hours after gonadal microinjection of a potent RNA Polymerase II transcriptional inhibitor, α-amanitin, P granules disappeared in a pachytene-specific manner. To determine if Mutator foci also require RNA for their stability, we microinjected 200 μg/mL α-amanitin into the gonads of adults expressing fluorescently-tagged MUT-16 and PGL-1. We found that both P granules and Mutator foci began to disperse by three hours, forming fewer foci in both the mid- and late-pachytene regions (Figure 4A and 4B). In late pachytene, P granules appeared rounder and more detached from the nuclear pore environment (Figure 4B). Mutator foci remain adjacent to P granules despite the overall reduction in foci number.

By five and seven hours post α-amanitin injection, P granules and Mutator foci are nearly completely dissolved in the mid-pachytene region (Figure 4A). In late pachytene, P granules and Mutator foci also dissipate with increasing severity (Figure 4B). Interestingly however, we consistently observe faint Mutator foci remaining at seven hours, despite no detectible PGL-1 foci. This result suggests that Mutator foci may only partially rely on RNA for stability.

To further explore the effect of mRNA loss on Mutator foci, we placed L4 animals on RNAi targeting ama-1, the largest subunit of RNA polymerase II, required for mRNA transcription (Bird and Riddle 1989). Phenotypes from some RNAi can be first observed after 24 hr of feeding (Kamath et al. 2001). We did not observe any noticeable perturbation of either P granules or Mutator foci at 24 hr on ama-1 RNAi, but after 30 hr, PGL-1 granules in mid pachytene were faint and fluorescent signal was largely cytoplasmic (Figure S2). Similarly, MUT-16 foci dissipate after 30 hr on ama-1 RNAi. Though it is possible that the inhibition of transcription resulted in reduction of PGL-1, MUT-16, or other major protein constituents of these granules, the dispersed cytoplasmic signal of PGL-1 and MUT-16 is more suggestive of foci loss due to lack of mRNA than due to lack of protein components. These data support our hypothesis that Mutator foci at least partially rely on RNA for foci integrity.

**Mutator foci intensity is dictated by stage of germ cell progression**

To examine other elements of Mutator foci regulation, we directed our attention to germ cell stage and morphology. The C. elegans germline is a syncytium of cells organized in two branching “arms” extending from a central somatic uterus. The distal tip of each arm contains actively dividing mitotic cells that progress through meiotic S phase before transitioning to the leptotene/zygotene stage of meiosis. In this transition zone, nuclei appear crescent-shaped as chromosomes polarize to find homologs, a trait that is readily apparent by DAPI staining. The transition zone is followed by early, mid, and late pachytene, where crossing over of homologous chromosomes occurs. Cells then progress proximally through the diplotene and diakinesis stages, where chromosomes condense and the nuclear envelope begins to break down (Figure S3A). By live and immunofluorescent imaging of endogenously tagged MUT-16, we noticed the early- and mid-pachytene regions of adult germlines contain dim Mutator foci, whereas the mitotic tip, transition zone, and late-pachytene regions have large, bright Mutator foci (Figure 5A). We refer to this quality of brightness as “Mutator foci intensity”. To quantify Mutator foci intensity, we divided each gonad into 10 equal sections and performed a granule count on images processed with a uniform threshold (Figure S3B). In this manner, dim foci fell below the threshold and only bright foci were counted, allowing us to...
determine the distribution of the brightest foci throughout the germline. To help delineate meiotic stage, we utilized a central component of the synaptonemal complex, SYP-1, which loads during the transition zone and is present on synapsed chromosomes through the pachytene region (MacQueen et al. 2002). We discovered that the first two regions of the gonad, which corresponds to the mitotic zone, contains high percentages of above-threshold Mutator foci (Figure 5B, regions 1-2). However, Mutator foci intensity peaks in a single region coinciding with the onset of the transition zone (Figure 5B, region 3). This peak in Mutator foci intensity is followed by a dramatic reduction in intensity in the regions corresponding to early and mid pachytene (Figure 5B, regions 4-7). Consistent with our qualitative observations, the foci-depleted region is followed by an increase in foci intensity in regions corresponding to the late pachytene (Figure 5B, regions 8-10). Our quantification shows Mutator foci intensity fluctuates in a consistent bright-dim-bright pattern within adult wild-type germlines.

Mutator foci intensity is associated with mitotic and not transition zone nuclei
Because the number of above-threshold Mutator foci peaks near the transition zone, we sought to determine if foci intensity was associated with the polarization of chromosomes that produces the unique crescent-shaped morphology of transition zone nuclei. To first examine the effects of an extended transition zone, we utilized a mutation in the him-8 gene, required to promote X chromosome pairing and synapsis (Phillips et al. 2005). Cells that fail to complete synapsis cannot exit the condensed transition state, resulting in polarized nuclei well into the pachytene region of the germline. Despite having an extended transition zone region, the brightest Mutator foci in a him-8 mutant gonad remain restricted to the mitotic region and beginning of the transition zone, with an overall pattern similar to wild-type animals (Figure S3C). Next, we aimed to determine if Mutator foci intensity was affected by eliminating the Mutator foci integrity partially relies on RNA. (A-B) Germlines of young adult mut-16::mCherry, pgl-1::gfp animals were injected with either a vehicle control or 200 μg/mL α-amanitin, a transcriptional inhibitor. (A) Live images of the mid-pachytene region at three, five, or seven hours post injection. MUT-16 foci (red) and PGL-1 granules (green) are unperturbed in vehicle control injections but dissipate with increasing severity over time. At seven hours, some MUT-16 foci remain (white arrows) though PGL-1 granules are absent. Scale bars, 5 μm.

Figure 4 Mutator foci integrity partially relies on RNA. (A-B) Germlines of young adult mut-16::mCherry, pgl-1::gfp animals were injected with either a vehicle control or 200 μg/mL α-amanitin, a transcriptional inhibitor. (A) Live images of the mid-pachytene region at three, five, or seven hours post injection. MUT-16 foci (red) and PGL-1 granules (green) are unperturbed in vehicle control injections but dissipate with increasing severity over time. At seven hours, some MUT-16 foci remain (white arrows) though PGL-1 granules are absent. Scale bars, 5 μm.

Because the mitotic region is associated with some of the highest numbers of above-threshold Mutator foci, we next sought to investigate whether the mitotic cell state determines Mutator foci intensity. To this end, we knocked down the KH motif-containing RNA-binding protein GLD-1 and the poly(A) polymerase GLD-2, which are redundantly required to promote meiotic entry and, when disrupted, produce a tumorous mitotic germline (Kadyk and Kimble 1998). Because RNAi knockdown was not completely penetrant, we observe some nuclei entering meiosis, marked by SYP-1 staining (Figure 5C). Despite this variation in penetrance, the gld-2 gld-1 RNAi gonads consistently produce a proximal mitotic tumor containing very bright Mutator foci. Quantification reveals more above-threshold foci in the proximal tumor compared to the distal mitotic zone. Additionally, no peak of fluorescence is found at the onset of SYP-1 loading, in contrast with the peak near wild-type transition zones (Figure 5D). In gonads with fewer meiotic nuclei and a larger mitotic tumor, bright Mutator foci are found more uniformly throughout the germline (Figure S3E). These observations indicate that the mitotic cell state is a contributing factor of Mutator foci intensity.

Finally, we sought to manipulate germline morphology and cell state in additional ways to test if Mutator foci intensity experienced similar perturbations. We knocked down ATX-2, which functions to promote germ cell proliferation and prevent premature meiotic entry; RNAi of atx-2 was previously observed to produce small germlines with truncated mitotic and transition zones (Maine et al. 2004). As expected, immunostained gonads were smaller than wild-type and had reduced mitotic tip and transition zones (Figure 5E). Interestingly, Mutator foci appear bright in the mitotic tip but do not form a distinct intensity peak preceding the transition zone and, in the ensuing pachytene region, foci generally appear brighter than in the mid pachytene of wild-type animals. Quantification reveals that...
above-threshold foci are more evenly distributed throughout the germline (Figures 5F and S3F). Thus, distribution of above-threshold Mutator foci can also be altered by general perturbations of germline morphology and cell state.

**DISCUSSION**

Mutator foci are hubs of secondary siRNA biogenesis in the *C. elegans* germline. Here we show that Mutator foci first appear in PGCs beginning around the 100-cell stage of embryogenesis, and that these foci persist in germ cells through all subsequent developmental stages.

Additionally, we find MUT-16 present in post-meiotic spermatids. We further demonstrate that both the presence of RNA and the germline cell cycle play key roles in promoting assembly of Mutator foci. Therefore, our work begins to define both the developmental stages and regulatory factors that shape Mutator foci integrity and intensity.

**Mutator foci in embryos**

Our first observation of robust Mutator foci occurs around the 100-cell stage, after the P4 cell divides into the PGCs Z2 and Z3. Interestingly, this timing coincides with the appearance of Z granules,
which are germline-specific phase-separated condensates required for RNAi inheritance. At this time, Z granules de-mix from P granules to form adjacent foci (Wan et al. 2018). MUT-16, however, does not appear significantly enriched in P granules prior to Mutator foci formation and is therefore unlikely to be de-mixing from P granules, but rather forming foci de novo. The birth of the Z2 and Z3 PGCs is also marked by the disappearance of MEG-3 and MEG-4, which surround the PGL phase of P granules in early embryogenesis and are crucial for P granule assembly (Wang et al. 2014). Together, these data suggest that there may be a coordinated reorganization of germ granule components at this time. While the exact mechanism for this reorganization remains unknown, the timing coincides with a burst of transcription in the PGCs (Seydoux and Dunn 1997). It is possible that an increase in mut-16 transcript, and thus protein levels, leads to the emergence of Mutator foci; however, detectable levels of cytoplasmic MUT-16 protein can be observed in all cells throughout embryonic development. Therefore, we favor the possibility that newly synthesized transcripts emerging from the nuclear pores necessitate and perhaps directly promote assembly of the P granule/Z granule/Mutator foci multi-condensate structures.

Regulation of Mutator foci by RNA
Because the appearance of Mutator foci in embryos coincides with the onset of transcription in the PGCs, we investigated the effect of transcriptional inhibition in the adult germline. It was previously shown that injection of a transcriptional inhibitor causes pachytene-specific loss of P granules (Sheth et al. 2010). We found that Mutator foci similarly rely on RNA for their stability in the mid-pachytene region. However, in late pachytene we observed differences in the dissolution of Mutator foci and P granules following transcriptional inhibition. Specifically, we observed persistent Mutator foci in the late pachytene, despite visible dissipation of all P granules at seven hours post-transcriptional inhibition. The sustained presence of Mutator foci in late pachytene, but not mid pachytene, could arise for multiple reasons. One possibility is that Mutator foci may be interacting with more stable classes of RNAs. A recent discovery shows that long stretches of alternating uridine (U) and guanosine (G), termed polyUG (pUG) tails, are added to siRNA-targeted transcripts to mark them as templates for secondary siRNA synthesis (Shukla et al. 2020). These pUG tails, added by the Mutator complex protein MUT-2, are hypothesized to confer stability to targeted transcripts. If these stabilized transcripts are associating with Mutator foci in higher quantities in late pachytene, it may explain the resistance of late-pachytene Mutator foci to dissolution after transcriptional inhibition. An alternate explanation may reside in the phase-separation properties of Mutator foci. We previously tested Fluorescence Recovery After Photobleaching on Mutator foci in the late-pachytene region, and showed that, although there was rapid recovery of a highly-mobile fraction within Mutator foci, there was also a large non-mobile fraction of MUT-16 that failed to recover after photobleaching. We have not yet identified the molecular basis of these distinct phases within Mutator foci, but phase separated granules can be regulated by post-translational modifications (Li et al. 2013; Wang et al. 2014), making this a tantalizing avenue for future exploration. Nonetheless, this non-mobile fraction may be in a more gel-like state and therefore resistant to dissolution after transcriptional inhibition. A direct comparison of the liquid properties of mid- vs. late-pachytene Mutator foci is necessary to address this possibility.

We also noticed that P granules became more rounded at three hours post-transcriptional inhibition in late pachytene compared to mid pachytene. Typically, P granules are intimately associated with nuclear pores, likely through the interaction of the FG-repeats in DEAD-box helicases GLH-1, GLH-2, and GLH-4, with nuclear pore-containing FG-repeat proteins (Updike et al. 2011; Marnik et al. 2019). This non-spherical appearance of P granules due to association with nuclear pores has been described as a “wetting” of the nuclear membrane (Brangwynne et al. 2009). Interestingly, a mutation in GLH-1 also creates large, round P granules, possibly caused by an inability to release captured RNA (Marnik et al. 2019). As first proposed by Sheth et al. (2010), our data suggests that P granule association with the nuclear periphery may also depend on the sustained presence of transcripts exiting the nucleus.

Mutator foci in the cell cycle and inheritance
Finally, we explored why Mutator foci brightness varies within different regions of the germline and determined that perturbations to the cell cycle affected the intensity and distribution of Mutator foci. We found that nuclei in mitosis tend to be associated with large, bright Mutator foci, whereas Mutator foci in the early and mid-pachytene are much dimmer. The exact mechanism governing Mutator foci robustness remains elusive. Interestingly, the mid-pachytene region is also where Mutator foci are most sensitive to transcriptional inhibition, again suggesting that more stable RNAs, or perhaps higher concentrations of small RNA-target transcripts, in the mitotic and late-pachytene regions may promote condensation of larger foci.

It has been well-documented that small RNAs can be inherited through both the maternal and paternal germline (Grishok et al. 2000; Alcazar et al. 2008; Lev et al. 2019). This transgenerational epigenetic inheritance can promote a memory of self and non-self transcripts across generations as well as transmit gene regulatory information in response to environmental conditions (Ashe et al. 2012; Luteijn et al. 2012; Shirayama et al. 2012; Conine et al. 2013; Rechavi et al. 2014; de Albuquerque et al. 2015; Phillips et al. 2015). While the Argonaute WAGO-4 and helicase ZNFX-1 have been implicated in the transmission of maternal small RNAs (Ishidate et al. 2018; Wan et al. 2018; Xu et al. 2018), little is known about how small RNAs are packaged and transmitted through paternal lineages. Here, we observed Mutator foci throughout spermatogenesis and detected MUT-16 in the cytoplasm of post-meiotic spermatids. A similarly granular expression pattern in spermatids was seen for WAGO-1 (Conine et al. 2010); if these granules coincide, further work will be necessary to determine if they act together to promote paternal inheritance and whether they promote paternal deposition of not just small RNAs, but also small-RNA targeted mRNAs. Ultimately, Mutator foci morphology and regulation may influence efficacy of RNAi in certain cell stages or environments, an avenue that warrants further investigation.

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