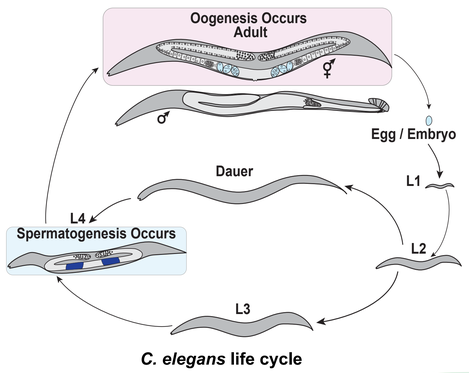
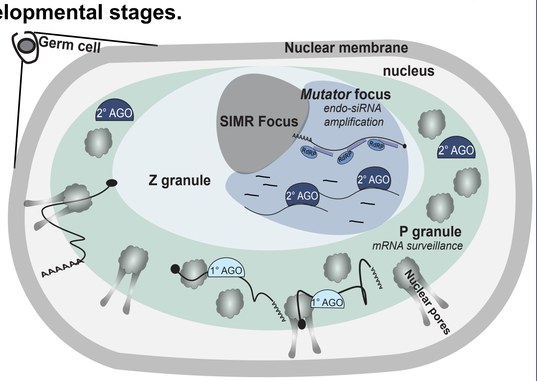
Control of spermatogenesis developmental program via RNAi-mediated regulation of ALG-3/4 small RNA pathway in *C.elegans*

Trilotma Sen*, Cara McCormick* and Alicia K. Rogers
The Department of Biology, The University of Texas at Arlington, Texas
(*co-first authors)

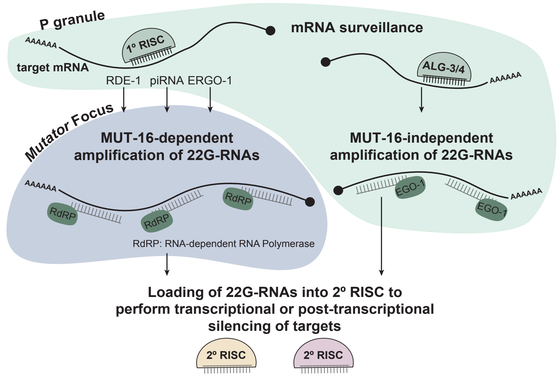
Gene regulation is necessary during development to ensure proper fertility. Precise activation and silencing of genes within totipotent germ cells is crucial for gametogenesis, and yet the molecular mechanisms that govern these actions remain elusive. Gametogenesis is a thermosensitive process and small RNAs play a pivotal role in maintaining regulation during thermotolerant fertility. In hermaphroditic *C. elegans*, spermatogenesis occurs during the L4 developmental stage, followed by oogenesis during the adult life stage. RNA interference (RNAi) pathways restrict spermatogenesis during the L4 stage and permit the expression of oogenesis genes in the adult stage. To examine how RNAi pathways protect sperm reproductive potential during stress, we used *mut-16 (pk710)* mutants, which exhibit sperm-based infertility at elevated temperature (25ºC). We found that sperm genes were developmentally mis-coordinated in *mut-16 (pk710)* mutant hermaphrodites, particulary during heat stress. Bioinformatic analyses revealed the mis-regulated genes largely overlapped with ALG-3/4 pathway targets, which are canocically involved in spermatogenesis. Further analysis revealed developmental mis-regulation of *alg-3* and *alg-4* gene expression and ALG-3::GFP and ALG-4::GFP proteins in heat-stressed *mut-16* mutants. This indicates that *mutator* complex mediated small RNA regulation is essential, despite ALG-3/4 small RNA pathway being independent of the *mutator* complex for amplification of its downstream 22G-RNAs. These discoveries suggest that small RNA pathways have an intricate RNAi-RNAi cascade that play a role in activating and deactivating the ALG-3/4 pathway at the proper developmental stages.



C. elegans life cycle



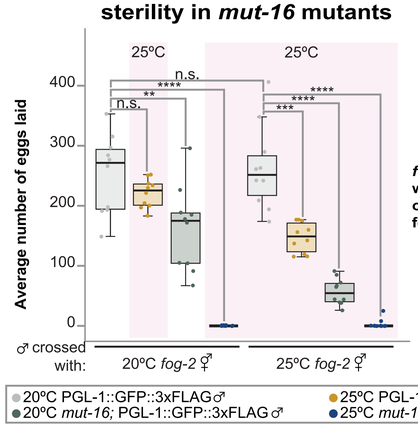
Germ granule organization and nuage assembly



ALG-3/4 small RNA pathway

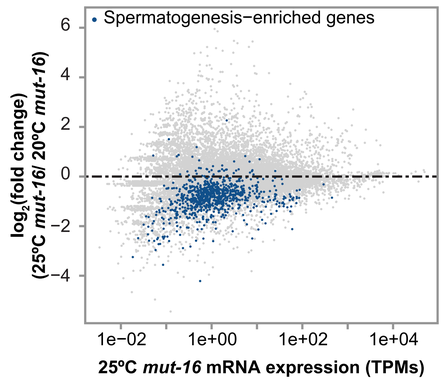
Results

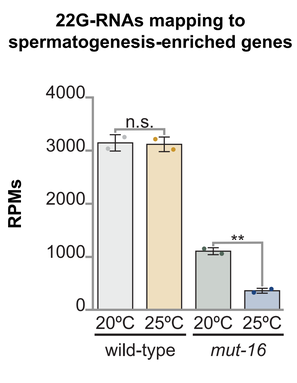
1. Temperature sensitive sperm-based sterility in *mut-16* mutants



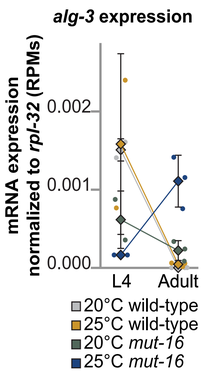
***fog-2* are a feminized strain, which cannot produce their own sperm and have to be fertilized from external sperm donors.**

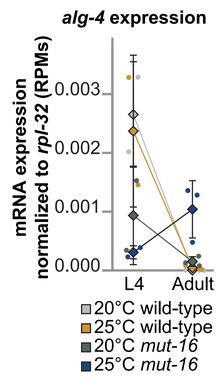
2. mRNA & 22G-RNA levels of sperm genes are downregulated in heat-stressed L4 *mut-16* mutants

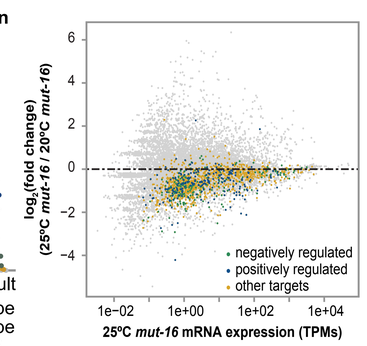




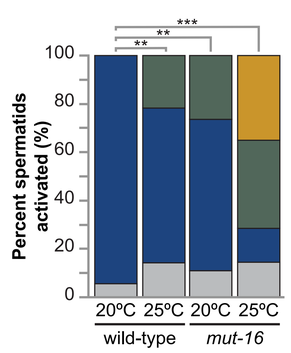
3. *Mutator* focus is necessary for developmental coordinatin of *alg-3* and *alg-4* genes and thus ALG-3/4 pathway function

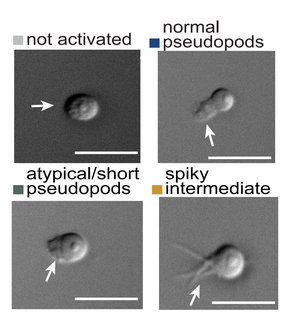




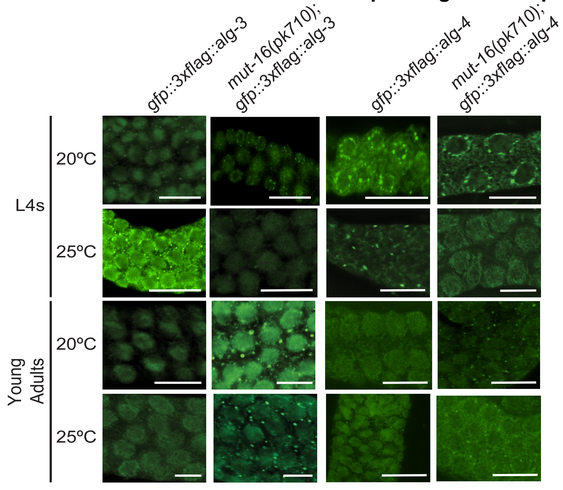


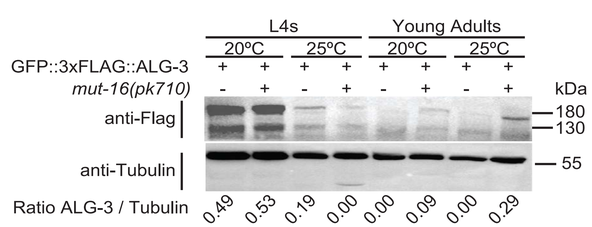
4. Spermiogenesis defects in heat stressed *mut-16* mutants which phenocpy *alg-3/4* mutants

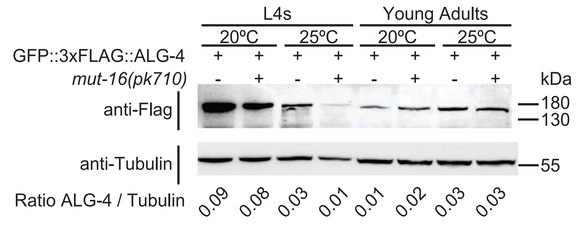




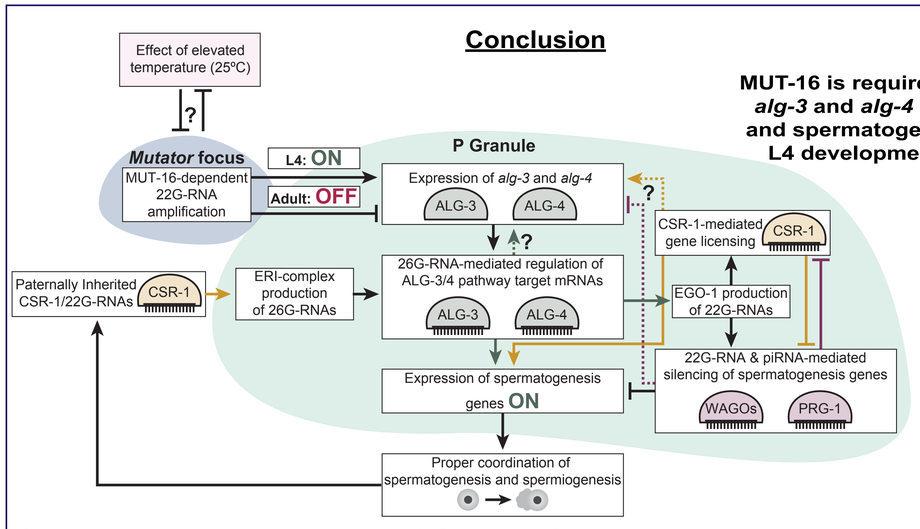
5. The *mutator* complex regulates expression of functional proteins ALG-3 and ALG-4







Conclusion



MUT-16 is required to restrict *alg-3* and *alg-4* expression and spermatogenesis to the L4 developmental stage

Our model suggets that *mutator*-dependent 22G-RNA amplifcation, along with putative auto-regulatory feedback from ALG-3/4 bound 26G-RNAs, is crucial for promoting *alg-3* and *alg-4* expression during the L4 stage. This mechanism is essential for mitigating heat stress effects, maintaining robust sperm-based fertility, and coordinating the sperm-matogenesis-to-oognesis switch.

Methods

- **Brood size assay to determine sterility defect in *mut-16 mutants*.**
- **RNA extraction of *mut-16 (*pk710*)* & WT of L4s and young adults., and bioinformatic analysis on small RNA and mRNA libraries.**
- **In vitro sperm activation assay of *mut-16 (*pk710*)* vs WT.**
- **Western blots of *mut-16 (*pk710*)* vs WT L4s and young adults.**
- **Fluorescence microscopy of *mut-16 (*pk710*)* vs WT L4s and young adults dissected germlines.**

Future Directions

- **Our goal is identify how the ALG-3/4 pathway is regulated and how do these pathways control sperm development?**
- **We wish to employ high throughput bioinformatics on mRNA and small sequencing data on *alg-3/4* double mutant and WT.**
- **Perform co-IP on ALG-3 an ALG-4 in *mut-16* mutatns vs WT**

Broad Impact

- **Identifying and understanding germline small RNA pathways can be crucial in developing effective therapeutic approaches for heritable diseases and infertility.**
- **This research would add to our incomplete understanding of underlying factors contributing to infertiliy in both males and females.**

References

- Conine, C. C. et al. Argonautes ALG-3 and ALG-4 are required for spermatogenesis-specific 26G-RNAs and thermotolerant sperm in *Caenorhabditis elegans*. Proc Natl Acad Sci U S A 107, 3588-3593 (2010).
- Rogers, A. K. & Phillips, C. M. A Small-RNA-Mediated Feedback Loop Maintains Proper Levels of 22G-RNAs in *C.elegans*. Cell Rep 33 (2020).