

Sexually Dimorphic Argonaute Localization Underlies Sex-Specificity of Small
RNA Pathways During Germ Cell Development

by

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A dissertation accepted and approved in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy
in Biology

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Spring 2025

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DISSERTATION ABSTRACT

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Doctor of Philosophy in Biology

Title: Sexually Dimorphic Argonaute Localization Underlies Sex-Specificity of Small RNA Pathways During Germ Cell Development

Meiosis, a specialized form of cell division that produces haploid gametes, requires precise gene regulation to mediate proper germ cell development. Utilized during both oogenesis and spermatogenesis, small RNA pathways tune expression during germ cell development using functional Argonaute (AGO) proteins. AGOs mediate gene expression by both licensing genes required for meiosis and epigenetic inheritance, while simultaneously silencing deleterious genomic elements. The function and localization of AGOs during *C. elegans* oogenesis has been extensively characterized but less is known about mechanisms of AGO-mediated gene regulation during spermatogenesis, and if they differ from egg development. Here, I demonstrate that the cytoplasmic Argonaute proteins, WAGO-1 and WAGO-4, are sexually dimorphic in their localization throughout egg and sperm development, and that function of WAGO-1 protein structural features affects sex-specific gene regulation during *C. elegans* germ cell development. Using a whole germline image analysis pipeline, I characterize the morphology of WAGO-1 and WAGO-4 foci, as well as co-localization with germ granule components PGL-1 and ZNFX-1, through all stages of meiotic prophase I. These analyses highlight the highly dynamic properties of AGOs, and how these biophysical attributes differ between oogenesis and spermatogenesis at distinct stages of germ cell development. I further reveal that native WAGO-1 and WAGO-4 structures are required for sex-specific maintenance of germ granule structure during oogenesis and spermatogenesis. Finally, I link WAGO-1-mediated disruption of germ granule structure to sex-specific regulation of mRNA expression and small RNA profiles. Overall, these data highlight that sexual dimorphism in WAGO-1 and WAGO-4 structure are imperative for mediating germ granule structure, and mutations to WAGO-1 disrupt not only the germ granule, but also gene regulation in a sexually dimorphic manner. Taken together my thesis work provides a foundation for our understanding of sex-specific roles of conserved Argonaute proteins in properly regulating gene expression and maintaining germ granule structure during oogenesis and spermatogenesis.

This dissertation includes previously unpublished co-authored material.

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PUBLICATIONS:

DiNardo, A.L., Kurhanewicz, N.A., Wilson, H.R., Berg, V., Libuda, D.E. 2025. WAGO-1 is a sexually dimorphic Argonaute protein required for proper germ granule structure and gametogenesis. Manuscript submitted for publication in *Development*.

- Garcia, D.M., Campbell, E.A., Jakobson, C.M., Tsuchiya, M., Shaw, E.A., DiNardo, A., Kaeberlein, M., Jarosz, D.F. 2020. A prion accelerates proliferation at the expense of lifespan. *eLife* 202, e60917.
- Toraason, E., Adler, V.L., Kurhanewicz, N.A., DiNardo, A., Saunders, A.M., Cahoon, C.K., Libuda, D.E. 2021. Automated and customizable quantitative image analysis of whole *Caenorhabditis elegans* germlines. *Genetics* 217.

ACKNOWLEDGMENTS

I would like to express special thanks to my advisor Dr. Diana Libuda, for all her support and guidance throughout my PhD. Her mentorship has pushed me to become the best scientist I can be, and her constant belief in me has been incomparable. Diana has set the bar for how the scientific community should support each other and the importance of creativity and passion for driving science. Her commitment to creating a welcoming and supportive lab environment will be something I will strive to bring in every step of my career. Thank you to my committee members, Dr. David Garcia, Dr. Scott Hansen, Dr. Alice Barkan, and Dr. Bruce Bowerman for their advice and guidance throughout my PhD.

To the past and present members of the Libuda Lab, thank you so much for all your support, conversations, scientific and otherwise, and friendship over these past six years. A special thanks to Nicole Kurhanewicz, Zac Bush, and Cori Cahoon for allowing me to voice every wild idea, moment of doubt and everything in between that has ever crossed my mind. Your friendship was vital for preserving my sanity and finishing my studies. I would also like to thank my fellow graduate students, Jordan Brown, Caroline Crahan, and Hannah Wilson for our friendship and ability to talk about everything that made going to lab a highlight of my day.

I would like to thank my amazing community of friends in Eugene, who provided me with so much love, support, and adventures away from the bench. Thank you to Emily Dennis, Sam Horst, Max Horracks, William Crow, Rachael Giersch, Dr. Kona Orlandi, Dr. Ethan Shaw, Brenden Campbell, and Hannah Wilson for the constant camping trips, ski adventures, 6 am tailgates, dinners, movie nights, and everything in between. Thank you to Karen Ramming, Jackson Mestler, Eli Rosen-Duran, Jenn Randall, and Peter Stice for the game nights, long runs, and all the constant track talk. Special thank you to Molly Shallow and (soon to be Dr.) Sophia Phillips for being the best friends I could have ever asked for through this journey.

I would like to give special thanks to my parents, Ed and Jen DiNardo. Your constant support through the years has helped me become the person and scientist I am today. Also thank you to my brother Bryce, you have always been my rock and adventure buddy for everything we have done over the past 24 years. I cannot wait to see where all your adventures and work take you next.

Finally, I owe a very special thanks to my partner (almost Dr.) Jared Freedman and our dog Whiskey. I could not have asked for a better best friend over these past 9 years. You have

always been my biggest supporter, and you have pushed me to become the best scientist and version of myself. I will forever be grateful for those Sunday recovery runs and our hatred of doing dishes that has led to our life together. I cannot wait for our next adventures, wherever they may take us.

This work was supported by the National Institutes of Health R35GM128890 grant to Dr. Diana Libuda, and the National Institutes of Health T32GM0070431 grant and Raymod Stevens Fellowship awarded to me.

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CHAPTER 1: INTRODUCTION

The survival of a species depends on individual organisms being able to replicate and passage their genome from one generation to the next. Throughout history, fertility has been a focal point of humanity, vital for the continuation of families and legacies that in turn shape culture and religion. Today, politicization of fertility has turned it into a key issue of geopolitical affairs (Blum, 2024; Ongweso Jr, 2022). Through the turn of the 21st century, numerous countries have seen dropping fertility rates, in part due to increased access to birth control and family planning but also due to increased rates of infertility (Borumandnia et al., 2021; Mascarenhas et al., 2012; World Health Organization, 2023). Infertility is medically defined as a disease of the reproductive system characterized by the failure to achieve a successful pregnancy following 12 or more months of regular unprotected sexual intercourse (World Health Organization, 2023; Zegers-Hochschild et al., 2017). Struggles with infertility have been tied to numerous societal issues, such as social stigma, economical and mental health hardship, and gender-based violence throughout the globe (Greil et al., 2010; Sangster and Lawson, 2015; Thoma et al., 2021; Wang et al., 2022). In part, these social issues arise because the underlying causes of medical infertility remains so poorly defined (Greil et al., 2010). The medical definition of infertility focuses on absence of a pregnancy rather than the mechanisms that prevent successful reproduction. This, in part, may be the cause of one of the largest misconceptions historically surrounding infertility: that infertility is a disease that primarily affects females (Agarwal et al., 2015; Hasanpoor-Azghady et al., 2019; Taebi et al., 2021; Wang et al., 2022). Indeed, numerous studies have found that across the globe, and especially in patriarchal societies, females bear the majority of the blame (Taebi et al., 2021; Thoma et al., 2021; Wang et al., 2022). In actuality, 50% of infertility cases are due to sperm specific factors (Huang et al., 2023; Leslie et al., 2025), however mechanisms driving male-specific infertility remain understudied.

Research into mechanisms of infertility is an important topic of public health. Better understanding of the mechanisms that confer fertility, as well as the underlying genetic conditions and molecular mechanisms that may impair fertility aids in creating novel methods of prevention or interventions (Matzuk and Lamb, 2008). The work presented in this dissertation aims to better understand mechanisms underlying infertility during sperm development, and how these mechanisms may differ or be conserved with egg development. Specifically, my work

reveals differences in gene regulation during meiosis, the specialized cellular process that results in the formation of gametes, such as egg and sperm. Proper progression through meiosis is essential for an organism to successfully reproduce. Sexual reproduction occurs in three distinct steps: 1) the formation of gametes by meiosis, 2) fertilization through the fusion of gametes, and 3) embryogenesis, the development of a zygote into a multicellular organism able to itself successfully sexually reproduce (Gheldof et al., 2019; Gray and Cohen, 2016; Handel and Schimenti, 2010; Siu et al., 2021). Infertility can arise through errors in any of these steps, although nearly half of these issues can be traced back to improper formation of egg or sperm cells (Martin, 2008; Zorrilla and Yatsenko, 2013). As such, gametogenesis is highly regulated due to the fact that the genetic information packaged in each egg and sperm cell is directly passaged to the next generation and influences the success of a gamete to undergo fertilization, as well as providing genetic information required for proper development of progeny (Bourc'his and Voinnet, 2010; Gheldof et al., 2019; Handel and Schimenti, 2010). Better understanding of meiosis and the formation of gametes is therefore imperative to provide better understanding of mechanisms causing infertility.

Meiosis and the worm

The formation of gametes, such as egg and sperm cells, is vital for sexually reproducing organisms. Meiosis is a specialized process of cell division that creates haploid gametes (eggs and sperm) from germ cells. Unlike mitosis, germ cells undergoing meiosis go through two cellular divisions following a single round of DNA replication. The first division halves the genome from a diploid ($2N$) state to a haploid ($1N$) state through the segregation of homologous chromosomes (meiosis I), while the second division segregates sister chromatids similar to mitosis (meiosis II). In addition to segregating homologous chromosomes rather than sister chromatids, meiosis I also has a prolonged prophase stage that allows for the physical connection of homologs through the formation of crossovers (Gray and Cohen, 2016; Zickler and Kleckner, 2023).

Meiotic prophase I is divided into five distinct stages: leptotene, zygotene, pachytene, diplotene, and diakinesis (Fig. 1.1; Reviewed in Garcia-Muse and Boulton, 2007; Zickler and Kleckner, 2015). These stages can be determined cytologically and are underpinned by structural changes to the chromosomes through meiotic progression (Zickler and Kleckner, 2015, 1998).

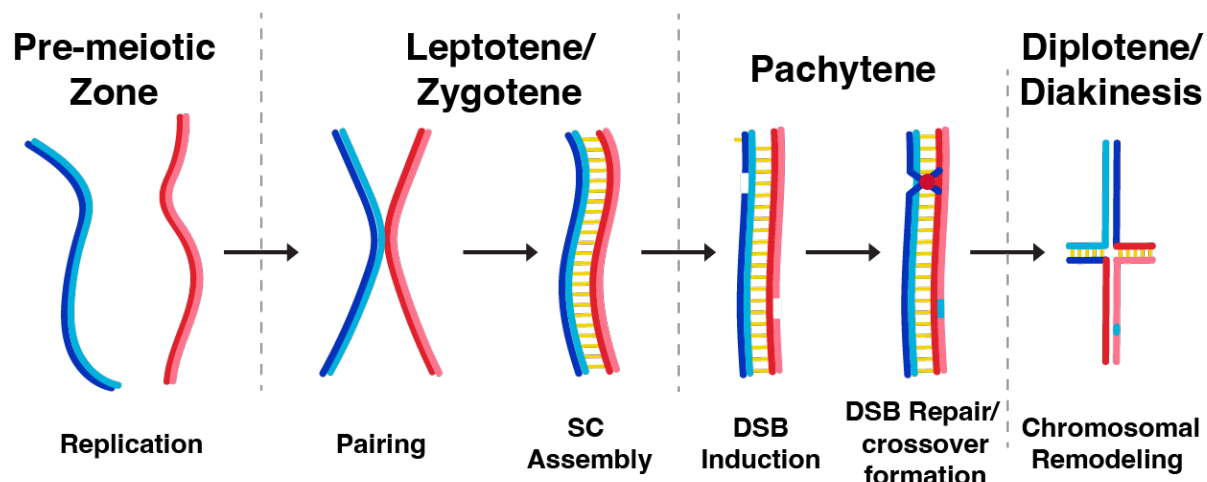


Figure 1.1: Key steps of extended meiotic prophase I. Representative cartoon depicting key changes to chromosomal structures of germ cell DNA from replication in the pre-meiotic region to the further compaction and chromosomal remodeling at the end of meiotic prophase I.

During leptotene and zygotene homologous chromosomes find each other within the nucleus, align, and pair. A proteinaceous structure known as the synaptonemal complex (SC) then forms between paired homologous chromosomes, creating a physical link (Page and Hawley, 2004). Concurrently, meiotic recombination, a well conserved mechanism for ensuring accurate homologous chromosome segregation, is initiated through the creation of an excess number of pre-programmed double-stranded breaks (DSBs) across the genome (Dernburg et al., 1998; Keeney et al., 1997). Upon entrance into pachytene, synapsis between homologous chromosomes is complete and the DSBs undergo repair. A subset of DSBs are repaired utilizing homologous recombination, which leads to the formation of crossovers. Crossovers allow for the exchange of genetic material between the homologs and physically links them together, which is imperative for successful meiotic divisions (Gray and Cohen, 2016). Crossovers are fully formed by the end of pachytene, allowing for SC disassembly and further chromosomal rearrangements in diplotene and diakinesis (Albertson et al., 1997; Page and Hawley, 2004). These chromosomal rearrangements occur around crossovers, forming meiotic bivalents, which aid in both meiosis I and meiosis II divisions (Albertson and Thomson, 1993).

While developing eggs and sperm both undergo the above steps of meiotic prophase I to proceed through meiosis and form haploid gametes, a growing body of evidence suggests that meiotic mechanisms differ between the sexes. From differences in the resulting gametes size, eggs are the largest cells in humans while sperm are the smallest, to the mechanisms of meiotic prophase I, egg and sperm development (oogenesis and spermatogenesis respectively) is sexually

dimorphic (Cahoon and Libuda, 2019; Ishiguro, 2023; Morelli and Cohen, 2005). Drivers of sexually dimorphic meiotic progression has become an area of interest, in part due to sex-specific infertility associated with environmental stressors across species (Abd El-Emam et al., 2023; Hoang-Thi et al., 2022; Kurhanewicz et al., 2020; Prasad et al., 2016; Saini et al., 2017; Sen et al., 2024). Small RNA pathways are well-conserved mediators of gene expression during both egg and sperm development and are central for preserving fertility during periods of environmental stress (Cecere, 2021; Houri-Zeevi et al., 2021; Kurhanewicz et al., 2020; Sen et al., 2024). Disruption to these pathways causes male specific infertility during periods of heat stress, suggesting that despite their sex-independent role in regulating germ cell development, mechanisms mediating small RNA pathway functions are sexually dimorphic (Conine et al., 2013, 2010; Ketting and Cochella, 2021; Kurhanewicz et al., 2020; Sen et al., 2024).

The nematode *Caenorhabditis elegans* has established itself as an ideal model system for studying germ cell development and meiosis (Fig. 1.2) (Hillers et al., 2018; Hubbard and Greenstein, 2005). The study of meiosis and the regulatory aspects that govern proper germ cell

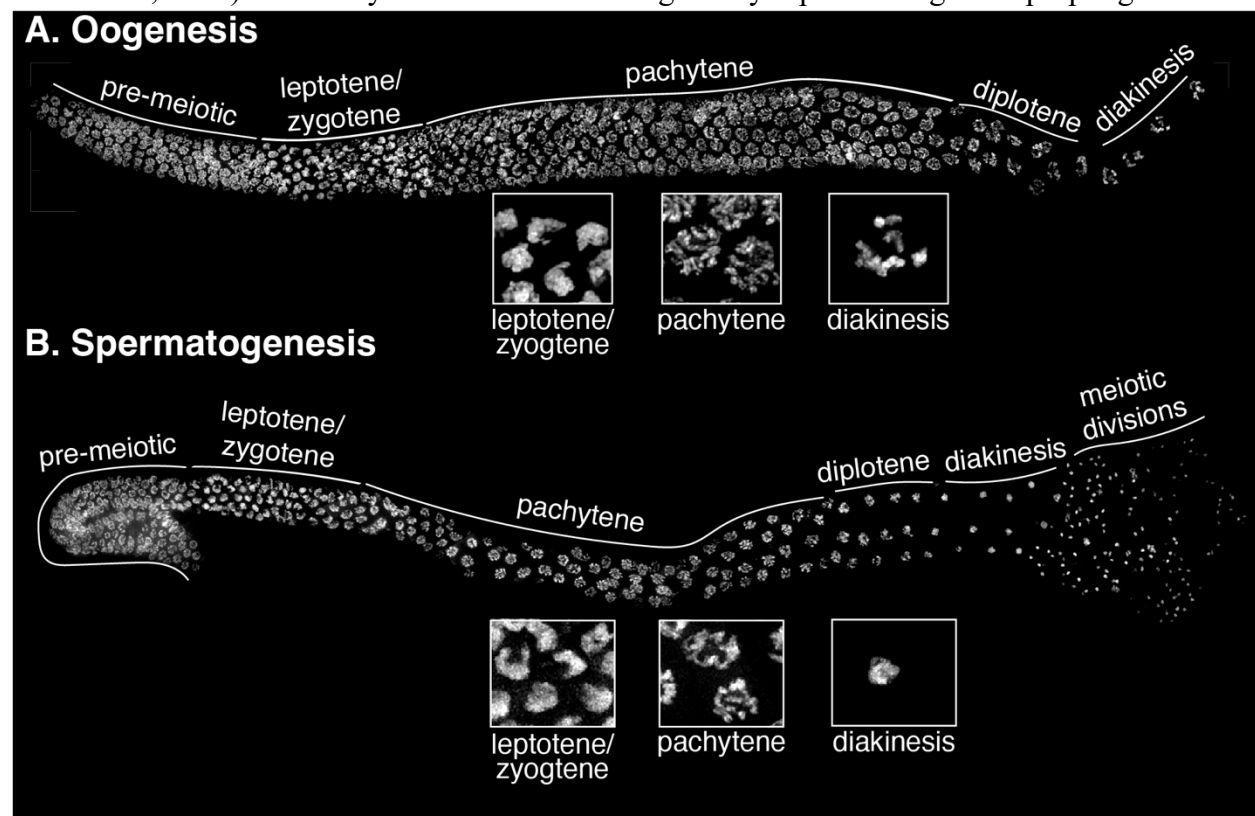


Figure 1.2: Organization of *C. elegans* germline. Dissected gonads stained with the DNA dye DAPI from (A) an adult hermaphrodite undergoing oogenesis and (B) an adult male undergoing spermatogenesis. Nuclei in both germlines are arranged in a spatiotemporal gradient and changes in chromosome morphology permit easy identification of each stage of meiotic prophase I.

development is limited in mammalian systems due to the bulk of female meiosis I occurring during fetal development and the mixing of somatic and meiotic germ cells within the gonad of both sexes (Morelli and Cohen, 2005; Roig et al., 2011). *C. elegans* are an ideal system to bypass these limitations and study sexually dimorphic aspects of meiosis. *C. elegans* exist as two sexes: hermaphrodites that undergo spermatogenesis during an early larval stage and oogenesis as an adult, and males that undergo spermatogenesis. Unlike in mammalian systems, both spermatogenesis and oogenesis occur during the adult life stages in hermaphrodite and male worms respectively, negating the need to study these processes during the organisms own development (Lui and Colaiácovo, 2013). Furthermore, the gonad of both sexes contains all stages of meiosis I concurrently and is arranged in a spatiotemporal manner (Fig. 1.2) (Tzur et al., 2018). This organization allows for interrogation of 3D cytological features of meiotic progression through staining of fixed germlines for proteins of interest and visualization using immunofluorescent microscopy.

The physiological features of *C. elegans* make it powerful system for understanding how small RNA pathways differentially regulate key genes required for proper germ cell development during oogenesis and spermatogenesis. In this thesis, I exploit *C. elegans* to further our understanding of sexual dimorphisms underlying organization of small RNA pathways and determine how changes to this structure affects gene regulation during meiosis.

Small RNA pathways: Regulators of gene expression during gametogenesis

Conserved across most eukaryotes, with the notable exception of *Saccharomyces cerevisiae*, small RNA pathways are key regulators of gene expression during gametogenesis and development (Chapman and Carrington, 2007; Drinnenberg et al., 2009). Central to these pathways are the RNA binding proteins Argonautes (AGOs), which engage distinct classes of small RNAs (18-30 nucleotides in length) for targeted regulation of recently transcribed mRNAs, non-coding RNAs (ncRNAs), and chromatin structure (Reviewed in: Ketting and Cochella,

2021; Seroussi et al., 2022; Youngman and Claycomb, 2014). AGOs have a well conserved, bilobed structure that contain four domains: the N-terminal lobe is comprised of an intrinsically disordered region (IDR) and PAZ (PIWI-Argonaute-Zwille) domain, while the C-terminal lobe contains the MID (middle) and PIWI (P-element-induced wimpy testis) domains (Fig. 1.3) (Sheu-Gruttadauria and MacRae, 2017).

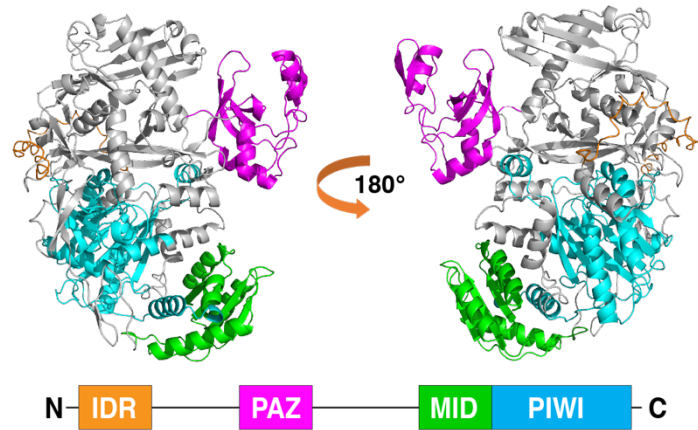


Figure 1.3: Conserved structure of Argonaute proteins.

Cartoon schematic of representative Argonaute, WAGO-1, with conserved protein domains colored as follows. Orange, intrinsically disordered region (IDR); pink, PIWI/Argonaute/Zwille domain (PAZ); green, Middle domain (MID); light blue, P-element-induced wimpy testis domain (PIWI).

The central channel between the two lobes is highly basic and positively charged, which allows for binding of negatively charged mRNAs and small RNAs within the cleft (Wang et al., 2008). The PAZ domain specifically binds the 3' end of loaded small RNAs, while the MID domain anchors the 5' end of the small RNA within the binding pocket (Jinek and Doudna, 2009). AGOs loaded with a small RNA comprise the RNA-induced silencing complex (RISC), which can then bind target RNA and DNA sequences through direct Watson-Crick base pairing interactions for subsequent silencing or licensing of gene expression (Dueck and Meister, 2014). The PIWI domain adopts a confirmation structurally similar to RNase H and, in a subset of AGOs, cleave bound mRNA (Dueck and Meister, 2014; Elkayam et al., 2012; Jinek and Doudna, 2009). Silencing mechanisms by AGOs lacking functional cleavage capabilities remains an open area of investigation, especially for AGOs known regulate transcripts post-transcriptionally (Yigit et al., 2006; Youngman and Claycomb, 2014).

Diversity in gene regulation by small RNA pathways. Argonautes engage a variety of small RNAs (sRNAs) for targeted gene regulation during germ cell development. In *C. elegans* 16 AGOs (Fig. 1.4) engage at least four distinct endogenously encoded sRNAs for gene regulation: micro RNAs (miRNAs), PIWI RNAs (piRNAs), 26G-RNAs, and 22G-RNAs (Fig. 1.5).

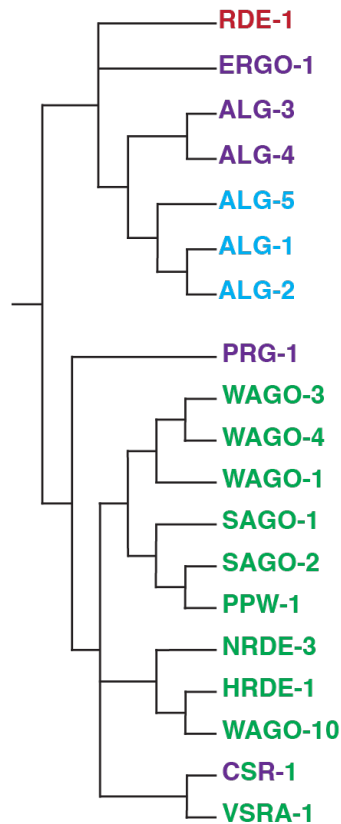


Figure 1.4: Phylogenetic tree of functional Argonaute proteins in *C. elegans*. AGOs in light blue are used in miRNA pathways, AGOs in purple represent primary AGOs used by piRNA and siRNA pathways, AGOs in green are secondary WAGO proteins. RDE-1 is colored in red to highlight its role in exogenous and endogenous small RNA pathways

Pathways employing these sRNAs utilize two main mechanisms for target gene regulation: direct sRNA/target site binding interactions and a feedforward amplification of sRNAs triggered by primary sRNA interactions with AGOs (Ketting and Cochella, 2021). The miRNA pathway utilizes this first mechanism, known as miRNA-mediated repression, which relies on specific sRNA/target transcript interactions to trigger mRNA degradation and translation repression (Fig. 1.5A) (Duchaine and Fabian, 2019; Ketting and Cochella, 2021). These specific miRNA/target interactions rely on the processing of miRNAs from duplexed, hairpin RNA structures to mature miRNAs which are loaded onto AGOs ALG-2, ALG-5, and RDE-1 in the germline (Brown et al., 2017; Dueck and Meister, 2014; Kotagama and McJunkin, 2024; Seroussi et al., 2023; Vasquez-Rifo et al., 2012). Known to be expressed throughout development and in every tissue, gene regulation by miRNAs is imperative for proper development and cellular function. miRNA-associated AGOs are similarly crucial for reproductive success of *C. elegans*, with depletion of any of these AGOs causing a decrease in fertility (Brown et al., 2017; Seroussi et al., 2023; Vasquez-Rifo et al., 2012). As such, both ALG-2 and RDE-1 contain PIWI domains with functional slicer activity, which is essential for efficient regulation by the miRNA pathway (Bouasker and Simard, 2012; Steiner et al., 2009).

piRNAs, 26G-RNAs, and 22G-RNAs are all utilized in pathways that amplify small RNAs against target transcripts through the use of primary and secondary AGOs and RNA-dependent RNA polymerases (RdRPs) (Fig. 1.5B). piRNAs, also known as 21U-RNAs in *C. elegans*, and 26G-RNAs are both upstream small RNAs that are bound by well-conserved primary AGOs. PRG-1 is the only primary AGO that binds piRNAs, and as such is the sole PIWI Argonaute in the *C. elegans* genome. Targeted regulation by germline piRNAs occurs during both oogenesis and spermatogenesis to silence deleterious genomic elements such as transposons and pseudogenes (Fig. 1.5B) (Batista et al., 2008; Das et

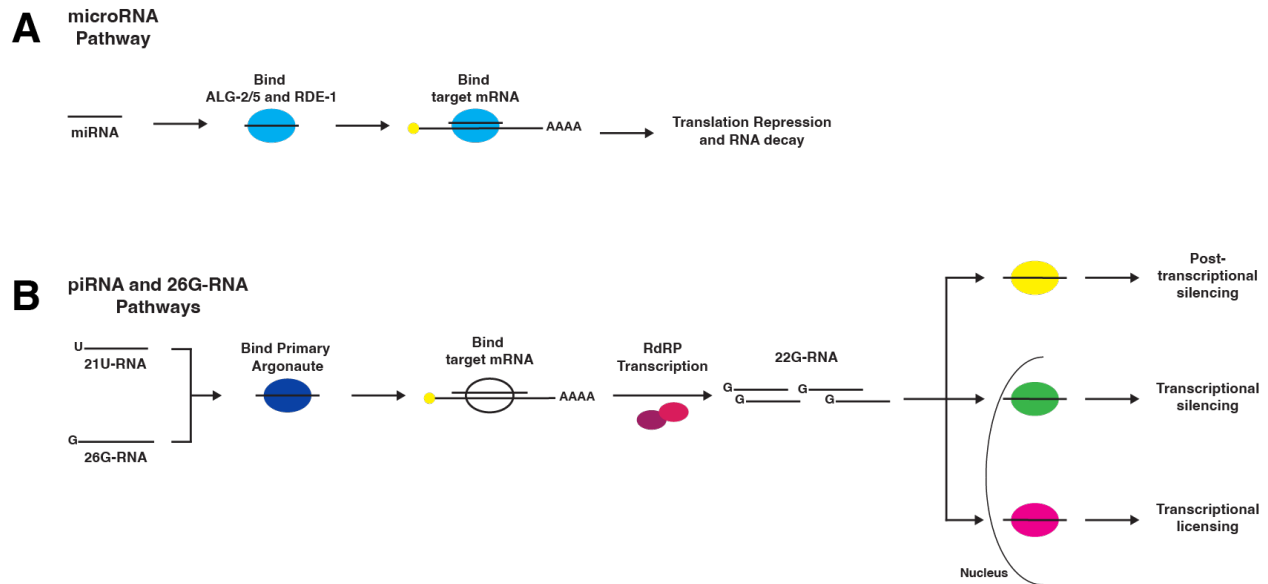


Figure 1.5: Key steps of gene regulation by miRNA, piRNA, and siRNA pathways during germ cell development. (A) Cartoon schematic depicting gene regulation utilizing miRNA-mediated repression. (B) Cartoon schematic depicting feedforward amplification mechanism of gene regulation by piRNA and siRNA pathways.

al., 2008; Spichal et al., 2021; Wang and Reinke, 2008). In contrast, 26G-RNAs are bound by three different primary AGOs that act in a sexually dimorphic manner. ERGO-1 is expressed during oogenesis and early embryogenesis, binding 26G-RNAs that primary target pseudogenes, somatic genes, and improperly expressed spermatogenetic genes for silencing. The paralogs ALG-3 and ALG-4 bind 26G-RNAs during spermatogenesis in adult males and larval stage 4 hermaphrodites. These 26G-RNAs target genes required for spermatogenesis for licensing and an additional, distinct, subset of protein coding genes for silencing (Conine et al., 2013, 2010; Han et al., 2009; Seroussi et al., 2023).

The targeting of transcripts by both piRNA-directed PRG-1 and 26G-RNA-directed ERGO-1 and ALG-3/4 converge onto a worm specific family of Argonautes, the WAGOs (Fig. 1.4, green; Fig. 1.5B). WAGOs (Worm-specific Argonautes) are an expanded clade of AGOs that are present in all but one species in the genus *Caenorhabditis*, as well as in a number of closely related nematodes (Buck and Blaxter, 2013; Fusca et al., 2024). WAGOs bind to 22G-RNAs, a secondary class of siRNAs that are transcribed from sequences targeted by primary AGOs using RdRPs (Gent et al., 2010; Gu et al., 2009; Ketting and Cochella, 2021). Regulation by WAGOs occur both post- and co-transcriptionally, with a subset directly engaging target mRNAs and other ncRNAs, while others re-localize to the nucleus and aid in the placement of chromatin marks (Fig. 1.5B) (Buckley et al., 2012; Charlesworth et al., 2021; Claycomb et al.,

2009; Frolows and Ashe, 2021; Gu et al., 2009; Guang et al., 2008; Ishidate et al., 2018; Nguyen and Phillips, 2021; Seroussi et al., 2023; Xu et al., 2018).

WAGOs engage distinct groups of 22G-RNAs based on their original template and primary AGO, clustering into four distinct subgroups. The ERGO-1 Cluster includes somatic WAGOs, SAGO-1 and SAGO-2, as well as WAGOs expressed in both oogenetic germlines and somatic cells: PPW-1, VSRA-1, and NRDE-3 (Seroussi et al., 2023). As the cluster name suggests, these 22G-RNAs are primarily transcribed from targets of ERGO-1 bound 26G-RNAs and regulate gene expression of long non-coding RNAs (lincRNAs) and mutator genes in the germline both post-transcriptionally (PPW-1 and VSRA-1) and co-transcriptionally (NRDE-3) (Guang et al., 2008; Seroussi et al., 2023; Tijsterman et al., 2002b). ALG-3/4 Cluster WAGOs engage 22G-RNAs primarily transcribed from spermatogenic ALG-3/4 targets and include nuclear WAGO-10, the sperm-specific CSR-1a isoform, and RDE-1. Genes targeted by CSR-1a are licensed for expression through unknown mechanism(s)¹, while WAGO-10 and RDE-1 target lincRNAs, pseudogenes, and transposons for repression (Charlesworth et al., 2021; Nguyen and Phillips, 2021; Seroussi et al., 2023). The final two clusters, the WAGO Cluster and the CSR-1 Cluster, act in the germline independent of meiotic process (Seroussi et al., 2023). The WAGO Cluster primarily targets transcripts engaged by PRG-1 and piRNAs, leading to the repression of genes associated with genomic instabilities including transposons and pseudogenes, (Ortega et al., 2024; Seroussi et al., 2023; Spichal et al., 2021). WAGOs utilized in this cluster include the highly related paralogs, WAGO-1 and WAGO-3 (PPW-2), nuclear HRDE-1, and PPW-1 (Frolows and Ashe, 2021; Gu et al., 2009; Schreier et al., 2022; Seroussi et al., 2023). The final secondary AGO cluster is headed by CSR-1 and regulates both the licensing and repression of protein coding genes (Seroussi et al., 2023). The mechanisms of regulation remain the least understood of the four clusters due to CSR-1 acting as the primary AGO despite only engaging 22G-RNAs. Indeed, previous works has found that CSR-1 binds a unique subset of 22G-RNAs that allows for targeted binding and subsequent cleavage through slicer activity of the CSR-1 PIWI domain (Singh et al., 2021). These RNA fragments aid in priming downstream RdRPs for synthesis of new 22G-RNAs for loading onto secondary WAGOs (Singh et al., 2021). In addition to acting as the primary AGO within this cluster, CSR-1 is also thought to act as a secondary

¹ CSR-1 both demonstrates nuclear association, as well as functionality in regulating genes post-transcriptionally (Charlesworth et al., 2021; Claycomb et al., 2009; Nguyen and Phillips, 2021; Singh et al., 2021)

WAGO that aids in the regulation of protein coding genes along with WAGO-4 and VSRA-1 (Seroussi et al., 2023).

Sexually dimorphic small RNA pathways: In *C. elegans*, both the primary and secondary Argonautes associated with piRNAs and siRNAs (26G- and 22G-RNAs) display gamete-specific gene regulation. Most evident is the sex-specific expression and engagement of primary AGOs which bind 26G-RNAs. Indeed, ERGO-1 is only expressed in adult hermaphrodites undergoing oogenesis, while ALG-3/4 are only expressed by adult males and larval stage 4 hermaphrodites undergoing spermatogenesis (Conine et al., 2013, 2010; Seroussi et al., 2023; Vasale et al., 2010). Furthermore, CSR-1, which acts as both a primary and secondary AGO that uniquely engages 22G-RNAs at both steps, is transcribed as two isoforms: CSR-1a that is exclusively expressed during spermatogenesis and CSR-1b that is expressed during both meiotic processes (Charlesworth et al., 2021; Nguyen and Phillips, 2021; Seroussi et al., 2023). Regulation of CSR-1 is therefore sex-specific, with regulation of spermatogenetic genes only occurring through use of the sperm-specific CSR-1a isoform (Charlesworth et al., 2021).

Regulation by the piRNA pathway mediated by PRG-1 is similarly sexually dimorphic, despite engagement of PRG-1 during oogenesis and spermatogenesis (Cornes et al., 2022; Ortega et al., 2024; Reed et al., 2020; Reinke et al., 2000). Across species PIWI homologs are required for maintaining male fertility while being relatively expendable for oogenesis (Deng and Lin, 2002; Kuramochi-Miyagawa et al., 2004). Recent works have found that in *C. elegans* proper piRNA and PRG-1 expression is similarly required for the effective regulation of genes during oogenesis, but does not directly disrupt meiotic progression (Barucci et al., 2020; Reed et al., 2020; Wahba et al., 2021). Rather, the transgenerational infertility observed in adult hermaphrodites may be caused by improper regulation of small RNA pathways which leads to the generational misregulation of histone and ribosomal genes, eventually leading to sterility (Barucci et al., 2020; Reed et al., 2020; Wahba et al., 2021). In contrast, during spermatogenesis improper expression of piRNAs and/or PRG-1 leads to dysregulation of important meiotic processes, including homologous chromosome pairing, as well as increased expression of deleterious genomic elements such as transposons (Cornes et al., 2022; Ortega et al., 2024; Wang and Reinke, 2008).

Multiple secondary WAGOs also display sex-specific roles in regulating germ cell development. As with the primary AGOs associated with 26G-RNAs, certain WAGOs are only expressed during oogenesis or spermatogenesis. Indeed, WAGO-10 and CSR-1a are both only present during spermatogenesis and directly interact with transcripts targeted by ALG-3/4 (Charlesworth et al., 2021; Conine et al., 2013, 2010; Seroussi et al., 2023). VRSA-1, PPW-1, and NRDE-1, in contrast, are only expressed during oogenesis and primarily target improperly expressed spermatogenic and somatic genes within the germline (Guang et al., 2008; Seroussi et al., 2023; Tijsterman et al., 2002b). Sexual dimorphisms in WAGOs that are utilized in both germlines are less well studied. Works have identified that there may be sex-specific gene regulation by these WAGOs, for example WAGO-1 and WAGO-4 22G-RNAs are enriched for oogenic transcripts while WAGO-3 associated 22G-RNAs are enriched for spermatogenic transcripts in adult hermaphrodites undergoing oogenesis (Seroussi et al., 2023). While these data suggest that cytoplasmic WAGOs may aid in sex-specific regulation, the mechanisms leading to sex-specific usage of WAGO-1, WAGO-3, and WAGO-4 remain largely unknown.

It's not just a phase: germ granules in the germline

Numerous components of small RNA pathways, including cytoplasmic AGOs, RNA-dependent RNA polymerases, and functional small RNAs all localize to a specialized sub-compartment of the germline, the germ granule. Cells contain numerous sub-compartments within the cytoplasm allowing for specialized storage and/or biochemical reactions to occur. The most well studied of these compartments have membranes that allow for a clear separation of the organelle from the cytoplasm. More recently, there has been the identification of a plethora of membrane-less organelles, that contain proteins and RNAs and form through liquid-liquid phase separation (Banani et al., 2017; Shin and Brangwynne, 2017). Germ granules are one such biomolecular condensate that forms in the germ cells of nearly all metazoans. First detected in 1890, over 80 species across the animal kingdom have germ granules (Eddy, 1975; Ritter, 1890). For a subsection of species, these granules are present throughout development and are inherited by the embryo through the germ plasma; while in others, most notably mammals, germ granules form *de novo* in primordial germ cells following their specification (Reviewed in Voronina et al., 2011).

Germ granules form through liquid-liquid phase separation (LLPS), a process similar to how oil separates from water (Banani et al., 2017; Brangwynne et al., 2009; Shin and Brangwynne, 2017). LLPS occurs when a subset of molecules de-mixes from the surrounding cytoplasm to form a dense phase and a dilute phase (Hyman et al., 2014). The dense phase results in the formation of spherical droplets, or condensates, with liquid-like properties that are enriched for specific molecules compared to surrounding cytoplasm (dilute phase) (Alberti et al., 2019; Thomas et al., 2023). LLPS is primarily driven by multivalent interactions between proteins and RNAs, and often involves proteins with intrinsically disordered domains (Boeynaems et al., 2018; Shin and Brangwynne, 2017). Resulting condensates, unlike membrane-bound organelles, can still dynamically exchange biomolecules with the cytoplasm (Brangwynne et al., 2009). This dynamic structure makes condensates especially responsive to changes in protein or RNA concentration, post-translational modifications, and temperature (Brangwynne, 2013; Brangwynne et al., 2011; Dodson and Kennedy, 2020; Liu et al., 2024). During meiosis, germ granules respond to meiotic progression, displaying distinct biophysical properties and internal configuration depending on meiotic stage (Ortega et al., 2024; Uebel et al., 2023, 2020).

In *C. elegans*, the germ granule was first discovered by Strome and Wood in 1982 as occurring only in the P lineage of developing embryos that eventually become the germline (Strome and Wood, 1982). While initially dispersed throughout the cytoplasm of the developing embryo, by the 16-cell embryo stage, germ granules are associated with the nuclear periphery of the P4 cell. Germ granules remain nuclear-associated through the development of the germline and meiotic prophase I of spermatocytes and oocytes, only detaching during late stages of maturation (Hird et al., 1996; Pitt et al., 2000; Wolf et al., 1983). Upon reaching the 100-cell stage of the embryo, *C. elegans* germ granules de-mix further into sub-compartments, including the P-granule, Z-granule, *Mutator* foci, E-granule, SIMR-foci, and D compartment (Fig. 1.6) (Chen et al., 2024; Ishidate et al., 2018; Lu et al., 2025; Manage et al., 2020; Strome and Wood, 1982; Uebel et al., 2020; Wan et al., 2018). Each of these compartments contain distinct populations of proteins and RNAs, including messenger RNAs (mRNAs) and small RNAs (sRNAs), that when perturbed affect germ cell development and subsequent fertility of the organism. Below, I summarize key proteins and RNAs found within each germ granule sub-

compartments in *C. elegans* and how they aid in gene regulation during gametogenesis through cooperation with small RNA pathways (Fig. 1.6).

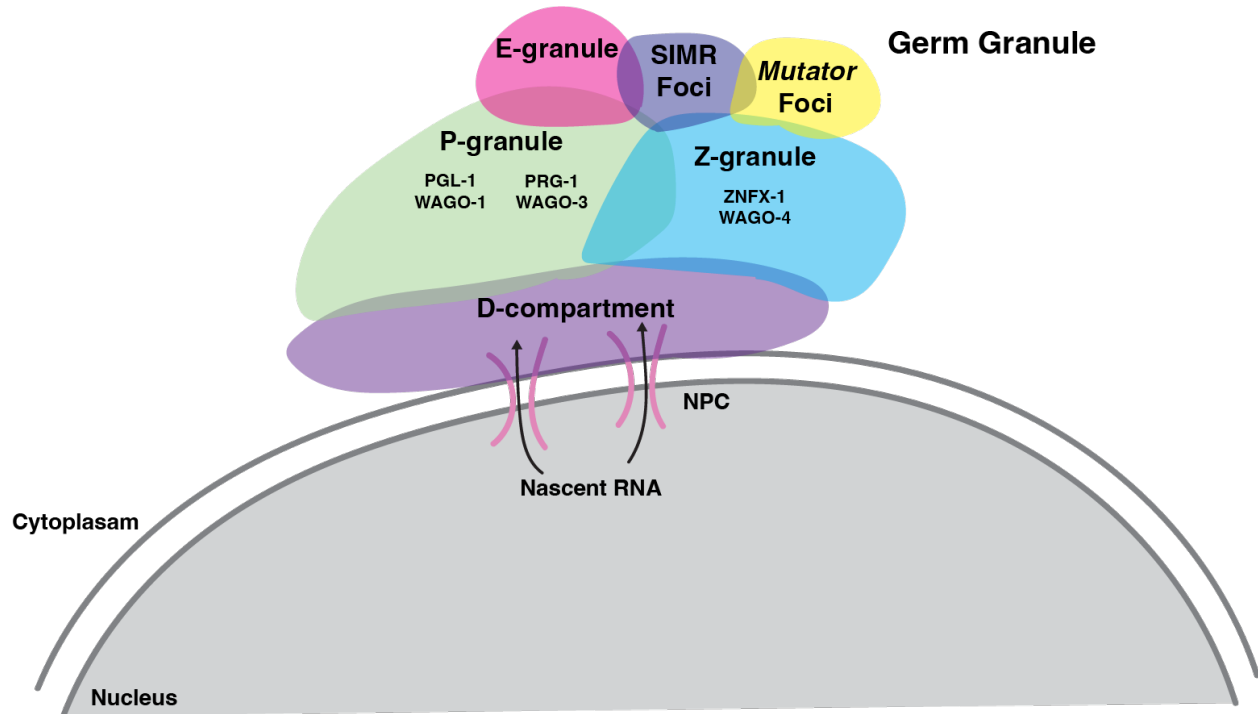


Figure 1.6: Current model of germ granule organization during *C. elegans* oogenesis. Cartoon schematic depicting primary localization patterns observed during oogenesis. Key proteins to this thesis and where they localize based on previous literature are labeled.

P- and Z-granules– Argonaute mediated gene regulation: The most well studied sub-compartment of the *C. elegans* germ granule is the P-granule (Strome and Wood, 1982). Numerous proteins localize with the P-granule transiently through early embryo development, germline establishment, and gametogenesis, with only a sub-set constitutively localizing to the sub-compartment. These include novel *C. elegans* proteins PGL-1 and PGL-3, Vasa RNA helicase homologs GLH-1, 2, 3, and 4, and cytoplasmic Argonautes including CSR-1, PRG-1, WAGO-1, and WAGO-3 (Phillips and Updike, 2022; Schreier et al., 2022; Updike and Strome, 2010). PGL-1 and PGL-3 scaffold the P-granule and drive the phase separation of the sub-compartment. Through dimerization, PGL-1 and PGL-3 have RNA endonuclease activity and are known to repress localized mRNAs within the compartment during oogenesis through interactions with small RNA pathways (Aoki et al., 2021, 2016). Loss of *pgl-1*, and to a lesser extent its paralog *pgl-3*, causes defects in fertility and germ cell proliferation, as well as an

increase in somatic gene expression and a sex-specific increase in spermatogenetic genes during oogenesis (Campbell and Updike, 2015; Kawasaki et al., 2004; Updike et al., 2014).

Multiple small RNAs and recently transcribed mRNAs transiently localize to the P-granule, in part due to the P-granules perinuclear localization. Indeed, over 75% of nuclear pore complexes (NPCs) of developing oocytes and spermatocytes associate with a P-granule foci (Pitt et al., 2000; Sheth et al., 2010). P-granules within the germ line transiently house most mRNAs that are maternally inherited by the embryo and are enriched for in the P-lineage cells (Schisa et al., 2001; Seydoux and Fire, 1994; Sheth et al., 2010). Intriguingly, ribosomal RNAs (rRNAs) and maternally inherited mRNAs of genes expressed in both the soma and germline, including actin and tubulin, are excluded from P-granules, suggesting that these transcripts may be exported from the nucleus through the remaining ~25% of NPCs (Marnik et al., 2019; Schisa et al., 2001; Seydoux and Fire, 1994). In addition to germline transcribed mRNAs, the P-granule houses two distinct classes of small RNAs: piRNAs and 22G-RNAs. piRNAs (PIWI interacting RNAs) are bound by the Argonaute PRG-1 and play a role in silencing transcripts that can damage the genome, such as transposons (Batista et al., 2008; Reed et al., 2020; Shen et al., 2018; Shirayama et al., 2012; Wang and Reinke, 2008). 22G-RNAs associate with AGOs CSR-1, WAGO-1, and WAGO-3 and play a role in both silencing improperly expressed transcripts of somatic genes and transposons, and increasing expression of genes required for meiotic progression (Aoki et al., 2021; Campbell and Updike, 2015; Cecere et al., 2014; Charlesworth et al., 2021; Claycomb et al., 2009; Gu et al., 2009; Seroussi et al., 2023). Disruption to these Argonautes leads to transgenerational infertility during oogenesis.

The Z-granule is the only other sub-compartment in *C. elegans* canonically known to house Argonaute proteins and has been tied to the transgenerational viability of the germline. The germline of sexually reproducing organisms is considered “immortal” due to the ability of germ cells to be passed down continuously through generations (Smelick and Ahmed, 2005). Disruption to defining proteins of the Z-granule, including the well conserved RNA helicase and zinc-finger domain protein, ZNFX-1, and the secondary AGO, WAGO-4, causes a mortal germline phenotype, with more extreme sterility at higher temperatures (Seroussi et al., 2023; Wan et al., 2018; Xu et al., 2018). Furthermore, at physiological rearing temperatures, perturbations to the Z-granule demonstrate defects in the transgenerational epigenetic inheritance (TEI) (Ishidate et al., 2018; Wan et al., 2018; Xu et al., 2018). The inability to passage silencing

signals from developing oocytes to an embryo may be tied to the role of the Z-granule in mediating the formation of 22G-RNAs (Ishidate et al., 2018; Ouyang et al., 2022; Shukla et al., 2020). Specifically, the Z-granule is believed to house mRNAs used as templates for 22G-RNA transcription, ensuring their inheritance for continued silencing by the next generation, as well as mediating the region of the mRNA utilized as a template for 22G-RNAs (Ishidate et al., 2018; Ouyang et al., 2022; Shukla et al., 2020).

***Mutator* foci and E-granules– small RNA production:** The *Mutator* foci houses components required for proper function of small RNA pathways but lacks Argonaute proteins required for the physical act of gene regulation. Assembly of *Mutator* foci is driven by the scaffolding protein MUT-16, which both drives phase separation of the foci from the cytoplasm and aids in the recruitment of other MUT proteins which form the *mutator* complex to the granule (Phillips et al., 2012; Uebel et al., 2018). The *mutator* complex is comprised of proteins that function in the production of 22G-RNAs bound by the Argonautes WAGO-1, WAGO-3, and WAGO-4, and 26G-RNAs bound by the oocyte specific AGO, ERGO-1 (Chen et al., 2024; Gu et al., 2009; Ketting et al., 1999; Kim et al., 2005; Phillips et al., 2012, 2014; Alicia K. Rogers and Phillips, 2020; Tijsterman et al., 2002; Tops et al., 2005; Tsai et al., 2015; Vastenhouw et al., 2003). Disruption of this granule sub-compartment has been tied to the transgenerational sterility of oogenetic germlines and the complete sterility of spermatogenetic germlines when exposed to acute heat stress (Rogers and Phillips, 2020). In addition to housing the actively forming siRNA species, *Mutator* foci also contain mRNAs recruited to the granule to serve as templates for 22G- and 26G-RNAs amplification (Phillips et al., 2012; Shukla et al., 2020; Uebel et al., 2018).

The E-granule is hypothesized to serve a similar role as the *Mutator* foci in the creation of 22G-RNAs from target mRNAs through housing an RNA-dependent RNA polymerase (RdRP) (Chen et al., 2024). While the *Mutator* foci houses the RdRP RRF-1 that synthesizes 22G-RNAs known to bind WAGO Cluster Argonautes, WAGO-1 and WAGO-3 (Gent et al., 2010; Gu et al., 2009; Phillips et al., 2012), the CSR-1 Cluster RdRP, EGO-1, localizes to the E-granule (Chen et al., 2024). While both RdRPs synthesize 22G-RNAs, the resulting species have little overlap between those transcribed by EGO-1 versus RRF-1 (Chen et al., 2024; Claycomb et al., 2009; Gu et al., 2009).

SIMR Foci – a bridge between the granules: The SIMR foci mediates the transcription of small RNAs within the *Mutator* foci triggered by piRNA targeting by PRG-1 and their eventual association with WAGO-1 (22G-RNAs) in the P-granule. Imperative for SIMR foci formation is the protein SIMR-1 (siRNA defective and mortal germline), which primarily localizes adjacent to the P-granule and the *Mutator* foci (Manage et al., 2020; Uebel et al., 2023). This localization pattern establishes the SIMR foci as a mediator of the production and binding of piRNAs by PRG-1 within the P-granule and the creation of 22G-RNAs transcribed from piRNA-targeted mRNAs in the *Mutator* foci (Manage et al., 2020; Uebel et al., 2023). Additionally, the localization of both the Z-granule and the P-granule within the same germ granule is required for the recruitment of SIMR and *Mutator* foci, suggesting that components of the Z-granule may also aid in SIMR-1 mediated piRNA to 22G-RNA transcription (Uebel et al., 2023). This is further supported by the mortal germline phenotype observed with both perturbed Z-granule components and with loss of the SIMR foci in both spermatogenic and oogenic germlines, suggesting that regulation by the small RNA pathways is reliant on proper SIMR-1 function (Manage et al., 2020).

The D-compartment – anchor to the nuclear pores: The most recently identified sub-compartment of the *C. elegans* germ granule is the D-compartment (Huang et al., 2024; Lu et al., 2025). Unlike the other sub-granules described above that directly regulate distinct steps in gene regulation by small RNA pathways, the D-compartment anchors the other compartments of the germ granule to the nuclear pore of developing germ cells (Lu et al., 2025). The loss of this anchoring coincides with phenotypes associated with mRNAs escaping surveillance by small RNA pathways including misregulation of genes, defects in transgenerational epigenetic inheritance, and loss of germline immortality (Lu et al., 2025). The requirement of the D-compartment for transgenerational fertility suggests that the localization of germ granules to the nuclear periphery of developing germ cells may serve as a mechanism for ensuring transgenerational epigenetic inheritance of gene expression mediated by small RNA pathways.

In summary, components of the *C. elegans* germ granule are vital for preserving fertility through generations, independent of sex. Within the germline this may be mediated through organizing components of small RNA pathways into distinct sub-compartments of the germ granule. Indeed,

proteins known to be necessary for gene regulation by small RNA pathways are found within each sub-compartment. Disruption of these sub-compartments through loss of structural germ granule components leads to similar defects observed in organisms lacking small RNA pathway components, such as loss of transgenerational epigenetic inheritance and mortal germlines. Previous works looking germ granules and how they organize small RNA pathway components throughout meiosis have primarily occurred during oogenesis. Studies examining germ granules during spermatogenesis have focused on identifying if small RNA and structural proteins are conserved between the process and have aided in finding sex-specific players. Most germ granules in *C. elegans* though, are comprised of the same proteins independent of sex. How small RNA pathways and their localization within the germ granule confer sex-specific gene regulation therefore remains an open area of research.

Illuminating the interplay between small RNA pathways and germ granule organization

The majority of work determining AGO function within the germline has built off the hypotheses that the localization of AGOs and other small RNA pathway components to the germ granule is vital for mediating gene regulation (Reviewed in: Dodson and Kennedy, 2020; Phillips and Updike, 2022; Sundby et al., 2021). Indeed, the model presented above (Fig. 1.6) postulates that germ granules are functional condensates and that their localization to the nuclear periphery and the internal organization of small RNA pathway components inside mediates function. Recent works, though, suggests an alternative hypothesis for germ granule structure and function: the germ granule and its resulting sub-compartments are incidental condensates and are expandable for proper gene regulation by small RNA pathways in the germline (Lee et al., 2020; Ouyang and Seydoux, 2022; Putnam et al., 2023). Indeed, a growing body of work finds that the localization of the germ granule to the nuclear pore complex is not required for proper regulation of target transcripts (Thomas et al., 2025). Furthermore, CSR-1 and its associated RdRP EGO-1 do not require localization to the germ granule for 22G-RNA biogenesis, nor do mRNAs destined for degradation by sRNA pathways (Lee et al., 2020; Singh et al., 2021). The formation and biophysical properties of the germ granule therefore may not rely on active gene regulation within the phase-separated compartment but rather act as a physical read out of how different proteins within small RNA pathways may interact within the dilute phase of the cytoplasm.

As recent work has further interrogated the interplay between gene regulation by small RNA pathways and organization within the germ granule during gametogenesis, novel models have emerged suggesting that the localization of Argonautes within these sub-compartments are less defined than previously believed. A number of these studies suggest that certain Argonautes ascribed to localize within just one sub-compartment, actually span multiple (Charlesworth et al., 2021; Huang et al., 2024; Seroussi et al., 2023). PRG-1 and CSR-1 were previously hypothesized to localize within the P-granule during oogenesis, but recent works have found that both localize with multiple germ granule sub-compartments (Batista et al., 2008; Charlesworth et al., 2021; Claycomb et al., 2009; Huang et al., 2024; Nguyen and Phillips, 2021; Seroussi et al., 2023; Spichal et al., 2021). PRG-1 associates with both the P- and Z-granule during oogenesis, while CSR-1 associates with the P- and Z- granule, as well as the D-compartment (Huang et al., 2024; Seroussi et al., 2023). These changes in protein localization from single to multiple sub-compartments are in part due to improvements in microscopy techniques that better resolve the divisions of these granules (Huang et al., 2024; Suen et al., 2023; Uebel et al., 2023). However, these same techniques have challenged the paradigm of distinct boundaries existing between well-established sub-compartments, specifically the Z- and P-granule (Suen et al., 2023). Additionally, most studies utilize bulky fluorescent tags to study AGOs and other germ granule components within the germline, due to initial works aiming to determine if these proteins behave as liquid-like condensates *in vivo* (Brangwynne et al., 2009). Recent observations have found that use of fluorescent tags on phase-separated germ granule components leads to tag-dependent biophysical properties, as well as fertility defects (Seroussi et al., 2023; Uebel and Phillips, 2019). Utilization of these tags may therefore disrupt native interactions and properties required for localization of these proteins within the germ granule.

In these dissertation studies I utilize *C. elegans* as an ideal model system to determine sexually dimorphic attributes of how AGO proteins, WAGO-1 and WAGO-4, influence gene regulation during gametogenesis. To probe the discrete localization patterns of WAGO-1 and WAGO-4 without interference from possibly detrimental tags, I generated antibodies directly against native WAGO-1 and WAGO-4 structure. Using these antibodies, I demonstrate that WAGO-1 and WAGO-4 display dynamic and sexually dimorphic localization patterns and biophysical properties throughout meiotic prophase I. I further determine that both WAGO-1 and WAGO-4 localize in a sexually dimorphic manner to the germ granule that fails to adhere to

previous studies demonstrating discrete sub-compartmentalization of these proteins within the P- and Z-granules respectively. Additionally, by exploiting the genetic toolkit of *C. elegans*, I interrogate the role of WAGO-1 structural domains on mediating both germ granule structure, as well as gene regulation during oogenesis and spermatogenesis. I determine that disruptions to WAGO-1 protein structure leads to sex-specific dysregulation germ granule structure, as well as of mRNA and small RNA transcriptional landscapes. Taken together my thesis work highlights sexual dimorphisms in small RNA pathway gene regulation during egg and sperm development, and how these differences may be driven by sex-specific configurations and biophysical properties of components of the germ granule.

Bridge to Chapter 2:

In Chapter 1, I introduced key concepts in meiosis and germ cell development and present the nematode *Caenorhabditis elegans* as an ideal model system for understanding sex-specific differences of these processes. I expanded upon these concepts by presenting the role of small RNA pathways and germ granules in regulating gene expression germ cell development. I highlight that while these pathways and their localization to germ granules has been highly characterized during oogenesis, little is known about mechanisms of small RNA pathway gene regulation during spermatogenesis. In Chapter 2, I utilize a whole germline analysis pipeline to characterize the structure and localization of an Argonaute protein used by multiple small RNA pathways, WAGO-1, during egg and sperm development. I further couple these cytological analyses with mutant and genomic techniques to demonstrate that proteins within small RNA pathways localize to distinct sub-compartments of the germ granule, and disruption to germ granule structure leads to sex-specific misregulation of genes and infertility.

CHAPTER 2: WAGO-1 IS A SEXUALLY DIMORPHIC ARGONAUTE PROTEIN REQUIRED FOR PROPER GERM GRANULE STRUCTURE AND GAMETOGENESIS

(Submitted to *Development*)

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keywords: germ granules, small RNAs, germ cell development, sexual dimorphisms, gametogenesis, oogenesis, spermatogenesis, Argonautes, PIWI pathway, *C. elegans*

Abstract:

Germ cell proliferation and proper genome inheritance are critical for maintaining fertility through generations. To promote proper germ cell development, small RNA pathways employ Argonaute proteins (AGOs) to modulate gene expression and protect against deleterious genomic elements while not silencing against self. Here we identify the Argonaute WAGO-1 is sexually dimorphic in its localization and the function of its protein structural features that affects sex-specific gene regulation during *C. elegans* germ cell development. During meiotic prophase I progression, we find that germ granule structural proteins and the PIWI AGO, PRG-1, display dynamic and distinct localization patterns between egg and sperm development which coincide with differential WAGO-1 localization and biophysical properties. Sexually dimorphic functions of specific WAGO-1 protein structural domains underpin these differences. Disruption or modification to the N-terminus intrinsically disordered region (IDR) of WAGO-1 leads to loss of PGL-1 phase separation only during spermatogenesis. Further, we find that these germ granule disruptions are likely due to prolonged association of the IDR with the RNA-binding pocket of WAGO-1. In addition, deletion of the MID and part of the PIWI domains causes male-specific sterility and disruption to WAGO-1 localization with PGL-1 during oogenesis. Finally, we demonstrate that these disruptions to WAGO-1 protein structure dynamically change the mRNA and sRNA landscape of adult males and hermaphrodites, in which the AGOs ALG-3/4 and VSRA-1 are misregulated. Together, these data suggest that WAGO-1 differentially regulates genes during oogenesis versus spermatogenesis, and that these differences in gene regulation may be due to the sex-specific configuration and biophysical properties of WAGO-1 within the germ granule.

Introduction

Germ cell proliferation and immortality are critical for fertility and proper genome inheritance by the next generation. To generate viable egg and sperm cells, the regulation of the genome is finely tuned to express genes vital for meiotic progression and gametogenesis, while simultaneously suppressing deleterious genomic elements, such as pseudogenes, transposons, and somatic genes (Choi and Lee, 2020; Fischer et al., 2011; Kurhanewicz et al., 2020; Reinke et al., 2000; Roberts and Morris, 2013). During oogenesis and spermatogenesis, the licensing and repression of genes is regulated by small RNA (sRNA) pathways. Widely conserved in eukaryotes, sRNA pathways modulate gene expression from transcription through translation both during periods of homeostasis and stress, and are essential for persevering fertility (Cornes et al., 2022; Malone and Hannon, 2009; Ortega et al., 2024; Wang and Reinke, 2008).

Functionality of small RNA pathways rely on the Argonaute (AGO) family of proteins. AGOs are bilobed proteins that contain four structural domains: the N-terminal lobe is comprised of the intrinsically disordered (IDR) N-terminus and PAZ (PIWI-Argonaute-Zwille) domains, while the MID (middle), and PIWI (P element-induced wimpy testis) domains comprise the C-terminal lobe (Fig. S2.1) (Sheu-Gruttadauria and MacRae, 2017). Small RNAs bind within the hydrophilic binding pocket formed between the two lobes, with the 3' and 5' ends of small RNAs binding with the PAZ and MID domains, respectively. Through binding of small RNAs, AGOs target sequences for licensing or silencing through direct Watson-Crick base-pairing interactions with complementary target RNA or DNA sequences (Dueck and Meister, 2014). The PIWI domain has structural similarities to RNase H, although the majority of AGOs lack catalytically active RNase function (Nakanishi et al., 2012). Mutations in Argonaute proteins and other machinery required for proper regulation by sRNA pathways have been tied to birth defects, developmental disorders, and infertility (Conine et al., 2013; Duan et al., 2024; Gou et al., 2017; Lessel et al., 2020; Seroussi et al., 2023; Spichal et al., 2021; Wan et al., 2018).

Small RNA pathways employ a variety of non-coding sRNAs (18-30nt) for directed targeting of specific transcript populations during germ cell development. In *Caenorhabditis elegans*, at least four distinct classes of endogenously encoded small RNAs are utilized for gene regulation: miRNAs, piRNAs, 26G- and 22G-RNAs. Pathways employing these sRNAs utilize two main mechanisms for target gene regulation: direct sRNA/target site binding interactions and a feedforward amplification of sRNAs triggered by primary sRNA interactions with AGOs

(Ketting and Cochella, 2021). The miRNA pathway utilizes this first mechanism, known as miRNA-mediated repression, which relies on specific sRNA/binding site interactions on the target transcript to trigger mRNA degradation and translation repression (Duchaine and Fabian, 2019; Ketting and Cochella, 2021). In contrast, piRNAs, 26G-RNAs, and 22G-RNAs are all utilized by small RNA pathways that amplify sRNAs when regulating target genes using primary and secondary AGOs.

piRNAs, also known as 21U-sRNAs in *C. elegans*, and 26G-RNAs are upstream sRNAs that are loaded onto well-conserved primary AGOs. PRG-1, the only PIWI AGO in the *C. elegans* genome, specifically binds to piRNAs and primarily targets transposons and pseudogenes in the germline (Batista et al., 2008; Das et al., 2008; Spichal et al., 2021; Wang and Reinke, 2008). ERGO-1, ALG-3, and ALG-4 all bind 26G-RNAs, a class of small interfering RNAs (siRNAs) that are primarily complementary to protein coding genes expressed in the germline (Conine et al., 2013, 2010; Han et al., 2009; Seroussi et al., 2023; Vasale et al., 2010). Following primary AGO binding, the sRNA-AGO complexes are recruited to target mRNA transcripts and transcribe a secondary class of small interfering RNAs, 22G-RNAs, with RNA-dependent RNA Polymerases (RdRPs) (Bagijn et al., 2012; Claycomb et al., 2009; Gent et al., 2010; Gu et al., 2009). 22G-RNAs are then loaded onto a distinct, worm specific clade of AGOs, termed WAGOs, for targeted gene regulation post- and co-transcriptionally (Buckley et al., 2012; Charlesworth et al., 2021; Claycomb et al., 2009; Gu et al., 2009; Guang et al., 2008; Nguyen and Phillips, 2021; Seroussi et al., 2023; Xu et al., 2018; Yigit et al., 2006). WAGOs bind specific 22G-RNAs based on the primary AGO utilized for mRNA targeting. For example, 22G-RNAs derived from sequences targeted by PRG-1 and piRNAs are primarily loaded onto the secondary AGOs from the WAGO Cluster, which includes WAGO-1 and PPW-2 (Barucci et al., 2020; Buckley et al., 2012; Reed et al., 2020; Schreier et al., 2022; Seroussi et al., 2023; Shirayama et al., 2012; Spichal et al., 2021). In contrast, 22G-RNAs derived from ALG-3/4 target sequences, which are primarily comprised of genes expressed during spermatogenesis, are loaded onto WAGO-10 and a sperm-specific CSR-1 isoform (Charlesworth et al., 2021; Conine et al., 2010; Nguyen and Phillips, 2021; Seroussi et al., 2023).

In *C. elegans* both the piRNA and the 26G-RNA pathways display gamete-specific gene regulation. Most evident is the expression of the primary AGOs associated with the 26G-RNA pathway: ERGO-1, ALG-3, and ALG-4. ERGO-1 is only expressed in germlines undergoing

oogenesis (adult hermaphrodites) while ALG-3 and ALG-4 are only expressed in germlines undergoing spermatogenesis (adult males and larval stage 4 hermaphrodites) (Conine et al., 2013, 2010; Seroussi et al., 2023; Vasale et al., 2010). Regulation by the piRNA pathways is similarly sexually dimorphic. Previous work in mammalian systems have identified proper regulation of piRNAs by PIWI-homologs is required for spermatocyte development, while being expendable for egg development (Deng and Lin, 2002; Kuramochi-Miyagawa et al., 2004). Similarly in *C. elegans*, the role of piRNAs and the PIWI AGO PRG-1 is sex specific, with proper PRG-1 function being required for spermatogenesis and male fertility (Cornes et al., 2022; Ortega et al., 2024; Reed et al., 2020; Reinke et al., 2000). Regulation by PRG-1 and downstream WAGOs, specifically WAGO-1 and CSR-1, ensures that meiotic genes are properly expressed during pachytene while simultaneously silencing deleterious genomic elements, such as transposons, in the male germline (Barucci et al., 2020; Charlesworth et al., 2021; Cornes et al., 2022; Nguyen and Phillips, 2021; Ortega et al., 2024). In contrast, during oogenesis PRG-1 and the piRNA pathway regulates transgenerational fertility by ensuring proper histone and ribosomal RNA expression (Barucci et al., 2020; Reed et al., 2020; Wahba et al., 2021).

Numerous components of small RNA pathways are known to localize to germ granules; liquid-like, phase separated compartments perinuclear to developing germ cells. These components include, but are not limited to: primary AGOs PRG-1, ALG-3 and ALG-4, secondary AGO WAGO-1, and RdRPs used for 22G-RNA transcription (Batista et al., 2008; Chen et al., 2024; Conine et al., 2013, 2010; Gu et al., 2009; Huang et al., 2024; Ortega et al., 2024; Phillips et al., 2012; Seroussi et al., 2023). During germ cell development, germ granules separate into at least six distinct sub-compartments: the P-granule, the Z-granule, *Mutator* foci, SIMR foci, the E-granule, and the D-compartment (Chen et al., 2024; Huang et al., 2024; Lu et al., 2025; Manage et al., 2020; Ouyang et al., 2022; Phillips and Updike, 2022; Seroussi et al., 2023; Uebel et al., 2023, 2020; Updike and Strome, 2010; Wan et al., 2018). Interestingly, the transgenerational sterility observed in *prg-1* mutants appears to be caused by disruption specifically to the P-granule structure (Spichal et al., 2021).

The P-granule is the most well studied of the germ granules and is identified by the presence of PGL-1, a scaffolding protein known to aid in the repression of mRNA translation in developing germ cells (Aoki et al., 2021; Kawasaki et al., 2004, 1998; Strome and Wood, 1982). Deletion of *pgl-1* has been tied to transgenerational loss of fertility, underproliferation of

germlines, and improper expression of somatic and sex-specific genes (Kawasaki et al., 2004, 1998; Updike et al., 2014). PGL-1 helps maintain fertility through translation suppression of mRNA mediated by WAGO-1 and PRG-1 (Aoki et al., 2021; Batista et al., 2008; Gu et al., 2009; Ouyang et al., 2019; Seroussi et al., 2023; Shirayama et al., 2012; Updike et al., 2014). WAGO-1 specifically has been implicated as a secondary AGO whose dysregulation causes transgenerational infertility due to erroneous mRNA silencing by the P-granule (Aoki et al., 2021; Barucci et al., 2020; Shirayama et al., 2012). Loss of *wago-1* causes loss of 22G-RNAs against transposons, pseudogenes, and other WAGO class genes suggesting WAGO-1 is required for proper repression of deleterious elements targeted by sRNA pathways (Gu et al., 2009; Seroussi et al., 2023). Additionally, WAGO-1 demonstrates a sexually dimorphic targeting of transcripts by the piRNA pathway. Specifically, within the WAGO Cluster of secondary AGOs, WAGO-1 primarily targets transcripts required for proper progression through oogenesis, while PPW-2 targets spermatogenetic genes (Schreier et al., 2022; Seroussi et al., 2023). WAGO-1 is also required for persevering fertility of *C. elegans* hermaphrodites. Tagging of the intrinsically disordered N-terminus of WAGO-1 causes a decrease in fertility, suggesting wild-type WAGO-1 structure is required for proper germ cell development (Seroussi et al., 2023). The mechanisms leading to sex-specific usage of WAGO-1, however, remain largely unclear.

WAGO-1 and PGL-1 are also known to interact with ZNFX-1, a well conserved zinc finger helicase that marks the Z-granule (Ishidate et al., 2018; Wan et al., 2018). Loss of ZNFX-1 similarly causes transgenerational loss of fertility, as well as defects in both endogenous and exogenous targeting by small RNA pathways through interaction with secondary AGO, WAGO-4 (Ishidate et al., 2018; Ouyang et al., 2022; Seroussi et al., 2023; Wan et al., 2018). Both ZNFX-1 and PGL-1 are hypothesized to play a role in ensuring proper gene regulation by small RNA pathways during germ cell development. Most studies examining the interplay of ZNFX-1 and PGL-1 with WAGO-1 in monitoring transcript expression has occurred during oogenesis (Aoki et al., 2021; Barucci et al., 2020; Gu et al., 2009; Ishidate et al., 2018; Seroussi et al., 2023; Wan et al., 2018). Work looking at the role of ZNFX-1, PGL-1, and WAGO-1 during spermatogenesis has found that these proteins are present and co-localize to the germ granule (Campbell and Updike, 2015; Charlesworth et al., 2021; Conine et al., 2013, 2010; Nguyen and Phillips, 2021; Seroussi et al., 2023), but their role and how it may differ between the sexes remains unknown.

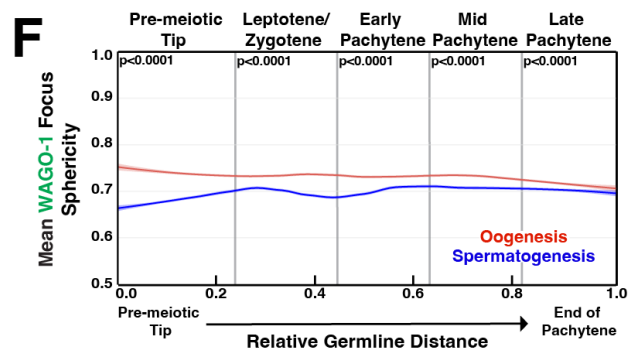
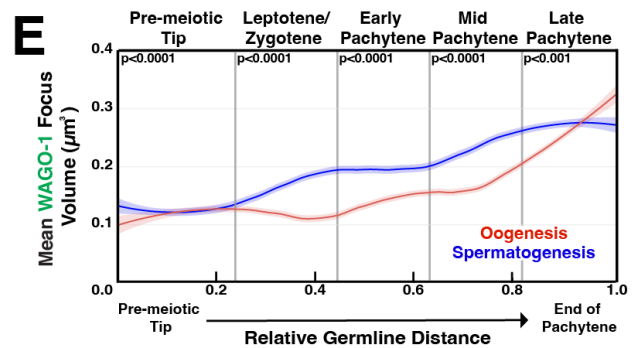
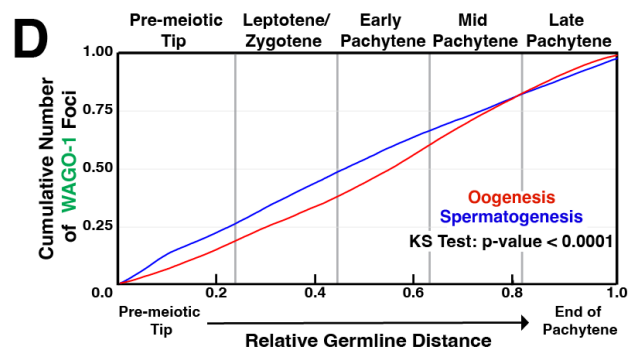
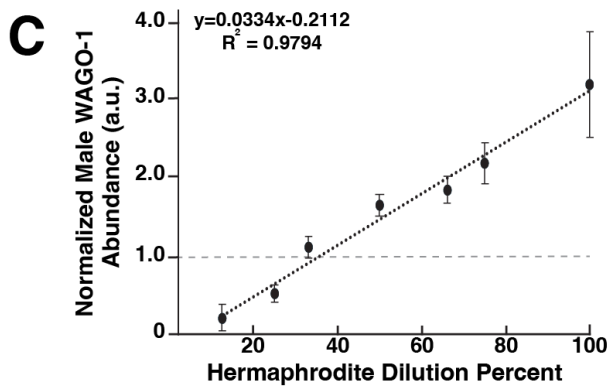
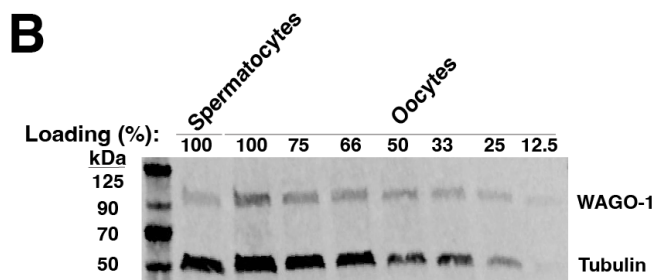
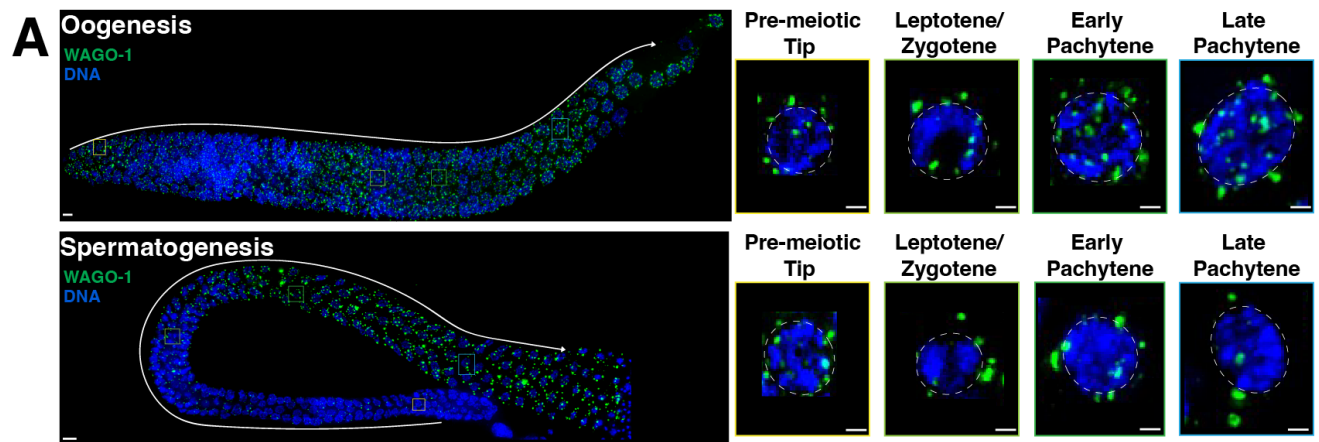
Here we demonstrate that WAGO-1 is a sexually dimorphic Argonaute, with distinct dynamics and localization with germ granule components ZNFX-1, PGL-1, as well as with the PIWI AGO, PRG-1, during oogenesis and spermatogenesis. Further, we find that the structural domains of WAGO-1 are sexually dimorphic in their role for maintaining germ granule formation, fertility, and gene expression. Together, our findings indicate that small RNA pathways are sexually dimorphic in their modulation of germ cell development, and that these differences may be due to sexually dimorphic AGO localization and interactions within the germ granule.

Results

WAGO-1 abundance and accumulation within the germ granule is sexually dimorphic during oogenesis and spermatogenesis

The Argonaute WAGO-1 is a known germ granule component in larval stage 4 (L4) hermaphrodites undergoing spermatogenesis (Seroussi et al., 2023) and adult hermaphrodites undergoing oogenesis (Gu et al., 2009; Seroussi et al., 2023; Aoki et al., 2021). To examine the expression and localization of WAGO-1 in adult males undergoing spermatogenesis compared to adult hermaphrodites undergoing oogenesis, we raised an antibody against native WAGO-1 (Fig. S2.2). Utilizing this antibody, we performed fixed immunofluorescent microscopy to examine WAGO-1 localization and morphology throughout germlines undergoing either oogenesis or spermatogenesis. We found WAGO-1 forms foci in the cytoplasm around the periphery of

Figure 2.1: WAGO-1 localization and protein levels are sexually dimorphic within the germline. (A) Representative immunofluorescence images of WAGO-1 (green) throughout meiosis of wild-type adult hermaphrodites (oogenesis, top) and adult males (spermatogenesis, bottom). Enlarged panels display individual nuclei from four distinct stages. Scale bars represent 1 μ m in insert panels and 10 μ m in full gonad images. (B) Dilution series Western blot with adult hermaphrodite samples to determine the dilution level necessary for hermaphrodite (oogenesis) WAGO-1 amounts to equal male (spermatogenesis) WAGO-1 amounts. The 100% loading is the undiluted sample for both hermaphrodites and males. (C) Quantification of WAGO-1 band normalized to the averaged undiluted male WAGO-1 amounts. (D) Empirical cumulative distribution function (ECDF) plot of WAGO-1 foci per germline through oogenesis (red) and spermatogenesis (blue) as a function of meiotic progression. (E) Quantification of mean WAGO-1 foci volume through oogenesis (red) and spermatogenesis (blue). p-values calculated using Mann-Whitney *U* tests. (F) Quantification of mean WAGO-1 foci sphericity through oogenesis (red) and spermatogenesis (blue). p-values calculated using Mann-Whitney *U* tests.



developing germ cell nuclei starting in the pre-meiotic tip (PMT) and persisting through late pachytene (LP) during oogenesis (Fig. 2.1A, top) and spermatogenesis (Fig. 2.1A, bottom). Despite the presence of WAGO-1 foci during both processes, males undergoing spermatogenesis have 36% of the protein levels compared to hermaphrodites undergoing oogenesis (Fig. 2.1B, C). WAGO-1 transcripts are similarly downregulated in adult males compared to adult hermaphrodites (Fig. S2.3). Despite lower WAGO-1 protein levels, relatively more WAGO-1 foci accumulate during spermatogenesis through mid-pachytene (MP) compared to oogenesis (Fig. 2.1D). This difference could be attributed to the higher proportion of WAGO-1 within foci compared to diffuse throughout the cytoplasm during spermatogenesis compared to oogenesis (Fig. S2.4). Taken together, our data suggest that while WAGO-1 transcript and protein levels are higher during oogenesis, WAGO-1 foci accumulate faster during early spermatogenesis.

WAGO-1 displays sexual dimorphism in biophysical properties during meiotic prophase I

The formation of phase separated condensates is often driven by interactions between multivalent proteins with intrinsically disordered regions (IDRs) and RNAs, and is fine-tuned by the protein concentrations within the cytoplasm (Alberti et al., 2019; André and Spruijt, 2020; Boeynaems et al., 2018; Brangwynne et al., 2009; Shin and Brangwynne, 2017; Thomas et al., 2023). Additionally, the volume of condensates can act as a read out on the overall concentration of phase-separated protein, with larger volume being tied to higher protein concentrations within the cytoplasm (Brangwynne, 2013; Brangwynne et al., 2011; Folkmann et al., 2021). To better understand dynamics of WAGO-1 concentration and its ability to phase separate throughout meiosis, we calculated the volume and sphericity of each individual WAGO-1 focus throughout germlines undergoing either oogenesis (Fig. 2.1A, top) or spermatogenesis (Fig. 2.1A, bottom). We found that WAGO-1 foci volume is different between sexes and this difference depends on the meiotic stage (Fig. 2.1E; Table S2.1). In the PMT, WAGO-1 foci were significantly larger during spermatogenesis compared to oogenesis, primarily driven by differences very early in the region (Fig. 2.1E, PMT oogenesis = $0.124\mu\text{m}^3$, spermatogenesis = $0.126\mu\text{m}^3$, Mann Whitney U test $p < 0.0001$; Table S2.1). Upon entering meiosis I, as marked by the leptotene/zygotene (L/Z) zone, WAGO-1 foci rapidly gained volume during spermatogenesis compared to oogenesis (Fig. 2.1E, L/Z oogenesis = $0.117\mu\text{m}^3$, spermatogenesis = $0.172\mu\text{m}^3$, Mann Whitney U test $p < 0.0001$; Table S2.1), and remained significantly larger during spermatogenesis, despite increases in foci

volume in both sexes through early and mid-pachytene (Fig. 2.1E, EP oogenesis = $0.143\mu\text{m}^3$, spermatogenesis = $0.194\mu\text{m}^3$, Mann Whitney U test $p < 0.0001$; MP oogenesis = $0.168\mu\text{m}^3$, spermatogenesis = $0.237\mu\text{m}^3$, Mann Whitney U test $p < 0.0001$; Table S2.1). By late pachytene, though, WAGO-1 foci volume plateaus during spermatogenesis while continuing to grow during oogenesis (Fig. 2.1E). Overall spermatogenetic WAGO-1 foci are larger during LP, despite oogenesis having larger WAGO-1 foci in the final third of the zone (Fig. 2.1E, LP oogenesis = $0.261\mu\text{m}^3$, spermatogenesis = $0.274\mu\text{m}^3$, Mann Whitney U test $p < 0.001$; Table S2.1). These results suggest that WAGO-1 foci size is dynamic and sexually dimorphic through oogenesis and spermatogenesis, and the magnitude of these differences are not consistent throughout meiotic I progression.

To assess sexual dimorphisms in the liquid-like properties of these condensates during germ cell development, we interrogated the sphericity of WAGO-1 foci. Previous work looking at proteins that undergo liquid-liquid phase separation (LLPS) have correlated sphericity of foci formed with liquid-like nature of the protein and associated RNA (Alberti et al., 2019; Brangwynne et al., 2009; Folkmann et al., 2021; Ma et al., 2021; Molliex et al., 2015). Less spherical foci tend to contain proteins or RNAs that are more gel-like in nature, leading to less protein turnover with the cytoplasm compared to more spherical, and therefore liquid-like, foci (Alberti and Hyman, 2021; Brangwynne et al., 2009; Cochard et al., 2022; Folkmann et al., 2021; Kroschwald et al., 2015; Molliex et al., 2015). Our quantitative image analysis found that WAGO-1 foci are significantly more spherical during oogenesis compared to spermatogenesis across all stages of meiotic prophase I sampled (Fig. 2.1F; Table S2.3). Additionally, we found that WAGO-1 foci in both sexes display opposite trends in sphericity as they progress through meiosis, with foci growing less spherical through oogenesis and more spherical through spermatogenesis, converging by the end of pachytene (Fig. 2.1F; Table S2.3). Taken together, our data suggests that WAGO-1 foci are biophysically distinct between oogenesis and spermatogenesis. These differences suggest that the structure of germ granules is distinct between the sexes and rapidly responds to meiotic progression.

Sexually dimorphic colocalization of WAGO-1 with structural germ granule components ZNFX-1 and PGL-1

Prior studies in L4 hermaphrodites (spermatogenesis) and adult hermaphrodites (oogenesis) identified WAGO-1 as a germ granule component in *C. elegans* that co-localizes and directly interacts with structural germ granule component PGL-1, a marker of the P-granule (Aoki et al., 2021; Gu et al., 2009; Seroussi et al., 2023). Additionally, WAGO-1 was found to interact with the Z-granule component ZNFX-1, and displayed a modest amount of overlap with the Z-granule during oogenesis (Ishidate et al., 2018; Seroussi et al., 2023). To determine if WAGO-1 localizes with PGL-1 and ZNFX-1 in adult males undergoing spermatogenesis and how these localization patterns compare to oogenesis, we analyzed the percent of WAGO-1 foci volume that overlaps with PGL-1 and ZNFX-1 during three distinct meiotic stages: leptotene/zygotene (L/Z), early pachytene (EP), and late pachytene (LP). During spermatogenesis and oogenesis, we found that majority of WAGO-1 foci overlaps with PGL-1 (Fig. 2.2A-B). Furthermore, in both sexes, the percent of WAGO-1 foci that overlapped with PGL-1 increased from L/Z to LP, suggesting that WAGO-1 localization within the germ granule is dynamic through meiosis. Despite similar localization patterns with PGL-1 during both meiotic processes, WAGO-1 localized significantly more with PGL-1 during spermatogenesis compared to oogenesis in L/Z and LP (Fig. 2.2B L/Z Chi-square test $p < 0.0001$; EP Chi-square test $p = 0.0575$; LP Chi square test $p = 0.0001$; Fig. S2.5A). Additionally, WAGO-1 foci were significantly more likely to have greater than 25% of their volume overlapping with PGL-1 during all stages of spermatogenesis compared to oogenesis (Fig. 2.2B L/Z oogenesis =

Figure 2.2: WAGO-1 co-localization with PGL-1 and ZNFX-1 is sexually dimorphic and dynamic through meiotic progression. (A,C) Representative images of (A) WAGO-1 (green) and PGL-1 (green) or (C) WAGO-1 (green) and ZNFX-1 (magenta) undergoing oogenesis (top) or spermatogenesis (bottom) during L/Z, EP, and LP. Scale bars represent 1 μ m. (B,D) Bar graphs indicating percentage of WAGO-1 foci volume overlapping with (B) PGL-1 foci and (D) ZNFX-1 foci between oogenesis and spermatogenesis from meiotic regions L/Z, EP, and LP. The numbers reported indicate the percentage of WAGO-1 foci that have 0% volume overlap with p-values of Chi-square test with Bonferroni correction. (E, F, G) Representative images of WAGO-1 (green), PGL-1 (yellow), and ZNFX-1 (magenta) localization around single nuclei undergoing oogenesis (top) and spermatogenesis (bottom) during (E) L/Z, (F) EP, and (G) LP. Venn Diagrams show percent WAGO-1 foci overlapping with ZNFX-1 foci (top), PGL-1 foci (bottom), and with both PGL-1 and ZNFX-1 foci (middle).

21.9%, spermatogenesis = 41.4% Chi square test $p < 0.0001$; EP oogenesis = 26.9%, spermatogenesis = 39.0% Chi-square test $p < 0.0001$; LP oogenesis = 31.2%, spermatogenesis = 50.7% Chi-square test $p < 0.0001$; Table S2.5). We found that most WAGO-1 foci also co-localized with ZNFX-1 during oogenesis and spermatogenesis (Fig. 2.2C-D). Unlike with PGL-1, we found distinct patterns of WAGO-1 overlap with ZNFX-1 through meiotic progression dependent on the sex. During L/Z, significantly more WAGO-1 foci localized with ZNFX-1 during spermatogenesis compared to oogenesis (Fig. 2.2D, L/Z Chi-square test $p < 0.0001$; Fig. S2.5B). Oogenesis saw a significant increase in WAGO-1 foci overlapping with ZNFX-1 from L/Z to EP, leading to significantly more overlap compared during oogenesis to spermatogenesis (Fig. 2.2D, oogenesis L/Z vs EP Chi-square $p = 0.004$; EP spermatogenesis vs oogenesis Chi-square $p < 0.0001$). By LP, the percent of WAGO-1 foci overlapping with ZNFX-1 during spermatogenesis increased to similar levels to oogenesis (Fig. 2.2D, LP Chi-square $p = 0.25$). Together, these data suggest that oogenesis and spermatogenesis display similar trends of WAGO-1 overlap with other germ granule components. Indeed, most WAGO-1 foci have some volume overlapping with ZNFX-1 and/or PGL-1 foci throughout meiosis, with the percent of WAGO-1 overlapping increasing through meiotic progression. Despite this overlap, the percent of WAGO-1 foci having any overlap with, as well as the percent overlapping WAGO-1 volume with PGL-1 and ZNFX-1, was sexually dimorphic and varied with meiotic prophase I progression.

Dynamic co-localization of WAGO-1 with PGL-1 and ZNFX-1 during meiotic progression

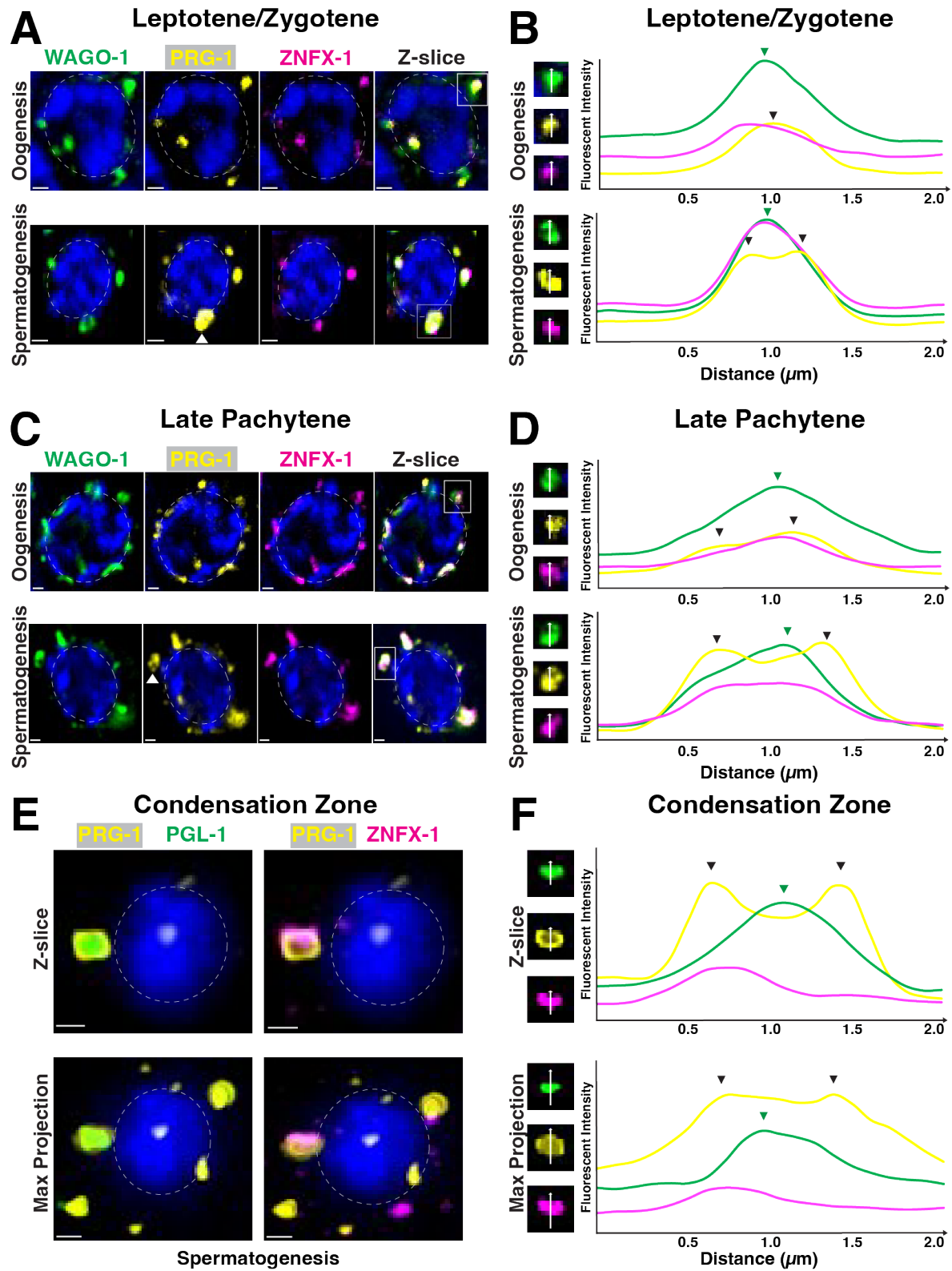
PGL-1 and ZNFX-1 preserve the transgenerational integrity of the germline and ensure proper silencing by Argonaute proteins (Ishidate et al., 2018; Kawasaki et al., 1998; Lev et al., 2019; Wan et al., 2018). Previous studies also found that PGL-1 and ZNFX-1 co-localize with distinct Argonautes and that these localization patterns inform AGO function within small RNA pathways (Aoki et al., 2021; Ishidate et al., 2018; Seroussi et al., 2023; Wan et al., 2018). To better understand WAGO-1 localization with PGL-1 and ZNFX-1, we compared the percent of WAGO-1 foci that overlapped with: only ZNFX-1, only PGL-1, or with both proteins between oogenesis and spermatogenesis. We found that throughout oogenesis and spermatogenesis WAGO-1 was most likely to co-localize with both ZNFX-1 and PGL-1, rather than localize with a single structural component (Fig. 2.2E-G). Interestingly, when WAGO-1 localized with only a

single component, it primarily co-localized with ZNFX-1 independent of meiotic stage and sex (Fig. 2.2E-G; magenta). These data suggest that WAGO-1 localization is not restricted to only the P-granule as marked by PGL-1. Rather, WAGO-1 displays versatile localization patterns within the germ granule, which may broaden possible WAGO-1 protein interactions.

WAGO-1 and the PIWI Argonaute PRG-1 form dynamic toroidal rings only during spermatogenesis

Previous work established WAGO-1 as a secondary Argonaute that acts within multiple small RNA pathways in *C. elegans* including the piRNA pathway, that is required for silencing of transposons, pseudogenes, and fast evolving genes in developing germ cells (Barucci et al., 2020; Das et al., 2008; Gu et al., 2009; Reed et al., 2020; Seroussi et al., 2023; Shirayama et al., 2012). To determine how WAGO-1 and PRG-1, the primary AGO of the piRNA pathway, co-localize during germ cell development, we stained for WAGO-1, PRG-1, and ZNFX-1 in adult males undergoing spermatogenesis and adult hermaphrodites undergoing oogenesis. Notably, we found a subset of PRG-1 foci forms toroidal rings during spermatogenesis and that these rings became more prevalent and defined in later meiotic prophase I stages (Fig. 2.3A, C, white arrows; Fig S2.6). We first identified ring-like PRG-1 structures during the leptotene/zygotene stage of spermatogenesis (Fig. 2.3A, white arrows). The presence of these rings was verified by line scans, where PRG-1 fluorescent intensity has two distinct peaks on either side of a local minima across an individual germ granule (Fig. 2.3B, black arrows; Fig. S2.7). In germ granules that contained PRG-1 in this ring-like structure, we found that both WAGO-1 and ZNFX-1 foci were present but only contained a single intensity maximum between peaks in PGL-1 intensity

Figure 2.3: PRG-1 and WAGO-1 form toroidal structure around ZNFX-1 during spermatogenesis. (A,C) Representative Z-slice of single nuclei undergoing oogenesis(top) and spermatogenesis(bottom) during (A) L/Z and (C) LP stained for WAGO-1(green), PRG-1(yellow), and ZNFX-1(magenta). Scale bars represent 1µm. (B, D) Left: Inset of single channels from representative germ granules boxed in (B) A and (D) C. Right: Averaged line scans of pixel intensity across germ granules during (B) L/Z and (D) LP. (E) Representative Z-slice(top) and max projection(bottom) of single condensing spermatid stained for PRG-1(yellow), PGL-1(green), and ZNFX-1(magenta). Scale bars represent 1µm. (F) Left: Inset of single channels from representative germ granules with toroidal PRG-1 structure. Right: Averaged line scans of pixel intensity across germ granules with toroidal PRG-1 structure in single Z-slice(top) and max projection(bottom). Line scan direction(white arrows). Local maxima of PRG-1 intensity(black arrows). Local maxima of WAGO-1 intensity(green arrows).



(Fig. 2.3B, bottom). Additionally, while we found very few ring-like structures of PRG-1 in oocytes during L/Z (Fig. S2.6A), numerous germ granules contained PRG-1, WAGO-1, and ZNFX-1, all with single maxima (Fig. 2.3B, top). In late pachytene, we noted a germ granule containing at least one ring-like PRG-1 foci on nearly every developing spermatocyte (Fig. S2.6B). These PRG-1 rings were more defined compared to those observed in L/Z (Fig. 2.3C, bottom), with two sharply defined peaks of intensity around a local minimum (Fig. 2.3D, bottom, black arrows). Further, we found that with these PRG-1 rings, WAGO-1 formed a half-moon structure, where line scans across the WAGO-1 foci found a single maximum (Fig. 2.3D, green arrow), while line scans perpendicular to the granule had two maxima around a local minimum that coincided with PRG-1 peaks (Fig. S2.7). In oocytes during LP, we also noted a subset of PRG-1 foci forming ring structures, albeit less defined than in spermatocytes, primarily towards the end of the region (Fig. 2.3C-D, black arrows). These rings were independent of pocket-like germ granule structures that were also noted on developing oocytes, similar to previous observations from Uebel *et al.* 2023. In the condensation zone of spermatogenesis, PRG-1 rings convert into a “shell-like” casing structure that encompasses both PGL-1 and ZNFX-1 foci (Fig. 2.3E). Line scans reveal that PGL-1 specifically displays a local maximum where PRG-1 experiences a local minimum in intensity (Fig. 2.3F, top, green arrow). Unlike the rings formed by PRG-1 during earlier stages of meiotic prophase I (Fig. S2.8C-D), line scans across maximum projections of these condensation zone rings lack local PRG-1 minimums suggesting PRG-1 forms a shell rather than a toroidal ring (Fig. 2.3F, bottom: max projection). Taken together, our data suggests a sexually dimorphic organization of a subset of PRG-1 foci, which can dynamically change from a toroidal ring-like structure localizing around WAGO-1 and ZNFX-1 into a shell-like complex that surrounds ZNFX-1 and PGL-1 only during spermatogenesis.

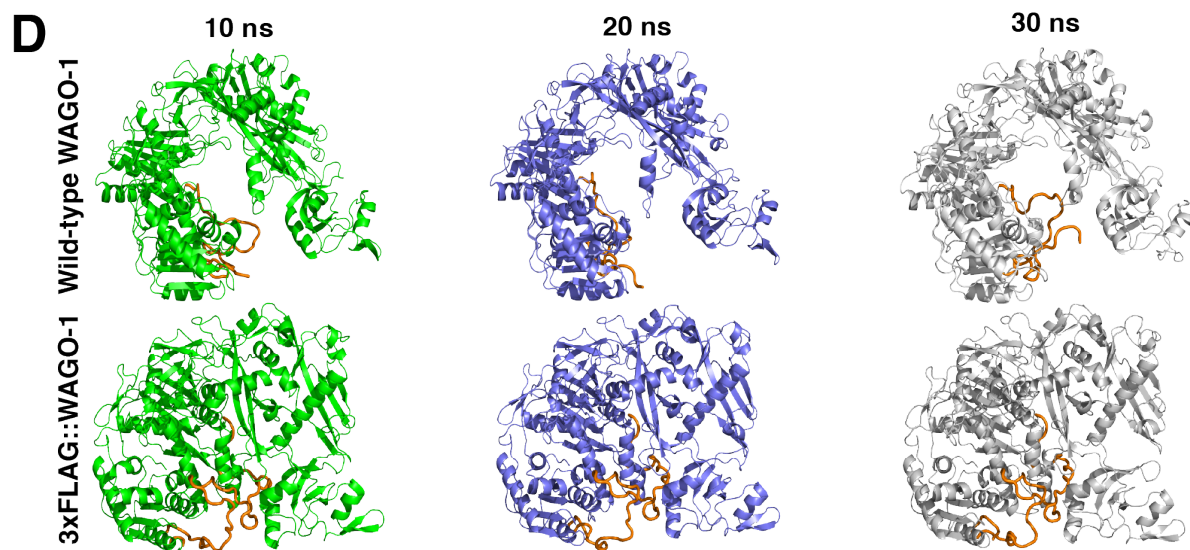
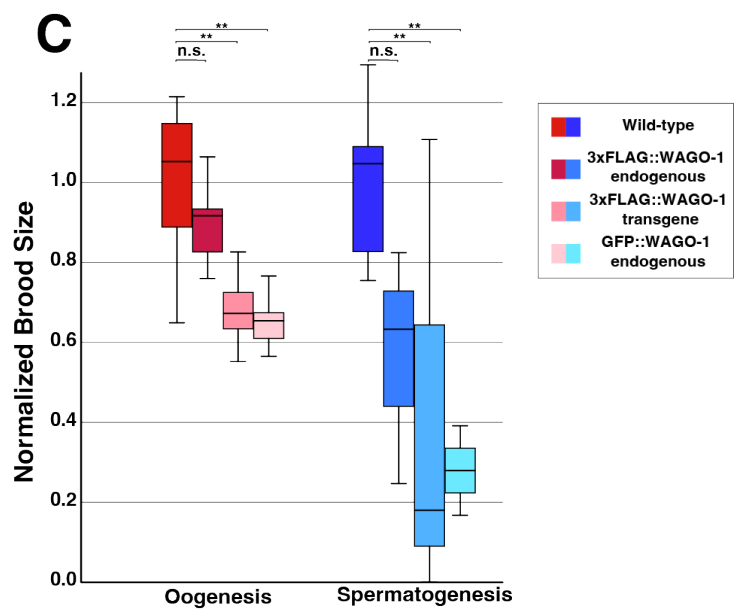
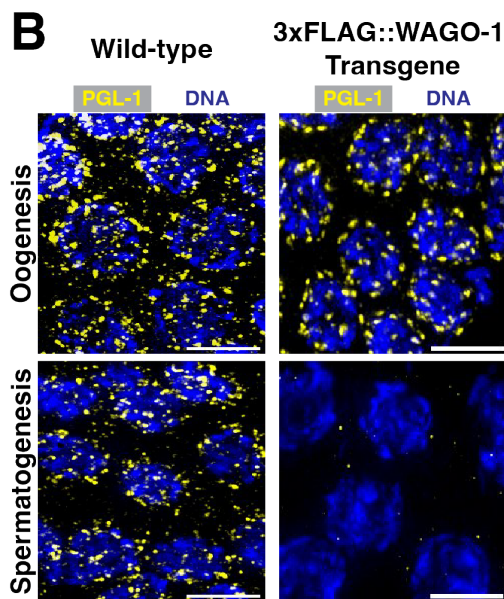
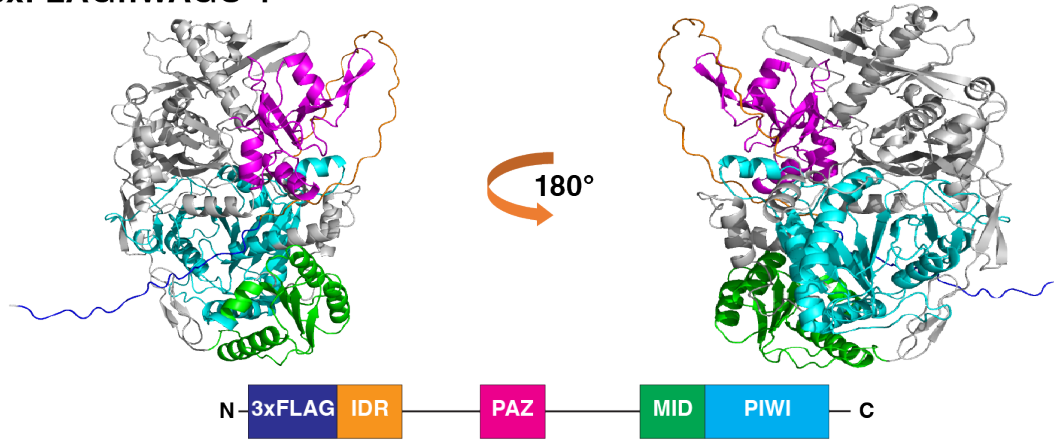
Sexually dimorphic roles of the WAGO-1 N-terminus in PGL-1 phase separation

Previous work looking at WAGO-1 localization has relied on tagging both endogenous and transgenic copies of WAGO-1 with 3xFLAG or GFP on the N-terminus of the protein (Dokshin et al., 2018; Gu et al., 2009; Seroussi et al., 2023). Predictive structures of 3xFLAG tag on the N-terminal of WAGO-1 by AlphasFold3 (Abramson et al., 2024) suggest these tags may disrupt the intrinsically disordered region (IDR) of WAGO-1, but otherwise does not change the overall protein structure (Fig. 2.4A). To determine if germ granule structure is perturbed in

tagged WAGO-1 strains we stained for PGL-1 in adult males undergoing spermatogenesis and adult hermaphrodites undergoing oogenesis. Notably, in all the tagged WAGO strains assessed, 30-50% of germlines undergoing spermatogenesis lose PGL-1 phase separation (Fig. 2.4B; Fig. S2.9). Additionally, we found that tagging of WAGO-1 on the N-terminus, independent of the tag used, caused a significant decrease in the brood size of both hermaphrodites (Fig. 2.4C, red) and males (Fig. 2.4C, blue). These drops in brood size are also significantly larger in adult males compared to adult hermaphrodites for all genotypes (Fig. 2.4C; Fig. S2.10). We also noted that endogenous tagging of WAGO-1 with 3xFLAG tag caused a transgenerational silencing of *wago-1* transcripts and WAGO-1 protein expression (Fig. S2.11). We hypothesized that these mutant phenotypes were due to the modified IDRs blocking sRNA and/or mRNA access to the RNA binding pocket of WAGO-1. WAGO-1 is known to bind sRNAs and target mRNAs within its positively charged binding pocket (Fig. S2.12; Dueck and Meister, 2014; Gu et al., 2009; Seroussi et al., 2023; Sheu-Gruttadauria and MacRae, 2017). FLAG tags are primarily comprised of aspartic acid, which is negatively charged and, when added onto the IDR of WAGO-1, could allow the flexible disordered domain to dock within the RNA binding pocket, blocking any mRNA or small RNA interactions. To test this hypothesis, we utilized Molecular Dynamic (MD) simulations to test the accessibility of the RNA binding pocket with and without the 3xFLAG tag added onto the N-terminus of WAGO-1. We found that 3xFLAG::WAGO-1 had significantly lower solvent accessible surface area (SASA) compared to wild-type WAGO-1, suggesting that when the IDR of WAGO-1 contains a 3xFLAG-tag, the binding pocket of WAGO-1 is less

Figure 2.4: N-terminus tags of WAGO-1 cause sex-specific infertility and loss of PGL-1 phase separation. (A) Ribbon diagram of endogenous 3xFLAG::WAGO-1 AlphaFold3 structure (top) and gene structure (bottom). 3xFLAG tag (dark blue); N-terminus IDR (orange); PAZ domain (pink); MID domain (green); PIWI domain (light blue). (B) Representative immunofluorescence images of germ cells during LP undergoing oogenesis (top) and spermatogenesis (bottom) from wild-type (left) and a 3xFLAG::WAGO-1 transgene (right) strain stained for PGL-1 (yellow). Scale bars represent 5µm. (C) Normalized brood size of adult hermaphrodites undergoing oogenesis (red; left) and adult males undergoing spermatogenesis (blue; right) of wild-type, endogenously tagged 3xFLAG::WAGO-1, 3xFLAG::WAGO-1 transgene, and endogenously tagged GFP::WAGO-1. * $p < 0.05$, ** $p < 0.001$, Two-way ANOVA with Tukey's multiple comparison test. (D) Stills from MD simulation of wild-type WAGO-1 (top) and 3xFLAG::WAGO-1 (bottom) taken 10 nanoseconds (ns) apart. IDR and 3xFLAG tag (orange), remaining protein in green (10ns), blue (20ns), and white (30ns).

A 3xFLAG::WAGO-1



available for interface with target sRNAs or mRNAs (Fig. 2.4D; Fig. S2.13). Additionally, comparing the structures across multiple frames of the MD simulation, we observed the 3xFLAG::IDR within the binding pocket more often compared to the wild-type IDR (Fig. 2.4D; Fig. S2.14; Fig. S2.15). Taken together, our data suggests proper N-terminus structure of WAGO-1 is required for maintaining fertility and WAGO-1 expression in both hermaphrodites and males, as well as PGL-1 phase separation during spermatogenesis.

C-terminal truncation of WAGO-1 leads to complete infertility in males

WAGO-1 has a bilobed structure, with a N- terminal lobe containing the IDR and PAZ domains and a C-terminal lobe containing MID and PIWI domains (Fig. S2.1; Sheu-Gruttadauria and MacRae, 2017). Loss of the MID domain and truncation of the PIWI domain in the *wago-1(tm1414)* allele revealed that both domains are required for proper WAGO-1 function (Fig. 2.5A; Gu et al., 2009; Seroussi et al., 2023). To determine whether loss of these domains affects fertility, we compared the brood sizes of the *wago-1(tm1414)* allele in both sexes (Fig. 2.5B). We found that, similar to tagging of the N-terminus of WAGO-1, truncation of the C-terminus causes a significant decrease in fertility for both adult hermaphrodites and males (Fig. 2.5B). *wago-1(tm1414)* males specifically failed to produce any living progeny or dead eggs when mated with obligate females, suggesting that the C-terminus of WAGO-1 is required for fertility during spermatogenesis. Our analysis of the adult hermaphrodite germlines of the *wago-1* truncation mutants found significantly reduced levels of germ cells in comparison to age-matched wild-type animals (Fig. S2.16). Despite the decreased germ cell population of adult hermaphrodite germlines, developing oocytes still properly progressed through all stages of prophase I (Fig.S2.16A). We next looked at localization of WAGO-1 and PGL-1 within adult hermaphrodites that these C-terminus truncations of WAGO-1. Since the pachytene region of oogenesis was reduced in *wago-1(tm1414)* compared to wild-type, we analyzed all of pachytene as a singular region (Fig. 2.5C-D). We found that during both leptotene/zygotene and pachytene, WAGO-1 and PGL-1 foci are still recruited the periphery of *wago-1(tm1414)* developing oocytes but rarely co-localize compared to wild-type germlines (Fig. 2.5C-F). These data suggest a sexually dimorphic role for the MID and PIWI domains of WAGO-1 in modulating fertility and germ granule structure. Proper MID and PIWI structure are required for male fertility, while loss of these WAGO-1 protein domains during oogenesis still allows some progeny to develop.

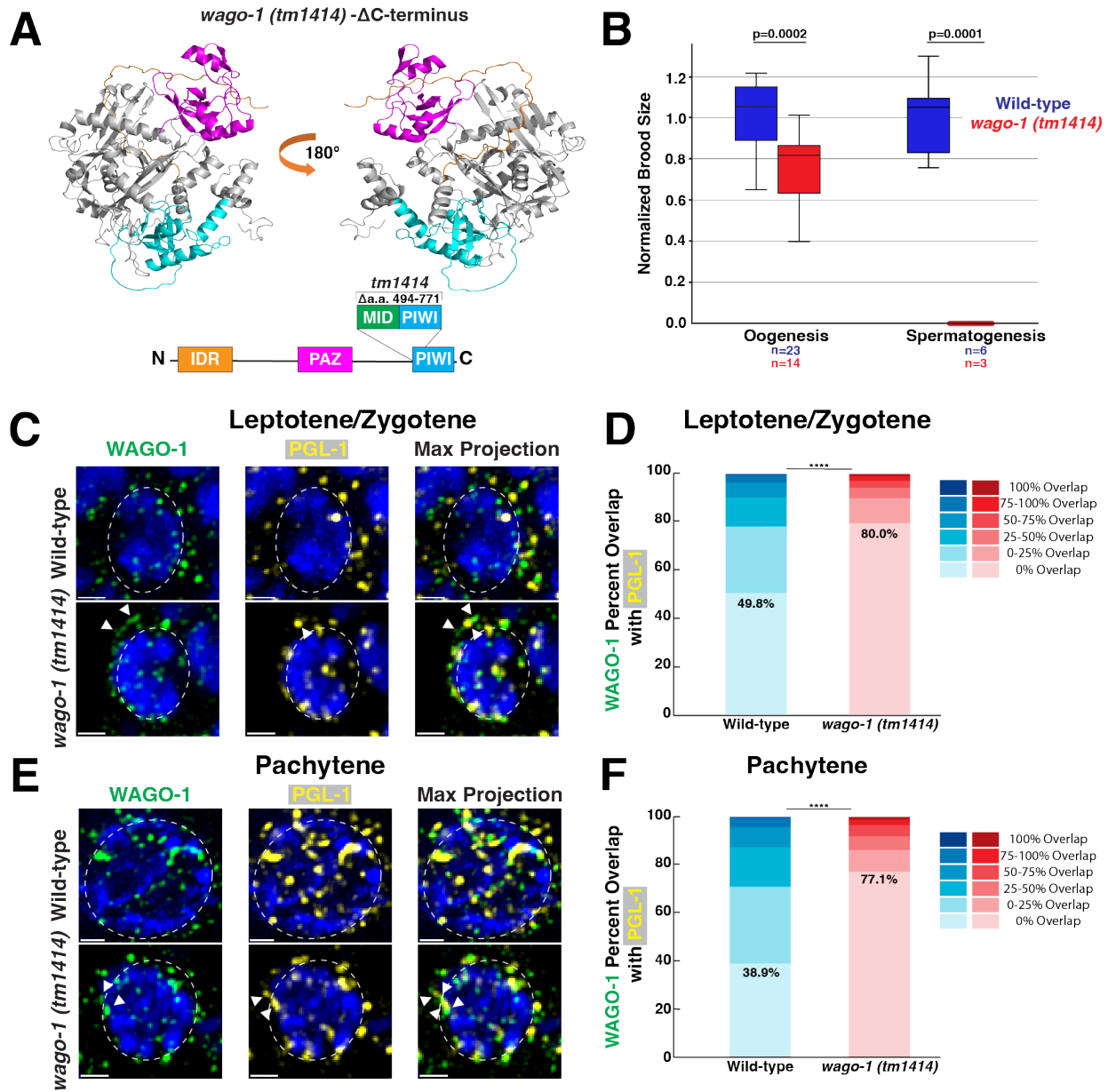


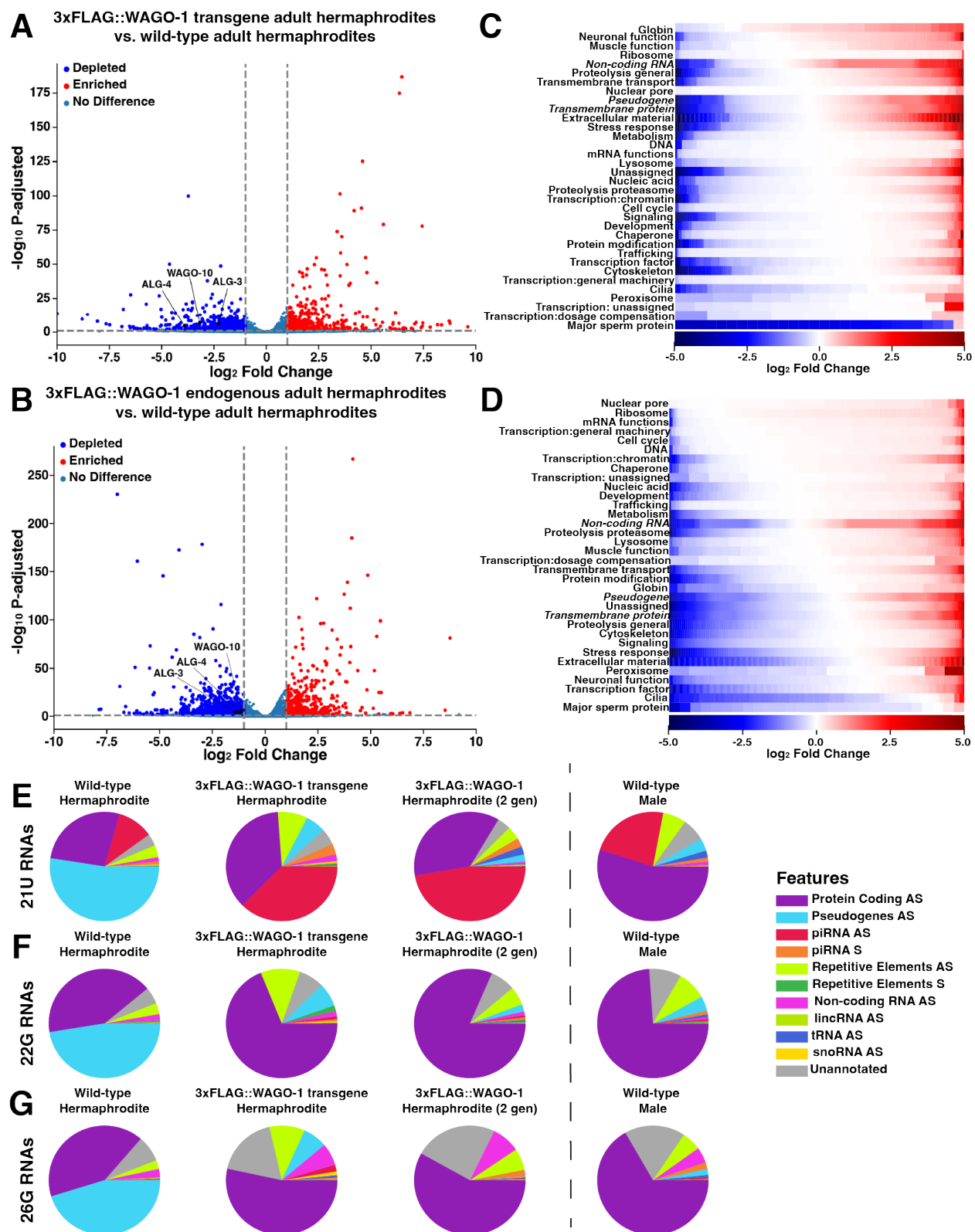
Figure 2.5: MID deletion and PIWI truncation of WAGO-1 causes male specific infertility and disrupts WAGO-1 localization with PGL-1. (A) Ribbon diagram of *wago-1(tm1414)* allele AlphaFold3 structure (top) and gene structure (bottom). Orange, N-terminus IDR; pink, PAZ domain; green, MID domain; light blue, PIWI domain. (B) Normalized brood size of adult hermaphrodites undergoing oogenesis (left) and adult males undergoing spermatogenesis (right) of wild-type and *wago-1(tm1414)* strains. p-values calculated using one-Way ANOVA with Bonferroni correction. (C, E) Representative immunofluorescence images of single nuclei undergoing oogenesis from wild-type (top) and *wago-1(tm1414)* strains during (C) L/Z and (E) pachytene stained for WAGO-1 (green) and PGL-1 (yellow). Scale bars represent 1 μ m. (D, F) Bar graphs indicating percentage of WAGO-1 foci volume overlap with PGL-1 foci from wild-type (blue) and *wago-1(tm1414)* strains during (D) L/Z and (F) pachytene. **** p<0.0001, Chi-square test with Bonferroni correction post-hoc.

Combined with the disruption of the germ granule observed with tagging of WAGO-1 IDR and the coinciding decreases in fertility, these data suggest that proper germ granule structure may be critical for preserving male and hermaphrodite fertility.

N-terminus tagging of WAGO-1 causes misregulation of sperm specific AGOs during oogenesis

To determine how disruption to native N-terminus WAGO-1 structure affects the transcriptional landscape, we performed mRNA-seq on adult hermaphrodites undergoing oogenesis and adult males undergoing spermatogenesis on a subset of N-terminally tagged mutants. We specifically examined the gene expression profile of adult male and hermaphrodite *C. elegans* with an endogenous 3xFLAG::WAGO-1 tag and adult hermaphrodite *C. elegans* with a transgene of 3xFLAG::WAGO-1 (see Materials and Methods) and compared them to the expression levels of adult male and hermaphrodite *C. elegans* with wild-type WAGO-1 structure. In all three conditions, we found a significant number of genes that are misregulated between wild-type and the mutant context (Fig. 2.6A-B; Fig. S2.17). Additionally, both endogenous 3xFLAG::WAGO-1 and 3xFLAG::WAGO-1 transgene hermaphrodites showed an over two-fold depletion of *alg-3*, *alg-4*, and *wago-10* compared to wild-type (Fig. 2.6A-B; Supplemental Data 1). ALG-3 and ALG-4 are paralogs known to bind 26G-RNAs and act as the primary Argonautes for licensing and silencing of transcripts during spermatogenesis utilizing secondary AGOs WAGO-10, CSR-1a, and RDE-1 (Charlesworth et al., 2021; Conine et al., 2013, 2010; Nguyen and Phillips, 2021; Seroussi et al., 2023). Mutations to *alg-3/4* cause downstream sperm specific

Figure 2.6: 3xFLAG::WAGO-1 hermaphrodites downregulate genes required for spermatogenesis and proper sperm maturation. (A-B) Volcano plot of mRNA-sequencing data depicting genes up-regulated (red), downregulated (blue), or have no change (blue-grey) between wild-type and **(A)** 3xFLAG::WAGO-1 transgene hermaphrodites or **(B)** endogenously tagged 3xFLAG::WAGO-1 hermaphrodites. **(C-D)** Heat map depicting individual genes, grouped by gene ontology (GO) terms, that are upregulated (red) or downregulated (blue) based on log2Fold Change in **(C)** 3xFLAG::WAGO-1 transgene hermaphrodites or **(D)** 3xFLAG::WAGO-1 endogenously tagged hermaphrodites compared to wild-type. GO groups are vertically sorted in descending order based on the proportion of genes that are upregulated. White indicates no significant change. **(E-G)** Pie charts depicting the proportion of **(E)** 21U-, **(F)** 22G-, and **(G)** 26G-RNAs mapping to specific genetic elements in wild-type, 3xFLAG::WAGO-1 transgene, and 3xFLAG::WAGO-1 endogenously tagged hermaphrodites and wild-type males. AS=antisense, S=sense.



infertility tied to improper pseudopod formation and sperm motility, with complete sterility when *csr-1a* and *wago-10* are also knocked out (Charlesworth et al., 2021; Conine et al., 2013, 2010; Seroussi et al., 2023). Indeed, when we looked at the gene ontology (GO) terms of differentially expressed genes between wild-type and WAGO-1 tagged hermaphrodites, we found that both tagged strains were depleted of transcripts associated with Major Sperm Protein (MSP) compared to wild-type, with the transgene 3xFLAG::WAGO-1 showing a larger depletion compared to the endogenous 3xFLAG::WAGO-1 population (Fig. 2.6C-D). This depletion in MSP-associated transcripts could suggest that sperm present in adult hermaphrodites lack the proteins required for proper sperm activation to fertilize oocytes, driving the decrease in brood size observed in the hermaphrodites (Fig. 2.4B).

While we found no change in AGO expression levels between wild-type and endogenously tagged 3xFLAG::WAGO-1 males, numerous genes displayed changes in expression (Fig. S2.17). In both sexes, we found that the one of the most misregulated sets of genes were pseudogenes, a gene class known to be regulated by WAGO-1 (Fig. S2.18; Seroussi et al., 2023). Misexpression of pseudogenes, as well as genes from the other two highest classes of misregulated genes, non-coding RNAs and transmembrane proteins, was not directional (Fig. 2.6C-D). Rather than an overarching trend towards up- versus down-regulation, we found that a subset of these misregulated genes significantly decreased in expression in the mutant compared to the wild-type (Fig. 2.6C-D, blue), while a different, distinct subset saw a significant increase in gene expression in the mutant (Fig. 2.6C-D, red). Overall, our data suggests that the addition of 3xFLAG tag on the N-terminus of WAGO-1 disrupts proper gene expression in adult hermaphrodites and males. Specifically, adult hermaphrodites with tagged WAGO-1 are depleted for sperm-specific transcripts, including *alg-3/4*, *wago-10*, and MSPs, which may drive the reduction in brood size. Additionally, we find that numerous WAGO-1 targets, including pseudogenes, are misregulated in both sexes, possibly leading to observed decreases in brood size and disruption to germ granule structure.

N-terminus tagging of WAGO-1 causes disruption to hermaphrodite and male small RNA profiles

Pseudogenes are primarily targeted by secondary AGOs within the WAGO cluster of the endogenous small interfering RNA pathway, which includes WAGO-1 (Seroussi et al., 2023). To

determine if dysregulation of pseudogenes is due to changes in small RNA profiles when WAGO-1 structure is perturbed, we performed small RNA-seq on the same samples utilized for mRNA-seq analysis. We first looked at the global expression of different types of small RNAs based on length, ranging from 18 to 30 nucleotides (nt), and the 5' nucleotide end of each small RNA (Fig. S2.19). We found that in the 3xFLAG::WAGO-1 transgene hermaphrodites, the proportion of 22G-RNAs is depleted compared to the wild-type (Fig. S2.19A, C, teal). This lower share of 22G-RNAs resembles the proportions we observed in the wild-type male small RNA profile (Fig. S2.19D). We also found that in both adult male and adult hermaphrodite endogenously tagged 3xFLAG::WAGO-1 populations, there is a significant increase in the proportion of 26G-RNAs compared to wild-type (Fig. S2.19B, E). Following passage of the endogenously tagged 3xFLAG::WAGO-1 strain for 100 generations, we found that the relative levels of 26G-RNAs return to baseline (Fig. S2.20).

Due to the misregulation of 22G- and 26G-RNAs and their role in small RNA pathways, we next wanted to look at the proportion of sRNAs that associate with specific genetic elements. These populations, and their misregulation, especially interested us due to 22G-RNAs being the sRNA species primarily associated with WAGO-1 and can be derived from 26G-RNAs. We also interrogated the genomic features present in the 21U-RNA populations, a sRNA class that associates with PRG-1 and also templates WAGO-1 bound 22G-RNAs. We found that the overall sRNA profiles of each class are sexually dimorphic when comparing wild-type hermaphrodites (Fig. 2.6E far left) to wild-type males (Fig. 2.6E far right). For all three classes of sRNAs in hermaphrodites, we found that the majority were antisense to pseudogenes (light blue), while in males they are primarily antisense to protein coding genes (purple). Additionally, in the male 21U-RNA population we see significantly larger proportion of antisense piRNAs in males compared to hermaphrodites (Fig. 2.6E, red).

We next compared the small RNA profiles of wild-type hermaphrodites to that of the hermaphrodite populations with endogenously tagged or a transgene 3xFLAG::WAGO-1. We found that in both mutant contexts, the largest proportion of 21U-RNAs were antisense piRNAs, while 22G- and 26G-RNAs were primarily anti-sense to protein coding genes (Fig. 2.6E-G). In contrast, pseudogenes were much less prevalent in 21U-, 22G-, and 26G-RNA species for both 3xFLAG::WAGO-1 tagged hermaphrodite strains, suggesting a possible mechanism for the change in pseudogene expression we observed. Additionally, small RNAs antisense to repetitive

elements (lime green), which includes transposons, were specifically prevalent for all classes of sRNAs in the 3xFLAG::WAGO-1 transgene, suggesting that the mechanisms causing sRNA misregulation between the mutant N-terminally tagged strains may differ (Fig. 2.6E-G, middle left). For males with endogenously tagged 3xFLAG::WAGO-1, we found that the proportions of sRNAs for each class were highly similar to endogenous 3xFLAG::WAGO-1 hermaphrodites (Figure S2.21).

We finally compared the small RNA profiles of endogenously tagged 3xFLAG::WAGO-1 males and hermaphrodites following 100 generations of passaging to determine how the transgenerational silencing of WAGO-1 changes the small RNA landscape (Fig. S2.11). We found that, similar to with the global small RNA levels, all three classes of sRNAs appeared to move towards wild-type proportions following 100 generations of passaging (Fig. S2.21). Taken together, our data suggests that disruption to the IDR of WAGO-1 through the addition of a 3xFLAG tag dramatically changes the small RNA profiles of adult hermaphrodites but transgenerational silencing of endogenously tagged WAGO-1 shift small RNA profiles back towards wild-type.

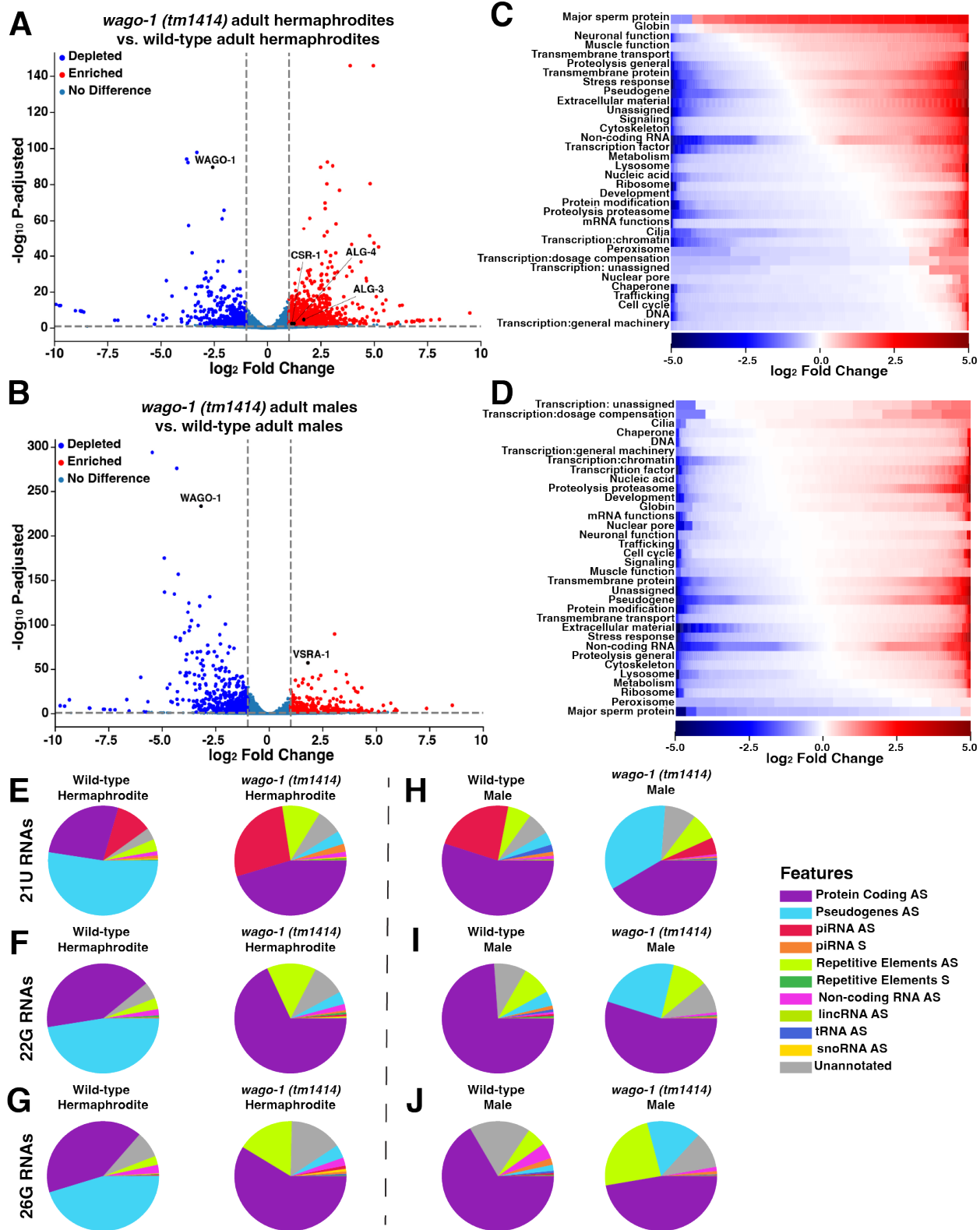
***wago-1 (tm1414)* hermaphrodites upregulate mRNAs enriched in adult males**

Similar to N-terminus modified WAGO-1, truncation of the WAGO-1 protein with the *tm1414* allele causes male specific infertility (Fig. 2.5B) but preserves hermaphrodite fertility despite underproliferation of the germline and disruption to both WAGO-1 and PGL-1 localization (Fig. 2.5C-D; Fig. S2.16). To determine how mRNA expression differs in *wago-1 (tm1414)* mutants compared to wild-type, we performed mRNA-seq on *wago-1 (tm1414)* adult hermaphrodites and adult males undergoing oogenesis and spermatogenesis respectively. Due to the severe infertility of *wago-1 (tm1414)* males (Fig. 2.5B), we performed HIM-8 RNAi to generate a large enough population of males for sequencing (see Materials and Methods). We found that transcript levels were perturbed in both *wago-1 (tm1414)* adult males and adult hermaphrodites (Fig. 2.7A-B). Additionally, *wago-1* expression in both sexes was depleted compared to the wild-type but was still transcribed at significantly higher levels compared to the 3xFLAG::WAGO-1 strain after *wago-1* silencing (Fig. 2.7A-B; Fig. S2.11; Supplemental Data 1; 3xFLAG::WAGO-1 endogenous hermaphrodite *wago-1* log₂Fold Change = -7.18, *wago-1 (tm1414)* hermaphrodite *wago-1* log₂ Fold Change = -2.58; 3xFLAG::WAGO-1 endogenous

male *wago-1* log₂Fold Change = -6.37, *wago-1 (tm1414)* male *wago-1* log₂ Fold Change = -3.19).

In addition to depletion of *wago-1* transcripts, we found that *wago-1 (tm1414)* hermaphrodites had increased expression of the Argonaute genes *csr-1*, *alg-3*, and *alg-4* (Fig. 2.7A). Paralogs ALG-3 and ALG-4, and the CSR-1a isoform are AGOs primarily known to be expressed in germlines undergoing spermatogenesis (Charlesworth et al., 2021; Conine et al., 2013, 2010; Nguyen and Phillips, 2021; Seroussi et al., 2023), and are upregulated in adult males compared to adult hermaphrodites (Fig. S2.3). All three are known to play a role in not only repressing oogenesis genes, but also licensing the expression of genes required for proper sperm development and maturation (Campbell and Updike, 2015; Charlesworth et al., 2021; Conine et al., 2013, 2010; Nguyen and Phillips, 2021). Genes targeted for upregulation by ALG-3/4 include ones that are required for sperm mobility, such as those associated with Major Sperm Protein (MSP) (Conine et al., 2013). Indeed, when we compared which genes classified by GO terms were up- versus down-regulated, we found that genes associated with MSP were primarily upregulated in *wago-1 (tm1414)* hermaphrodites versus wild-type hermaphrodites (Fig. 2.7C). In males harboring the *wago-1 (tm1414)* truncation allele, we observed an increase in expression of *vsra-1*, a secondary AGO primarily expressed during oogenesis that acts downstream of both ERGO-1 and CSR-1 to target protein coding genes and lincRNAs (Seroussi et al., 2023). Increased expression of *vsra-1* suggests that *wago-1 (tm1414)* males may repress genes required for proper sperm development despite wild-type levels of spermatogenic-specific AGOs, *alg-3/4* and *csr-1*. Indeed, when we looked at differential expression of genes associated with MSP, the majority were downregulated in *wago-1 (tm1414)* males compared to wild-type males (Fig. 2.7D). Together with the *wago-1 (tm1414)* hermaphrodites, our data suggests that loss of the

Figure 2.7: *wago-1(tm1414)* hermaphrodites and males upregulate AGOs specific to sperm and egg development. (A-B) Volcano plot of mRNA-sequencing data depicting genes upregulated (red), downregulated (blue), or have no change (blue-grey) between wild-type and (A) *wago-1(tm1414)* hermaphrodites or (B) *wago-1(tm1414)* males. (C-D) Heat map depicting individual genes, grouped by GO terms, that are up-regulated (red) or down-regulated (blue) based on log₂ Fold Change in (C) *wago-1(tm1414)* hermaphrodites or (D) *wago-1(tm1414)* males compared to wild-type. GO groups are vertically sorted in descending order based on the proportion of genes that are upregulated. White indicates no significant change. (E-J) Pie charts depicting the proportion of (E,H) 21U-, (F, I) 22G-, and (G, J) 26G- RNAs mapping to specific genetic elements in wild-type and *wago-1(tm1414)* (E-G) hermaphrodites and (H-J) males. AS=antisense, S=sense.



MID domain and truncation to the PIWI domain of WAGO-1 causes a misregulation of sex-specific genes required for proper oogenesis and spermatogenesis. Specifically, adult males repress genes associated with spermatogenesis while adult hermaphrodites are enriched for spermatogenic genes.

***wago-1 (tm1414)* hermaphrodite small RNA profiles are reminiscent of wild-type males**

To determine if the small RNA landscape of *wago-1 (tm1414)* males and hermaphrodites is also perturbed, we performed sRNA-seq on the same samples utilized for mRNA-seq. When looking at the global profile of small RNAs, we found that the proportion of 22G-RNAs compared to all 22-nucleotide sRNAs had the most striking changes between wild-type and *wago-1 (tm1414)* (Fig. S2.22). *wago-1 (tm1414)* hermaphrodites had significantly lower proportion of 22G-RNAs compared to wild-type hermaphrodites (Fig. S2.22A-B, teal), reminiscent of the 22G population observed in wild-type males (Fig. S2.22C). In contrast, the global small RNA profile of *wago-1 (tm1414)* males had a higher proportion of 22G-RNAs compared to wild-type males (Fig. S2.22C-D).

Due to the significant changes in relative 22G-RNA levels between *wago-1 (tm1414)* and wild-type *C. elegans*, we next looked at the proportion of sRNAs mapping to specific regions of the genome. We looked at 22G-RNAs, as well as upstream 21U- and 26G-RNAs from which 22G-RNAs are derived. Strikingly, we found differences in the profiles of all three sRNA classes in both adult male and adult hermaphrodite *wago-1 (tm1414)* populations. A significant proportion of 21U-RNAs from *wago-1(tm1414)* hermaphrodites were antisense to piRNAs, a class not highly prevalent in wild-type hermaphrodites (Fig. 2.7E) but are in wild-type males (Fig. 2.7H, left). We similarly saw that for 22G- and 26G-RNAs, *wago-1 (tm1414)* hermaphrodites had a profile more similar to wild-type males compared to wild-type hermaphrodites, where the primary species are antisense to protein coding genes rather than pseudogenes (Fig. 2.7F-J). We also noted a significant increase of the proportion of 22G- and 26G-RNAs against repetitive elements, including transposons, in the *wago-1 (tm1414)* hermaphrodites compared to wild-type (Fig. 2.7F-G, light green).

We found that the proportions of 21U-, 22G-, and 26G-RNAs present in *wago-1 (tm1414)* males were substantially different than wild-type males or *wago-1 (tm1414)* hermaphrodites (Fig. 2.7H-J). The proportion of 21U-RNAs antisense to piRNAs (red) in *wago-1 (tm1414)*

males was significantly lower compared to wild-type males (Fig. 2.7H). Additionally, a higher proportion of all three sRNA classes were antisense to pseudogenes compared to wild-type males (Fig. 2.7H-J) but still made up a lower proportion of the sRNAs than observed in wild-type hermaphrodites (Fig. 2.7E-G, left). Taken together, our data indicates that truncation of WAGO-1 on the C-terminus leads to small RNA profiles that are more similar to wild-type males in *wago-1 (tm1414)* hermaphrodites, while *wago-1 (tm1414)* males display a unique small RNA expression profile suggesting sex-specific misregulation of small RNAs.

Discussion:

Sexually dimorphic germ granule structure

Mechanisms underlying how specific sRNA pathways achieve sexually dimorphic gene regulation has remained an open area of investigation. Here we find that the organization and interactions of WAGO-1 within the germ granule may play a role in sexually dimorphic gene regulation by sRNA pathways during germ cell development. There are currently several hypotheses regarding the function of germ granules. First, germ granules are hypothesized as a location for mRNA regulation by sRNA pathways due to their localization and protein composition (Ketting and Cochella, 2021; Manage et al., 2020; Phillips and Updike, 2022; Uebel et al., 2023). Following transcription and processing, mRNA is exported out of the nucleus through nuclear pore complexes (NPCs) to the cytoplasm. Nearly 75% of these NPCs are associated with a germ granule during germ cell development (Pitt et al., 2000; Sheth et al., 2010). Actively transcribed genes required for meiosis as well as embryogenesis are primarily exported through NPCs that are directly associated with germ granules (Schisa et al., 2001; Sheth et al., 2010). While germ granule localization may prime small RNA pathways by mediating direct interactions with nascent transcripts exiting the nucleus through NPCs, another hypothesis indicates that this perinuclear localization may not be required. Indeed, recent works suggest that docking of the germ granule to the NPC is expendable for regulation of most transcripts as long as all functional small RNA and germ granule components are still properly expressed (Thomas et al., 2025). Other works have similarly found that the silencing of target mRNAs does not depend on localization to germ granules during embryogenesis, supporting the hypothesis that these phase-separated compartments may be incidental condensates, and gene regulation by small RNA pathways can still occur in the dilute, cytoplasmic phase (Lee et al., 2020; Putnam et

al., 2023). In this study, we observed that WAGO-1 expression is higher in the dilute, cytoplasmic phase compared to germ granules (Fig. S2.4), suggesting that indeed WAGO-1 complexes, both with RNAs and other proteins, may likely form and function in the cytoplasm. Phase-separation, as well as localization of germ granules to the nuclear periphery, may not be required for gene regulation but rather aid in increasing the probability that recently transcribed mRNAs encounter small RNA machinery, including WAGO-1, for efficient gene regulation. Overall, sexually dimorphic structure of WAGO-1 within the germ granule suggests potential sex-specific roles of germ granule regulation in monitoring recently transcribed mRNAs and/or sex-specific protein and RNA interactions with WAGO-1 within the cytoplasm.

Our data suggest that the N- and C-terminus of WAGO-1 have sexually dimorphic roles for regulating different aspects of germ cell development. Proper IDR structure, possibly due to access to the RNA-binding domain of WAGO-1, appears critical for proper spermatogenesis both in adult males and in L4 hermaphrodites. Despite this conserved requirement, changes to the IDR causes sex-specific changes to gene expression, possibly due to PGL-1 no longer forming functional germ granule compartments and/or functional cytoplasmic interactions with WAGO-1 in adult males undergoing spermatogenesis. By contrast, the C-terminus of WAGO-1 likely aids in the ability of WAGO-1 to properly localize with PGL-1 despite still being recruited to the nuclear periphery during oogenesis (Fig. 2.5C). Indeed, we found that these interactions are critical for proper gene regulation, with loss of this localization leading to similar increase in *alg-3/4* transcripts as seen when *pgl-1* is knocked down (Campbell and Updike, 2015). Males with C-terminus truncations are sterile, which is possibly due to the upregulation of *vsra-1*, an AGO known to repress spermatogenic genes during oogenesis. To determine if this upregulation is similarly due to disruption or loss of WAGO-1 interaction with PGL-1, future works conditionally expressing VRSA-1 or by looking at *pgl-1* mutant males should be pursued.

Function of the WAGO-1 IDR in PGL-1 phase separation

Our work uncovers novel roles of the IDR domain of WAGO-1 for maintaining germ granule stability specifically during spermatogenesis. Previous work had found that WAGO-1 and PGL-1 recruitment and stability within the germ granule is independent of each other during oogenesis (Aoki et al., 2021). Through manipulation of the N-terminus of WAGO-1 by both 1) extending the disordered region and 2) inserting negatively charged amino acids, we found that

altered WAGO-1 structure can indeed affect PGL-1 localization within the germ granule. Specifically, we found that proper IDR structure of WAGO-1 mediates PGL-1 phase separation during spermatogenesis. Interestingly, the loss of PGL-1 phase separation within the male germline is limited to mid-pachytene through the condensation zone. This pattern of PGL-1 phase separation loss observed in tagged-WAGO-1 germlines matches the expression profile of the *C. elegans* extracellular-signal-regulated kinase (ERK) ortholog, MPK-1, within spermatogenic germlines (Lee et al., 2007).

MPK-1, the *C. elegans* ortholog to human MAPK-1, is well known to act within the germline to regulate pachytene progression of germ cells during oogenesis and spermatogenesis, as well as chromosomal dynamics during oogenesis through phosphorylation of key proteins (Das et al., 2020; Lee et al., 2007; Nadarajan et al., 2016). Despite the conserved requirement for MPK-1 expression during germ cell development, protein levels between the two processes differ significantly. During spermatogenesis, MPK-1 levels fall to near zero following germ cell entry into early pachytene. In contrast, MPK-1 accumulates during oogenesis, in which MPK-1 levels increase following meiotic entry and stay relatively constant through the end of pachytene (Lee et al., 2007). The depletion of MPK-1 protein levels during spermatogenesis spatially aligns without our observation of spermatocyte-specific loss of PGL-1 phase separation, which may explain why PGL-1 remains within the germ granule through oogenesis. The post-translational modification of phosphorylation has long been associated with regulating germ granule aggregation and dissolution (Li et al., 2022; Liu et al., 2024; Shin and Brangwynne, 2017; Wang et al., 2014; Zhang et al., 2018). Additionally, previous works found that phosphorylation of PGL-1 during embryogenesis drives more of the protein into a phase-separated granule, even during periods of heat, a physical stressor that often causes phase-separated states to dissolve (Brangwynne, 2013; Brangwynne et al., 2009; Zhang et al., 2018). Overall, the IDR of WAGO-1 may play a role in maintaining PGL-1 phosphorylation, and therefore phase separation, during germ cell development.

Small RNA pathways, germ granules, and meiotic progression

Previous work has found that multiple components of the germ granule are also dynamic during germ cell progression, including the Mutator foci structural protein MUT-16, PGL-1, ZNFX-1, and PRG-1 (Ortega et al., 2024; Uebel et al., 2023, 2020). Our work builds upon these

findings with WAGO-1 displaying distinct biophysical confirmations and localization patterns within the germ granule that changes through meiotic progression. The changes of how WAGO-1 localizes within the germline through meiotic progression suggests a dynamic role for small RNA pathways in regulating meiosis. Additionally, our work suggests that the role of WAGO-1 differs between the sexes, with WAGO-1 foci displaying distinct configurations not only dependent on meiotic stage, but also on meiotic process.

Small RNA pathways are known to be highly active during meiosis to both repress deleterious elements of the genome that can induce instabilities linked to infertility and birth defects and ensure that meiosis progresses properly (Batista et al., 2008; Ortega et al., 2024; Seroussi et al., 2023; Spichal et al., 2021). The spatiotemporal regulation required for proper germ cell development, such as the timing of chromosome pairing, DNA double strand break induction and repair, and eventual chromosome segregation, relies on a coordinated regulation of transcripts and translation (Diag et al., 2018; Tzur et al., 2018). Small RNA pathways provide a possible mechanism for modulating protein levels as specific stages of meiosis through post-transcriptional gene regulation. Post-transcriptional gene regulation can allow for the accumulation of mRNAs required for certain processes without translation occurring until reaching a specific meiotic check point. Germ granules house not only the machinery required for proper sRNA pathway function, but also recently transcribed mRNAs and translation initiation factors (Amiri et al., 2001; Schisa et al., 2001; Sheth et al., 2010). Specifically, IFE-1 and IFE-3, two *C. elegans* isoforms of eIF4E, are known to localize to the germ granule and mediate sex-specific germ cell development (Amiri et al., 2001). In most cells, eIF4E is the limiting initiation factor, positioning the protein as an important regulator of translation rates (Raught and Gingras, 1999). The localization of eIF4E components within the germ granule could exist as a mechanism to help regulate overall translation rates within the germline.

Here we find that WAGO-1, an Argonaute in the small RNA pathway, is important for maintaining proper germ granule structure throughout meiotic progression. In mutant 3xFLAG::WAGO-1 we found that while PGL-1 foci form normally from the pre-meiotic tip through leptotene/zygotene, it becomes diffuse upon entering pachytene. These changes to germ granule structure caused by WAGO-1 perturbations may be explained by WAGO-1 dynamics throughout germline progression in wild-type strains. We found that WAGO-1 foci both grow in volume and change in sphericity during meiosis, possibly aiding in reorganization of granule

structure or modulating gene regulation done by other components of the germ granule. Additionally, we found that these changes in the biophysical properties of WAGO-1 differs between the sexes, specifically becoming less spherical during oogenesis and more spherical during spermatogenesis, suggesting a possible change in liquid-like dynamics (Fig. 2.1E). Changes in the liquid-like nature of WAGO-1 could affect the ability of WAGO-1, as well as other proteins within the germ granule to interface within the compartments, as well as with the cytoplasm to properly regulate transcripts. Future works comparing the liquid-like turnover of WAGO-1 proteins within the germ granule during oogenesis and spermatogenesis could aid better understanding the overall effects of these differing WAGO-1 foci structures. As we further study the sexually dimorphic properties and localization patterns of other germ granule components, we may find more insights into the mechanisms of gene regulation by these proteins.

Taken together, our work uncovers sexually dimorphic functions for specific Argonaute protein structural features within germlines to regulate gene expression, and future studies of other Argonautes or sRNA pathway components may uncover further dimorphic structures and functions that enable sexual dimorphic gene regulation.

Methods and Materials:

Strains:

All strains were generated from the N2 background and maintained at 20°C under standard conditions of nematode growth media (NGM) with OP50 *Escherichia coli*. Strains were maintained as mating stocks unless otherwise mentioned. All spermatogenesis experiments were conducted in adult males to ensure spermatocyte-only producing populations for analysis. Mutant and tagged WAGO-1 strains were back crossed five times with N2 prior to work. The following strains were using in this study:

N2: Bristol wildtype strain

WM616: *wago-1(ne4435[3xflag::wago-1]) I.*

WM192: *nels21 [wago-1::3xflag+ unc-119(+)] III.*

C184: *wago-1(tm1414) I.*

YY1325: *wago-4(gg620[3xflag::gfp::wago-4]) II.*

YY916: *znfx-1(gg554[3xflag::gfp::znfx-1]) II.*

WM205: *unc-119(ed3) III; nels22[wago-1::GFP + unc-119(+)]*

JMC250: *prg-1 (tor149[prg1::gfp::flag]) I; znfx-1(gg634[ha::tagRFP::znfx-1]) II.*

CB4108: *fog-2(q71) V.*

WAGO-1 Antibody Production

Peptides for the last fourteen amino acids (Cys-GYKQTDLNQKRVNA) of the C-terminus of WAGO-1 were produced by Biomatik. Antibodies against the WAGO-1 peptides were produced in chicken and affinity purified by Pocono Rabbit Farms. Antibody specificity was verified utilizing western blotting (Figure S2.2) and immunofluorescence.

Immunofluorescence:

Immunofluorescence was performed as described in Claycomb *et al.*, 2009 with the following adaptations. Briefly, gonads from adult male and hermaphrodite worms at 18-22 hour post-L4 stage were dissected together into 1x sperm salts (50 mM PIPES, pH 7.0, 25mM KCl, 1 mM MgSO₄, 45 mM NaCl, 2 mM CaCl₂) on the same VWR Superfrost Plus slides, frozen and cracked on dry ice for 10 minutes and then fixed at -20°C for 5 minutes in 100% methanol, 5 minutes in 50% methanol/50% acetone, and 5 minutes in 100% acetone. Samples were blocked in 1xPBST + 3% BSA at room temperature for 15 minutes. Primary antibody dilutions were made in 1xPBST + 3% BSA and added to the slides. Slides were covered with a parafilm cover slip and incubated overnight at 4°C in a humid chamber. Slides were washed 3 x 10 minutes in 1xPBST and then blocked in 1xPBST + 3% BSA for 15 minutes at room temperature. Secondary antibody dilutions were made at 1:200 in 1x PBST + 3% BSA using Invitrogen goat Alexa Fluoro-labeled antibodies and were added to the slides. Secondary antibody incubation occurred for 1 hour at room temperature in a dark humid chamber. Slides were then washed 3x10 minutes in 1xPBS in the dark and then incubated with 2 µg/ mL DAPI for 5 minutes in a humid chamber in the dark. Slides were then washed with 1xPBS for 5 minutes in the dark and then mounted with Vectashield. Slides were sealed with nail polish immediately following mounting and then stored at 4°C prior to imaging. All slides were imaged within 2 weeks of preparation. The following primary antibody dilutions were used: WAGO-1 anti-chicken (1:1000, this study), monoclonal mouse aPGL-1 K76 (1:20, Developmental Studies Hybridoma Bank), rabbit anti-PGL-1 (1:1000, Susan Strome Lab), mouse anti-FLAG (1:1000, Millipore Sigma F1804-50UG), ChromoTek

GFP-booster Alexa Fluor® 488 (1:200, ChromoTek gb2AF488), rabbit anti-HA (1:500, Abcam ab9110).

Imaging

Immunofluorescence slides were imaged at 1024 x 1024 pixel dimensions on an Applied Precision DeltaVision microscope with a 63x lens and a 1.5x optivar. Images were acquired as Z stacks at 0.2 μ m intervals and deconvolved with Applied Precisions softWoRx deconvolution software.

Foci quantification and Overlap

Foci quantification adapted from Toraason *et al.*, 2021. Briefly, germ granule foci were defined as Surface objects in Imaris (Bitplane) with the following settings: Smooth (not enabled), Background 0.513, and Seed Point Diameter (not enabled). Foci were analyzed if larger than 0.034 μ m² and smaller than 10 μ m² in volume. Counts, volume, and sphericity of granule structures were then aligned along an X-Y axis utilizing a Gonad Linearization Algorithm as described in Toraason *et al.*, 2021 and normalized from the pre-meiotic tip to the end of pachytene. Differences in counts were quantified using two sample Kolmogorov-Smirnov tests. Volume and sphericity were graphed using the plotnine.geom_smooth metric and the loess method. Counts were binned along meiotic progression utilizing well established nuclei morphology criteria to indicate meiotic stage: pre-meiotic tip, leptotene/zygotene, early pachytene, mid pachytene, late pachytene (Hillers et al., 2018). Differences in distributions were calculated utilizing Mann-Whitney U test with a Bonferroni correction using SciPy stats.

To determine if foci colocalized, we applied the Surface-to-Surface Percent Overlap function in Imaris to identify and replicate surfaces with greater than 0.1% volume overlap. These overlapping surfaces were then given unique colocalization identity intensity channels. A focus was considered co-localized with another in all analyses if two or more foci of different types (*i.e.* PGL-1 and WAGO-1) with the same unique colocalization intensity value could be identified in the exported data. Protein foci were considered to have no overlap if the primary protein foci had no volume overlapping with a secondary protein focus. 50% overlap corresponds with half of a single primary foci's volume being internal to a secondary protein's foci, while 100% occurs when the entirety of a single primary protein focus resides within a secondary protein focus. The percent overlap of the primary foci, though, does not report the percent of the secondary foci volume associating with the primary. Co-localized foci were then

aligned on an X-Y axis utilizing the Gonad Linearization Algorithm and binned based on normalized progression through the germline. Differences in the percent co-localization of foci within each bin was calculated utilizing Chi Squared Test with a Bonferroni Adjustment.

Percent of WAGO-1 protein in germ granules

To quantify WAGO-1 protein amount in germ granules versus diffuse in the cytoplasm Surface objects were created for the entire germline in addition to the germ granule foci described above. The germline was then separated into five regions, pre-meiotic tip, leptotene/zygotene, early pachytene, mid pachytene, and late pachytene, for individual stage analysis. The total fluorescence sum intensity of the germ granules was divided by the total sum fluorescence intensity of the entire region to determine the percent of total WAGO-1 present in the germ granule. To normalize for variations between stains as well as the possibility of secondary antibodies forming granule-like structures, the ratio of total fluorescence sum intensity of granule-like structures to total sum fluorescence intensity of the entire region for samples only stained with secondary antibodies was subtracted from the calculated experimental ratios.

Male and hermaphrodite fertility assays

For hermaphrodite fertility assays, hermaphrodites of each genotype were isolated at the L4 developmental stage and aged 18-22 hours to adult stage. Individual adult hermaphrodites were then singled on small plates (35 x 10 mm) with a small OP50 dot and allowed to lay for 24 hours. Following 24 hours, the adult hermaphrodite was moved to a fresh small plate (35 x 10 mm) with a small OP50 dot and allowed to lay for an additional 24 hours. This was repeated for a total of 5 plates. Following 24 hours on the fifth plate, the adult hermaphrodite was flamed off. 1-2 days following the removal of the adult hermaphrodite, plates were counted for number of living progeny, number of dead eggs, and number of unfertilized eggs. Plates where the hermaphrodite had run away or died were excluded. Differences in the ability of *wago-1* mutant hermaphrodites to produce viable progeny were analyzed using Two-way ANOVA with Tukey's multiple comparison test.

For male fertility assays, males of each genotype and obligate females (CB4108: *fog2Δ*) were isolated at the L4 developmental stage and aged 18-22 hours to adult stage. Individual obligate females were then singled and paired with an individual male on small plates (35 x 10 mm) with a small OP50 dot ringed with garlic extract to keep males from leaving the plate for 24 hours. After 24 hours males were moved to a new plate with an additional 18-22 hours post-L4

stage obligate female and allowed to mate for another 24 hours. After removal of the male, obligate females were allowed to lay eggs for 24 additional hours and then scored for living progeny, dead eggs, and unfertilized eggs. Pairs with no unfertilized eggs or where either the male or obligate female died before egg counting were excluded. Differences in the ability of *wago-1* mutant males to produce viable progeny were analyzed using Two-way ANOVA with Tukey's multiple comparison test.

HIM-8 RNAi male production

Due to the low brood sizes produced by males following mating with hermaphrodites for tagged and *wago-1 (tm1414)* strains, to increase male populations for mRNA- and sRNA- sequencing we utilized HIM-8 RNAi. Briefly, bleached synchronized *C. elegans* were allowed to grow on NGM+AMP+IPTG plates seeded with *E. coli* expressing the pLT 653 plasmid starting from L1s. Worms were grown at 20°C on RNAi plates until 18-22 hours post-L4 before being placed onto NGM plates with OP50 and grown at 20°C. Plates were then screened daily for male progeny. During this RNAi treatment, we found that WM192 failed to produce males at higher rates than seen naturally. We therefore were unable to collect enough WM192 for downstream mRNA- and sRNA-sequencing experiments.

Total RNA extraction and isolation for mRNA and sRNA-sequencing

300 adult hermaphrodites or 300 adult males 18-22 hours post-L4 stage were picked onto unseeded plates and washed off with 1 mL of cold M9. Worms were then spun down for 1 minute at 2000 x g and placed on ice for 3 minutes. Supernatant was then aspirated off to not disturb the worm pellet and washed with 200 µL of M9 and again pelleted. Worms were washed one final time with 200 µL of DNase and RNase free UltraPure distilled water (Invitrogen), spun for 1 minute at 2000 RPM and all but 50uL of liquid was removed. 500 µL of TRIzol (ThermoFisher) was added and tubes were flash frozen and placed in -80°C freezer for 15 minutes. Samples were then removed and vortexed for 15 minutes at room temperature with 100 µm acid washed, RNase free beads (Sigma-Aldrich, <106 µm). Freeze-thaw and vortexing was then repeated twice more for a total of 3 times. 100 µL of chloroform was then added to samples and tubes were shaken vigorously for 15 seconds and incubated at room temperature for 3 minutes. Tubes were then centrifuged at 12,000 x g for 15 minutes at 4°C. The top aqueous layer was then removed and transferred to a new tube. Phenol: chloroform: isoamyl alcohol was added 1:1 in the new tube and mixed for 15 seconds. Samples were then centrifuged at 12,000 x g for

15 minutes at 4°C. Top aqueous phase was again transferred to a new tube and 20 µg of GlycoBlue (15 mg/mL, ThermoFisher) and 1:1 ratio of isopropanol was added to new tube. Samples were then incubated at -20°C overnight or -80°C for 1 hour. Samples were then centrifuged at 16,000 x g for 30 minutes at 4°C and supernatant then removed. The pellet was then washed with 900 µL of 70% ice-cold ethanol for 10 minutes and then centrifuged at 16,000 x g for 10 minutes at 4°C. Supernatant was again removed and washing and centrifugation was repeated. Following last centrifugation, as much ethanol as possible was removed and pellet was left to air dry for 10 minutes at room temperature. Pellet was then resuspended in 12 µL of RNase-free water and purity and amount was quantified using Nanodrop. Samples were then diluted to 1 µg of RNA per 50 µL and used for downstream preparations.

Western Blotting

To generate protein lysates, 300 adult hermaphrodites or 300 adult males 18-22 hours post-L4 stage were picked onto unseeded plates and washed off with 1 mL of cold M9. Worms were then pelleted at 2000 x g for 1 minute and incubated on ice for 3 minutes. Supernatant was removed and worms were washed in 200 µL of M9 and again pelleted twice. Following the second pelleting step, all but 35 µL was removed, and worms were then mixed with NuPAGE LDS sample buffer (4x, Invitrogen) and DTT for a final concentration of 1xLDS and 100 mM DTT and boiled for 10 minutes at 95°C with intermittent vortexing. Samples were then run on SDS-PAGE gel (BioRad) at 100 V for 1:15 in 1x Laemmli Running Buffer. Samples were transferred at 100 V for 2 hours onto 0.45 µM nitrocellulose membrane (Thermo) in 1x Transfer Buffer (3.027 g Tris, 14.4 g Glycine, 200 mL MeOH). Membrane was then blocked for 1 hour at room temp in 5% milk powder + 1x PBST. Primary antibodies were then diluted in the milk powder solution and left at 4°C overnight with agitation. Membrane was then washed 3 x 5 minutes in 1x PBST and then incubated with Licor secondary antibodies in 1x PBST for 1 hour under agitation in the dark. Membrane was then washed in 1x PBST for 30 minutes in the dark before imaging using the Licor Imager. The following primary antibody dilutions were used: WAGO-1 anti-chicken (1:500, this study), alpha Tubulin anti-mouse (1:1000, Abcam ab7291).

Structure predictions

Structures for WAGO-1, 3xFLAG::WAGO-1, and *wago-1 (tm1414)* were all created utilizing AlphaFold3 (Abramson et al., 2024). Sequences for *wago-1* and *wago-1(tm1414)* were taken from WormBase and spliced and translated using aPe (Davis and Jorgensen, 2022). The sequence for 3xFLAG::WAGO-1 was derived from the WM616 strain from Dokshin *et al.* The electrostatics were predicted utilizing the APBS-PDB2PQR software suite and PyMol plugin (Jurrus et al., 2018).

Model construction for molecular dynamic simulations

For wildtype and 3xFLAG::WAGO-1 simulations, we started with the top three predicted AlphaFold3 models utilizing the sequence of WM616 from Dokshin *et al.*, 2018 with and without the 3xFLAG tag (Figure S1; Figure 4A). For all simulation parameters we utilized GROMACS 2023.4 (Abraham et al., 2015; Van Der Spoel et al., 2005) with the CHARMM36 2021 forcefield (Juan et al., 1995) with TIP3P waters (Jorgensen et al., 1983). All simulations were done in the NPT ensemble. We ran each simulation for a minimum of 500ns. All scripts for running simulations are available on github (https://github.com/harmslab/setup_md). We did all calculations using the talapas high-performance computing cluster at the University of Oregon.

Analysis of MD simulation trajectories

For analysis of MD simulations we visualized results using Visual Molecular Dynamics (Humphrey et al., 1996), python scripts using the MDAnalysis library (Gowers et al., 2019; Michaud-Agrawal et al., 2011), and PyMol (Schrodinger, 2015). We calculated the solvent-accessible surfaces areas using python scripts integrating the FreeSASA library (Mitternacht, 2016).

mRNA library preparation and sequencing

Samples were prepared for sequencing using the KAPA mRNA HyperPrep Kit for Illumina Platforms (KAPA Biosystems) following the protocol provided by manufacturer. The resulting DNA library was visualized and checked for quality using the 5200 Fragment Analyzer System (Agilent). Samples were then sequenced on the NovaSeq 6000 with a coverage of 25-30 million reads per a sample.

Small RNA library preparation and sequencing

Samples were prepared for sequencing using the Revvity small RNA kit following the protocol provided by manufacturer. The resulting DNA library was visualized and checked for quality

using the 5200 Fragment Analyzer System (Agilent). Samples were then sequenced on the NovaSeq 6000 with a coverage of 10 million reads per a sample.

mRNA-seq analysis

The mRNA sequences obtained from the sequencer were first assed for quality using MultiQC (Ewels et al., 2016). Adapter sequences were then removed using Trimmomatic for paired ends (version 0.36; Bolger et al., 2014) and run through MultiQC again to asses quality. The trimmed reads were then aligned to the *C. elegans* PRJNA13758 ce11 genome assembly (WormBase version WS280) using STAR (version 2.7.11b; Dobin et al., 2013). Reads were then counted using HTSeq (version 2.0.3; Anders et al., 2015) and differential expression was determined utilizing DESeq2 (version 3.20; Love et al., 2014). Genes were considered differentially expressed if their Log₂ Fold Change was great than 1 or less than -1 and had a p-adjusted value of less than 0.5. GO terms for each gene were assigned using WormCat 2.0 (Holdorf et al., 2020).

sRNA-seq analysis

The sRNA sequences obtained from the sequencer were first assed for quality using MultiQC (Ewels et al., 2016). Adapter sequences were then removed using Trimmomatic for paired ends (version 0.36; Bolger et al., 2014) and run through MultiQC again to asses quality. The trimmed reads were then aligned to the *C. elegans* PRJNA13758 ce11 genome assembly (WormBase version WS280) using STAR (version 2.7.11b; Dobin et al., 2013). Reads were then counted using the custom R-script created by Seroussi *et al.*, 2023 aligned to the following genome annotations: WormBase version WS280 PRJNA13758 ce11, *C. elegans* miRNAs from miRbase (release 22.1), repats and transposons annotations from RepeatMasker+Dfam (ce10, October 2010, RepeatMasker open-4.0.6, Dfam 2.0). Counts for different gene biotypes were then utilized for total small RNA sequencing landscape. For specific enrichment of 21U, 22G, and 26G small RNAs, we removed all sense reads to non-coding RNAs, rRNAs, snoRNAs, snRNAs, tRNAs, lincRNAs, protein coding genes, and pseudogenes due to the likely event they were caused by degradation. We also removed all RNAs transcribed in high copy in *C. elegans* including miRNAs and anti-sense rRNA and snRNAs to enrich for small RNAs that would be bound by AGOs in the endogenous RNAi pathways (Blumenfeld and Jose, 2016).

Data Availability.

The mRNA-sequencing and small RNA-sequencing data sets generated in this study are available on the NCBI BioProject database (<https://www.ncbi.nlm.nih.gov/bioproject/>) under accession number PRJNA1252786. Strains and WAGO-1 antibodies are available upon request.

Acknowledgments:

We thank Z. Bush, J. Brown, C. Crahan, C. Cahoon, and J. Freedman for thoughtful discussion and comments on the manuscript. Special thanks to M. Harms for helping with MD simulations and SASA analysis. We also thank A. Barkan, B. Bowerman, D. Garcia, and S. Hansen for feedback on figures. We thank the Claycomb lab for PRG-1::GFP::FLAG; HA::tagRFP::ZNFX-1 strain, the Mello Lab for sharing the 3xFLAG::WAGO-1 endogenously tagged, 3xFLAG::WAGO-1 transgene, and *wago-1(tm1414)* strains, and the Strome Lab for sharing their PGL-1 α rabbit antibody. We are grateful to the University of Oregon's Genomics and Cell Characterization Core Facility for small RNA and mRNA sample prep and sequencing. We thank the CGC for providing multiple strains for this study (funded by National Institutes of Health P40 OD010440).

Funding Sources:

This work was supported by the National Institutes of Health T32GM007431 to A.L.D., National Institutes of Health R03HD110785 to N.A.K., National Institutes of Health T32HD007348 to H.R.W., and National Institutes of Health R35GM128890 to D.E.L.

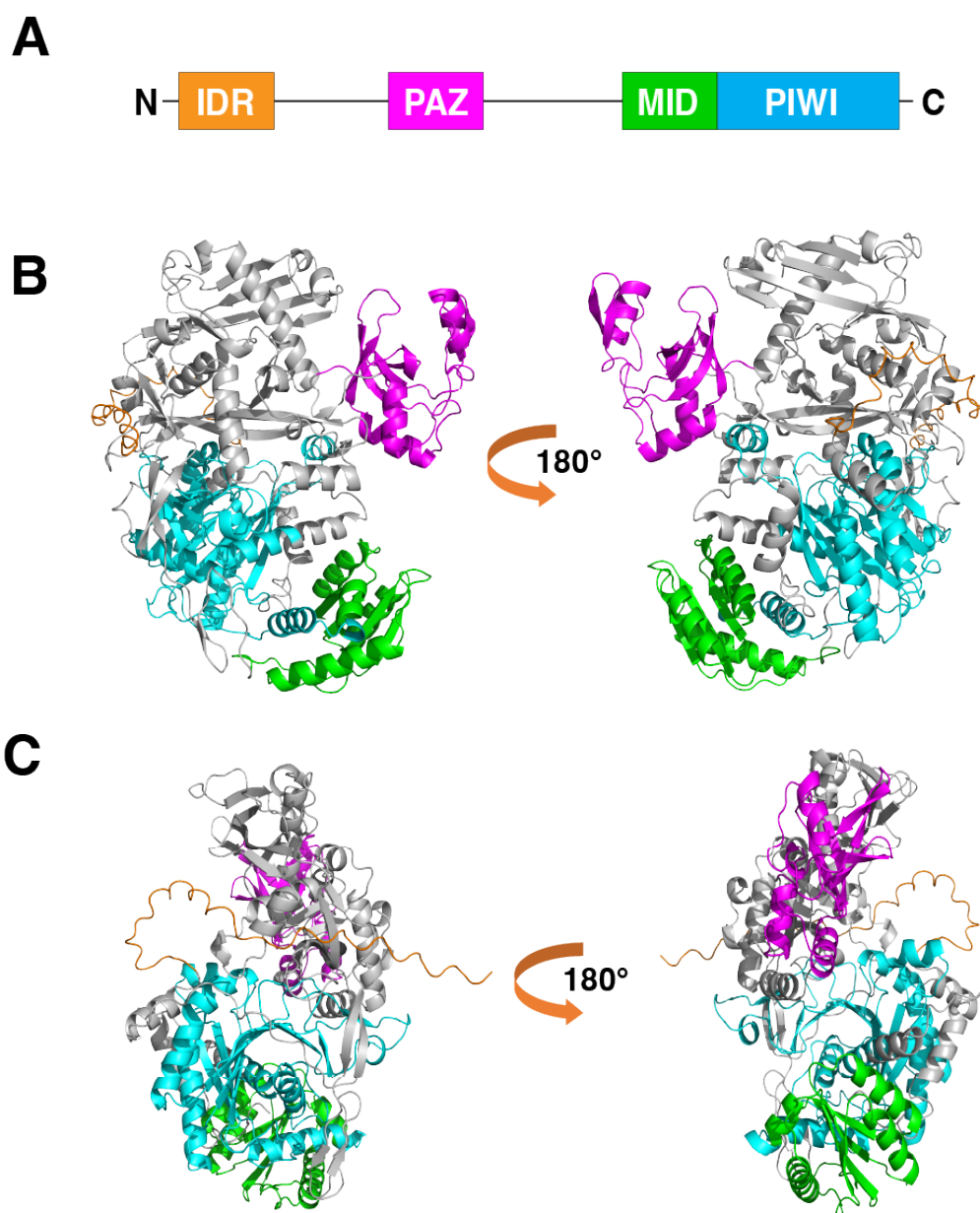


Figure S2.1: WAGO-1 predicted protein structure. (A) Cartoon schematic of WAGO-1 protein structure. Orange, IDR; pink, PAZ domain; green, MID domain; light blue, PIWI domain. (B) Ribbon diagram of WAGO-1 AlphaFold3 predicted structure. (C) Front views of WAGO-1 ribbon diagram of AlphaFold3 predicted structure.

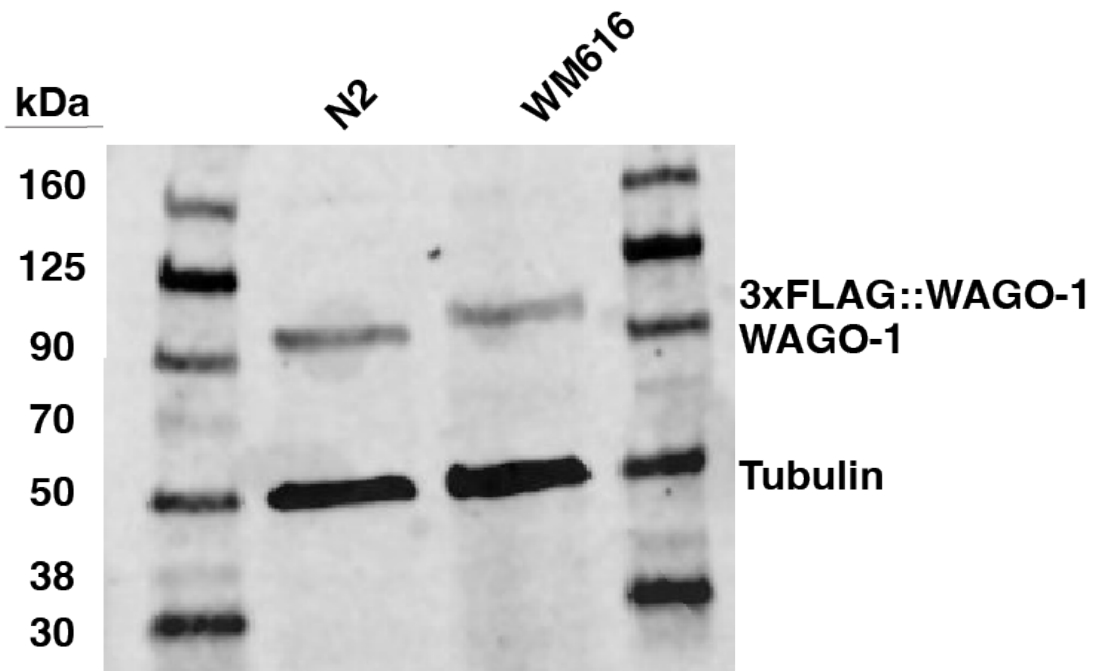


Figure S2.2: Western blot confirmation of WAGO-1 anti-chicken antibody. Representative western blot for WAGO-1 protein size in wild-type (N2) and endogenously tagged 3xFLAG::WAGO-1 (WM616) strains. Wild-type WAGO-1 size = 105kDa; endogenously tagged 3xFLAG::WAGO-1 size = 108kDa.

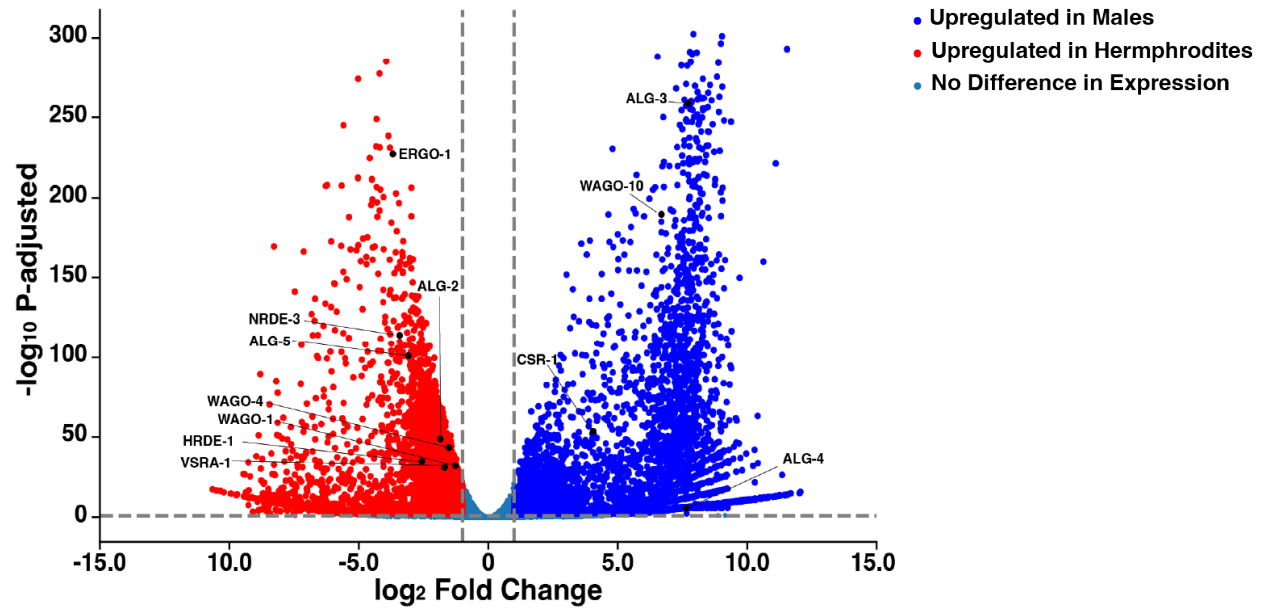


Figure S2.3: *wago-1* mRNA upregulated in hermaphrodites compared to males. Volcano plot of mRNA-seq between wild-type adult males and hermaphrodites. Genes upregulated in hermaphrodites are in red. Genes upregulated in males are in blue. Blue-grey dots represent genes with no statistical difference in regulation between the sexes. Black dots and labels denote differentially regulated AGOs between adult hermaphrodites and adult males.

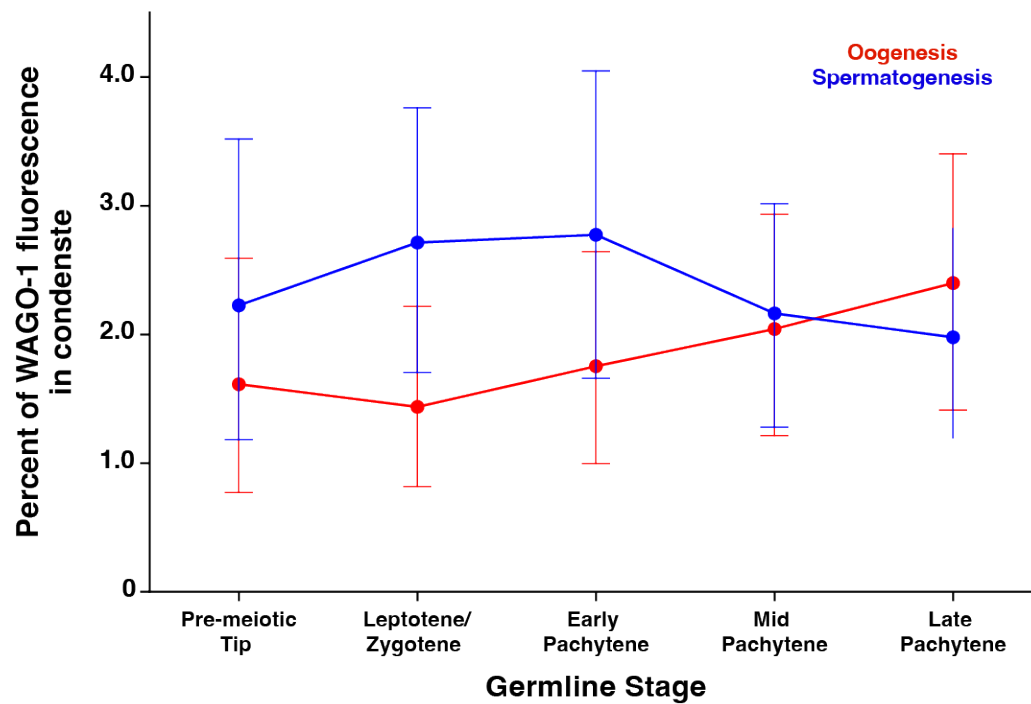


Figure S2.4: Meiotic-stage specific effect on WAGO-1 phases during oogenesis and spermatogenesis. Line graph depicting average WAGO-1 fluorescent intensity within germ granules as a percentage of total WAGO-1 fluorescent intensity for PMT, L/Z, EP, MP, and LP regions of the germline undergoing oogenesis (red) and spermatogenesis (blue). For each stage n=9 gonads were analyzed.

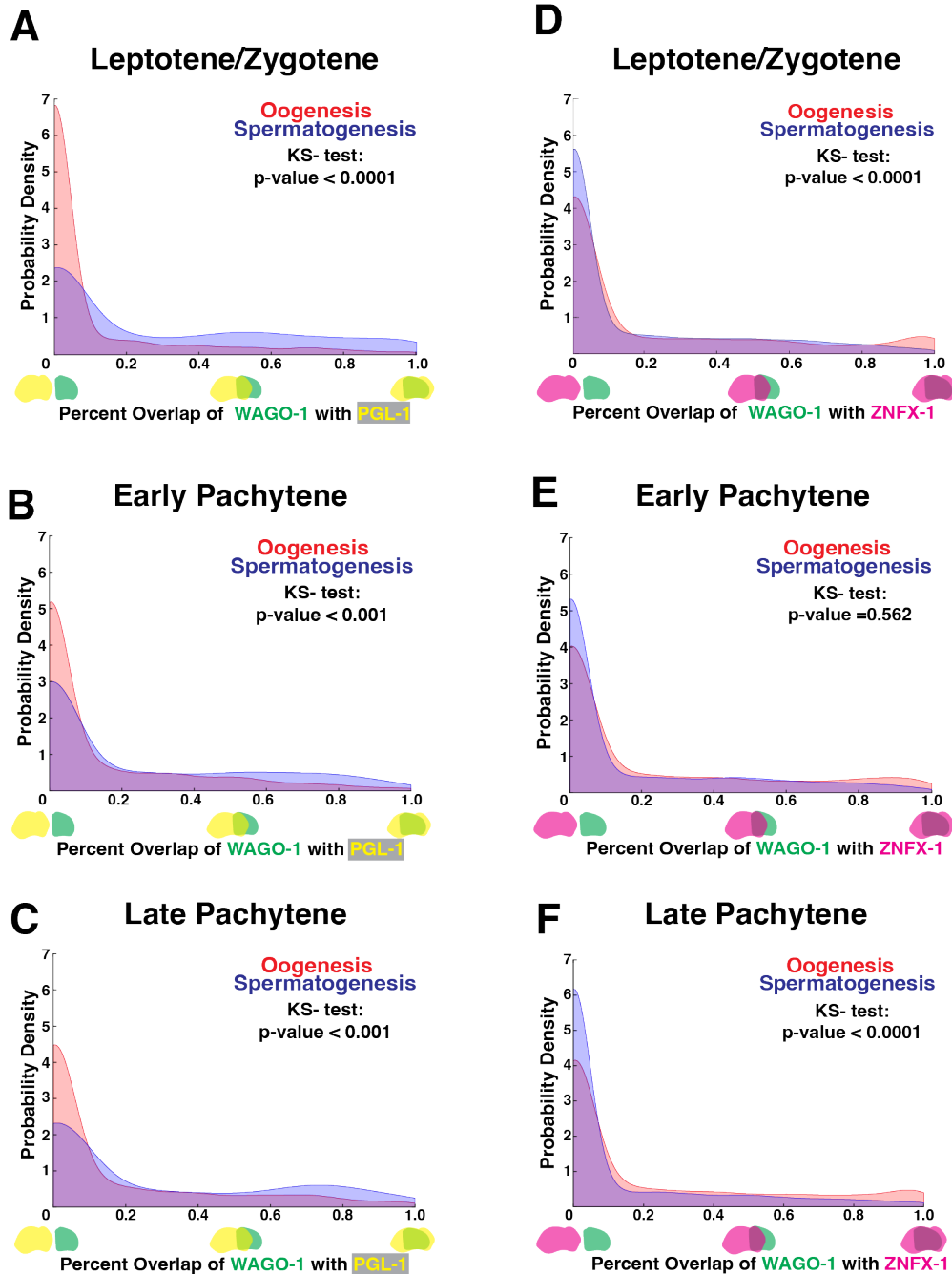


Figure S2.5: Kernel density estimate plots of percent of WAGO-1 foci volume overlap with PGL-1 and ZNFX-1. (A,B,C) Kernel density estimate (KDE) plots of the percent of WAGO-1 foci volume overlapping with PGL-1 foci undergoing oogenesis (red) and spermatogenesis (blue) in the (A) L/Z, (B) EP, and (C) LP regions of meiosis. The p-values were calculated using two-sample Kolmogorov-Smirnov test. **(D,E,F)** KDE plots of the percent of WAGO-1 foci volume overlapping with ZNFX-1 foci undergoing oogenesis (red) and spermatogenesis (blue) in the (D) L/Z, (E) EP, and (F) LP stages of meiosis. The p-values were calculated using two-sample Kolmogorov-Smirnov test.

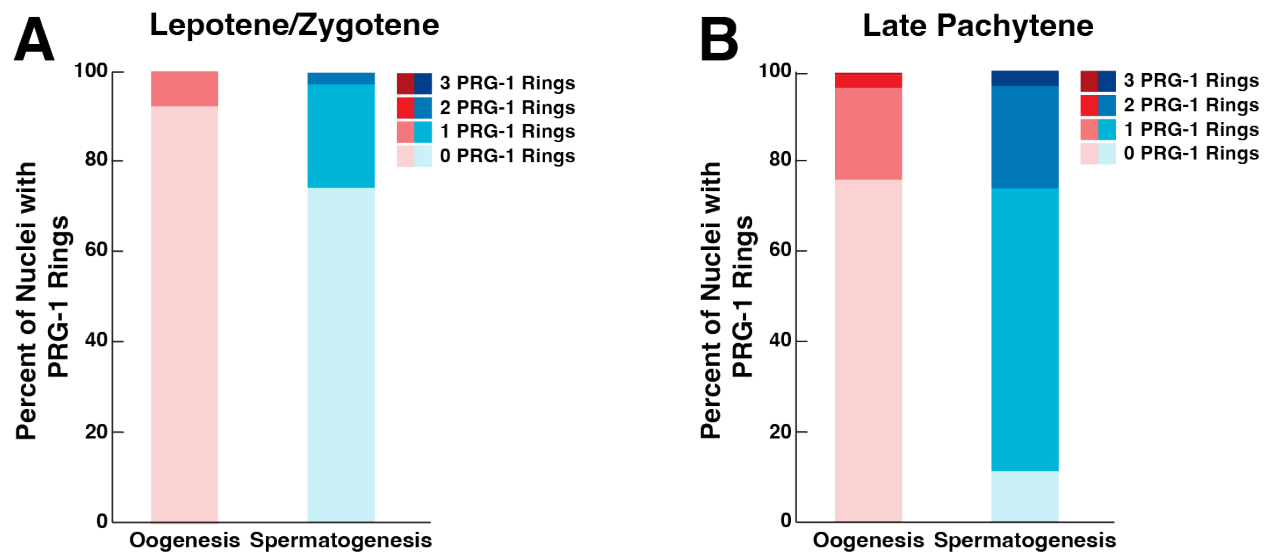


Figure S2.6: PRG-1 rings are most prevalent in spermatogenesis during late pachytene. (A) Bar graphs indicating the percent of nuclei undergoing oogenesis (red) and spermatogenesis (blue) with 0, 1, 2, or 3 PRG-1 rings during L/Z. **(B)** Bar graphs indicating the percent of nuclei undergoing oogenesis (red) and spermatogenesis (blue) with 0, 1, 2, or 3 PRG-1 rings during LP. For each stage n=9 gonads were analyzed. For total number of nuclei see Table S2.9.

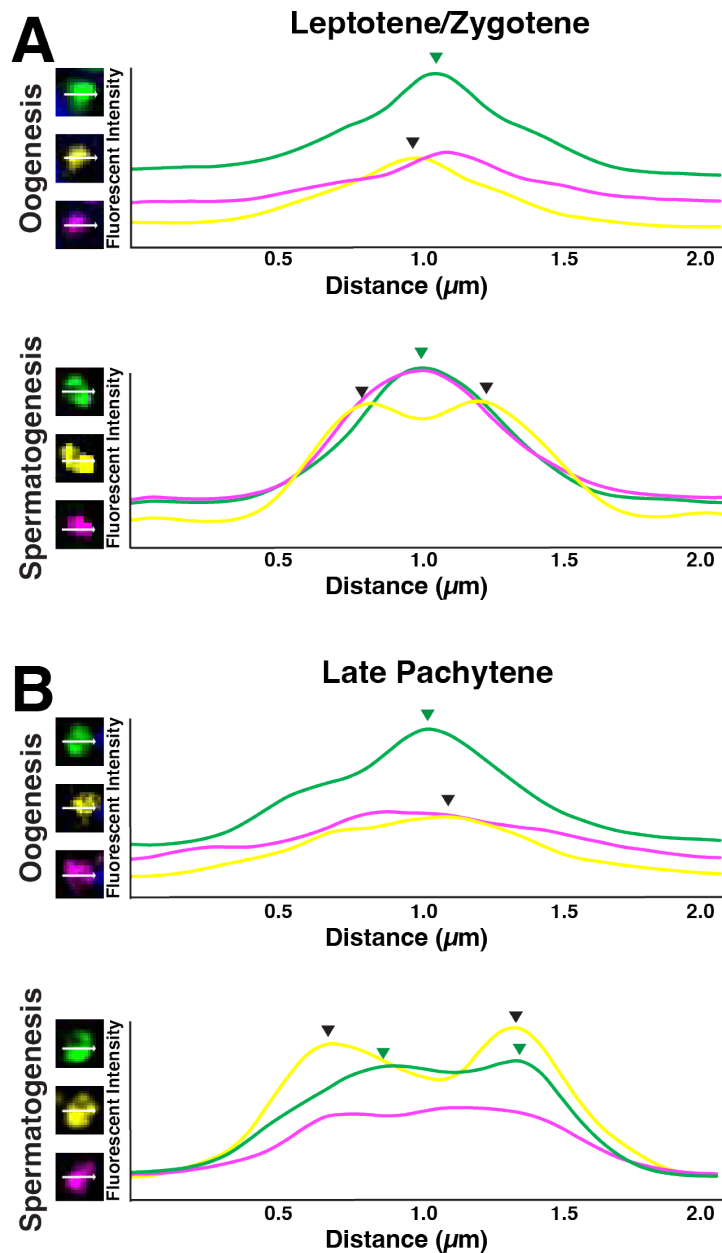


Figure S2.7: PRG-1 forms ring-like structure and WAGO-1 forms half-moon structure during spermatogenesis. (A) Left: Zoom in panels of single channels from representative germ granules boxed in Fig. 2.3A. White arrow indicates line scan direction. Right: Averaged line scans of pixel intensity across germ granules from oogenesis (top) and spermatogenesis (bottom) during L/Z. **(B)** Left: Zoom in panels of single channels from representative germ granules boxed in Fig. 2.3C. White arrow indicates line scan direction. Right: Averaged line scans of pixel intensity across germ granules from oogenesis (top) and spermatogenesis (bottom) during late pachytene. Black arrows indicate local maxima of PRG-1 intensity, green arrows indicate local maxima of WAGO-1 intensity.

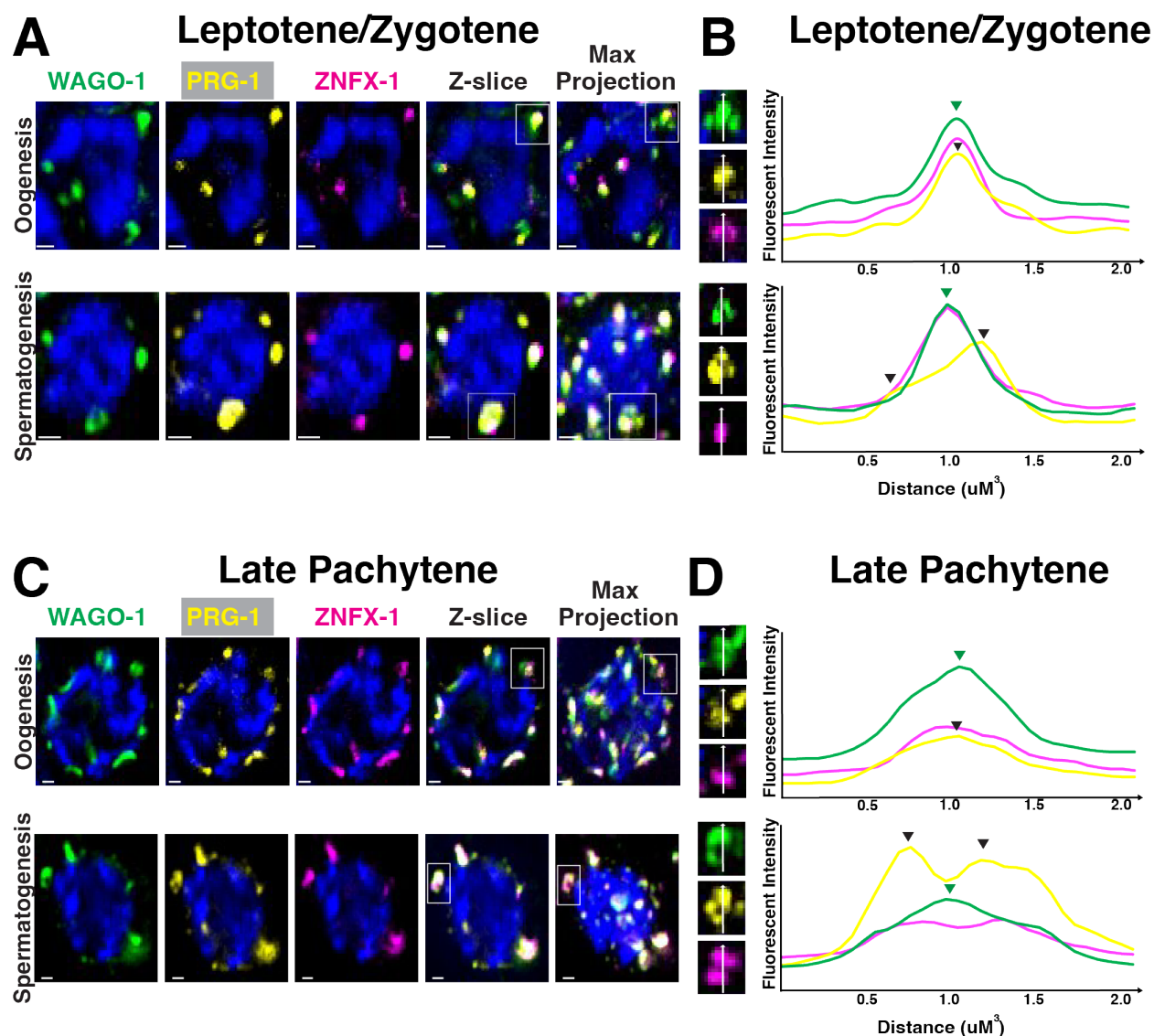


Figure S2.8: PRG-1 forms hollow rings during spermatogenesis. (A,C) Representative Z-slice and max-projection of single nucleus either undergoing oogenesis (top) or spermatogenesis (bottom) during (A) L/Z and (C) LP stained for WAGO-1 (green), PRG-1 (yellow), and ZNFX-1 (magenta). Scale bars represent $1\mu\text{m}$. (B,D) Left: Zoom panels of single channels from representative germ granules boxed in (B) A and (D) C. White arrow indicates line scan direction. Right: Averaged line scans of pixel intensity across max projection of germ granules from oogenesis (top) and spermatogenesis (bottom) during (B) L/Z and (D) LP. Black arrows indicate local maxima of PRG-1 intensity, green arrows indicate local maxima of WAGO-1 intensity.

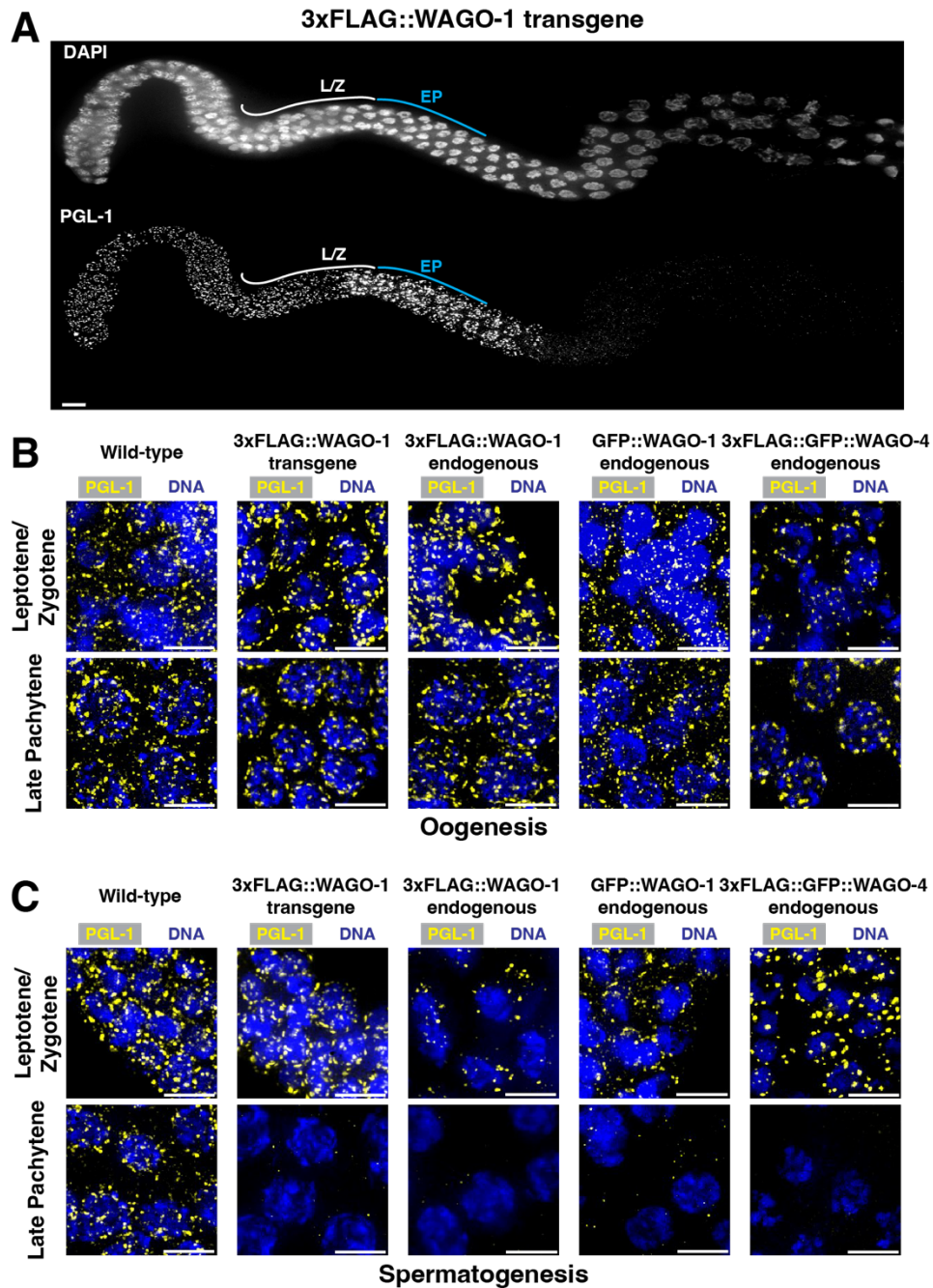


Figure S2.9: Tagging WAGO-1 and WAGO-4 on N-terminus causes male specific loss of PGL-1 phase separation during late pachytene. (A) Representative immunofluorescence images of nuclei (top) and PGL-1 (bottom) throughout spermatogenesis from an adult male with 3xFLAG::WAGO-1 transgene. Leptotene/zygotene (L/Z, white) and early pachytene (EP, light blue) zones are highlighted and identified based on nuclei morphology. Scale bars represent 10µm. (B,C) Max projection images of L/Z (top) and LP (bottom) nuclei from (B) adult hermaphrodites undergoing oogenesis and (C) adult males undergoing spermatogenesis stained for PGL-1 (yellow) from the following strains (L to R): wild-type, 3xFLAG::WAGO-1

transgene, endogenously tagged 3xFLAG::WAGO-1, endogenously tagged GFP::WAGO-1, and endogenously tagged 3xFLAG::GFP::WAGO-4. Scale bars represent 5 μ m.

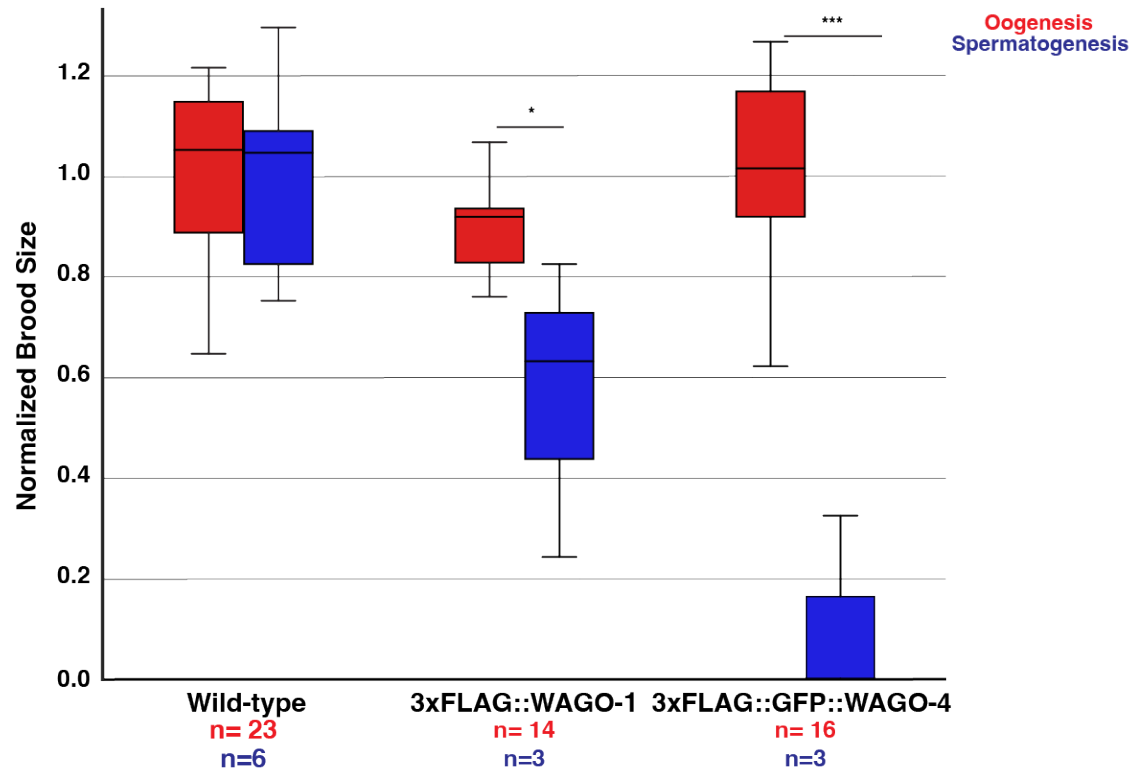


Figure S2.10: Endogenous N-terminus tagging of WAGO-1 and WAGO-4 causes male specific decrease in brood size. Normalized brood size of adult hermaphrodites undergoing oogenesis (red) and adult males undergoing spermatogenesis (blue) from wild-type, endogenously tagged 3xFLAG::WAGO-1, and endogenously tagged 3xFLAG::GFP::WAGO-4 strains. * $p < 0.05$, *** $p < 0.0001$, Two-way ANOVA with Tukey's multiple comparison test.

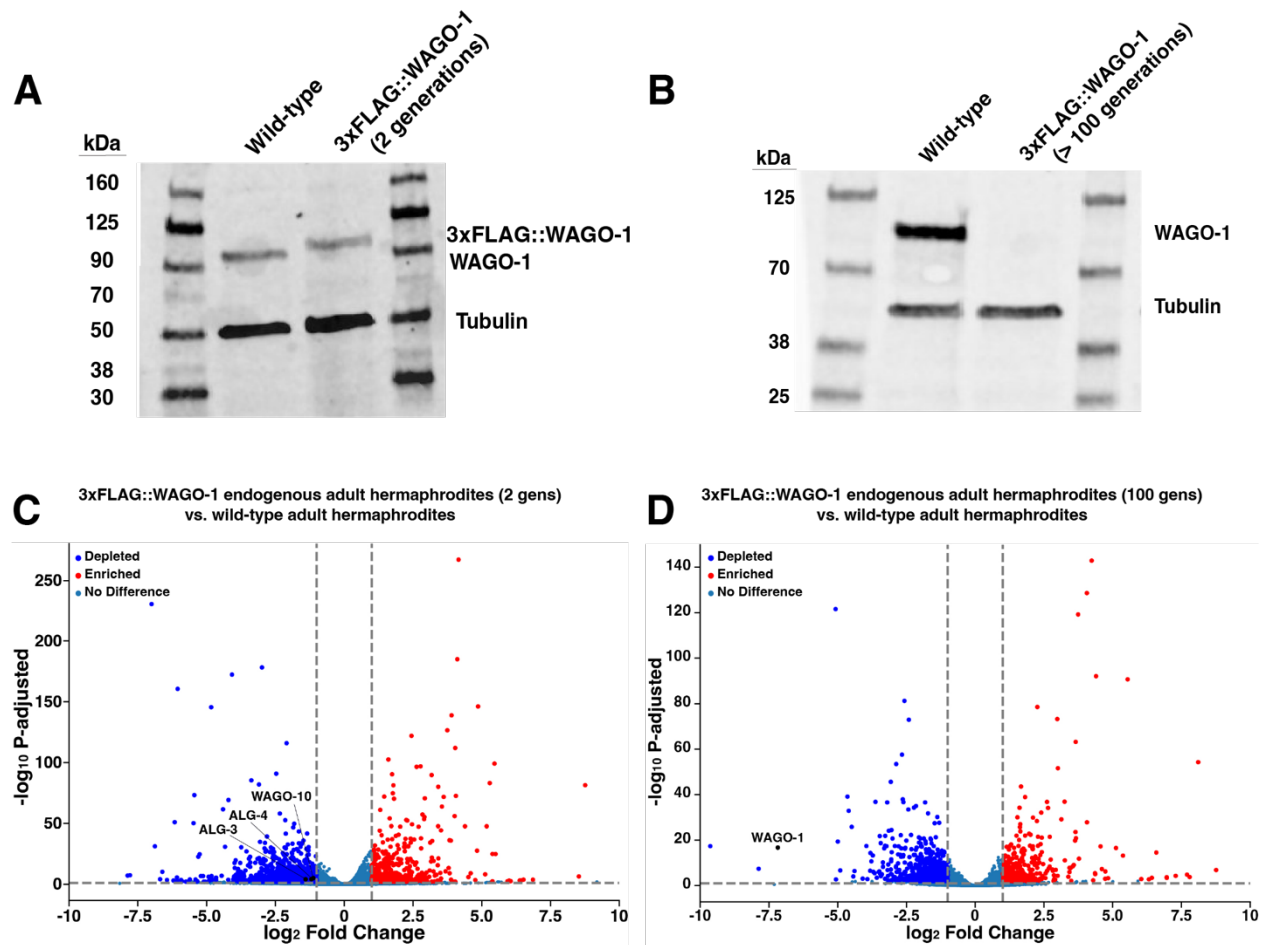


Figure S2.11: N-terminus tagging of endogenous WAGO-1 cause transgenerational loss of *wago-1* gene expression and WAGO-1 protein expression. (A,B) Representative western blot showing WAGO-1 expression in wild-type (left) and 3xFLAG::WAGO-1 adult hermaphrodites (right) that have been passaged for (A) two generations and (B) 100 generations. (C,D) Volcano plot displaying differential expression from mRNA-sequencing of wild-type and endogenously tagged 3xFLAG::WAGO-1 adult hermaphrodites after (C) two generations and (D) 100 generations. Blue denotes genes significantly depleted in 3xFLAG::WAGO-1 hermaphrodites compared to wild-type. Red dots represent gene significantly enriched in 3xFLAG::WAGO-1 hermaphrodites compared to wild-type. Differentially expressed AGOs labeled based on decreased expression compared to wild-type labeled in black.

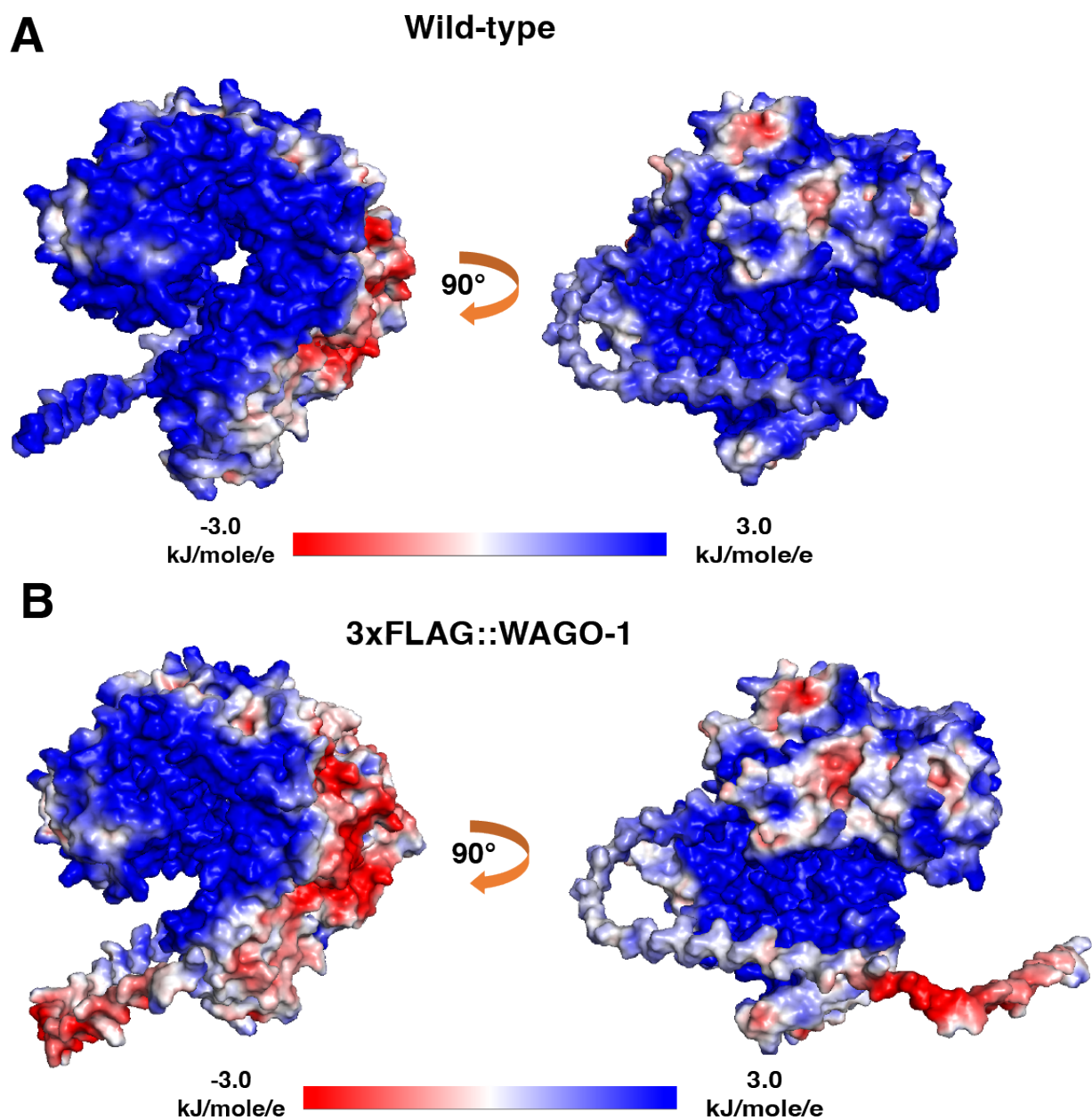


Figure S2.12: Local electrostatic potential of wild-type and 3xFLAG::WAGO-1. (A-B) Electrostatic map of AlphaFold3 predicted (A) wild-type WAGO-1 and (B) 3xFLAG::WAGO-1 structure created utilizing APBS and PDB2PQR Pymol extensions100. Potentials are on a -3.0 to 3.0 red-white-blue color map with units kJ/mol/e, where blue represents regions with a more positive charge and red represents regions with a more negative charge.

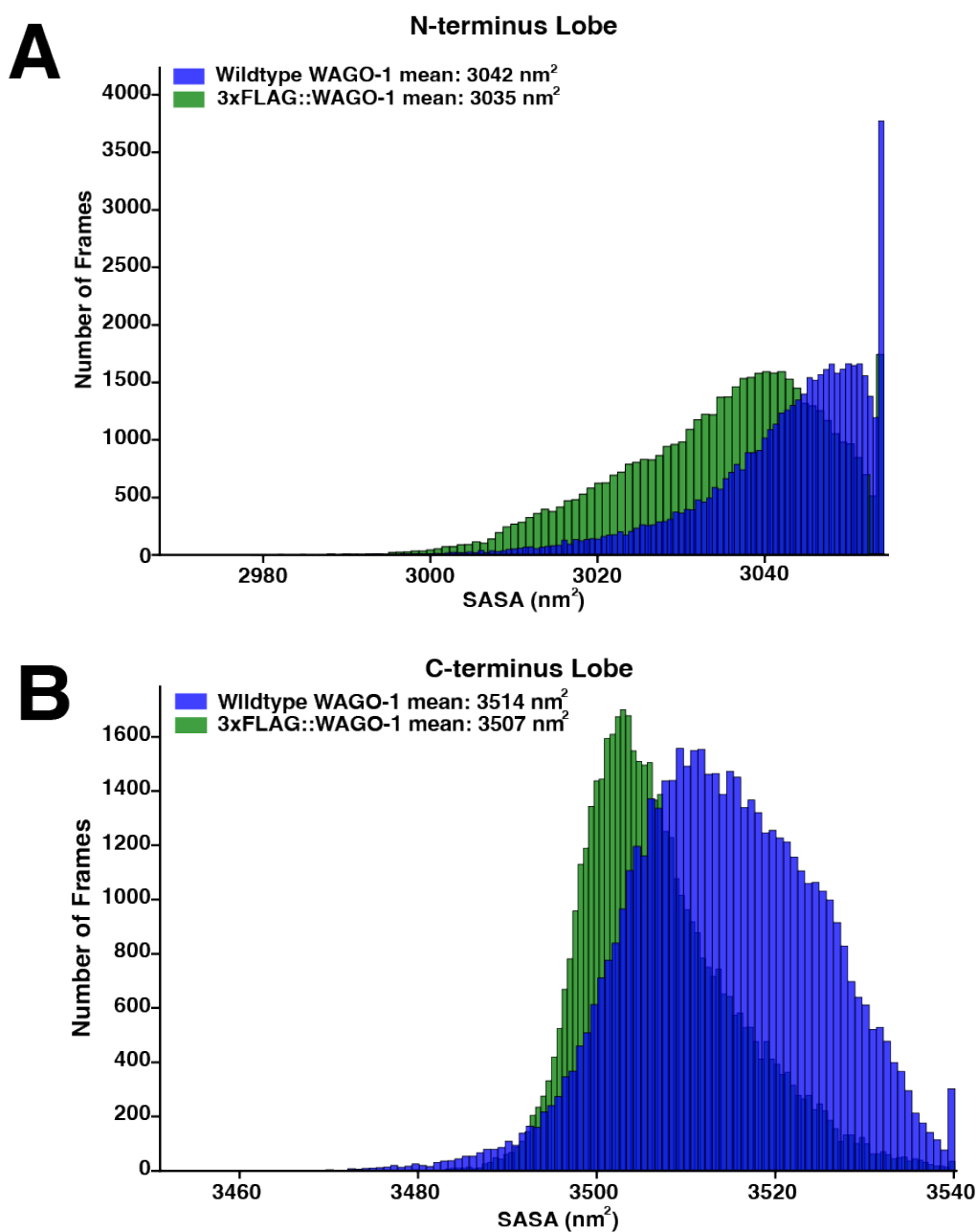
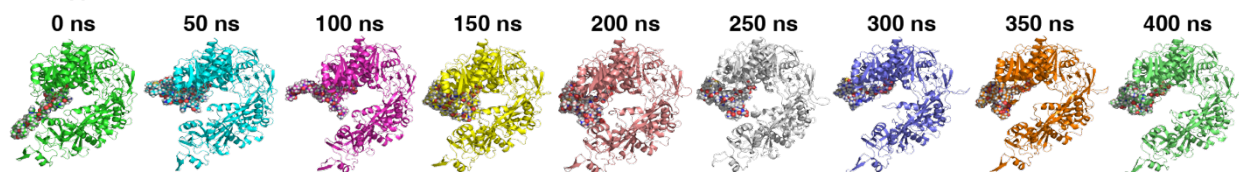
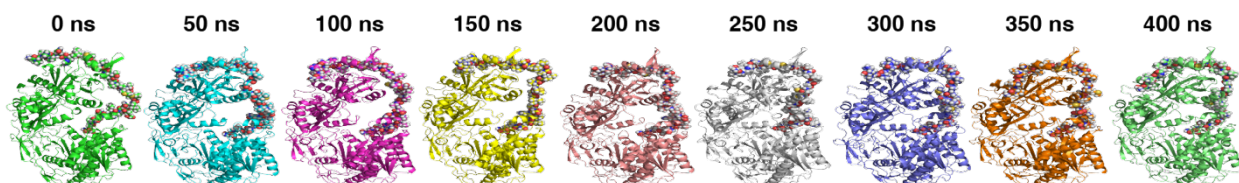


Figure S2.13: Histogram of average solvent accessible surface area (SASA) of wild-type and 3xFLAG::WAGO-1. (A) Histogram depicting the average number of frames from three molecular dynamic simulations where the N-terminal lobe wild-type WAGO-1 (blue) and 3xFLAG::WAGO-1 (green) have a specific SASA. **(B)** Histogram depicting the average number of frames from three molecular dynamic simulations where the C-terminal lobe wild-type WAGO-1 (blue) and 3xFLAG::WAGO-1 (green) have a specific SASA.

Wild-type trial 1



Wild-type trial 2



Wild-type trial 3

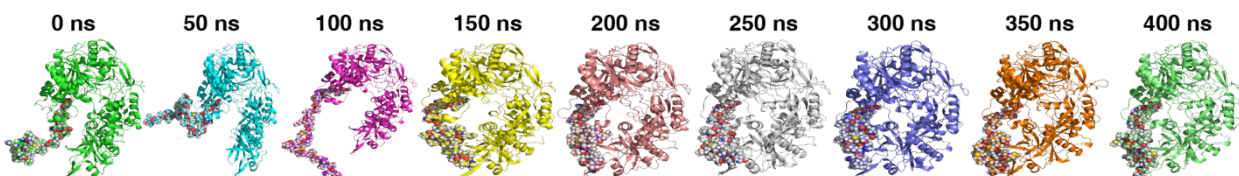


Figure S2.14: Stills from three wild-type WAGO-1 molecular dynamic simulations. Stills from three molecular dynamic simulations of wild-type WAGO-1 taken 50 nanoseconds apart. The IDR is depicted as ball-and-sphere model while the core of the protein is depicted as ribbon model. See Methods and Materials for determining starting configuration.

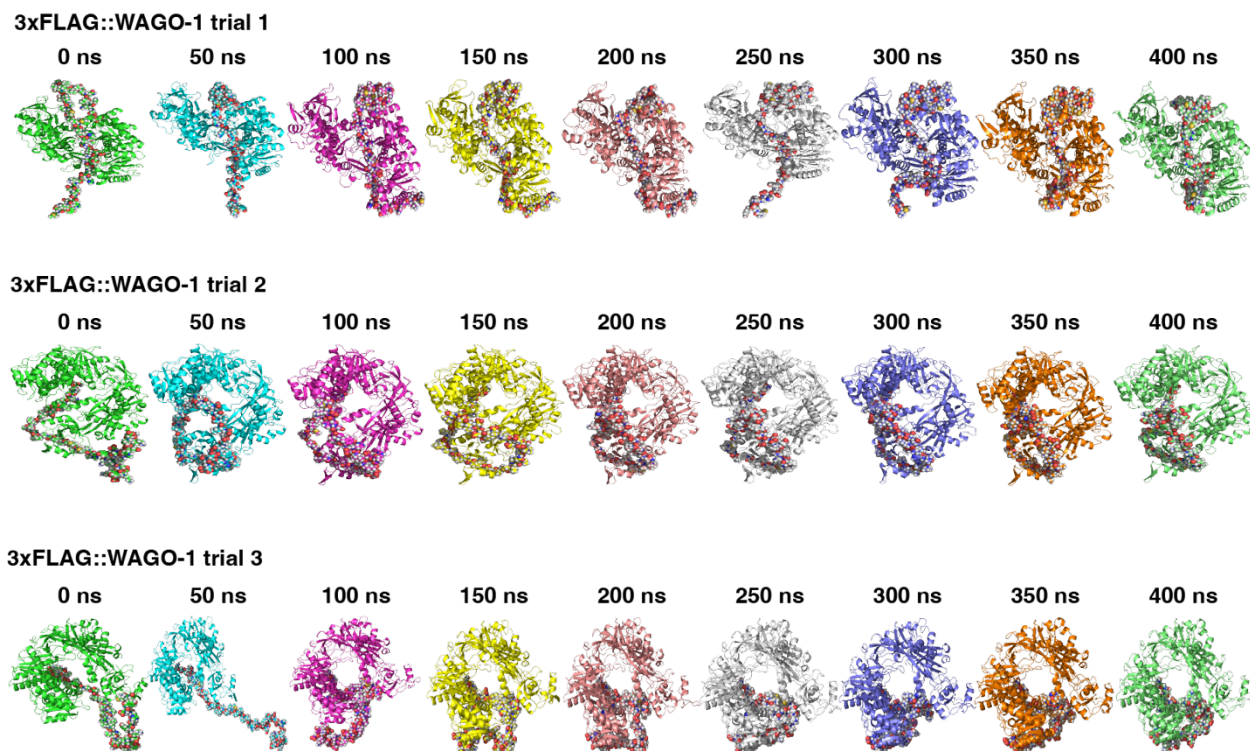
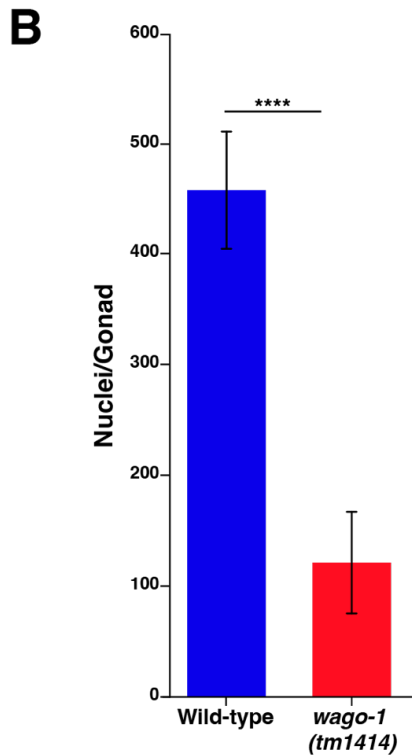
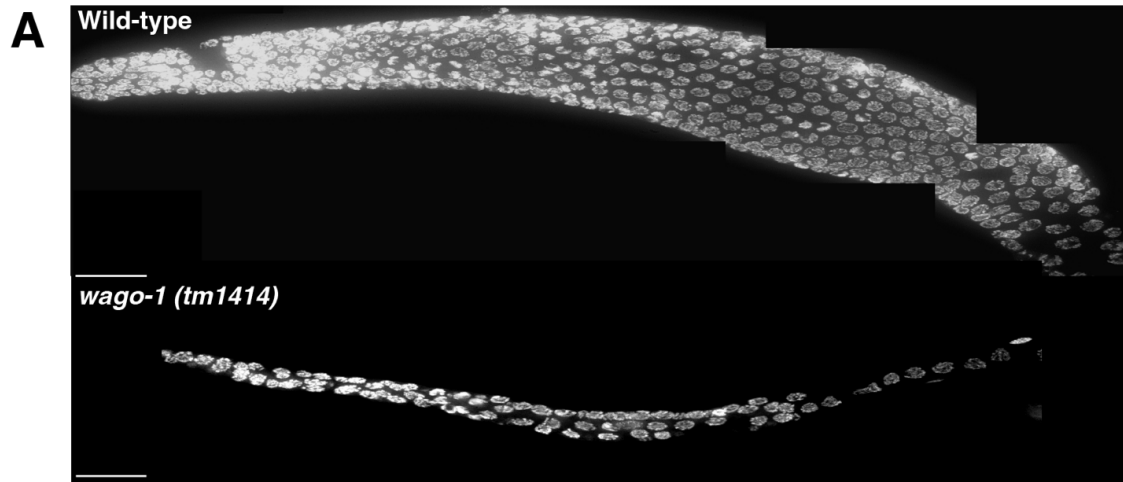


Figure S2.15: Stills from three 3xFLAG::WAGO-1 molecular dynamic simulations. Stills from three molecular dynamic simulations of 3xFLAG::WAGO-1 taken 50 nanoseconds apart. The IDR is depicted as ball-and-sphere model while the core of the protein is depicted as ribbon model. See Methods and Materials for determining starting configuration.



Figure

S2.16: *wago-1 (tm1414)* causes a decrease in germ cell number in germlines undergoing oogenesis. (A) Representative immunofluorescence images of nuclei in germlines undergoing oogenesis from wild-type and *wago-1(tm1414)* adult hermaphrodites. Scale bars represent 10µm. (B) Bar graph showing average number of nuclei counts from halved wild-type and *wago-1(tm1414)* adult hermaphrodite germlines undergoing oogenesis. For each genotype n=9 gonads were analyzed. ****p<0.00001, one-way Anova.

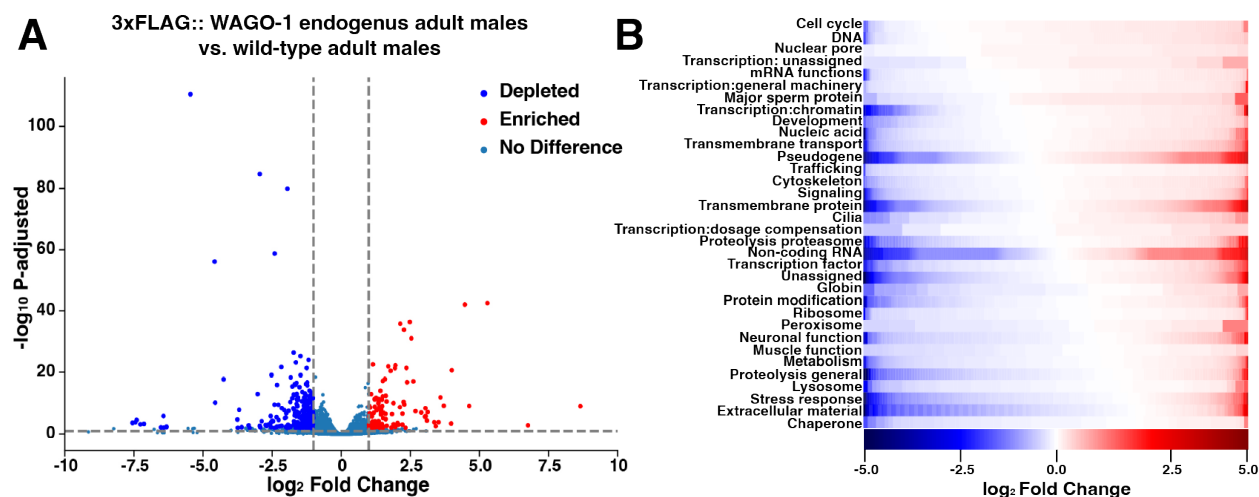
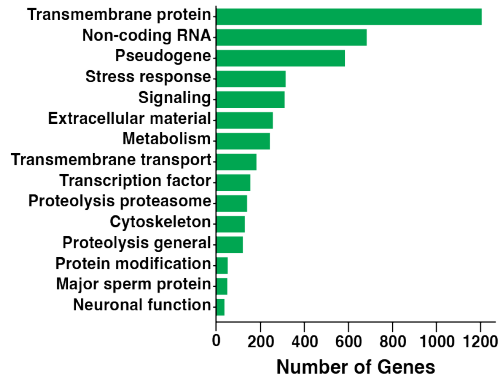
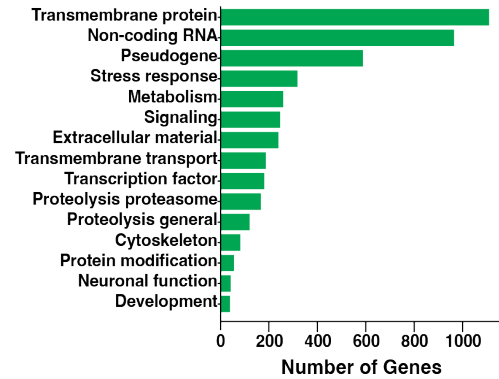


Figure S2.17: Endogenously tagged 3xFLAG::WAGO-1 males misregulates gene expression compared to wild-type males (A) Volcano plot of mRNA-sequencing data between wild-type and endogenously tagged 3xFLAG::WAGO-1 males. Genes depleted in 3xFLAG::WAGO-1 males are in blue. Genes enriched in 3xFLAG::WAGO-1 males are in red. Genes with less than a $\log_2 \text{Fold Change}$ of 1 and a p-adjusted value less than 0.05 are in blue-gray. **(B)** Heat map depicting individual genes that are up (red) or down (blue) regulated based on $\log_2 \text{Fold Change}$ in 3xFLAG::WAGO-1 compared to wild-type WAGO-1 grouped by gene ontology (GO) terms. GO groups are vertically sorted in descending order based on the proportion of genes that are upregulated. GO terms were assigned using WormCat 2.0 (Holdorf et al., 2020). White indicates no significant change in expression.

A Top 15 GO terms 3xFLAG::WAGO-1 Transgene Hermaphrodites



B Top 15 GO terms 3xFLAG:: WAGO-1 Endogenous Hermaphrodites



C Top 15 GO terms 3xFLAG:: WAGO-1 Endogenous Males

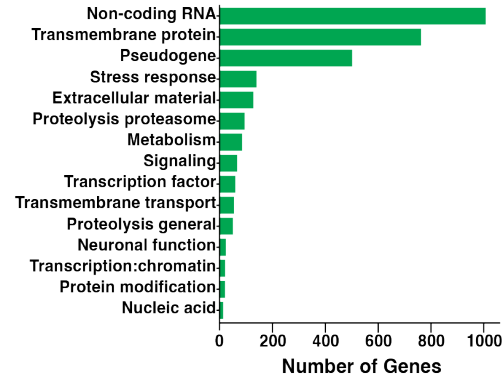


Figure S2.18: Top 15 GO terms that are misregulated in 3xFLAG::WAGO-1 strains. (A-C) Top 15 gene ontology (GO) terms of genes misregulated in (A) adult hermaphrodites with 3xFLAG::WAGO-1 transgene, (B) adult hermaphrodites with 3xFLAG::WAGO-1 endogenously tagged, (C) adult males with 3xFLAG::WAGO-1 endogenously compared to wild-type adult hermaphrodites or males. GO terms are vertically sorted in descending order based on the number of genes misregulated. GO terms were assigned using WormCat 2.0 (Holdorf et al., 2020).

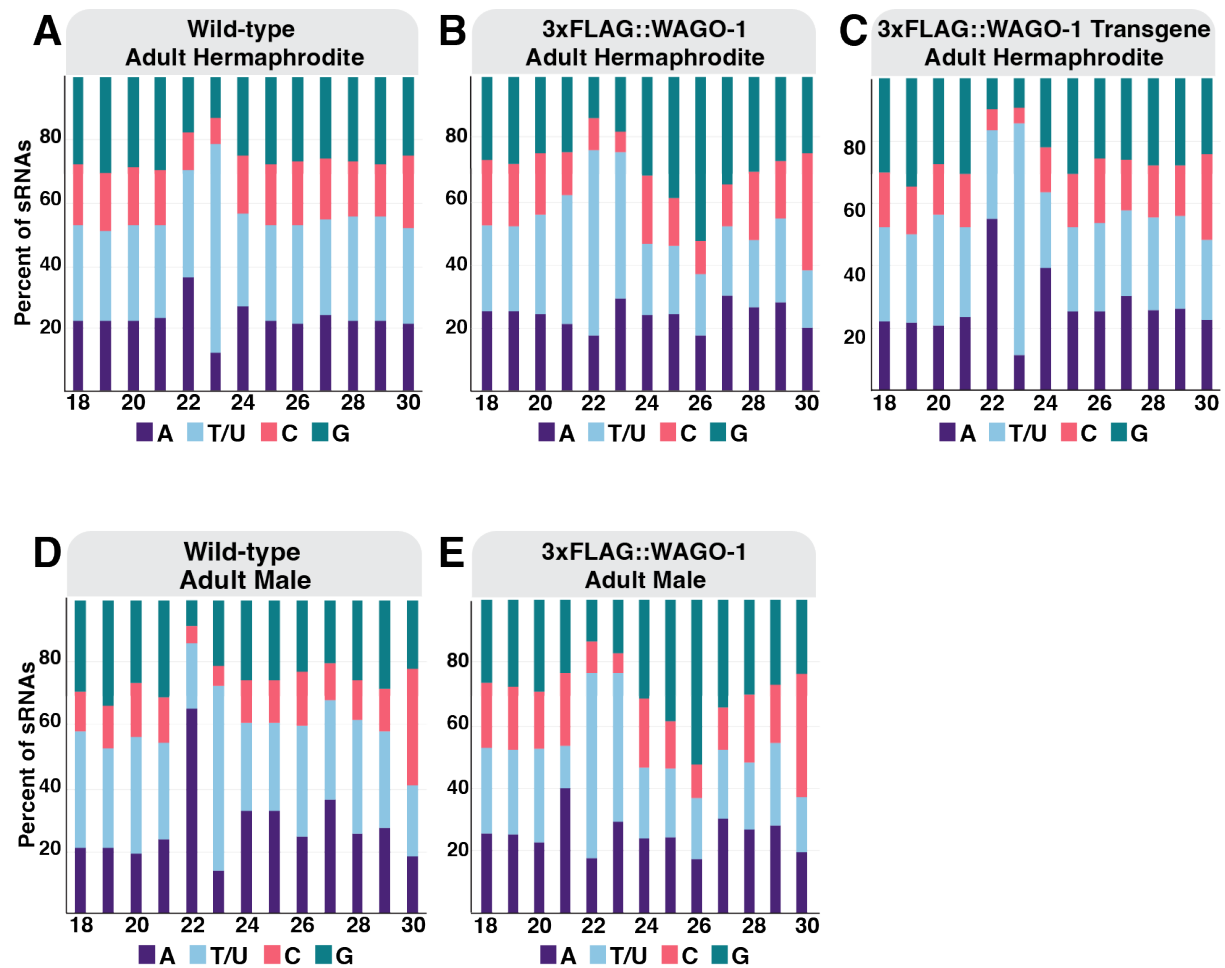


Figure S2.19: Global small RNA profile of wild-type and 3xFLAG::WAGO-1 tagged strains. (A-E) Small RNAs grouped by nucleotide length from 18 to 30, with percent sRNAs with an A (purple), T/U (light blue), C (coral), or G (teal) on the 5' end for (A) wild-type adult hermaphrodites, (B) 3xFLAG::WAGO-1 endogenously tagged adult hermaphrodites, (C) adult hermaphrodites with 3xFLAG::WAGO-1 transgene, (D) wild-type adult males, and (E) 3xFLAG::WAGO-1 endogenously tagged adult males.

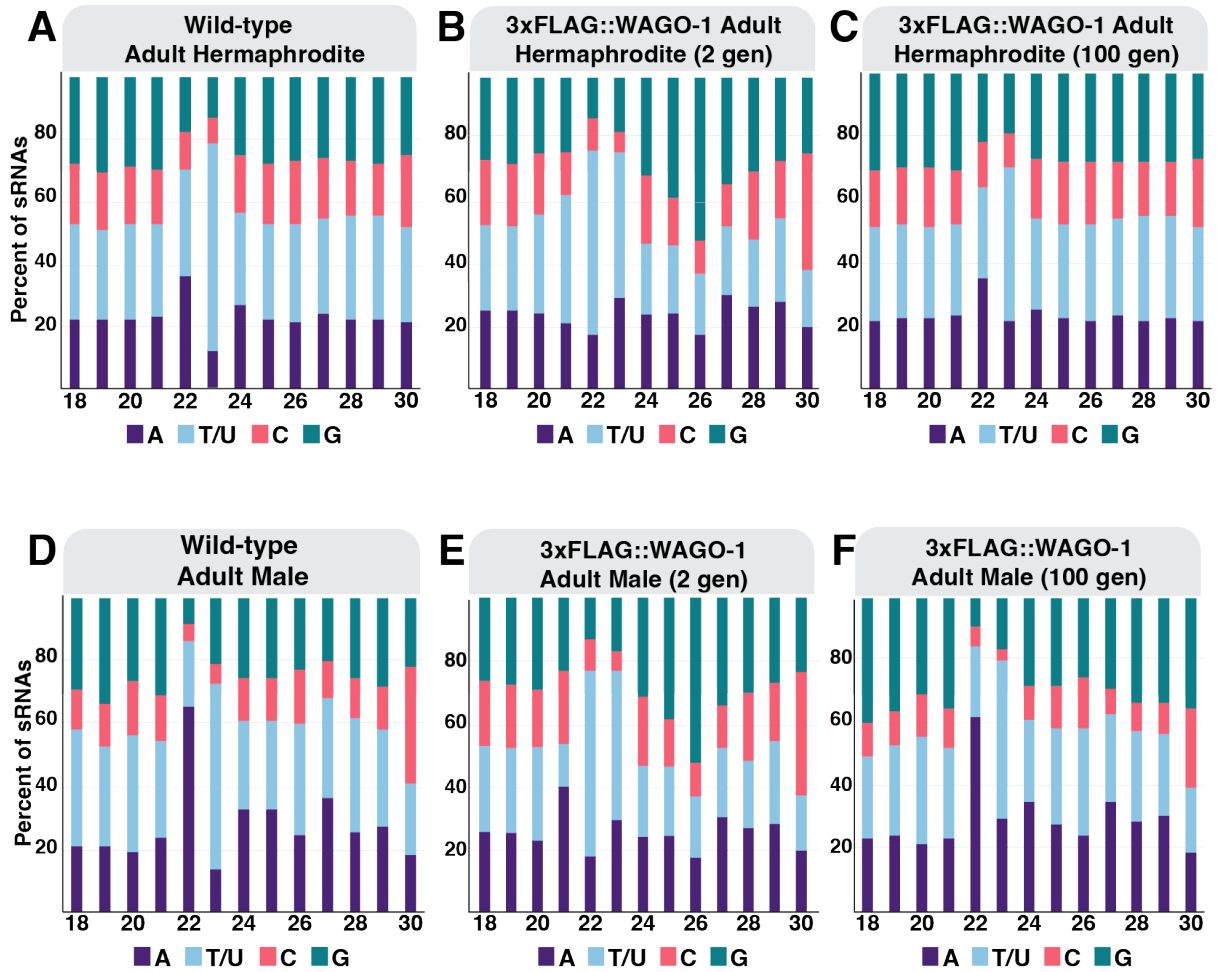


Figure S2.20: Global small RNA profile of wild-type and silenced *wago-1* 3xFLAG::WAGO-1 tagged strains. (A-F) Small RNAs grouped by nucleotide length from 18 to 30, with percent sRNAs with an A (purple), T/U (light blue), C (coral), or G (teal) on the 5' end for (A) wild-type adult hermaphrodites, (B) 3xFLAG::WAGO-1 endogenously tagged adult hermaphrodites passed for two generations, (C) 3xFLAG::WAGO-1 endogenously tagged adult hermaphrodites passed for 100 generations, (D) wild-type adult males, (E) 3xFLAG::WAGO-1 endogenously tagged adult males passed for two generations, and (F) 3xFLAG::WAGO-1 endogenously tagged adult males passed for 100 generations.

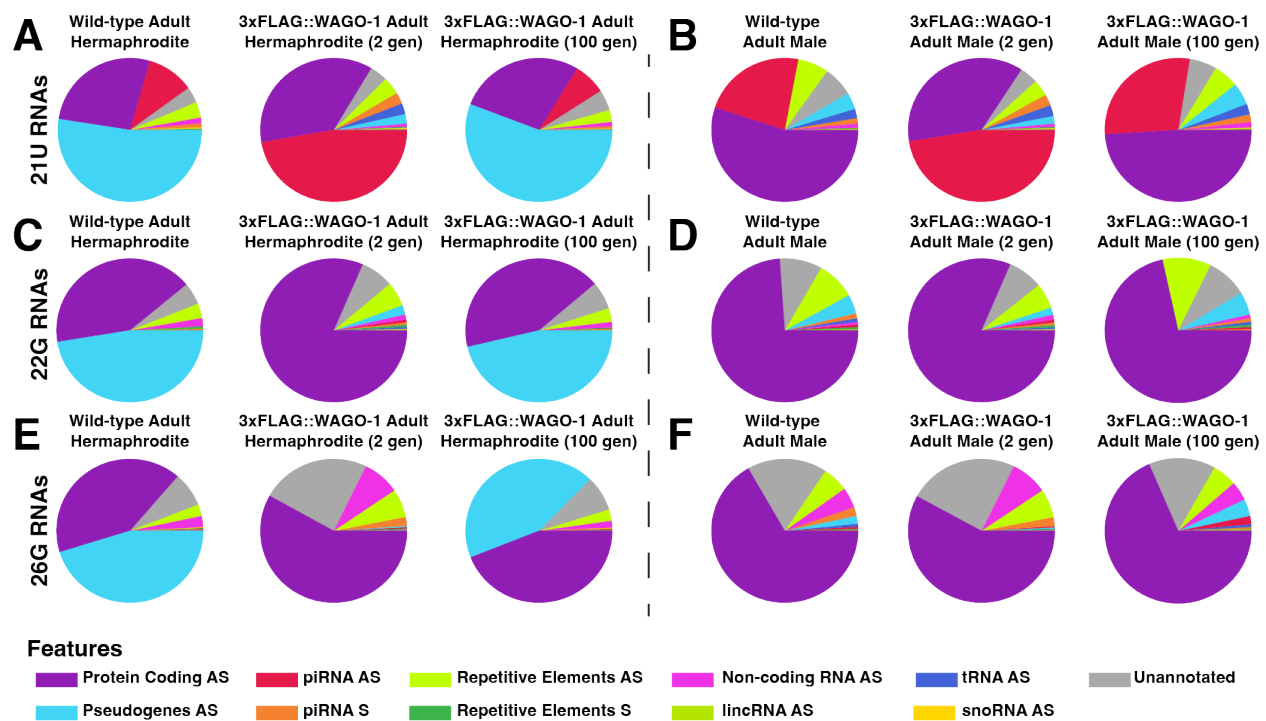


Figure S2.21: Proportion of small RNAs mapping to specific genetic elements in silenced *wago-1* 3xFLAG::WAGO-1 strains. (A-F) Pie charts depicting the proportion of (A-B) 21U-, (C-D) 22G-, and (E-F) 26G-RNAs mapping to specific genetic elements in wild-type, 3xFLAG::WAGO-1 passaged for two generations, and 3xFLAG::WAGO-1 passaged for 100 generations (A,C,E) adult hermaphrodites and (B,D,F) adult males. AS = antisense, S=sense.

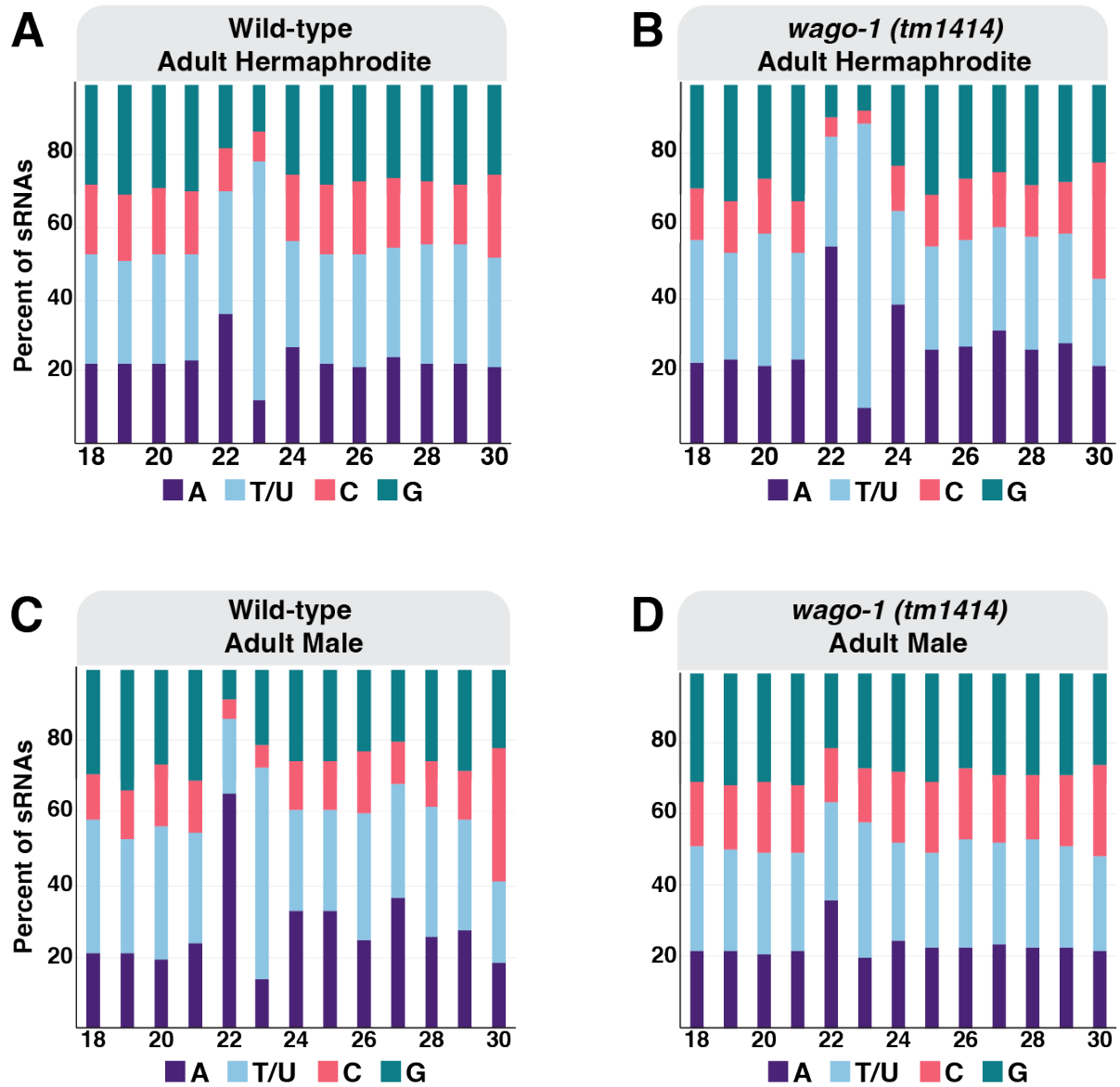


Figure S2.22: Global small RNA profile of wild-type and *wago-1 (tm1414)* hermaphrodites and males. (A-D) Small RNAs grouped by nucleotide length from 18 to 30, with percent sRNAs with an A (purple), T/U (light blue), C (coral), or G (teal) on the 5' end for (A) wild-type adult hermaphrodites, (B) *wago-1 (tm1414)* adult hermaphrodites, (C) wild-type adult males, and (D) *wago-1 (tm1414)* adult males.

Table S2.1: Means and standard deviations of WAGO-1 foci volumes throughout meiotic progression. Hermaphrodite germlines n=9, male germlines n=9.

Sex	Stage	Mean Volume (μm^3)	STD	Number of foci
Hermaphrodite	PMT	0.124	0.17	5722
Male	PMT	0.126	0.12	7116
Hermaphrodite	L/Z	0.117	0.15	6448
Male	L/Z	0.172	0.17	5959
Hermaphrodite	EP	0.143	0.15	7014
Male	EP	0.194	0.20	4449
Hermaphrodite	MP	0.168	0.23	7114
Male	MP	0.237	0.31	3913
Hermaphrodite	LP	0.261	0.37	4552
Male	LP	0.274	0.36	3775

Table S2.2: Mann-Whitney *U* statistics and p-values with Bonferroni correction comparing WAGO-1 foci volume distribution between hermaphrodites and males throughout meiosis.

Stage	Statistic	p-value
PMT	18166008.5	4.04E-26
L/Z	14258864	1.33E-136
EP	13047742.5	7.62E-50
MP	12160562	2.11E-28
LP	8221293.0	0.00034

Table S2.3: Means and standard deviations of WAGO-1 foci sphericity throughout meiotic progression.

Sex	Stage	Mean Sphericity	STD
Hermaphrodite	PMT	0.738	0.101
Male	PMT	0.680	0.101
Hermaphrodite	L/Z	0.734	0.101
Male	L/Z	0.698	0.103
Hermaphrodite	EP	0.732	0.093
Male	EP	0.704	0.101
Hermaphrodite	MP	0.731	0.096
Male	MP	0.706	0.101
Hermaphrodite	LP	0.715	0.100
Male	LP	0.702	0.105

Table S2.4: Mann-Whitney *U* statistics and p-values with Bonferroni correction comparing WAGO-1 foci sphericity distribution between hermaphrodites and males throughout meiosis.

Stage	Statistic	p-value
PMT	13774769	1.05E-218
L/Z	15217242.0	1.22E-89
EP	12992480.5	6.22E-52
MP	11715351.0	1.81E-43
LP	7883386.0	4.35E-11

Table S2.5: Binning of percent volume of WAGO-1 foci overlap with PGL-1 and ZNFX-1

Process	Classification	Protein	Zone	Foci Counts	Percent
Oogenesis	0%	PGL-1	L/Z	3128	0.498
Oogenesis	1-25%	PGL-1	L/Z	1775	0.283
Oogenesis	26-50%	PGL-1	L/Z	780	0.124
Oogenesis	51-75%	PGL-1	L/Z	396	0.063
Oogenesis	76-99%	PGL-1	L/Z	190	0.030
Oogenesis	100%	PGL-1	L/Z	10	0.002
Oogenesis	Total	PGL-1	L/Z	6279	1.000
Spermatogenesis	0%	PGL-1	L/Z	1787	0.328
Spermatogenesis	1-25%	PGL-1	L/Z	1373	0.252
Spermatogenesis	26-50%	PGL-1	L/Z	1035	0.190
Spermatogenesis	51-75%	PGL-1	L/Z	808	0.148
Spermatogenesis	76-99%	PGL-1	L/Z	422	0.078
Spermatogenesis	100%	PGL-1	L/Z	18	0.003
Spermatogenesis	Total	PGL-1	L/Z	5443	1.000
Oogenesis	0%	PGL-1	EP	2120	0.420
Oogenesis	1-25%	PGL-1	EP	1574	0.312
Oogenesis	26-50%	PGL-1	EP	768	0.152
Oogenesis	51-75%	PGL-1	EP	436	0.086
Oogenesis	76-99%	PGL-1	EP	144	0.029
Oogenesis	100%	PGL-1	EP	8	0.002
Oogenesis	Total	PGL-1	EP	5050	1.000
Spermatogenesis	0%	PGL-1	EP	960	0.389
Spermatogenesis	1-25%	PGL-1	EP	545	0.221
Spermatogenesis	26-50%	PGL-1	EP	372	0.151
Spermatogenesis	51-75%	PGL-1	EP	386	0.156
Spermatogenesis	76-99%	PGL-1	EP	198	0.080
Spermatogenesis	100%	PGL-1	EP	7	0.003
Spermatogenesis	Total	PGL-1	EP	2468	1.000
Oogenesis	0%	PGL-1	LP	1966	0.361
Oogenesis	1-25%	PGL-1	LP	1778	0.327
Oogenesis	26-50%	PGL-1	LP	963	0.177
Oogenesis	51-75%	PGL-1	LP	458	0.084
Oogenesis	76-99%	PGL-1	LP	264	0.048
Oogenesis	100%	PGL-1	LP	15	0.003
Oogenesis	Total	PGL-1	LP	5444	1.000
Spermatogenesis	0%	PGL-1	LP	617	0.309

Table S2.5 (continued).

Process	Classification	Protein	Zone	Foci Counts	Percent
Spermatogenesis	1-25%	PGL-1	LP	459	0.230
Spermatogenesis	26-50%	PGL-1	LP	292	0.146
Spermatogenesis	51-75%	PGL-1	LP	422	0.211
Spermatogenesis	76-99%	PGL-1	LP	200	0.100
Spermatogenesis	100%	PGL-1	LP	10	0.005
Spermatogenesis	Total	PGL-1	LP	2000	1.000
Oogenesis	0%	ZNFX-1	L/Z	2691	0.380
Oogenesis	1-25%	ZNFX-1	L/Z	1766	0.249
Oogenesis	26-50%	ZNFX-1	L/Z	1350	0.191
Oogenesis	51-75%	ZNFX-1	L/Z	918	0.130
Oogenesis	76-99%	ZNFX-1	L/Z	349	0.049
Oogenesis	100%	ZNFX-1	L/Z	11	0.002
Oogenesis	Total	ZNFX-1	L/Z	7085	1.000
Spermatogenesis	0%	ZNFX-1	L/Z	1784	0.304
Spermatogenesis	1-25%	ZNFX-1	L/Z	1092	0.186
Spermatogenesis	26-50%	ZNFX-1	L/Z	1088	0.185
Spermatogenesis	51-75%	ZNFX-1	L/Z	1089	0.186
Spermatogenesis	76-99%	ZNFX-1	L/Z	772	0.132
Spermatogenesis	100%	ZNFX-1	L/Z	45	0.008
Spermatogenesis	Total	ZNFX-1	L/Z	5870	1.000
Oogenesis	0%	ZNFX-1	EP	1401	0.260
Oogenesis	1-25%	ZNFX-1	EP	1425	0.265
Oogenesis	26-50%	ZNFX-1	EP	1269	0.236
Oogenesis	51-75%	ZNFX-1	EP	876	0.163
Oogenesis	76-99%	ZNFX-1	EP	396	0.074
Oogenesis	100%	ZNFX-1	EP	14	0.003
Oogenesis	Total	ZNFX-1	EP	5381	1.000
Spermatogenesis	0%	ZNFX-1	EP	895	0.317
Spermatogenesis	1-25%	ZNFX-1	EP	450	0.159
Spermatogenesis	26-50%	ZNFX-1	EP	459	0.162
Spermatogenesis	51-75%	ZNFX-1	EP	552	0.195
Spermatogenesis	76-99%	ZNFX-1	EP	453	0.160
Spermatogenesis	100%	ZNFX-1	EP	18	0.006
Spermatogenesis	Total	ZNFX-1	EP	2827	1.000
Oogenesis	0%	ZNFX-1	LP	1550	0.266
Oogenesis	1-25%	ZNFX-1	LP	1741	0.299
Oogenesis	26-50%	ZNFX-1	LP	1442	0.248

Table S2.5 (continued).

Process	Classification	Protein	Zone	Foci Counts	Percent
Oogenesis	51-75%	ZNFX-1	LP	752	0.129
Oogenesis	76-99%	ZNFX-1	LP	306	0.053
Oogenesis	100%	ZNFX-1	LP	33	0.006
Oogenesis	Total	ZNFX-1	LP	5824	1.000
Spermatogenesis	0%	ZNFX-1	LP	570	0.245
Spermatogenesis	1-25%	ZNFX-1	LP	290	0.125
Spermatogenesis	26-50%	ZNFX-1	LP	359	0.154
Spermatogenesis	51-75%	ZNFX-1	LP	545	0.234
Spermatogenesis	76-99%	ZNFX-1	LP	528	0.227
Spermatogenesis	100%	ZNFX-1	LP	37	0.016
Spermatogenesis	Total	ZNFX-1	LP	2329	1.000

Table S2.6: Chi-square test with Bonferroni correction comparing percent of WAGO-1 foci volume overlap with PGL-1 or ZNFX-1 between oogenesis and spermatogenesis.

Comparison	Classification	Protein	Zone	Chi-squared statistic	p-value
Oogenesis vs. Spermatogenesis	0%	PGL-1	L/Z	344.78	2.91E-76
Oogenesis vs. Spermatogenesis	1-25%	PGL-1	L/Z	13.6	1.15E-03
Oogenesis vs. Spermatogenesis	26-50%	PGL-1	L/Z	96.34	4.84E-22
Oogenesis vs. Spermatogenesis	51-75%	PGL-1	L/Z	229.69	3.49E-51
Oogenesis vs. Spermatogenesis	76-99%	PGL-1	L/Z	130.71	1.44E-29
Oogenesis vs. Spermatogenesis	100%	PGL-1	L/Z	2.91	4.40E-01
Oogenesis vs. Spermatogenesis	0%	PGL-1	EP	6.39	5.75E-02
Oogenesis vs. Spermatogenesis	1-25%	PGL-1	EP	67.16	1.25E-15
Oogenesis vs. Spermatogenesis	26-50%	PGL-1	EP	0.014	4.53E+00
Oogenesis vs. Spermatogenesis	51-75%	PGL-1	EP	83.36	3.43E-19
Oogenesis vs. Spermatogenesis	76-99%	PGL-1	EP	100.91	4.82E-23

Table S2.6 (continued):

Comparison	Classification	Protein	Zone	Chi-squared statistic	p-value
Oogenesis vs. Spermatogenesis	100%	PGL-1	EP	0.75	1.98E+00
Oogenesis vs. Spermatogenesis	0%	PGL-1	LP	17.64	1.33E-04
Oogenesis vs. Spermatogenesis	1-25%	PGL-1	LP	65.14	3.49E-15
Oogenesis vs. Spermatogenesis	26-50%	PGL-1	LP	9.74	9.00E-03
Oogenesis vs. Spermatogenesis	51-75%	PGL-1	LP	224.63	4.41E-50
Oogenesis vs. Spermatogenesis	76-99%	PGL-1	LP	65.51	2.89E-15
Oogenesis vs. Spermatogenesis	100%	PGL-1	LP	1.58	1.05E+00
Oogenesis vs. Spermatogenesis	0%	ZNFX-1	L/Z	81.33	9.55E-19
Oogenesis vs. Spermatogenesis	1-25%	ZNFX-1	L/Z	74.19	3.55E-17
Oogenesis vs. Spermatogenesis	26-50%	ZNFX-1	L/Z	0.53	2.34E+00
Oogenesis vs. Spermatogenesis	51-75%	ZNFX-1	L/Z	76.41	1.16E-17
Oogenesis vs. Spermatogenesis	76-99%	ZNFX-1	L/Z	274.12	7.20E-61
Oogenesis vs. Spermatogenesis	100%	ZNFX-1	L/Z	20	3.87E-05
Oogenesis vs. Spermatogenesis	0%	ZNFX-1	EP	28.37	5.00E-07
Oogenesis vs. Spermatogenesis	1-25%	ZNFX-1	EP	116.75	1.63E-26
Oogenesis vs. Spermatogenesis	26-50%	ZNFX-1	EP	59.74	5.40E-14
Oogenesis vs. Spermatogenesis	51-75%	ZNFX-1	EP	13.38	1.30E-03
Oogenesis vs. Spermatogenesis	76-99%	ZNFX-1	EP	149.11	1.36E-33
Oogenesis vs. Spermatogenesis	100%	ZNFX-1	EP	5.83	8.00E-02

Table S2.6 (continued)

Comparison	Classification	Protein	Zone	Chi-squared statistic	p-value
Oogenesis vs. Spermatogenesis	0%	ZNFX-1	LP	3.85	2.50E-01
Oogenesis vs. Spermatogenesis	1-25%	ZNFX-1	LP	296.64	6.80E-60
Oogenesis vs. Spermatogenesis	26-50%	ZNFX-1	LP	83.88	2.63E-19
Oogenesis vs. Spermatogenesis	51-75%	ZNFX-1	LP	136.03	9.85E-31
Oogenesis vs. Spermatogenesis	76-99%	ZNFX-1	LP	547.66	2.03E-120
Oogenesis vs. Spermatogenesis	100%	ZNFX-1	LP	19.23	5.80E-05

Table S2.7: WAGO-1 foci overlap counts with ZNFX-1, PGL-1, or both germ granule components

Stage	Sex	ZNFX-1	PGL-1	Both	Total	% ZNFX-1	% PGL-1	% Both
L/Z	Hermaphrodite	1123	648	2329	4100	27.4	15.8	56.8
L/Z	Male	842	655	2571	4068	20.7	16.1	63.2
EP	Hermaphrodite	1065	263	2330	3658	29.1	7.2	63.7
EP	Male	476	254	1253	1983	24.0	12.8	63.2
LP	Hermaphrodite	868	406	2744	4018	21.6	10.1	68.3
LP	Male	400	160	1223	1783	22.4	9.0	68.6

Table S2.8: Chi-square test with Bonferroni correction comparing percent of WAGO-1 foci that overlap with PGL-1, ZNFX-1, or both foci during oogenesis vs. spermatogenesis.

Comparison	Protein	Zone	Chi-squared statistic	p-value
Oogenesis vs. Spermatogenesis	Both	L/Z	34.53	1.2564E-08
Oogenesis vs. Spermatogenesis	PGL-1	L/Z	59.17	4.32E-14
Oogenesis vs. Spermatogenesis	ZNFX-1	L/Z	0.113	2.211
Oogenesis vs. Spermatogenesis	Both	EP	0.123	2.178
Oogenesis vs. Spermatogenesis	PGL-1	EP	16.66	0.0001344
Oogenesis vs. Spermatogenesis	ZNFX-1	EP	48.1	1.2159E-11
Oogenesis vs. Spermatogenesis	Both	LP	0.038	2.535
Oogenesis vs. Spermatogenesis	PGL-1	LP	0.452	1.503
Oogenesis vs. Spermatogenesis	ZNFX-1	LP	1.668	0.588

Table S2.9: PRG-1 ring counts per a nuclei. n=6 germlines per a sex taken from three distinct technical replicates.

Stage	Sex	Number of Rings	Counts	Percent
Leptotene/Zygotene	Hermaphrodite	0	285	92.2
Leptotene/Zygotene	Hermaphrodite	1	24	7.8
Leptotene/Zygotene	Hermaphrodite	2	0	0
Leptotene/Zygotene	Hermaphrodite	3	0	0
Leptotene/Zygotene	Male	0	208	74.0
Leptotene/Zygotene	Male	1	65	23.2
Leptotene/Zygotene	Male	2	8	2.8
Leptotene/Zygotene	Male	3	0	0
Late Pachytene	Hermaphrodite	0	165	76.0
Late Pachytene	Hermaphrodite	1	45	20.7
Late Pachytene	Hermaphrodite	2	6	2.8
Late Pachytene	Hermaphrodite	3	1	0.5
Late Pachytene	Male	0	17	11.0
Late Pachytene	Male	1	97	63.0
Late Pachytene	Male	2	35	22.7
Late Pachytene	Male	3	5	3.3

Table S2.10: Sex specific normalized fertility of tagged WAGO-1 and WAGO-4 strains

Strain	Counts	Sex	Genotype	Average	Normalized Count
N2	318	Hermaphrodite	Wild-type	298.77	1.06
N2	353	Hermaphrodite	Wild-type		1.18
N2	310	Hermaphrodite	Wild-type		1.03
N2	304	Hermaphrodite	Wild-type		1.02
N2	346	Hermaphrodite	Wild-type		1.16
N2	315	Hermaphrodite	Wild-type		1.05
N2	345	Hermaphrodite	Wild-type		1.15
N2	346	Hermaphrodite	Wild-type		1.16
N2	346	Hermaphrodite	Wild-type		1.16
N2	345	Hermaphrodite	Wild-type		1.15
N2	337	Hermaphrodite	Wild-type		1.13
N2	250	Hermaphrodite	Wild-type		0.84
N2	314	Hermaphrodite	Wild-type		1.05
N2	363	Hermaphrodite	Wild-type		1.21
N2	321	Hermaphrodite	Wild-type		1.07

Table S2.10 (continued)

Strain	Counts	Sex	Genotype	Average	Normalized Count
N2	335	Hermaphrodite	Wild-type		1.12
N2	293	Hermaphrodite	Wild-type		0.98
N2	286	Hermaphrodite	Wild-type		0.96
N2	218	Hermaphrodite	Wild-type		0.73
N2	194	Hermaphrodite	Wild-type		0.65
N2	246	Hermaphrodite	Wild-type		0.82
N2	319	Hermaphrodite	Wild-type		1.07
N2	226	Hermaphrodite	Wild-type		0.76
N2	282	Hermaphrodite	Wild-type		0.94
N2	196	Hermaphrodite	Wild-type		0.66
N2	260	Hermaphrodite	Wild-type		0.87
WM205	196	Hermaphrodite	GFP::WAGO-1 endogenous	199.69	0.66
WM205	153	Hermaphrodite	GFP::WAGO-1 endogenous		0.51
WM205	195	Hermaphrodite	GFP::WAGO-1 endogenous		0.65
WM205	293	Hermaphrodite	GFP::WAGO-1 endogenous		0.98

Table S2.10 (continued).

Strain	Counts	Sex	Genotype	Average	Normalized Count
WM205	241	Hermaphrodite	GFP::WAGO-1 endogenous		0.81
WM205	200	Hermaphrodite	GFP::WAGO-1 endogenous		0.67
WM205	229	Hermaphrodite	GFP::WAGO-1 endogenous		0.77
WM205	198	Hermaphrodite	GFP::WAGO-1 endogenous		0.66
WM205	188	Hermaphrodite	GFP::WAGO-1 endogenous		0.63
WM205	188	Hermaphrodite	GFP::WAGO-1 endogenous		0.63
WM205	169	Hermaphrodite	GFP::WAGO-1 endogenous		0.57
WM205	203	Hermaphrodite	GFP::WAGO-1 endogenous		0.68
WM205	180	Hermaphrodite	GFP::WAGO-1 endogenous		0.60
WM205	183	Hermaphrodite	GFP::WAGO-1 endogenous		0.61
WM205	201	Hermaphrodite	GFP::WAGO-1 endogenous		0.67
WM205	178	Hermaphrodite	GFP::WAGO-1 endogenous		0.60
WM616	265	Hermaphrodite	3xFLAG::WAGO-1 endogenous	264.06	0.89
WM616	55	Hermaphrodite	3xFLAG::WAGO-1 endogenous		0.18
WM616	243	Hermaphrodite	3xFLAG::WAGO-1 endogenous		0.81

Table S2.10 (continued).

Strain	Counts	Sex	Genotype	Average	Normalized Count
WM616	247	Hermaphrodite	3xFLAG::WAGO-1 endogenous		0.83
WM616	269	Hermaphrodite	3xFLAG::WAGO-1 endogenous		0.90
WM616	275	Hermaphrodite	3xFLAG::WAGO-1 endogenous		0.92
WM616	318	Hermaphrodite	3xFLAG::WAGO-1 endogenous		1.06
WM616	305	Hermaphrodite	3xFLAG::WAGO-1 endogenous		1.02
WM616	277	Hermaphrodite	3xFLAG::WAGO-1 endogenous		0.93
WM616	346	Hermaphrodite	3xFLAG::WAGO-1 endogenous		1.16
WM616	246	Hermaphrodite	3xFLAG::WAGO-1 endogenous		0.82
WM616	227	Hermaphrodite	3xFLAG::WAGO-1 endogenous		0.76
WM616	274	Hermaphrodite	3xFLAG::WAGO-1 endogenous		0.92
WM616	332	Hermaphrodite	3xFLAG::WAGO-1 endogenous		1.11
WM616	276	Hermaphrodite	3xFLAG::WAGO-1 endogenous		0.92
WM616	279	Hermaphrodite	3xFLAG::WAGO-1 endogenous		0.93
WM616	255	Hermaphrodite	3xFLAG::WAGO-1 endogenous		0.85
WM192	214	Hermaphrodite	3xFLAG::WAGO-1 transgene	208.20	0.72

Table S2.10 (continued).

Strain	Counts	Sex	Genotype	Average	Normalized Count
WM192	165	Hermaphrodite	3xFLAG::WAGO-1 transgene		0.55
WM192	183	Hermaphrodite	3xFLAG::WAGO-1 transgene		0.61
WM192	305	Hermaphrodite	3xFLAG::WAGO-1 transgene		1.02
WM192	247	Hermaphrodite	3xFLAG::WAGO-1 transgene		0.83
WM192	219	Hermaphrodite	3xFLAG::WAGO-1 transgene		0.73
WM192	211	Hermaphrodite	3xFLAG::WAGO-1 transgene		0.71
WM192	199	Hermaphrodite	3xFLAG::WAGO-1 transgene		0.67
WM192	190	Hermaphrodite	3xFLAG::WAGO-1 transgene		0.64
WM192	214	Hermaphrodite	3xFLAG::WAGO-1 transgene		0.72
WM192	194	Hermaphrodite	3xFLAG::WAGO-1 transgene		0.65
WM192	175	Hermaphrodite	3xFLAG::WAGO-1 transgene		0.59
WM192	240	Hermaphrodite	3xFLAG::WAGO-1 transgene		0.80
WM192	188	Hermaphrodite	3xFLAG::WAGO-1 transgene		0.63
WM192	203	Hermaphrodite	3xFLAG::WAGO-1 transgene		0.68
WM192	180	Hermaphrodite	3xFLAG::WAGO-1 transgene		0.60

Table S2.10 (continued).

Strain	Counts	Sex	Genotype	Average	Normalized Count
WM192	234	Hermaphrodite	3xFLAG::WAGO-1 transgene		0.78
WM192	191	Hermaphrodite	3xFLAG::WAGO-1 transgene		0.64
WM192	216	Hermaphrodite	3xFLAG::WAGO-1 transgene		0.72
WM192	196	Hermaphrodite	3xFLAG::WAGO-1 transgene		0.66
YY1325	322	Hermaphrodite	GFP::WAGO-4 endogenous	276.31	1.08
YY1325	295	Hermaphrodite	GFP::WAGO-4 endogenous		0.99
YY1325	277	Hermaphrodite	GFP::WAGO-4 endogenous		0.93
YY1325	239	Hermaphrodite	GFP::WAGO-4 endogenous		0.80
YY1325	172	Hermaphrodite	GFP::WAGO-4 endogenous		0.56
YY1325	279	Hermaphrodite	GFP::WAGO-4 endogenous		0.93
YY1325	292	Hermaphrodite	GFP::WAGO-4 endogenous		0.98
YY1325	350	Hermaphrodite	GFP::WAGO-4 endogenous		1.17
YY1325	282	Hermaphrodite	GFP::WAGO-4 endogenous		0.94
YY1325	325	Hermaphrodite	GFP::WAGO-4 endogenous		1.09
YY1325	279	Hermaphrodite	GFP::WAGO-4 endogenous		0.93

Table S2.10 (continued).

Strain	Counts	Sex	Genotype	Average	Normalized Count
YY1325	219	Hermaphrodite	GFP::WAGO-4 endogenous		0.73
YY1325	327	Hermaphrodite	GFP::WAGO-4 endogenous		1.09
YY1325	326	Hermaphrodite	GFP::WAGO-4 endogenous		1.09
YY1325	259	Hermaphrodite	GFP::WAGO-4 endogenous		0.87
YY1325	178	Hermaphrodite	GFP::WAGO-4 endogenous		0.60
N2	520	Male	Wild-type	401.67	1.29
N2	413	Male	Wild-type		1.03
N2	428	Male	Wild-type		1.07
N2	303	Male	Wild-type		0.75
N2	305	Male	Wild-type		0.76
N2	441	Male	Wild-type		1.10
WM205	67	Male	GFP::WAGO-1 endogenous	112.00	0.17
WM205	157	Male	GFP::WAGO-1 endogenous		0.39
WM616	254	Male	3xFLAG::WAGO-1 endogenous	228.00	0.63
WM616	331	Male	3xFLAG::WAGO-1 endogenous		0.82

Table S2.10 (continued).

Strain	Counts	Sex	Genotype	Average	Normalized Count
WM616	99	Male	3xFLAG::WAGO-1 endogenous		0.25
WM192	72	Male	3xFLAG::WAGO-1 transgene	172.33	0.18
WM192	445	Male	3xFLAG::WAGO-1 transgene		1.11
WM192	0	Male	3xFLAG::WAGO-1 transgene		0
YY1325	0	Male	GFP::WAGO-4 endogenous	41.00	0
YY1325	2	Male	GFP::WAGO-4 endogenous		0.005
YY1325	121	Male	GFP::WAGO-4 endogenous		0.30

Table S2.11: Sex-specific normalized fertility of wild-type and *wago-1(tm1414)*

Strain	Counts	Sex	Genotype	Average	Normalized Count
N2	318	Hermaphrodite	Wild-type	298.77	1.06
N2	353	Hermaphrodite	Wild-type		1.18
N2	310	Hermaphrodite	Wild-type		1.04
N2	304	Hermaphrodite	Wild-type		1.02
N2	346	Hermaphrodite	Wild-type		1.16
N2	315	Hermaphrodite	Wild-type		1.05
N2	345	Hermaphrodite	Wild-type		1.15
N2	346	Hermaphrodite	Wild-type		1.16
N2	346	Hermaphrodite	Wild-type		1.16
N2	345	Hermaphrodite	Wild-type		1.15
N2	337	Hermaphrodite	Wild-type		1.13
N2	250	Hermaphrodite	Wild-type		0.84
N2	314	Hermaphrodite	Wild-type		1.05
N2	363	Hermaphrodite	Wild-type		1.21
N2	321	Hermaphrodite	Wild-type		1.07
N2	335	Hermaphrodite	Wild-type		1.12
N2	293	Hermaphrodite	Wild-type		0.98
N2	286	Hermaphrodite	Wild-type		0.96
N2	218	Hermaphrodite	Wild-type		0.73
N2	194	Hermaphrodite	Wild-type		0.65
N2	246	Hermaphrodite	Wild-type		0.82
N2	319	Hermaphrodite	Wild-type		1.07
N2	226	Hermaphrodite	Wild-type		0.76
N2	282	Hermaphrodite	Wild-type		0.94
N2	196	Hermaphrodite	Wild-type		0.66
N2	260	Hermaphrodite	Wild-type		0.87

Table S2.11 (continued).

Strain	Counts	Sex	Genotype	Average	Normalized Count
C184	193	Hermaphrodite	<i>wago-1 (tm1414)</i>	216.29	0.65
C184	0	Hermaphrodite	<i>wago-1 (tm1414)</i>		0.00
C184	119	Hermaphrodite	<i>wago-1 (tm1414)</i>		0.40
C184	302	Hermaphrodite	<i>wago-1 (tm1414)</i>		1.01
C184	144	Hermaphrodite	<i>wago-1 (tm1414)</i>		0.48
C184	189	Hermaphrodite	<i>wago-1 (tm1414)</i>		0.63
C184	279	Hermaphrodite	<i>wago-1 (tm1414)</i>		0.93
C184	292	Hermaphrodite	<i>wago-1 (tm1414)</i>		0.98
C184	273	Hermaphrodite	<i>wago-1 (tm1414)</i>		0.91
C184	258	Hermaphrodite	<i>wago-1 (tm1414)</i>		0.86
C184	258	Hermaphrodite	<i>wago-1 (tm1414)</i>		0.86
C184	210	Hermaphrodite	<i>wago-1 (tm1414)</i>		0.70
C184	244	Hermaphrodite	<i>wago-1 (tm1414)</i>		0.82
C184	254	Hermaphrodite	<i>wago-1 (tm1414)</i>		0.85
C184	249	Hermaphrodite	<i>wago-1 (tm1414)</i>		0.83
C184	170	Hermaphrodite	<i>wago-1 (tm1414)</i>		0.57
C184	243	Hermaphrodite	<i>wago-1 (tm1414)</i>		0.81
N2	520	Male	Wild-type	401.67	1.29
N2	413	Male	Wild-type		1.03
N2	428	Male	Wild-type		1.07
N2	303	Male	Wild-type		0.75
N2	305	Male	Wild-type		0.76
N2	441	Male	Wild-type		1.10
C184	0	Male	<i>wago-1 (tm1414)</i>	0.00	0.00
C184	0	Male	<i>wago-1 (tm1414)</i>		0.00
C184	0	Male	<i>wago-1 (tm1414)</i>		0.00

Table S2.12: Binning of percent WAGO-1 volume overlap with PGL-1 in wild-type and truncated *wago-1(tm1414)* hermaphrodites.

Genotype	Classification	Protein	Zone	Foci Counts	Percent
Wild-type	0%	PGL-1	L/Z	3128	0.498
Wild-type	1-25%	PGL-1	L/Z	1775	0.283
Wild-type	26-50%	PGL-1	L/Z	780	0.124
Wild-type	51-75%	PGL-1	L/Z	396	0.063
Wild-type	76-99%	PGL-1	L/Z	190	0.030
Wild-type	100%	PGL-1	L/Z	10	0.002
Wild-type	Total	PGL-1	L/Z	6279	1.000
<i>wago-1 (tm1414)</i>	0%	PGL-1	L/Z	1721	0.800
<i>wago-1 (tm1414)</i>	1-25%	PGL-1	L/Z	222	0.103
<i>wago-1 (tm1414)</i>	26-50%	PGL-1	L/Z	92	0.043
<i>wago-1 (tm1414)</i>	51-75%	PGL-1	L/Z	62	0.029
<i>wago-1 (tm1414)</i>	76-99%	PGL-1	L/Z	40	0.019
<i>wago-1 (tm1414)</i>	100%	PGL-1	L/Z	15	0.007
<i>wago-1 (tm1414)</i>	Total	PGL-1	L/Z	2152	1.000
Wild-type	0%	PGL-1	Pachytene	4086	0.389
Wild-type	1-25%	PGL-1	Pachytene	3352	0.319
Wild-type	26-50%	PGL-1	Pachytene	1731	0.165
Wild-type	51-75%	PGL-1	Pachytene	894	0.085
Wild-type	76-99%	PGL-1	Pachytene	408	0.039
Wild-type	100%	PGL-1	Pachytene	23	0.002
Wild-type	Total	PGL-1	Pachytene	10494	1.000
<i>wago-1 (tm1414)</i>	0%	PGL-1	Pachytene	2690	0.771
<i>wago-1 (tm1414)</i>	1-25%	PGL-1	Pachytene	318	0.091
<i>wago-1 (tm1414)</i>	26-50%	PGL-1	Pachytene	199	0.057
<i>wago-1 (tm1414)</i>	51-75%	PGL-1	Pachytene	154	0.044
<i>wago-1 (tm1414)</i>	76-99%	PGL-1	Pachytene	80	0.023
<i>wago-1 (tm1414)</i>	100%	PGL-1	Pachytene	47	0.013
<i>wago-1 (tm1414)</i>	Total	PGL-1	Pachytene	3488	1.000

Table S2.13: Chi-square test with Bonferroni correction of comparing percent WAGO-1 foci volume overlap with PGL-1 in wild-type versus *wago-1 (tm1414)* hermaphrodites

Comparison	Classification	Protein	Zone	Chi-squared statistic	p-Value
Wild-type vs. <i>wago-1(tm1414)</i>	0%	PGL-1	L/Z	59.556	1.18E-14
Wild-type vs. <i>wago-1(tm1414)</i>	1-25%	PGL-1	L/Z	36.49	1.53E-09
Wild-type vs. <i>wago-1(tm1414)</i>	26-50%	PGL-1	L/Z	5.85	0.0156
Wild-type vs. <i>wago-1(tm1414)</i>	51-75%	PGL-1	L/Z	9.81	0.0017
Wild-type vs. <i>wago-1(tm1414)</i>	76-99%	PGL-1	L/Z	6.46	0.011
Wild-type vs. <i>wago-1(tm1414)</i>	100%	PGL-1	L/Z	3.2	0.074
Wild-type vs. <i>wago-1(tm1414)</i>	0%	PGL-1	Pachytene	460.07	4.63E-102
Wild-type vs. <i>wago-1(tm1414)</i>	1-25%	PGL-1	Pachytene	240.43	3.17E-54
Wild-type vs. <i>wago-1(tm1414)</i>	26-50%	PGL-1	Pachytene	72.14	2.00E-17
Wild-type vs. <i>wago-1(tm1414)</i>	51-75%	PGL-1	Pachytene	40.71	1.77E-10
Wild-type vs. <i>wago-1(tm1414)</i>	76-99%	PGL-1	Pachytene	22.26	2.38E-06
Wild-type vs. <i>wago-1(tm1414)</i>	100%	PGL-1	Pachytene	21.28	3.96E-06

Table S2.14: 21U-RNA biotypes for wild-type and mutant WAGO-1 males and hermaphrodites

Genotype	Sex	Biotype	Counts
Wild-type	Hermaphrodite	lincRNA AS	4
Wild-type	Hermaphrodite	tRNA AS	5
Wild-type	Hermaphrodite	Repetitive Elements S	25
Wild-type	Hermaphrodite	snoRNA AS	33
Wild-type	Hermaphrodite	piRNA S	94
Wild-type	Hermaphrodite	ncRNA AS	133
Wild-type	Hermaphrodite	Repetitive Elements AS	374
Wild-type	Hermaphrodite	No Feature	378
Wild-type	Hermaphrodite	piRNA AS	1112
Wild-type	Hermaphrodite	Protein CodingAS	2841
Wild-type	Hermaphrodite	Pseudogene AS	5507
Wild-type	Male	lincRNA AS	18
Wild-type	Male	snoRNA AS	42
Wild-type	Male	Repetitive Elements S	49
Wild-type	Male	ncRNA AS	207
Wild-type	Male	piRNA S	266
Wild-type	Male	tRNA AS	459
Wild-type	Male	Pseudogene AS	829
Wild-type	Male	No Feature	1471

Table S2.14 (continued).

Genotype	Sex	Biotype	Counts
Wild-type	Male	Repetitive Elements AS	1493
Wild-type	Male	piRNA AS	5032
Wild-type	Male	Protein Coding AS	12060
3xFLAG::WAGO-1 transgene	Hermaphrodite	lincRNA AS	43
3xFLAG::WAGO-1 transgene	Hermaphrodite	tRNA AS	55
3xFLAG::WAGO-1 transgene	Hermaphrodite	Repetitive Elements S	88
3xFLAG::WAGO-1 transgene	Hermaphrodite	snoRNA AS	107
3xFLAG::WAGO-1 transgene	Hermaphrodite	ncRNA AS	380
3xFLAG::WAGO-1 transgene	Hermaphrodite	piRNA S	598
3xFLAG::WAGO-1 transgene	Hermaphrodite	No Feature	899
3xFLAG::WAGO-1 transgene	Hermaphrodite	Pseudogene AS	1115
3xFLAG::WAGO-1 transgene	Hermaphrodite	Repetitive Elements AS	1626
3xFLAG::WAGO-1 transgene	Hermaphrodite	Protein Coding AS	6847
3xFLAG::WAGO-1 transgene	Hermaphrodite	piRNA AS	7024
3xFLAG::WAGO-1 endogenous	Hermaphrodite	lincRNA AS	53
3xFLAG::WAGO-1 endogenous	Hermaphrodite	snoRNA AS	65
3xFLAG::WAGO-1 endogenous	Hermaphrodite	Repetitive Elements S	76
3xFLAG::WAGO-1 endogenous	Hermaphrodite	ncRNA AS	252

Table S2.14 (continued).

Genotype	Sex	Biotype	Counts
3xFLAG::WAGO-1 endogenous	Hermaphrodite	Pseudogene AS	741
3xFLAG::WAGO-1 endogenous	Hermaphrodite	tRNA AS	844
3xFLAG::WAGO-1 endogenous	Hermaphrodite	piRNA S	898
3xFLAG::WAGO-1 endogenous	Hermaphrodite	Repetitive Elements AS	1356
3xFLAG::WAGO-1 endogenous	Hermaphrodite	No Feature	1412
3xFLAG::WAGO-1 endogenous	Hermaphrodite	Protein Coding AS	12579
3xFLAG::WAGO-1 endogenous	Hermaphrodite	piRNA AS	16366
3xFLAG::WAGO-1 endogenous	Male	lincRNA AS	36
3xFLAG::WAGO-1 endogenous	Male	snoRNA AS	61
3xFLAG::WAGO-1 endogenous	Male	Repetitive Elements S	80
3xFLAG::WAGO-1 endogenous	Male	ncRNA AS	241
3xFLAG::WAGO-1 endogenous	Male	Pseudogene AS	566
3xFLAG::WAGO-1 endogenous	Male	tRNA AS	811
3xFLAG::WAGO-1 endogenous	Male	piRNA S	828
3xFLAG::WAGO-1 endogenous	Male	Repetitive Elements AS	1210
3xFLAG::WAGO-1 endogenous	Male	No Feature	1375
3xFLAG::WAGO-1 endogenous	Male	Protein Coding AS	12047
3xFLAG::WAGO-1 endogenous	Male	piRNA AS	15581
3xFLAG::WAGO-1 endogenous (100 generations)	Hermaphrodite	lincRNA AS	2

Table S2.14 (continued).

Genotype	Sex	Biotype	Counts
3xFLAG::WAGO-1 endogenous (100 generations)	Hermaphrodite	tRNA AS	6
3xFLAG::WAGO-1 endogenous (100 generations)	Hermaphrodite	Repetitive Elements S	11
3xFLAG::WAGO-1 endogenous (100 generations)	Hermaphrodite	snoRNA AS	13
3xFLAG::WAGO-1 endogenous (100 generations)	Hermaphrodite	piRNA S	47
3xFLAG::WAGO-1 endogenous (100 generations)	Hermaphrodite	ncRNA AS	89
3xFLAG::WAGO-1 endogenous (100 generations)	Hermaphrodite	Repetitive Elements AS	249
3xFLAG::WAGO-1 endogenous (100 generations)	Hermaphrodite	No Feature	433
3xFLAG::WAGO-1 endogenous (100 generations)	Hermaphrodite	piRNA AS	665
3xFLAG::WAGO-1 endogenous (100 generations)	Hermaphrodite	Protein Coding AS	2595
3xFLAG::WAGO-1 endogenous (100 generations)	Hermaphrodite	Pseudogene AS	5194
3xFLAG::WAGO-1 endogenous (100 generations)	Male	lincRNA AS	6
3xFLAG::WAGO-1 endogenous (100 generations)	Male	Repetitive Elements S	45
3xFLAG::WAGO-1 endogenous (100 generations)	Male	snoRNA AS	66
3xFLAG::WAGO-1 endogenous (100 generations)	Male	ncRNA AS	255
3xFLAG::WAGO-1 endogenous (100 generations)	Male	piRNA S	359
3xFLAG::WAGO-1 endogenous (100 generations)	Male	tRNA AS	502
3xFLAG::WAGO-1 endogenous (100 generations)	Male	Pseudogene AS	1051
3xFLAG::WAGO-1 endogenous (100 generations)	Male	Repetitive Elements AS	1164
3xFLAG::WAGO-1 endogenous (100 generations)	Male	No feature	1262

Table S2.14 (continued).

Genotype	Sex	Biotype	Counts
3xFLAG::WAGO-1 endogenous (100 generations)	Male	piRNA AS	5971
3xFLAG::WAGO-1 endogenous (100 generations)	Male	Protein Coding AS	10272
<i>wago-1 (tm1414)</i>	Hermaphrodite	lincRNA AS	18
<i>wago-1 (tm1414)</i>	Hermaphrodite	tRNA AS	65
<i>wago-1 (tm1414)</i>	Hermaphrodite	Repetitive Elements S	74
<i>wago-1 (tm1414)</i>	Hermaphrodite	snoRNA AS	102
<i>wago-1 (tm1414)</i>	Hermaphrodite	ncRNA AS	330
<i>wago-1 (tm1414)</i>	Hermaphrodite	piRNA S	532
<i>wago-1 (tm1414)</i>	Hermaphrodite	Pseudogene AS	876
<i>wago-1 (tm1414)</i>	Hermaphrodite	No Feature	1664
<i>wago-1 (tm1414)</i>	Hermaphrodite	Repetitive Elements AS	2471
<i>wago-1 (tm1414)</i>	Hermaphrodite	piRNA AS	6110
<i>wago-1 (tm1414)</i>	Hermaphrodite	Protein Coding AS	10141
<i>wago-1 (tm1414)</i>	Male	lincRNA AS	3
<i>wago-1 (tm1414)</i>	Male	snoRNA AS	17
<i>wago-1 (tm1414)</i>	Male	Repetitive Elements S	24
<i>wago-1 (tm1414)</i>	Male	tRNA AS	46
<i>wago-1 (tm1414)</i>	Male	piRNA S	61
<i>wago-1 (tm1414)</i>	Male	ncRNA AS	76

Table S2.14 (continued).

Genotype	Sex	Biotype	Counts
<i>wago-1 (tm1414)</i>	Male	piRNA AS	579
<i>wago-1 (tm1414)</i>	Male	Repetitive Elements AS	924
<i>wago-1 (tm1414)</i>	Male	No Feature	1065
<i>wago-1 (tm1414)</i>	Male	Pseudogene AS	4076
<i>wago-1 (tm1414)</i>	Male	Protein Coding AS	4855

Table S2.15: 22G-RNA biotypes for wild-type and mutant WAGO-1 males and hermaphrodites

Genotype	Sex	Biotype	Counts
Wild-type	Hermaphrodite	tRNA AS	1
Wild-type	Hermaphrodite	lincRNA AS	3
Wild-type	Hermaphrodite	piRNA S	4
Wild-type	Hermaphrodite	piRNA AS	8
Wild-type	Hermaphrodite	snoRNA AS	10
Wild-type	Hermaphrodite	Repetitive Elements S	38
Wild-type	Hermaphrodite	ncRNA AS	125
Wild-type	Hermaphrodite	Repetitive Elements AS	223
Wild-type	Hermaphrodite	No feature	333
Wild-type	Hermaphrodite	Protein Coding AS	2798
Wild-type	Hermaphrodite	Pseudogene AS	3195
Wild-type	Male	lincRNA AS	6

Table S2.15 (continued).

Genotype	Sex	Biotype	Counts
Wild-type	Male	snoRNA AS	20
Wild-type	Male	Repetitive Elements S	65
Wild-type	Male	piRNA AS	70
Wild-type	Male	ncRNA AS	78
Wild-type	Male	tRNA AS	98
Wild-type	Male	piRNA S	139
Wild-type	Male	Pseudogene AS	538
Wild-type	Male	Repetitive Elements AS	1073
Wild-type	Male	No Feature	1167
Wild-type	Male	Protein Coding AS	9208
3xFLAG::WAGO-1 transgene	Hermaphrodite	tRNA AS	9
3xFLAG::WAGO-1 transgene	Hermaphrodite	lincRNA AS	19
3xFLAG::WAGO-1 transgene	Hermaphrodite	snoRNA AS	28
3xFLAG::WAGO-1 transgene	Hermaphrodite	piRNA S	43
3xFLAG::WAGO-1 transgene	Hermaphrodite	piRNA AS	65
3xFLAG::WAGO-1 transgene	Hermaphrodite	ncRNA AS	123
3xFLAG::WAGO-1 transgene	Hermaphrodite	Repetitive Elements S	134
3xFLAG::WAGO-1 transgene	Hermaphrodite	Pseudogene AS	546
3xFLAG::WAGO-1 transgene	Hermaphrodite	No Feature	571

Table S2.15 (continued).

Genotype	Sex	Biotype	Counts
3xFLAG::WAGO-1 transgene	Hermaphrodite	Repetitive Elements AS	896
3xFLAG::WAGO-1 transgene	Hermaphrodite	Protein Coding AS	5378
3xFLAG::WAGO-1 endogenous	Hermaphrodite	lincRNA AS	20
3xFLAG::WAGO-1 endogenous	Hermaphrodite	snoRNA AS	30
3xFLAG::WAGO-1 endogenous	Hermaphrodite	tRNA_AS	74
3xFLAG::WAGO-1 endogenous	Hermaphrodite	Repetitive Elements S	80
3xFLAG::WAGO-1 endogenous	Hermaphrodite	piRNA S	91
3xFLAG::WAGO-1 endogenous	Hermaphrodite	piRNA AS	96
3xFLAG::WAGO-1 endogenous	Hermaphrodite	ncRNA AS	186
3xFLAG::WAGO-1 endogenous	Hermaphrodite	Pseudogene AS	348
3xFLAG::WAGO-1 endogenous	Hermaphrodite	Repetitive Elements AS	892
3xFLAG::WAGO-1 endogenous	Hermaphrodite	No Feature	1189
3xFLAG::WAGO-1 endogenous	Hermaphrodite	Protein Coding AS	13201
3xFLAG::WAGO-1 endogenous	Male	lincRNA AS	18
3xFLAG::WAGO-1 endogenous	Male	snoRNA AS	22
3xFLAG::WAGO-1 endogenous	Male	tRNA AS	67
3xFLAG::WAGO-1 endogenous	Male	Repetitive Elements S	77
3xFLAG::WAGO-1 endogenous	Male	piRNA S	88
3xFLAG::WAGO-1 endogenous	Male	piRNA AS	96

Table S2.15 (continued).

Genotype	Sex	Biotype	Counts
3xFLAG::WAGO-1 endogenous	Male	ncRNA AS	171
3xFLAG::WAGO-1 endogenous	Male	Pseudogene AS	251
3xFLAG::WAGO-1 endogenous	Male	Repetitive Elements AS	874
3xFLAG::WAGO-1 endogenous	Male	No Feature	1189
3xFLAG::WAGO-1 endogenous	Male	Protein Coding AS	12491
3xFLAG::WAGO-1 endogenous (100 generations)	Hermaphrodite	tRNA AS	1
3xFLAG::WAGO-1 endogenous (100 generations)	Hermaphrodite	lincRNA AS	2
3xFLAG::WAGO-1 endogenous (100 generations)	Hermaphrodite	snoRNA AS	4
3xFLAG::WAGO-1 endogenous (100 generations)	Hermaphrodite	piRNA S	6
3xFLAG::WAGO-1 endogenous (100 generations)	Hermaphrodite	piRNA AS	9
3xFLAG::WAGO-1 endogenous (100 generations)	Hermaphrodite	Repetitive Elements S	15
3xFLAG::WAGO-1 endogenous (100 generations)	Hermaphrodite	ncRNA AS	81
3xFLAG::WAGO-1 endogenous (100 generations)	Hermaphrodite	Repetitive Elements AS	195
3xFLAG::WAGO-1 endogenous (100 generations)	Hermaphrodite	No Feature	397
3xFLAG::WAGO-1 endogenous (100 generations)	Hermaphrodite	Protein Coding AS	2670

Table S2.15 (continued).

Genotype	Sex	Biotype	Counts
3xFLAG::WAGO-1 endogenous (100 generations)	Hermaphrodite	Pseudogene AS	2913
3xFLAG::WAGO-1 endogenous (100 generations)	Male	lincRNA AS	8
3xFLAG::WAGO-1 endogenous (100 generations)	Male	snoRNA AS	20
3xFLAG::WAGO-1 endogenous (100 generations)	Male	piRNA AS	76
3xFLAG::WAGO-1 endogenous (100 generations)	Male	Repetitive Elements S	79
3xFLAG::WAGO-1 endogenous (100 generations)	Male	tRNA AS	89
3xFLAG::WAGO-1 endogenous (100 generations)	Male	piRNA S	112
3xFLAG::WAGO-1 endogenous (100 generations)	Male	ncRNA AS	119
3xFLAG::WAGO-1 endogenous (100 generations)	Male	Pseudogene AS	728
3xFLAG::WAGO-1 endogenous (100 generations)	Male	No Feature	1303
3xFLAG::WAGO-1 endogenous (100 generations)	Male	Repetitive Elements AS	1520
3xFLAG::WAGO-1 endogenous (100 generations)	Male	Protein Coding AS	10192
<i>wago-1 (tm1414)</i>	Hermaphrodite	tRNA AS	14
<i>wago-1 (tm1414)</i>	Hermaphrodite	lincRNA AS	16
<i>wago-1 (tm1414)</i>	Hermaphrodite	snoRNA AS	42

Table S2.15 (continued).

Genotype	Sex	Biotype	Counts
<i>wago-1 (tm1414)</i>	Hermaphrodite	piRNA AS	64
<i>wago-1 (tm1414)</i>	Hermaphrodite	Repetitive Elements S	65
<i>wago-1 (tm1414)</i>	Hermaphrodite	piRNA S	65
<i>wago-1 (tm1414)</i>	Hermaphrodite	ncRNA AS	206
<i>wago-1 (tm1414)</i>	Hermaphrodite	Pseudogene AS	408
<i>wago-1 (tm1414)</i>	Hermaphrodite	No Feature	1064
<i>wago-1 (tm1414)</i>	Hermaphrodite	Repetitive Elements AS	1570
<i>wago-1 (tm1414)</i>	Hermaphrodite	Protein Coding AS	7525
<i>wago-1 (tm1414)</i>	Male	lincRNA AS	3
<i>wago-1 (tm1414)</i>	Male	piRNA AS	5
<i>wago-1 (tm1414)</i>	Male	tRNA AS	6
<i>wago-1 (tm1414)</i>	Male	snoRNA AS	7
<i>wago-1 (tm1414)</i>	Male	piRNA S	38
<i>wago-1 (tm1414)</i>	Male	Repetitive Elements S	40
<i>wago-1 (tm1414)</i>	Male	ncRNA AS	85
<i>wago-1 (tm1414)</i>	Male	No Feature	928
<i>wago-1 (tm1414)</i>	Male	Repetitive Elements AS	1016
<i>wago-1 (tm1414)</i>	Male	Pseudogene AS	2362
<i>wago-1 (tm1414)</i>	Male	Protein Coding AS	5471

Table S2.16: 26G-RNA biotypes for wild-type and mutant WAGO-1 males and hermaphrodites

Genotype	Sex	Biotype	Count
Wild-type	Hermaphrodite	tRNA AS	6
Wild-type	Hermaphrodite	piRNA S	6
Wild-type	Hermaphrodite	piRNA AS	8
Wild-type	Hermaphrodite	Repetitive Elements S	9
Wild-type	Hermaphrodite	snoRNA AS	9
Wild-type	Hermaphrodite	ncRNA AS	105
Wild-type	Hermaphrodite	Repetitive Elements AS	110
Wild-type	Hermaphrodite	No Feature	325
Wild-type	Hermaphrodite	Protein Coding AS	1722
Wild-type	Hermaphrodite	Pseudogene AS	1895
Wild-type	Male	lincRNA AS	2
Wild-type	Male	snoRNA AS	19
Wild-type	Male	Repetitive Repeats	51
Wild-type	Male	piRNA AS	99
Wild-type	Male	tRNA AS	132
Wild-type	Male	Pseudogene AS	362
Wild-type	Male	piRNA S	409
Wild-type	Male	ncRNA AS	936
Wild-type	Male	Repetitive Elements AS	1163

Table S2.16 (continued).

Genotype	Sex	Biotype	Count
Wild-type	Male	No Feature	3617
Wild-type	Male	Protein Coding AS	13612
3xFLAG::WAGO-1 transgene	Hermaphrodite	lincRNA AS	3
3xFLAG::WAGO-1 transgene	Hermaphrodite	Repetitive Elements S	9
3xFLAG::WAGO-1 transgene	Hermaphrodite	piRNA S	22
3xFLAG::WAGO-1 transgene	Hermaphrodite	tRNA AS	30
3xFLAG::WAGO-1 transgene	Hermaphrodite	snoRNA AS	47
3xFLAG::WAGO-1 transgene	Hermaphrodite	piRNA AS	83
3xFLAG::WAGO-1 transgene	Hermaphrodite	ncRNA AS	280
3xFLAG::WAGO-1 transgene	Hermaphrodite	Pseudogene AS	301
3xFLAG::WAGO-1 transgene	Hermaphrodite	Repetitive Elements AS	436
3xFLAG::WAGO-1 transgene	Hermaphrodite	No Feature	764
3xFLAG::WAGO-1 transgene	Hermaphrodite	Protein Coding AS	2259
3xFLAG::WAGO-1 endogenous	Hermaphrodite	lincRNA AS	8
3xFLAG::WAGO-1 endogenous	Hermaphrodite	snoRNA AS	32
3xFLAG::WAGO-1 endogenous	Hermaphrodite	tRNA AS	81
3xFLAG::WAGO-1 endogenous	Hermaphrodite	Repetitive Elements	119
3xFLAG::WAGO-1 endogenous	Hermaphrodite	piRNA AS	178
3xFLAG::WAGO-1 endogenous	Hermaphrodite	Pseudogene AS	181

Table S2.16 (continued).

Genotype	Sex	Biotype	Count
3xFLAG::WAGO-1 endogenous	Hermaphrodite	piRNA	1203
3xFLAG::WAGO-1 endogenous	Hermaphrodite	Repetitive Elements AS	3864
3xFLAG::WAGO-1 endogenous	Hermaphrodite	ncRNA AS	4992
3xFLAG::WAGO-1 endogenous	Hermaphrodite	No Feature	14629
3xFLAG::WAGO-1 endogenous	Hermaphrodite	Protein Coding AS	35084
3xFLAG::WAGO-1 endogenous	Male	lincRNA AS	8
3xFLAG::WAGO-1 endogenous	Male	snoRNA AS	33
3xFLAG::WAGO-1 endogenous	Male	tRNA AS	90
3xFLAG::WAGO-1 endogenous	Male	Repetitive Elements S	113
3xFLAG::WAGO-1 endogenous	Male	Pseudogene AS	141
3xFLAG::WAGO-1 endogenous	Male	piRNA AS	168
3xFLAG::WAGO-1 endogenous	Male	piRNA S	1199
3xFLAG::WAGO-1 endogenous	Male	Repetitive Elements AS	3822
3xFLAG::WAGO-1 endogenous	Male	ncRNA AS	4885
3xFLAG::WAGO-1 endogenous	Male	No Feature	14486
3xFLAG::WAGO-1 endogenous	Male	Protein Coding AS	34412
3xFLAG::WAGO-1 endogenous (100 generations)	Hermaphrodite	lincRNA AS	1
3xFLAG::WAGO-1 endogenous (100 generations)	Hermaphrodite	piRNA S	2

Table S2.16 (continued).

Genotype	Sex	Biotype	Count
3xFLAG::WAGO-1 endogenous (100 generations)	Hermaphrodite	tRNA AS	3
3xFLAG::WAGO-1 endogenous (100 generations)	Hermaphrodite	Repetitive Elements S	5
3xFLAG::WAGO-1 endogenous (100 generations)	Hermaphrodite	piRNA AS	7
3xFLAG::WAGO-1 endogenous (100 generations)	Hermaphrodite	snoRNA AS	8
3xFLAG::WAGO-1 endogenous (100 generations)	Hermaphrodite	ncRNA AS	57
3xFLAG::WAGO-1 endogenous (100 generations)	Hermaphrodite	Repetitive Elements AS	94
3xFLAG::WAGO-1 endogenous (100 generations)	Hermaphrodite	No Feature	291
3xFLAG::WAGO-1 endogenous (100 generations)	Hermaphrodite	Pseudogene AS	1596
3xFLAG::WAGO-1 endogenous (100 generations)	Hermaphrodite	Protein Coding AS	1621
3xFLAG::WAGO-1 endogenous (100 generations)	Male	lincRNA AS	9
3xFLAG::WAGO-1 endogenous (100 generations)	Male	snoRNA AS	35
3xFLAG::WAGO-1 endogenous (100 generations)	Male	Repetitive Elements S	45
3xFLAG::WAGO-1 endogenous (100 generations)	Male	piRNA AS	86
3xFLAG::WAGO-1 endogenous (100 generations)	Male	tRNA AS	151

Table S2.16 (continued).

Genotype	Sex	Biotype	Count
3xFLAG::WAGO-1 endogenous (100 generations)	Male	piRNA S	433
3xFLAG::WAGO-1 endogenous (100 generations)	Male	Pseudogene AS	898
3xFLAG::WAGO-1 endogenous (100 generations)	Male	ncRNA AS	1013
3xFLAG::WAGO-1 endogenous (100 generations)	Male	Repetitive Elements AS	1275
3xFLAG::WAGO-1 endogenous (100 generations)	Male	No Feature	3492
3xFLAG::WAGO-1 endogenous (100 generations)	Male	Protein Coding AS	16172
<i>wago-1 (tm1414)</i>	Hermaphrodite	lincRNA AS	2
<i>wago-1 (tm1414)</i>	Hermaphrodite	Repetitive Elements S	18
<i>wago-1 (tm1414)</i>	Hermaphrodite	tRNA AS	25
<i>wago-1 (tm1414)</i>	Hermaphrodite	piRNA S	45
<i>wago-1 (tm1414)</i>	Hermaphrodite	snoRNA AS	50
<i>wago-1 (tm1414)</i>	Hermaphrodite	piRNA AS	61
<i>wago-1 (tm1414)</i>	Hermaphrodite	ncRNA AS	154
<i>wago-1 (tm1414)</i>	Hermaphrodite	Pseudogene AS	251
<i>wago-1 (tm1414)</i>	Hermaphrodite	No Feature	971
<i>wago-1 (tm1414)</i>	Hermaphrodite	Repetitive Elements AS	1056
<i>wago-1 (tm1414)</i>	Hermaphrodite	Protein Coding AS	3758

Table S2.16 (continued).

Genotype	Sex	Biotype	Count
<i>wago-1 (tm1414)</i>	Male	lincRNA AS	2
<i>wago-1 (tm1414)</i>	Male	snoRNA AS	7
<i>wago-1 (tm1414)</i>	Male	piRNA AS	8
<i>wago-1 (tm1414)</i>	Male	Repetitive Elements S	12
<i>wago-1 (tm1414)</i>	Male	tRNA AS	16
<i>wago-1 (tm1414)</i>	Male	piRNA S	78
<i>wago-1 (tm1414)</i>	Male	ncRNA AS	107
<i>wago-1 (tm1414)</i>	Male	No Feature	897
<i>wago-1 (tm1414)</i>	Male	Pseudogene AS	1314
<i>wago-1 (tm1414)</i>	Male	Repetitive Elements AS	1994
<i>wago-1 (tm1414)</i>	Male	Protein Coding AS	3972

Bridge to Chapter 3:

In Chapter 2, I found that WAGO-1 is a sexually dimorphic component of small RNA pathways. By leveraging a whole germline analysis pipeline and a novel antibody against native WAGO-1 structure, I quantitatively characterize biophysical attributes of WAGO-1 foci throughout both oogenesis and spermatogenesis. Furthermore, I provide an in-depth analysis of WAGO-1 localization with structural markers of the P- and Z-granule and elucidate sexual dimorphisms within germ granule structure. By combining my cytological analysis of wild-type WAGO-1 with functional assessment of germ granule structure and function of mutant variants of WAGO-1, I determine that WAGO-1 differentially regulates fertility and germ granule organization during germ cell development. Finally, I use genomic techniques to uncover sexually dimorphic perturbations to mRNA and small RNA profiles of adult *C. elegans* through misregulation of other, sex-specific Argonautes in mutant WAGO-1 contexts. These studies demonstrate the power of our approach in uncovering sex-specific mechanisms of gene regulation by conserved small RNA pathways. Further, our work creates a foundation for future studies of other Argonautes or sRNA pathway components which may uncover additional dimorphic structures and functions that enable sexual dimorphic gene regulation. In Chapter 3, we begin these future works by applying our whole germline analysis of biophysical characterization and localization of two additional Argonaute proteins, WAGO-3 and WAGO-4, during egg and sperm development. We also expand our process to interrogating sexual dimorphisms in key structural components of the germ granule, PGL-1 and ZNFX-1.

CHAPTER 3: SEXUAL DIMORPHISMS IN GERM GRANULE MORPHOLOGY AND ORGANIZATION IN *C. ELEGANS*

Abstract

In sexually reproducing organisms, germ cells faithfully transmit both the genome and epigenetic information from one generation to the next through the formation of haploid gametes, eggs and sperm. Small RNA pathways tune gene expression during germ cell development and facilitate transgenerational inheritance of epigenetic information. In *Caenorhabditis elegans*, components of small RNA pathways localize to germ granules, liquid-like membraneless organelles within the cytoplasm of developing germ cells. During oogenesis, germ granules form hierarchical sub-compartments that are believed to be required for proper germ cell development and epigenetic inheritance. However, germ granule structure during spermatogenesis has been largely overlooked, and how sexual dimorphisms in germ granule structure underpin sex-specific function of small RNA pathways remains largely undescribed. In Chapter 2, I established that gene regulation by the Argonaute WAGO-1 is sexually dimorphic and likely mediated by sex-specific localization with germ granule structural proteins, PGL-1 and ZNFX-1. Here we determine that PGL-1 and ZNFX-1 also display sexually dimorphic biophysical properties, which may drive their sex-specific co-localization observed with WAGO-1. Further, we define the extent of co-localization of these two proteins within the germ granule, determining, as suggested in Chapter 2, that PGL-1 and ZNFX-1 do not form distinct foci. Rather PGL-1 and ZNFX-1 overlap independent of sex, with the extent of co-localization dependent on meiotic stage. We also determine that WAGO-3 and WAGO-4, two cytoplasmic Argonaute proteins central to gene regulation by small RNA pathways, are similarly sexually dimorphic in their biophysical properties during germ cell development. These dimorphisms likely influence localization with PGL-1 and ZNFX-1, with both Argonautes primarily localizing with PGL-1 during spermatogenesis and ZNFX-1 during oogenesis. Finally, we reveal that WAGO-3 and WAGO-4 co-localization with each other is dependent on both meiotic stage and processes. Together, our studies reveal four additional sexually dimorphic components of the germ granule and that these dimorphisms may underpin sex-specific and Argonaute specific gene regulation by WAGO-3 and WAGO-4.

Introduction

Sexually reproducing organisms faithfully transmit their genome from one generation to the next through meiosis, the specialized cell division that produces germ cells. Both oogenesis and spermatogenesis lead to the formation of haploid gametes, eggs and sperm respectively. While both of these gametes contain exactly half the number of chromosomes compared to their parental cell, the mechanisms that lead to their formation are sexually dimorphic (Cahoon and Libuda, 2019; Ishiguro, 2023). Indeed, a growing body of evidence suggests that oogenesis and spermatogenesis differ despite the conservation of numerous meiotic proteins (Cahoon et al., 2023; Checchi et al., 2014; Jaramillo-Lambert et al., 2016; Li et al., 2020; Ortega et al., 2024; Rourke and Jaramillo-Lambert, 2022).

Gametes can transmit epigenetic information to the next generation in a form of inheritance termed transgenerational epigenetic inheritance (TEI). TEI is vital for preserving the immortality of the germline, as well as ensuring proper meiotic progression, fertilization, and embryogenesis (Ben Maamar et al., 2021; Heard and Martienssen, 2014). Epigenetic information can be maintained between generations through mechanisms such as DNA methylation, histone modifications, and non-coding RNAs (Fitz-James and Cavalli, 2022). Many multicellular organisms, though, including humans, dynamically alter DNA methylation and histone marks during gametogenesis and fertilization, making direct transmission of epigenetic information between generations difficult (Gu et al., 2010; Messerschmidt et al., 2014; Nilsson et al., 2022; Samson et al., 2014). Non-coding RNAs may serve as a memory of these epigenetic DNA methylation and histone modification marks, as they provide a template for re-establishment and directly target transcripts for silencing or licensing (Aravin and Bourc'his, 2008; Barucci et al., 2020; Buckley et al., 2012; Kuramochi-Miyagawa et al., 2008; Seroussi et al., 2022). Small RNAs, such as PIWI-interacting RNAs (piRNAs) and endogenous small interfering RNAs (endo-siRNAs), are inherited through the cytoplasm of eggs (Brennecke et al., 2008; Roovers et al., 2015; Tam et al., 2008) or sperm (Conine et al., 2018; Schreier et al., 2022; Sharma et al., 2016), and are required for proper embryonic development and regulation of gene expression both transcriptionally and post-transcriptionally. Despite the use of small RNAs during both spermatogenesis and oogenesis for TEI, whether these mechanisms are sexually dimorphic is largely unknown.

In *C. elegans*, small RNAs are crucial for TEI and regulate proper gene expression through generations utilizing an evolutionally well-conserved process known as RNA-interference (RNAi) (Fire et al., 1998; Ketting, 2011). RNAi employs Argonaute proteins (AGOs) to bind small RNAs, 18 to 30 nucleotides in length, to target exogenous or endogenous sequences for post-transcriptional or transcriptional gene regulation (Almeida et al., 2019; Cernilogar et al., 2011; Ketting and Cochella, 2021; Okamura et al., 2004; Seroussi et al., 2022; Youngman and Claycomb, 2014). Proper expression and regulation of these AGOs is required for meiotic progression, TEI, and stress response in germ cells (Kurhanewicz et al., 2020; Ortega et al., 2024; Sen et al., 2024; Wan et al., 2018; Xu et al., 2018).

C. elegans have 16 AGOs that are active within the germline and engage with three distinct classes of small RNAs: micro RNAs (miRNAs), PIWI interacting RNAs (piRNAs) and small interfering RNAs (siRNAs). siRNAs can further be broken into two distinct classes: 1) primary siRNAs which are bound by primary AGOs including ERGO-1, RDE-1, and the paralogs ALG-3/4, and 2) secondary siRNAs which are templated from primary siRNA or piRNA targets and bound by a secondary class of worm-specific AGOs, termed WAGOs (Batista et al., 2008; Conine et al., 2010; Gu et al., 2009; Seroussi et al., 2023; Vasale et al., 2010; Wang and Reinke, 2008). Secondary AGOs are especially crucial for transgenerational silencing of transcripts, as well as paternal and maternal epigenetic inheritance through the cytoplasm (Ishidate et al., 2018; Schreier et al., 2022; Wan et al., 2018; Xu et al., 2018). Indeed, loss of any of the three cytoplasmic WAGOs (*wago-1*, *wago-3*, or *wago-4*) causes a mortal germline (Mrt) phenotype, leading to complete sterility within 10 generations when reared at temperatures above standard physiological conditions (Schreier et al., 2022; Seroussi et al., 2023; Wan et al., 2018; Xu et al., 2018).

WAGO-1, WAGO-3, and WAGO-4, as well as primary AGOs and numerous machineries required for RNAi all localize to germ granules during gametogenesis. Germ granules are liquid-like phase-separated organelles that are present within developing germ cells of most sexually reproducing organisms and play numerous roles in small RNA processing and regulation (Dodson and Kennedy, 2020; Gao and Arkov, 2013; Phillips and Updike, 2022; Thomas et al., 2023). Germ granules are a type of biomolecular condensate, whose formation is driven by biophysical interactions between RNA and multivalent proteins with intrinsically disordered domains (Alberti et al., 2019; Alberti and Hyman, 2021; Boeynaems et al., 2018; Putnam et al.,

2023), and is fine-tuned by concentrations of these macromolecules (Banani et al., 2017; Dodson and Kennedy, 2020; Shin and Brangwynne, 2017). Condensates are hypothesized to act as sub-compartments within the cytoplasm or nucleus that function to mediate biochemical processes required for proper cellular functions (Boeynaems et al., 2019; Feric et al., 2016; Hnisz et al., 2017; Shin and Brangwynne, 2017). Indeed, germ granules have been found to contain numerous components of small RNA pathways, and disruption to their localization and ability to phase separate during oogenesis and spermatogenesis has been tied to instances of infertility (Campbell and Updike, 2015; Spichal et al., 2021). Foci morphology of phase separated condensates can act as a read out of the biophysical properties of the proteins and RNAs within, with larger size being tied to higher protein concentrations and irregular aspherical surface area acting as a proxy for more gel-like or glassy condensates (Alberti and Hyman, 2021; Brangwynne, 2013; Putnam et al., 2023; Shin and Brangwynne, 2017). Previous work has found that germ granules are primarily spherical and only change in shape due to localization around the nucleus or following fusion events (Brangwynne et al., 2009; Folkmann et al., 2021).

The germ granules of *C. elegans* are organized into sub-compartments that occur in non-random configurations and dynamically change through oogenesis and early germline development (Chen et al., 2024; Huang et al., 2024; Lu et al., 2025; Manage et al., 2020; Uebel et al., 2020, 2023; Wan et al., 2018). Currently, six distinct compartments of the germ granule have been identified, but only two are known to house secondary WAGOs associated with small RNA pathways and TEI. These two compartments are the P-granule, marked by the RNA-binding protein PGL-1 (Aoki et al., 2021; Kawasaki et al., 2004, 1998), and the Z-granule, which houses the zinc-finger protein ZNFX-1 (Ishidate et al., 2018; Wan et al., 2018). WAGO-3 has been found to primarily localize with PGL-1 during oogenesis and spermatogenesis, while WAGO-4 primarily localizes with ZNFX-1 during oogenesis (Ishidate et al., 2018; Schreier et al., 2022; Seroussi et al., 2023; Wan et al., 2018). Additionally, in Chapter 2 I found that while WAGO-1 co-localizes with both proteins during oogenesis and spermatogenesis, it has higher association with ZNFX-1 compared to PGL-1 (DiNardo et al., 2025).

Most studies on WAGOs and their localization within germ granules focuses on presence versus absence of these proteins within the biomolecular condensate (Ishidate et al., 2018; Schreier et al., 2022; Seroussi et al., 2023; Wan et al., 2018). Additionally, studies that have investigated dynamics of the germ granule throughout gametogenesis have primarily focused on

the localization and dynamics of major sub-compartment components, such as PGL-1 and ZNFX-1 (Huang et al., 2024; Lu et al., 2025; Uebel et al., 2023, 2020; Wan et al., 2018). In Chapter 2, I identified WAGO-1 as a dynamic component of the germ granule, whose biophysical properties and localization differs between sexes and underlies sexually dimorphic gene regulation by small RNA pathways (DiNardo et al., 2025). Due to similarities in WAGO-1 protein structure and localization to the germ granule during oogenesis, I hypothesized that WAGO-3 and WAGO-4 may have sexually dimorphic structures and functions during germ cell development.

Here we examine germ granule dynamics through egg and sperm development by assessing WAGO-3, WAGO-4, PGL-1, and ZNFX-1 foci morphology and localization utilizing quantitative immunofluorescent microscopy. We find that structural PGL-1 and ZNFX-1 and Argonautes WAGO-3 and WAGO-4 display sexually dimorphic biophysical properties and localization within the germ granule throughout meiotic prophase I. Furthermore, PGL-1 and ZNFX-1 localization in reference to each other is sexually dimorphic, building upon our model presented in Chapter 2 that the sub-compartmentalization of the germ granule is more dynamic and fluid than previously hypothesized. Additionally, we find that WAGO-3 and WAGO-4 display distinct sex-dependent localization patterns with each other during germ cell development, providing possible mechanisms for targeted gene regulation during oogenesis and spermatogenesis. Overall, these findings build upon our model of the sexually dimorphic germ granule and its role in organizing internal small RNA pathway components.

Results:

PGL-1 and ZNFX-1 are sexually dimorphic components of the germ granule

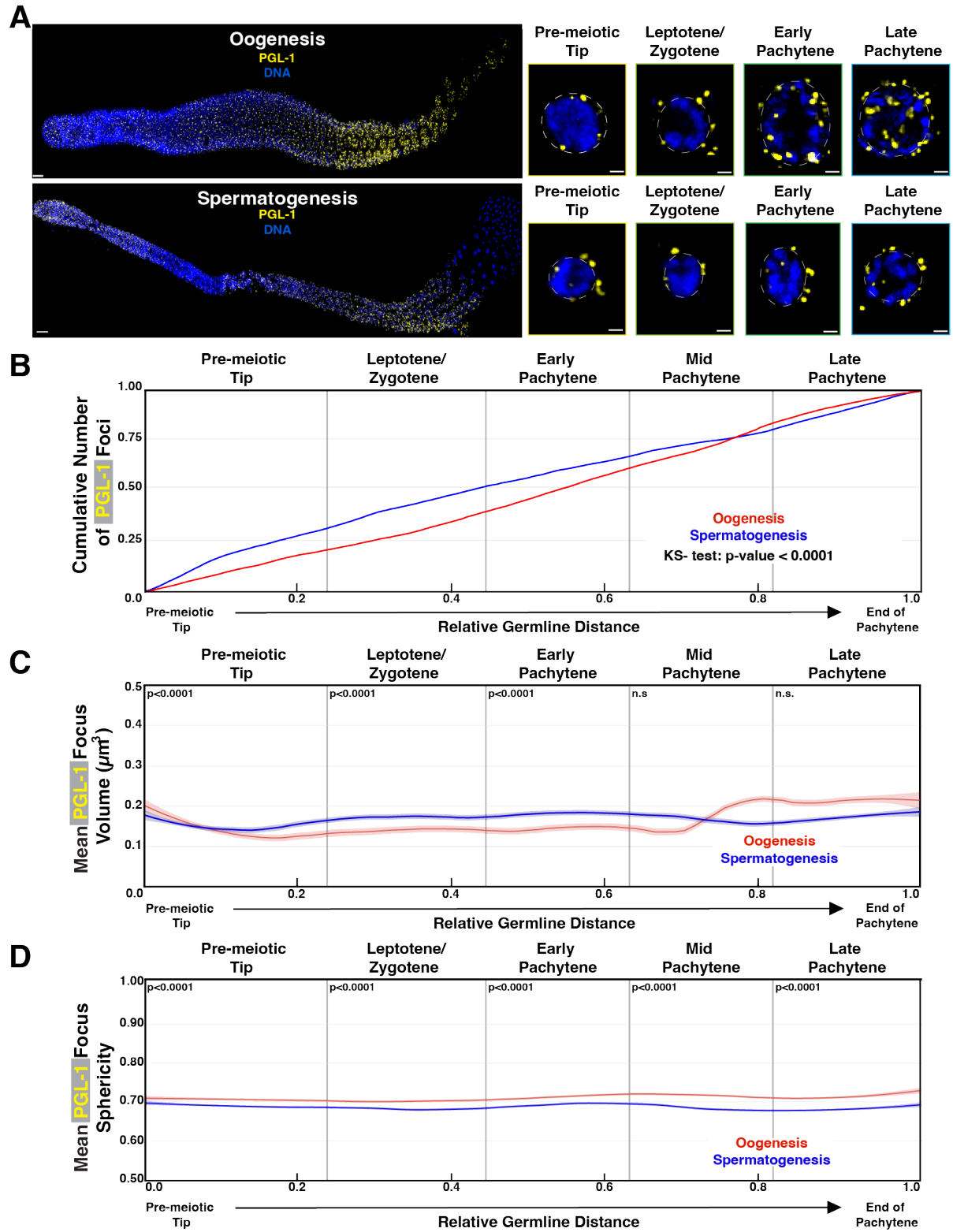
PGL-1 and ZNFX-1 are fundamental components of germ granules during oogenesis and spermatogenesis, and form distinct sub-compartments within germ granules: the P-granule and Z-granule respectively (Ishidate et al., 2018; Kawasaki et al., 2004, 1998; Wan et al., 2018). In Chapter 2, we identified that WAGO-1 co-localization with PGL-1 and ZNFX-1 is sex-specific, suggesting that these structural components may also display sexually dimorphic attributes. Through mRNA-seq analysis we found that PGL-1 is upregulated in adult hermaphrodites compared to adult males, while ZNFX-1 expression is relatively equal between the two sexes (Fig. S3.1). To determine if these two constitutive components of the germ granule are sexually

dimorphic, we looked at their localization and biophysical properties during oogenesis and spermatogenesis utilizing quantitative immunofluorescence microscopy.

We found PGL-1 foci localize around the nuclear periphery of developing germ cells starting in the premeiotic tip and persisting into late pachytene during oogenesis (Fig. 3.1A, top) and spermatogenesis (Fig. 3.1A, bottom). During spermatogenesis, PGL-1 foci accumulation is elevated compared to oogenesis during early stages of meiotic progression through the leptotene/zygotene (L/Z) stage (Fig. 3.1B). We also found that PGL-1 foci volume was sexually dimorphic and dynamic throughout meiotic progression. From the pre-meiotic tip (PMT) through early pachytene (EP), PGL-1 foci were significantly larger during spermatogenesis compared to oogenesis (Fig. 3.1C PMT oogenesis = $0.142\mu\text{m}^3$, spermatogenesis = $0.154\mu\text{m}^3$ Mann Whitney U test $p < 0.0001$; L/Z oogenesis = $0.142\mu\text{m}^3$, spermatogenesis = $0.173\mu\text{m}^3$ Mann Whitney U test $p < 0.0001$; EP oogenesis = $0.144\mu\text{m}^3$, spermatogenesis = $0.181\mu\text{m}^3$ Mann Whitney U test $p < 0.0001$; Table S3.1). Upon onset of mid-pachytene (MP), PGL-1 foci in spermatogenesis shrank in volume, while PGL-1 foci in oogenesis grew (Fig. 3.1C oogenesis EP = $0.140\mu\text{m}^3$, MP = $0.181\mu\text{m}^3$ Mann Whitney U test $p < 0.0001$; spermatogenesis EP = $0.181\mu\text{m}^3$, MP = $0.166\mu\text{m}^3$ Mann Whitney U test $p < 0.0001$). This male-specific decrease in PGL-1 volume observed in mid-pachytene coincides with the eventual loss of PGL-1 phase separation around the germ cell that occurs in the condensation zone of spermatogenesis (Updike and Strome, 2010).

We next compared the sphericity of PGL-1 foci throughout meiotic progression. Previous works have correlated sphericity of phase-separated compartments with liquid-like properties of

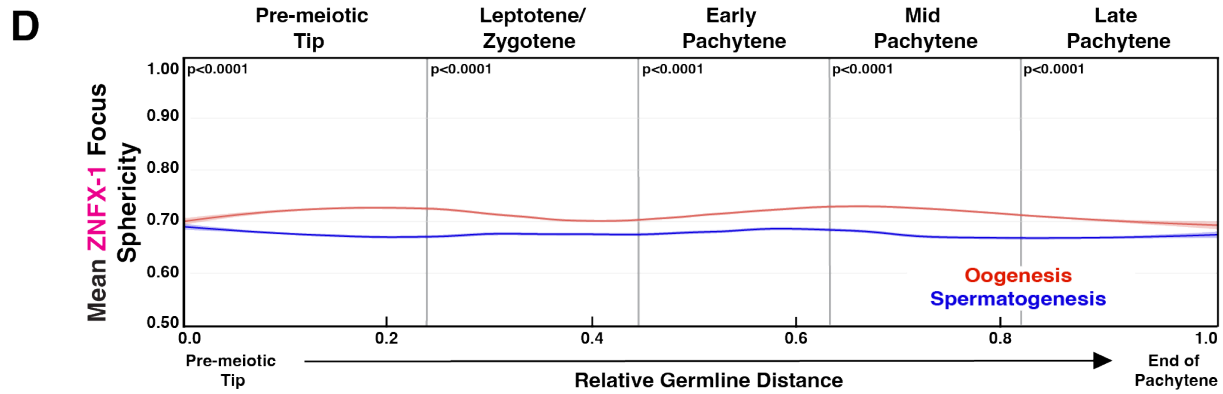
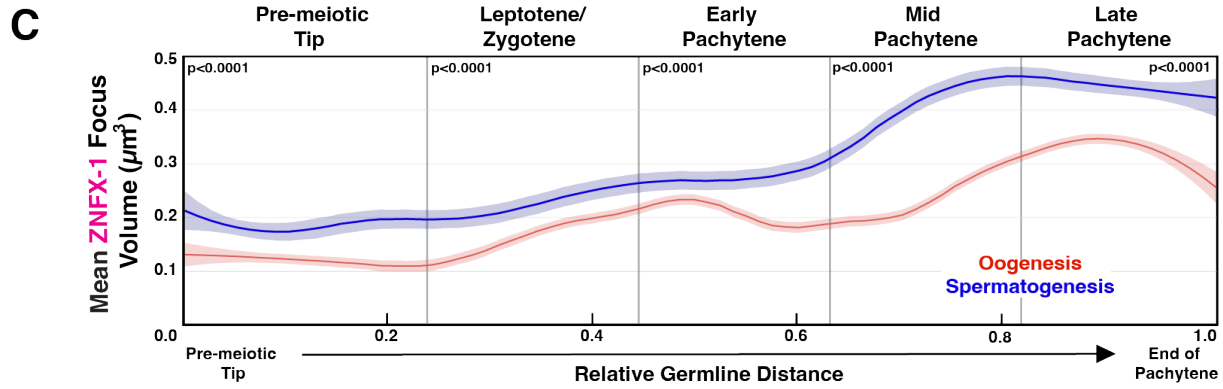
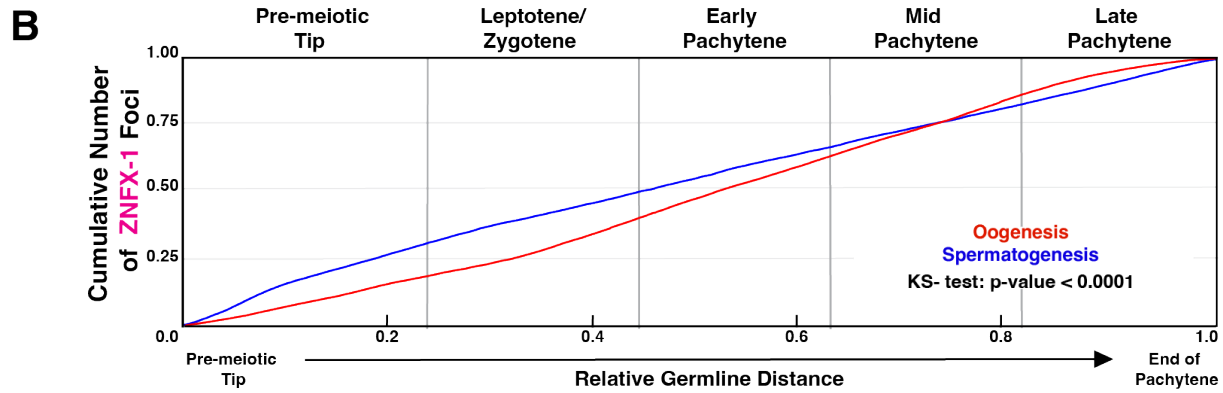
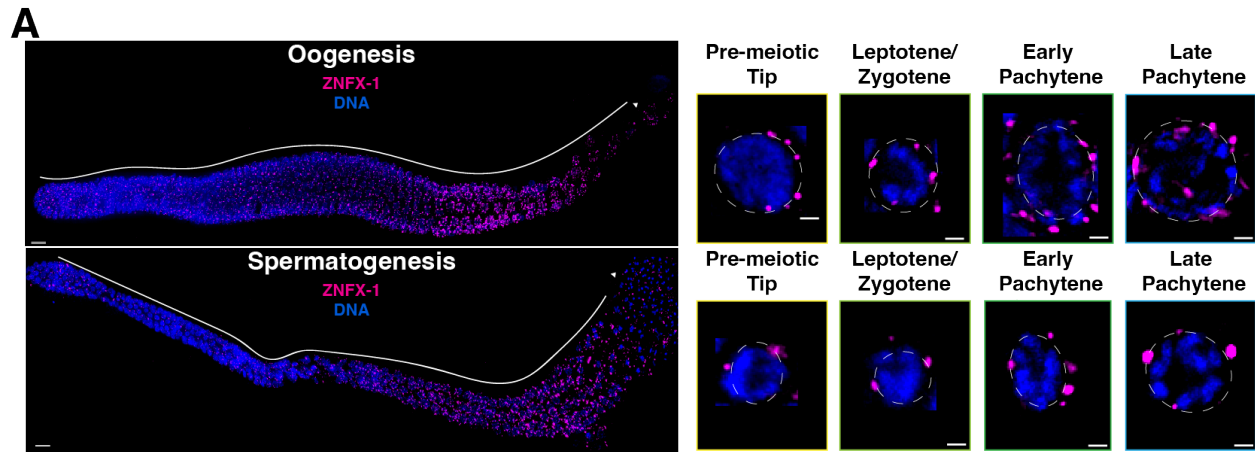
Figure 3.1: PGL-1 displays dynamic sexually dimorphic biophysical properties throughout meiosis (A) Representative immunofluorescence images of PGL-1 (yellow) throughout meiosis of wild-type adult hermaphrodite (oogenesis, top) and adult males (spermatogenesis, bottom). Enlarged panels display individual nuclei from four distinct stages. Scale bars represent $1\mu\text{m}$ in insert panels and $10\mu\text{m}$ in full gonad images. (B) Empirical cumulative distribution function (ECDF) plot of PGL-1 foci throughout oogenesis (red) and spermatogenesis (blue). (C) Quantification of mean PGL-1 foci volume throughout oogenesis (red, pale red band is the standard error of the mean) and spermatogenesis (blue, pale blue band is the standard error of the mean). p-values for each region were calculated using Mann-Whitney *U* tests with Bonferroni correction for multiple comparisons. (D) Quantification of PGL-1 foci sphericity throughout oogenesis (red, pale red band is the standard error of the mean) and spermatogenesis (blue, pale blue band is the standard error of the mean). p-values for each region were calculated using Mann-Whitney *U* tests with Bonferroni correction for multiple comparisons.



the protein (Alberti et al., 2019; Alberti and Hyman, 2021; Brangwynne et al., 2009; Folkmann et al., 2021; Ma et al., 2021; Molliex et al., 2015). Less spherical condensates tend to house proteins that are more gel-like in nature, leading to lower turnover with diffuse protein in the cytoplasm compared to more spherical, and therefore liquid-like, foci (Alberti and Hyman, 2021; Brangwynne et al., 2009; Cochard et al., 2022; Folkmann et al., 2021; Kroschwald et al., 2015; Molliex et al., 2015). Throughout meiotic progression, PGL-1 foci surrounding developing oocytes were significantly more spherical compared to PGL-1 foci associated with spermatocytes, suggesting sex-specific liquid-like properties within the granule (Fig. 3.1D; Table S3.3). Taken together, PGL-1 is a sexually dimorphic component of the germ granule that dynamically changes in focus size and morphology through meiotic progression.

We next analyzed the localization and dynamics of ZNFX-1 through oogenesis and spermatogenesis. We found that ZNFX-1 is perinuclear to developing oocytes and spermatocytes throughout the *C. elegans* germline (Figure 3.2A; Ishidate et al., 2018; Uebel et al., 2023; Wan et al., 2018). Similar to PGL-1, males undergoing spermatogenesis have significantly more ZNFX-1 foci compared to hermaphrodites undergoing oogenesis from the PMT until MP region (Fig. 3.2B). Following MP, ZNFX-1 foci rapidly accumulated in oogenesis compared to spermatogenesis. We also found that throughout germ cell development, ZNFX-1 foci from adult males were significantly larger than those observed in adult hermaphrodite germlines undergoing oogenesis (Fig. 3.2C; Table S3.5). Despite this size discrepancy, foci volume in both sexes

Figure 3.2: ZNFX-1 displays dynamic sexually dimorphic biophysical properties throughout meiosis (A) Representative immunofluorescence images ZNFX-1 (magenta) throughout meiosis of wild-type adult hermaphrodite (oogenesis, top) and adult males (spermatogenesis, bottom). Enlarged panels display individual nuclei from four distinct stages of meiosis. Scale bars represent 1µm in insert panels and 10µm in full gonad images. (B) Empirical cumulative distribution function (ECDF) plot of ZNFX-1 foci throughout oogenesis (red) and spermatogenesis (blue). (C) Quantification of ZNFX-1 foci volume throughout oogenesis (red, pale red band is the standard error of the mean) and spermatogenesis (blue, pale blue band is the standard error of the mean). p-values for each region were calculated using Mann-Whitney *U* tests with Bonferroni correction for multiple comparisons. (D) Quantification of ZNFX-1 foci sphericity throughout oogenesis (red, pale red band is the standard error of the mean) and spermatogenesis (blue, pale blue band is the standard error of the mean). p-values for each region were calculated using Mann-Whitney *U* tests with Bonferroni correction for multiple comparisons.



increased throughout meiotic progression until late pachytene, where ZNFX-1 foci associated with spermatocytes remained constant in volume while those derived from oogenesis decreased in volume (Fig. 3.2C oogenesis MP = $0.241\mu\text{m}^3$, LP = $0.332\mu\text{m}^3$ Mann Whitney U test $p < 0.0001$; spermatogenesis MP = $0.410\mu\text{m}^3$, LP = $0.440\mu\text{m}^3$ Mann Whitney U test $p = 1.0$). We also found that ZNFX-1 foci, similar to PGL-1, are significantly more spherical throughout oogenesis compared to spermatogenesis (Fig. 3.1D; Fig. 3.2D; Table S3.3; Table S3.7). Taken together, our data identifies two additional components of the germ granule with sexually dimorphic biophysical properties that are responsive to progression through meiosis.

ZNFX-1 and PGL-1 association is protein specific and sexually dimorphic

In Chapter 2, we found that WAGO-1 foci were associated with both PGL-1 and ZNFX-1 independent of sex (Fig. 2.2), suggesting that a subset of PGL-1 and ZNFX-1 foci may co-localize rather than form distinct foci. To determine the extent of PGL-1 and ZNFX-1 sub-compartmentalization throughout meiosis I and if their co-localization differs between oogenesis and spermatogenesis, we analyzed the overlap of the two proteins. We specifically probed the percentage of ZNFX-1 and PGL-1 volume overlap in three distinct stages of meiosis: L/Z, EP, and LP. We found that in all three stages, independent of sex, the majority of PGL-1 and ZNFX-1 foci had some degree of overlap with each other (Fig. 3.3A). To better understand this overlap, we examined both ZNFX-1 overlap with PGL-1 and PGL-1 overlap with ZNFX-1. We first looked at the percent of ZNFX-1 volume overlapping with PGL-1 during spermatogenesis compared to oogenesis. 100% overlap corresponds to all the volume of a ZNFX-1 foci overlapping with the volume of a singular PGL-1 foci (Fig. 3.3Bi). We found that during L/Z, significantly more ZNFX-1 foci overlapped with PGL-1 during spermatogenesis compared to oogenesis (Fig. 3.3Bii; Table S3.9). As the germ cells enter pachytene, the number of ZNFX-1 foci overlapping with PGL-1 decreases during spermatogenesis and increases during oogenesis, leading to a similar percent of ZNFX-1 foci displaying any overlap with PGL-1 between the two

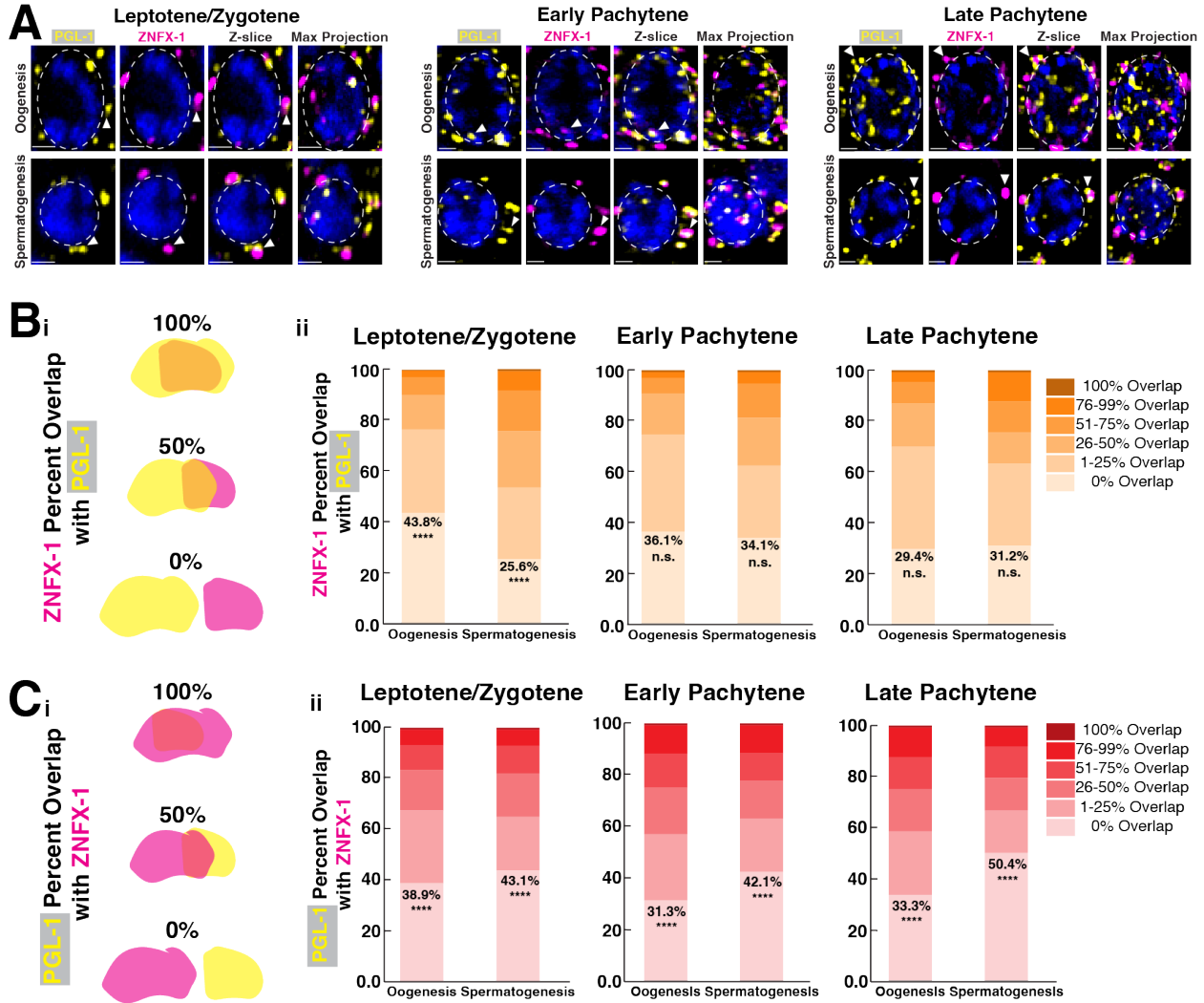


Figure 3.3: PGL-1 and ZNFX-1 co-localization is sexually dimorphic and dependent on meiotic stage. (A) Representative images of single nuclei stained for PGL-1 (yellow) and ZNFX-1 (magenta) undergoing oogenesis (top) or spermatogenesis (bottom) during L/Z, EP, and LP. Scale bars represent 1 μ m. (B) Percent ZNFX-1 volume overlap with PGL-1. (B_i) Schematic depicting the binning of percent ZNFX-1 (magenta) volume overlap with PGL-1 (yellow). (B_{ii}) Bar graphs indicating percentage of ZNFX-1 volume overlapping with PGL-1 foci between oogenesis and spermatogenesis from meiotic regions L/Z, EP, and LP. The numbers reported indicate the percentage of ZNFX-1 foci that have 0% overlap with PGL-1 foci. n.s. indicates a p-value > 0.05, **** indicates a p-value < 0.0001, Chi-squared tests with Bonferroni correction. (C) Percent PGL-1 volume overlap with ZNFX-1. (C_i) Schematic depicting the binning of percent PGL-1 (yellow) volume overlap with ZNFX-1 (magenta). (C_{ii}) Bar graphs indicating percentage of PGL-1 foci volume overlapping with ZNFX-1 foci between oogenesis and spermatogenesis during L/Z, EP, and LP. Reported numbers indicate the percentage of PGL-1 foci that have 0% overlap with a ZNFX-1 foci. **** indicates a p-value < 0.0001, Chi-squared tests with Bonferroni correction.

sexes through early and late pachytene (Fig. 3.3Bii; Table S3.9). Despite similar probability of ZNFX-1 foci co-localizing with PGL-1 between the sexes, during spermatogenesis significantly more ZNFX-1 foci had greater than 25% of their volume overlapping with PGL-1 compared to oogenesis (Fig. 3.3Bii, EP oogenesis = 25.4%, spermatogenesis = 37.1% Chi-square test $p < 0.0001$; LP oogenesis = 30.4%, spermatogenesis = 36.4% Chi-square test $p < 0.0001$). Together, these data suggest that while ZNFX-1 localizes with PGL-1 independent of sex throughout meiosis I, the extent of overlap is sexually dimorphic, with oogenesis having significantly less volume overlap compared to spermatogenesis.

We next probed the reverse association of PGL-1 with ZNFX-1. Here, we looked at the percent of PGL-1 volume that is overlapping with ZNFX-1 between oogenesis and spermatogenesis (Fig. 3.3Ci). We found that throughout meiotic prophase I, PGL-1 association with ZNFX-1 was significantly higher during oogenesis compared to spermatogenesis (Fig. 3.3Cii; Table S3.11). This trend differed significantly from what we observed with ZNFX-1 overlap with PGL-1, where during L/Z we found that spermatogenesis had significantly both more instances of co-localization between the two proteins and higher percent of ZNFX-1 volume overlapping with PGL-1 compared to oogenesis. (Fig. 3.3Bii). These differences in percent overlap of each protein with the other may be driven by the higher prevalence of unassociated PGL-1 foci compared to unassociated ZNFX-1 foci specifically during spermatogenesis (Fig. 3.3Bii; Fig. 3.3Cii; L/Z PGL-1= 43.1%, ZNFX-1=25.6% Chi-square test $p < 0.0001$; EP PGL-1 = 42.1%, ZNFX-1=34.1% Chi-square test $p < 0.0001$; LP PGL-1=50.4%, ZNFX-1=31.2%, Chi-square test $p < 0.0001$). Together, our data suggests that ZNFX-1 and PGL-1 primarily localize to the same germ granule independent of sex. The degree of co-localization differs between the sexes and is dependent on meiotic stage, suggesting that sex-specific sub-compartmentalization of these germ granule components does occur.

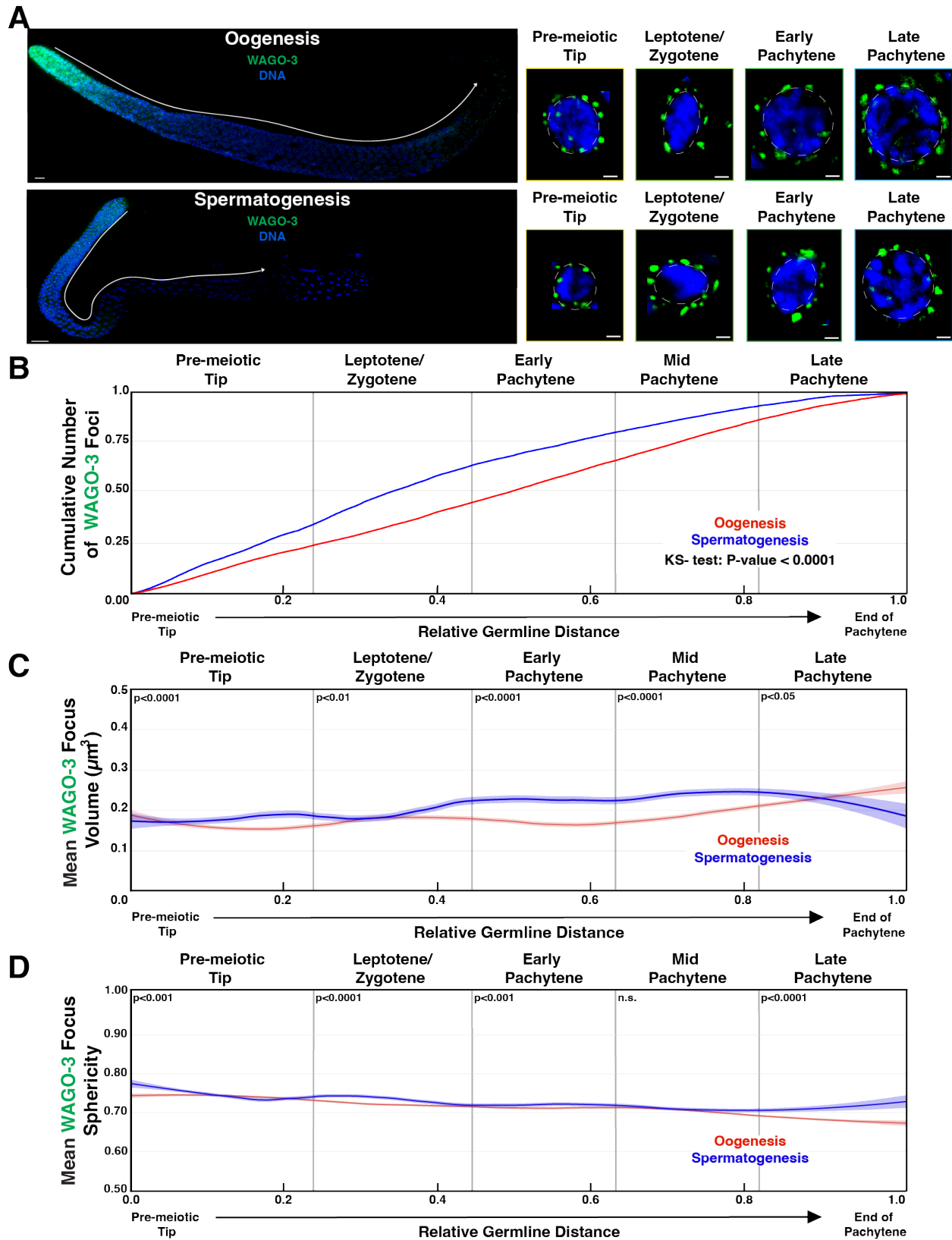
WAGO-3 displays sexually dimorphic properties throughout germ cell development.

WAGO-3 is a secondary AGO that acts within multiple small RNA pathways to silence erroneously expressed pseudogenes and repetitive elements that can threaten genomic integrity (Schreier et al., 2022; Seroussi et al., 2023; Vastenhouw et al., 2003). *wago-3* is expressed similarly in adult hermaphrodites and adult males (Fig. S3.1), and null mutations to *wago-3* compromise fertility in both adult hermaphrodites undergoing oogenesis (Fig. S3.2, left) and

adult males undergoing spermatogenesis (Fig. S3.2, right). Despite these similarities, WAGO-3 plays a direct role in paternal epigenetic inheritance (Schreier et al., 2022).

To determine if this role of WAGO-3 for paternal epigenetic inheritance may be associated with sexual dimorphisms in WAGO-3 foci behavior, we compared WAGO-3 localization and biophysical properties between oogenesis and spermatogenesis. We found that WAGO-3 was present in both adult hermaphrodite (oogenesis) and adult male (spermatogenesis) germlines throughout meiotic progression (Fig. 3.4A). Additionally, we noted that fluorescent intensity of WAGO-3 decreased during both meiotic processes but was still present in late pachytene (Fig. 3.4A, LP inset; Fig. S3.3). Despite similarities in intensity patterns, WAGO-3 displayed sexually dimorphic rates of foci accumulation, with males accumulating significantly more foci during the pre-meiotic tip and leptotene/zygotene regions of the germline compared to hermaphrodites (Fig. 3.4B). Upon entry into pachytene, these accumulation rates slow in spermatocytes (Fig. 3.4B, blue), but remain relatively constant throughout oogenesis (Fig. 3.4B, red). We additionally found that WAGO-3 foci volume dynamics were distinct between oogenesis and spermatogenesis. Specifically, throughout all five binned stages of meiotic progression, the mean foci volume is significantly larger during spermatogenesis compared to oogenesis. Despite larger mean foci volume, WAGO-3 volume decreases in the last third of late pachytene only in spermatocytes (Fig. 3.4C; Table S3.13). We also compared the sphericity of WAGO-3 during oogenesis and spermatogenesis (Fig. 3.4D; Table S3.15). Through meiotic

Figure 3.4: WAGO-3 displays dynamic sexually dimorphic biophysical properties throughout meiosis (A) Representative immunofluorescence images WAGO-3 (magenta) throughout meiosis of wild-type adult hermaphrodite (oogenesis, top) and adult males (spermatogenesis, bottom). Enlarged panels display individual nuclei from four distinct stages of meiosis. Scale bars represent 1 μ m in insert panels and 10 μ m in full gonad images. (B) Empirical cumulative distribution function (ECDF) plot of WAGO-3 foci throughout oogenesis (red) and spermatogenesis (blue). The p-value reported on the plot is based on comparison between distribution of foci using two-sample Kolmogorov-Smirnov test. (C) Quantification of WAGO-3 foci volume throughout oogenesis (red, pale red band is the standard error of the mean) and spermatogenesis (blue, pale blue band is the standard error of the mean). p-values for each region were calculated using Mann-Whitney *U* tests with Bonferroni correction for multiple comparisons. (D) Quantification of WAGO-3 foci sphericity throughout oogenesis (red, pale red band is the standard error of the mean) and spermatogenesis (blue, pale blue band is the standard error of the mean). p-values for each region were calculated using Mann-Whitney *U* tests with Bonferroni correction for multiple comparisons.



progression in both germlines, we found that WAGO-3 foci decrease in sphericity until late pachytene. Within LP WAGO-3 foci sphericity during spermatogenesis remains constant, while foci from oogenesis continue to grow more aspherical (Fig. 3.4D oogenesis MP = 0.700, LP = 0.676 Mann Whitney U test $p < 0.0001$; spermatogenesis MP = 0.707, LP = 0.707 Mann Whitney U test $p = 0.0668$; Table S3.15). These data suggest that similar to WAGO-1, WAGO-3 foci behave in a sexually dimorphic manner and may drive sex-specific organization of the germ granule.

WAGO-3 foci localize to distinct sub-compartments of the germ granule based on sex

We next determined WAGO-3 localization within the sub-compartments of the germ granule. We had previously found that the germ granule structural proteins, PGL-1 and ZNFX-1, primarily co-localize, although sex-specific sub-compartmentalization of the two foci does occur (Fig. 3.3). Further, despite the association of the two germ granule proteins, we found that a larger portion of PGL-1 foci localize independently of ZNFX-1 than ZNFX-1 foci independent of PGL-1 specifically during spermatogenesis (Fig. 3.3B-C). To determine how WAGO-3 interfaces with these two proteins, we looked at WAGO-3 foci percent volume overlap with PGL-1 (Fig. 3.5A-B) and ZNFX-1 (Fig. 3.5C-D). We found that most WAGO-3 foci co-localized with PGL-1 during both oogenesis (Fig. 3.5A, top) and spermatogenesis (Fig 3.5A, bottom). Despite this conservation, significantly more WAGO-3 foci associated with PGL-1 during spermatogenesis compared to oogenesis (Fig. 3.5B; Table S3.17). Indeed, in all stages of spermatogenesis, more than 90% of WAGO-3 foci co-localized with PGL-1, with the majority

Figure 3.5: WAGO-3 co-localization with PGL-1 and ZNFX-1 is sexually dimorphic and dynamic through meiotic progression. (A,C) Representative images of (A) WAGO-3 (green) and PGL-1 (yellow) or (C) WAGO-1 (green) and ZNFX-1 (magenta) undergoing oogenesis (top) or spermatogenesis (bottom) during L/Z, EP, and LP. Scale bars represent 1 μm . (B,D) Bar graphs indicating percentage of WAGO-3 foci volume overlapping with (B) PGL-1 foci and (D) ZNFX-1 foci between oogenesis and spermatogenesis during L/Z, EP, and LP. The numbers within each bar indicate the percentage of WAGO-3 foci that have 0% overlap with PGL-1 foci. *** indicates a p -value < 0.001 , **** indicates a p -value < 0.0001 , Chi-squared test with Bonferroni correction. (E) Representative images of WAGO-3 (green), PGL-1 (yellow), and ZNFX-1 (magenta) localization around single nuclei undergoing oogenesis (top) and spermatogenesis (bottom) during L/Z, EP, and LP. Venn Diagrams show percent of WAGO-3 foci overlapping with just ZNFX-1 foci (top, magenta), just PGL-1 foci (bottom, yellow), and with both PGL-1 and ZNFX-1 foci (middle).

having greater than 25% foci volume overlapping with PGL-1. In contrast, we found less than 75% of WAGO-3 foci co-localized with PGL-1 during oogenesis (Fig. 3.5B; Table S3.17). We next compared WAGO-3 foci overlap with ZNFX-1. Here, we found that throughout meiotic progression, WAGO-3 foci from oogenesis were significantly more likely to localize with ZNFX-1 than WAGO-3 foci from spermatogenesis (Fig. 3.5C-D; Table S3.17). Additionally, WAGO-3 association with ZNFX-1 was dynamic throughout both meiotic processes. Indeed, significantly more WAGO-3 foci localized with ZNFX-1 in late pachytene compared to leptotene/zygotene and early pachytene during oogenesis. In contrast spermatogenesis saw a constant increase in ZNFX-1-associated WAGO-3 foci throughout meiotic. Together our data suggests that WAGO-3 localization with structural PGL-1 and ZNFX-1 is sex-specific and dynamically changes through meiosis.

Dynamic co-localization of WAGO-3 with PGL-1 and ZNFX-1 during meiotic progression

In Chapter 2, we determined that most WAGO-1 foci that localized with one structural protein (PGL-1 or ZNFX-1) within the germ granule also localized with the other (Fig. 2.2). To better understand the localization of WAGO-3 within germ granule sub-compartments marked by PGL-1 and ZNFX-1, we compared the percent of WAGO-3 foci that overlapped with: only PGL-1, only ZNFX-1, or with both proteins (Fig. 3.5E). We found that independent of sex and meiotic stage, two-thirds of WAGO-3 foci overlap with both PGL-1 and ZNFX-1 (Fig. 3.3E, middle; Table S3.19). Interestingly, we found that the other third of WAGO-3 foci specifically overlapped with either PGL-1 or ZNFX-1 in a sex-specific manner, with WAGO-3 primarily co-localizing with just PGL-1 during spermatogenesis (Fig. 3.3E, yellow) and just ZNFX-1 during oogenesis (Fig. 3.3E, magenta). Together, these data suggest that a subset of germ granules that house WAGO-3 demonstrate a sub-compartmentalization of the AGO to either the Z- or P-granule. Furthermore, which sub-compartment this subset of WAGO-3 foci localizes to is dependent on sex.

WAGO-4 foci are sexually dimorphic and dynamic through meiotic progression

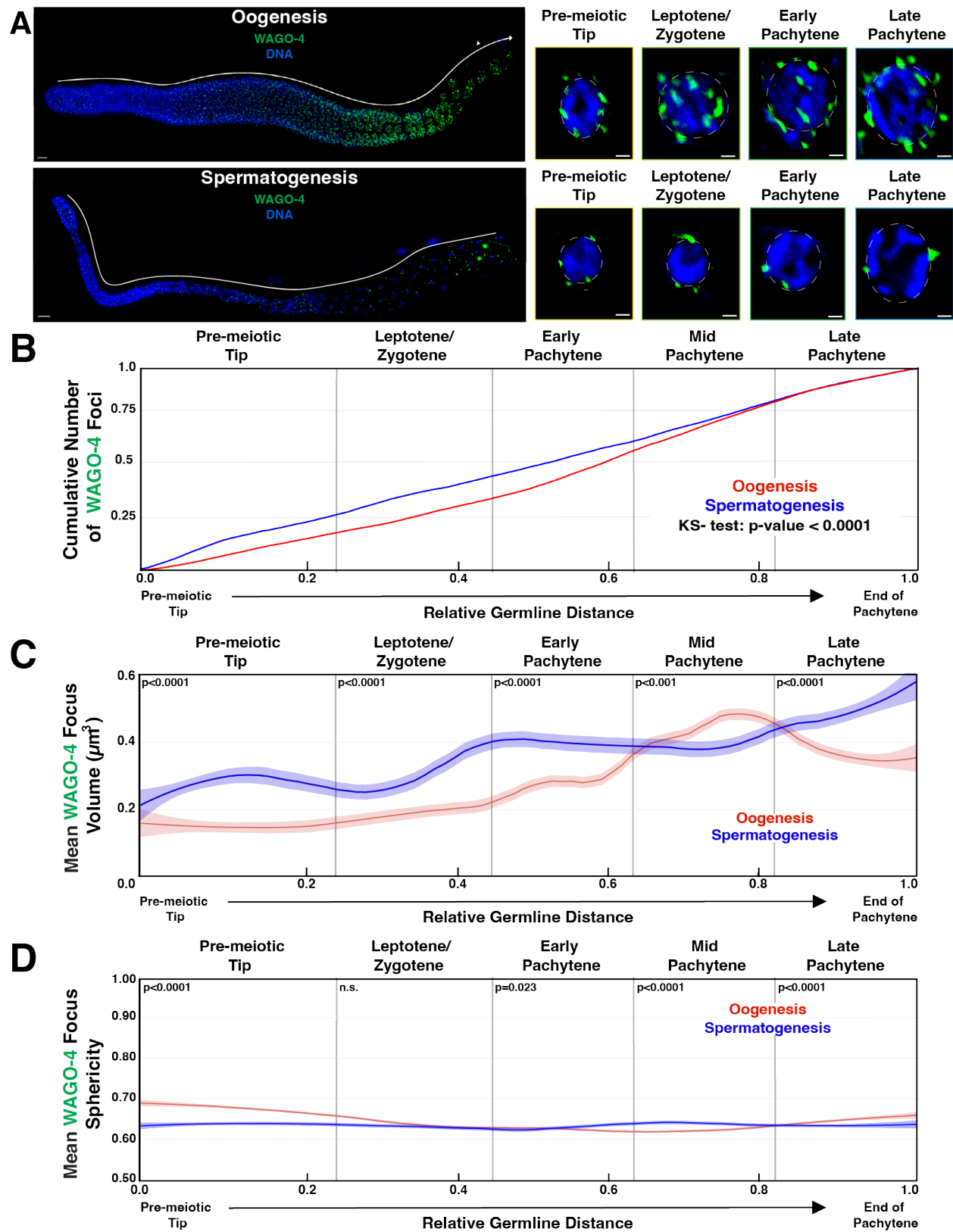
We next looked to determine if WAGO-4, another secondary AGO known to act within multiple small RNA pathways in the germline, is sexually dimorphic (Ishidate et al., 2018; Wan et al., 2018; Xu et al., 2018). Unlike WAGO-3, WAGO-4 expression is sexually dimorphic, with

adult hermaphrodites enriched for *wago-4* transcripts compared to adult males (Fig. S3.1). Additionally, we identified that tagging of the N-terminus of WAGO-4 causes male specific fertility defects in brood size (Fig. S3.4), suggesting that while WAGO-4 may be present at higher levels in hermaphrodites versus males, proper WAGO-4 structure is imperative for preserving male fertility.

To determine if these sexually dimorphic phenotypes may be driven by WAGO-4 localization, we utilized immunofluorescent microscopy to determine WAGO-4 localization during oogenesis and spermatogenesis (Fig. 3.6A). Due to our own and other reported observations of WAGO-4 tagging affecting fertility in adult hermaphrodites and males (Fig. S3.4, Gudipati et al., 2021), we raised antibodies specifically against the N-terminus region of WAGO-4 (Fig. S3.5). We found that throughout the germline, WAGO-4 accumulation was sexually dimorphic. WAGO-4 foci accumulated more rapidly in the pre-meiotic tip and leptotene/zygotene regions during spermatogenesis compared to oogenesis (Fig. 3.6B). Upon entering pachytene, though, we found more WAGO-4 foci accumulated during oogenesis compared to spermatogenesis. This coincided with our observation in spermatogenesis that the pre-meiotic tip had more nuclear associated WAGO-4 foci than during late pachytene (Fig. 3.6A, bottom panels).

We next looked at the biophysical properties of WAGO-4 through oogenesis and spermatogenesis. We found that WAGO-4 foci during spermatogenesis gained volume in a stepwise fashion, while WAGO-4 in oogenesis saw constant growth through mid-pachytene

Figure 3.6: WAGO-4 displays dynamic sexually dimorphic biophysical properties throughout meiosis (A) Representative immunofluorescence images WAGO-4 (magenta) throughout meiosis of wild-type adult hermaphrodite (oogenesis, top) and adult males (spermatogenesis, bottom). Enlarged panels display individual nuclei from four distinct stages of meiosis. Scale bars represent 1 μ m in insert panels and 10 μ m in full gonad images. **(B)** Empirical cumulative distribution function (ECDF) plot of WAGO-4 foci throughout oogenesis (red) and spermatogenesis (blue) as a function of meiotic progression. **(C)** Quantification of WAGO-4 foci volume throughout oogenesis (red, pale red band is the standard error of the mean) and spermatogenesis (blue, pale blue band is the standard error of the mean). p-values for each region were calculated using Mann-Whitney *U* tests with Bonferroni correction for multiple comparisons. **(D)** Quantification of WAGO-4 foci sphericity throughout oogenesis (red, pale red band is the standard error of the mean) and spermatogenesis (blue, pale blue band is the standard error of the mean). p-values for each region were calculated using Mann-Whitney *U* tests with Bonferroni correction for multiple comparisons.

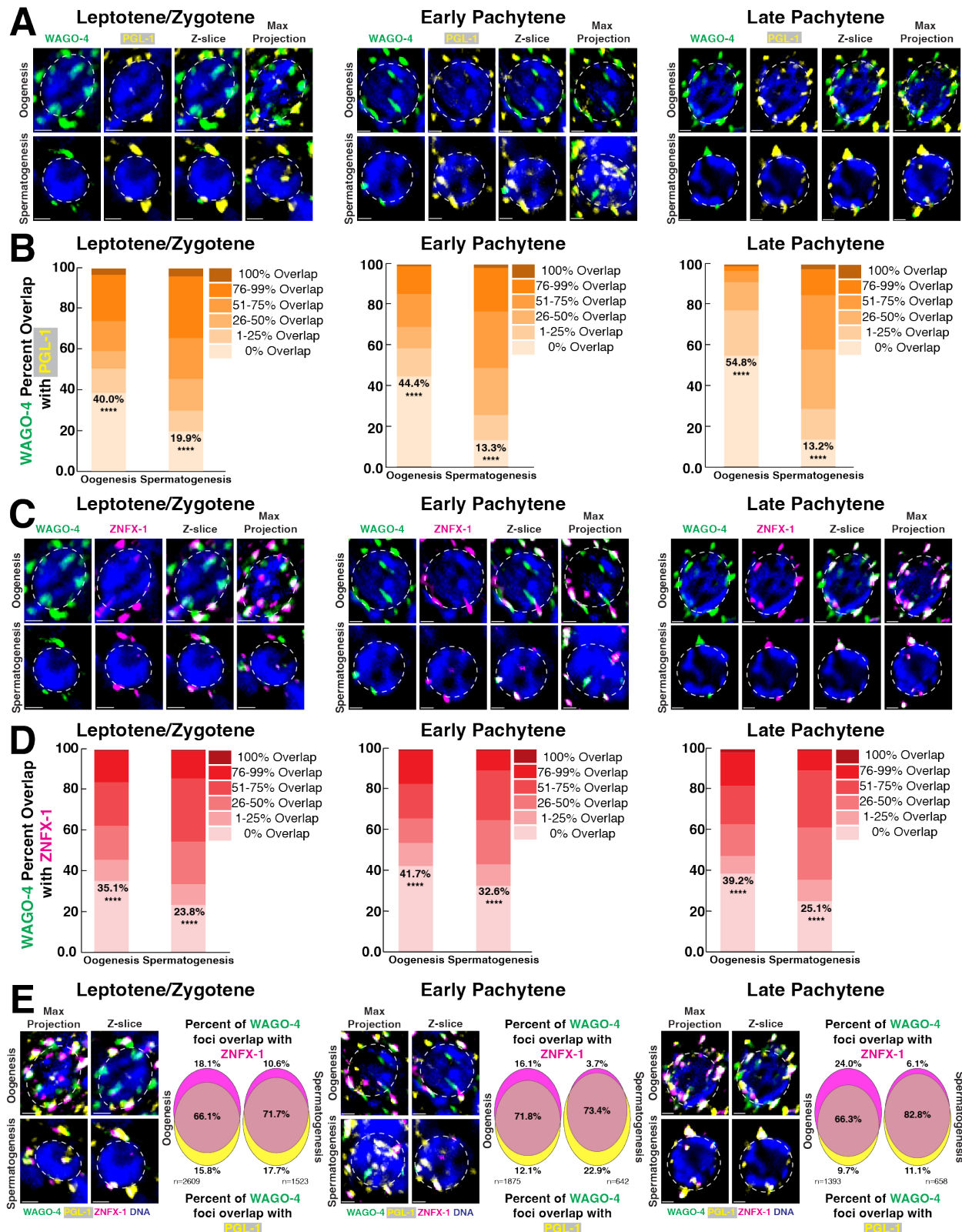


(Figure 3.6C; Table S3.21). Additionally, we found that WAGO-4 foci volume peaks during mid-pachytene of oogenesis (Fig 3.6C, red, MP = $0.442\mu\text{m}^3$), while during spermatogenesis the largest volumes arise in late pachytene (Fig 3.6C, blue, LP = $0.485\mu\text{m}^3$). We also found that changes in WAGO-4 sphericity were unique compared to PGL-1, ZNFX-1 and WAGO-3 (Fig. 3.1D; Fig. 3.2D; Fig 3.4D), with significantly more fluctuations in WAGO-4 sphericity between the two sexes. In the pre-meiotic tip region, WAGO-4 foci were significantly more spherical during oogenesis compared to spermatogenesis (Fig. 3.6D oogenesis = 0.680, spermatogenesis = 0.639 Mann Whitney U test $p < 0.0001$; Table S3.23). Upon meiotic entry, WAGO-4 foci from oogenesis decreased in sphericity to similar levels as observed during spermatogenesis (Fig 3.6D L/Z oogenesis = 0.639, spermatogenesis = 0.633, Mann Whitney U test $p = 0.308$; Table S3.23). This similarity in foci morphology persisted until mid-pachytene where we saw the foci sphericity fluctuated from larger during spermatogenesis in mid-pachytene (Fig 3.6D oogenesis = 0.623, spermatogenesis = 0.641, Mann Whitney U test $p < 0.0001$) to larger during oogenesis in late pachytene (Fig. 3.6D oogenesis = 0.650, spermatogenesis = 0.634, Mann Whitney U test $p < 0.0001$). Taken together, our data suggests that WAGO-4 is a dynamic component of the germ granule that displays distinct properties between sexes, as well as from other WAGOs.

WAGO-4 foci display significantly more localization with germ granule components during spermatogenesis.

Previous work has found that WAGO-4 localizes and interacts with ZNFX-1 during oogenesis to coordinate transgenerational inheritance of gene regulation by small RNA pathways

Figure 3.7: WAGO-4 co-localization with PGL-1 and ZNFX-1 is sexually dimorphic and dynamic through meiotic progression. (A,C) Representative images of single nuclei stained for (A) WAGO-4 (green) and PGL-1 (yellow) or (C) WAGO-1 (green) and ZNFX-1 (magenta) undergoing oogenesis (top) or spermatogenesis (bottom) during L/Z, EP, and MP. Scale bars represent $1\mu\text{m}$. (B,D) Bar graphs indicating percentage of WAGO-4 volume overlapping with (B) PGL-1 foci and (D) ZNFX-1 foci between oogenesis and spermatogenesis during L/Z, EP, and MP. The numbers within each bar indicate the percentage of WAGO-4 foci that have 0% overlap with (B) PGL-1 foci or (D) ZNFX-1 foci. **** indicates a p-value < 0.0001 , Chi-squared test with Bonferroni correction. (E) Representative images of WAGO-4 (green), PGL-1 (yellow), and ZNFX-1 (magenta) localization around single nuclei undergoing oogenesis (top) and spermatogenesis (bottom) during L/Z, EP, and LP. Venn Diagrams show percent of WAGO-4 foci overlapping with just ZNFX-1 foci (top), just PGL-1 foci (bottom), and with both PGL-1 and ZNFX-1 foci (middle).



(Wan et al., 2018; Xu et al., 2018). We wanted to determine if WAGO-4 displays similar localization with ZNFX-1 during spermatogenesis and how WAGO-4 localizes with PGL-1 throughout meiosis in both sexes. To achieve this, we first looked at the percent volume overlap of WAGO-4 with PGL-1 during oogenesis and spermatogenesis in three distinct meiotic stages: leptotene/zygotene, early pachytene, and late pachytene (Fig. 3.7A-B). We found that, similar to WAGO-3, WAGO-4 foci are significantly more likely to overlap with PGL-1 foci during spermatogenesis compared to oogenesis in all meiotic stages assessed (Fig. 3.7B; Table S3.25.). Additionally, we found a reverse trend in the WAGO-4 association with PGL-1 through meiotic progression between the sexes: WAGO-4 foci association with PGL-1 increases through spermatogenesis and decreases through oogenesis (Fig. 3.7B; Table S3.25).

We next analyzed WAGO-4 percent overlap with ZNFX-1 during germ cell development (Fig. 3.7C). Similar to WAGO-4 overlap with PGL-1, we found significantly more WAGO-4 foci overlapped with ZNFX-1 foci in spermatogenesis compared to oogenesis (Fig. 3.7D; Table S3.25). Additionally, we found that for both sexes early pachytene had the lowest levels of WAGO-4 association with ZNFX-1 (Figure 3.7D, middle). This loss of co-localization in early pachytene could suggest a change in the germ granule structure unique to the entry of the germ cell into the pachytene region. To determine how WAGO-4 foci localization within the germ granule changes through meiotic progression, we looked at the proportion of WAGO-4 foci that localized with just PGL-1 (Fig. 3.7E, yellow), just ZNFX-1 (Fig. 3.7E, magenta), or both foci (Fig. 3.7E, middle region). We found that WAGO-4 foci were most likely to overlap with both PGL-1 and ZNFX-1 throughout oogenesis and spermatogenesis, rather than with either germ granule component individually (Fig. 3.7E; Table S3.27). For WAGO-4 foci that associated with either PGL-1 or ZNFX-1, we found that throughout oogenesis WAGO-4 was more likely to localize with only ZNFX-1 (Fig. 3.7E, magenta; Table S3.27), while during spermatogenesis, WAGO-4 foci primarily localized with PGL-1 (Fig. 3.7E, yellow; Table S3.27). These data suggest that although WAGO-4 is a core component of both the P- and Z- granules, WAGO-4 displays sexually dimorphic associations with both granules.

WAGO-4 foci associate with WAGO-3 foci during spermatogenesis

We finally looked at WAGO-3 and WAGO-4 co-localization during spermatogenesis and oogenesis (Fig. 3.8A). While both proteins are known secondary AGOs acting within

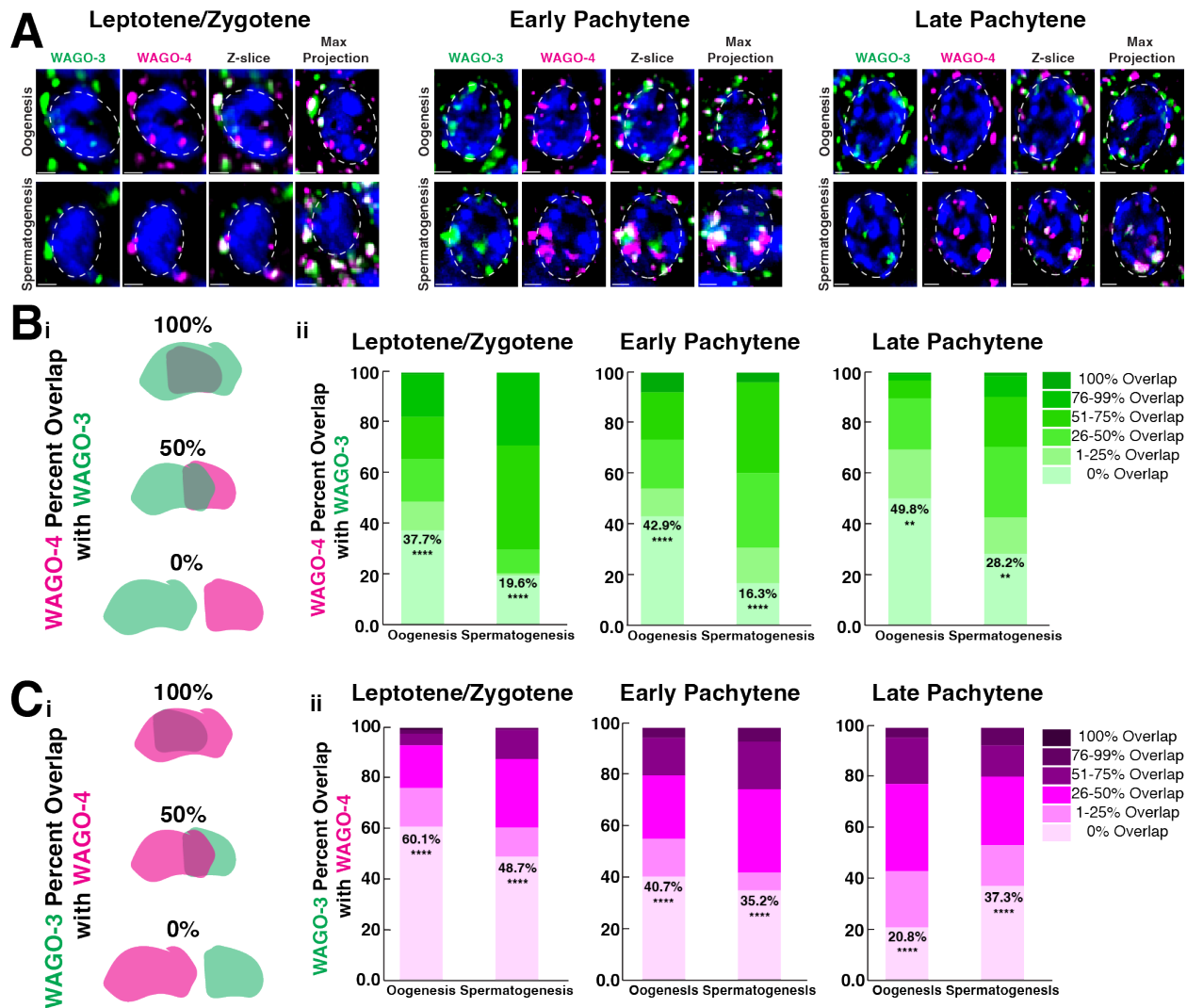


Figure 3.8: WAGO-3 and WAGO-4 co-localization is sexually dimorphic and dependent upon meiotic stage. (A) Representative images of single nuclei stained for WAGO-3 (green) and WAGO-4 (magenta) undergoing oogenesis (top) or spermatogenesis (bottom) during L/Z, EP, and LP. Scale bars represent 1µm. (B) Percent WAGO-4 volume overlap with WAGO-3. (Bi) Schematic depicting the binning of WAGO-4 (magenta) overlap with WAGO-3 (green). (Bii) Bar graphs indicating percentage of WAGO-4 foci volume overlapping with WAGO-3 foci between oogenesis and spermatogenesis during L/Z, EP, and MP. The numbers within each bar indicate the percentage of WAGO-4 foci that have 0% overlap with WAGO-3 foci. ** indicates a p-value < 0.01, **** indicates a p-value < 0.0001, Chi-squared test with Bonferroni correction. (C) Percent WAGO-3 volume overlap with WAGO-4. (Ci) Schematic depicting the binning of WAGO-3 (green) overlap with WAGO-4 (magenta). (Cii) Bar graphs indicating percentage of WAGO-3 foci volume overlapping with WAGO-4 foci between oogenesis and spermatogenesis during L/Z, EP, and LP. The numbers within each bar indicate the percentage of WAGO-3 foci that have 0% overlap with WAGO-4 foci. **** indicates a p-value < 0.0001, Chi-squared test with Bonferroni correction.

endogenous sRNA pathways (Gu et al., 2009; Schreier et al., 2022; Seroussi et al., 2023; Xu et al., 2018), we found that the proportions of the two WAGOs that localize with PGL-1 and ZNFX-1 differ (Fig. 3.5, Fig 3.7). Further, we identified that both WAGO-3 and WAGO-4 foci that associate with only one germ granule structural component (PGL-1 or ZNFX-1), primarily co-localize with ZNFX-1 during oogenesis and PGL-1 during spermatogenesis. To determine whether WAGO-3 and WAGO-4 occupy distinct regions within the germ granule, we looked at the percent volume overlap of WAGO-4 with WAGO-3 (Fig. 3.8B) and of WAGO-3 with WAGO-4 (Fig. 3.8C). We first assessed the percent of WAGO-4 foci volume overlapping with WAGO-3 foci during oogenesis and spermatogenesis (Fig. 3.8Bi). We found that throughout meiotic progression, significantly more WAGO-4 foci overlapped with WAGO-3 foci in spermatogenesis compared to oogenesis (Fig. 3.8Bii). Additionally, WAGO-4 foci were less likely to overlap with WAGO-3 foci during late pachytene compared to other stages of meiosis independent of sex.

We next evaluated the percent volume of WAGO-3 foci that overlapped with WAGO-4 (Fig. 3.8Ci). We found that percent volume overlap of WAGO-3 foci with WAGO-4 had the opposite trend than what we observed for WAGO-4 foci association with WAGO-3. Indeed, when comparing the percent WAGO-3 foci volume that overlapped with WAGO-4 in both sexes had higher amounts of co-localization during pachytene versus leptotene/zygotene (Fig. 3.8Cii). Strikingly during the leptotene/zygotene region of oogenesis, we found that the majority of WAGO-3 failed to overlap with any WAGO-4 foci (Fig. 3.8Cii, left), suggesting that WAGO-3 foci often form without a coinciding WAGO-4 foci. Following entry into pachytene, though, the majority of WAGO-3 foci do overlap with WAGO-4 (Fig. 3.8A; 3.8Cii), possibly driven by there being significantly more WAGO-3 foci compared to WAGO-4 until early pachytene (Fig. S3.6). Taken together, these data suggest that localization dynamics of WAGO-3 and WAGO-4 are both sexually dimorphic and independent from one and other.

Discussion

Sexual dimorphisms within the small RNA pathway

PGL-1 and ZNFX-1 play distinct roles from each other during meiosis. ZNFX-1 is required for proper transgenerational epigenetic silencing of transcripts through association with small RNA pathway machinery. Indeed, ZNFX-1 has been found to maintain TEI by storing

pUGylated transcripts, which are utilized as templates for secondary sRNA transcription (Shukla et al., 2021), within the germ granule, as well as preventing drift of secondary sRNA transcription from the 3' end to the 5' end of templates (Ishidate et al., 2018; Ouyang et al., 2022). In contrast, proper PGL-1 structure is required for PRG-1 and WAGO-1 mediated regulation of transcripts within the germline and silencing of spermatogenic specific genes during oogenesis (Aoki et al., 2021; Campbell and Updike, 2015; Spichal et al., 2021; Updike et al., 2014). The majority of previous research describing germline specific functions of ZNFX-1 and PGL-1 have been done predominately in adult hermaphrodites undergoing oogenesis (Aoki et al., 2021; Campbell and Updike, 2015; Ishidate et al., 2018; Wan et al., 2018), however, numerous meiotic proteins function differently during oogenesis versus spermatogenesis to mediate conserved processes (Cahoon et al., 2023; DiNardo et al., 2025; Li et al., 2020; Rourke and Jaramillo-Lambert, 2022). We found that ZNFX-1 and PGL-1 have sexually dimorphic foci morphology throughout germ cell development, which coincides with sex-specific co-localization of these structural components (Fig. 3.1; Fig. 3.2; Fig. 3.3). Differences in ZNFX-1 and PGL-1 localization within the germ granule described here may suggest that mechanisms of TEI and gene silencing mediated by germ granules also differ between the sexes. We additionally noted that the majority of PGL-1 and ZNFX-1 foci co-localized with each other independent of sex (Fig. 3.3). Despite PGL-1 and ZNFX-1 primarily localizing together within the same granule, the relative volume amounts of overlap were sexually dimorphic. These results, in conjunction with our observations in Chapter 2 that most WAGO-1 foci overlap with both PGL-1 and ZNFX-1 during oogenesis and spermatogenesis, suggest that the majority of germ granules do not have distinct Z- and P-granules. In depth characterization of the localization and organization of each individual granule containing both PGL-1 and ZNFX-1 throughout all stages of meiosis could uncover the frequency of previously reported sub-compartmentalized germ granules. Coupled with *in vivo* work visualizing mRNA association with ZNFX-1 and PGL-1 could aid in determining if differences in these germ granule structures are functional, as well as if they are utilized in a sex-specific manner.

In addition to TEI and WAGO-1 mediated transcript regulation, both ZNFX-1 and PGL-1 directly interact with key AGOs required for functional gene regulation during germ cell development (Aoki et al., 2021; Gu et al., 2009; Ishidate et al., 2018a; Schreier et al., 2022; Wan et al., 2018). Sexual dimorphisms within AGO expression during meiosis have been well

documented in *C. elegans*. Numerous AGOs including ALG-3/4, ERGO-1, VSRA-1 and WAGO-10 are expressed only during oogenesis or spermatogenesis (Charlesworth et al., 2021; Conine et al., 2013, 2010; Nguyen and Phillips, 2021; Seroussi et al., 2023; Vasale et al., 2010), and those constitutively expressed, including PRG-1 and WAGO-1, have sex-specific roles or localization patterns during pachytene (Ortega et al., 2024; DiNardo et al., 2025). Here, we identify two additional sex-constitutive AGOs, WAGO-3 and WAGO-4, that exhibit sexually dimorphic features within the pachytene region of meiosis. Notably, we find that both WAGO-3 and WAGO-4 foci are on average larger during spermatogenesis compared to oogenesis (Fig. 3.4, Fig. 3.6). These changes in size correlate with co-localization patterns observed with PGL-1 and ZNFX-1. For both WAGO-3 and WAGO-4 foci, we found significantly more volume overlapped with PGL-1 during spermatogenesis compared to oogenesis (Fig. 3.5, Fig. 3.7). Additionally, we found that sub-compartmentalization of WAGO-3 and WAGO-4 was distinct between the sexes. Both proteins predominantly localized with ZNFX-1 during oogenesis, while in spermatogenesis the WAGOs primarily localized with PGL-1 (Fig. 3.5E, Fig. 3.7E). In Chapter 2 of this dissertation, we similarly identify sexually dimorphic interactions of PGL-1 and ZNFX-1 with WAGO-1. Mutations introduced to WAGO-1 protein structure causes disruption to PGL-1 localization, as well as misexpression of genes during oogenesis and spermatogenesis in a sex-dependent manner. Due to the structural and functional similarities between WAGO-1, WAGO-3, and WAGO-4, we hypothesize that disruption to WAGO-3 and WAGO-4 likely causes similar phenotypes including mislocalization of germ granule components and dysregulated gene expression. Future works should aim to examine if indeed wild-type WAGO-3 and WAGO-4 protein structure is required for proper localization of PGL-1 and ZNFX-1 and how changes to germ granule localization may coincide with gene misexpression. Further, the data presented in Chapter 3 suggests that WAGO-3 and WAGO-4 may play sex-specific roles in regulating TEI and piRNA-directed silencing through interactions with PGL-1 and ZNFX-1 (Ishidate et al., 2018a; Schreier et al., 2022; Seroussi et al., 2023; Wan et al., 2018). Future work probing if the sex-specific localization of WAGO-3 and WAGO-4 is required for TEI and piRNA-mediated silencing during both oogenesis and spermatogenesis will aid in better understanding the importance of WAGO localization within the germ granule for mediating germ cell development.

Stage specific function of WAGO proteins

This work highlights not only sexually dimorphic WAGO-3 and WAGO-4 foci morphology and localization, but also that germ granule morphology is dynamic through meiotic progression. We find that both proteins experience a change in the rate of foci accumulation between oogenesis and spermatogenesis during early pachytene. We also identify regions of local fluctuations in foci volume and sphericity throughout meiotic progression. Specifically, oocyte-associated WAGO-4 foci volume peaks in mid-pachytene before significantly shrinking in late pachytene (Fig. 3.6C, red), while spermatocyte-associated WAGO-4 foci consistently increases in volume following entry into pachytene (Fig. 3.6C, blue). Interestingly, WAGO-3 intensity significantly reduces in both sexes through meiotic progression (Fig. S3.2). These responses suggests that WAGO-3 and WAGO-4 function, as well as their interacting proteins, may change dynamically over the course of meiosis, potentially in a sexually dimorphic fashion. *C. elegans* provides an excellent model system to try and better define the sexually dimorphic spatiotemporal interactions, functions, and regulation by WAGO-3 and WAGO-4 during germ cell development (Tzur et al., 2018). Future work parsing specific WAGO-3 and WAGO-4 interactions during distinct stages of meiotic progression and how their disruption changes the transcriptional landscape will greatly aid in our understanding of stage specific roles of small RNA mediated regulation.

Together this work uncovers novel sexually dimorphic morphology and localization patterns of both AGOs and constitutive germ granule components during germ cell development. These differences in germ granule sub-compartmentalization suggest a new avenue of study in understanding mechanisms of small RNA pathway regulation and transmission of epigenetic information during oogenesis and spermatogenesis.

Future directions

Our work characterizing sexually dimorphic morphology and localization patterns of AGOs and constitutive germ granule components during germ cell development represents foundational evidence contributing to a larger continuum of research illuminating sex and gamete-specific features of small RNA pathways in the germline. Future work will focus on expanding these preliminary datasets to produce robust results to better understand sexual

dimorphisms in germ granule structure during egg and sperm development. We will also expand our work to include analysis of strains lacking these germ granule components to parse out the resultant function of sexually dimorphic germ granule structure. Lastly, to more clearly link changes in germ granule structure with functional changes in genomic regulation by germline small RNA pathways, we plan to expand our analysis to encompass previous work interrogating WAGO-1 structure and function (Chapter 2).

Expand existing preliminary datasets. A subset of the comparisons presented have been carried out in a limited number of germlines and technical replicates, and as such should be considered preliminary. These findings include: WAGO-3 localization with PGL-1 during spermatogenesis (Fig. 3.5), WAGO-4 localization with PGL-1 during spermatogenesis (Fig. 3.7), and WAGO-3 and WAGO-4 co-localization during oogenesis and spermatogenesis (Fig. 3.8). Future work on this project will expand the existing analysis to include additional germlines undergoing both oogenesis and spermatogenesis to more fully describe the sexually dimorphic localization of WAGO-3 and WAGO-4 within the germ granule.

Illuminate relationship between sexually dimorphic germ granule structure and small RNA pathway function. The presented work is derived from quantitative microscopy experiments comparing foci morphology and localization in wildtype contexts. While this work is imperative for determining how the germ granule changes and responds to meiotic progression, it does not provide insight into the mechanisms which may differ between the sexes. To remedy this, future work looks to determine how loss of germ granule components PGL-1 and ZNFX-1 effects the morphology and the relative localization of WAGO proteins, WAGO-1, WAGO-3, and WAGO-4. To better determine the role of WAGO structure and proper localization for mediating germ granule structure, we also aim to look a PGL-1, ZNFX-1, and reciprocal WAGO foci morphology and localization in mutant *wago* contexts between sexes. Finally, we will determine how disruption to both the germ granule structure through the introduction of structural mutations, as well as how loss of WAGO proteins differentially changes the transcriptional landscape in adult males and adult hermaphrodites. Together, this work will build upon our understanding of mechanistic differences that underlie sexually dimorphic germ granule

organization and how these differences may drive sexually dimorphic transgenerational epigenetic inheritance (TEI) and gene regulation by small RNA pathways.

Define a role for WAGO-1 in sexually dimorphic small RNA pathways. We plan to expand our analysis to better understand where WAGO-1 fits into the sexually dimorphic small RNA landscape. Chapter 2 of this dissertation focuses solely on WAGO-1, another cytoplasmic secondary AGO present during both oogenesis and spermatogenesis (DiNardo et al., 2025). Previous research has found that WAGO-1 may play a redundant role in regulating transcripts to WAGO-3 (Seroussi et al., 2023), although mutations to only WAGO-1 causes extreme fertility defects and disrupts germ granule structure in both oocytes and spermatocytes. To determine if WAGO-1 and WAGO-3 are indeed redundant, as well as better understand the role of all three cytoplasmic secondary AGOs in egg and sperm development, we plan to interrogate WAGO-1 localization with WAGO-3 and WAGO-4 in wild-type and mutant contexts. Together, we hope to propose a more comprehensive model of the role of sexually dimorphic germ granule structure in small RNA pathway regulation and transgenerational epigenetic inheritance.

Methods and Materials

Strains:

All strains were generated for the N2 background and maintained at 20°C under standard conditions of nematode growth media (NGM) with OP50 *Escherichia coli*. All mutant and tagged strains with fertility defects were backcrossed five times with N2 prior to work.

The following strains were using in this study:

N2: Bristol wildtype strain

YY1325: *wago-4(gg620[3xflag::gfp::wago-4]) II*.

YY916: *znfx-1(gg554[3xflag::gfp::znfx-1]) II*.

JMC249: *ppw-2 (tor115[ppw-2::GFP::FLAG]) I*; *znfx-1(gg634[ha::tagRFP::znfx-1]) II*.

WM155: *ppw-2 (tm1120)I*.

DLW185: *wago-4 (tm1019)II*; *mnC1 [dpy-10(e128) unc-52(e444) nIs190 let-?] II*

CB4108: *fog-2(q71) V*.

WAGO-4 antibody production:

Peptides within the IDR domain (RSEYQTSNDACIKRLEE-Cys) of the N-terminus of WAGO-4 were produced by Biomatik. Antibodies against the WAGO-4 peptides were produced in guinea pig and chicken and affinity purified by Pocono Rabbit Farms. Antibody was confirmed utilizing immunofluorescence.

Immunofluorescence:

Immunofluorescence was performed as described in Claycomb *et al.*, 2009 with the following adaptations. Briefly, gonads from adult male and hermaphrodite worms at 18-22 hour post-L4 stage were dissected together into 1x sperm salts (50 mM PIPES, pH 7.0, 25mM KCl, 1 mM MgSO₄, 45 mM NaCl, 2 mM CaCl₂) on the same VWR Superfrost Plus slides, frozen and cracked on dry ice for 10 minutes and then fixed at -20°C for 5 minutes in 100% methanol, 5 minutes in 50% methanol/50% acetone, and 5 minutes in 100% acetone. Samples were blocked in 1xPBST + 3% BSA at room temperature for 15 minutes. Primary antibody dilutions were made in 1xPBST + 3% BSA and added to the slides. Slides were covered with a parafilm cover slip and incubated overnight at 4°C in a humid chamber. Slides were washed 3 x 10 minutes in 1xPBST and then blocked in 1xPBST + 3% BSA for 15 minutes at room temperature. Secondary antibody dilutions were made at 1:200 in 1x PBST + 3% BSA using Invitrogen goat Alexa Fluoro-labeled antibodies and were added to the slides. Secondary antibody incubation occurred for 1 hour at room temperature in a dark humid chamber. Slides were then washed 3x10 minutes in 1xPBS in the dark and then incubated with 2 µg/ mL DAPI for 5 minutes in a humid chamber in the dark. Slides were then washed with 1xPBS for 5 minutes in the dark and then mounted with Vectashield. Slides were sealed with nail polish immediately following mounting and then stored at 4°C prior to imaging. All slides were imaged within 2 weeks of preparation. The following primary antibody dilutions were used: WAGO-4 anti-guinea pig (1:500, this study), monoclonal mouse aPGL-1 K76 (1:20, Developmental Studies Hybridoma Bank), rabbit anti-PGL-1 (1:1000, Susan Strome Lab), mouse anti-FLAG (1:1000, Millipore Sigma F1804-50UG), ChromoTek GFP-booster Alexa Fluor® 488 (1:200, ChromoTek gb2AF488), rabbit anti-HA (1:500, Abcam ab9110).

Imaging

Immunofluorescence slides were imaged at 1024 x 1024 pixel dimensions on an Applied Precision DeltaVision microscope with a 63x lens and a 1.5x optivar. Images were acquired as Z stacks at 0.2 μ m intervals and deconvolved with Applied Precisions softWoRx deconvolution software.

Foci quantification and Overlap

Foci quantification adapted from Toraason *et al.*, 2021. Briefly, germ granule foci were defined as Surface objects in Imaris (Bitplane) with the following settings: Smooth (not enabled), Background 0.513, and Seed Point Diameter (not enabled). Foci were analyzed if larger than 0.034 μ m² and smaller than 10 μ m² in volume. Counts, volume, and sphericity of granule structures were then aligned along an X-Y axis utilizing a Gonad Linearization Algorithm as described in Toraason *et al.*, 2021 and normalized from the pre-meiotic tip to the end of pachytene. Differences in counts were quantified using two sample Kolmogorov-Smirnov tests. Volume and Sphericity were graphed using the plotnine.geom_smooth metric and the loess method. Counts were binned along meiotic progression utilizing well established nuclei morphology criteria to indicate meiotic stage: pre-meiotic tip, leptotene/zygotene, early pachytene, mid pachytene, late pachytene (Hillers *et al.*, 2018). Differences in distributions were calculated utilizing Mann-Whitney U test with a Bonferroni correction using SciPy stats.

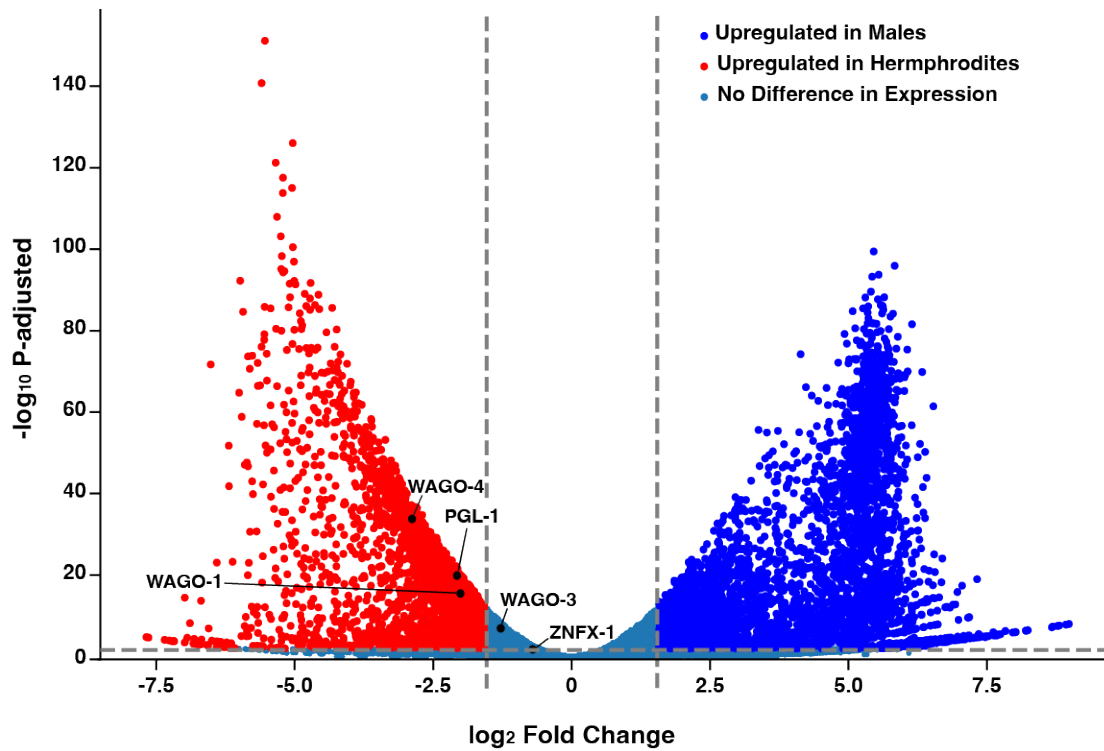
To determine if foci colocalized, we applied the Surface-to-Surface Percent Overlap function in Imaris to identify and replicate surfaces with greater than 5% volume overlap. These overlapping surfaces were then given unique colocalization identity intensity channels. A focus was considered co-localized with another in all analyses if two or more foci of different types (*i.e.* PGL-1 and WAGO-3) with the same unique colocalization intensity value could be identified in the exported data. Protein foci were considered to have no overlap if the primary protein foci had no volume overlapping with a secondary protein focus. 50% overlap corresponds with half of a single primary foci's volume being internal to a secondary protein's foci, while 100% occurs when the entirety of a single primary protein focus resides within a secondary protein focus (refer to Fig. 3Bi for a visual representation). The percent overlap of the primary foci, though, does not report the percent of the secondary foci volume associating with the primary. Co-

localized foci were then aligned on an X-Y axis utilizing the Gonad Linearization Algorithm and binned based on normalized progression through the germline. Differences in the percent co-localization of foci within each bin was calculated utilizing Chi Squared Test with a Bonferroni Adjustment.

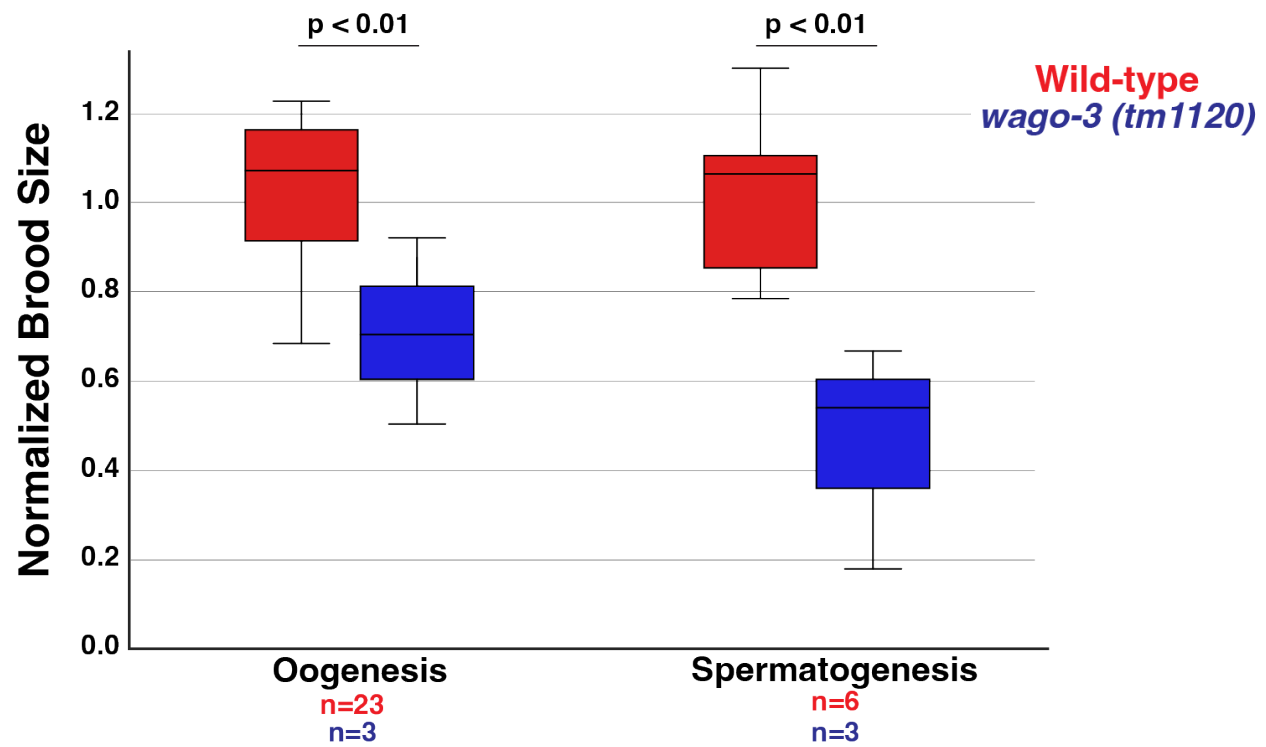
Male and hermaphrodite fertility assays

For hermaphrodite fertility assays, hermaphrodites of each genotype were isolated at the L4 developmental stage and aged 18-22 hours to adult stage. Individual adult hermaphrodites were then singled on small plates (35 x 10 mm) with a small OP50 dot and allowed to lay for 24 hours. Following 24 hours, the adult hermaphrodite was moved to a fresh small plate (35 x 10 mm) with a small OP50 dot and allowed to lay for an additional 24 hours. This was repeated for a total of 5 plates. Following 24 hours on the fifth plate, the adult hermaphrodite was flamed off. 1-2 days following the removal of the adult hermaphrodite, plates were counted for number of living progeny, number of dead eggs, and number of unfertilized eggs. Plates where the hermaphrodite had run away or died were excluded. Differences in the ability of *wago-3* and *wago-4* mutant hermaphrodites to produce viable progeny were analyzed using Two-way ANOVA with Tukey's multiple comparison test.

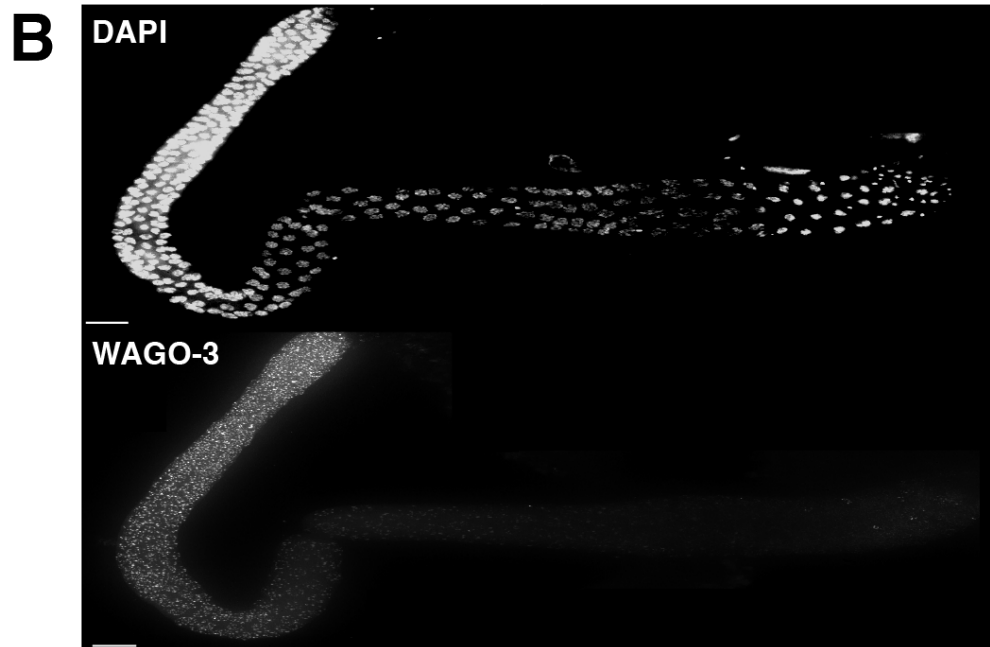
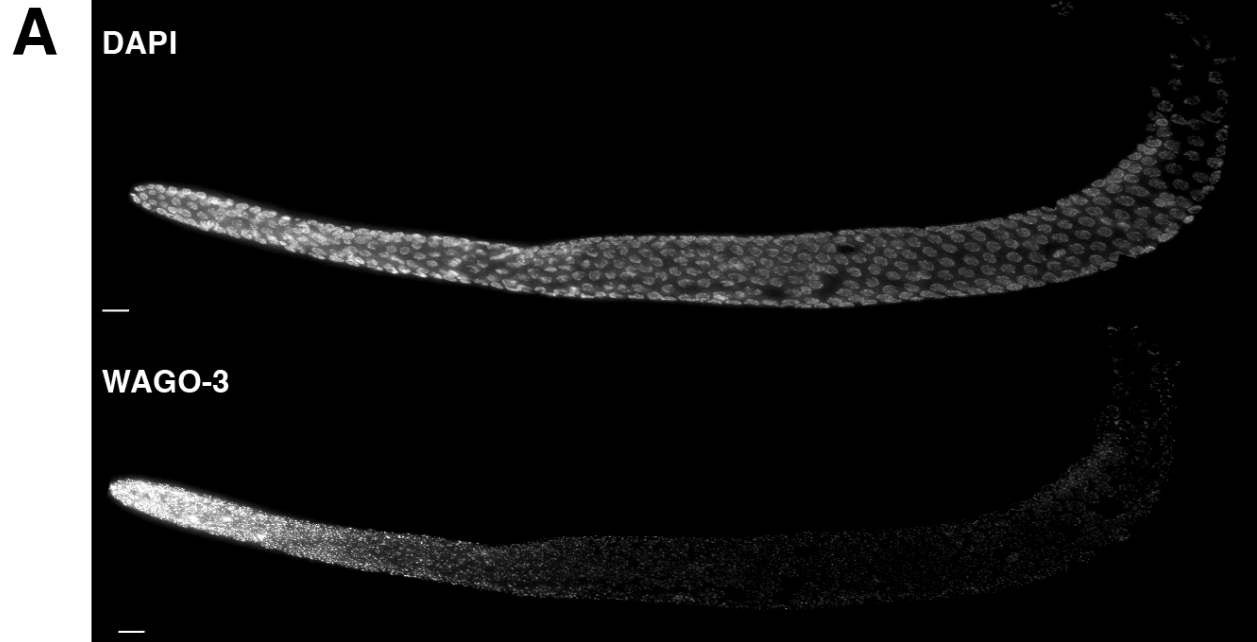
For male fertility assays, males of each genotype and obligate females (CB4108: *fog2Δ*) obligate females were isolated at the L4 developmental stage and aged 18-22 hours to adult stage. Individual obligate females were then singled and paired with individual males on small plates (35 x 10 mm) with a small OP50 dot ringed with garlic extract to keep males from leaving the plate for 24 hours. After 24 hours males were moved to a new plate with an additional 18-22 hours post-L4 stage obligate female and allowed to mate for another 24 hours. After removal of the male, obligate females were allowed to lay eggs for 24 additional hours and then scored for living progeny, dead eggs, and unfertilized eggs. Pairs with no unfertilized eggs or where either the male or obligate female died before egg counting were excluded. Differences in the ability of *wago-3* and *wago-4* mutant males to produce viable progeny were analyzed using Two-way ANOVA with Tukey's multiple comparison test.



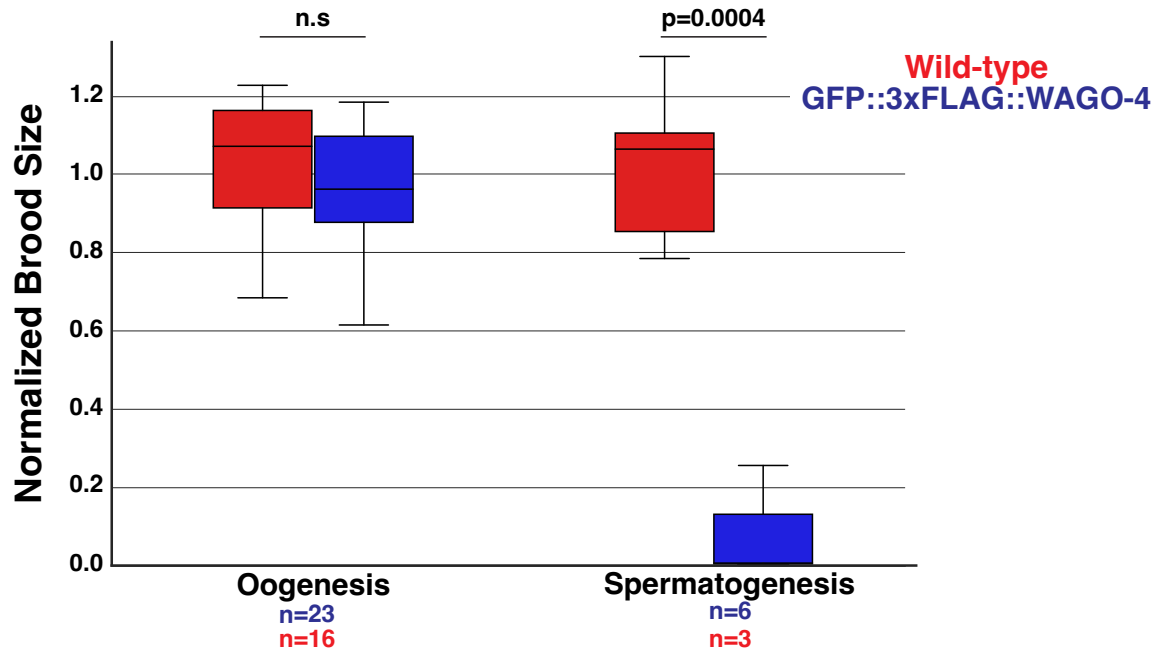
Supplemental Figure S3.1: A subset of germ granule components display sexually dimorphic gene expression. Volcano plot of mRNA-seq between wild-type adult males and hermaphrodites. Genes upregulated in hermaphrodites are in red. Genes upregulated in males are in blue. Blue-grey dots represent genes with no statistical difference in regulation between the sexes. Labels denote genes of interest further explored in this study.



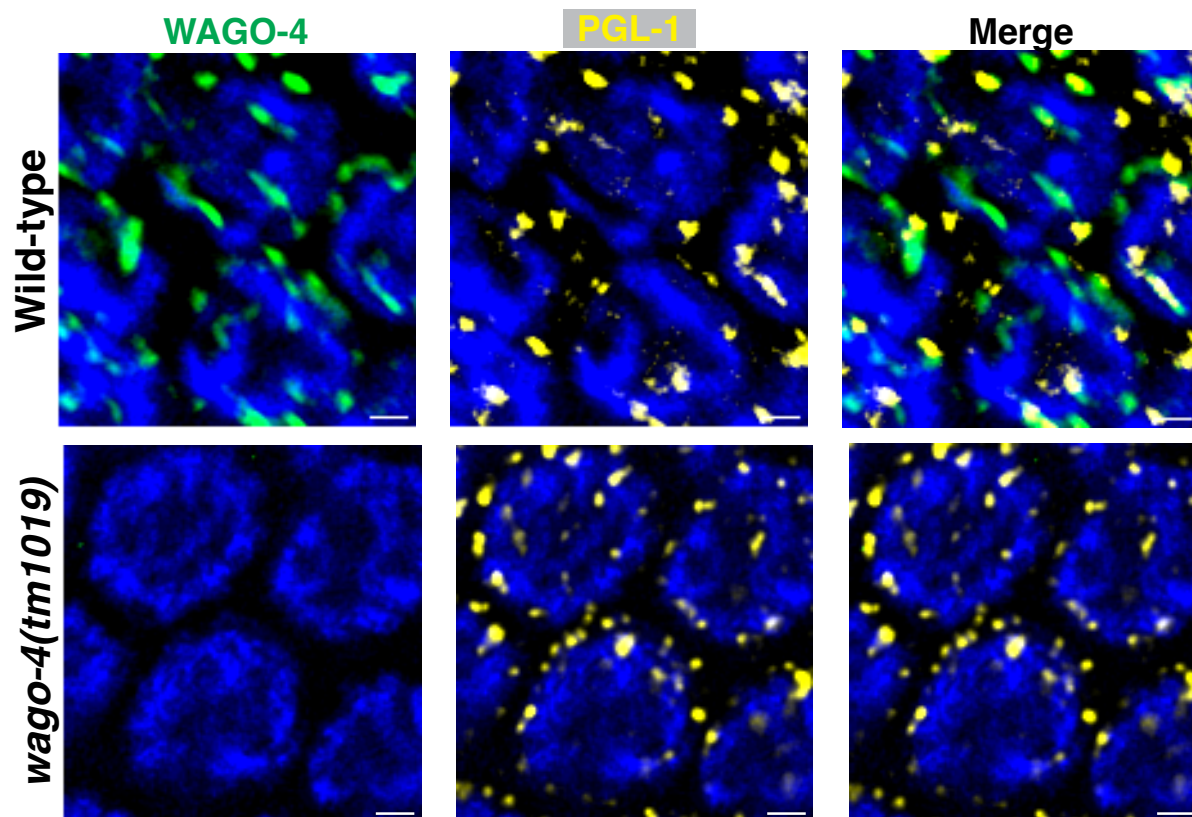
Supplemental Figure S3.2: Loss of *wago-3* causes significant decrease in hermaphrodite and male brood sizes. (A) Normalized brood size of adult hermaphrodites undergoing oogenesis (left) and adult males undergoing spermatogenesis (right) of wild-type and *wago-3(tm1120)* strains. Reported p-values calculated using one-way ANOVA with a Bonferroni correction.



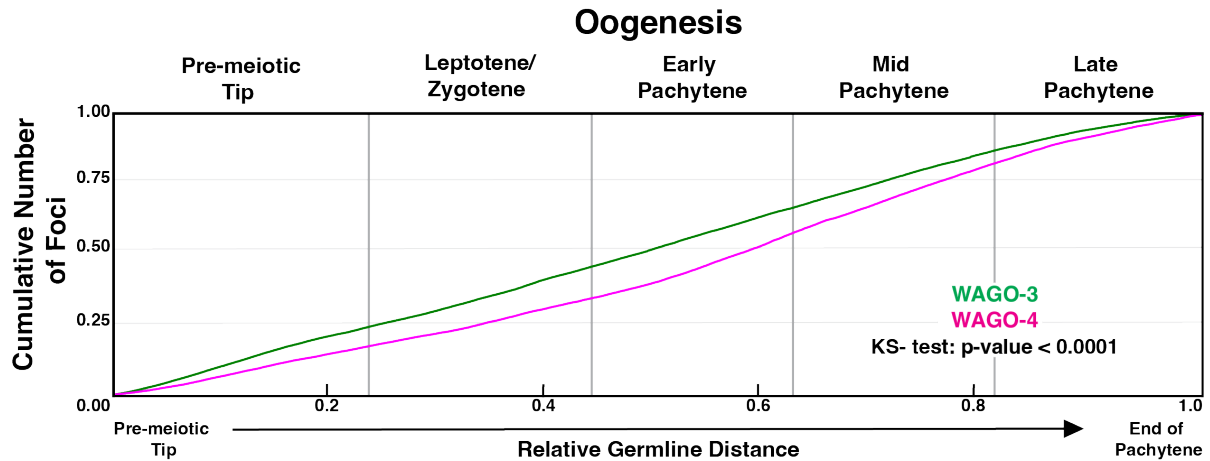
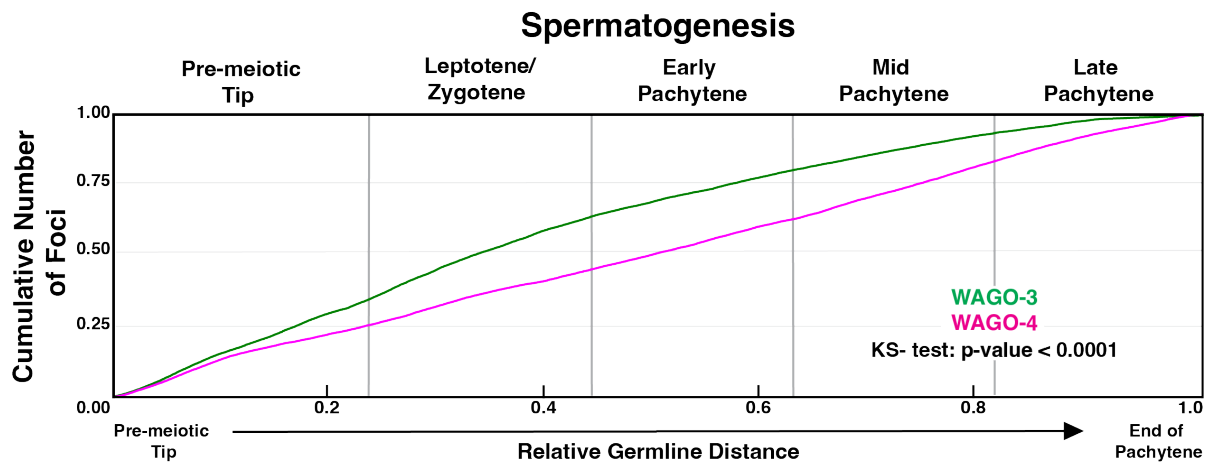
Supplemental Figure S3.3: WAGO-3 foci intensity decreases throughout meiotic progression. (A, B) Split channels of WAGO-3 intensity from germlines undergoing (A) oogenesis or (B) spermatogenesis. Images were taken as tiles at the same exposure settings for WAGO-3 stained for in the 488 channel across the entire gonad.



Supplemental Figure S3.4: N-terminus tagging of WAGO-4 causes male specific infertility.
(A) Normalized brood size of adulate hermaphrodites undergoing oogenesis (red) and adult males undergoing spermatogenesis (blue) of *C. elegans* with wild-type WAGO-4 (left) and 3xFLAG::GFP::WAGO-4 (right). Reported p-values calculated using one way ANOVA with a Bonferroni correction.



Supplemental Figure S3.5: Immunofluorescent confirmation of WAGO-4 anti-guinea pig antibody. Representative images of oocyte nuclei from wild-type (top) and *wago-4(tm1019)* during LP stained for WAGO-4 (green) and PGL-1 (yellow). Scale bars represent 1 μ m.

A**B**

Supplemental Figure S3.6: WAGO-3 and WAGO-4 foci accumulate at different rates through meiotic progression. (A) ECDF plot of the cumulative number of WAGO-3 (green) and WAGO-4 (magenta) foci as a function of meiotic progression through oogenesis. **(B)** ECDF plot of the cumulative number of WAGO-3 (green) and WAGO-4 (magenta) foci as a function of meiotic progression through spermatogenesis.

Table S3.1: Mean PGL-1 foci volume during spermatogenesis and oogenesis. Hermaphrodite germlines n=8, male germlines n=8.

Protein	Sex	Stage	Mean Volume (μm^3)	Standard Deviation
PGL-1	Hermaphrodite	PMT	0.142	0.158
PGL-1	Hermaphrodite	L/Z	0.142	0.148
PGL-1	Hermaphrodite	EP	0.144	0.16
PGL-1	Hermaphrodite	MP	0.181	0.254
PGL-1	Hermaphrodite	LP	0.211	0.39
PGL-1	Male	PMT	0.154	0.146
PGL-1	Male	L/Z	0.173	0.173
PGL-1	Male	EP	0.181	0.206
PGL-1	Male	MP	0.166	0.21
PGL-1	Male	LP	0.175	0.207

Table S3.2: Mann-Whitney *U* statistics and p-values with Bonferroni correction comparing PGL-1 foci volume distribution between hermaphrodites and males through meiosis.

Stage	Statistic	p-value
PMT	22785550	6.55E-28
L/Z	115822771	1.245E-29
EP	9536726	3.63E-18
MP	10600540.5	1.05
LP	9855760	0.79

Table S3.3: Mean PGL-1 foci sphericity during spermatogenesis and oogenesis. Hermaphrodite germlines n=8, male germlines n=8.

Protein	Sex	Stage	Mean Sphericity	Standard Deviation
PGL-1	Hermaphrodite	PMT	0.701	0.111
PGL-1	Hermaphrodite	L/Z	0.698	0.097
PGL-1	Hermaphrodite	EP	0.71	0.1
PGL-1	Hermaphrodite	MP	0.71	0.103
PGL-1	Hermaphrodite	LP	0.708	0.113
PGL-1	Male	PMT	0.687	0.106
PGL-1	Male	L/Z	0.676	0.095
PGL-1	Male	EP	0.69	0.094
PGL-1	Male	MP	0.68	0.096
PGL-1	Male	LP	0.678	0.098

Table S3.4: Mann-Whitney *U* statistics and p-values with Bonferroni correction comparing PGL-1 foci sphericity distribution between hermaphrodites and males throughout meiosis.

Stage	Statistic	p-value
PMT	23444550.5	1.28E-16
L/Z	11476655.5	3.12E-33
EP	9462092	1.4105E-22
MP	8783119	1.92E-48
LP	8375976	5.6E-45

Table S3.5: Mean ZNFX-1 foci volume during spermatogenesis and oogenesis. Hermaphrodite germlines n=8, male germlines n=8.

Protein	Sex	Stage	Mean Volume (μm^3)	Standard Deviation
ZNFX-1	Hermaphrodite	PMT	0.120	0.120
ZNFX-1	Hermaphrodite	L/Z	0.179	0.200
ZNFX-1	Hermaphrodite	EP	0.206	0.266
ZNFX-1	Hermaphrodite	MP	0.241	0.304
ZNFX-1	Hermaphrodite	LP	0.332	0.515
ZNFX-1	Male	PMT	0.187	0.204
ZNFX-1	Male	L/Z	0.227	0.217
ZNFX-1	Male	EP	0.276	0.298
ZNFX-1	Male	MP	0.410	0.723
ZNFX-1	Male	LP	0.440	0.658

Table S3.6: Mann-Whitney *U* statistics and p-values with Bonferroni correction comparing ZNFX-1 foci volume distribution between hermaphrodites and males throughout meiosis.

Stage	Statistic	p-value
PMT	16646775	2.3E-126
L/Z	11589080	4.9E-47
EP	10197046.5	3.1E-57
MP	10723086	2.8E-40
LP	6725988.5	7.6E-19

Table S3.7: Mean ZNFX-1 foci sphericity during spermatogenesis and oogenesis.
Hermaphrodite germlines n=8, male germlines n=8.

Protein	Sex	Stage	Mean Sphericity	Standard Deviation
ZNFX-1	Hermaphrodite	PMT	0.717	0.0925
ZNFX-1	Hermaphrodite	L/Z	0.699	0.102
ZNFX-1	Hermaphrodite	EP	0.712	0.101
ZNFX-1	Hermaphrodite	MP	0.716	0.096
ZNFX-1	Hermaphrodite	LP	0.695	0.101
ZNFX-1	Male	PMT	0.671	0.099
ZNFX-1	Male	L/Z	0.671	0.091
ZNFX-1	Male	EP	0.678	0.088
ZNFX-1	Male	MP	0.665	0.101
ZNFX-1	Male	LP	0.668	0.103

Table S3.8: Mann-Whitney *U* statistics and p-values with Bonferroni correction comparing ZNFX-1 foci sphericity distribution between hermaphrodites and males throughout meiosis.

Stage	Statistic	p-value
PMT	16183328	3.0E-149
L/Z	11534441.5	3.1E-49
EP	9960441.5	6.9E-69
MP	9002085.5	3.0E-135
LP	6350824	9.7E-37

Table S3.9: Binning of percent ZNFX-1 foci volume overlap with PGL-1.

Process	Percent Volume Overlap	Zone	Foci Counts	Percent
Oogenesis	0%	L/Z	2710	0.438
Oogenesis	1-25%	L/Z	2051	0.332
Oogenesis	26-50%	L/Z	848	0.137
Oogenesis	51-75%	L/Z	428	0.069
Oogenesis	76-99%	L/Z	148	0.024
Oogenesis	100%	L/Z	2	0.000
Oogenesis	Total	L/Z	6187	1.000
Spermatogenesis	0%	L/Z	1170	0.256
Spermatogenesis	1-25%	L/Z	1291	0.282
Spermatogenesis	26-50%	L/Z	1010	0.221
Spermatogenesis	51-75%	L/Z	749	0.164
Spermatogenesis	76-99%	L/Z	332	0.073
Spermatogenesis	100%	L/Z	18	0.004
Spermatogenesis	Total	L/Z	4570	1.000
Oogenesis	0%	EP	1992	0.361
Oogenesis	1-25%	EP	2120	0.384
Oogenesis	26-50%	EP	904	0.164
Oogenesis	51-75%	EP	369	0.067
Oogenesis	76-99%	EP	126	0.023
Oogenesis	100%	EP	4	0.001
Oogenesis	Total	EP	5515	1.000
Spermatogenesis	0%	EP	742	0.341
Spermatogenesis	1-25%	EP	628	0.288
Spermatogenesis	26-50%	EP	414	0.190
Spermatogenesis	51-75%	EP	294	0.135
Spermatogenesis	76-99%	EP	90	0.041
Spermatogenesis	100%	EP	9	0.004
Spermatogenesis	Total	EP	2177	1.000
Oogenesis	0%	LP	1619	0.294
Oogenesis	1-25%	LP	2220	0.403
Oogenesis	26-50%	LP	939	0.170
Oogenesis	51-75%	LP	498	0.090
Oogenesis	76-99%	LP	225	0.041
Oogenesis	100%	LP	11	0.002
Oogenesis	Total	LP	5512	1.000
Spermatogenesis	0%	LP	782	0.312

Table S3.9 (continued).

Process	Percent Volume Overlap	Zone	Foci Counts	Percent
Spermatogenesis	1-25%	LP	812	0.324
Spermatogenesis	26-50%	LP	306	0.122
Spermatogenesis	51-75%	LP	305	0.122
Spermatogenesis	76-99%	LP	293	0.117
Spermatogenesis	100%	LP	9	0.004
Spermatogenesis	Total	LP	2507	1.000

Table S3.10: Chi-square test with Bonferroni correction comparing percent of ZNFX-1 foci volume overlap with PGL-1 between oogenesis and spermatogenesis.

Stage	Percent Volume Overlap	Chi-square Statistic	p-value
L/Z	0%	376.77	6.27E-84
L/Z	1-25%	29.25	6.37E-08
L/Z	26-50%	129.04	6.65E-30
L/Z	51-75%	241.02	2.35E-54
L/Z	76-99%	145.25	1.89E-33
L/Z	100%	16.62	0.0000457
EP	0%	2.74	0.098
EP	1-25%	62.14	3.19E-15
EP	26-50%	7.39	0.0065
EP	51-75%	91.15	1.33E-21
EP	76-99%	18.9	0.0000138
EP	100%	8.82	0.003
LP	0%	2.64	0.104
LP	1-25%	45.25	1.74E-11
LP	26-50%	30.28	3.74E-08
LP	51-75%	18.4	0.0000179
LP	76-99%	163.7	1.76E-37
LP	100%	1.18	0.278

Table S3.11: Binning of percent PGL-1 foci volume overlap with ZNFX-1.

Process	Percent Volume Overlap	Zone	Foci Counts	Percent
Oogenesis	0%	L/Z	1913	0.389
Oogenesis	1-25%	L/Z	1454	0.296
Oogenesis	26-50%	L/Z	775	0.158
Oogenesis	51-75%	L/Z	480	0.098
Oogenesis	76-99%	L/Z	280	0.057
Oogenesis	100%	L/Z	18	0.004
Oogenesis	Total	L/Z	4920	1.000
Spermatogenesis	0%	L/Z	2652	0.431
Spermatogenesis	1-25%	L/Z	1306	0.212
Spermatogenesis	26-50%	L/Z	1064	0.173
Spermatogenesis	51-75%	L/Z	691	0.112
Spermatogenesis	76-99%	L/Z	410	0.067
Spermatogenesis	100%	L/Z	26	0.004
Spermatogenesis	Total	L/Z	6149	1.000
Oogenesis	0%	EP	1360	0.313
Oogenesis	1-25%	EP	1122	0.258
Oogenesis	26-50%	EP	769	0.177
Oogenesis	51-75%	EP	579	0.133
Oogenesis	76-99%	EP	470	0.108
Oogenesis	100%	EP	41	0.009
Oogenesis	Total	EP	4341	1.000
Spermatogenesis	0%	EP	1044	0.421
Spermatogenesis	1-25%	EP	519	0.209
Spermatogenesis	26-50%	EP	361	0.146
Spermatogenesis	51-75%	EP	277	0.112
Spermatogenesis	76-99%	EP	262	0.106
Spermatogenesis	100%	EP	17	0.007
Spermatogenesis	Total	EP	2480	1.000
Oogenesis	0%	LP	1829	0.333
Oogenesis	1-25%	LP	1379	0.251
Oogenesis	26-50%	LP	884	0.161
Oogenesis	51-75%	LP	689	0.126
Oogenesis	76-99%	LP	656	0.120
Oogenesis	100%	LP	48	0.009
Oogenesis	Total	LP	5485	1.000
Spermatogenesis	0%	LP	1792	0.504

Table S3.11 (continued).

Process	Percent Volume Overlap	Zone	Foci Counts	Percent
Spermatogenesis	1-25%	LP	602	0.169
Spermatogenesis	26-50%	LP	447	0.126
Spermatogenesis	51-75%	LP	417	0.117
Spermatogenesis	76-99%	LP	272	0.077
Spermatogenesis	100%	LP	23	0.006
Spermatogenesis	Total	LP	3553	1.000

Table S3.12: Chi-square test with Bonferroni correction comparing percent of PGL-1 foci volume overlap with ZNFX-1 between oogenesis and spermatogenesis.

Stage	Percent Volume Overlap	Chi-square Statistic	p-value
L/Z	0%	376.77	0.0000071
L/Z	1-25%	29.25	1.19E-23
L/Z	26-50%	129.04	0.0312
L/Z	51-75%	241.02	0.0129
L/Z	76-99%	145.25	0.038
L/Z	100%	16.62	0.748
EP	0%	2.74	4.34E-19
EP	1-25%	62.14	0.00000555
EP	26-50%	7.39	0.00083
EP	51-75%	91.15	0.0103
EP	76-99%	18.9	0.767
EP	100%	8.82	0.325
LP	0%	2.64	7.77E-59
LP	1-25%	45.25	4.49E-20
LP	26-50%	30.28	0.00000417
LP	51-75%	18.4	0.256
LP	76-99%	163.7	5.78E-11
LP	100%	1.18	0.282

Table S3.13: Mean WAGO-3 foci volume during spermatogenesis and oogenesis.
Hermaphrodite germlines n=9, male germlines n=6.

Protein	Sex	Stage	Mean Volume (μm^3)	Standard Deviation
WAGO-3	Hermaphrodite	PMT	0.159	0.205
WAGO-3	Hermaphrodite	L/Z	0.181	0.202
WAGO-3	Hermaphrodite	EP	0.166	0.19
WAGO-3	Hermaphrodite	MP	0.188	0.252
WAGO-3	Hermaphrodite	LP	0.234	0.327
WAGO-3	Male	PMT	0.179	0.211
WAGO-3	Male	L/Z	0.193	0.219
WAGO-3	Male	EP	0.221	0.251
WAGO-3	Male	MP	0.241	0.281
WAGO-3	Male	LP	0.248	0.395

Table S3.14: Mann-Whitney *U* statistics and p-values with Bonferroni correction comparing WAGO-3 foci volume distribution between hermaphrodites and males throughout meiosis.

Stage	Statistic	p-value
PMT	24053603.5	5.815E-23
L/Z	15852716.5	0.001
EP	7762136	1.535E-26
MP	6623606.5	2.0825E-14
LP	1970574.5	0.01

Table S3.15: Mean WAGO-3 foci sphericity during spermatogenesis and oogenesis. Hermaphrodite germlines n=9, male germlines n=6.

Protein	Sex	Stage	Mean Sphericity	Standard Deviation
WAGO-3	Hermaphrodite	PMT	0.737	0.11
WAGO-3	Hermaphrodite	L/Z	0.714	0.109
WAGO-3	Hermaphrodite	EP	0.707	0.101
WAGO-3	Hermaphrodite	MP	0.7	0.104
WAGO-3	Hermaphrodite	LP	0.676	0.109
WAGO-3	Male	PMT	0.741	0.121
WAGO-3	Male	L/Z	0.717	0.132
WAGO-3	Male	EP	0.698	0.127
WAGO-3	Male	MP	0.707	0.128
WAGO-3	Male	LP	0.707	0.128

Table S3.16: Mann-Whitney *U* statistics and p-values with Bonferroni correction comparing WAGO-3 foci sphericity distribution between hermaphrodites and males throughout meiosis.

Stage	Statistic	p-value
PMT	25578682	0.00014005
L/Z	1504729	2.6535E-15
EP	8414718.5	2.1385E-07
MP	7419252.5	1.00
LP	1776812	6.41E-13

Table S3.17: Binning of WAGO-3 overlap with PGL-1 or ZNFX-1. Hermaphrodite germlines with PGL-1 n=4, male germlines with PGL-1 n=1. Hermaphrodite germlines with ZNFX-1 n=7, male germlines with ZNFX-1 n=5.

Process	Percent Volume Overlap	Protein	Zone	Counts	Percent
Oogenesis	0%	PGL-1	L/Z	562	0.334
Oogenesis	1-25%	PGL-1	L/Z	454	0.269
Oogenesis	26-50%	PGL-1	L/Z	375	0.223
Oogenesis	51-75%	PGL-1	L/Z	233	0.138
Oogenesis	76-99%	PGL-1	L/Z	60	0.036
Oogenesis	100%	PGL-1	L/Z	1	0.001
Oogenesis	0%	PGL-1	L/Z	1685	1.000
Spermatogenesis	1-25%	PGL-1	L/Z	31	0.057
Spermatogenesis	26-50%	PGL-1	L/Z	92	0.169
Spermatogenesis	51-75%	PGL-1	L/Z	167	0.306
Spermatogenesis	76-99%	PGL-1	L/Z	177	0.325
Spermatogenesis	100%	PGL-1	L/Z	78	0.143
Spermatogenesis	0%	PGL-1	L/Z	0	0.000
Spermatogenesis	1-25%	PGL-1	L/Z	545	1.000
Oogenesis	26-50%	PGL-1	EP	517	0.344
Oogenesis	51-75%	PGL-1	EP	475	0.316
Oogenesis	76-99%	PGL-1	EP	311	0.207
Oogenesis	100%	PGL-1	EP	148	0.098
Oogenesis	0%	PGL-1	EP	51	0.034
Oogenesis	1-25%	PGL-1	EP	2	0.001
Oogenesis	26-50%	PGL-1	EP	1504	1.000
Spermatogenesis	51-75%	PGL-1	EP	9	0.044
Spermatogenesis	76-99%	PGL-1	EP	38	0.187
Spermatogenesis	100%	PGL-1	EP	60	0.296
Spermatogenesis	0%	PGL-1	EP	68	0.335
Spermatogenesis	1-25%	PGL-1	EP	28	0.138
Spermatogenesis	26-50%	PGL-1	EP	0	0.000
Spermatogenesis	51-75%	PGL-1	EP	203	1.000
Oogenesis	76-99%	PGL-1	LP	417	0.272
Oogenesis	100%	PGL-1	LP	585	0.382
Oogenesis	0%	PGL-1	LP	328	0.214
Oogenesis	1-25%	PGL-1	LP	154	0.100
Oogenesis	26-50%	PGL-1	LP	49	0.032
Oogenesis	51-75%	PGL-1	LP	0	0.000
Oogenesis	76-99%	PGL-1	LP	1533	1.000

Table S3.17 (continued).

Process	Percent Volume Overlap	Protein	Zone	Counts	Percent
Spermatogenesis	100%	PGL-1	LP	1	0.029
Spermatogenesis	0%	PGL-1	LP	10	0.294
Spermatogenesis	1-25%	PGL-1	LP	7	0.206
Spermatogenesis	26-50%	PGL-1	LP	11	0.324
Spermatogenesis	51-75%	PGL-1	LP	5	0.147
Spermatogenesis	76-99%	PGL-1	LP	0	0.000
Spermatogenesis	100%	PGL-1	LP	34	1.000
Oogenesis	0%	ZNFX-1	L/Z	1755	0.314
Oogenesis	1-25%	ZNFX-1	L/Z	2299	0.412
Oogenesis	26-50%	ZNFX-1	L/Z	1069	0.191
Oogenesis	51-75%	ZNFX-1	L/Z	379	0.068
Oogenesis	76-99%	ZNFX-1	L/Z	83	0.015
Oogenesis	100%	ZNFX-1	L/Z	0	0.000
Oogenesis	0%	ZNFX-1	L/Z	5585	1.000
Spermatogenesis	1-25%	ZNFX-1	L/Z	1391	0.390
Spermatogenesis	26-50%	ZNFX-1	L/Z	1285	0.360
Spermatogenesis	51-75%	ZNFX-1	L/Z	540	0.151
Spermatogenesis	76-99%	ZNFX-1	L/Z	258	0.072
Spermatogenesis	100%	ZNFX-1	L/Z	90	0.025
Spermatogenesis	0%	ZNFX-1	L/Z	1	0.000
Spermatogenesis	1-25%	ZNFX-1	L/Z	3565	1.000
Oogenesis	26-50%	ZNFX-1	EP	1659	0.329
Oogenesis	51-75%	ZNFX-1	EP	1954	0.388
Oogenesis	76-99%	ZNFX-1	EP	999	0.198
Oogenesis	100%	ZNFX-1	EP	367	0.073
Oogenesis	0%	ZNFX-1	EP	60	0.012
Oogenesis	1-25%	ZNFX-1	EP	0	0.000
Oogenesis	26-50%	ZNFX-1	EP	5039	1.000
Spermatogenesis	51-75%	ZNFX-1	EP	693	0.367
Spermatogenesis	76-99%	ZNFX-1	EP	598	0.317
Spermatogenesis	100%	ZNFX-1	EP	300	0.159
Spermatogenesis	0%	ZNFX-1	EP	247	0.131
Spermatogenesis	1-25%	ZNFX-1	EP	50	0.026
Spermatogenesis	26-50%	ZNFX-1	EP	0	0.000
Spermatogenesis	51-75%	ZNFX-1	EP	1888	1.000
Oogenesis	76-99%	ZNFX-1	LP	1287	0.261

Table S3.17 (continued).

Process	Percent Volume Overlap	Protein	Zone	Counts	Percent
Oogenesis	100%	ZNFX-1	LP	2227	0.452
Oogenesis	0%	ZNFX-1	LP	1035	0.210
Oogenesis	1-25%	ZNFX-1	LP	338	0.069
Oogenesis	26-50%	ZNFX-1	LP	44	0.009
Oogenesis	51-75%	ZNFX-1	LP	0	0.000
Oogenesis	76-99%	ZNFX-1	LP	4931	1.000
Spermatogenesis	100%	ZNFX-1	LP	381	0.351
Spermatogenesis	0%	ZNFX-1	LP	401	0.369
Spermatogenesis	1-25%	ZNFX-1	LP	135	0.124
Spermatogenesis	26-50%	ZNFX-1	LP	114	0.105
Spermatogenesis	51-75%	ZNFX-1	LP	55	0.051
Spermatogenesis	76-99%	ZNFX-1	LP	0	0.000
Spermatogenesis	100%	ZNFX-1	LP	1086	1.000

Table S3.18. Chi-square test with Bonferroni correction comparing percent of WAGO-3 foci volume overlap with PGL-1 or ZNFX-1 between oogenesis and spermatogenesis. N/A coincides if the reads for both oogenesis and spermatogenesis for that bin was 0. Hermaphrodite germlines with PGL-1 n=4, male germlines with PGL-1 n=1. Hermaphrodite germlines with ZNFX-1 n=7, male germlines with ZNFX-1 n=5

Comparison	Percent Volume Overlap	Protein	Zone	Chi-squared statistic	p-value
Oogenesis vs. Spermatogenesis	0%	PGL-1	L/Z	160.044	1.107E-36
Oogenesis vs. Spermatogenesis	1-25%	PGL-1	L/Z	22.012	2.709E-06
Oogenesis vs. Spermatogenesis	26-50%	PGL-1	L/Z	15.292	0.0000921
Oogenesis vs. Spermatogenesis	51-75%	PGL-1	L/Z	94.209	2.839E-22
Oogenesis vs. Spermatogenesis	76-99%	PGL-1	L/Z	80.15	3.471E-19
Oogenesis vs. Spermatogenesis	100%	PGL-1	L/Z	0.354	0.552
Oogenesis vs. Spermatogenesis	0%	PGL-1	EP	73.814	8.582E-18
Oogenesis vs. Spermatogenesis	1-25%	PGL-1	EP	13.473	0.000242
Oogenesis vs. Spermatogenesis	26-50%	PGL-1	EP	7.775	0.0053

Table S3.18 (continued).

Comparison	Percent Volume Overlap	Protein	Zone	Chi-squared statistic	p-value
Oogenesis vs. Spermatogenesis	51-75%	PGL-1	EP	88.439	5.242E-21
Oogenesis vs. Spermatogenesis	76-99%	PGL-1	EP	41.522	1.165E-10
Oogenesis vs. Spermatogenesis	100%	PGL-1	EP	0.328	0.567
Oogenesis vs. Spermatogenesis	0%	PGL-1	LP	8.807	0.003
Oogenesis vs. Spermatogenesis	1-25%	PGL-1	LP	0.741	0.389
Oogenesis vs. Spermatogenesis	26-50%	PGL-1	LP	0.00957	0.9221
Oogenesis vs. Spermatogenesis	51-75%	PGL-1	LP	15.28	0.0000926
Oogenesis vs. Spermatogenesis	76-99%	PGL-1	LP	10.009	0.00156
Oogenesis vs. Spermatogenesis	100%	PGL-1	LP	N/A	N/A
Oogenesis vs. Spermatogenesis	0%	ZNFX-1	L/Z	55.297	1.036E-13
Oogenesis vs. Spermatogenesis	1-25%	ZNFX-1	L/Z	23.716	1.116E-06
Oogenesis vs. Spermatogenesis	26-50%	ZNFX-1	L/Z	23.668	1.144E-06
Oogenesis vs. Spermatogenesis	51-75%	ZNFX-1	L/Z	0.615	0.433
Oogenesis vs. Spermatogenesis	76-99%	ZNFX-1	L/Z	12.096	0.00505
Oogenesis vs. Spermatogenesis	100%	ZNFX-1	L/Z	0.0512	0.821
Oogenesis vs. Spermatogenesis	0%	ZNFX-1	EP	8.593	0.00337
Oogenesis vs. Spermatogenesis	1-25%	ZNFX-1	EP	29.482	5.645E-08
Oogenesis vs. Spermatogenesis	26-50%	ZNFX-1	EP	13.704	0.000214
Oogenesis vs. Spermatogenesis	51-75%	ZNFX-1	EP	56.466	5.717E-14
Oogenesis vs. Spermatogenesis	76-99%	ZNFX-1	EP	17.75	0.0000252
Oogenesis vs. Spermatogenesis	100%	ZNFX-1	EP	N/A	N/A

Table S3.18 (continued).

Comparison	Percent Volume Overlap	Protein	Zone	Chi-squared statistic	p-value
Oogenesis vs. Spermatogenesis	0%	ZNFX-1	LP	35.393	2.694E-09
Oogenesis vs. Spermatogenesis	1-25%	ZNFX-1	LP	24.233	8.58E-07
Oogenesis vs. Spermatogenesis	26-50%	ZNFX-1	LP	1687.269	1E-100
Oogenesis vs. Spermatogenesis	51-75%	ZNFX-1	LP	16.477	0.0000493
Oogenesis vs. Spermatogenesis	76-99%	ZNFX-1	LP	93.17	4.798E-22
Oogenesis vs. Spermatogenesis	100%	ZNFX-1	LP	N/A	N/A

Table S3.19: WAGO-3 foci overlap with ZNFX-1, PGL-1, or both germ granule components

Stage	Sex	ZNFX-1	PGL-1	Both	Total	% ZNFX-1	% PGL-1	% Both
L/Z	Hermaphrodite	428	124	999	1551	27.6	8.0	64.4
L/Z	Male	7	157	357	521	1.3	30.1	68.5
EP	Hermaphrodite	390	107	880	1377	28.3	7.8	63.9
EP	Male	8	48	146	202	4.0	23.8	72.3
LP	Hermaphrodite	326	121	995	1442	22.6	8.4	69.0
LP	Male	1	1	32	34	2.9	2.9	94.1

Table S3.20: Chi-square test with Bonferroni correction comparing percent of WAGO-3 foci that overlap with PGL-1, ZNFX-1, or both

Comparison	Protein	Zone	Chi-squared statistic	p-value
Oogenesis vs. Spermatogenesis	ZNFX-1	L/Z	160.46	2.697E-36
Oogenesis vs. Spermatogenesis	PGL-1	L/Z	161.19	1.869E-36
Oogenesis vs. Spermatogenesis	Both	L/Z	2.74	0.294
Oogenesis vs. Spermatogenesis	ZNFX-1	EP	54.17	5.52E-13
Oogenesis vs. Spermatogenesis	PGL-1	EP	49.1	7.29E-12
Oogenesis vs. Spermatogenesis	Both	EP	5.06	0.0732
Oogenesis vs. Spermatogenesis	ZNFX-1	LP	29.08	2.079E-7
Oogenesis vs. Spermatogenesis	PGL-1	LP	2.49	0.342
Oogenesis vs. Spermatogenesis	Both	LP	4.95	0.078

Table S3.21: Mean WAGO-4 foci volume during spermatogenesis and oogenesis.
Hermaphrodite germlines n=7, male germlines n=7.

Protein	Sex	Stage	Mean Volume (μm^3)	Standard Deviation
WAGO-4	Hermaphrodite	PMT	0.151	0.156
WAGO-4	Hermaphrodite	L/Z	0.192	0.189
WAGO-4	Hermaphrodite	EP	0.279	0.392
WAGO-4	Hermaphrodite	MP	0.442	0.781
WAGO-4	Hermaphrodite	LP	0.377	0.493
WAGO-4	Male	PMT	0.275	0.348
WAGO-4	Male	L/Z	0.301	0.375
WAGO-4	Male	EP	0.404	0.54
WAGO-4	Male	MP	0.388	0.525
WAGO-4	Male	LP	0.485	0.613

Table S3.22: Mann-Whitney *U* statistics and p-values with Bonferroni correction comparing WAGO-4 foci volume distribution between hermaphrodites and males throughout meiosis.

Stage	Statistic	p-value
PMT	7495245.5	2.885E-89
L/Z	55466.43	8.28E-26
EP	7362308.5	1.4295E-07
MP	9717179	0.00002943
LP	4926048	1.5398E-07

Table S3.23: Mean WAGO-4 foci sphericity during spermatogenesis and oogenesis.

Protein	Sex	Stage	Mean Sphericity	Standard Deviation
WAGO-4	Hermaphrodite	PMT	0.680	0.11
WAGO-4	Hermaphrodite	L/Z	0.639	0.109
WAGO-4	Hermaphrodite	EP	0.627	0.119
WAGO-4	Hermaphrodite	MP	0.623	0.133
WAGO-4	Hermaphrodite	LP	0.650	0.121
WAGO-4	Male	PMT	0.639	0.112
WAGO-4	Male	L/Z	0.633	0.115
WAGO-4	Male	EP	0.631	0.116
WAGO-4	Male	MP	0.641	0.115
WAGO-4	Male	LP	0.634	0.116

Table S3.24. Mann-Whitney *U* statistics and p-values with Bonferroni correction comparing WAGO-4 foci sphericity distribution between hermaphrodites and males throughout meiosis.

Stage	Statistic	p-value
PMT	7873541.50	3.037E-64
L/Z	6357228.5	0.3075
EP	7668011.5	0.023
MP	9336989.5	6.385E-13
LP	4992098	0.000012055

Table S3.25: Binning of WAGO-4 overlap with PGL-1 or ZNFX-1. Hermaphrodite germlines with PGL-1 n=4, male germlines with PGL-1 n=4. Hermaphrodite germlines with ZNFX-1 n=5, male germlines with ZNFX-1 n=6.

Process	Percent Volume Overlap	Protein	Zone	Counts	Percent
Oogenesis	0%	PGL-1	L/Z	1364	0.390
Oogenesis	1-25%	PGL-1	L/Z	420	0.120
Oogenesis	26-50%	PGL-1	L/Z	307	0.088
Oogenesis	51-75%	PGL-1	L/Z	521	0.149
Oogenesis	76-99%	PGL-1	L/Z	792	0.226
Oogenesis	100%	PGL-1	L/Z	96	0.027
Oogenesis	0%	PGL-1	L/Z	3500	1.000
Spermatogenesis	1-25%	PGL-1	L/Z	336	0.198
Spermatogenesis	26-50%	PGL-1	L/Z	167	0.098
Spermatogenesis	51-75%	PGL-1	L/Z	263	0.155
Spermatogenesis	76-99%	PGL-1	L/Z	343	0.202
Spermatogenesis	100%	PGL-1	L/Z	523	0.308
Spermatogenesis	0%	PGL-1	L/Z	66	0.039
Spermatogenesis	1-25%	PGL-1	L/Z	1698	1.000
Oogenesis	26-50%	PGL-1	EP	1257	0.444
Oogenesis	51-75%	PGL-1	EP	400	0.141
Oogenesis	76-99%	PGL-1	EP	302	0.107
Oogenesis	100%	PGL-1	EP	467	0.165
Oogenesis	0%	PGL-1	EP	386	0.136
Oogenesis	1-25%	PGL-1	EP	18	0.006
Oogenesis	26-50%	PGL-1	EP	2830	1.000
Spermatogenesis	51-75%	PGL-1	EP	95	0.133
Spermatogenesis	76-99%	PGL-1	EP	90	0.126
Spermatogenesis	100%	PGL-1	EP	165	0.231
Spermatogenesis	0%	PGL-1	EP	199	0.279
Spermatogenesis	1-25%	PGL-1	EP	152	0.213
Spermatogenesis	26-50%	PGL-1	EP	12	0.017
Spermatogenesis	51-75%	PGL-1	EP	713	1.000
Oogenesis	76-99%	PGL-1	LP	1281	0.548
Oogenesis	100%	PGL-1	LP	535	0.229
Oogenesis	0%	PGL-1	LP	329	0.141
Oogenesis	1-25%	PGL-1	LP	132	0.056
Oogenesis	26-50%	PGL-1	LP	61	0.026
Oogenesis	51-75%	PGL-1	LP	1	0.000
Oogenesis	76-99%	PGL-1	LP	2339	1.000

Table S3.25 (continued).

Process	Percent Volume Overlap	Protein	Zone	Counts	Percent
Spermatogenesis	100%	PGL-1	LP	94	0.132
Spermatogenesis	0%	PGL-1	LP	105	0.147
Spermatogenesis	1-25%	PGL-1	LP	210	0.295
Spermatogenesis	26-50%	PGL-1	LP	195	0.274
Spermatogenesis	51-75%	PGL-1	LP	92	0.129
Spermatogenesis	76-99%	PGL-1	LP	16	0.022
Spermatogenesis	100%	PGL-1	LP	712	1.000
Oogenesis	0%	ZNFX-1	L/Z	1405	0.351
Oogenesis	1-25%	ZNFX-1	L/Z	408	0.102
Oogenesis	26-50%	ZNFX-1	L/Z	679	0.170
Oogenesis	51-75%	ZNFX-1	L/Z	867	0.217
Oogenesis	76-99%	ZNFX-1	L/Z	622	0.156
Oogenesis	100%	ZNFX-1	L/Z	17	0.004
Oogenesis	0%	ZNFX-1	L/Z	3998	1.000
Spermatogenesis	1-25%	ZNFX-1	L/Z	813	0.238
Spermatogenesis	26-50%	ZNFX-1	L/Z	348	0.102
Spermatogenesis	51-75%	ZNFX-1	L/Z	715	0.209
Spermatogenesis	76-99%	ZNFX-1	L/Z	1072	0.314
Spermatogenesis	100%	ZNFX-1	L/Z	441	0.129
Spermatogenesis	0%	ZNFX-1	L/Z	26	0.008
Spermatogenesis	1-25%	ZNFX-1	L/Z	3415	1.000
Oogenesis	26-50%	ZNFX-1	EP	1181	0.417
Oogenesis	51-75%	ZNFX-1	EP	327	0.116
Oogenesis	76-99%	ZNFX-1	EP	356	0.126
Oogenesis	100%	ZNFX-1	EP	491	0.173
Oogenesis	0%	ZNFX-1	EP	464	0.164
Oogenesis	1-25%	ZNFX-1	EP	11	0.004
Oogenesis	26-50%	ZNFX-1	EP	2830	1.000
Spermatogenesis	51-75%	ZNFX-1	EP	496	0.326
Spermatogenesis	76-99%	ZNFX-1	EP	159	0.105
Spermatogenesis	100%	ZNFX-1	EP	334	0.220
Spermatogenesis	0%	ZNFX-1	EP	374	0.246
Spermatogenesis	1-25%	ZNFX-1	EP	148	0.097
Spermatogenesis	26-50%	ZNFX-1	EP	10	0.007
Spermatogenesis	51-75%	ZNFX-1	EP	1521	1.000
Oogenesis	76-99%	ZNFX-1	LP	1083	0.392

Table S3.25 (continued).

Process	Percent Volume Overlap	Protein	Zone	Counts	Percent
Oogenesis	100%	ZNFX-1	LP	232	0.084
Oogenesis	0%	ZNFX-1	LP	446	0.161
Oogenesis	1-25%	ZNFX-1	LP	518	0.188
Oogenesis	26-50%	ZNFX-1	LP	449	0.163
Oogenesis	51-75%	ZNFX-1	LP	34	0.012
Oogenesis	76-99%	ZNFX-1	LP	2762	1.000
Spermatogenesis	100%	ZNFX-1	LP	374	0.251
Spermatogenesis	0%	ZNFX-1	LP	159	0.107
Spermatogenesis	1-25%	ZNFX-1	LP	388	0.260
Spermatogenesis	26-50%	ZNFX-1	LP	420	0.282
Spermatogenesis	51-75%	ZNFX-1	LP	148	0.099
Spermatogenesis	76-99%	ZNFX-1	LP	2	0.001
Spermatogenesis	100%	ZNFX-1	LP	1491	1.000

Table S3.26: Chi-square test with Bonferroni correction comparing percent of WAGO-4 foci volume overlap with PGL-1 or ZNFX-1 between oogenesis and spermatogenesis. N/A coincides if the reads for both was 0. Hermaphrodite germlines with PGL-1 n=4, male germlines with PGL-1 n=4. Hermaphrodite germlines with ZNFX-1 n=5, male germlines with ZNFX-1 n=6.

Comparison	Percent Volume Overlap	Protein	Zone	Chi-squared statistic	p-value
Oogenesis vs. Spermatogenesis	0%	PGL-1	L/Z	189.65	3.789E-43
Oogenesis vs. Spermatogenesis	1-25%	PGL-1	L/Z	5.14	0.0234
Oogenesis vs. Spermatogenesis	26-50%	PGL-1	L/Z	52.16	5.127E-13
Oogenesis vs. Spermatogenesis	51-75%	PGL-1	L/Z	22.92	0.0000017
Oogenesis vs. Spermatogenesis	76-99%	PGL-1	L/Z	39.97	2.57E-10
Oogenesis vs. Spermatogenesis	100%	PGL-1	L/Z	4.58	0.0323
Oogenesis vs. Spermatogenesis	0%	PGL-1	EP	232.00	2.179E-52
Oogenesis vs. Spermatogenesis	1-25%	PGL-1	EP	0.97	0.325
Oogenesis vs. Spermatogenesis	26-50%	PGL-1	EP	76.31	2.429E-18
Oogenesis vs. Spermatogenesis	51-75%	PGL-1	EP	47.82	4.68E-12

Table S3.26 (continued)

Comparison	Percent Volume Overlap	Protein	Zone	Chi-squared statistic	p-value
Oogenesis vs. Spermatogenesis	76-99%	PGL-1	EP	25.48	4.467E-07
Oogenesis vs. Spermatogenesis	100%	PGL-1	EP	6.24	0.0124
Oogenesis vs. Spermatogenesis	0%	PGL-1	LP	379.24	1.825E-84
Oogenesis vs. Spermatogenesis	1-25%	PGL-1	LP	21.26	0.0000040
Oogenesis vs. Spermatogenesis	26-50%	PGL-1	LP	88.27	5.705E-21
Oogenesis vs. Spermatogenesis	51-75%	PGL-1	LP	267.44	4.112E-60
Oogenesis vs. Spermatogenesis	76-99%	PGL-1	LP	119.73	7.234E-28
Oogenesis vs. Spermatogenesis	100%	PGL-1	LP	43.97	3.324E-11
Oogenesis vs. Spermatogenesis	0%	ZNFX-1	L/Z	112.33	3.021E-26
Oogenesis vs. Spermatogenesis	1-25%	ZNFX-1	L/Z	0.00	0.986
Oogenesis vs. Spermatogenesis	26-50%	ZNFX-1	L/Z	18.60	0.0000162
Oogenesis vs. Spermatogenesis	51-75%	ZNFX-1	L/Z	89.31	3.372E-21
Oogenesis vs. Spermatogenesis	76-99%	ZNFX-1	L/Z	10.27	0.00135
Oogenesis vs. Spermatogenesis	100%	ZNFX-1	L/Z	3.05	0.081
Oogenesis vs. Spermatogenesis	0%	ZNFX-1	EP	34.36	4.571E-09
Oogenesis vs. Spermatogenesis	1-25%	ZNFX-1	EP	1.10	0.294
Oogenesis vs. Spermatogenesis	26-50%	ZNFX-1	EP	64.53	9.52E-16
Oogenesis vs. Spermatogenesis	51-75%	ZNFX-1	EP	32.10	1.467E-08
Oogenesis vs. Spermatogenesis	76-99%	ZNFX-1	EP	35.81	2.173E-09
Oogenesis vs. Spermatogenesis	100%	ZNFX-1	EP	0.98	0.322
Oogenesis vs. Spermatogenesis	0%	ZNFX-1	LP	85.18	2.731E-20

Table S3.26 (continued).

Comparison	Percent Volume Overlap	Protein	Zone	Chi-squared statistic	p-value
Oogenesis vs. Spermatogenesis	1-25%	ZNFX-1	LP	5.68	0.0172
Oogenesis vs. Spermatogenesis	26-50%	ZNFX-1	LP	59.27	1.372E-14
Oogenesis vs. Spermatogenesis	51-75%	ZNFX-1	LP	49.38	2.112E-12
Oogenesis vs. Spermatogenesis	76-99%	ZNFX-1	LP	31.63	1.863E-08
Oogenesis vs. Spermatogenesis	100%	ZNFX-1	LP	12.60	0.0038

Table S3.27: WAGO-4 foci overlap with ZNFX-1, PGL-1, or both germ granule components

Stage	Sex	ZNFX-1	PGL-1	Both	Total	% ZNFX-1	% PGL-1	% Both
L/Z	Hermaphrodite	473	412	1724	2609	18.1	15.8	66.1
L/Z	Male	161	270	1092	1523	10.6	17.7	71.7
EP	Hermaphrodite	302	226	1347	1875	16.1	12.1	71.8
EP	Male	24	147	471	642	3.7	22.9	73.4
LP	Hermaphrodite	335	135	923	1393	24.0	9.7	66.3
LP	Male	40	73	545	658	6.1	11.1	82.8

Table S3.28: Chi-square test with Bonferroni correction comparing the percent WAGO-4 foci that overlap with PGL-1, ZNFX-1, or both

Comparison	Protein	Zone	Chi-squared statistic	p-value
Oogenesis vs. Spermatogenesis	ZNFX-1	L/Z	41.71	3.18E-10
Oogenesis vs. Spermatogenesis	PGL-1	L/Z	2.48	0.345
Oogenesis vs. Spermatogenesis	Both	L/Z	13.74	0.00063
Oogenesis vs. Spermatogenesis	ZNFX-1	EP	63.8	4.14E-15
Oogenesis vs. Spermatogenesis	PGL-1	EP	43.7	1.152E-10
Oogenesis vs. Spermatogenesis	Both	EP	0.481	1.00
Oogenesis vs. Spermatogenesis	ZNFX-1	LP	95.4	4.68E-22
Oogenesis vs. Spermatogenesis	PGL-1	LP	0.817	1.00
Oogenesis vs. Spermatogenesis	Both	LP	59.48	3.72E-14

Table S3.29: Binning of WAGO-4 overlap with WAGO-3. Hermaphrodite germlines n=2, male germlines n=1.

Process	Percent Volume Overlap	Zone	Foci Counts	Percent
Oogenesis	0%	L/Z	238	0.377
Oogenesis	1-25%	L/Z	72	0.114
Oogenesis	26-50%	L/Z	107	0.170
Oogenesis	51-75%	L/Z	107	0.170
Oogenesis	76-99%	L/Z	106	0.168
Oogenesis	100%	L/Z	1	0.002
Oogenesis	Total	L/Z	631	1.000
Spermatogenesis	0%	L/Z	49	0.196
Spermatogenesis	1-25%	L/Z	3	0.012
Spermatogenesis	26-50%	L/Z	24	0.096
Spermatogenesis	51-75%	L/Z	103	0.412
Spermatogenesis	76-99%	L/Z	71	0.284
Spermatogenesis	100%	L/Z	0	0.000
Spermatogenesis	Total	L/Z	250	1.000

Table S3.29 (continued).

Process	Percent Volume Overlap	Zone	Foci Counts	Percent
Oogenesis	0%	EP	323	0.429
Oogenesis	1-25%	EP	82	0.109
Oogenesis	26-50%	EP	149	0.198
Oogenesis	51-75%	EP	142	0.189
Oogenesis	76-99%	EP	57	0.076
Oogenesis	100%	EP	0	0.000
Oogenesis	Total	EP	753	1.000
Spermatogenesis	0%	EP	16	0.163
Spermatogenesis	1-25%	EP	14	0.143
Spermatogenesis	26-50%	EP	29	0.296
Spermatogenesis	51-75%	EP	35	0.357
Spermatogenesis	76-99%	EP	4	0.041
Spermatogenesis	100%	EP	0	0.000
Spermatogenesis	Total	EP	98	1.000
Oogenesis	0%	LP	548	0.498
Oogenesis	1-25%	LP	219	0.199
Oogenesis	26-50%	LP	228	0.207
Oogenesis	51-75%	LP	79	0.072
Oogenesis	76-99%	LP	25	0.023
Oogenesis	100%	LP	2	0.002
Oogenesis	Total	LP	1101	1.000
Spermatogenesis	0%	LP	31	0.282
Spermatogenesis	1-25%	LP	16	0.145
Spermatogenesis	26-50%	LP	31	0.282
Spermatogenesis	51-75%	LP	22	0.200
Spermatogenesis	76-99%	LP	9	0.082
Spermatogenesis	100%	LP	1	0.009
Spermatogenesis	Total	LP	110	1.000

Table S3.30: Chi-square test with Bonferroni correction comparing percent of WAGO-4 foci volume overlap with WAGO-3 between oogenesis and spermatogenesis. Hermaphrodite germlines n=2, male germlines n=1.

Stage	Percent Volume Overlap	Chi-square Statistic	p-value
L/Z	0%	25.94	3.518E-07
L/Z	1-25%	22.68	1.918E-06
L/Z	26-50%	7.09	0.00777
L/Z	51-75%	56.64	5.24E-14
L/Z	76-99%	14.30	0.00156
L/Z	100%	0.23	0.631
EP	0%	24.44	7.65E-07
EP	1-25%	0.69	0.407
EP	26-50%	4.46	0.0346
EP	51-75%	13.95	0.00188
EP	76-99%	1.74	0.187
EP	100%	N/A	N/A
LP	0%	17.83	0.00002417
LP	1-25%	1.50	0.2204
LP	26-50%	2.89	0.0889
LP	51-75%	19.87	8.282E-06
LP	76-99%	10.73	0.001015
LP	100%	0.21	0.647

Table S3.31: Binning of WAGO-3 overlap with WAGO-4. Hermaphrodite germlines n=2, male germlines n=1.

Process	Percent Volume Overlap	Zone	Foci Counts	Percent
Oogenesis	0%	L/Z	599	0.610
Oogenesis	1-25%	L/Z	156	0.159
Oogenesis	26-50%	L/Z	166	0.169
Oogenesis	51-75%	L/Z	47	0.048
Oogenesis	76-99%	L/Z	13	0.013
Oogenesis	100%	L/Z	1	0.001
Oogenesis	Total	L/Z	982	1.000
Spermatogenesis	0%	L/Z	187	0.487
Spermatogenesis	1-25%	L/Z	46	0.120
Spermatogenesis	26-50%	L/Z	104	0.271
Spermatogenesis	51-75%	L/Z	46	0.120
Spermatogenesis	76-99%	L/Z	1	0.003
Spermatogenesis	100%	L/Z	0	0.000
Spermatogenesis	Total	L/Z	384	1.000
Oogenesis	0%	EP	286	0.407
Oogenesis	1-25%	EP	104	0.148
Oogenesis	26-50%	EP	175	0.249
Oogenesis	51-75%	EP	107	0.152
Oogenesis	76-99%	EP	31	0.044
Oogenesis	100%	EP	0	0.000
Oogenesis	Total	EP	703	1.000
Spermatogenesis	0%	EP	44	0.352
Spermatogenesis	1-25%	EP	9	0.072
Spermatogenesis	26-50%	EP	41	0.328
Spermatogenesis	51-75%	EP	24	0.192
Spermatogenesis	76-99%	EP	7	0.056
Spermatogenesis	100%	EP	0	0.000
Spermatogenesis	Total	EP	125	1.000
Oogenesis	0%	LP	140	0.208
Oogenesis	1-25%	LP	153	0.227
Oogenesis	26-50%	LP	232	0.345
Oogenesis	51-75%	LP	124	0.184
Oogenesis	76-99%	LP	24	0.036
Oogenesis	100%	LP	0	0.000
Oogenesis	Total	LP	673	1.000
Spermatogenesis	0%	LP	47	0.373

Table S3.31 (continued)

Process	Percent Volume Overlap	Zone	Foci Counts	Percent
Spermatogenesis	1-25%	LP	20	0.159
Spermatogenesis	26-50%	LP	34	0.270
Spermatogenesis	51-75%	LP	16	0.127
Spermatogenesis	76-99%	LP	9	0.071
Spermatogenesis	100%	LP	0	0.000
Spermatogenesis	Total	LP	126	1.000

Table S3.32: Chi-square test with Bonferroni correction comparing percent of WAGO-3 foci volume overlap with WAGO-4 between oogenesis and spermatogenesis. Hermaphrodite germlines n=2, male germlines n=1.

Stage	Percent Volume Overlap	Chi-square Statistic	p-value
L/Z	0%	16.595	0.00004628
L/Z	1-25%	22.012	2.708E-06
L/Z	26-50%	15.292	0.00009208
L/Z	51-75%	94.209	2.839E-22
L/Z	76-99%	80.149	3.471E-19
L/Z	100%	0.354	0.552
EP	0%	1.112	0.292
EP	1-25%	13.473	0.000242
EP	26-50%	7.775	0.0053
EP	51-75%	88.439	5.242E-21
EP	76-99%	41.522	1.165E-10
EP	100%	N/A	N/A
LP	0%	15.209	0.00009624
LP	1-25%	0.741	0.389
LP	26-50%	0.0095	0.9221
LP	51-75%	0.0095	0.922
LP	76-99%	10.009	0.00156
LP	100%	N/A	N/A

Bridge to Chapter 4:

In Chapter 3, I present a whole germline analysis of the biophysical dynamics of two additional Argonaute proteins, WAGO-3 and WAGO-4, and two constitutive components of the germ granule, PGL-1 and ZNFX-1, during sperm and egg development. I determine that all four proteins demonstrate sexual dimorphisms in their foci morphology that is reminiscent of differences observed in WAGO-1 from Chapter 2. Further, I find that these differences in biophysical properties coincide with sexually dimorphic localization patterns of WAGO-3 and WAGO-4 with the structural germ granule components, as well as with each other. Finally, I suggest that PGL-1 and ZNFX-1 may not form fully distinct sub-compartments within the germ granule and rather, multiple germ granule configurations may exist around a single developing germ cell nucleus. In Chapter 4, I summarize the findings of Chapter 2 and Chapter 3 and place these findings in the largest context of each other and the small RNA field as a whole. Additionally, I highlight future directions for research both in understanding the roles of the proteins studied in this dissertation, as well as the roles of other components of small RNA pathways.

CHAPTER 4: CONCLUSIONS

Germ granules are phase-separated sub-compartments of the germ cell that house functional components of small RNA pathways during oogenesis and spermatogenesis. Previous works in *C. elegans* characterized the organization of key structural and functional proteins within germ granules and their role in regulating oogenesis (Uebel et al., 2023, 2020). Similar studies characterizing the role of small RNA pathways and germ granules during spermatogenesis are limited. The work presented in this dissertation characterizes the structure and function of Argonaute proteins (AGOs) localized within the germ granule during spermatogenesis. I identify three AGOs that function within small RNA pathways, WAGO-1, WAGO-3, and WAGO-4, which display distinct foci morphology and localization with constitutive components of the germ granule that dynamically responds to meiotic progression through spermatogenesis (Chapter 2 and 3). Through comparison of the biophysical characteristics and localization of these AGO proteins within the germ granule to oogenesis, I identify that all three Argonautes are sexually dimorphic components of the small RNA pathways. Finally, I reveal that the sexually dimorphic configuration and biophysical properties of WAGO-1 may drive sex-specific gene regulation during germ cell development (Chapter 2). Together, my thesis identifies sexually dimorphic roles of conserved Argonaute proteins in properly regulating gene expression and maintaining germ granule structure during oogenesis and spermatogenesis. These sex-specific differences in gene regulation by small RNA pathways suggests a new avenue of research for understanding mechanisms of how Argonautes mediate gene expression and epigenetic inheritance during egg and sperm development.

My data provides an in-depth analysis of multiple AGOs, WAGO-1, WAGO-3, and WAGO-4, as well as structural components of the germ granule, PGL-1 and ZNFX-1, for morphology and localization throughout meiotic prophase I of developing egg and sperm cells (Chapter 2, Chapter 3). This work highlights the highly dynamic attributes of the germ granule, and how changes in meiotic process and stage affects germ granule structure. Meiosis is a highly coordinated process, with transcription and translation of specific genes only occurring in discrete stages of germ cell development (Gray and Cohen, 2016; Zickler and Kleckner, 2015). Argonaute proteins play a role in gene regulation during meiosis, and previous works demonstrated that infertility associated with AGO mutations may partially be driven by

prolonged expression of meiotic genes (Ortega et al., 2024). My work demonstrates that WAGO-1 regulates gene expression in a sex-specific manner (Chapter 2) and provides preliminary evidence that WAGO-3 and WAGO-4 may also mediate sex- and stage-specific gene expression (Chapter 3). Analysis of WAGO-3 and WAGO-4 relies primarily on wild-type characterization of these proteins as phase-separated foci and their localization within the germ granule (Chapter 3), revealing similar patterns of sex-specific behaviors as observed with WAGO-1. Sexual dimorphisms in WAGO-1 foci structure and localization underpins germ granule stability and sex-specific gene regulation during gametogenesis (Chapter 2). WAGO-3 and WAGO-4 may therefore play a similar role in mediating sexually dimorphic germ cell development. Future works will investigate the role of WAGO-3 and WAGO-4 protein structure in mediating germ granule organization and gene regulation between egg and sperm development.

WAGO-1, WAGO-3, and WAGO-4, are highly related paralogs within the *C. elegans* genome (Fig. 1.4). Disruption to the IDR of both WAGO-1 and WAGO-4 causes similar defects in fertility, as well as sex-specific loss of PGL-1 phase separation only during spermatogenesis (Chapter 2). Despite the similarities in native protein structure and phenotypes associated with structural mutants, WAGO-1, WAGO-3, and WAGO-4 are all hypothesized to bind distinct pools of 22G-RNAs for gene regulation (Gu et al., 2009; Ishidate et al., 2018; Schreier et al., 2022; Seroussi et al., 2023). Mechanisms for how WAGOs target distinct transcript populations, and if they are conserved between oogenesis and spermatogenesis remains unknown. Our work finds that WAGO-1, WAGO-3, and WAGO-4 have distinct sex-specific foci dynamics and demonstrate protein specific localization patterns within the germ granule that is dependent both on meiotic stage and sex (Chapter 2 and 3). I additionally identify that WAGO-3 and WAGO-4 localize to distinct regions of the germ granule, and that their co-localization is sex-specific (Chapter 3). Based on these preliminary results, I propose the testable model that slight variations in WAGO structure drives protein-specific interactions with other small RNA pathway and germ granule components, causing WAGOs to localize to distinct regions of the germ granule. These interactions and localization patterns, in turn, mediate loading of independent populations of 22G-RNAs, and allows for WAGO-specific targeting of recent transcripts. Mechanisms driving WAGO-specific gene regulation likely differ between the sexes due to sexually dimorphic biophysical properties and interactions with other components of the germ granule. Future work will determine the localization patterns of all three WAGO proteins concurrently within the germ

granule in wild-type germlines undergoing oogenesis and spermatogenesis. Additional studies utilizing mutant *wago* contexts may help determine if individual WAGOs play a role in regulating the interactions of the other AGOs during germ cell development.

Our work presented in Chapter 3 additionally found that canonical markers of germ granule sub-compartments are also sexually dimorphic both in biophysical properties as well as localization as distinct foci within the germ granule. Previous works looking at PGL-1 and ZNFX-1, markers of the P- and Z-granule respectively, suggest that these proteins exist in distinct configurations in relation to each other (Chen et al., 2024; Huang et al., 2024b; Uebel et al., 2023; Wan et al., 2018). Our data, however, identifies numerous configurations of PGL-1 and ZNFX-1 foci with varying degrees of overlap both with each other (Chapter 3), as well as with AGO proteins required for small RNA pathway function (Chapter 2 and 3). The germ granule, therefore, may exist in numerous configurations around the same developing germ cell, suggesting that highly specialized sub-compartmentalization of the germ granule is dispensable for proper regulation of transcripts. Recent work has also suggested that the localization of the germ granule to the nuclear periphery, which drives formation of these distinct sub-granules, may be expendable for proper regulation of transcripts by small RNA pathways during germ cell development (Thomas et al., 2025). Future works should interrogate the different configuration of germ granules that are present throughout germ cell development. Use of *in vivo* techniques probing the localization of recently transcribed mRNAs may aid in determining if certain transcripts are sorted to differently organized germ granules within the same germ cell.

Despite our observations in both Chapter 2 and Chapter 3 that the germ granule does not exist in a singular confirmation during a meiotic stage, we found that disruptions to germ structure do occur and are often associated with fertility defects. Specifically, we found that disruption to the IDR of WAGO-1 or WAGO-4 can cause loss of PGL-1 phase-separation during spermatogenesis, while truncation of the C-terminus of WAGO-1 leads to less interactions between WAGO-1 and PGL-1 foci (Chapter 2). The idea that some characteristics of germ granule structure are dispensable while others are required for preserving fertility and proper gene regulation may be reconciled through the hypothesis that germ granules are incidental rather than functional condensates (Putnam et al., 2023). Recent studies have found that a number of processes mediated by small RNA pathway proteins that localize to the germ granule

actually happen in the dilute phase of the cytoplasm, including mRNA silencing in early embryos and CSR-1 mediated 22G-RNA transcription in developing germ cells (Lee et al., 2020; Singh et al., 2021). Additionally, we found that the majority of WAGO-1 protein expression occurred within the dilute phase of the cytoplasm rather than in the enriched phase of the germ granule (Chapter 2), suggesting that WAGO-1 may also function and bind target transcripts within the cytoplasm. Phase separation is a natural phenomenon that occurs when concentrations of proteins and/or RNAs reaches a certain threshold (Putnam et al., 2023; Shin and Brangwynne, 2017). Proteins associated with small RNA pathways may be expressed at such high levels during germ cell development to ensure proper gene regulation that phase separation of a subset of these molecules occurs. Changes in germ granule composition and organization may therefore be driven by changes in how these proteins are acting within the cytoplasm, rather than within the germ granule. A more comprehensive examination into how disruption to phase separation of proteins required for small RNA pathway-mediated gene regulation would provide a clearer model of the germ granule as a functional versus incidental condensate. Additionally, future studies that look at germ granule structure should also integrate other techniques that would allow for understanding of how proteins associated with the germ granule mediate cellular process both in the dilute cytoplasmic phase and the condensed, granular phase.

Taken together, this dissertation provides valuable insight into sexually dimorphic gene regulation during meiosis. Through use of cytological, genetic, and genomic analysis of oogenesis and spermatogenesis we identify Argonaute proteins as imperative mediators of sex-specific germ cell development. Furthermore, through interrogation of germ granule structure in response to mutations in Argonaute proteins we determine that AGOs likely drive stability of the germ granule throughout gametogenesis. This research lays the groundwork for future experiments elucidating sexually dimorphic mechanisms of small RNA-mediated gene regulation during germ cell development.



Automated and customizable quantitative image analysis of whole *Caenorhabditis elegans* germlines

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Abstract

Arranged in a spatial-temporal gradient for germ cell development, the adult germline of *Caenorhabditis elegans* is an excellent system for understanding the generation, differentiation, function, and maintenance of germ cells. Imaging whole *C. elegans* germlines along the distal-proximal axis enables powerful cytological analyses of germ cell nuclei as they progress from the pre-meiotic tip through all the stages of meiotic prophase I. To enable high-content image analysis of whole *C. elegans* gonads, we developed a custom algorithm and pipelines to function with image processing software that enables: (1) quantification of cytological features at single nucleus resolution from immunofluorescence images; and (2) assessment of these individual nuclei based on their position within the germline. We show the capability of our quantitative image analysis approach by analyzing multiple cytological features of meiotic nuclei in whole *C. elegans* germlines. First, we quantify double-strand DNA breaks (DSBs) per nucleus by analyzing DNA-associated foci of the recombinase RAD-51 at single-nucleus resolution in the context of whole germline progression. Second, we quantify the DSBs that are licensed for crossover repair by analyzing foci of MSH-5 and COSA-1 when they associate with the synaptonemal complex during meiotic prophase progression. Finally, we quantify P-granule composition across the whole germline by analyzing the colocalization of PGL-1 and ZNFX-1 foci. Our image analysis pipeline is an adaptable and useful method for researchers spanning multiple fields using the *C. elegans* germline as a model system.

Keywords: microscopy; image analysis; *C. elegans*; worms; germline; gonad; meiosis; germ cells; P granules; DNA repair

Introduction

Reproduction in many sexually reproducing organisms requires the formation of haploid gametes. Gametes originate from germ cells that divide and differentiate to generate a germline, which is also known as the “totipotent” or “immortal” cell lineage due to its ability to pass on its genetic information to the next generation (Hubbard and Schedl 2019). Studies of germ cells in multiple systems have revealed molecular mechanisms of germ cell development, function, and maintenance. Over the past several decades, the use of genetics and cytology has been instrumental for understanding fundamental aspects of germ cell biology.

For germ cell studies, the *Caenorhabditis elegans* germline provides unique manipulation and visualization advantages (Hubbard and Greenstein 2000, 2005). In adult hermaphrodites, there are two complete tube-shaped gonads that each form a U-shape when contained within the adult animal (Hubbard and Schedl 2019). Within the adult hermaphrodite germline, ~1000 germ cell nuclei are positioned around the circumference of the tube and are arranged in a spatial-temporal gradient according to developmental stage along the distal-proximal axis. At the distal end of the gonad (pre-meiotic tip or proliferative zone), mitotically-cycling nuclei move proximally until they reach the

leptotene/zygotene region that commits them to enter meiosis, the specialized cell division that generates haploid gametes. This entry into meiosis is termed the “transition zone” and the germ cells begin differentiating to form mature oocytes. The transition zone is classically identified by crescent-shaped DAPI morphology due to the polarized active movement of chromosomes; however, in certain mutant situations that affect chromosome pairing or germ cell proliferation, this region with distinct DAPI morphology may be either absent or extended (e.g., *hal-2* and *syp-1*) (MacQueen et al. 2002; Zhang et al. 2012). Following the transition zone, germ cell nuclei enter pachytene stage where chromosomes are no longer undergoing rapid polarized movement and instead assume a cage-like appearance. After pachytene, chromosomes begin the condensation process in the diplotene stage and eventually fully condense to form six DAPI-staining bodies (one for each set of homologs) at diakinesis. This “pipeline” of germ cell development in the *C. elegans* gonad has enabled the visualization of all stages of germ cell development simultaneously within a single germline, thereby making this model system a powerful tool for cytological approaches.

Cytological studies of the *C. elegans* germline illuminate key aspects of meiosis, including chromosome pairing,

Received: November 16, 2020. Accepted: January 11, 2021

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recombination, regulation of DNA damage responses, and apoptosis in gamete production (Garcia-Muse and Boulton 2007; Hillers et al. 2017; Cahoon and Libuda 2019). The spatial-temporal organization of the germline can be used to define the timing and/or progression of these events throughout meiotic prophase I in *C. elegans* (Garcia-Muse and Boulton 2007; Jaramillo-Lambert et al. 2007; Hillers et al. 2017). For example, localization and quantification of foci composed of meiotic recombination proteins established the timing and steps of DNA repair events in the *C. elegans* germline (Yokoo et al. 2012; Schvarzstein et al. 2014; Woglar and Villeneuve 2018; Cahoon and Libuda 2019). Further, quantification of these foci within the germ cell nuclei can indicate changes in the frequency of these specific DNA repair events both in wild type and mutant contexts. Overall, quantitative image analysis of whole germlines has been instrumental in revealing roles for specific genes in meiotic DNA repair (Garcia-Muse and Boulton 2007).

Germ cell differentiation and fertility in *C. elegans* require the germline to assemble RNA/protein condensates called P granules. These membraneless organelles are perinuclear during the majority of germ cell development and are involved in silencing germline transcription via small RNA pathways (Seydoux 2018; Ouyang et al. 2019; Putnam et al. 2019). For nearly 40 years, cytology and genetics have played critical roles in studies of P granules. In 1982, P granules were originally identified by immunofluorescence imaging that revealed the existence of granules in the *C. elegans* P cell lineage, which exclusively gives rise to the germline (Strome and Wood 1982). Subsequent high-resolution microscopy, live imaging, and fluorescence recovery after photobleaching studies have revealed the components, dynamics, and liquid-like properties of P granules (Seydoux 2018; Ouyang et al. 2019; Putnam et al. 2019). Further, analysis of whole adult gonads stained for P granule structures reveals that some components of these membraneless organelles can undergo morphological changes during meiotic prophase I progression (Uebel et al. 2020), further suggesting possible changes in function during oogenesis.

Although both qualitative and quantitative microscopy approaches are currently used to study the *C. elegans* germline, the variation in the chromosome morphology throughout the germline and technical variability from affixing dissected gonads affixed to microscope slides have limited high-content automated analysis of germline features. Some powerful computational methods exist for analyzing images taken of specific regions of the germline, such as the distal region where germ cell proliferation occurs (Lee et al. 2016; Crittenden et al. 2019), but these methods are limited to germlines (whole or specific regions) that affix in a straight/linear orientation. Due to limitations of existing automated image analysis methods for non-linear whole gonads, many research groups still rely on time-consuming and laborious manual efforts for quantifying features of germ cells within whole *C. elegans* germlines. To expedite and expand quantitative image analysis of the whole *C. elegans* germline, we developed a high-content, automated method using custom algorithms that function with image processing software. This method enables quantitative image analysis of cytological features of single nuclei within non-linear whole *C. elegans* gonads. Further, this computational pipeline permits analysis and data visualization of individual nuclei based on their position within the germline. Here we describe and validate our computational method by analyzing images of multiple features of germ cell nuclei undergoing meiotic prophase I progression within the context of an entire *C. elegans* germline.

Materials and methods

Caenorhabditis elegans strains and maintenance

Caenorhabditis elegans strains were maintained under standard conditions on nematode growth medium (NGM) plates at 20°C with OP50 *Escherichia coli* bacteria lawns. All experiments were performed in the N2 background of *C. elegans* (CGC).

Strains used in this study include N2 (wild type), AV157 (*spo-11(me44)/hT1 [unc-?(n754) let-? qIs50]* (IV; V), AV630 (*meIs8[unc-119(+)] pie-1p::GFP::cosa-1*) II), VC531 (*rad-54&snx-3(ok615) I/hT2 [bli-4(e937) let-?(q782) qIs48]* I; III), and YY916 (*znfx-1(gg544[3xflag::GFP::znfx-1])* II.).

Immunofluorescence

Immunofluorescence was performed as in Libuda et al. (2013). At 18–22 h before dissection, L4 stage hermaphrodite worms were isolated and maintained at 20°C on NGM plates seeded with OP50. Gonads were dissected in 30 µL of egg buffer (118 mM NaCl, 48 mM KCl₂, 2 mM CaCl₂, 2 mM MgCl₂, 25 mM HEPES pH7.4, 0.1% Tween20) and were fixed in egg buffer with 1% paraformaldehyde for 5 min on a Superfrost Plus slide (VWR). Gonads were then flash frozen in liquid N₂ and the cover slip was removed. For germlines stained for DSB-2, RAD-51, MSH-5, or GFP::COSA-1, the slide was placed in –20°C MeOH for 1 min and then was washed in PBST (1× PBS, 0.1% Tween20). For germlines stained for PGL-1 and ZNFX-1, the slide was placed in –20°C MeOH for 10 min, then in –20°C acetone for 5 min, and then was washed in PBST. Slides were washed 3× in 1× PBST for 5 min before being placed in block (1× PBS, 0.1% Tween20, 0.7% Bovine Serum Albumin) for 1 h. 50 µL of diluted primary antibody (in 1× PBST; see below for individual antibody concentrations) was applied to each slide and allowed to stain overnight in a dark humidifying chamber with a parafilm coverslip. At 16–18 h after application of primary antibody, slides were washed 3× in PBST for 10 min. 50 µL of diluted secondary antibody (in 1× PBST; see below for individual antibody concentrations) was applied to each slide and allowed to stain for 2 h in a dark humidifying chamber with a parafilm coverslip. Slides were washed 3× in PBST for 10 min in a dark chamber and then 50 µL of 2 µg/mL of DAPI in ddH₂O was added to each slide and incubated for 5 min in a dark humidifying chamber with a parafilm coverslip. Slides were washed in PBST for 5 min in a dark chamber and then were mounted in VectaShield with a No. 1.5 coverslip (VWR) and sealed with nail polish. Slides were maintained at 4°C prior to imaging (as described below). The following primary antibodies were used in this study at the listed concentrations: polyclonal chicken αRAD-51 (1:1000, this study, see below), αDSB-2 (1:5000; Rosu et al. 2013), αMSH-5 (1:10,000, Novus #3875.00.02), polyclonal chicken αGFP (1:2000, Abcam #ab13790), monoclonal mouse αPGL-1 K76 (1:20, Developmental Studies Hybridoma Bank), polyclonal guinea pig SYP-1 (1:250; MacQueen and Villeneuve 2001), and polyclonal rabbit GFP (1:1000; Yokoo et al. 2012). Secondary staining was performed with goat antibodies conjugated to Alexa Fluors 488 and 555 targeting the primary antibody species (1:200, Invitrogen).

Antibody production

Our RAD-51 antibody was generated from a His-tagged fusion protein expressed by Genscript from plasmid pET30a containing the entire RAD-51S coding sequence (1385 bp, GenBank accession number AF061201; Rinaldo et al. 1998; Colaiácovo et al. 2003). Antibodies were produced in chicken and affinity purified by Pocono Rabbit Farms.

Image acquisition

Immunofluorescence slides were imaged at 512×512 or 1024×1024 pixel dimensions on an Applied Precision DeltaVision microscope with a $63\times$ lens and a $1.5\times$ optivar. To ensure analysis of the highest resolution germline images, we imaged the top ~quarter of the germline along the dorsal-ventral axis that encompassed whole nuclei closest to the coverslip, but our pipeline can be used for analysis of gonads imaged through entire dorsal-ventral axis. Images were acquired as Z-stacks at $0.2\mu\text{m}$ intervals and deconvolved with Applied Precision softWoRx deconvolution software.

Gonad Analysis Pipeline

Below is a detailed section describing the method. A step-by-step protocol published with the manuscript is available as Supplementary File S1. For the latest step-by-step protocol of this method, please go to the publication section of www.libudalab.org. The ability to resolve foci or cellular features using this protocol depends on the microscopy method used to capture the images. For this study, widefield microscopy combined with deconvolution was used to generate the gonad images for a resolution limit of 200nm (see above "Image Acquisition" method section). To resolve objects less than 200nm apart with this protocol, users will need to use superresolution microscopy techniques, such as structured illumination microscopy (SIM), stochastic optical reconstruction microscopy (STORM), or stimulated depletion microscopy (STED).

Identification of nuclei within whole gonad images

Three-dimensional images were tiled using the Imaris Stitcher software (Bitplane) or the Grid/Collection Stitching plugin in FIJI with regression threshold of 0.7 (this value was raised or lowered depending on the stitching results) (Preibisch et al. 2009). If images were not accurately aligned by the Imaris Stitcher algorithm, they were manually adjusted before proceeding with analysis. Individual nuclei within stitched gonads were identified by DAPI as Surface objects. When using DAPI staining to define Surface objects, the changing morphology of nuclei within the germline required different sets of parameters to be used. Nuclei spanning from the distal premeiotic tip through the final 5 rows of pachytene were defined using Smooth 0.15, Background 3.5, Seed Point Diameter 2–3, and Volume Filter 8–55. Late pachytene nuclei (nuclei in the 5 rows preceding diplotene) were defined using Smooth 0.15, Background 4, Seed Point Diameter 3–4, and Volume Filter 10–50. Manual thresholding and specific values for Seed Point Diameter and Volume Filter were defined for each gonad within the indicated ranges. Defined Surfaces were then split to designate individual nuclei using the Imaris Surfaces Split module. Nuclei that were either partially imaged or overlapping with another nucleus were eliminated from analysis.

Identification of SYP-1 surfaces in whole gonad images

In 3D stitched gonad images (see "Identification of nuclei within whole gonad images", above) Individual SYP surfaces were defined using Absolute Intensity (enabled), Smooth (0.22), Background (N/A), Seed Point Diameter (N/A), and Volume Filter (deleted surfaces less than $0.5\mu\text{m}$). If multiple individual surfaces were generated to represent the SYP-1 staining of a single given nucleus, then these surfaces were manually unified.

Quantification of DSB-2 normalized mean staining intensity

DSB-2 mean staining intensity per nucleus was calculated in Imaris following definition of single nuclei as surface objects using DAPI signal (see "Identification of nuclei within whole gonad images" section). As image acquisition settings differed between imaged germlines but were consistent within the same germline, the DSB-2 mean intensity of each nucleus was normalized by dividing the mean intensity of each nucleus by the highest mean intensity among nuclei within a gonad.

Quantification of meiotic recombination foci

RAD-51, MSH-5, and GFP::COSA-1 foci were defined from stitched whole gonad images (see "Identification of nuclei within whole gonad images" section) using the "Create Spots" tool in Imaris (Bitplane) with the settings Estimated XY Diameter 0.1, Model PSF-elongation 1.37, and Background Subtraction enabled. To determine the number of RAD-51 foci per nucleus by determining based on proximity of defined Spots to Surfaces, we used a custom "Find Spots Close to Surface" MATLAB module (Threshold value 1; see "Data and Code Availability" section for link to download module). The number of SYP-1 associated MSH-5 or GFP::COSA-1 foci per nucleus was also determined using the "Find Spots Close to Surface" module (Threshold value 0.1).

Quantification of PGL-1 and ZNFX-1 foci

PGL-1 and ZNFX-1 foci were defined as Surface objects in Imaris (Bitplane) with the settings Smooth (Not enabled), Background 0.513, Seed Point Diameter (Not enabled), and Volume Filter (foci $> 0.1\mu\text{m}$). In late pachytene, the large variance in different P granule sizes required the generation of a separate additional set of "large" surfaces with the settings Smooth (Not enabled), Background 0.513, Seed Point Diameter (Not Enabled), and Volume Filter A ($0.1\text{--}2\mu\text{m}$) for Filter B ($0.1\mu\text{m}\text{--}12\mu\text{m}$). To ensure that moderately sized PGL-1 and ZNFX-1 foci were not counted twice in this analysis, we generated unique intensity channels to identify overlapping "small" and "large" PGL-1 and ZNFX-1 surfaces. If a "small" and "large" PGL-1 or ZNFX-1 surface was found to share a unique intensity value, indicating that they were occupying the same volume in the 3D reconstructed image, the "small" surface was excluded, as the "large" surface better represented the image.

Colocalization analysis

To determine if foci (PGL-1 and ZNFX-1 Figure 4, RAD-51 and MSH-5 Supplementary Figure S6) colocalized, we applied the Shortest Distance Calculation function in Imaris to identify and replicate surfaces separated by $0\mu\text{m}$. These overlapping surfaces were then given unique colocalization identity intensity channels. A focus was considered "co-localized" in all analyses if two or more foci of different types (PGL-1 and ZNFX-1; or, MSH-5 and RAD-51) with the same unique colocalization intensity value could be identified in the exported data. For colocalization analysis, MSH-5 and RAD-51 foci were identified as surface objects, following a protocol identical to the one described in "Quantification of PGL-1 and ZNFX-1 foci."

Gonad Linearization Algorithm

To assess nuclei based on their position within the gonad, we used an algorithm (called "Gonad Linearization Algorithm") implemented in R to approximate the progression of nuclei through the *C. elegans* germline as a linearly ordered sequence

beginning at the premeiotic tip and terminating at the end of pachytene. For a link to download the Gonad Linearization Algorithm, see “Data and Code Availability” section of *Materials and Methods*. To delineate the orientation of the gonad, a series of connected line segments marking the approximate center of the gonad were drawn on the stitched germline image using the Imaris Measurement tool. When possible, specific measurement points were placed at positions indicating transitions between meiotic stages based on DAPI nuclei morphology, specifically marking the beginning of the premeiotic zone, transition zone, pachytene, and end of pachytene.

Each line segment drawn through the germline was defined by the coordinates of its respective start (x_i, y_i) and end (x_j, y_j) points. The standard equation [$0 = Ax + By + C$] of each line segment $\overline{ij}$ was calculated such that:

$$\begin{aligned} A_{\overline{ij}} &= \frac{(y_j - y_i)}{(x_j - x_i)} \\ B_{\overline{ij}} &= -1 \\ C_{\overline{ij}} &= y_i - x_i \left(\frac{y_j - y_i}{x_j - x_i} \right). \end{aligned}$$

To determine whether the position of a nucleus within the gonad could be well approximated as a position on a given line segment, we calculated the perpendicular intersection point of a vector drawn from the position of the nucleus to each line segment. The perpendicular intersection point (x_p, y_p) of a nucleus at position (x_n, y_n) to a line $\overline{ij}$ was calculated as follows:

$$\begin{aligned} x_p &= \frac{y_n - \left(\frac{-x_n}{A_{\overline{ij}}} \right) - C_{\overline{ij}}}{A_{\overline{ij}} - \left(\frac{-1}{A_{\overline{ij}}} \right)} \\ y_p &= \left(\frac{-x_p}{A_{\overline{ij}}} \right) + y_n - \left(\frac{-x_n}{A_{\overline{ij}}} \right). \end{aligned}$$

The transformed coordinate position (x_p, y_p) of a nucleus was considered well approximated if the distances from the start position of the line segment (x_i, y_i) to (x_p, y_p) and the distance from the end position of the line segment (x_j, y_j) to (x_p, y_p) were smaller than the total length of the line segment $\overline{ij}$. If multiple line segments met these criteria, the correct line segment was inferred to be the one for which the distance from the nucleus' original position (x_n, y_n) to its perpendicular intersection point (x_p, y_p) was the shortest.

The above method of assigning nuclei to segments was sufficient for all germlines analyzed in this study. However, the specific arrangement of nuclei around the central gonad axis in the context of the whole germline conformation may lead to nuclei being incorrectly aligned according to these criteria. To ameliorate this potential problem, we included a stringency parameter in our algorithm, which increases the permissible distance nuclei may be assigned to a particular line segment. If increasing the stringency parameter from its default value of 0 is not sufficient to enable more accurate nuclei assignment, nuclei can also be manually assigned to line segments.

Once all nuclei had been assigned transformed coordinate positions, the sum length of all contiguous line segments drawn through a germline, as well as the sum distance of all line segments from the most proximal point to each transformed nucleus position, were calculated. Each length measurement was normalized to the total length of all line segments drawn through

the germline to calculate relative gonad position, where a position of 0 corresponded to the start of the premeiotic tip and position 1 corresponded to the end of late pachytene.

Validation of nucleus positioning by the Gonad Linearization Algorithm

100 “gonads” were simulated by iteratively generating six consecutive line segments with lengths $\sim \text{Normal}(50, 5)$ and angles of intersection $\sim \text{Normal}(180, 30)$. 100 points were simulated along the sum length of the line segments for each gonad $\sim \text{Uniform}(0, \text{sum line segment lengths})$. Each point was then transposed perpendicularly to its line segment a distance $\sim \text{Normal}(10, 3)$. These transposed “nucleus” positions were then realigned to the line segments using the Gonad Linearization Algorithm and were subsequently analyzed to determine goodness of fit.

Statistics

All statistics were calculated in R (v4.0.3). Data wrangling was performed using the Tidyverse package (v1.3.0). Nonparametric correlations between DSB-2 normalized staining intensity and RAD-51 focus counts (Supplementary Figure S3) were assessed by Spearman correlation tests with confidence intervals calculated using the DescTools package (v0.99.30). Comparisons of RAD-51 focus manual and automated quantification (Figure 2B) and the rank order of simulated nucleus position data (Supplementary Figure S1C) were performed by linear regression analysis. The 95% Binomial confidence interval for the proportion of colocalized PGL-1 and ZNFX-1 granules (Figure 4C) was calculated using the DescTools package. Volumes of PGL-1 and ZNFX-1 (Figure 4D) foci were compared by Mann-Whitney U test.

Data and code availability

All strains and antibodies are available upon request. File S1 contains a step-by-step protocol for the Gonad Analysis Pipeline at the time of this manuscript's publication. The latest step-by-step protocol for the Gonad Analysis Pipeline can be found at www.libudalab.org in the publication section. The “Gonad Linearization Algorithm” and “Find Spots Close to Surface” MATLAB module are available at github.com/libudalab/Gonad-Analysis-Pipeline. Supplementary Figure S1 displays bar plots representing the proportion of nuclei identified from each region of the germline by the Whole Gonad Pipeline. Supplementary Figure S2A displays plots of the simulated “germlines” and “nuclei” use to validate the Gonad Linearization Algorithm. Supplementary Figure S2B displays a density plot of the proportion of “nuclei” in simulated “germlines” which were accurately assigned to central line segments. Supplementary Figure S2C displays a plot comparing the rank order of simulated “nuclei” correctly assigned to central line segments within simulated “germlines” to their known simulated rank order. Supplementary Figure S2D displays density plots showing the relative deviation of simulated “nuclei” from their known simulated positions relative to the alignment performed by the Gonad Linearization Algorithm. Supplementary Figure S3D displays dot plots assessing the association of DSB-2 staining intensity and RAD-51 focus counts in germlines analyzed by the Gonad Analysis Pipeline. Supplementary Figure S4AB displays immunofluorescence images on whole gonads of *spo-11(me44)* and *rad-54(ok615)* whole germlines. Supplementary Figure S4C displays dot plots assessing RAD-51 focus counts per nucleus in *spo-11(me44)* and *rad-54(ok615)* whole germlines analyzed by the Gonad Analysis Pipeline. Supplementary Figure S5 displays dot plots assessing “bright” MSH-5 foci counts per nucleus in germline analyzed by

the Gonad Analysis Pipeline. Supplementary Figure S6A displays immunofluorescence image of MSH-5 and RAD-51 staining in germline. Supplementary Figure S6B displays a bar graph of the frequency of colocalization between MSH-5 and RAD-51 foci. Supplementary Figure S6C displays a density plot of the distance between an MSH-5 focus and the nearest RAD-51 focus considered colocalized versus not colocalized. Supplementary material (Supplementary File S1 and Supplementary Figures S1–S6) is available at figshare: <https://doi.org/10.25386/genetics.13571210>.

Results

Gonad analysis pipeline for fluorescent image analysis of whole *C. elegans* germlines

The *C. elegans* germline presents many challenges for automated quantification of cytological data. Due to the non-linear three-dimensional (3D) shape of both undissected and dissected gonads, it has been difficult to computationally: (1) distinguish individual nuclei within an imaged gonad; and (2) contextualize quantitative features of individual nuclei based on their position in the gonad and during specific stages of meiotic prophase I. Further, the freedom of dissected gonads to adopt multiple shape conformations when affixed to a microscope slide or coverslip presents an additional challenge for automating computational analysis of large numbers of dissected gonads. To overcome these challenges, we constructed a Gonad Analysis Pipeline using image quantification software in conjunction with custom scripts implemented in MATLAB and R to enable high-throughput quantification of germline features at single nucleus resolution, while maintaining information regarding the relative position of these nuclei within the *C. elegans* germline. For all of our analyses, we acquired and used 3D immunofluorescence images of dissected, fixed *C. elegans* germlines using established protocols that preserve the 3D architecture of the germline (Figure 1, A and B; Materials and Methods). Since high-resolution analysis of whole *C. elegans* gonads requires acquisition of multiple 3D images to encompass their entire distal-proximal length, we stitched the individual 3D images together into a single reconstruction of the imaged germline using either Imaris Stitcher or an image stitching plugin in Fiji (see Materials and Methods; (Preibisch et al. 2009)). Individual nuclei within the gonad were defined using Surface in Imaris with the DNA stain DAPI (see below). Due to the arrangement of nuclei in some germlines, a subset of nuclei (~23%) was unable to be computationally identified and were subsequently removed from the dataset (Figure 1, B and C). A caveat of removing these nuclei is that specific germline regions could be under sampled ($P < 0.001$ chi-square test of goodness of fit, Supplementary Figure S1, A and B); however, we found that combining the datasets of at least two germlines was sufficient to enable even sampling of nuclei across the germline from the premeiotic tip to the end of late pachytene ($P = 0.422$, chi-square test of goodness of fit, Supplementary Figure S1C). From our imaged gonads (which capture the top 25–30% of the germline along the dorsal-ventral axis; see Materials and Methods), we computationally identified an average of 146.3 ± 16.9 nuclei per germline ($n = 4$ gonads). Overall, ~15% of the germline nuclei contained in a single gonad arm are captured by this method using our particular images of the top dorsal portion of each gonad, and are evenly sampled from all regions of the germline. These results indicate the ability of this pipeline to identify and analyze large numbers of nuclei from whole gonads.

To demarcate the conformation of each gonad from the distal tip (premeiotic) to proximal end (late pachytene), we drew contiguous line segments down the center of each germline (Figure 1D). Using this method, we designated each stage of meiotic prophase I along this segmented line based on DNA morphology: the premeiotic zone, transition zone (encompassing leptotene and zygotene), and pachytene. Since some mutant germlines lack some of these cytological features (e.g., absence of polarized chromosomes characteristic of transition zone nuclei), we developed an algorithm to approximate the relative germline position of each nucleus independent of DNA morphology (Figure 1E). This algorithm (called the “Gonad Linearization Algorithm”) approximates the position of each nucleus along the length of the germline based on its orientation relative to the line drawn along the center of the gonad. To calculate the position of each nucleus, the Gonad Linearization Algorithm identifies the best fit perpendicular intersection point for the position of each nucleus relative to the central line segments (see perpendicular arrows projecting from each nucleus to the central line in Figure 1D). This analysis allows us to recontextualize individual nuclei from 3D space into a one-dimensional (1D) space, enabling assessment of nucleus features based on position in the gonad as nuclei progress through meiotic prophase I.

To assess the ability of the Gonad Linearization Algorithm to accurately align nuclei through the germline, we applied the algorithm to a simulated dataset of 100 “germlines.” Each simulated “germline” contained 100 simulated “nuclei” dispersed along the lengths of the “germline” (Supplementary Figure S2A). We found that, for most simulated “germlines,” >90% of the “nuclei” were accurately assigned to the correct line segment, and that correctly aligned nuclei recapitulated the order in which they were simulated along the length of the “germline” ($P < 0.001$, $R^2 = 1$, Linear regression analysis, Supplementary Figure S2, B and C). Even in the case of incorrect assignment of a “nucleus” to a line segment, the deviation of the placement of each “nucleus” in the context of the whole gonad was $0.00 \pm 3.7\%$ on average, and all were <10% (Supplementary Figure S2D). In addition, we have included within the algorithm a way to manually correct the assignment of these incorrectly assigned nuclei. These data illustrate the accuracy and customizability of the Gonad Linearization Algorithm for analysis of diverse conformations of dissected gonads.

Quantification of DNA-associated proteins at single nucleus resolution

Manual quantification of foci within nuclei from whole *C. elegans* gonads is a laborious, rate-limiting step during image analysis. To validate our Gonad Analysis Pipeline’s automated quantification of meiotic features, we first quantified classic markers that are involved in double-strand DNA break (DSB) formation and repair. The recombinase RAD-51 loads at sites of DSBs in meiotic nuclei (Colaiácovo et al. 2003; Lemmens and Tijsterman 2011). The number of RAD-51 foci within germline nuclei can indicate either the extent of DSB induction and/or the efficiency of DSB repair during meiotic prophase I progression (Bickel et al. 2010; Rosu et al. 2013). DSB-2 promotes DSB induction, and accumulates on meiotic chromatin in the final stages of the transition zone and early pachytene when RAD-51 forms numerous foci (Rosu et al. 2013; Stamper et al. 2013).

To quantify RAD-51 within an entire germline, we implemented our Gonad Analysis Pipeline adapted with a custom MATLAB script in combination with the Gonad Linearization Algorithm. First, we identified nuclei within the germline using

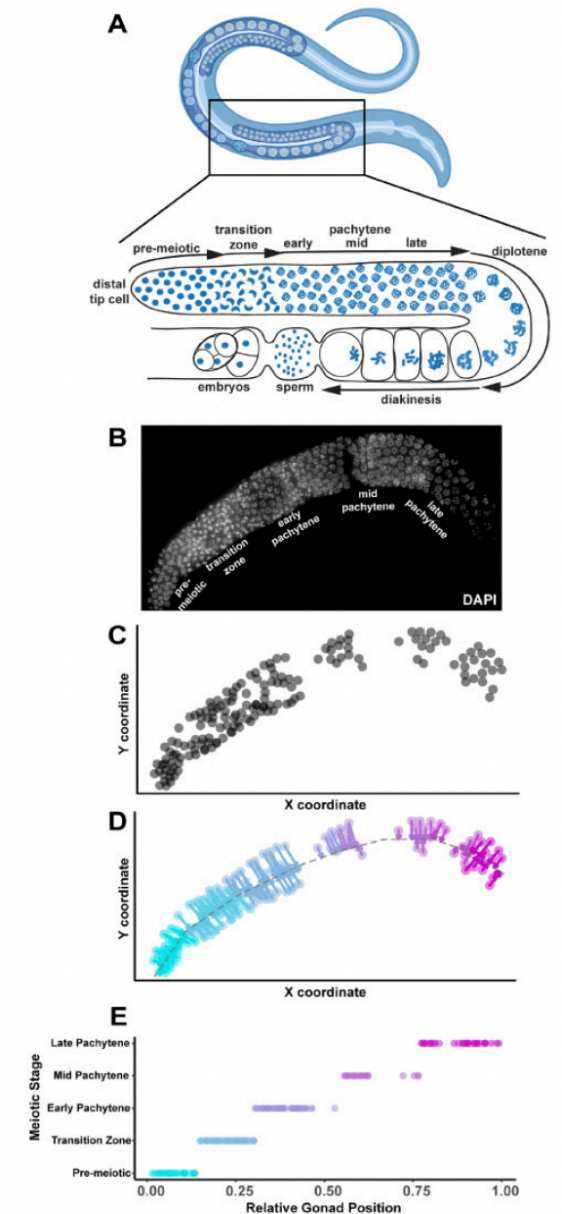


Figure 1 Gonad Linearization Algorithm transforms and orients 3D-nuclei within a non-linear *C. elegans* gonad onto a one-dimensional axis. We designed a custom algorithm (called the “Gonad Linearization Algorithm”) to enable the assessment of individual *C. elegans* nuclei relative to their position within a germline. (A) Cartoon of adult hermaphrodite worm (top panel; made with Biorender) with zoom in of one gonad arm (lower panel) with nuclei (blue) and indicated stages of meiosis based on DNA morphology (B) Dissected *C. elegans* hermaphrodite germline with DNA stained using DAPI (white). Specific meiotic stages were determined by DNA morphology. (C) 2D coordinate positions (units arbitrary) of individual whole nuclei (gray circles) within a *C. elegans* germline. Whole nuclei and respective coordinate positions were defined using Imaris. Nuclei found to be overlapping or only partially imaged were eliminated from analysis. Nuclei that were not able to be computationally oriented were also removed from analysis. (D) Application of the Gonad Linearization Algorithm transforms the coordinates of nuclei onto a central axis line drawn through the

DAPI (see Methods for details). We developed a custom MATLAB script (called “Find Spots Close to Surface”) to: (1) identify the RAD-51 foci (spots) that were associated with each individual nucleus (surface); and (2) provide a readout of foci per nucleus. Then after drawing line segments along the length of the gonad, the Gonad Linearization Algorithm was used to transform the position of each nucleus and the RAD-51 foci (spots) associated with that nucleus on to that 1D line. This transformation generated data from a single germline that contained both the number of spots associated with each nucleus and the relative position of each nucleus along the length of the germline. In addition to scoring the number of RAD-51 foci for each nucleus, we further calculated the mean intensity of DSB-2 staining with each nucleus using Imaris (Figure 2A). From these analyses, we are able to observe the complete dynamics of DNA repair at a single nucleus resolution.

To determine the accuracy of our high-throughput Gonad Analysis Pipeline method for nuclear-associated foci quantification, we manually scored RAD-51 foci in a blinded subset of representative nuclei taken from whole gonad images (Figure 2B; $n=70$ nuclei). The mean deviation between automated and manual foci quantification was 0.05 ± 1.21 , and the number of foci per nucleus quantified by Imaris software correlated well with the number of foci scored manually ($P < 0.001$, Adjusted $R^2 = 0.91$, Linear Regression Analysis, Figure 2B). Thus, across a population of nuclei, our Gonad Analysis Pipeline yields reliable statistics for the number of foci associated with individual *C. elegans* germline nuclei.

Using the Gonad Analysis Pipeline, we assessed the distribution of RAD-51 along the length of the germline (Figure 2C) in wild-type and mutant contexts. In concordance with previous studies of wild-type germlines (Colaiácovo et al. 2003; Rosu et al. 2013; Stamper et al. 2013), we observe most nuclei with one or more RAD-51 foci within the central ~50% of the germline (Figure 2C), corresponding to the end of the transition zone through mid-pachytene stages of meiosis I (Figure 1D). In addition, we assessed mutants that displayed aberrant meiotic recombination. Null mutants for the topoisomerase-like protein SPO-11 are unable to generate endogenous meiotic DSBs and show few RAD-51 foci throughout meiotic prophase I (Colaiácovo et al. 2003; Supplementary Figure S4A). Our quantification of a *spo-11(me44)* null mutant hermaphrodite germline reflects this phenotype, with 93.3% of nuclei analyzed (125/139 nuclei) showing no RAD-51 foci (Supplementary Figure S4C, top). Null mutants of the recombination protein RAD-54 are unable to unload RAD-51 from DSBs during pachytene, resulting in elevated and persistent RAD-51 foci (Mets and Meyer 2009; Rosu et al. 2011; Nottke et al. 2011; Supplementary Figure S4B). When we quantified RAD-51 foci per nucleus in a *rad-54(ok615)* null mutant hermaphrodite germline using the Whole Gonad Pipeline, we also observed elevated and persistent RAD-51 foci during meiotic prophase I progression (Supplementary Figure S4C, bottom). Our dataset illustrates that the number of DSBs per nucleus in a *rad-*

Figure 1 Continued

germline, approximating the progression of nuclei through the germline based on their position along that line. (E) Normalizing the total length of line segments drawn through the center of the gonad enables standardized assessment of individual nuclei contextualized by their progression through the germline. Line segments were specifically placed to delineate the premeiotic zone and transition zone based on DAPI morphology of chromosomes. Early, mid, and late pachytene were defined on this graph by dividing the remaining normalized germline length into equal thirds.

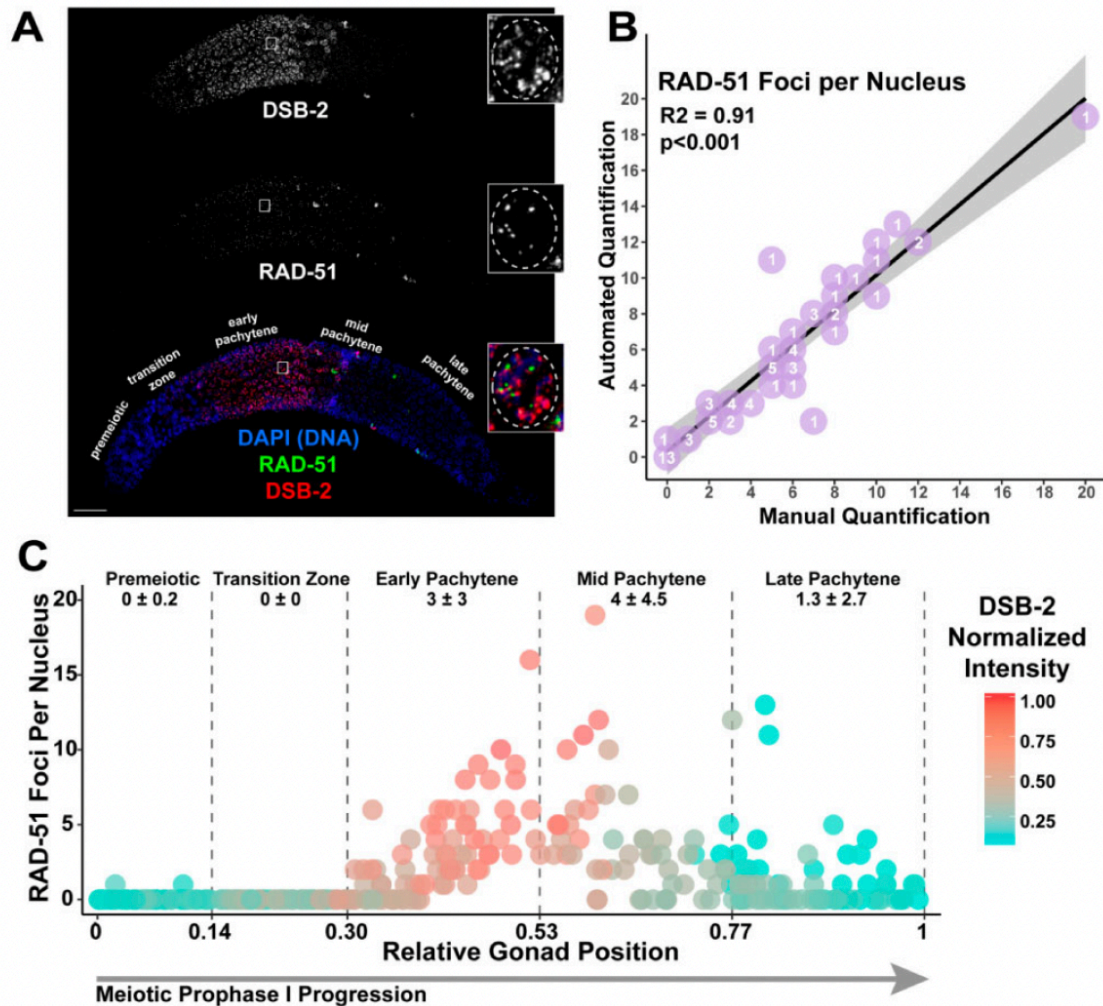


Figure 2 Gonad Analysis Pipeline enables germline-wide single nucleus assessment of double-strand DNA break (DSB) levels. (A) Immunofluorescence image of a *C. elegans* hermaphrodite germline stained with DAPI (DNA; blue), DSB-2 (red), and RAD-51 (green). Scale bar represents 20 μ m. Inset images display a representative mid-pachytene nucleus with characteristic DSB-2 and RAD-51 staining. The location of inset image within the whole germline image is indicated by a white box on the whole germline image. For clarity, a dashed line is used in the inset image to emphasize the region of the image containing the nucleus of interest. Specific meiotic stages were determined by DNA morphology. (B) Comparison of data from automated quantification of RAD-51 foci associated with individual nuclei to data from manual quantification of RAD-51 foci within those same nuclei analyzed by the automated system ($n=70$ nuclei derived from three separate germlines). The number listed on each point (purple) indicates the number of nuclei scored with that result. The linear regression line is displayed as a black line, whereas the gray-shaded area represents the 95% confidence interval of this analysis. (C) Visualization of RAD-51 foci counts and DSB-2 normalized mean fluorescence intensity of individual nuclei across gonads ($n=2$). The RAD-51 foci counts and DSB-2 normalized intensity values of 295 individual nuclei are displayed. DSB-2 mean intensity was normalized within analyzed gonads by the highest recorded DSB-2 mean fluorescence intensity among nuclei. Normalized DSB-2 intensity is indicated with a color gradient from red (highest intensity) to blue (lowest intensity). Vertical dashed lines indicate the average position in which nuclei within each gonad transition between each successive stage of meiotic prophase I (as indicated by text in the figure), determined by DAPI morphology. The deviation between these transition points was <0.01 between the germlines. Numbers below the text demarcating each respective meiotic stage in the germline indicate the mean number of RAD-51 foci $\pm$ the standard deviation of RAD-51 foci among nuclei within that region.

54 mutant is dramatically higher than wild-type (Supplementary Figure S4C), with the highest number of DSBs in a single nucleus (83 RAD-51 foci in a single nucleus) located most proximally in the germline. Taken together, these data show the flexibility of using the Gonad Analysis Pipeline to quantify DNA damage repair foci across different mutant contexts.

To further assess the functionality and accuracy of our image quantification method, we quantified the association between

RAD-51 and DSB-2 on a single nucleus basis. Similar to RAD-51, the per-nucleus normalized mean intensity of DSB-2 within germlines was also highest in the central 50% of the germline (Figure 2C). To dissect this relationship further, we binned the DSB-2 and RAD-51 data into two bins based on when DSB-2 is loaded to chromatin in early prophase (transition zone-early pachytene) or offloaded from chromatin in late prophase (mid-late pachytene) (Rosu et al. 2013). Overall, higher DSB-2 intensity

is correlated with increased numbers of RAD-51 foci (Supplementary Figure S3). Notably, we observed a stronger correlation in early prophase (Spearman's ρ 0.785 95% CI 0.721–0.836, $P < 0.001$, Spearman's rank correlation test) than in late prophase (Spearman's ρ 0.389 95% CI 0.225–0.532, $P < 0.001$, Spearman's rank correlation test), supporting the reported function of DSB-2 to promote DSB induction during early pachytene by creating a DSB-permissive state when loaded onto chromatin (Rosu et al. 2013). Taken together, these results show the capability of the Gonad Analysis Pipeline to quantify the relationships of cytological features at single nucleus resolution.

Quantification of meiotic chromosome structure-associated foci at single nucleus resolution

Next we used the Gonad Analysis Pipeline to quantify foci associated with specific steps in DSB repair that occur along meiotic chromosome axis structures. Although many proteins are involved in establishing a crossover during meiosis, we focused on quantifying the localization pattern of two proteins that are loaded after the initial strand invasion steps of recombination. The MutS homolog MSH-4/5 and cyclin-like COSA-1 localize to intermediate steps in the meiotic DSB repair process and are required for crossover recombination events between homologous chromosomes (Kelly et al. 2000; Colaiácovo et al. 2003; Yokoo et al. 2012; Woglar and Villeneuve 2018). In early-mid pachytene, MSH-5 has been observed to form many dim foci before late pachytene, when both COSA-1 and MSH-5 localize to 6 foci, marking the positions of the obligate crossover for each of the six *C. elegans* chromosomes. Studies have shown that the synaptonemal complex—a proteinaceous structure that assembles between homologous chromosomes during meiosis—recruits MSH-5 and COSA-1 in *C. elegans* (MacQueen and Villeneuve 2001; Colaiácovo et al. 2003; Jantsch et al. 2004; Bhalla et al. 2008; Libuda et al. 2013; Cahoon et al. 2019).

We adapted the Gonad Analysis Pipeline to determine the number of MSH-5 and COSA-1 foci associated with the synaptonemal complex protein, SYP-1 throughout the germline (Figure 3, A and B). For this approach, SYP-1 staining was used instead of DAPI to generate surfaces for each individual nucleus. Next, we identified MSH-5 or GFP::COSA-1 foci, then used the “Find Spots Close to Surface” MATLAB script to identify the foci associated with each SYP-1 surface, and finally approximated the positions of these SYP-1 surfaces along the germline using the Gonad Linearization Algorithm. As the synaptonemal complex is not fully assembled until the end of the transition zone, we did not identify any SYP-1 objects in the first segmented portion of each analyzed germline, which corresponds to the pre-meiotic region (Figure 3C). In total, we identified the SYP-1 surfaces of 167 individual nuclei in a single germline stained with SYP-1 and MSH-5, and 168 individual nuclei in a single germline stained with SYP-1 and GFP::COSA-1. As previously reported (Yokoo et al. 2012; Woglar and Villeneuve 2018), MSH-5 forms >6 foci per meiotic nucleus in early-mid pachytene. Then in late pachytene (the final ~25% of the germline), GFP::COSA-1 forms bright, robust foci and both MSH-5 and COSA-1 foci counts converge to ~6 foci per nucleus, which corresponds to the 6 total crossovers formed per nucleus (Yokoo et al. 2012). Notably, MSH-5 and COSA-1 foci in late pachytene have been found to be brighter than those observed earlier in meiotic prophase I (Yokoo et al. 2012; Woglar and Villeneuve 2018). As Imaris software enables flexible thresholding of puncta based on signal intensity, it is possible to limit analysis specifically to these bright foci by changing the threshold cutoff (Supplementary Figure S5). Overall, these results show

the capability of our approach to identify nuclear structures, as well as to quantitate the subnuclear association of specific meiotic proteins with specific chromosome structures at single-nucleus resolution.

Quantification of perinuclear structures across the *C. elegans* germline

To show the ability of our method to assess extranuclear features of the *C. elegans* germline, we adapted our Gonad Analysis Pipeline to identify and quantify P granule structures that assemble within the perinuclear space of germ cells. P granules are liquid-like condensates associated with nuclear pore complexes in the *C. elegans* germline that process small RNAs (Seydoux 2018). For our analysis of P granules, we analyzed two components of P granules: PGL-1 and ZNFX-1. PGL-1 is a core component of P granules that is required for fecundity (Strome and Wood 1982; Kawasaki et al. 1998). ZNFX-1 is a P granule component required for effective transcript silencing in the germline and colocalizes with PGL-1 perinuclear foci in the germline (Ishidate et al. 2018; Wan et al. 2018).

To analyze the localization of PGL-1 and ZNFX-1 P granule components throughout the adult germline (Figure 4A), we adapted our Gonad Analysis Pipeline to initially identify and quantify the number of individual perinuclear PGL-1 and ZNFX-1 foci by creating surfaces of each focus in Imaris (Figure 4A). In total, we identified $n=4543$ PGL-1 foci and $n=3842$ ZNFX-1 foci (Figure 4B). Then, we applied the Gonad Linearization Algorithm to approximate the position of these foci relative to their progression through the germline (Figure 4B). To understand the relationship between PGL-1 and ZNFX-1, we determined the proportion of colocalized PGL-1 and ZNFX-1 along the germline in a sliding window representing 10% of total gonad length (Figure 4C). Throughout meiotic prophase I, >50% of PGL-1 and ~75% of ZNFX-1 foci are consistently colocalized. However, in late prophase I, the frequency of PGL-1 colocalization increases to ~75%. From our analysis, we also found that throughout the germline, PGL-1 foci were more frequently found unassociated with ZNFX-1 than ZNFX-1 was found unassociated with PGL-1 (Figure 4D). Together, these results agree with previous findings indicating the colocalization of these two components within the *C. elegans* hermaphrodite germline (Ishidate et al. 2018; Wan et al. 2018).

To assess the accuracy of the Gonad Analysis Pipeline in determining colocalization frequencies, we also compared the association between MSH-5 and RAD-51 foci, which mark distinct recombination intermediates and rarely interact in germ nuclei (Schwarzstein et al. 2014; Woglar and Villeneuve 2018) (Supplementary Figure S6). We found that ~6–7% of MSH-5 and RAD-51 are colocalized (Supplementary Figure S6B). Importantly, we found that the distributions of distances between colocalized MSH-5 foci with their associated RAD-51 focus, as well as between non-colocalized MSH-5 foci with the nearest RAD-51 focus, are not discretely separated (Supplementary Figure S6C). This result indicates that proximity alone does not determine colocalization, therefore supporting the accuracy of the Gonad Analysis Pipeline in identifying interactions between germline features. Overall, our data show the adaptability and customizability of the Gonad Analysis Pipeline to quantitate the changes in colocalization frequency throughout the whole *C. elegans* germline.

To test whether our method could quantify additional structural features of P granules, we quantified and compared the volume/size of individual PGL-1 and ZNFX-1 P granules to the

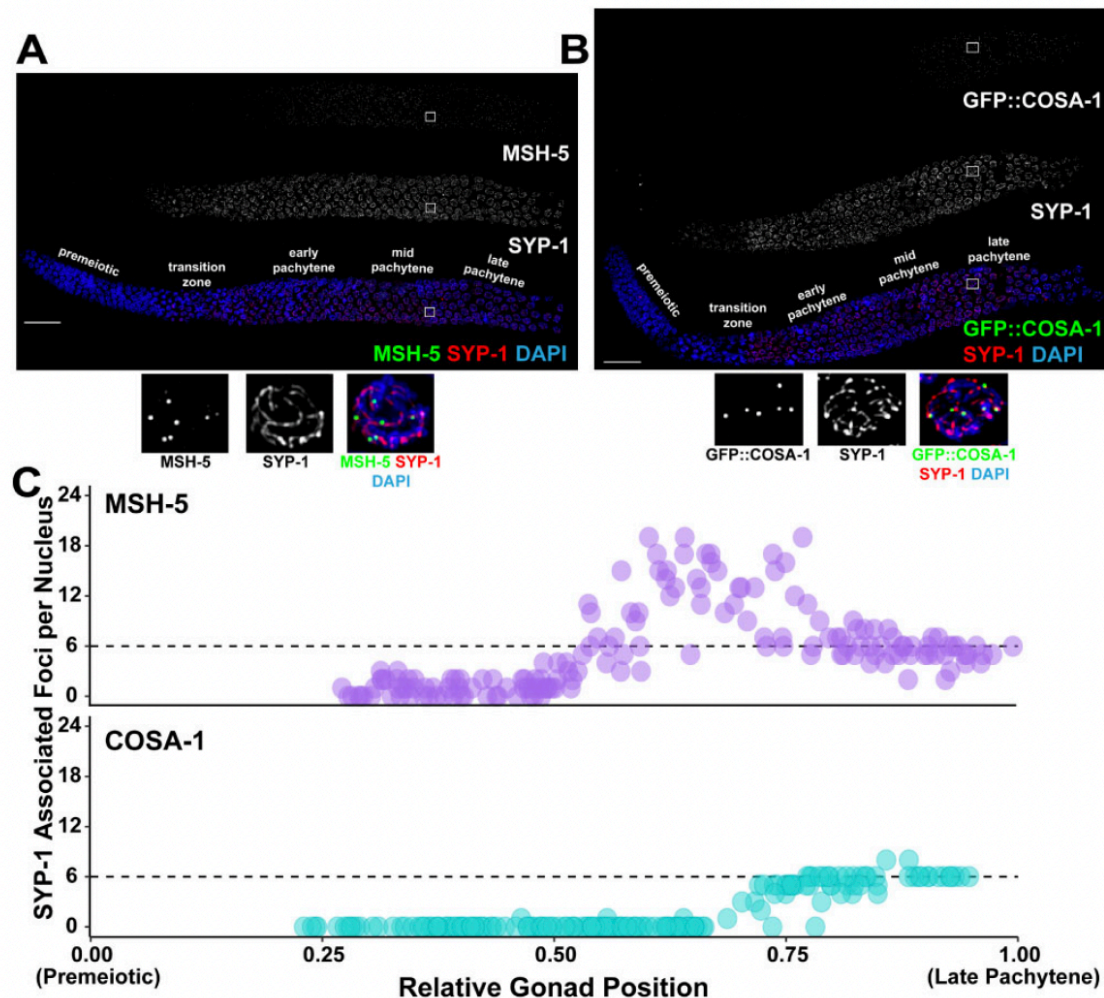


Figure 3 Single-nucleus analysis of meiotic recombination markers along the meiotic chromosome axis. Immunofluorescence images of a *C. elegans* hermaphrodite germline stained with either (A) DAPI (DNA; blue), MSH-5 (green), and SYP-1 (red), or (B) DAPI (blue), GFP::COSA-1 (green), and SYP-1 (red). Scale bar represents 20 μ m. Inset images display representative late pachytene nuclei. The location of these inset images within the germline image are indicated by white boxes on the whole gonad images. Specific meiotic stages were determined by DNA morphology. (C) Visualization of numbers of MSH-5 (purple) or GFP::COSA-1 (blue) foci associated with SYP-1 within individual nuclei across the germlines displayed in A-B. As nuclei progress through meiotic prophase I, the number of MSH-5 and COSA-1 spots converge at 6 foci per nucleus in the latter part of the germline, consistent with the reported number of MSH-5 and COSA-1 foci marking the 6 crossover sites in late pachytene (Yokoo et al. 2012).

volume/size of P granules with colocalized PGL-1 and ZNFX-1. From our analysis across meiotic prophase I, we found that the volume of foci that were colocalized were larger than individualized foci for both proteins assessed ($P < 0.001$, Mann-Whitney U test, Figure 4D). When we examined the mean volume of PGL-1 and ZNFX-1 foci in a sliding window representing 10% of total gonad length (Figure 4E), we observed that P granules with colocalization of PGL-1 and ZNFX-1 were consistently larger in volume than those granules that did not have both components present. This result may indicate that the inclusion of multiple P granule components possibly results in a synergistic increase the volume of a granule. Taken together, we have shown that our approach enables high-throughput analysis of germline granules and provides support for a model in which the composition and features

of individual P granules may change throughout meiotic prophase I progression.

Discussion

In this study, we show the utility of a customizable computational pipeline, called the Gonad Analysis Pipeline, developed to perform automated quantification of features within (or associated with) individual nuclei with reference to the position of the nuclei in the *C. elegans* gonad. Specifically, we adapt and use the Gonad Analysis Pipeline to quantify foci per nucleus, foci associated with chromosome structures, and foci colocalization frequencies across whole adult *C. elegans* hermaphrodite gonads from the pre-meiotic tip to late pachytene. This pipeline yields

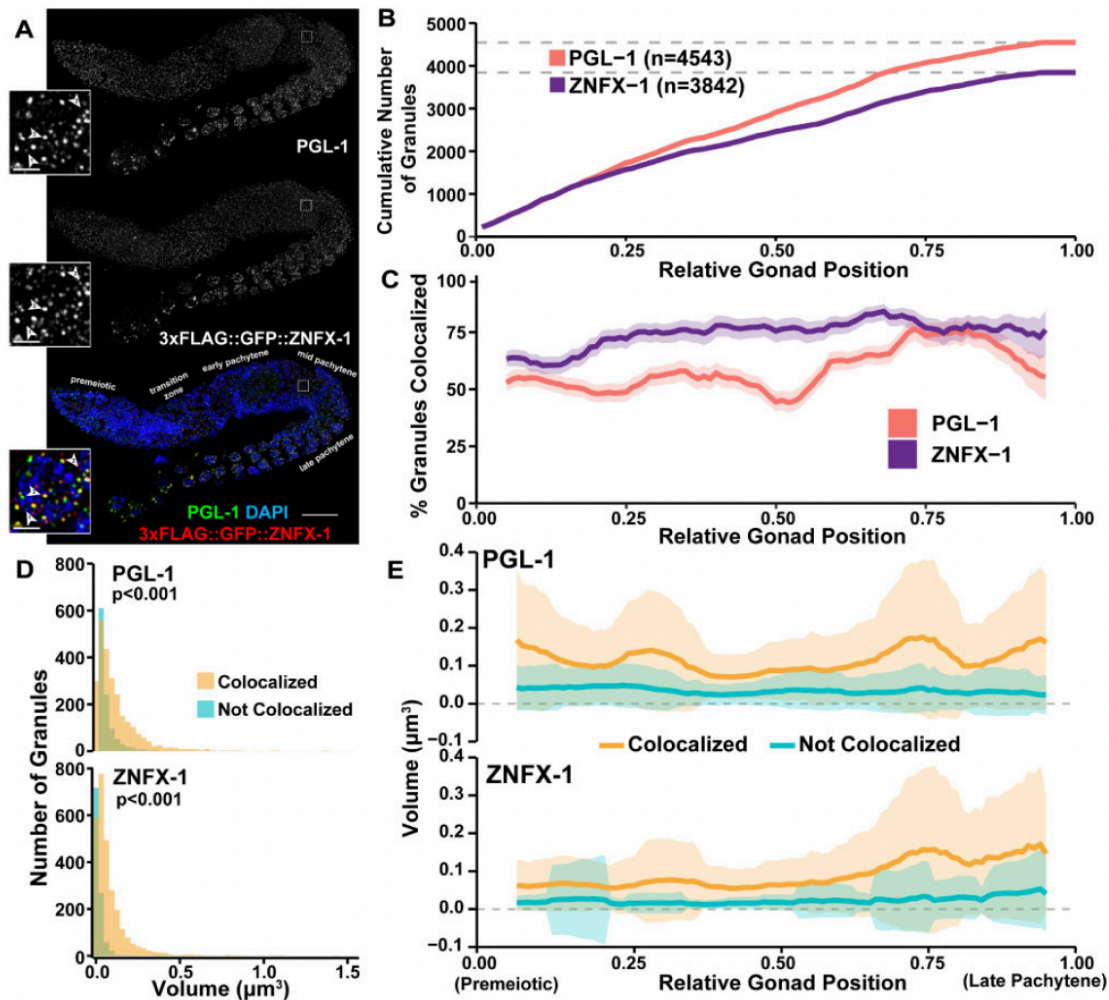


Figure 4 Assessment of P-granule components across meiotic prophase I. (A) Immunofluorescence image of a *C. elegans* hermaphrodite germline stained with DAPI (DNA), PGL-1 (green), and 3xFLAG::GFP::ZNFX-1 (red). Specific meiotic stages were determined by DNA morphology. Inset images show a single mid-pachytene nucleus. The location of these inset nuclei within the original whole germline image are indicated by white boxes on the whole gonad images. Numbered arrowheads, respectively, indicate examples of: (1) a PGL-1 focus not colocalized with ZNFX-1, (2) a ZNFX-1 focus not colocalized with PGL-1, and (3) colocalized PGL-1 and ZNFX-1 foci. The scale bar in the whole germline image represents 20 μm , whereas the scale bars in the insets represent 2 μm . (B) Cumulative number of PGL-1 and ZNFX-1 foci identified across the germline. (C) Percent of total PGL-1 and ZNFX-1 foci which are, respectively, colocalized within a sliding window representing 10% of total germline length. Shaded area represents 95% Binomial Confidence Interval. (D) Histograms displaying the distribution of PGL-1 and ZNFX-1 foci volumes, distinguishing between foci colocalized (yellow) or not colocalized (blue) with other respective protein. P values were calculated from comparisons between colocalized and non-colocalized focus volumes by Mann-Whitney U test. (E) Mean volume of PGL-1 and ZNFX-1 foci in a sliding window representing 10% of total germline length, distinguishing between foci which are (yellow) or are not (blue) colocalized. Shaded area represents standard deviation.

datasets concordant with previous observations for known features of meiotic prophase I (Colaiácovo et al. 2003; Rosu et al. 2011; Nottke et al. 2011; Yokoo et al. 2012; Rosu et al. 2013; Schvarzstein et al. 2014; Ishidate et al. 2018; Wan et al. 2018; Woglar and Villeneuve 2018). Additionally, many *C. elegans* mutants defective in key meiotic events such as synapsis and pairing can have aberrant DNA morphology and disruption of normal meiotic stage progression. These defects make it difficult to use DNA morphology to discern the specific transitions between meiotic stages and challenging to categorically delineate nuclei within those germline contexts. Our automated Gonad Analysis Pipeline provides a consistent metric using position

along the normalized gonad length for comparative analysis of mutants to wildtype germlines.

Although analyses presented here assess nuclei from the premeiotic tip to late pachytene of the *C. elegans* germline, our pipeline can also be extended to include more proximal portions of the germline for quantitative analyses of other germline features. For example, P granules display a dynamic localization pattern throughout the germline, changing from cytoplasmic localization in the distal region of the germline to a more perinuclear localization in the more proximal region of the germline (Seydoux 2018). Our computational pipeline could be adapted to quantify these changes in P granule localization across the entire *C. elegans*

germline and perform comparative studies of these nucleus-cytoplasm localization dynamics between wild type and mutant contexts. Additionally, several studies have found dynamic changes to the localization of specific synaptonemal complex components during meiotic prophase progression (Martinez-Perez et al. 2008; Severson et al. 2009; Tzur et al. 2012; Nadarajan et al. 2016). Our pipeline can also be used to quantify these changes in the chromosome axis and the synaptonemal complex from transition zone through diakinesis.

Our analyses show how small customizable changes to the Gonad Analysis Pipeline can enable quantification at multiple levels from the entire germline to single nuclei. Further changes can enable the additional quantifications of cytological objects, such as sphericity, intensity, and relative distance between objects. Use of these other quantifiable metrics enables a comprehensive analysis of many germ cell features, including the quantification of chromosome pairing for fluorescence in situ hybridization (FISH) experiments, assembly and disassembly of chromosome structures, and protein dynamics during live cell imaging. In particular for live imaging, the pipeline could assess changes in numerous metrics such as velocity, mean square displacement, duration, volume, and sphericity of objects over time for all nuclei during oogenesis and contextualize these statistics based on nuclear position within the germline. These types of adaptations of our Gonad Analysis Pipeline for live imaging may prove particularly powerful for quantification of the liquid-like properties and dynamics of P granules in the adult germline, especially in response to different stresses or aging.

The present study focuses on adult hermaphrodite germlines; however, the Gonad Analysis Pipeline can also be used to analyze larval germlines and adult male germlines. An increasing number of studies are showing the power of performing comparative analyses between oogenesis and spermatogenesis in *C. elegans* to identify important sexual dimorphic features of meiosis (Jaramillo-Lambert et al. 2007; Van et al. 2016; Cahoon and Libuda 2019; Li et al. 2020). Spermatogenesis in the germlines of *C. elegans* males is also organized in a spatial-temporal gradient (Shakes et al. 2009) and can easily be analyzed by our pipeline, thereby aiding both studies of spermatogenesis as well as sexual dimorphism of germ cell development. Additionally, this approach and protocol could be adapted to use with other tissues and organisms. For example, many developing tissues do not follow a straight line, such as the epithelium of the *Drosophila* wing disc, and this protocol can be used to linearize elements within the developing tissue that might help to reveal different aspects of cellular migration and development.

Taken together, we have generated and validated an automated and customizable image analysis resource for the *C. elegans* germline community. Our Gonad Analysis Pipeline enables standardized quantification of diverse features of the *C. elegans* gonad. Moreover, our approach is flexible and could be applied to analyze features of other tissues composed of cells organized along a linear gradient.

Acknowledgments

We thank A. Villeneuve for antibodies, the CGC [funded by National Institutes of Health (NIH) P40 OD010440] for strains, and Adele Adamo for providing the pET30a plasmid. We thank the members of the Libuda Lab for many insightful and helpful discussions in refining this method. We also thank A. Villeneuve for encouraging us to write up and publish this method.

Funding

This work was supported by the National Institutes of Health [grant number T32GM007413 to ET and AD]; Advancing Science in America (ARCS) Foundation Award [to ET]; National Institutes of Health [grant number F32GM130006 to NAK]; Jane Coffin Childs Postdoctoral Fellowship [to CKC]; and National Institutes of Health [grant number R35GM128890 to DEL]. DEL is also a recipient of a March of Dimes Basil O'Connor Starter Scholar award and Searle Scholar Award. The funders had no role in the research design, execution, analysis, interpretation, and/or reporting.

Conflicts of interest

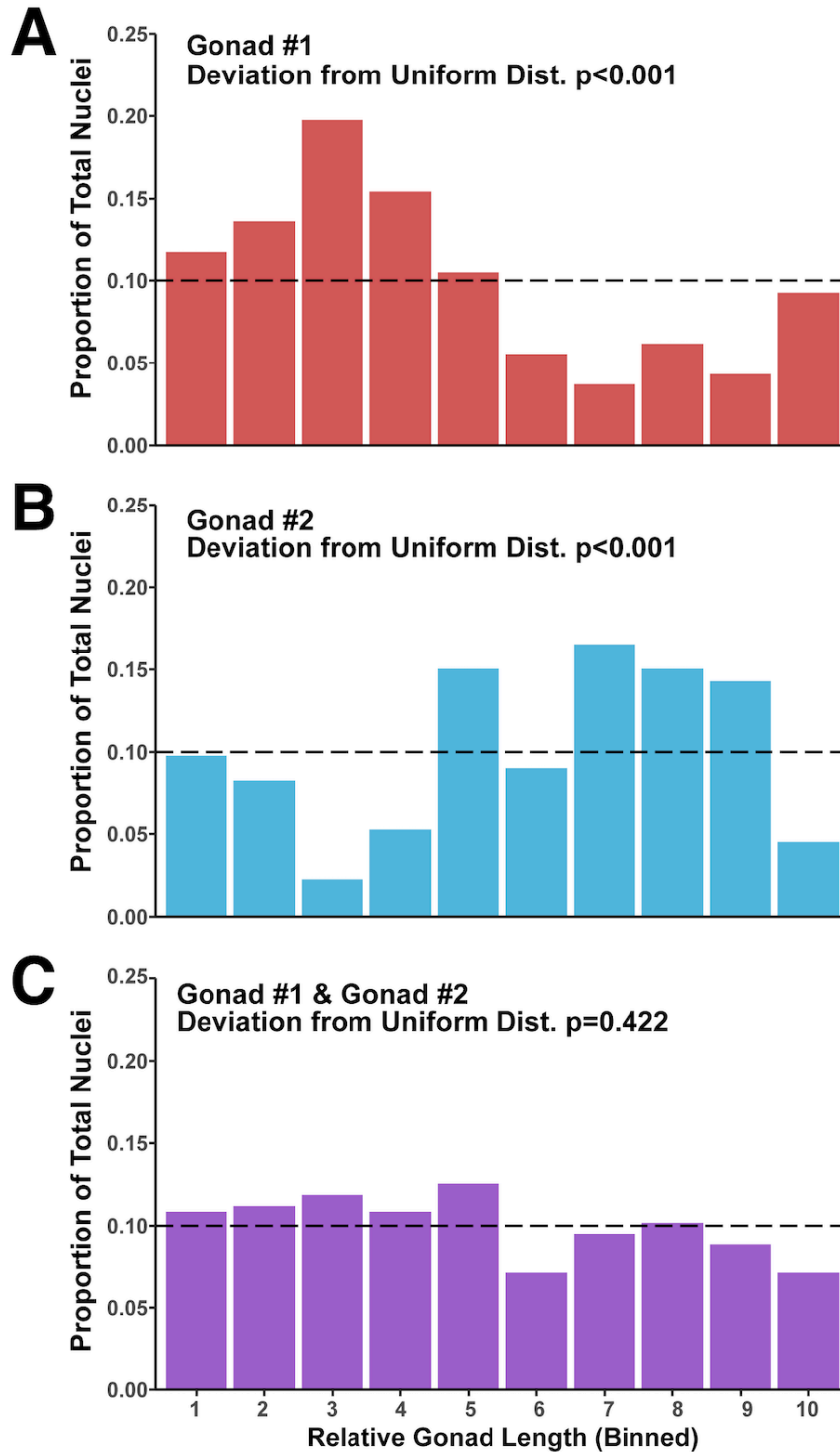
None declared.

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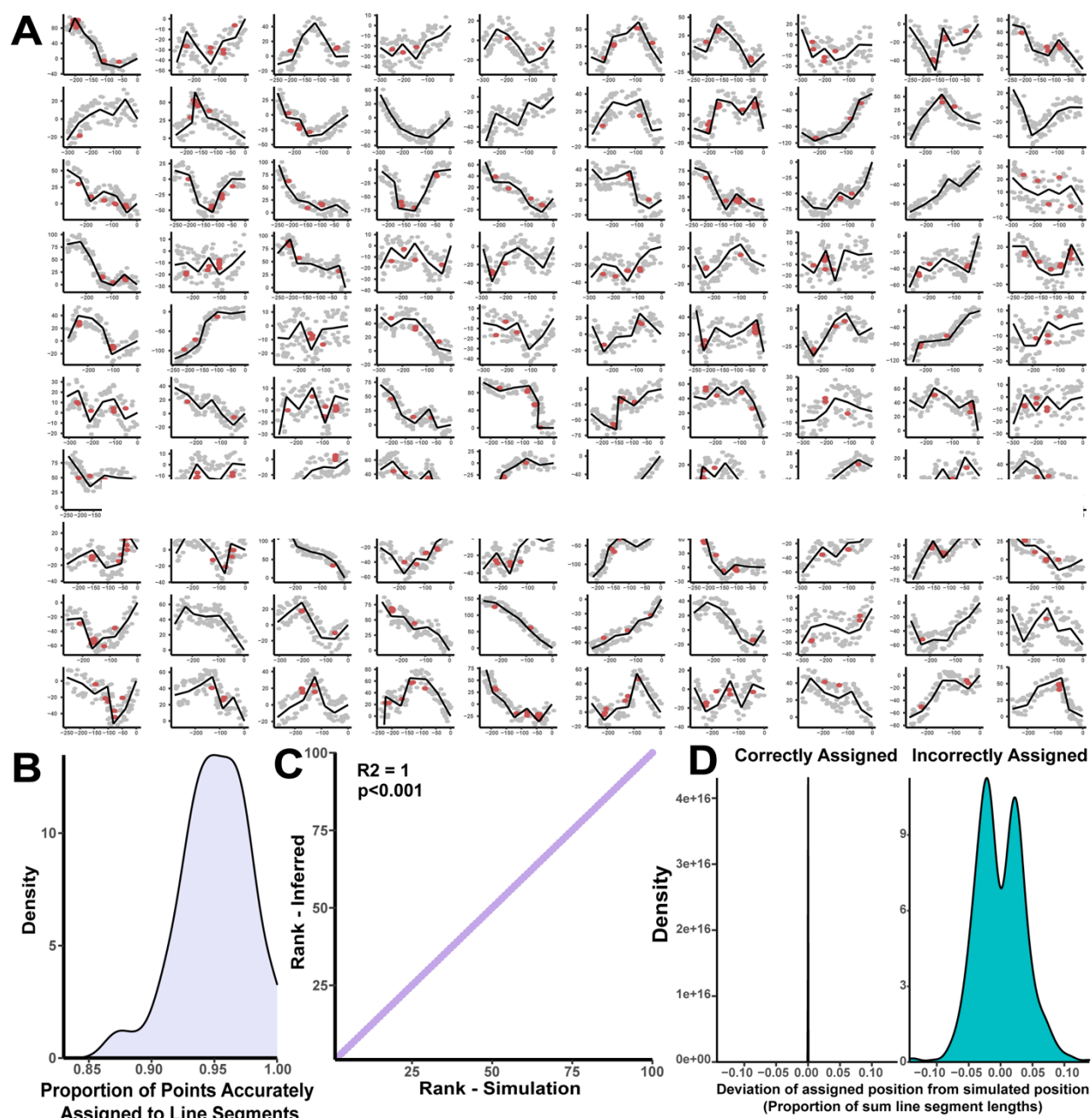
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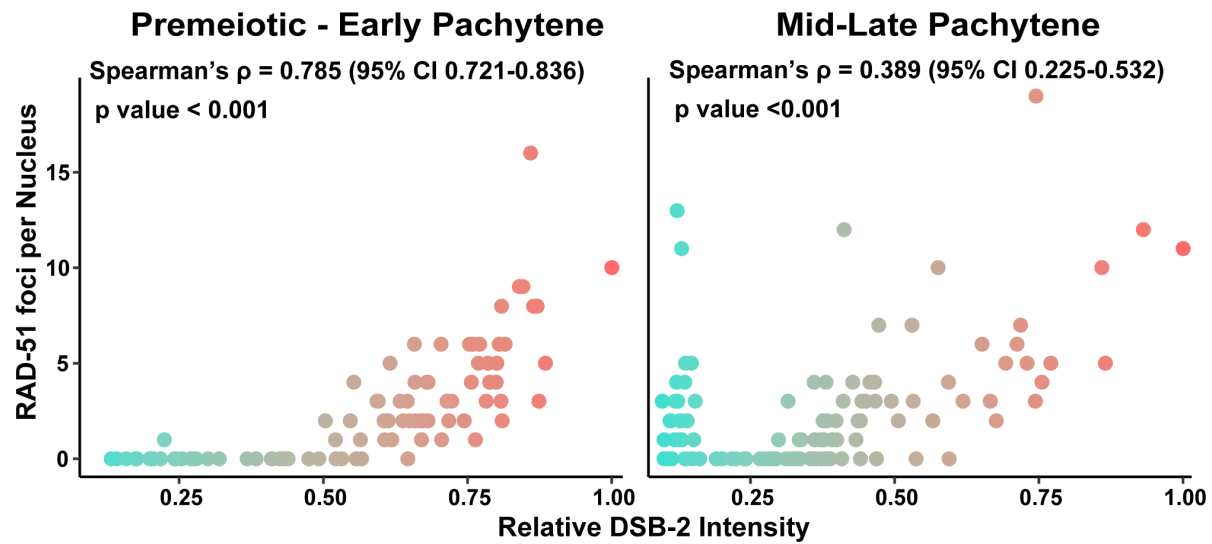
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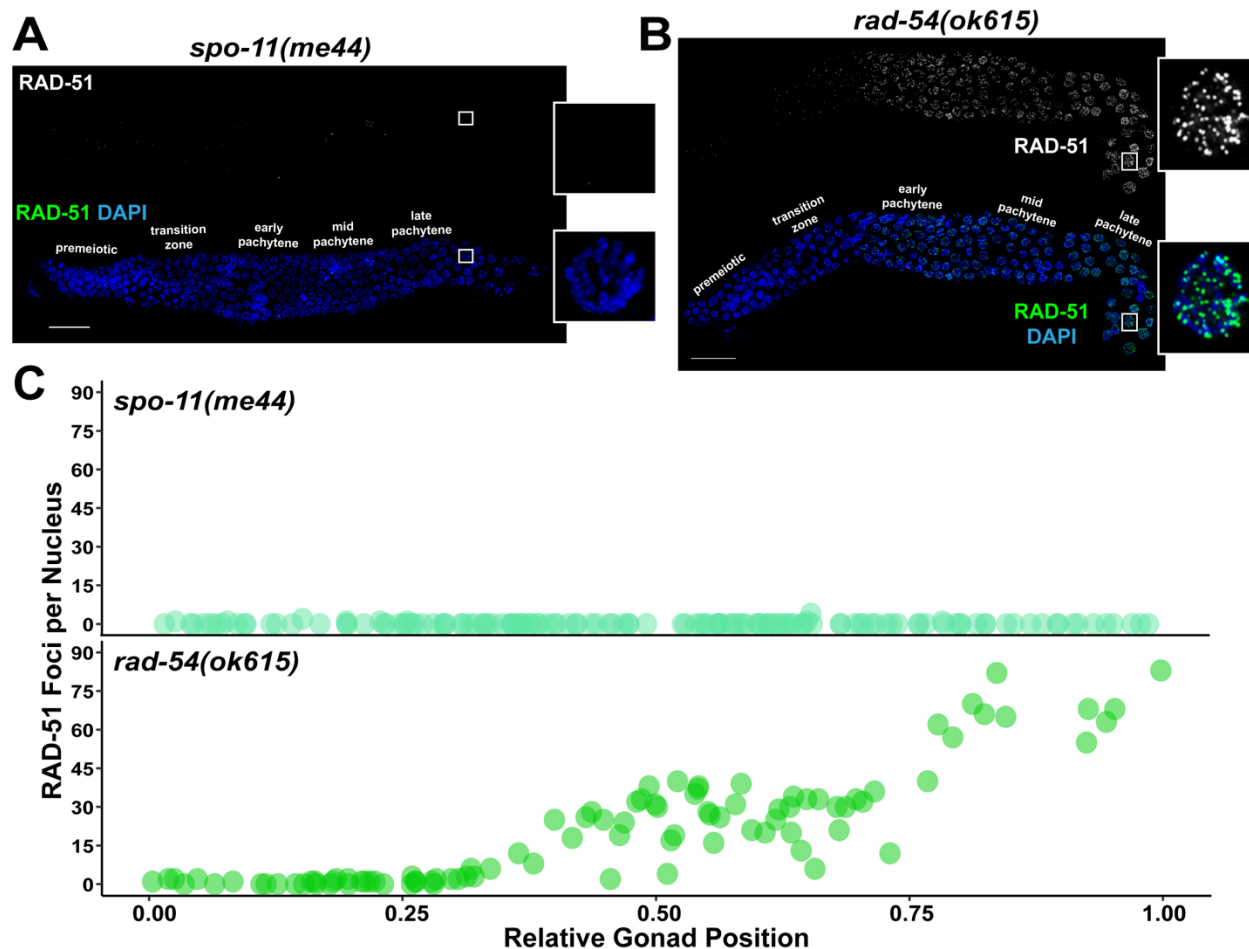
Supplemental Figure 1. Position of individual nuclei within whole germlines identified for computational analysis. Bar plots representing the proportion of nuclei in ten equal bins across the lengths of the two gonads analyzed in Figure 2. P values in all panels were calculated by Chi Square Test of Goodness of Fit (expected frequency 0.1 in each bin). (A-B) Number of nuclei within bins across the lengths of analyzed individual germlines. The distribution of nuclei computationally identify by the Whole Gonad Pipeline along the length of each gonad was nonuniform. (C) Number of nuclei within bins of combined gonads analyzed in panels A-B. The distribution of nuclei within bins is indistinguishable from a uniform distribution by this same test when the nuclei from the two germlines are taken together.



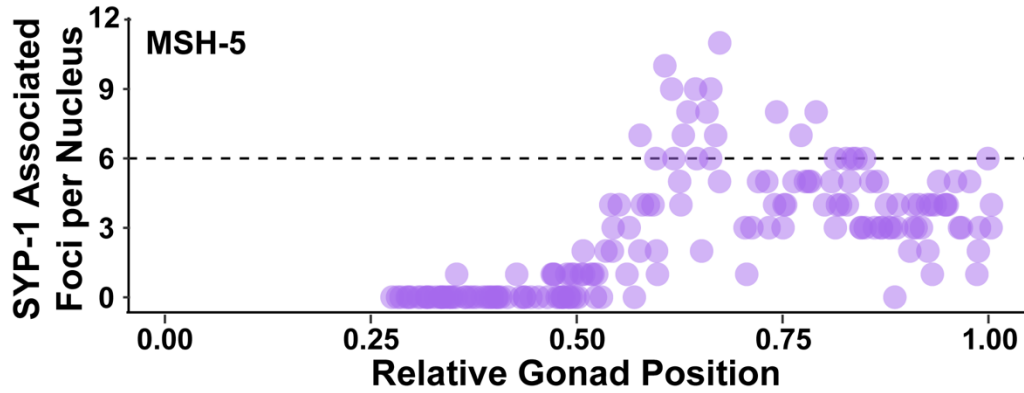
Supplemental Figure 2. Validation of Gonad Linearization algorithm. (A) Plots of simulated dataset of 100 'germlines' each with 100 'nuclei' points dispersed along their lengths. Points were realigned to the central lines using the Gonad Linearization algorithm, and points that were aligned to the correct line segment are marked in grey while points marked in red were aligned to the incorrect line segment. (B) Density plot demonstrating the distribution of accuracy of point alignment to line segments among the 100 individual simulated 'gonads'. (C) Comparison of the known rank order of correctly aligned spots to the rank order of spots as determined by the Gonad Linearization algorithm. R2 and p values were calculated by linear regression analysis. (D) Calculation of the deviation of assigned positions as determined by the Gonad Linearization algorithm from 'actual' known positions from the original simulation.



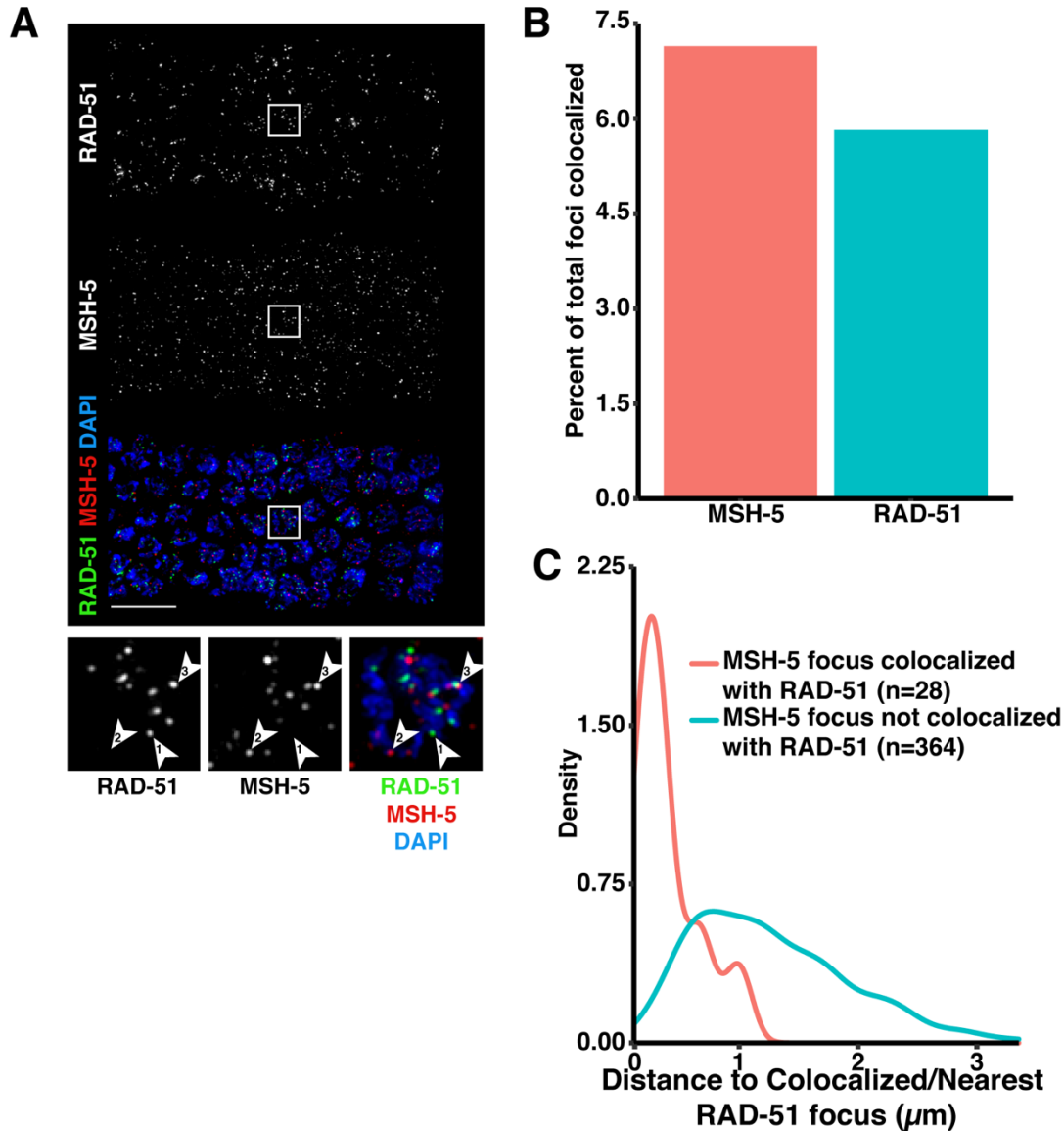
Supplemental Figure 3. DSB-2 normalized intensity per nucleus correlates with RAD-51 foci per nucleus. Assessment of nonparametric correlation by Spearman correlation tests between RAD-51 foci per nucleus and normalized DSB-2 staining intensity among nuclei within the premeiotic through early pachytene stages, and in mid- through late pachytene stages. Specific meiotic stages determined based on DNA morphology.



Supplemental Figure 4. RAD-51 foci per nucleus quantification in meiotic mutants. Immunofluorescence images of (A) a *spo-11(me44)* null mutant hermaphrodite germline (no endogenous DSBs made during meiotic prophase I progression), and (B) a *rad-54(ok615)* null mutant hermaphrodite germline (RAD-51 cannot be unloaded during meiotic prophase I progression) stained with DAPI (DNA; blue) and RAD-51 (green). Scale bar represents 20 μ m. Inset images display representative late pachytene nuclei. The position these nuclei are located within the germline are indicated by white boxes on the whole gonad images. Specific meiotic stages determined based on DNA morphology. Image in (B) is an immunofluorescence image from a supplemental figure in Rosu *et al.*, *Science* 2011 that has been requantified utilizing the method described in this manuscript. (C) Visualization of numbers of RAD-51 foci associated with individual nuclei across the germlines displayed in A-B. Mutants deficient in *spo-11* are unable to induce meiotic DNA breaks, resulting in very few DSBs observed within the germline (Colaiácovo *et al.* 2003). Mutants for *rad-54* are unable to complete DSB repair, and so exhibit elevated and persistent RAD-51 marked DSBs (Mets and Meyer 2009; Rosu *et al.* 2011; Nottke *et al.* 2011).



Supplemental Figure 5. Quantification of 'bright' MSH-5 foci associated with the chromosome axes of individual meiotic nuclei. Visualization of numbers of MSH-5 foci associated with SYP-1 within individual nuclei across the germline displayed in Figure 3A. As MSH-5 foci are known to become brighter in late prophase I, we restricted our analysis to account for only the brightest foci in the germline. This dataset, composed of identical nuclei to those quantified in the top panel of Figure 3C, demonstrates the flexible capacity of Imaris software in conjunction with our Whole Gonad Pipeline to specifically identify and quantify specific subpopulations of meiotic biological features.



Supplemental Figure 6. Colocalization analysis of meiotic features which infrequently colocalize.

To determine the accuracy of our colocalization analysis (Figure 4), we assessed the association of MSH-5 and RAD-51, which mark distinct recombination intermediates and rarely colocalize (Schwarzstein *et al.* 2014). (A) Immunofluorescence image of a *C. elegans* hermaphrodite germline stained with DAPI (DNA; blue), RAD-51 (green), and MSH-5 (red). Our analysis focused on early-mid pachytene where MSH-5 and RAD-51 foci are most frequently observed in the same nuclei. Scale bar represents 10 μm . Inset images display a representative early pachytene nucleus. The position at which this nucleus is located within the germline is indicated by white boxes on the gonad images. Numbered arrowheads respectively indicate examples of: (1) a RAD-51 focus not colocalized with MSH-5, (2) a MSH-5 focus not colocalized with RAD-51, and (3) colocalized RAD-51 and MSH-5 foci. (B) Proportion of MSH-5 (7.1%; 28/392) and RAD-51 (5.8%; 28/481 foci) foci determined to be colocalized with the respective other protein foci. (C) Density plot displaying the distributions of distances between MSH-5 foci and their respective colocalized RAD-51 focus, or between non-colocalized MSH-5 foci and the nearest RAD-51 focus.

A prion accelerates proliferation at the expense of lifespan

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Abstract In fluctuating environments, switching between different growth strategies, such as those affecting cell size and proliferation, can be advantageous to an organism. Trade-offs arise, however. Mechanisms that aberrantly increase cell size or proliferation—such as mutations or chemicals that interfere with growth regulatory pathways—can also shorten lifespan. Here we report a natural example of how the interplay between growth and lifespan can be epigenetically controlled. We find that a highly conserved RNA-modifying enzyme, the pseudouridine synthase Pus4/TruB, can act as a prion, endowing yeast with greater proliferation rates at the cost of a shortened lifespan. Cells harboring the prion grow larger and exhibit altered protein synthesis. This epigenetic state, [BIG⁺] (better in growth), allows cells to heritably yet reversibly alter their translational program, leading to the differential synthesis of dozens of proteins, including many that regulate proliferation and aging. Our data reveal a new role for prion-based control of an RNA-modifying enzyme in driving heritable epigenetic states that transform cell growth and survival.

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Competing interest: See page 32

Funding: See page 32

Received: 09 July 2020

Preprinted: 10 July 2020

Accepted: 12 August 2021

Published: 21 September 2021

Reviewing Editor: Weiwei Dang, Baylor College of Medicine, United States

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Introduction

Cell size and proliferation are fundamental determinants of development, survival, and disease (Su and O'Farrell, 1998). Although they can be independently controlled—for example, when cell division is disrupted, cell size continues to increase, whereas restricting cell growth does not completely inhibit division (Johnston et al., 1977)—coupling is common due to their dependence on the same biochemical building blocks (Su and O'Farrell, 1998; Turner et al., 2012). Changing cell size and proliferation genetically or through chemical perturbation—whether via diet, nutrient sensing (e.g., TOR signaling), or growth factor signaling and stress responses—also impacts lifespan across eukaryotes (Fontana et al., 2010; Kenyon, 2010; Pitt and Kaeberlein, 2015). The importance of tightly controlling each of these properties is underscored by the many mutations affecting cell size and proliferation that are pathogenic, leading to cancer, developmental abnormalities, and numerous diseases of age (reviewed in Hanahan and Weinberg, 2011; Saxton and Sabatini, 2017).

Organisms commonly alter the relationship between cell size and proliferation during development (Su and O'Farrell, 1998). In oocytes of *Drosophila melanogaster*, for example, a massive expansion in cytoplasmic volume without cellular division precedes a later step of numerous cellular divisions without cytoplasmic growth. The rates of cell size increase and proliferation can be strongly coupled to nutrient sensing and changes in metabolism (Efeyan et al., 2015; Turner et al., 2012). Thus, organisms can use genetically encoded signaling pathways to commit to different strategies depending on their needs and environment (Ivanov et al., 2015; Jung et al., 2018). Epigenetic tuning of cell

eLife digest Cells make different proteins to perform different tasks. Each protein is a long chain of building blocks called amino acids that must fold into a particular shape before it can be useful. Some proteins can fold in more than one way, a normal form and a 'prion' form. Prions are unusual in that they can force normally folded proteins with the same amino acid sequence as them to refold into new prions. This means that a single prion can make many more that are inherited when cells divide. Some prions can cause disease, but others may be beneficial.

Pus4 is a yeast protein that is typically involved in modifying ribonucleic acids, molecules that help translate genetic information into new proteins. Sometimes Pus4 can adopt a beneficial prion conformation called [BIG⁺]. When yeast cells have access to plenty of nutrients, [BIG⁺] helps them grow faster and larger, but this comes at the cost of a shorter lifespan.

Garcia, Campbell et al. combined computational modeling and experiments in baker's yeast (*Saccharomyces cerevisiae*) to investigate the role of [BIG⁺]. They found that the prion accelerated protein production, leading to both faster growth and a shorter lifespan in these cells, even without any changes in gene sequence.

Garcia, Campbell et al.'s findings explain the beneficial activity of prion proteins in baker's yeast cells. The results also describe how cells balance a tradeoff between growth and lifespan without any changes in the genome. This helps to highlight that genetics do not always explain the behaviors of cells, and further methods are needed to better understand cell biology.

size and proliferation could in principle provide a stable yet reversible mechanism to alter the relationship between these properties according to differing needs in fluctuating environments. Histone modifications can enable such adaptation in a way that can be heritable over several mitotic divisions. However, apart from a few notable exceptions (Catania et al., 2020; Grewal and Klar, 1996; Nakayama et al., 2000), most studied examples are erased during meiosis (Heard and Martienssen, 2014; Moazed, 2011).

Prions are distinct among epigenetic mechanisms in that they are faithfully transmitted through both mitotic and meiotic cell divisions (Brown and Lindquist, 2009; Cox, 1965; Garcia and Jarosz, 2014; Wickner, 1994). The unusual folding landscape of prion proteins, which allows the recruitment of proteins from the naïve to the prion fold, promotes a mode of inheritance that is both stable and reversible (Chakrabortee et al., 2016a; McKinley et al., 1983). For example, transient perturbations in molecular chaperone activity (Brown and Lindquist, 2009; Chernoff et al., 1995), specific environmental stressors (Garcia et al., 2016; Singh et al., 1979; Tuite et al., 1981), or regulated proteolysis (Ali et al., 2014; Kabani et al., 2014) can induce or eliminate prion states. It is now appreciated that this form of information transfer is far more common than previously realized, occurring not only in mammals but also in wild fungi, plants, and even bacteria (Chakrabortee et al., 2016b; Halfmann et al., 2012; Yuan and Hochschild, 2017).

Here we investigate the inheritance of a prion-based epigenetic state that alters yeast cell physiology, potentiating a trade-off between proliferation and lifespan. We first describe the [BIG⁺] prion—driven by the conserved pseudouridine synthase Pus4 (known as TruB in mammals and bacteria)—which increases proliferation but shortens lifespan. We then quantitatively model the adaptive value of this 'live fast, die young' growth strategy in fluctuating environments. [BIG⁺] cells are larger and exhibit increased protein synthesis while maintaining Pus4-dependent pseudouridylation activity. Finally, we find evidence for analogous Pus4-dependent epigenetic control of cell size in wild yeast. The epigenetic inheritance of an altered form of an RNA-modifying enzyme over long biological timescales, as occurs in [BIG⁺], reveals a new mechanism by which regulation of these activities can be perpetuated across generations.

Results

Cells bearing a prion-like epigenetic element live fast and die young

We recently discovered more than 40 protein-based epigenetic elements in *Saccharomyces cerevisiae* that are both heritable and reversible upon transient perturbation of protein chaperone

function (Chakrabortee *et al.*, 2016a). Many of the proteins that underlie this behavior have the potential to regulate growth. One of these epigenetic states was induced by transient overexpression of the highly conserved pseudouridine synthase *PUS4*/TRUB, which catalyzes the formation of a ubiquitous pseudouridine on U55 in tRNAs in bacteria, yeast, and humans (Becker *et al.*, 1997; Gutgsell *et al.*, 2000; Zucchini *et al.*, 2003). Mutation of U55 leads to large fitness deficits, second only to mutations in the tRNA anticodon loop, highlighting the functional importance of this nucleotide (Li *et al.*, 2016). Mutation of U55 is also linked to deafness and diabetes in humans (Wang *et al.*, 2016). We originally discovered this epigenetic state because transient overexpression of *PUS4* led to an enduring and heritable growth improvement in medium containing zinc sulfate (Chakrabortee *et al.*, 2016a). This phenotype could possibly be explained by the strong genetic interaction between *PUS4* and the zinc homeostasis regulator *ZRC1*—among all genes, *ZRC1* is the second strongest genetic interactor with *PUS4* (Koh *et al.*, 2010). Upon closer examination, we noticed that cells harboring the *Pus4*-induced element also achieved an ~60% faster maximal proliferation rate than naïve cells in the absence of zinc stress in standard rich medium (YPD; Figure 1A, $p=0.0095$, unpaired t-test). We therefore initially named this mitotically heritable element ‘Big⁺’ for better in growth.

We next tested whether this growth advantage from the Big⁺ epigenetic element was more pronounced during direct competition and oscillating nutrient availability—as a single-celled organism such as yeast often faces in nature. To do so, we performed a competition experiment that encompassed periods of abundant nutrient availability followed by starvation. Using resistance to canavanine as a marker to distinguish naïve and Big⁺ strains, we mixed equal numbers of cells from each strain. We propagated the mixed culture for approximately 100 generations, diluting into fresh medium and measuring the fraction of the population that harbored the canavanine resistance marker every 10 generations (Figure 1—figure supplement 1A). In these experiments, cells harboring Big⁺ invariably outcompeted the genetically identical naïve cells that lacked it—they live fast—with a selection coefficient of nearly 1% (Figure 1B). Competitions using reciprocally marked strains produced equivalent results. As a frame of reference, the fitness advantage that we measured for Big⁺ is larger than those conferred by >30% of non-essential genes (Breslow *et al.*, 2008), and the vast majority of natural genetic variants that have been quantified (Jakobson and Jarosz, 2019; Jakobson *et al.*, 2019; Sharon *et al.*, 2018; She and Jarosz, 2018).

Given the close link between proliferation and aging in many organisms (Bitto *et al.*, 2015), we investigated whether Big⁺ also influenced lifespan. Studies of aging in budding yeast have led to the discovery of numerous genes with conserved roles in aging of metazoans (Kaeberlein, 2010). These studies have measured two types of aging, both of which we tested here—chronological lifespan and replicative lifespan (RLS; Longo *et al.*, 2012). Chronological lifespan is a measure of post-mitotic viability, during which cells cease division under starvation conditions until nutrients become available again. These conditions occur commonly in the natural ecology of budding yeast (Landry *et al.*, 2006). To investigate, we aged cultures of naïve cells and genetically identical cells harboring Big⁺ (Figure 1—figure supplement 1B). Over the course of 80 days, Big⁺ cells had progressively lower viability than matched isogenic-naïve controls (Figure 1C).

RLS measures the number of cell divisions that yeast can undergo before death (Figure 1—figure supplement 1B). These experiments revealed a significant difference in replicative potential between Big⁺ and naïve cells—the median survival rate is reduced by seven generations (Figure 1D, $p<0.0001$, Gehan–Breslow–Wilcoxon test, and Figure 1—figure supplement 1C). This degree of shortening is comparable to that of classic lifespan-altering mutants: yeast lacking *SIR3* or *SIR4* have reductions of ~3–4 generations, and yeast lacking *SIR2* by ~10–11 generations (Kaeberlein *et al.*, 2005; Kaeberlein *et al.*, 1999). Thus, Big⁺ cells harboring this *Pus4*-induced element exhibit both a significantly decreased chronological lifespan and RLS: they die young.

Modeling trade-offs between proliferation and lifespan

We next investigated the adaptive value of the Big⁺ phenotype by quantitatively modeling its fitness consequences in fluctuating environments, where committing to a single strategy can impose limits on the long-term fitness of a population. For example, when nutrient-rich periods tend to greatly exceed starvation periods, the rapid growth of the Big⁺ phenotype might be favored, despite its die-young phenotype (Figure 2A). However, this same decision could be maladaptive if growth conditions

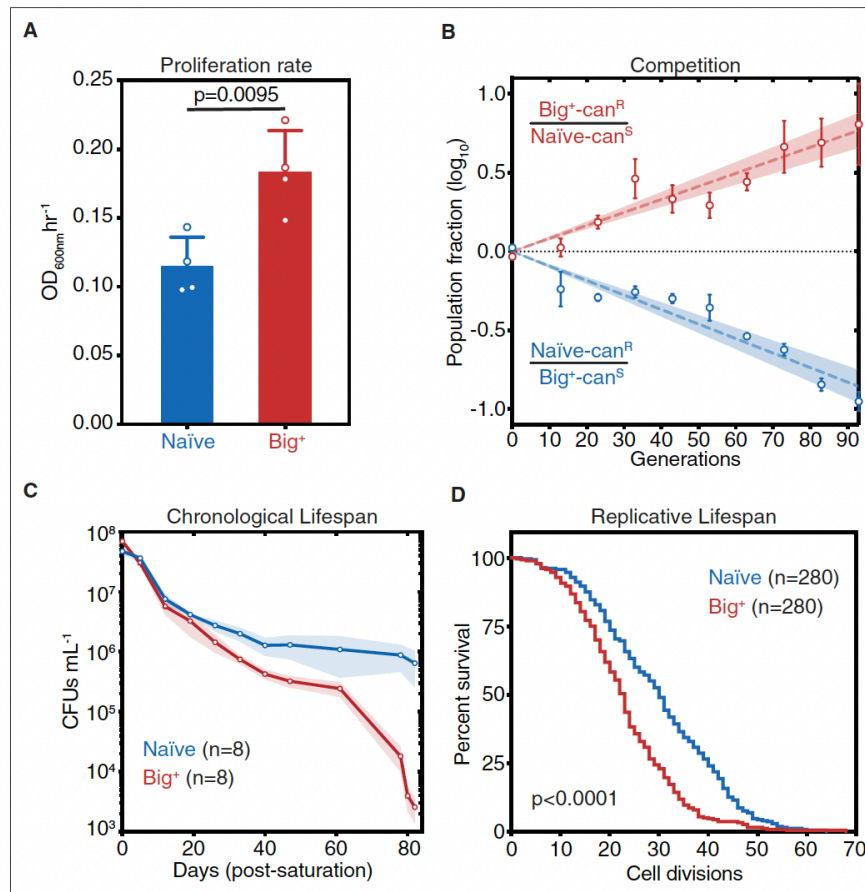


Figure 1. Cells bearing a prion-like epigenetic element live fast and die young. (A) Big⁺ cells proliferate faster than naïve cells. Bars represent the mean of four replicates of maximum growth rate in YPD medium (measured by the peak of the derivative of the growth data), error bars are standard deviation, $p=0.0095$, unpaired t-test. (B) In a direct growth competition, Big⁺ cells outcompete naïve cells. Raw data with standard error bars; trend line is dashed line showing shaded standard error; four replicates were performed for each competition. (C) Big⁺ cells have a reduced chronological lifespan (CLS). Cells were grown to saturation in rich medium and then transferred to nutrient-poor medium and allowed to age for up to 80 days. Periodically samples were replated onto rich medium to measure remaining viability via colony-forming units (CFUs). Thin lines are the average value from eight biological replicates with standard error represented by shading. (D) Big⁺ cells have a reduced replicative lifespan (RLS). Starting with virgin mother cells, at each cell division, daughter cells were separated, and the total number yielded was counted for each replicate. $n = 280$ per strain, combined from three independent experiments. p -value < 0.0001 , by Gehan–Breslow–Wilcoxon test. Median survival: naïve = 30 generations, Big⁺ = 23 generations.

The online version of this article includes the following figure supplement(s) for figure 1:

Figure supplement 1. Probing lifespan of Big⁺ cells.

skewed towards frequent and more extended periods of nutrient scarcity—where cells that grow slower and die older would instead have an advantage (Figure 2B).

To quantify these trade-offs, we considered a population in which individuals heritably adopted one of these two strategies (live-fast-die-young, *LF-DY*, like Big⁺ cells; or live-slow-die-old, *LS-DO*, like naïve cells), modeling their fates in alternating nutrient environments (Figure 2C). These simulations suggested that *LS-DO* cells should outcompete *LF-DY* cells when the periods of starvation are much longer than periods of nutrient abundance (Figure 2—figure supplement 1A). By contrast,

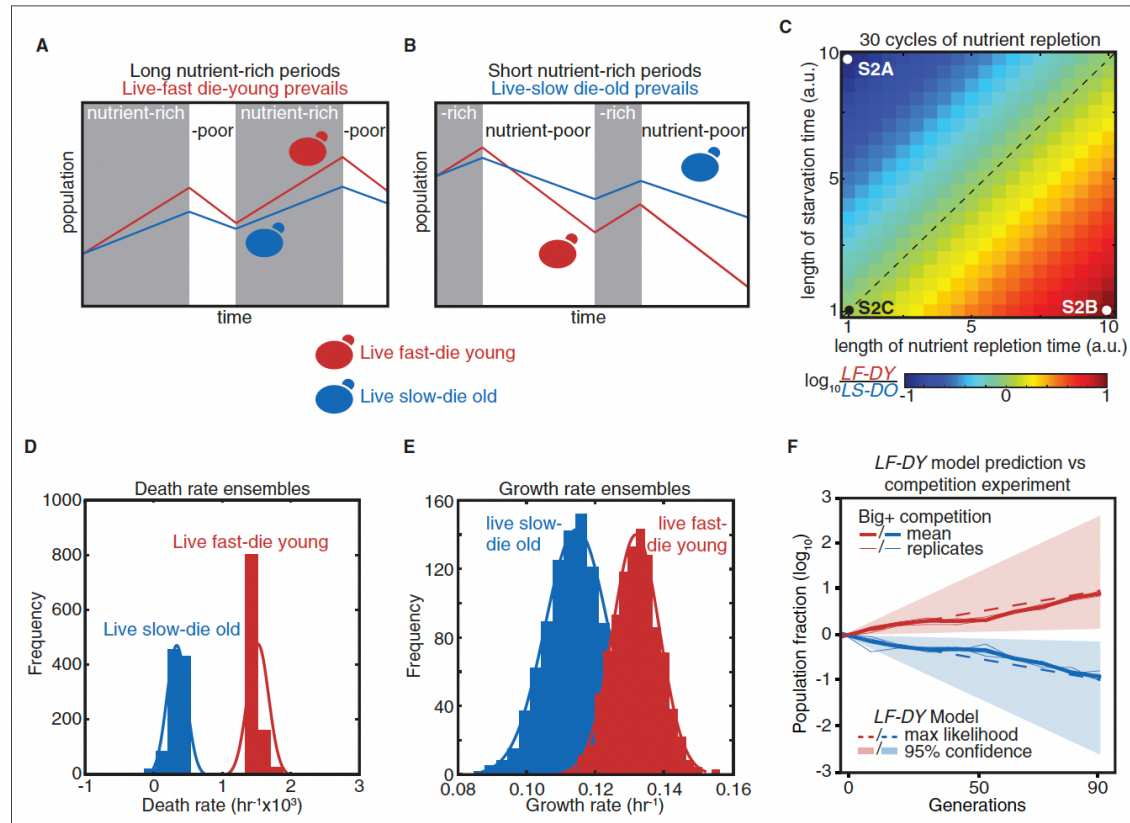


Figure 2. Modeling a reversible epigenetic live fast and die young strategy. (A) A live fast–die young epigenetic element is beneficial for survival in environments with regular, extended nutrient-rich periods. (B) A live slow–die old growth state is beneficial for survival during conditions of repeated and extended starvation. (C) Simulated final population fraction (ratio of live-fast-die-young [LF-DY] to live-slow-die-old [LS-DO]) after 30 cycles of nutrient repletion and starvation, assuming a 1% growth advantage, a 1% higher death rate, and equal starting population sizes. Note log scale. (D) Monte Carlo sampling of exponential decay constant and (E) exponential growth constant distributions used to generate the ensemble of simulations shown in (F). (F) Monte Carlo simulation (dashed lines) of growth competition between LF-DY to LS-DO cells under parameters sampled from experimental growth and lifespan measurements of the Big⁺ element (Figure 2D and E). 95% confidence interval indicated by shaded areas. Shown in solid lines are the results of competitive growth between Big⁺ and naïve cells as shown in Figure 1B (mean: bold line; n = 4 biological replicates: thin lines).

The online version of this article includes the following figure supplement(s) for figure 2:

Figure supplement 1. Modeling a reversible epigenetic live fast and die young strategy.

LF-DY cells come to dominate the simulated culture when periods of nutrient abundance are much longer than periods of starvation (Figure 2—figure supplement 1B). When periods of starvation and nutrient repletion are of equal duration, both populations are equally fit (Figure 2—figure supplement 1C). When we varied the growth advantage and lifespan cost of the LF-DY sub-population and the periods of nutrient availability and starvation, we noted that each phase space contained regimes in which either strategy could be advantageous (Figure 2C and Figure 2—figure supplement 1D).

Regimes in which either the LF-DY or LS-DO strategies would be strongly adaptive (and the other maladaptive) frequently arose under physiologically relevant environmental parameters. Oscillations within these regimes (i.e., between feast and famine) are common in nature (Broach, 2012), and withstanding them is essential for survival. Theory predicts that reversible epigenetic mechanisms, such

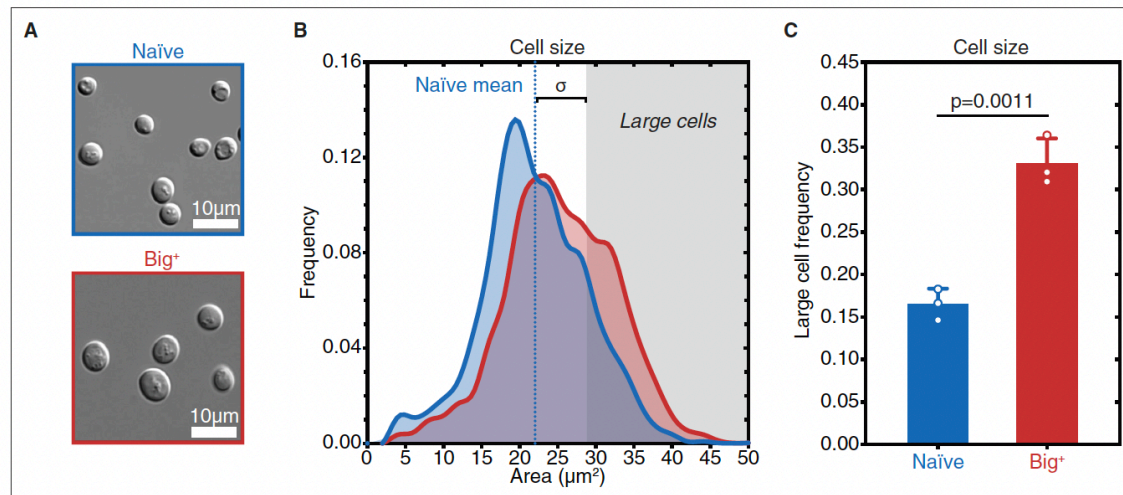


Figure 3. Big⁺ cells are large. (A) Micrographs of naïve and Big⁺ haploid yeast cells. (B) Cell size distributions for thousands of naïve and Big⁺ haploid cells (100% of distribution is shown, $n = 4678$ for naïve, $n = 5501$ for Big⁺, dotted line indicates naïve mean). Large cell threshold begins at one standard deviation above the naïve mean. (C) The frequency of haploid cells above the large cell threshold. Bars represent the mean of three replicate strains, for which thousands of cells are measured for each strain, error bars are standard deviation. $p = 0.0011$, unpaired t-test.

The online version of this article includes the following figure supplement(s) for figure 3:

Figure supplement 1. The large cell phenotype of Big⁺ emerges during the growth of a culture.

as prions, could confer a selective advantage in fluctuating environments (King and Masel, 2007). Importantly, our model demonstrates that this advantage could derive not only from a trade-off between improved stress resistance and impaired growth in normal conditions but also from a growth advantage in times of plenty coupled with a disadvantage under stress.

Notably, the growth advantages we observed in our competition experiment were quantitatively consistent with Monte Carlo simulations sampled from our experimental measurements of the death rates (from chronological lifespan measurements, Figure 2D) and growth rates (from proliferation rate measurements, Figure 2E) of individual cultures in nutrient-starved and replete conditions, respectively (Figure 2—figure supplement 1E). That is, the adaptive advantages that we measured in competition were equivalent to those that we predicted for a hypothetical *LF-DY* population after dozens of generations (Figure 2F). Thus, selection on these properties could be alone sufficient to favor maintenance of the Big⁺ state under the conditions we examined.

Big⁺ cells are large

Positive correlations between growth rate and cell size have long been noted (Johnston et al., 1977; Schaechter et al., 1958; Su and O’Farrell, 1998; Turner et al., 2012). To determine if the faster-growing Big⁺ cells were also larger, we examined them microscopically, employing a widely used image masking pipeline (Carpenter et al., 2006). This approach allowed us to measure the sizes of thousands of cells from multiple biological replicates and define size distributions for both naïve and Big⁺ populations.

During exponential growth, populations of Big⁺ cells had a similar size distribution to naïve control cells. However, as the cultures became denser, naïve control cells remained the same size whereas isogenic haploid Big⁺ cells became larger (Figure 3A and Figure 3—figure supplement 1A). To simplify these comparisons, we scored distributions by the fraction of very large cells that we observed (one standard deviation or larger than the mean naïve size; Figure 3B, $n = 4678$ and 5501 cells shown for naïve and Big⁺, respectively). This increase in mean area from 22.01 μm² to 25.36 μm² corresponds to a 23% larger volume (approximating the yeast cell as a sphere, naïve cell mean radius = 2.65 μm,

Big⁺ cell mean radius = 2.84 μm). In Big⁺ cultures 33.1% $\pm$ 2.8% of cells exceeded this threshold, whereas 16.5% $\pm$ 1.8% did in naïve cultures ($p=0.0011$ by unpaired t-test; **Figure 3C**). We note that this difference is not as large as that between haploid and diploid yeast; the naïve diploid mean area is 36.88 μm^2 . Moreover, in mRNA-sequencing—discussed in more detail below—we observed expression profiles in Big⁺ cells consistent with being haploid and mating type Mat a (like their naïve counterparts) (**Figure 3—figure supplement 1B and C**). We therefore conclude that the Big⁺ state does not represent a simple autodiploidization.

[BIG⁺] is a prion transmitted through mating and meiosis

Big⁺ was initially induced by transient *PUS4* overexpression in a screen to identify prion-like epigenetic elements (**Chakrabortee et al., 2016a**). We therefore tested whether the increased cell size associated with this state was transmitted through genetic crosses with the unusual patterns of inheritance that characterize prion-based phenotypes (**Brown and Lindquist, 2009; Cox, 1965; Wickner, 1994**). We began by mating the large haploid Big⁺ cells to naïve haploids of the opposite mating type, selecting diploid cells, and measuring their size. The resulting diploids were significantly larger than those derived from control crosses with two naïve parents (**Figure 4A and B**), establishing that the trait is dominant.

We next investigated the meiotic inheritance of the large cell size trait. Because prions are not driven by changes in nucleic acid sequence, they have unusual patterns of inheritance that defy Mendel's laws. In addition to dominance in genetic crosses, prion-based traits can be passed to *all* progeny of meiosis, in contrast to DNA-based traits, which are inherited by half (**Figure 4—figure supplement 1A; Garcia and Jarosz, 2014; Li and Kowal, 2012; Liebman and Chernoff, 2012; Wickner, 2016**). We first mated control naïve cells to naïve cells of the opposite mating type, sporulated the resulting diploids, and dissected their meiotic progeny. We then grew clonal cultures of these haploid meiotic progeny and examined their size distributions, which were indistinguishable from their haploid-naïve parents (**Figure 4C**).

In contrast, all cultures derived from the meiotic progeny of Big⁺ $\times$ naïve crosses were large (**Figure 4C and Figure 4—figure supplement 1B**). This non-Mendelian pattern of inheritance differs strongly from the expected behavior for genetic mutants or chromatin-based epigenetic elements but is consistent with a prion-based mechanism of transmission. We therefore term this state '[BIG⁺]' (with capital letters indicating dominance and brackets denoting its non-Mendelian pattern of segregation).

[BIG⁺] propagation requires the Hsp70 chaperone

In contrast to those driven by genetic mutations, the inheritance of prion-based phenotypes is strongly dependent upon the protein homeostasis network (**Garcia and Jarosz, 2014; Harvey et al., 2018; Liebman and Chernoff, 2012; Shorter and Lindquist, 2005**). As a consequence, transient inhibition of molecular chaperones can lead to the *permanent* elimination of prion-based traits. We therefore examined whether the large size of [BIG⁺] cells also depended on protein chaperone activity (**Figure 4—figure supplement 1C**). Transient inhibition of the Hsp104 disaggregase, which regulates the inheritance of many amyloid prions (**Chernoff et al., 1995; Eaglestone et al., 2000; Halfmann et al., 2012; Shorter and Lindquist, 2004**), did not affect cell size in either naïve or [BIG⁺] cells (**Figure 4D**). Transient inhibition of the Hsp90 foldase, which regulates the transmission of a different subset of prions (**Chakrabortee et al., 2016a**), also had no impact on [BIG⁺] transmission (**Figure 4E**). By contrast, transient inhibition of Hsp70 via expression of a dominant-negative SSA1^{K69M} allele (**Chakrabortee et al., 2016a; Jarosz et al., 2014; Lagaudriere-Gesbert et al., 2002**) caused [BIG⁺] cells to lose their large size phenotype permanently (**Figure 4F**). Thus like other prions (**Brown and Lindquist, 2009; Chakrabortee et al., 2016a; Chakravarty et al., 2020**), and unlike genetic mutations, propagation of [BIG⁺] is dependent on the activity of this ubiquitous molecular chaperone.

We note that Hsp70 expression drops dramatically as yeast reach saturation and begin to starve (**Werner-Washburne et al., 1989; Werner-Washburne and Craig, 1989**) and also decreases as cells age (**Janssens et al., 2015**). These are two scenarios in which [BIG⁺] is disadvantageous. Therefore, environmental conditions that favor growth, during which Hsp70 is abundant, also favor prion propagation. By contrast, conditions known to reduce Hsp70 expression and thereby increase prion elimination are also those in which prion loss would be favored.

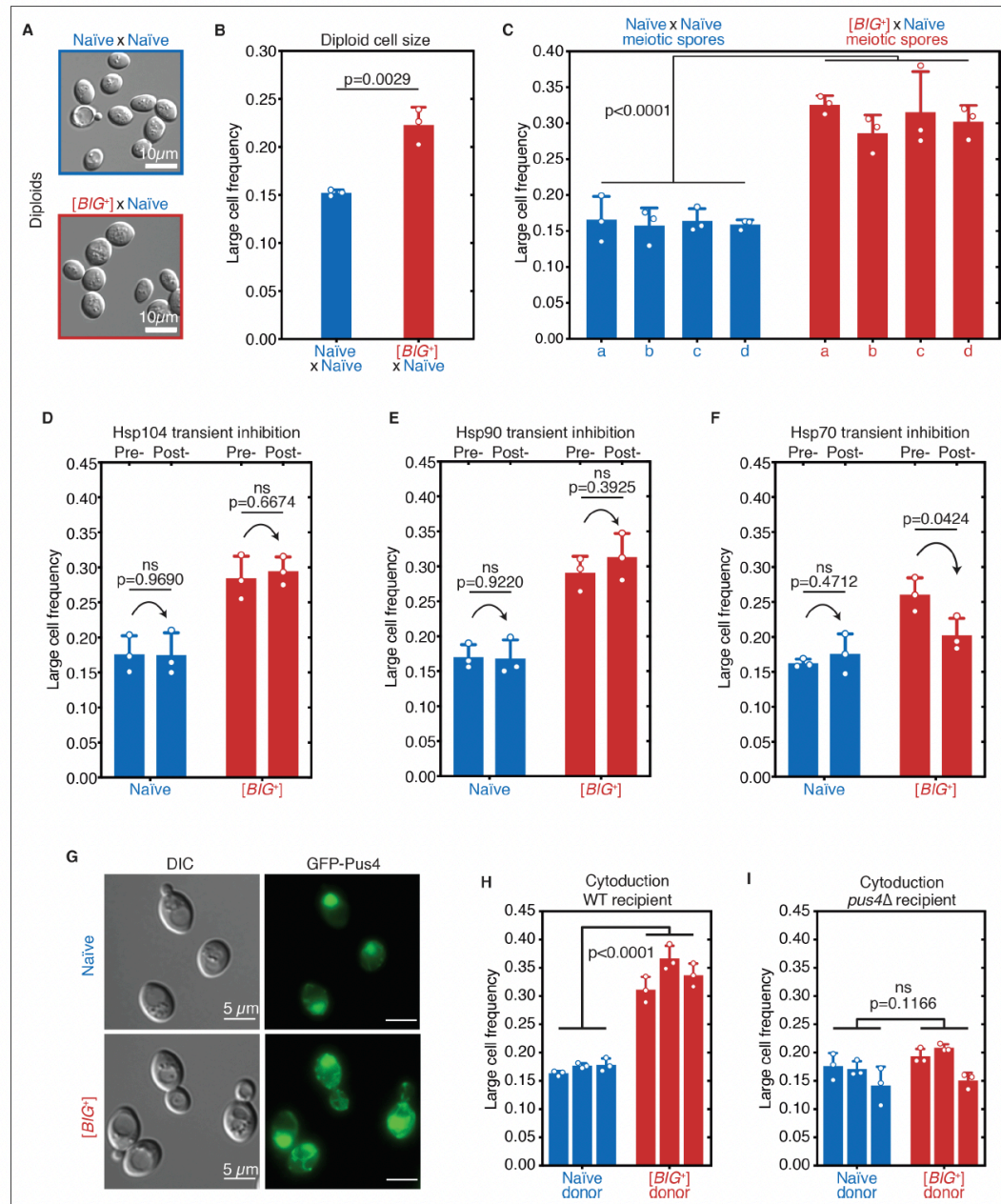


Figure 4. [B/G⁺] has prion-like patterns of inheritance. (A) Micrographs of diploid yeast cells resulting from crosses of naïve and naïve parents, or naïve and [B/G⁺] parents. (B) The frequency of diploid cells above the large cell threshold. Bars represent the mean of three replicate strains, for which thousands of cells were measured for each strain, error bars are standard deviation. $p=0.0029$, unpaired t-test. (C) Inheritance of large cell frequency to all meiotic spores. Bars represent the mean frequency of cells above the large cell threshold from three replicates, for which thousands of cells were

Figure 4 continued on next page

Figure 4 continued

measured for each replicate, error bars are standard deviation. Difference between the means of four tetrad spores between naïve and $[BIG^+]$, $p < 0.0001$, unpaired t-test. (D) Transient inhibition of Hsp104 chaperone activity using guanidinium hydrochloride (GdnHCl) does not heritably alter the cell size trait. Bars represent the mean frequency of cells above the large cell threshold from three replicates, for which thousands of cells were measured for each replicate, error bars are standard deviation. Control samples (left bars of each pair) were propagated in parallel on nutrient-matched agar plates not containing GdnHCl. Post-inhibition represents strains subjected to GdnHCl treatment followed by recovery prior to cell size measurements (Materials and methods). Naïve $p = 0.9690$, $[BIG^+]$ $p = 0.6674$; unpaired t-test for both. (E) Transient inhibition of Hsp90 chaperone activity using radicicol does not heritably alter the cell size trait. Bars represent the mean frequency of cells above the large cell threshold from three replicates, for which thousands of cells were measured for each replicate, error bars are standard deviation. Control samples (left bars of each pair) were propagated in parallel on nutrient-matched agar plates not containing radicicol. Post-inhibition represents strains subjected to radicicol treatment followed by recovery prior to cell size measurements (Materials and methods). Naïve $p = 0.9220$, $[BIG^+]$ $p = 0.3925$; unpaired t-test for both. (F) Transient inhibition of Hsp70 chaperone activity by expression of a dominant-negative allele of *SSA1* permanently eliminates the $[BIG^+]$ cell size trait. Bars represent the mean frequency of cells above the large cell threshold from three replicates, for which thousands of cells were measured for each replicate, error bars are standard deviation. Control samples (left bars of each pair) did not contain the *SSA1^{K99M}* constitutive expression plasmid but were propagated in parallel on non-dropout but otherwise nutrient-matched agar plates. Post-inhibition represents strains subjected to plasmid expression followed by plasmid removal and recovery prior to cell size measurements (Materials and methods). Naïve $p = 0.4712$, $[BIG^+]$ $p = 0.0424$; unpaired t-test for both. (G) The expression pattern of Pus4 is altered in $[BIG^+]$ cells. (H) $[BIG^+]$ can be transmitted via cytoduction into a wild-type recipient cell, consistent with a prion-based mechanism. Each bar represents the mean frequency of cells above the large cell threshold from three biological replicates, for which thousands of cells were measured for each replicate, error bars are standard deviation. Bars for three independent cytoductants are shown for each donor strain. Difference between the means of the three cytoductants between naïve and $[BIG^+]$ donors: $p < 0.0001$, unpaired t-test. (I) $[BIG^+]$ is not transmitted via cytoduction into a *pus4Δ* recipient cell, indicating that prion transmission depends on continuous endogenous expression of Pus4. Each bar represents the mean frequency of cells above the large cell threshold from three biological replicates, for which thousands of cells were measured for each replicate, error bars are standard deviation. Bars for three independent cytoductants are shown for each donor strain. Difference between the means of the three cytoductants between naïve and $[BIG^+]$ donors: $p = 0.1166$, unpaired t-test.

The online version of this article includes the following figure supplement(s) for figure 4:

Figure supplement 1. Tests for prion-like patterns of inheritance and dependence on Pus4 for $[BIG^+]$.

Pus4 protein has a different expression pattern in $[BIG^+]$ cells

Prion states often impact the localization of the proteins that encode them. To visualize endogenous levels of Pus4 expression in naïve and $[BIG^+]$ cells, we employed a strain in which an N-terminal GFP tag was appended at the endogenous *PUS4* locus, and therefore subject to endogenous regulation (i.e., not overexpressed; Weill et al., 2018; Yofe et al., 2016). We did not observe large fluorescent foci typical of amyloid prions (Alberti et al., 2009), as might be expected given that Pus4 is not enriched in glutamine and asparagine residues typically abundant in amyloid prion proteins (Chakrabortee et al., 2016a). We did, however, observe altered localization of Pus4. In naïve cells, Pus4 consistently localized to the nucleolus, as has been previously reported (Huh et al., 2003). In $[BIG^+]$ cells, the Pus4 protein was also present in the nucleolus, but we also observed substantial fluorescence in a fragmented network throughout the cytoplasm (Figure 4G). Although a high-resolution structure awaits determination, our data are consistent with an altered state of Pus4 in $[BIG^+]$ cells.

Endogenous Pus4 is required for propagation of $[BIG^+]$

Although $[BIG^+]$ was induced by a transient increase in Pus4 abundance and was stable after eliminating the inducing plasmid, we wanted to exclude the possibility that this prior overexpression event might have established a positive feedback loop leading to an enduring increase in Pus4 levels. We therefore constructed naïve and $[BIG^+]$ strains with a seamless N-terminal 3X-FLAG tag endogenously encoded at the *PUS4* locus. Using immunoblots to detect the FLAG epitope in naïve and $[BIG^+]$ cells, we observed equivalent Pus4 levels, indicating that the phenotypes we observed in $[BIG^+]$ cells were not simply due to increased expression of this RNA-modifying enzyme (Figure 4—figure supplement 1D).

Prion proteins can be inherited through the cytoplasm and do not require the exchange of genetic material for propagation. To test this, we performed a cytoduction, in which we mated $[BIG^+]$ cells to naïve recipient cells of the opposite mating type carrying the *kar1-Δ15* mutation. Upon mating, this mutation prevents nuclear fusion, permitting the mixing of cytoplasm, but not nuclei, between donor and recipient cells (Figure 4—figure supplement 1E, Materials and methods; Vallen et al., 1992). The transfer of $[BIG^+]$ cytoplasm into naïve recipient cells resulted in the transfer of the $[BIG^+]$ cell size

phenotype (Figure 4H). However, this was only the case for wild-type recipients. Naïve recipients lacking *PUS4* did not acquire the [BIG⁺] cell size phenotype (Figure 4I).

It remained formally possible that a multi-protein prion state could be maintained by other cellular factors, even if prion-based phenotypes depended on Pus4. Therefore, we tested whether transient loss of *PUS4* was sufficient to eliminate [BIG⁺] permanently. We first deleted *PUS4* from [BIG⁺] and naïve cells. Upon *PUS4* deletion, the sizes of the mutants derived from naïve and [BIG⁺] parents were equivalent (Figure 4—figure supplement 1F). Together with our cytoduction data, this suggested that continuous production of Pus4 is required to maintain this trait. Furthermore, *PUS4* deletion did not increase the size of naïve cells, suggesting that [BIG⁺] does not inactivate Pus4, in contrast to many well-characterized prions that phenocopy loss-of-function alleles of their underlying proteins (Byers and Jarosz, 2014). Finally, we restored the *PUS4* gene to its native locus in these same cells by homology-directed integration. Even after the re-introduction of *PUS4*, the size distributions of both populations remained equivalent (Figure 4—figure supplement 1F). Thus, both the expression and the propagation of the [BIG⁺] phenotype require the continual presence of a *PUS4* gene product.

These various lines of evidence—transmission to all meiotic progeny, dependence on molecular chaperones, altered protein localization, and requirement for continuous expression of the protein that initiated the epigenetic trait—lead us to propose that [BIG⁺] is a protein-based element of inheritance, a prion, formed by the Pus4 pseudouridine synthase.

Pus4-dependent pseudouridylation of tRNAs is maintained in [BIG⁺] cells

Because loss of *PUS4* did not phenocopy [BIG⁺] but did block propagation of the prion, we wondered whether the catalytic activity of Pus4 was maintained in [BIG⁺] cells. To measure pseudouridylation in naïve and [BIG⁺] cells, we employed a qPCR-based method that capitalizes on the enhanced susceptibility of pseudouridine to reacting with CMC (1-cyclohexyl-(2-morpholinoethyl)carbodiimide metho-p-toluene sulfonate); Figure 5A; Lei and Yi, 2017. When pseudouridines are 'labeled' by CMC, and these RNAs are used as templates for replication by reverse transcriptase, the enzyme generates nucleotide deletions and other mutations at CMC-labeled sites that can be detected by differences in the melting temperature of the derived nucleic acid duplexes (as compared to non-pseudouridylated or unlabeled controls). Using this approach, we examined the pseudouridylation of an archetypical Pus4 substrate, tRNA_{ACC} (Ala). When a pseudouridine is present and CMC is added, the melting curve shifts relative to an unlabeled control (no CMC). We observed a similar leftward shift in melting curves for both naïve and [BIG⁺] cells (Figure 5B–D).

In contrast, control cells lacking the Pus4 protein, and therefore lacking pseudouridylation at U55, did not produce this shift (Figure 5B–D). Because tRNAs bear pseudouridines at other positions—catalyzed by different pseudouridine synthase enzymes—which could potentially interfere with our analysis of Pus4 activity, we also directly examined the U55 position. We cloned the PCR products into plasmids and then used Sanger sequencing to identify positions with mutations indicating a CMC adduct. Within the T-arm stem-loop of tRNA_{ACC} (Figure 5—figure supplement 1A), we saw mutations originating at U55 in CMC-labeled samples from both naïve and [BIG⁺] cells, but not in unlabeled samples or *pus4Δ* samples (Figure 5—figure supplement 1B). These data establish that Pus4-dependent modification of tRNAs at U55 is maintained in [BIG⁺] cells.

Relative RNA levels are nearly unchanged in [BIG⁺] cells

In addition to its ubiquitous pseudouridylation activity on all tRNAs, Pus4 also modifies some mRNAs (Carlile et al., 2014; Lovejoy et al., 2014; Schwartz et al., 2014), which may impact mRNA stability (Zhao et al., 2017). We performed mRNA sequencing to discern whether the phenotypes of [BIG⁺] cells might be due to relative changes in mRNA levels. We grew naïve and [BIG⁺] cultures in YPD medium until late exponential phase, extracted total RNA, and enriched these samples for polyadenylated RNAs. Comparing four replicates each of naïve and [BIG⁺], the expression levels of only 47 genes changed significantly (28 decreased in expression and 19 increased as estimated using Kallisto and Sleuth; Bray et al., 2016; Pimentel et al., 2017; Benjamini–Hochberg-corrected q-value < 0.05 from the likelihood ratio test and |log₂ fold-change| > 1) (Supplementary file 2). Spike-in controls demonstrated quantitative scaling in our estimates of gene expression across a broad range of transcript abundance (Figure 5—figure supplement 1C). The upregulated genes in [BIG⁺] exhibited only

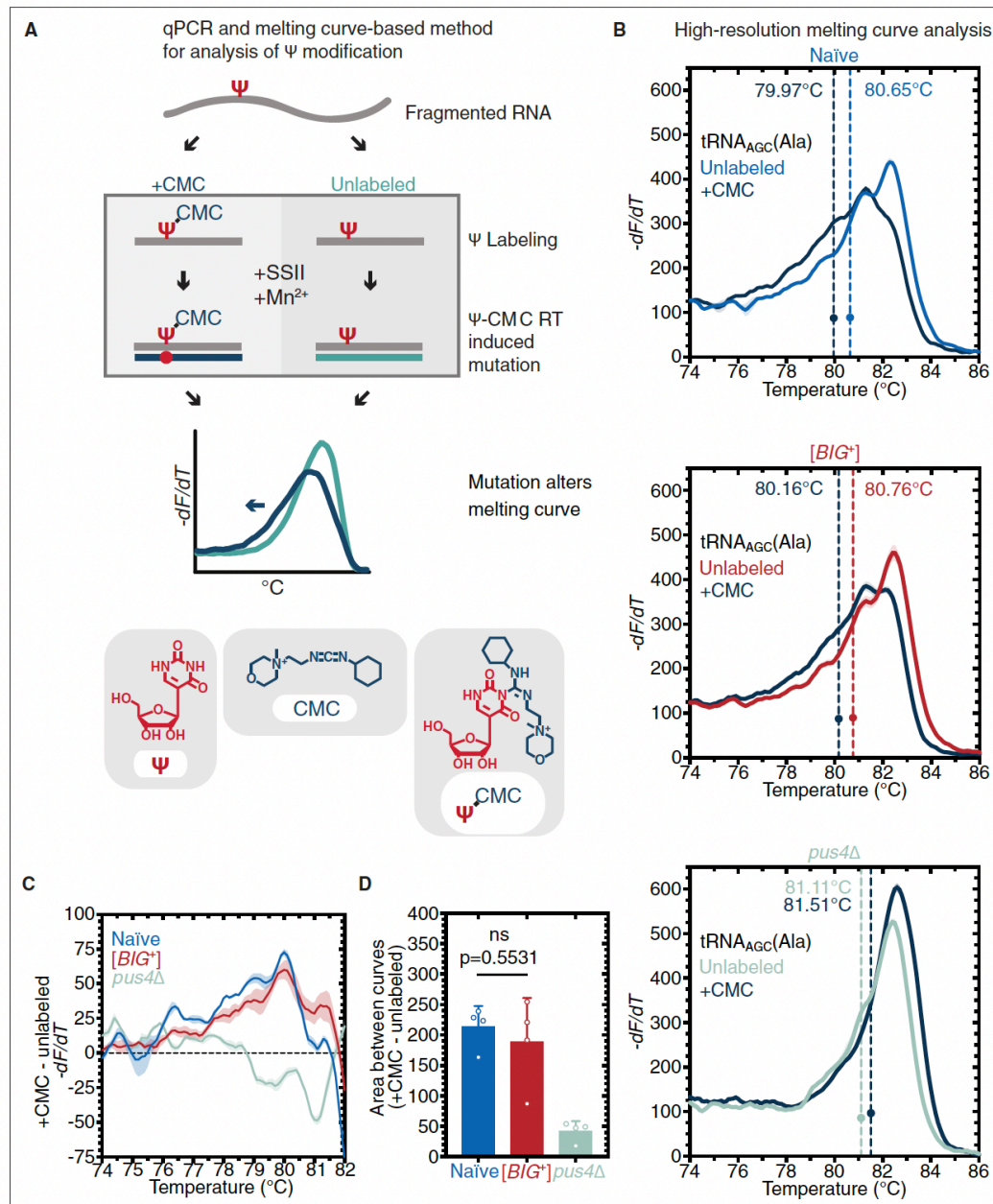


Figure 5. Pus4 activity is maintained in [BIG⁺]. (A) Radiolabeling-free, qPCR-based method for locus-specific pseudouridine detection. Illustration is adapted from Scheme 1 of *Lei and Yi, 2017*. (B) High-resolution melting curve analysis demonstrates that Pus4-dependent pseudouridylation of tRNA_{AGC}(Ala) is maintained in [BIG⁺] cells but not in cells that do not contain Pus4p. Top panel: naïve samples CMC-labeled (dark blue) or -unlabeled (light blue). Middle panel: [BIG⁺] samples CMC-labeled (dark blue) or -unlabeled (red). Bottom panel: *pus4*Δ samples CMC-labeled (dark blue) or -unlabeled (light blue). (C) Comparison of melting curves for Naïve, [BIG⁺], and *pus4*Δ strains. (D) Bar graph of the area between curves for Naïve, [BIG⁺], and *pus4*Δ strains. ns, not significant.

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-unlabeled (turquoise). Solid lines representing melting curves are the mean of four replicates, with shaded areas representing the standard error of the mean. Dots mark the geometric center of four replicates, bisected by a dashed line with a shaded area representing the standard error of the mean. The melting temperature (T_m) of this point is also displayed. The leftward shift of +CMC curves in naïve and [BIG⁺] but not *pus4Δ* samples indicates Pus4-dependent pseudouridylation of U55. (C) The difference in melting temperature behavior (dF/dT) between CMC-labeled and CMC-unlabeled tRNA_{AGC} (Ala) amplicons is similar between [BIG⁺] cells and naïve cells, suggesting that Pus4-dependent Ψ is present at position 55 in tRNA_{AGC} (Ala) in [BIG⁺] cells. Solid line represents the mean of four replicates, with shaded areas showing standard error of the mean. (D) The difference in area between the melting curves of CMC-labeled and CMC-unlabeled tRNA_{AGC} (Ala) amplicons is similar between [BIG⁺] cells and naïve cells, suggesting that Pus4-dependent Ψ is present in [BIG⁺] cells. Bars represent the mean of four replicates, error bars indicate standard deviation, $p=0.5531$, unpaired t-test.

The online version of this article includes the following figure supplement(s) for figure 5:

Figure supplement 1. tRNA pseudouridylation and quantitative scaling of gene expression in mRNA sequencing.

a modest enrichment for water transport (two aquaporin genes), while the downregulated genes were enriched for cell wall and plasma membrane components (Supplementary file 2; Szklarczyk et al., 2019), perhaps because larger [BIG⁺] cells have less surface area per unit volume. We conclude that the major effects of [BIG⁺] during exponential growth (e.g., on growth rate and RLS) likely do not occur via changes to steady-state mRNA levels.

To investigate whether the relative abundance of tRNAs is perturbed in [BIG⁺] cells, we ran total RNA from naïve and [BIG⁺] cells (grown to late-exponential phase) on a nucleic acid fragment analyzer and quantified tRNA levels relative to a similarly abundant RNA that is not a target of Pus4, 5.8 S rRNA (158 nt) (Figure 5—figure supplement 1D). We observed no significant differences. While we cannot exclude other effects on tRNA function or the relative abundance of particular tRNAs, our data show that bulk tRNA abundance differences are likely also not responsible for the phenotypes of actively growing cells containing [BIG⁺].

[BIG⁺] cells are resistant to inhibition of protein synthesis

Because the increased cell size and proliferation and reduced lifespan of [BIG⁺] cells occur in the absence of major alterations to relative abundances of mRNA or tRNA, we wondered whether a change in protein synthesis might be responsible. To test this, we employed two inhibitors: (1) cycloheximide, an inhibitor of translational elongation (Baliga et al., 1969; McKeehan and Hardesty, 1969), and (2) rapamycin, a natural product macrolide that inhibits the TOR kinase, blocking a conserved signaling cascade that promotes protein synthesis (Beretta et al., 1996; Chung et al., 1992; Kuo et al., 1992; Price et al., 1992; Urban et al., 2007).

[BIG⁺] cells grew nearly twofold better than naïve cells in a sub-inhibitory concentration of cycloheximide that was sufficient to impair proliferation (0.01 μg/mL, in which naïve cells needed 23.6 hours to reach their maximum proliferation rate vs. 21.3 hours in YPD; for comparison, 100 μg/mL is typically used for experiments that rapidly and completely arrest translation; Figure 6A). [BIG⁺] cells also proliferated faster in a concentration of rapamycin (10 μM) that inhibited growth (Figure 6B), suggesting that the pathway may be more active in cells harboring the prion. We found additional evidence in support of this hypothesis by examining the localization of Par32 (phosphorylated after rapamycin 32); the higher ratio of membrane-localized Par32 that we observed in [BIG⁺] cells is consistent with a more active TOR pathway (Figure 6—figure supplement 1A; Varlakhanova et al., 2018). These latter observations also provide a potential explanation for the decreased longevity of [BIG⁺] cells: loss of TOR pathway function is associated with extended lifespan in yeast (Dikicioglu et al., 2018; Fabrizio et al., 2001; Powers et al., 2006) and many other organisms including nematodes, fruit flies, and mice (Bjedov et al., 2010; Harrison et al., 2009; Robida-Stubbs et al., 2012).

We next investigated whether these inhibitors impacted the size of [BIG⁺] cells. When grown in cycloheximide or rapamycin to saturation, [BIG⁺] cells were no longer large compared to naïve controls (Figure 6C and D). Thus, unperturbed translation is necessary for the increased size of [BIG⁺] cells. These data could be explained by the inhibitors masking expression of the large cell trait or by reversion of the [BIG⁺] prion. To distinguish between these possibilities, we sub-cultured cells that had been treated with cycloheximide or rapamycin and allowed them to recover in rich medium for several dozen generations. We then examined their size distributions. Populations derived from [BIG⁺] ancestors were once again significantly larger than naïve controls that we subjected to the same

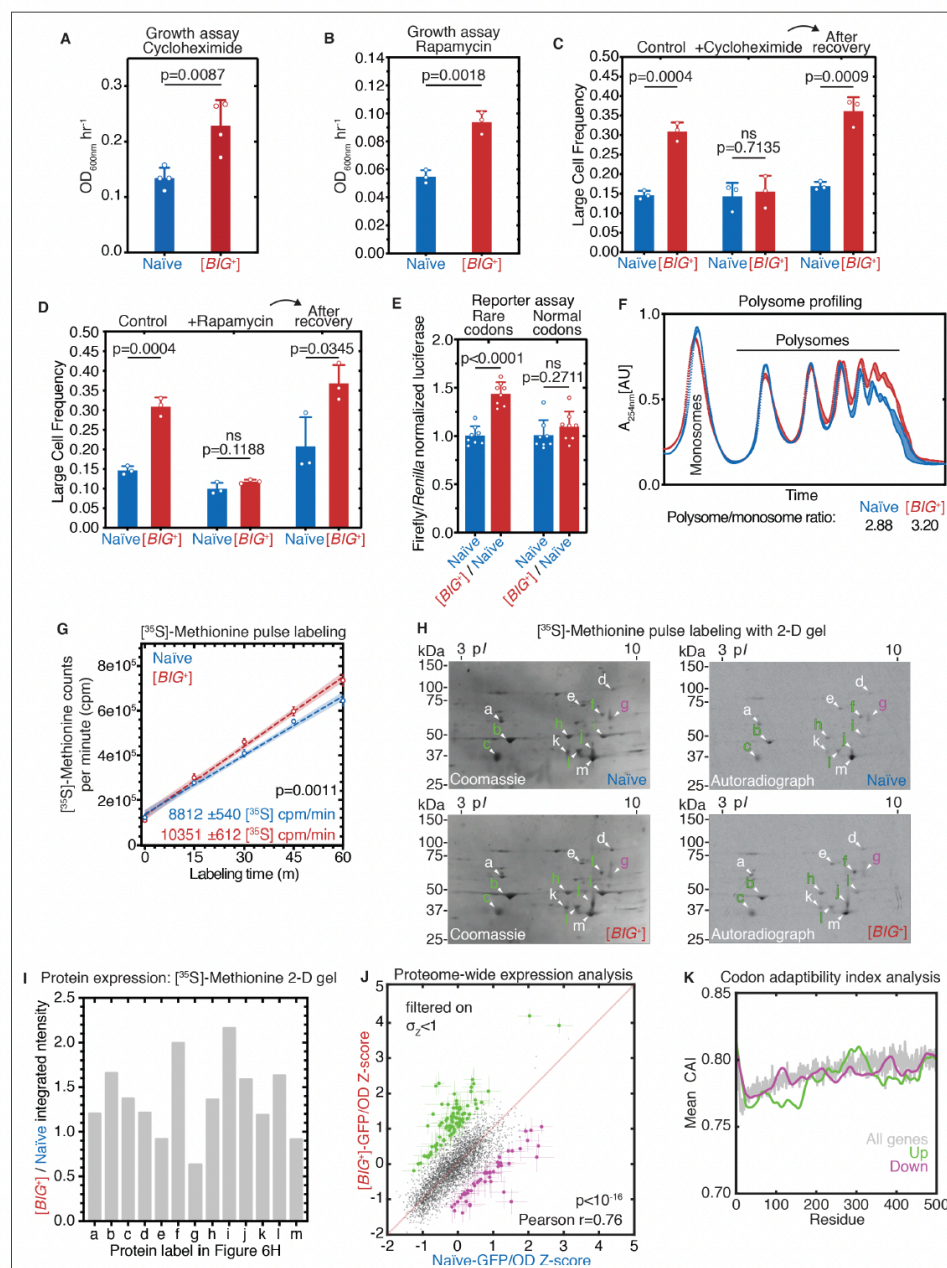


Figure 6. [BIG⁺] has altered protein synthesis. (A) [BIG⁺] cells are resistant to translation elongation inhibitor cycloheximide. Bars represent the mean of the maximum growth rate in YPD + cycloheximide (measured by the peak of the derivative of the growth data) of four replicates, error bars are standard deviation, p=0.0087, unpaired t-test. (B) [BIG⁺] cells are resistant to TOR inhibitor rapamycin. Bars represent the mean of the maximum growth rate in YPD + rapamycin (measured by the peak of the derivative of the growth data) of three replicates, error bars are standard deviation, p=0.0018, unpaired t-test.

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unpaired t-test. (C) $[BIG^+]$ cells grown in cycloheximide (0.05 $\mu\text{g/mL}$) are not larger than naïve cells. However, after recovery, they regain this phenotype. After treatment, cells were sub-cultured in YPD for ~75 generations before re-measuring the size in the absence of stress (see Materials and methods). Bars represent the mean frequency of cells above the large cell threshold from three replicates, for which thousands of cells were measured for each replicate, error bars are standard deviation. Difference between the means of naïve and $[BIG^+]$: YPD control $p=0.0004$; YPD + cycloheximide $p=0.7135$; YPD after recovery $p=0.0009$; unpaired t-test for all. (D) $[BIG^+]$ cells grown in rapamycin are not larger than naïve cells. However, after recovery they regain this phenotype. After treatment, cells were subcultured in YPD for ~75 generations before re-measuring the size in the absence of stress (see Materials and methods). Bars represent the mean frequency of cells above the large cell threshold from three replicates, for which thousands of cells were measured for each replicate, error bars are standard deviation. Difference between the means of naïve and $[BIG^+]$: YPD control $p=0.0004$ (same data presented in Figure 6C as experiments were done in parallel); YPD + rapamycin $p=0.1188$; YPD after recovery $p=0.0345$; unpaired t-test for all. (E) $[BIG^+]$ meiotic progeny translate more of a firefly luciferase reporter containing rare codons than naïve meiotic progeny do. This effect is not seen in an mRNA variant that encodes an identical protein but contains codons more frequently used in yeast. For each variant, normalized luciferase values in naïve cells are set to 1.0 to make presentation straightforward; however, the normal codon variant of firefly produces approximately five times more luciferase activity than the rare codon variant (data not shown; Chu et al., 2014). Bars represent mean normalized luciferase values (an invariable *Renilla* luciferase gene is co-expressed from the same plasmid) from eight replicates: rare codons $p<0.0001$, normal codons $p=0.2711$, unpaired t-tests for both. (F) $[BIG^+]$ cells have more polysomes than naïve cells, as measured by polysome profiling. Lines for two technical replicates for each sample are shown, with the area between them shaded. Ratios (average of two technical replicates) were calculated by taking the lowest point between the monosome and disome peak as zero, and then calculating the ratios of the areas under the sum of the polysome peaks to that under the monosome peaks. (G) Using $[^{35}\text{S}]$ -methionine pulse labeling, $[BIG^+]$ cells have an increased rate of translation relative to naïve cells (slope line fit: $p=0.0011$). Lines for three biological replicates for each sample are shown, with the area between them shaded. Timepoints were taken at 1, 15, 30, 45, and 60 min. (H) Separation of total protein by 2-D gel electrophoresis from cells pulse-labeled for 1 hr with $[^{35}\text{S}]$ -methionine demonstrates that many proteins are synthesized at a faster rate in $[BIG^+]$ cells, few at a slower rate, and some at an indistinguishably different rate, constituting an altered program of protein synthesis. For each gel, a Coomassie stain showing total protein and an autoradiography exposure newly synthesized protein are shown with a sampling of 13 distinguishable proteins labeled. Letters colored green or violet mark proteins whose expression was increased or decreased >1.25-fold, respectively, in the analysis presented in the next panel. (I) Relative protein levels measured by dot intensities from autoradiographs shown in Figure 6H. Shown are the values for 13 proteins quantified in both naïve and $[BIG^+]$ $[^{35}\text{S}]$ -methionine pulse-labeled samples. (J) Plot showing proteome-wide GFP::protein fusion expression in $[BIG^+]$ cells compared to naïve cells, highlighting ~130 proteins whose levels change. Each dot represents the mean of quadruplicate measurements of a single protein in naïve or $[BIG^+]$ cells: black dots are proteins that did not change significantly as measured by Z-score change of less than 1.0 ($\sigma_2<1$); green dots are protein fusions with higher fluorescence in $[BIG^+]$ cells; violet dots are protein fusions with lower fluorescence in $[BIG^+]$ cells. For colored dots, the standard error of the mean is shown for both measurements from four biological replicates each. Pearson correlation of naïve and $[BIG^+]$ cells, $r=0.76$, and $p<10^{-16}$ indicates that most proteins have correlated expression levels. OD_{600} was adjusted based on known blank wells, and the $\text{GFP}/\text{OD}_{600}$ measurements were normalized by Z-score $([x_i - \mu]/\sigma)$ within the naïve and $[BIG^+]$ populations independently. (K) Plot showing the protein residue number vs. the mean codon adaptation index (CAI) for all measured GFP-tagged proteins (gray line) and proteins whose levels were increased (green line) or decreased (violet line) in $[BIG^+]$ relative to naïve cells in the proteome-wide screen. Proteins whose levels were elevated in $[BIG^+]$ relative to naïve cells have a lower mean CAI in the 5' ends of their mRNAs.

The online version of this article includes the following figure supplement(s) for figure 6:

Figure supplement 1. Altered cell cycle and translation in $[BIG^+]$.

propagation regime (Figure 6C and D). We conclude that $[BIG^+]$ depends on protein synthesis to augment cell size but that the prion is stable to transient perturbations in translation.

$[BIG^+]$ cells have altered protein synthesis

Translation is rate-limiting for growth in nutrient-rich conditions (Kafri et al., 2016). We observed enhanced growth of $[BIG^+]$ cells in rich YPD medium (Figure 1A and B). However, in synthetic defined medium with identical carbon source abundance (2% glucose) but fewer amino acids, nucleosides, and other nutrients for optimum growth (SD-CSM), $[BIG^+]$ cells did not grow faster than naïve controls (Figure 6—figure supplement 1B). These data, combined with the resistance of $[PRION^+]$ cells to cycloheximide and rapamycin, suggested that protein synthesis might be enhanced in $[BIG^+]$.

Because a major component of translational regulation is the efficiency with which the ribosome translates each mRNA, we examined the impact of $[BIG^+]$ on mRNAs encoded with different codons using luciferase reporter assays. We transformed $[BIG^+]$ cells and isogenic naïve control cells with dual-luciferase plasmids encoding both *Renilla* and firefly luciferase genes. We tested two versions of the firefly luciferase gene: the first contained the standard suite of firefly mRNA codons; the second produced an identical protein product, but via codons that are rarer in *S. cerevisiae*, reducing steady-state protein levels by approximately five fold (Chu et al., 2014). Each plasmid contained an identical *Renilla* luciferase gene with its natural set of codons that served as an internal control (normalizing for copy number and general transcriptional activity). The firefly reporter with normal codons did not

produce more luciferase activity in $[BIG^+]$ cells than in naïve cells (Figure 6E). However, the firefly reporter with rarer codons produced normalized luciferase levels approximately 50% higher in $[BIG^+]$ cells than in isogenic naïve control cells (Figure 6E). These data suggest that $[BIG^+]$ cells may enhance the translation of some mRNAs, especially those containing a greater frequency of rare codons. We observed these effects in meiotic spores from naïve $\times$ $[BIG^+]$ crosses (Figure 6E) as well as in the original $[BIG^+]$ isolates (Figure 6—figure supplement 1C), showing that the altered translation phenotype, like cell size, is inherited by all progeny of meiosis.

We next investigated whether $[BIG^+]$ had global effects on the proteome by isolating total protein from cells harboring the prion and naïve controls. We reproducibly obtained more total protein per cell from the $[BIG^+]$ cultures (Figure 6—figure supplement 1D). We next loaded an equal mass of protein lysate from naïve and $[BIG^+]$ cells onto a denaturing polyacrylamide gel and separated them by electrophoresis. Coomassie staining of these gels showed no pronounced differences in banding patterns (Figure 6—figure supplement 1E), suggesting that $[BIG^+]$ does not exert substantial changes on the relative levels of the most highly expressed proteins, which are known to be efficiently translated (Gingold and Pilpel, 2011; Plotkin and Kudla, 2011).

We next performed polysome gradient analysis to assess ribosome density on mRNAs, a measure of global translation activity. We observed no change in monosomes or disomes in $[BIG^+]$ samples compared to naïve controls. However, polysomes—which are responsible for most protein synthesis (Noll, 2008; Warner and Knopf, 2002)—were increased in $[BIG^+]$ cells relative to naïve controls, with the polysome-to-monomosome ratio increasing from ~2.9 to 3.2 (Figure 6F).

To further investigate translation in $[BIG^+]$ cells, we performed a pulse-labeling experiment to measure the rate of newly synthesized protein. We grew naïve and $[BIG^+]$ cells in medium containing [35 S]-methionine and harvested protein lysates every 15 min for 1 hr, and then measured the extent of radiolabel incorporation by scintillation counting. These experiments established that the rate of newly synthesized total protein was higher in $[BIG^+]$ cells (10,179 cpm/min) compared to naïve cells (8812 cpm/min) (Figure 6G; $p < 0.0011$, unpaired t-test). In summary, we found that $[BIG^+]$ cells have a higher translation rate, which can increase total protein output, including the levels of some proteins translated from mRNAs enriched with codons generally associated with lower translation efficiency.

$[BIG^+]$ reduces time spent in the G1 phase of the cell cycle

Conditions that enhance protein synthesis also tend to reduce the fraction of time that cells spend in the G1 stage of the cell cycle (Jorgensen and Tyers, 2004). This is because commitment to S phase entry—budding of a daughter yeast cell—depends on sufficient production of proteins needed to replicate the genome and essential cellular structures. Accelerating the production of these factors can thus shorten this period. We measured the fraction of naïve and $[BIG^+]$ cells in the G1 phase of the cell cycle by counting the fraction of unbudded cells (i.e., cells in G1). For naïve cells, 36.2% were in G1, whereas only 27.2% of $[BIG^+]$ cells were in G1, a ~25% reduction (Figure 6—figure supplement 1F, $p = 0.0017$, unpaired t-test). These data suggest that the cell cycle checkpoint for progression to S phase remains intact, and that a shortened G1 stage in $[BIG^+]$ cells is consistent with their increased protein synthesis. $[BIG^+]$ cells are not larger during exponential phase growth (Figure 3—figure supplement 1A), suggesting that their cell size checkpoint remains intact. By contrast, cells in stationary phase, which do not have the nutrient content needed to progress to S phase, may continue to accumulate mass at a faster rate, contributing to their larger size.

$[BIG^+]$ cells enact an altered translational program

Because relative mRNA expression levels were only subtly altered in $[BIG^+]$ cells during exponential growth phase, but multiple measures of translation were altered, we considered the possibility that the phenotypes of the prion might be due to changes in protein levels of particular open reading frames. We tested this idea using two methods: (1) 2-D gel electrophoresis on pulse-labeled, newly synthesized proteins, and (2) a screen for differential expression of over 5000 GFP-tagged proteins.

We pulse-labeled [35 S]-methionine into newly synthesized proteins for 1 hr and then separated total protein using 2-D gel electrophoresis. Although labeling for less time than a cell division limited the number of newly synthesized proteins we could measure, we chose this time to minimize the contributions from factors other than protein synthesis, such as degradation. We observed [35 S]-methionine counts of 9.7 cpm/ μ g and 11.5 cpm/ μ g protein from naïve and $[BIG^+]$ cells, respectively, indicating that

cells harboring the prion synthesized proteins faster than naïve cells (Figure 6G). We also analyzed the [³⁵S]-methionine signal from 13 proteins that we could separate by 2-D gel electrophoresis. In [BIG⁺] cells, seven were increased, one was decreased, and five remained similar (Figure 6H and I). These data suggest that [BIG⁺] cells have an altered translation program, with variations in even the most rapidly synthesized proteins.

To identify specific proteins with altered levels in prion-containing cells, we capitalized on the dominance of [BIG⁺] in genetic crosses (Figure 4B), mating haploid cells harboring the prion to a genome-wide collection of superfolder-GFP gene fusions ('SWAT' library; ~ 5500 ORFs; fused seamlessly to retain endogenous promoter/regulatory elements; Weill et al., 2018). Equivalent matings between naïve strains and this genome-wide collection served as controls. To control for the potentially larger size of the [BIG⁺] cells, we assessed protein levels in these diploid strains in terms of the relative GFP levels (normalized by OD₆₀₀ and Z-scored) within the naïve and [BIG⁺] GFP-fusion collections separately. Mating and fluorescence measurements were performed in biological duplicate: the SWAT library was mated to two independent [BIG⁺] isolates alongside naïve controls. The reported OD₆₀₀-normalized GFP levels are the mean of two technical duplicates of each biological duplicate. Protein levels measured in this way were generally well correlated between naïve and [BIG⁺] strains (Pearson's $r = 0.76$; $p < 10^{-16}$; Figure 6J), in concordance with our results from electrophoresis of total cellular protein. However, many proteins were up- or downregulated in [BIG⁺] cells.

Of the 4233 fusions whose abundance could be robustly quantified across the four replicates (Z-score < 1 for both naïve and [BIG⁺]), ~130 were differentially expressed in [BIG⁺] cells. Consistent with a bias toward enhanced translation, 81 were upregulated and 46 were downregulated (Figure 6J and Supplementary file 3). These proteins did not show any strong enrichment in physicochemical properties, nor were they enriched in Pus4-dependent pseudouridylation sites (see Appendix 1 for further discussion). We also did not observe a widespread increase in the expression of ribosomal proteins. We did, however, observe that proteins that were increased in [BIG⁺] cells had a modest decrease in their codon adaptation index (CAI) at the 5' end of their mRNAs relative to all genes (Figure 6K), indicating that cells harboring the prion might more efficiently translate these messages. The dip in CAI that is typical in the 5' end of all genes, known as 'translational ramping,' is thought to reflect the bias toward translational control near the beginning of ORFs (Frumkin et al., 2018; Tuller et al., 2010). (Lower CAI can both reduce the speed of elongation by requiring rarer tRNAs and lead to differences in mRNA structure that might also affect initiation or elongation efficiency.) These data are consistent with our luciferase reporter data in which rarer codons throughout the message output more protein in [BIG⁺] than in naïve cells (Figure 6E and Figure 6—figure supplement 1C), as well as the resistance of prion cells to the elongation inhibitor cycloheximide (Figure 6A).

Many hits were logically connected to the enhanced translation, increased size, and shortened lifespan of [BIG⁺] cells. The 81 upregulated proteins included multiple ORFs whose deletions are associated with decreased cell size (such as *PHO5*, *MRPS28*, *KAP122*, and *SWE1*; Harvey and Kellogg, 2003; Jorgensen et al., 2002), increased chronological lifespan (*DIG2* and *UBX6*; Garay et al., 2014), and extended RLS (*ENO1*, *MUB1*, *PHO87*, *PGM2*, and *YRO2*; McCormick et al., 2015). Conversely, the 46 downregulated proteins included ORFs whose deletions are associated with increased cell size (*RNR4* and *RPL15B*; Jorgensen et al., 2002; Perlstein et al., 2005) and decreased chronological lifespan (including *BUD23*, *SMI1*, *MHP1*, and *CLG1*; Garay et al., 2014; Marek and Korona, 2013). Several proteins directly involved in translation control and ribosome biogenesis were also increased in [BIG⁺] cells, including *FHL1*, *MRPS28*, *MRPS16*, *RPL24B*, *RPS7A*, and *UTP10* (Figure 6—figure supplement 1G). The [BIG⁺]-regulated proteins also included 29 ORFs associated with differential sensitivity to rapamycin (including *SAS4*, *SNF1*, and *HCA4*; Butcher et al., 2006; Dudley et al., 2005; Kapitzky et al., 2010) and 14 ORFs that are known genetic or physical interactors with TOR1 (including *GCD14* and *PAR32*; Krogan et al., 2006; Varlakhanova et al., 2018). The functional breadth of these effects on the proteome and their logical connection to factors involved in the control of proliferation, cell size, and lifespan suggest that the phenotypes of [BIG⁺] are likely derived from an altered translational regulation program that favors increased cell size and proliferation at the expense of lifespan.

Pus4-dependent pseudouridylation of mRNAs in [BIG⁺] cells

Our finding that pseudouridylation on tRNA was maintained at similar levels in [BIG⁺] cells (Figure 5B–D) led us to wonder whether mRNA substrates of Pus4 were similarly modified. The

best-documented mRNA target of Pus4 is the translation elongation factor *TEF1/TEF2*—whose position U239 is robustly pseudouridylated in a Pus4-dependent manner (Carlile et al., 2014; Lovejoy et al., 2014; Schwartz et al., 2014). These paralogous genes encode identical copies of the eEF-1 α translation elongation factor, which binds to aminoacylated tRNAs—specifically the T arm stem-loop that is pseudouridylated by Pus4 (Dreher et al., 1999)—and delivers them to the A-site of the ribosome during translation elongation (Schirmaier and Philippsen, 1984). We used three different methods to measure pseudouridylation of U239 in *TEF1/TEF2* from naïve, [BIG⁺], and *pus4 Δ* cells. Sanger sequencing verified that the modified position was identical to that previously annotated in the literature (Figure 7—figure supplement 1A). Next, we used a quantitative RT-PCR-based method, known as CMC-RT and ligation-assisted PCR analysis of Ψ modification (CLAP) (Figure 7A; Zhang et al., 2019). We observed *TEF1/TEF2* U239 pseudouridylation in both naïve and [BIG⁺] cells, in contrast to very low/background levels in *pus4 Δ* cells (Figure 7B). Finally, using the aforementioned qPCR-based method for detecting pseudouridylation (Figure 5A), we found additional evidence that *TEF1/TEF2* was pseudouridylated in our wild-type cells (both naïve and [BIG⁺]) in a Pus4-dependent manner (Figure 7C).

Most studies of yeast prions have characterized them as decreasing or eliminating activity (Garcia and Jarosz, 2014). However, we recently discovered one notable exception in which the [SMAUG⁺] prion can increase the activity of the protein that encodes it, Vts1 (Chakravarty et al., 2020). We thus examined if there was an altered level of pseudouridylation of *TEF1/TEF2* mRNA. If altered levels affected protein activity, this could be one possible mechanism linked to the altered translation program we found in [BIG⁺] cells. By quantifying differences in the melt curve shift after CMC labeling—an analysis made simpler for *TEF1/TEF2* than for tRNAs by the absence of other pseudouridylated positions flanking U239—we observed an increase in the signal of pseudouridylation in [BIG⁺] cells relative to naïve (Figure 7D and E and Figure 7—figure supplement 1B).

Together these data demonstrate that the catalytic function of Pus4 is retained in [BIG⁺] cells and may be enhanced for specific substrates, contrasting with the classical view of prions as being loss-of-function protein conformations. They also provide a novel example of how RNA modification can be epigenetically controlled.

Epigenetic control of [BIG⁺]-like phenotypes in wild yeast

Finally, we tested whether protein-based epigenetic control of cell size, protein synthesis, or localization is present in wild yeast populations. Protein chaperones are essential regulators of prion propagation in wild strains, just as they are in laboratory strains (Halfmann et al., 2012; Jarosz et al., 2014). To block the passage of prions in wild yeasts, we transiently expressed a dominant-negative variant (*SSA1*^{K69M}) of Hsp70—the chaperone that is essential for [BIG⁺] propagation (Figure 4F)—in 20 wild *S. cerevisiae* strains isolated from various environments around the world (Cubillos et al., 2009; Itakura et al., 2020). We measured the size of cells before and after this chaperone curing and found that 20% (four isolates out of twenty)—‘273614 N’ (clinical, Newcastle, UK); ‘L-1528’ (fermentation, Cauquenes, Chile); ‘DBVPG1373’ (soil, Netherlands); ‘BC187’ (barrel fermentation, Napa Valley, California)—became smaller after curing (Figure 8A).

Given the wide array of genetic, epigenetic, and ecological diversity harbored in this collection, we also tested for three additional features associated with [BIG⁺] in laboratory strains: (1) increased protein synthesis, (2) differences in Pus4 localization between cured and uncured variants, and (3) a Pus4-dependent change in cell size.

First, to test whether the four isolates that became smaller upon curing had altered protein synthesis, we transformed them with the same luciferase reporters we tested in laboratory [BIG⁺] cells. We normalized the firefly reporters with variable codons to the *Renilla* control for cured strains and then normalized these to the corresponding uncured strain values. Two of the four isolates showed significant changes to translation (Figure 8B). BC187 yielded the largest change upon curing, an ~40% reduction in the translation of a firefly luciferase containing a ‘normal’ suite of codons, similar in magnitude to what we had observed with [BIG⁺] cells generated in the laboratory. Thus, since the loss of a chaperone-dependent epigenetic element reduced translational output from the reporter, the original, wild isolate (uncured) has higher protein synthesis, as we saw for [BIG⁺] in the BY4741 laboratory strains.

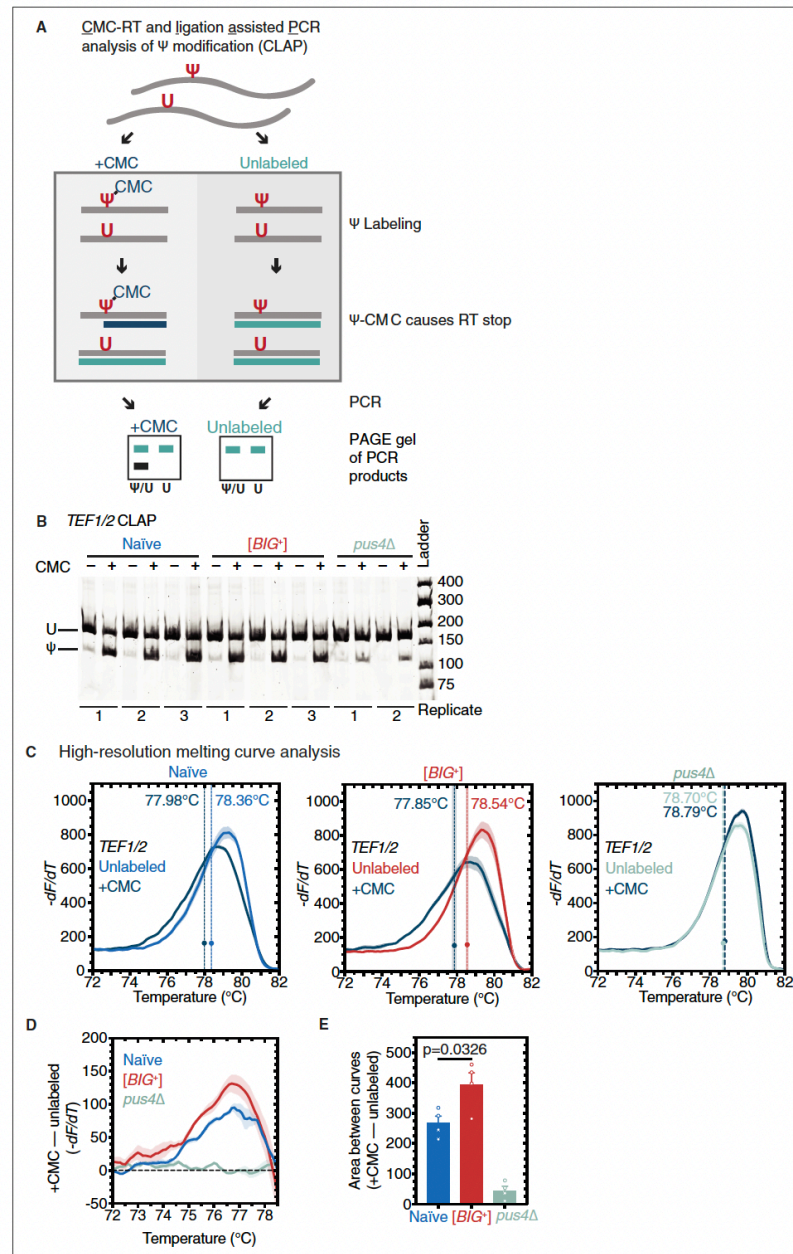


Figure 7. Pseudouridylation of mRNA in $[BIG^-]$ cells. **(A)** CMC-RT and ligation-assisted PCR analysis of Ψ modification (CLAP) (Zhang *et al.*, 2019). **(B)** Native PAGE showing the CLAP result for *TEF1/TEF2* mRNA from multiple replicates of naïve (3), $[BIG^-]$ (3), or *pus4Δ* (2). Shorter bands indicating pseudouridylation that appear in CMC-unlabeled or *pus4Δ* samples may reflect either background signal from spontaneous cleavage near

Figure 7 continued on next page

Figure 7 continued

target site or Pus4p-independent modification of this site. (C) High-resolution melting curve analysis shows that U239 is pseudouridylated in *TEF1/TEF2* mRNA in both naïve and [BIG⁺] cells but not in cells that do not contain Pus4p. Left panel: naïve samples CMC-labeled (dark blue) or -unlabeled (light blue). Middle panel: [BIG⁺] samples CMC-labeled (dark blue) or -unlabeled (red). Right panel: *pus4Δ* samples CMC-labeled (dark blue) or -unlabeled (turquoise). Dots mark the geometric center of four replicates, bisected by a dashed line with shaded area representing the standard error of the mean. The melting temperature (T_m) of this point is also displayed. Solid lines representing melting curves are the mean of four replicates, with shaded areas representing the standard error of the mean. (D) The difference in melting temperature behavior (df/dT) between CMC-labeled and CMC-unlabeled *TEF1/TEF2* mRNA amplicons is larger in [BIG⁺] cells than in naïve cells, suggesting higher levels of pseudouridylation of U239 in [BIG⁺] cells. Solid line represents the mean of four replicates, with shaded areas showing standard error of the mean. (E) The difference in area between the melting curves of CMC-labeled and CMC-unlabeled *TEF1/TEF2* mRNA amplicons is greater in [BIG⁺] cells than in naïve cells, suggesting higher levels of pseudouridylation of U239 in [BIG⁺] cells. Bars represent the mean of four replicates, error bars indicate standard deviation, $p=0.0326$, unpaired t-test.

The online version of this article includes the following figure supplement(s) for figure 7:

Figure supplement 1. Pseudouridylation of mRNA in [BIG⁺] cells.

Second, we examined the localization of Pus4 in BC187 and its Hsp70 cured derivative. We transformed uncured and cured strains with a plasmid expressing GFP-tagged Pus4 protein and imaged cells using epifluorescence microscopy. In uncured cells, we observed Pus4 in a fragmented network throughout the cytoplasm (Figure 8C), similar to what we observed in laboratory [BIG⁺] cells (Figure 4G). In contrast, in cured BC187 cells, the signal was more diffuse.

Finally, we investigated the relationship between Pus4 and cell size in BC187. The original [BIG⁺] trait identified in laboratory yeast strains (this work and Chakrabortee et al., 2016a) was induced after transient overexpression of Pus4 protein. Therefore, we tested whether transient overexpression of Pus4 protein could re-establish the large-cell phenotype in BC187 derivatives that had been subject to curing by transient Hsp70 inhibition. Indeed, cured BC187 cells treated in this way became large (Figure 8D). By contrast, transient overexpression of Pus4 did not increase the size of the already-large BC187 natural isolate (Figure 8—figure supplement 1). Thus, re-initiation of this epigenetic trait could be accomplished with transient Pus4 overexpression alone, thereby bearing strong similarities to [BIG⁺] in common laboratory strains and establishing Pus4 as a driver of epigenetic cell size control in this model eukaryote. Together, these observations suggest that epigenetic and potentially Pus4 prion-mediated control of mechanisms like [BIG⁺] that we have described in the laboratory is also likely present in nature.

Discussion

Epigenetic inheritance is most commonly thought to be driven by enzymes that modify chromatin and DNA. Here we show that an enzyme that catalyzes the epigenetic modification of RNA can itself be controlled by an extrachromosomal epigenetic process: a self-templating protein conformation that persists over long biological timescales. We found that prion cells not only maintain pseudouridylation activity but that activity can be increased, even without any detectable increase in *PUS4* expression. Future studies should address whether Pus4-dependent modification of *TEF1/TEF2* mRNA plays a role in the phenotypes of [BIG⁺] as this mRNA, which is the major non-tRNA substrate of Pus4, encodes a central elongation factor that binds to and escorts tRNAs to the ribosome. The modest changes we observed in the relative abundance of a few dozen mRNAs in actively growing cells do not appear to drive these growth or aging phenotypes based on their known functions.

Instead, we found that changes to the translational control of numerous genes are logically connected to these phenotypes. These include genes that affect cell size, chronological lifespan and replicative lifespan, translation control and ribosome biogenesis, and differential sensitivity to rapamycin (Supplementary file 3). Translation is a rate-limiting step for growth in many organisms (Polymenis and Schmidt, 1997; Sonenberg, 1993) and is often activated in human cancers (Sonenberg and Hinnebusch, 2009). Other prions in yeast also affect translation, including [PSI⁺] and [MOD⁺] (Baudin-Baillieu et al., 2014; Suzuki et al., 2012). However, in contrast to [BIG⁺], they lead to losses of their underlying protein activities, impairing translation and, in turn, growth in many conditions

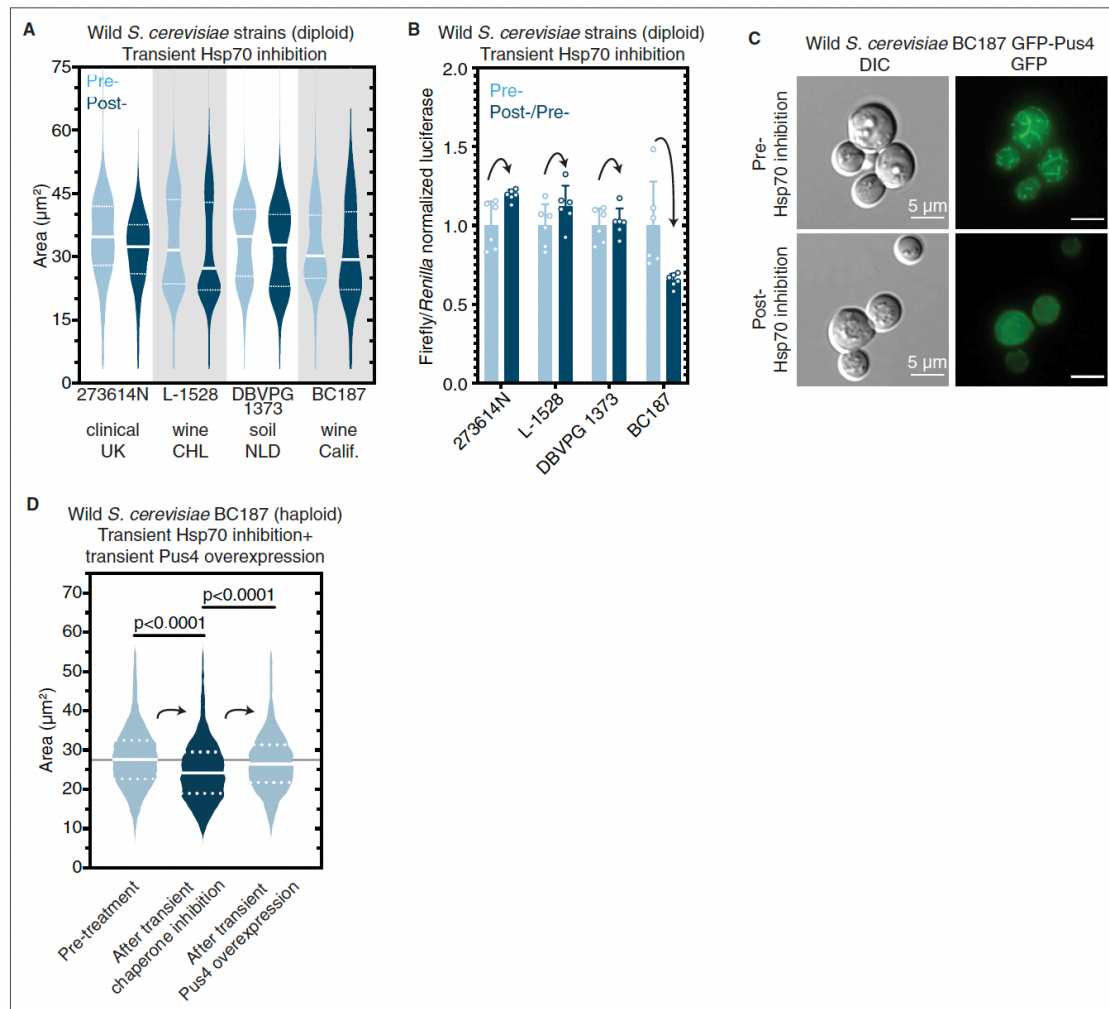


Figure 8. Epigenetic control of [BIG⁺]-like phenotypes in wild yeasts. **(A)** Transient inhibition of Hsp70 in diploid wild yeast strains from different niches around the globe leads to a permanent reduction in cell size. Violin plots show all data from thousands of cells from three biological replicates of each strain, light blue are cells before Hsp70 inhibition ('pre-'), dark blue are cells after transient Hsp70 inhibition and recovery ('post-'). The solid white line bisecting each distribution indicates mean; dotted lines indicate upper and lower quartiles. 273614 N: clinical isolate from the United Kingdom, pre- vs. post- $p < 0.0001$. L-1528: wine isolate from Chile, pre- vs. post- $p < 0.0001$. DBVPG 1373: soil isolate from the Netherlands, pre- vs. post- $p < 0.0001$. BC187: wine isolate from California, pre- vs. post- $p < 0.0001$. Kolmogorov–Smirnov test for all. **(B)** Transient inhibition of Hsp70 in wild yeast strains leads to permanent changes in protein synthesis capacity. Firefly reporter contains the 'normal' suite of codons, which is normalized to internal control Renilla luciferase. Bars represent the mean of normalized luciferase values for six biological replicates, error bars are standard deviation. Light blue 'pre-' are cells prior to Hsp70 inhibition, dark blue 'post-' are cells after transient Hsp70 inhibition and recovery. A value above 1.0 for the dark blue bars indicates that the normalized translation capacity has increased after prion curing, a value below 1.0 indicates that capacity has decreased. 273614 N $p = 0.0134$, L-1528 $p = 0.1397$, DBVPG 1373 $p = 0.7428$, BC187 $p = 0.0125$, unpaired t-test for all. **(C)** Transient inhibition of Hsp70 in BC187 wine isolate leads to permanent changes in Pus4p expression pattern, suggesting that its conformation may also be epigenetically regulated in wild strains. Prior to inhibition (top), cells show a distinct fragmented network of Pus4 expression; after transient inhibition and recovery (bottom), the expression pattern becomes much more diffuse. **(D)** Transient inhibition of Hsp70 in haploid wild BC187 yeast strains leads to a permanent reduction in cell size. After this regime, if

Figure 8 continued on next page

Figure 8 continued

Pus4p alone is transiently overexpressed, cells then permanently regain their original larger cell size. Violin plots show all data from thousands of cells from three biological replicates of each strain. The solid white line bisecting each distribution indicates mean; dotted lines indicate upper and lower quartiles. Kolmogorov–Smirnov tests.

The online version of this article includes the following figure supplement(s) for figure 8:

Figure supplement 1. Transient Pus4 overexpression does not permanently increase the size of uncured BC187 cells.

(Baudin-Baillieu et al., 2014; Cox, 1965; Suzuki et al., 2012). In contrast, in [BIG⁺] cells, translation appears to be amplified—it is faster, leading to higher expression levels of many proteins. We are unaware of an example in the literature of a mutation that enhances translation under nutrient-replete conditions.

More investigation is needed to determine the precise mechanism by which translation is altered. Increased polysome density can be associated with increased translation (more protein produced per mRNA) or decreased translation (due to defects in elongation or termination leading to accumulation of ribosomes). However, multiple lines of evidence lead us to favor the former interpretation. First, [BIG⁺] cells are resistant to the translation elongation inhibitor cycloheximide. Second, their rates of bulk translation as measured by [³⁵S]-methionine incorporation are also higher than naïve cells. General defects in translation elongation or termination, by contrast, would be expected to reduce this activity. Our luciferase reporter data shows an improved translation of mRNAs containing rare codons, which slow translation elongation. Finally, when measuring the proteome-wide effects on translation using our GFP screen, not only did we observe logical connections between the gene functions of altered proteins and the observed [BIG⁺] growth phenotypes, but it was the proteins whose expression increased that contained a feature consistent with enhanced elongation of their mRNAs: their ORF 5' ends had a lower CAI. Collectively, these data suggest an increase in translation elongation efficiency of many mRNAs as a parsimonious model.

The various possible mechanisms need not be mutually exclusive—some mRNAs in [BIG⁺] cells may indeed be translated faster to produce more protein without any defects and perhaps exhibit elevated rates of elongation and termination. In contrast, other mRNAs in the same cells may be subject to deficiencies at any of these steps. Examining protein synthesis at higher resolution, using methods like ribosome profiling, and in response to perturbations to ribosome-associated quality control pathways (Brandman and Hegde, 2016), may reveal the landscape of changes to protein synthesis in [BIG⁺] cells in greater detail.

Translation is also coupled to cell size, proliferation, and lifespan (Ecker and Schaechter, 1963; Kaeberlein and Kennedy, 2007; Lloyd, 2013; Steffen and Dillin, 2016; Tanenbaum et al., 2015). Cell size, which is determined in large part by the activity of the TOR pathway (Fingar et al., 2002; Zhang et al., 2000), has been inversely correlated with lifespan (Anzi et al., 2018; He et al., 2014; Yang et al., 2011), and older cells are larger (Egilmez et al., 1990). Moreover, molecules that extend lifespan, such as rapamycin, also influence cell size and proliferation by restricting the cell's translation capacity (Beretta et al., 1996; Terada et al., 1994). Here we describe an epigenetic paradigm that links all of these fundamental cellular properties: translation, cell size, proliferation, and lifespan. In future studies, we plan to explore to what extent the effect on lifespan may serve as material for selection to favor or disfavor [BIG⁺]. At present, we favor a hypothesis in which the aging defect of [BIG⁺] cells is due at least in part to pleiotropic consequences of their increased proliferation and translation capacity. Although these features have already been linked to aging in genetic studies, we note that such theories of antagonistic pleiotropy in aging are not without controversy (Hughes and Reynolds, 2005).

The replicative lifespan of wild budding yeast strains varies widely (Kaya et al., 2015) and has been associated with changes in oxidative phosphorylation, respiration, and differences in metabolite biosynthesis. Genetic screening has also offered some insight into the genetic basis of this variability (McCormick et al., 2015). Lastly, genetic mapping efforts have identified polymorphisms underlying natural variation in chronological lifespan variation (Kwan et al., 2011). The genetic architecture of natural lifespan, both chronological and replicative, remains obscure, however. Our data further demonstrate that it can be subject to strong epigenetic control.

We note that prior studies have characterized genetic links between cell size and lifespan in yeast—mutants that make cells larger tend to age faster, and older cells tend to be larger than younger cells

(Neurohr et al., 2019; Yang et al., 2011; Zdrag-Tecza et al., 2009). [BIG⁺] provides an epigenetic mechanism to alter these relationships heritably. Exerting epigenetic, rather than genetic, control over basic cell growth behaviors could be valuable in the face of fluctuations between nutrient-replete and nutrient-poor conditions. Theory predicts that such mechanisms can have substantial adaptive value when the frequency of environmental fluctuations is rare relative to the frequency of phenotypic change (King and Masel, 2007). In agreement with these inferences, our modeling quantitatively described the long-run selective advantage of this ‘live fast, die young’ prion state that we measured, illustrating the importance of considering not only steady-state phenotypes but also the ecological context in which prion states are expressed. Of particular relevance to this point is our data demonstrating that transient perturbation of Hsp70 activity can eliminate [BIG⁺]. Conditions in which the prion confers a growth benefit match those that promote its propagation. Conversely, conditions in which the prion is detrimental, shortening lifespan, are also known to reduce chaperone expression (Janssens et al., 2015; Werner-Washburne et al., 1989; Werner-Washburne and Craig, 1989) and could thereby cure the prion. The strong dependence of [BIG⁺] on chaperones therefore means that it is a natural epigenetic sensor of its environment.

Our data from wild yeast isolates demonstrates that cell size and protein synthesis can also be under epigenetic control in natural isolates and is influenced by Pus4. Exploring whether a [BIG⁺]-like epigenetic mechanism that promotes proliferation in nutrient-replete conditions is conserved in metazoans is a major goal for the future. Indeed, cancer cells also experience frequent oscillations in their environments: as tumors grow and metastasize, cells are exposed to shifting gradients of oxygen and glucose that influence their proliferation rate (Martinez-Outschoorn et al., 2017; Schito and Semenza, 2016). Regulation of cell physiology in these situations is best understood at the level of transcriptional changes due to the relative ease of profiling them. However, multiple lines of evidence suggest that translational hyperactivation can also fuel pathological proliferation (Robichaud et al., 2019). Our discovery of a protein-based mechanism that changes cell growth via engagement of an altered translational program argues for greater investigation into the epigenetic control of post-transcriptional processes in both normal biology and disease.

Materials and methods

Key resources table

Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
Strain, strain background (<i>Saccharomyces cerevisiae</i>)				See Supplementary file 4
Antibody	Anti-FLAG M2 mouse monoclonal antibody	Sigma	RRID:AB_262044	(1:1000)
Antibody	Goat anti-mouse IgG peroxidase	Sigma	RRID:AB_257993	(1:10,000)
Antibody	Mouse anti-PGK1 monoclonal antibody	Invitrogen	RRID:AB_2532235	(1:1000)
Recombinant DNA reagent				See Supplementary file 4
Sequence-based reagent				See Supplementary file 4
Commercial assay or kit	Dual-Luciferase Reporter Assay	Promega	Catalog# E1910	
Commercial assay or kit	ERCC ExFold RNA Spike-In Mixes	Invitrogen	Catalog# 4456739	
Chemical compound, drug	Cycloheximide	Sigma	Catalog# C1988	CAS# 66-81-9
Chemical compound, drug	Radicicol	Research Products International	Catalog#R20020-0.001	CAS# 12772-57-5
Chemical compound, drug	Rapamycin	LC Laboratories	Catalog# R-5000	CAS# 53123-88-9
Chemical compound, drug	Canavanine	Sigma	Catalog# C9758	CAS# 2219-31-0
Chemical compound, drug	5-Fluoroorotic acid monohydrate (5-FOA)	GoldBio	Catalog# F-230-2.5	CAS# 220141-70-8
Chemical compound, drug	Guanidine hydrochloride	Sigma	Catalog# G3272	CAS# 50-01-1

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Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
Chemical compound, drug	[³⁵ S]-methionine	Perkin Elmer	Catalog# NEG709A500UC	
Chemical compound, drug	N-Cyclohexyl-N'-(2-morpholinoethyl)carbodiimide methyl-p-toluenesulfonate (CMC)	Sigma	Catalog# C106402	CAS# 2491-17-0
Software, algorithm	ImageJ software	ImageJ (http://imagej.nih.gov/ij/)	RRID:SCR_003070	
Software, algorithm	GraphPad Prism software	GraphPad Prism (https://graphpad.com)	RRID:SCR_002798	
Software, algorithm	Model	https://github.com/cjakobson/liveFastDieYoung		

Model formulation

To model the fitness of an epigenetic element for which growth in nutrient-replete conditions is improved and survival in starvation conditions is worsened, we define the following growth equations in nutrient-replete conditions:

$$x_0 = x_{00} e^{\mu_0 t}$$

$$x_1 = x_{10} e^{\mu_1 t}$$

where x_0 represents the population of naïve cells and x_1 represents the population of $[BIG^+]$ cells. μ_0 and μ_1 are the growth rates of naïve and $[BIG^+]$ cells, respectively. We neglect the lag and stationary phases of growth, as the ratio between populations does not change during this time.

Likewise, in starvation,

$$x_0 = x_{00} e^{-\delta_0 t}$$

$$x_1 = x_{10} e^{-\delta_1 t}$$

where δ_0 and δ_1 are the death rates of naïve and $[BIG^+]$ cells, respectively.

We can furthermore define the times of nutrient repletion and starvation as τ_1 and τ_2 , respectively. Thus, for each cycle of nutrient/starvation, we can define the following recursion relations:

$$x_{0,j+1} = x_{0,j} e^{\mu_0 \tau_1}$$

$$x_{1,j+1} = x_{1,j} e^{\mu_1 \tau_1}$$

$$x_{0,j+2} = x_{0,j+1} e^{-\delta_0 \tau_2}$$

$$x_{1,j+2} = x_{1,j+1} e^{-\delta_1 \tau_2}$$

And we can write an analytical expression for the ratio of populations after one repletion/starvation cycle:

$$x_0(t + \tau_1 + \tau_2) = x_0(t) e^{\mu_0 \tau_1 - \delta_0 \tau_2}$$

$$x_1(t + \tau_1 + \tau_2) = x_1(t) e^{\mu_1 \tau_1 - \delta_1 \tau_2}$$

$$\frac{x_1}{x_0}(t + \tau_1 + \tau_2) = \frac{x_1(t)}{x_0(t)} e^{(\mu_1 \tau_1 - \delta_1 \tau_2) - (\mu_0 \tau_1 - \delta_0 \tau_2)}$$

For the purposes of the model, we consider only the ratio between populations in the two epigenetic states (neglecting the total carrying capacity of the environment). The above defines a recursion relation from which we can predict the ratio of naïve and [BIG⁺] cells after N cycles of nutrient repletion and starvation.

Parameter estimation and prediction of competitive fitness

The free parameters defining the growth advantage and starvation disadvantage attributable to the epigenetic element in the model above (μ_0 and μ_1 ; δ_0 and δ_1) were determined by Monte Carlo sampling of independent measurements of the growth and death rates (Figure 2D and E). To generate the ensemble of competitive fitness predictions shown in Figure 2C, we randomly sampled growth and death rates for naïve and [BIG⁺] cells according to their experimentally determined distributions. The sampling was conducted 1000 times, and the prediction shown in Figure 2F is the median and 95% confidence interval of the resulting ensemble of model predictions.

Code and data availability

All MATLAB code and data required to generate the model predictions are available at <http://github.com/cjakobson/liveFastDieYoung> (copy archived at [swh:1:rev:d86455c2e53f50644862690e22bfdcd080abdf63](https://www.swh.io/rev/d86455c2e53f50644862690e22bfdcd080abdf63), Jakobson, 2021). Code to generate the figures is available upon reasonable request to jarosz@stanford.edu.

Bacterial strain growth

Bacteria strains (for plasmid propagation) were cultured on LB agar or liquid (Research Products International (RPI), Mount Prospect, IL).

Yeast strains

Yeast strains were cultured on either YPD agar or liquid (RPI) or SD-Ura (Sunrise Scientific, Knoxville, TN) unless otherwise indicated. Strains were stored as glycerol stocks (25% glycerol [Amersco, Solon, OH] in appropriate media) at –80 °C and revived on YPD or amino acid dropout media before testing. Yeast were grown in YPD at 30 °C on a TC-7 roller drum wheel (New Brunswick) unless indicated otherwise. Yeast transformations were performed with a standard lithium–acetate protocol (Gietz *et al.*, 1992). The *pus4Δ* strain was sourced from the BY4741 MATa haploid knockout library (GE Dharmacon, Lafayette, CO).

Strain constructions

Most diploids were constructed by crossing indicated BY4741 haploids to the BY4742 parental strain (ATCC, Manassas, VA) by mixing a bead of cells of each strain (from a single colony) together on a YPD plate and growing overnight at 30 °C. A small globule of this cell mixture was then restreaked to single colonies on SD-Lys-Met agar plates to select for diploids.

Diploids constructed for the experiments presented in Figure 4G and Figure 6—figure supplement 1A were made by crossing [BIG⁺] or naïve haploids to the PCR-verified seamless GFP-Pus4 or seamless GFP-Par32 haploid strains, respectively. Due to overlapping auxotrophic makers in each parent strain, diploids could not be selected on dropout plates from cell mixtures. Instead, pools of the mated cells were grown in several successive competitions to allow diploids to outcompete haploid parents. Diploids were then isolated from single colonies, and verified using either microscopy to measure cell size (GFP-Pus4), which is significantly larger than either haploid parent, or PCR amplification of the GFP tag/ORF junction (GFP-Par32), which yielded two distinct bands matching the expected sizes of the wild-type allele and the tagged allele, in contrast to the haploid parental strains that yielded only one or the other band sizes. These isolated diploids were then imaged as described below.

Sporulations were performed inoculating single diploid colonies in Pre-SPO liquid media (YPD with 6% glucose) for 2 days at room temperature on a roller drum wheel. Cells were then pelleted and washed twice in SPO media (1% potassium acetate [Sigma, St. Louis, MO], 320 mg CSM-Met powder [Sunrise Scientific], 20 mg methionine [Sigma] per liter), and then diluted 10-fold into 3 mL cultures of SPO. These cultures were incubated on a rotary wheel for 1 week at room temperature, before dissecting tetrads on a Singer Instruments MSM400 (Somerset, England).

The 3XFLAG-Pus4 strains were constructed using PCR combined with the ‘Delitto Perfetto’ method (Storici and Resnick, 2006). Canavanine mutants were constructed by pelleting 500 μ L of a saturated YPD liquid culture, resuspending it in 100 μ L YPD and plating this on SD-Arg agar plates containing 60 μ g/mL canavanine (Sigma), and growing for 2 days at 30 °C. Single canavanine-resistant colonies were picked and re-tested for resistance before further testing.

Cytoductions were performed as described in Chakrabortee et al., 2016a. A BY4742 strain with a defective *KAR1* allele (*kar1-15*) was created as an initial recipient for cytoplasmic transfer. This allele prevents nuclear fusion during mating while permitting cytoplasmic transfer. The strain carries auxotrophic markers distinct from those in the putative [BIG⁺] or naïve donor strains, and was also converted to petite with growth on ethidium bromide (strains were grown in YPD with 25 μ g/mL ethidium bromide for approximately two-dozen generations before testing for growth on YP-glycerol). This allowed cytoplasmic transfer to be scored through the restoration of mitochondrial respiration, while selecting for auxotrophic markers unique to the recipient strain. The recipient and donor strains were mixed together on YPD-agar and grown overnight, followed by selection of heterokaryons and resulting haploid cytoductants on dropout media (selecting for the BY4742 recipient strain markers) containing glycerol as a carbon source. One more round of selection was used while replica-plating onto a dual-selection agar plate (SD-Lys-Met) to confirm that the colonies were not diploids. One additional round of propagation on a non-selective plate was performed before doing ‘reverse cytoductions,’ which were performed in the same way except selecting for BY4741 auxotrophy in recipient naïve strain. In the reverse cytoductions, the donors were naïve or putative-[BIG⁺] BY4742 *kar1-15* cytoductants from the first round, and the recipients were wild-type or *pus4* Δ naïve BY4741 petite cells.

Transient *PUS4* deletion experiment strains were made by the Delitto Perfetto method (Storici and Resnick, 2006). After deletion of *PUS4*, strains were propagated for ~75 generations before phenotyping. The *PUS4* gene was re-introduced by homologous recombination.

Transient overexpression of Pus4 in haploid BC187 yeast was performed using PDJ541 (CEN URA3 GAL::PUS4) (Supplementary file 4). Three independent transformants of BC187 (previously cured by transient Hsp70 inhibition) bearing this plasmid were grown in SGalactose-Ura for 48 hr at 30 °C. Overexpression was stopped by outgrowth in non-selective YPD medium for 48 hr. Strains lacking the overexpression plasmid were isolated by plating to single colonies on media containing 5-FOA. Uracil auxotrophy was confirmed before subsequent measurements of cell size.

Growth assays

Biological replicates of each yeast strain were pre-grown in rich media (YPD). We then diluted these saturated cultures 1:20 in sterile water and then inoculated 3 μ L into 96-well humidified plates (Nunc Edge Plates [Thermo Scientific, Waltham, MA]) with 150 μ L of YPD or SD-CSM per well. Cycloheximide (Sigma) was added to growth media at either 0.01 μ g/mL or 0.05 μ g/mL, as indicated. Rapamycin (LC Laboratories, Woburn, MA) was added to growth media at 10 μ M. Cell growth was monitored with continuous measurements of OD₆₀₀ (approximately every 10 min) at 30 °C over 96 hr using BioTek Eon or Synergy H1 microplate readers (Winooski, VT). Timepoints plotted in bar graphs correspond to the maximum proliferation rates calculated from growth data.

Measurement of chronological lifespan

For each strain, four single colonies were picked from freshly streaked YPD plate and grown in 5 mL of pre-sporulation media for 3 days on a roller drum wheel (New Brunswick Scientific, Edison, NJ) at 30 °C. Cultures were then pelleted and washed once with SPO media, and resuspended in 5 mL of SPO media, and placed back on the roller drum wheel at 30 °C. On days indicated, a dilution was made of each replicate to achieve dozens to hundreds of colonies on a YPD plate, which were then counted using a colony counter (Synbiosos Acolyte, Frederick, MD). Dilution was ~100,000 $\times$ at early stages of the experiment and was later empirically determined due to significant cell death. We note that aging the cells in SPO did not lead to significant acidification (pH of old cultures was found to be >5), as has been reported for cells aged in YPD, which contains high levels of glucose that upon metabolism leads to the secretion of organic acids (Murakami et al., 2011).

Measurement of replicative lifespan

RLS was assessed using the standard method of isolating virgin cells on agar YPD (2% glucose) plates, and then separating their daughter cells at each cell division by micromanipulation and counting the total number of daughters produced by each mother cell (Steffen *et al.*, 2009; Wasko *et al.*, 2013). Strains were streaked from glycerol stocks onto YPD plates and allowed to grow at 30 °C until individual colonies could be selected for each strain. Colonies were lightly patched onto fresh YPD overnight and 20 cells were isolated by microdissection from each patch. These cells were incubated at 30 °C for ~2 hr until they had formed daughter cells, at which time individual virgin daughter cells were selected and arrayed as previously described (Steffen *et al.*, 2009) for lifespan analysis. From these, daughter cells were removed by microdissection and counted approximately every 2 hr during the day. Plates were maintained at 30 °C during the day and placed at 4 °C overnight. At least four independent replicates (arising from different colonies) of 20 individual mother cells each were measured for each strain.

Curing

Three regimes of chaperone inhibition were tested: (1) transient exposure to a dominant-negative version of Hsp70 (Ssa1) to inhibit its activity (Chakrabortee *et al.*, 2016a; Jarosz *et al.*, 2014); (2) transient exposure to radicicol (RPI) to inhibit Hsp90 activity (Chakrabortee *et al.*, 2016a); and (3) transient exposure to guanidinium hydrochloride (Sigma) to inhibit Hsp104 activity (Ferreira *et al.*, 2001).

Regime 1 was performed by transforming cells with a plasmid, PDJ169, harboring a dominant-negative version of Hsp70 (Ssa1) as described previously (Chakrabortee *et al.*, 2016a; Jarosz *et al.*, 2014; Lagaudriere-Gesbert *et al.*, 2002). Transformants were picked and restreaked by hand or replica-pinned using a Singer HDA robot a total of 12 times on SD-Ura to promote Ssa1^{DN} expression. (Anecdotally, we note that prion phenotypes are frequently cured with fewer than 12 restreaks, but for reasons of technical throughput, 12 were used in this experiment.) Then plasmids were eliminated by plating on media containing 5-fluoroorotic acid (SD-Ura +0.1 % 5-FOA + 50 µg/mL uracil) and plasmid loss was verified by replating on SD-Ura. Colonies were then tested for the elimination of prion phenotypes. Tested strains were compared to control strains that were restreaked in parallel on SD-CSM plates.

Regime 2 was performed by replica-pinning cells six subsequent times on YPD agar plates containing 5 µM radicicol, using a Singer HDA robot. After replatings, cells were plated back onto YPD two subsequent times to facilitate recovery before being tested for the elimination of prion phenotypes. Tested strains were compared to control strains that were replica-pinned in parallel on YPD plates.

Regime 3 was performed like regime 2 but with SD-CSM plates containing 0.5 g/L guanidinium hydrochloride. Tested strains were compared to control strains that were replica-pinned in parallel on SD-CSM plates.

Wild yeast strains were cured by transforming a uracil-selectable 2micron plasmid (PDJ1222) encoding the aforementioned dominant-negative version of Hsp70 (Ssa1), under control of a constitutive promoter (*pGPD*). Transformants were passaged on selective media five times to allow the growth of single colonies. Transformants were then passaged three times on non-selective media (YPD) to permit plasmid loss, which was confirmed by the lack of growth on selective media (SD-Ura).

Strain competitions

Single colonies were used to inoculate 5 mL YPD cultures that were grown for 3 days on a roller drum wheel at 30 °C. Cells were diluted 1000-fold and then mixed in equal volumes to form 50:50 mixtures of either of the following: naïve Can^S and [BIG⁺] Can^R; or naïve Can^R and [BIG⁺] Can^S. Before mixing cells, saturated cultures were measured to have near equal cell densities, and 'time zero' measurement was made by plating the initial cell mixture and counting the number of canavanine-resistant colony-forming units (CFUs) relative to total CFUs. These initial strain mixtures were then grown for 2 days on a roller drum wheel at 30 °C, after which cells were diluted 50,000-fold or 25,000-fold and plated on YPD or canavanine plates, respectively. These plates were grown at 30 °C for 2 days before counting colonies. The liquid cultures were diluted 1:1000 in 5 mL of fresh YPD, and this process was repeated nine more times. Swapping of canavanine resistance between naïve and [BIG⁺] was done

to correct for the potential of canavanine resistance to influence cell growth. However, in our experiments the differences were negligible. The numbers of canavanine-resistant and total colonies were compared relative to a number of cells plated to determine the number of naïve or [BIG⁺] colonies arising at each timepoint.

Microscopy and cell size measurements

Most microscopy was performed using a Leica inverted epifluorescence microscope (DMI6000B) with a Hamamatsu Orca 4.0 camera. Cells were imaged after 3 days of growth in 5 mL YPD at 30 °C. Saturated cultures were diluted 10-fold with 1 X PBS and briefly sonicated to break up cell clumps. Differential interference contrast (DIC) images were taken at 20 ms exposure time using a 63× /1.40 oil objective. Cell area was calculated using CellProfiler (3.1.5) image analysis software (<http://www.cellprofiler.org>; Carpenter et al., 2006). The large cell threshold was set at one standard deviation above the cell area mean of the naïve cells.

GFP microscopy data presented in Figure 4G were obtained from cells were grown in YPD for 24 hr. Aliquots of 1 mL were spun down and resuspended in 200 µL 1 X PBS, 2 µL of which were prepared into an agar pad. Agar pad made with 2 % agar and PBS. Fluorescent images were taken at 400 ms exposure time and a Z-stack consisting of 25 steps for a total length of 4.83 µm in each channel using a 100× /1.40 oil objective.

Data presented in Figure 8C were imaged similarly to Figure 4G, except that cells were grown in SC-Ura media for retention of the GFP-Pus4 expression plasmid. This plasmid (Supplementary file 4) overexpresses Pus4 protein via a strong promoter driving the expression of GFP-Pus4 constitutively on a centromeric plasmid.

Data presented in Figure 6—figure supplement 1A were grown and imaged similarly to above, except cells were imaged in 1 X PBS using a GE DeltaVision Ultra microscope (Boston, MA).

Cell size experiments using protein synthesis inhibitors (Figure 6A–D): conditions were the same as above, with the following differences. Single colonies were inoculated into YPD, YPD + cycloheximide (0.05 µg/mL), or YPD + rapamycin (10 µM) and grown for 4 days before imaging. (Very similar results were observed after 3 days of growth.) The following day, cultures were diluted into liquid YPD and grown for 3 days, after which they were restreaked once onto YPD agar. Single colonies were then used to inoculate liquid YPD cultures, grown for 3 days before imaging to test for the reappearance of the large cell size phenotype.

Microscopy image processing

ImageJ version 2.0.0-rc-69/1.52 p, Build 269a0ad53f.

For GFP-Pus4 in laboratory *S. cerevisiae* and wild *S. cerevisiae* isolate BC187 (Figures 4G and 8C): (1) using full-sized images, created a maximum projection of 25-step Z-stack; (2) in ImageJ, set 50 × 50 pixel square in the area between cells and measure average intensity; (3) subtracted this intensity from the total image using the 'Math' function; (4) added 5 µm scale bar calculated from the original voxel size of 0.065 µm; and (5) enlarged Photos 6X using Topaz Gigapixel AI software version 5.3.2 (Topaz Labs).

For GFP-Par32 microscopy (Figure 6—figure supplement 1A), analysis was performed using ImageJ (imagej.nih.gov). All images were adjusted to a uniform contrast and illumination corrected using standard tools in ImageJ. The background was calculated using a morphological opening with a disc of radius 75 pixels, such that the structuring element was larger than cells in the foreground. In these background-corrected images, we quantified the ratio of membrane to cytoplasmic Par32-GFP within each cell by measuring the average intensity for a region at the plasma membrane and the average intensity for an equivalently sized region approximately 0.2 µm from the plasma membrane. For each cell, the 'Fraction membrane Par32 signal' was calculated by dividing the average Par32-GFP intensity in the membrane region by the average Par32 intensity in the cytoplasmic region.

Luciferase assays

Strains were transformed with PDJ512 and PDJ513. To maintain the plasmids, we grew these cells in a synthetic complete medium containing nutrient levels between those in SD and YPD formulations. Four independent transformants for each sample were grown for 1 day in 150 µL SC-Ura (Sunrise Scientific) per well in 96-well plates at 30 °C. Saturated cultures were then diluted 15 × into fresh

media in a new 96-well plate and grown until cultures reach OD₆₀₀ 0.6, as determined by a BioTek Eon plate reader. 20 μ L of each culture was added using a multichannel pipette into a white flat-bottom 96-well microplate (E&K Scientific, Santa Clara, CA) already containing 20 μ L of room temperature 1 X Passive Lysis Buffer from the Dual-Luciferase Reporter Assay System (Promega, Madison, WI). Cultures were then lysed by shaking at 300 rpm for 25 min at room temperature. *Renilla* and firefly luciferase activity was measured using 75 μ L injection volumes and otherwise default settings on a Veritas luminometer (Turner Biosystems). pTH726-CEN-RLuc/minCFLuc (PDJ512) and pTH727-CEN-RLuc/staCFLuc (PDJ513) were gifts from Tobias von der Haar (University of Kent; Addgene plasmids #38210 and #38211; *Chu et al., 2014*).

Final luciferase values were normalized to OD₆₀₀ measurements of cultures to account for cell density. We note, however, that [BIG⁺] cells did not have a general growth advantage over naïve cells in SC-Ura, and when comparing optical density measurements to those counting cells using a hemacytometer, we observed no perturbation in the relationship between cell number and optical density for [BIG⁺] cells.

For wild strains, we considered the possibility that curing could reverse multiple epigenetic elements affecting plasmid copy number, transcription, or other elements of gene expression apart from protein synthesis. Indeed, after normalizing *Renilla* or firefly luciferase values to cell density, some strains have several-fold differences after curing, although they were closely correlated irrespective of which firefly codon variant was compared. Therefore, as for data presented in *Figure 6E*, for *Figure 8B* we also normalized firefly luciferase values to *Renilla* luciferase, which is expressed from the same plasmid. This normalization procedure thus tests for differences in protein synthesis that are codon-frequency dependent, that is, a measure of translational efficiency.

Polysome profiling

Single colonies from two biological replicates per sample were used to inoculate 5 mL YPD cultures that were grown on a roller drum wheel at 30 °C overnight. Saturated cultures were added to 95 mL of YPD in 500 mL flasks and shaken at 225 rpm at 30 °C until cultures reached OD₆₀₀ 1.0. Five minutes prior to harvesting cells, we added cycloheximide (Sigma) to a final concentration of 100 μ g/mL to arrest translation, by adding 1 mL of a 10 mg/mL stock solution (in ethanol) per culture, then immediately swirling flask and putting back on a shaker for 5 min 225 rpm 30 °C to permit the chemical to enter cells and arrest protein synthesis. Cultures were pelleted in 50 mL conical tubes for 3 min at 5000 rpm. After decanting the supernatant, pellets were quickly resuspended in ice-cold Polysome Lysis Buffer (PLB; *Jan et al., 2014*) (20 mM Tris pH 8.0, 140 mM KCl, 1.5 mM MgCl₂, 100 μ g/mL cycloheximide, 1 % Triton X-100, RNase-free reagents), 250 μ L total PLB per sample. Resuspended pellets were then flash-frozen in liquid nitrogen. Pellets were weighed to ensure their weights were near equal and then thawed on ice. 250 μ L of additional ice-cold PLB was added per sample, making slightly over 0.5 mL per sample. Samples were then flash-frozen in tiny pellets ('yeast dippin' dots') by pipetting directly into a small dewar filled with liquid nitrogen and a wire mesh basket nested inside. Tiny pellets were then stored at -80 °C until lysis. Samples were lysed using a Retsch Cryomill (Haan, Germany) with 25 mL canisters and the following program: pre-cool, then 12 cycles of 15 Hz $\times$ 3 min. Smears of lysate were stained with Trypan blue and imaged under a microscope to verify efficient lysis. (We suspect with larger sample volume:canister volume ratios, fewer cycles would be necessary.)

Lysates were loaded onto 10–50% sucrose gradients pre-poured on a BioComp Gradient Master 108 (Fredericton, ND, Canada). Lysates generally contained RNA concentrations around 12–18 μ g/ μ L. 30 μ L of lysate was carefully pipetted onto the top of the sucrose gradient, and samples were spun in a Beckman SW41 Ti Rotor for 2.5 hr at 4 °C at 40,000 rpm. Gradients were analyzed on a Brandel fractionator (Gaithersburg, MD). Technical replicates (same lysate independently loaded onto separate gradients) showed a very high degree of similarity, as did biological replicates.

[³⁵S]-Methionine pulse labeling

Adapted from *Esposito and Kinzy, 2014*. Single colonies were inoculated into 5 mL complete media lacking methionine and grown overnight at 30 °C. The saturated starter culture was then inoculated into 75 mL of complete media lacking methionine to an OD₆₀₀ of 0.1 in a 250 mL flask. Flasks were grown at 30 °C shaking at 225 rpm until an OD₆₀₀ of 0.5–0.7 was reached at which point 37.5 μ L of 100 mM (cold) methionine (Sigma) and 7.32 μ L of 10.25 mCi/mL [³⁵S]-methionine (PerkinElmer) was

added for a final concentration of 50 μ M methionine and 1 μ Ci/mL [35 S]-methionine. At 15 min intervals from the time of methionine addition, OD₆₀₀ was determined by cuvette and 1 mL aliquots were obtained for scintillation counting. To the 1 mL aliquot, 200 μ L 50 % TCA (Sigma) was added and incubated on ice for 10 min. The samples were then incubated at 70 °C for 20 min and briefly spun down. In a 1225 Sampling Manifold (MilliporeSigma XX2702550), 25 mm glass microfiber filters (GE Healthcare Life Sciences) were arranged and pre-wetted with 5% TCA. Samples were then applied to the glass fiber filters under vacuum. Filters were then washed twice with 5 mL 5% TCA and once with 95% ethanol. Filters were then removed from the manifold, air-dried on Whatman paper, placed in scintillation vials with 5 mL ScintiSafe Econo 1 scintillation cocktail solution (Fisher Scientific SX20-5), and underwent six rounds of technical replicated counting on a Beckman LS 600SCS. Scintillation counts were then normalized to OD₆₀₀.

2-D gel electrophoresis

Following 1 hr of pulse labeling with [35 S]-methionine, 10 mL of cells were collected and centrifuged at 16 k $\times$ g at 4 °C for 10 min. The supernatant was removed and 300 μ L of TCA buffer was added (10 mM Tris-HCl pH 8.0; 10 % TCA; 25 mM NH₄OAc; 1 mM Na₂EDTA) along with glass beads. Samples were then vortexed for 1 min bursts with 5 min rests for five times at 4 °C. The slurry was transferred away from the glass beads to a fresh tube. 200 μ L of fresh TCA buffer was added to beads to pick up residue. Samples were then split, one half destined for resuspension in SDS for protein quantification and the other in CHAPS for the 2-D gel. Samples were centrifuged at 16 k $\times$ g at 4 °C for 10 min. The supernatant was then removed from the resulting pellets and washed twice with 1 mL acetone. The samples were then resuspended in SDS Resuspension Solution (0.1 M Tris-HCl pH 11.0; 3% SDS) or CHAPS Resuspension Buffer (8 M urea; 4% CHAPS; 2% IPG Buffer 3–10; 40 mM DTT). CHAPS samples were then adjusted to 1 μ g/ μ L. First-dimension resolution was conducted with 250 μ g with 13 cm 3–10 NL Immobiline DryStrip gels (Cytiva). Rehydration protocol started with 12 hr rehydration followed by 500 V for 0.5 kWh, 1000 V for 1.0 kWh, 16,000 V for 14.5 kWh. First dimension strips were then trimmed to fit the second-dimension gel and equilibrated in Equilibration Buffer (6 M urea; 30 % glycerol; 2 % SDS, 50 mM Tris-HCl pH 8.8; 0.002 % bromophenol blue; 65 mM DTT) for 30 min. Strips were rinsed with MOPS running buffer and loaded onto 11 cm 4–12% Bis-Tris Criterion XT gels (Bio-Rad). Strips were sealed in place with an agarose plug (0.5 % agarose; 0.0002 % bromophenol blue) and ran at 200 V for 1 hr. Second-dimension gels were then stained in Coomassie and dried in a cellulose sandwich consisting of a plastic wrap on one side of the gel to facilitate the removal of one of the cellulose layers for autoradiography. X-ray film was then exposed for 9 days. Autoradiography spots were quantified in ImageJ by tracing out the spot and quantifying the integrated density of the signal. Densities of prion cells were then normalized to the naïve sample, as reported in *Figure 6I*.

GFP-fusion measurements

Naïve or [B^{IG}+] cells were mated to the SWAT seamless-GFP library (Weill *et al.*, 2018; Yofe *et al.*, 2016) on solid YPD agar plates in 384-spot format for 24 hr at room temperature. Diploids were selected on media lacking both lysine and methionine (SD-Lys-Met) and propagated for 48 hr at room temperature. Diploids were inoculated into 60 μ L of liquid media lacking both lysine and methionine (SD-Lys-Met) in 384-well plates. All library manipulations were carried out using a Singer ROTOR HDA robotic pinning instrument. Cells were propagated in liquid medium for 24 hr at 30 °C (OD₆₀₀ ~ 1), at which time OD₆₀₀ and green fluorescence were measured using a BioTek Synergy H1 plate reader. OD₆₀₀ was adjusted based on known blank wells, and the GFP/OD₆₀₀ measurements were normalized by Z-score ($(x_i - \mu)/\sigma$) within the naïve and [B^{IG}+] populations independently.

Settings used for PWScan for predicted Pus4 target sites in silico

Matrix format: PFM-like matrix
 Motif length: 17
 Minimal log score: -10,000
 Log scaling factor: 100
 Genome assembly: sacCer3
 p-value threshold: 1.000e⁻⁰⁵
 Matrix score: -8265

Cut-off percentage: 93.88%
Background base composition: 0.31, 0.19, 0.19, 0.31
Search strand: both
Offset: 0
Non-overlapping matches: on

Western blots

For SDS-PAGE, immunoblots, and protein yield measurements, cells were lysed using a Retsch Cryo-mill using the following program: six 3 min cycles at 15 Hz with 2 min cooling cycles in between. Cell lysates were loaded onto GenScript ExpressPlus SDS-PAGE 4–20% gels (Piscataway, NJ) and stained using Coomassie blue. For western blots, anti-FLAG M2 monoclonal antibody (Sigma) was used to detect 3XFLAG-Pus4, and anti-PGK1 monoclonal antibody (Invitrogen) was used to detect the loading control.

RNA sequencing

Four independent colonies of naïve and [BIG⁺] cells were grown in 5 mL liquid YPD cultures on a culture roller drum wheel overnight at 30 °C. Saturated cultures were diluted 1:1000 in new 5 mL YPD cultures and grown until OD₆₀₀ 1.0 (exponentially growing). Cultures were pelleted in a table-top centrifuge with the swing-bucket rotor at 4300 × g at 4 °C for 5 min, washing once with ice-cold 1 X PBS. RNA isolation, poly-A selection, the addition of ERCC spike-in controls, and sequencing were performed by the Beijing Genomics Institute. Analysis was performed using Kallisto (Bray *et al.*, 2016) and Sleuth (Pimentel *et al.*, 2017). Transcripts Benjamini–Hochberg-corrected *q*-value <0.05 and |log₂ fold-change| > 1 were considered differentially expressed. Raw data and other experimental information are available on the Gene Expression Omnibus (Barrett *et al.*, 2013), accession: <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE176577>.

tRNA/rRNA quantification

Strains were grown up in 5 mL of YPD overnight at 30 °C. Saturated cultures were then diluted 15 × into fresh media and grown to an OD₆₀₀ 0.8. Cells were then pelleted, and total RNA was extracted by phenol-chloroform extraction. RNA concentration for each sample was then analyzed by Nanodrop and diluted to equal concentrations in RNase-free water. The extracted RNA was then run on a nucleic acid fragment analyzer, and total tRNA abundance was quantified relative to the abundance of 5.8 S rRNA.

Pseudouridine measurements: high-resolution melt curve analysis

Protocol adapted from Lei and Yi, 2017. For each sample, 40 µg of total RNA was fragmented in RNA fragmentation buffer (New England Bio Labs, Ipswich, MA) at 94 °C for 3 min. Following ethanol precipitation, fragmented RNA was resuspended in 80 µL of 5 mM EDTA, denatured at 80 °C for 5 min, and then immediately chilled on ice. Each sample was then split into two 40 µL samples for a CMC-labeled and non-labeled control. The 40 µL RNA sample destined for CMC-labeling was added to 400 µL BEU + CMC buffer (50 mM bicine, pH 8.5; 4 mM EDTA; 7 M urea; 200 mM CMC [Sigma]). The non-labeled 40 µL RNA sample was added to 400 µL BEU buffer (50 mM bicine, pH 8.5; 4 mM EDTA; 7 M urea). Both samples were incubated at 37 °C for 20 min to carry out the CMC-Ψ reaction, followed by ethanol precipitation. Each sample was then resuspended in 200 µL Na₂CO₃ buffer (50 mM Na₂CO₃, pH 10.4; 2 mM EDTA) and incubated at 37 °C for 6 hr. Following incubation, RNA was ethanol precipitated and resuspended in 40 µL H₂O. RNA was annealed to primers by the addition of 4 µL 100 µM Random Hexamer Primers (TaKaRa, Mountain View, CA) and incubation at 65 °C for 5 min. Samples were chilled on ice afterward. To perform the reverse transcription, 32 µL RT Buffer (125 mM Tris, pH 8.0; 15 mM MnCl₂; 187.5 mM KCl; 1.25 mM dNTPs; 25 mM DTT) was added to each sample. Samples were then incubated at 25 °C for 2 min. After, 0.5 µL SuperScript II reverse transcriptase (SSII, Invitrogen, Waltham, MA) was added to each sample followed by incubation at 25 °C for 10 min, 42 °C for 3 hr, and 70 °C for 15 min. To perform qPCR analysis, 2 µL of the sample was mixed with 10 µL 2 X SYBR Mix (Kapa Biosystems, Wilmington, MA), 0.4 µL of each 10 µM primer (IDT, Coralville, IA), and 7.2 µL H₂O for a total of 20 µL for each reaction. qPCR was performed in a Bio-Rad CFX Connect Real-Time System (Bio-Rad, Hercules, CA) using the following protocol: initial

incubation at 95 °C for 5 min, followed by 45 cycles of 95 °C for 0.5 min and 60 °C for 1 min. Following amplification, the reaction was brought down to 54 °C and held for 5 s, increasing in temperature by 0.1 °C increments until 95 °C is reached to obtain melt curve data.

Pseudouridine measurements: Sanger sequencing

Templates were *TEF1/TEF2* amplicons from the qPCR step above. Due to the short length of tRNA amplicons, the qPCR product was re-amplified with primers containing the restriction sites *NcoI* and *HindIII* and ligated into a pETm11 vector to allow for sequencing from the T7 promoter.

Pseudouridine measurements: CLAP

Adapted from *Zhang et al., 2019*.

CMC treatment of RNA

20 µg of total RNA in 12 µL of water was mixed with 24 µL of 1× TEU buffer (50 mM Tris-HCl pH 8.0, 4 mM EDTA, 7 M urea). Next, 1 M CMC was prepared fresh in TEU buffer, and 4 µL was added to +CMC samples while 4 µL of TEU buffer was added to –CMC samples for a total volume of 40 µL. To incorporate CMC, the samples were incubated at 30 °C for 16 hr. Following incubation, excess CMC was removed by the addition of 160 µL of 50 mM KOAc pH 5.5, 200 mM KCl, 3 µL 5 µg/µL GlycoBlue (Thermo Fisher, AM9515), and 550 µL of EtOH. The samples were incubated for 2 hr at –80 °C then centrifuged at 13 krpm at 4 °C for 35 min. The supernatant was removed and 500 µL of 75 % EtOH was added. The samples were incubated at –80 °C for 2 hr and centrifuged in the same manner as before. The EtOH precipitation and wash steps were repeated an additional time. After the second wash step, the samples were left at –80 °C overnight. The following day the samples were centrifuged again at 13 krpm at 4 °C for 35 min, and the supernatant was removed. To reverse the CMC-U/CMC-G adducts, the RNA pellet was mixed with 40 µL of 50 mM Na₂CO₃, 2 mM EDTA, and incubated at 37 °C for 6 hr. An additional 160 µL of 50 mM KOAc (pH 5.5), 200 mM KCl was added after incubation followed by ethanol precipitation.

RNA 5' phosphorylation and RNA-5 blocking oligo ligation

Performed similarly to *Zhang et al., 2019*. To reduce the signal derived from random RNA fragmentation during the CMC reaction, ±CMC-treated RNA in 6.5 µL H₂O was mixed with 0.5 µL RNase inhibitor (NEB, M0307L), 1 µL of 10× T4 PNK reaction buffer, 1 µL of 1 mM ATP, 1 µL of T4 polynucleotide kinase (PNK), and then incubated at 37 °C for 30 min. The RNA-5 oligo (5AmMC6/rArCrCrCrA; Integrated DNA Technologies) was ligated by addition of 1 µL of 10× T4 RNA Ligase Reaction Buffer, 1 µL of 100 µM RNA-5 oligo, 1 µL of 1 mM ATP, 1 µL of RNase inhibitor, 3 µL of DMSO, 2 µL of H₂O, and 1 µL of T4 RNA ligase I (NEB, M0437M), and incubated at 16 °C for 16 hr. The reaction was terminated by addition of 1.2 µL of 200 mM EDTA.

Reverse transcription and splint ligation

Performed similarly to *Zhang et al., 2019*. Reverse transcription was carried out using 3 µL of the above ligation mixture (~1 µg RNA) using AMV reverse transcriptase (NEB, M0277L) and target-specific primers. To anneal the primer, 1 µL of 10× annealing buffer (250 mM Tris-HCl [pH 7.4], 480 mM KCl) and 1 µL of 0.5 µM target-specific RT primer were added, and the mixture was incubated at 93 °C for 2 min. The RT reaction was performed by mixing 5 µL of 2× AMV RT reaction mixture to make 0.6 U/µL AMV RT, 1× AMV RT buffer, and 0.5 mM of each dNTP. The RT reaction was run at 42 °C for 1 hr. RT enzyme was inactivated by incubation at 85 °C for 5 min, and then 1 µL of 5 U/µL RNase H (NEB, M0297L) was added and the mixture incubated at 37 °C for 20 min to digest the RNA. RNase H was inactivated by incubating the mixture at 85 °C for 5 min.

To anneal the adaptor, 1 µL of the adaptor/splint oligos mixture (1.5 µM each) was added to the above RT mixture, and then incubated at 75 °C for 3 min. To ligate the adaptor, 4 µL of 4× ligation mixture was added for a final concentration of 10 U/µL of T4 DNA ligase (NEB, M0202L), 1× T4 DNA ligase reaction buffer, and 12.5 % DMSO. The ligation was performed at 16 °C for 16 hr. DNA ligase was inactivated by incubating the mixture at 65 °C for 10 min.

PCR amplification and gel electrophoresis

To perform PCR, 2 μ L of the above ligation mixture was mixed with various components for a final condition of 1 $\times$ Q5 reaction buffer, 1 $\times$ Q5 high GC enhancer, 200 μ M of each dNTP, 0.5 μ M forward and reverse primers, and 0.02 U/ μ L Q5 high-fidelity DNA polymerase (NEB, M0491L); the final PCR reaction volume was 35 μ L. PCR was done for 35 cycles. A PCR cleanup was performed, and 22 μ L of water was used to elute the final product.

About half of the PCR product, 9 μ L, was mixed with 7 μ L of water and 4 μ L of 6 $\times$ TriTrack DNA Loading Dye (Thermo Fisher, R1161). The entire mixture was loaded on a prerun 10 % nondenaturing gel containing 1 $\times$ TBE, together with Ultra Low Range DNA ladder (NEB, N0558S). The gel was stained with SYBR gold nucleic acid gel stain (Thermo Fisher, S11494) for 10 min. Product bands were visualized using the Amersham Typhoon imaging system and the bands quantified using ImageQuant TL.

Data display

Plots/graphs were made using PRISM 7/8/9 software (GraphPad, San Diego, CA).

Acknowledgements

We gratefully acknowledge Rebecca Freilich and Alan Itakura (Stanford University), Mike Harms (University of Oregon), and Gabriel Neurohr (ETH Zürich) for their review of the manuscript and members of the Jarosz laboratory for helpful discussions. We thank Maya Schuldiner (Weizmann Institute) for the generous gift of the SWAT GFP library; Kathrin Leppek, Gerald Tiu, and Maria Barna (Stanford University) for assistance with polysome gradients and profiling and the use of their instrument; Huili Guo (IMCB Singapore) for advice on presentation of polysome profiling data; José Aguilar Rodriguez (Stanford University) for creating the Position Weight Matrix and scanning the yeast genome for candidate pseudouridylation sites; and the Genomics and Cell Characterization Core Facility (University of Oregon) for assistance with tRNA quantification.

Additional information

Competing interests

Matt Kaeberlein: Reviewing editor, eLife. The other authors declare that no competing interests exist.

Funding

Funder	Grant reference number	Author
National Institute of General Medical Sciences	F32-GM109680	David M Garcia
Burroughs Wellcome Fund	Postdoctoral Enrichment Program 1015119	David M Garcia
National Institute on Aging	P30AG013280	Matt Kaeberlein
National Science Foundation	Graduate Research Fellowship	Edgar A Campbell
National Institute of General Medical Sciences	F32-GM125162	Christopher M Jakobson
National Institutes of Health	DP2-GM119140	Daniel F Jarosz
National Science Foundation	NSF-CAREER-MCB116762	Daniel F Jarosz
Kinship Foundation	Searle Scholar Award (14-SSP-210)	Daniel F Jarosz
Sidney Kimmel Foundation	Kimmel Scholar Award (SFK-15-154)	Daniel F Jarosz

Funder	Grant reference number	Author
David & Lucile Packard Foundation	Science and Engineering Fellowship	Daniel F Jarosz
Ford Foundation	Postdoctoral Fellowship	David M Garcia
Donald E. and Delia B. Baxter Foundation	Award	David M Garcia

The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

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David M Garcia, Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review and editing; Edgar A Campbell, Christopher M Jakobson, Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Resources, Software, Validation, Visualization, Writing – original draft, Writing – review and editing; Mitsuhiro Tsuchiya, Data curation, Formal analysis, Investigation, Methodology, Resources, Validation, Visualization; Ethan A Shaw, Investigation, Methodology, Resources, Validation, Visualization; Acadia L DiNardo, Investigation, Resources; Matt Kaeberlein, Funding acquisition, Supervision, Writing – review and editing; Daniel F Jarosz, Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review and editing

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Decision letter and Author response

Decision letter <https://doi.org/10.7554/eLife.60917.sa1>
Author response <https://doi.org/10.7554/eLife.60917.sa2>

Additional files

Supplementary files

- Supplementary file 1. See .docx file for parameter values used for the competitive fitness models shown in *Figure 2* and *Figure 2—figure supplement 1*.
- Supplementary file 2. RNA-sequencing results. See .xlsx file for transcripts per kilobase million for each gene in four replicates of naïve and four replicates of [BIG⁺]. Genes that showed significant differences are listed in a separate tab.
- Supplementary file 3. Proteins whose expression is changed in [BIG⁺] cells.
- Supplementary file 4. Yeast strains, plasmids, and oligonucleotides used in this study. See .xlsx file.
- Supplementary file 5. Yeast genes with predicted Pus4 target sites based on scanning the yeast genome using a position weight matrix. See .csv file.
- Transparent reporting form
- Reporting standard 1. Statistical reporting.

Data availability

Updated sequencing data have been deposited in GEO under accession code GSE176577.

The following dataset was generated:

Author(s)	Year	Dataset title	Dataset URL	Database and Identifier
Garcia DM, Campbell EA, Jakobson CM, Tsuchiya M, Shaw EA, DiNardo A, Kaerberlein M, Jarosz DF	2021	A prion accelerates proliferation at the expense of lifespan	https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE176577	NCBI Gene Expression Omnibus, GSE176577

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Appendix 1

We also examined the relationship between the proteins whose expression was altered in [BIG⁺] cells and other features related to gene expression, including mRNA secondary structure and pseudouridylation. Comparing the altered gene set to yeast transcripts with more double-stranded regions (Kertesz *et al.*, 2010), no relationship emerged. We also compared the gene list to mRNAs that have been experimentally validated to be pseudouridylated by Pus4 (Carlile *et al.*, 2014; Lovejoy *et al.*, 2014; Schwartz *et al.*, 2014), but did not see any significant correlation or changes among the limited number targets that contained Pus4-dependent pseudouridylation sites in these studies, and which were present in the SWAT gene collection (13 genes overlapped these two categories). Given the limited reproducibility of mapping pseudouridylation sites transcriptome-wide in yeast from past studies, with large variability sample to sample (Safra *et al.*, 2017; Zaringhalam and Papavasiliou, 2016), it is possible that the earlier studies missed possible Pus4 mRNA targets that could have been present in the list of altered genes. Therefore, we also created a position weight matrix (PWM) from an alignment of sequences centered around pseudouridine sites in tRNAs and mRNAs that were experimentally verified in the previous studies (41 sequences of 17 nucleotides in length), and then used this PWM to scan the yeast genome for other predicted Pus4 target sites. The scan produced 433 hits (Supplementary file 5), which, when compared to 127 proteins that changed from the SWAT gene collection, overlapped by only five genes. Therefore, we conclude that the changes we observe for the translation of ~130 messages cannot be explained by Pus4-dependent pseudouridylation of their mRNAs.

We also did not observe any relationship between genes with increased or decreased protein levels in [BIG⁺] and isoelectric point (pI), protein length, protein half-life, or GO category enrichments (process, component, or function). We did however observe several properties that were altered in both the genes that went up and those that went down: hydropathy score (GRAVY) (Kyte and Doolittle, 1982), aromaticity, and CAI, codon bias, and frequency of optimal codons. We observed some amino acid enrichments as well, with genes going up in [BIG⁺] being depleted in leucine, phenylalanine, and proline, and those down depleted in phenylalanine and aspartic acid.

Finally, given that many of the effects that we measured were post-transcriptional, we also examined genes with altered GFP levels (significantly up or down) in [BIG⁺] cells using the MEME Suite, specifically for the enrichment of predicted binding sites for RNA binding proteins (Bailey *et al.*, 2009). We found that binding sites for the CCR4-NOT deadenylase complex were significantly enriched in our hits (motif alt ID CNOT4; consensus motif ACACAWA; adjusted p-value 0.0135).

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