Dissecting the Factors Involved in mRNA Trafficking to Germ Granules

by

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A thesis submitted in conformity with the requirements for the degree of Master’s of Science
Department of Molecular Genetics
University of Toronto

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2019

Abstract

Small RNA pathways have emerged as key regulators of gene expression, and across animals small RNA pathway components localize to perinuclear sites of mRNA regulation termed germ granules. In the C. elegans germline, several small RNA pathways localize to germ granules and target unique subsets of the transcriptome. An important question that arises is: how do transcripts get sorted into specific small RNA pathways as they enter germ granules? To begin to address this broad question of transcript sorting, in this study I established a system to track transcripts of interest in their export and subsequent germ granule localization. I will discuss data derived from this pipeline in interrogating the role of a panel of mRNA processing/export, germ granule, nuclear pore and small RNA pathway factors that may contribute to the proper export and cytoplasmic localization of the target of a single small RNA pathway, the CSR-1 pathway.
Acknowledgements

I am not very good at being mushy so I will keep this short.

First of all I want to thank my supervisor Julie. When I started grad school I had no prior wet lab experience and she took a big chance on me. Thank you for teaching me basically everything. Thank you to my committee members, Amy, Karim and Andrew, for providing me with guidance along the way. Thank you to all the Claycomb lab members, past and present, and the friends I have made in grad school. Thank you for letting me constantly bounce ideas off of you and providing me with great laughs during stressful times. Thank you to all my non-science friends for listening to me complain about grad school and reminding me that a world exists outside of a lab. Finally, thank you to my family and my partner for serving as my emotional punching bag over the last two years. Would not have made it without you.
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<td>mRNP</td>
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<td>Mutator-16</td>
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<td>piRNA</td>
<td>PIWI-interacting RNA</td>
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<td>PIWI</td>
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<td>RNA immunoprecipitation</td>
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<td>RITS</td>
<td>RNA-induced transcriptional silencing</td>
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<td>RRM RNA binding domain containing-3</td>
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<td>siRNA</td>
<td>short-interfering RNA</td>
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<td>single molecule fluorescence <em>in situ</em> hybridization</td>
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<tr>
<td>snRNP</td>
<td>small nuclear ribonucleoprotein</td>
</tr>
<tr>
<td>SR</td>
<td>Serine/arginine rich</td>
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<tr>
<td>SSC</td>
<td>Standard saline citrate</td>
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<tr>
<td>TGS</td>
<td>Transcriptional gene silencing</td>
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<td>THO complex subunit 5</td>
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Chapter 1

1 Introduction

Regulation of gene expression during metazoan development is particularly important in the germline where changes in expression can be transmitted to future generations. Several layers of gene regulation exist. For example, in the nucleus, chromatin structure can influence a gene’s accessibility to the transcriptional machinery and RNA processing can influence the stability, export and cytoplasmic localization of a transcript. In the cytoplasm, a transcript can be selectively stabilized or degraded based on 3'UTR sequences, poly(A) tail length and associated RNA binding proteins deposited during processing. In recent decades, studies have unveiled another important mode of epigenetic gene regulation: small RNA pathways.

Small RNA-mediated gene regulation involves a small RNA molecule (ranging in size from 18-30 nucleotides) bound by an Argonaute effector protein. The small RNA directs the Argonaute to a target transcript based on sequence complementarity where the Argonaute can then affect gene expression through several mechanisms, both transcriptional and post-transcriptional.

In recent years, a great deal of progress has been made in understanding the genetic requirements for small RNA regulation. Epigenetic factors, RNA degradation machinery and germ granules have all been studied for their role in small RNA regulation. Notably, unbiased genome-wide screens together with candidate gene studies have implicated mRNA processing and export factors in small RNA regulation. For example, studies have found that loss of known or putative splicing factors leads to aberrant small RNA pathway activity, including loss of small RNA species and changes in the subcellular localization of small RNA target transcripts. The role of splicing factors in small RNA pathways across several organisms is well documented yet the underlying mechanisms remain to be fully explored. This thesis will focus on elucidating the relationship between mRNA processing and export factors and small RNA pathways.
1.1 Small RNA pathways and Argonaute proteins

1.1.1 Argonaute structure and function

The multidomain Argonaute proteins possess three domains that are highly conserved across species: the PAZ (Piwi-Argonaute-Zwille), the Mid (Middle) and the PIWI (P-element Induced Wimpy testis) domains\(^{30-34}\). The PAZ and Mid domains coordinate the binding of the Argonaute to the small RNA by interacting with the 3' and 5' termini of the small RNA molecule respectively\(^{32,35}\). In some Argonautes the C-terminal PIWI domain possesses four key catalytic residues (DEDX) that confer endonuclease activity to the Argonaute\(^{33,36}\). The N-terminal domain of Argonautes is the least conserved across phyla and contains sequences that often undergo posttranslational modifications which impact the function of the Argonaute protein\(^{37-39}\).

The number of functional Argonaute proteins encoded by an organism’s genome varies greatly. For example, the nematode *Caenorhabditis elegans* encodes 21 Argonaute proteins while fission yeast encodes only a single Argonaute\(^{40-42}\). In most species, Argonaute proteins can be broadly divided into two clades: the AGO clade which binds to microRNAs (miRNAs) and small-interfering RNAs (siRNAs), and the PIWI clade which binds to PIWI-interacting RNAs (piRNAs).

Argonaute proteins are guided to their target RNA via sequence complementarity of their associated small RNA molecule and can regulate expression through several different mechanisms. These include but are not limited to: endonucleolytic cleavage, inhibition of translation, RNA destabilization, and chromatin modification\(^{14,22,23,43}\). Further details regarding the different classes of small RNAs as well as the molecular mechanisms underlying these modes of regulation will be discussed below.

1.1.2 Classes of small RNAs

Several classes of small RNAs have emerged in recent decades and can generally be grouped into 3 main categories: siRNAs, piRNAs, and miRNAs. These classes differ in their biogenesis requirements, biochemical properties (i.e. terminal modifications and length) and Argonaute protein partners\(^{44-47}\).
miRNAs, first discovered in *C. elegans*, are a well-conserved RNA species approximately 18-23nt in length found in most eukaryotes. miRNAs are genomically-encoded, possess a 5' monophosphate and target protein-coding genes in germline and somatic tissues. The biogenesis of mature miRNAs has been well characterized. Briefly, the miRNA gene is transcribed by RNA polymerase II, processed in the nucleus by the RNase III type enzyme Drosha, exported out of the nucleus, and the pre-miRNA is further processed in the cytoplasm by the RNase III type enzyme Dicer to produce a mature miRNA duplex. The mature miRNA is loaded into an Argonaute from the AGO clade and one strand, the passenger strand, is degraded. The mature miRNA/Argonaute complex binds its target transcripts in the 3'UTR and regulates expression post-transcriptionally via transcript degradation or translational inhibition.

Endogenous siRNAs (endo-siRNAs) are a class of genomically encoded small RNAs that have been shown to target transposons, pseudogenes, and protein coding genes in germline and somatic tissue in many species. Biogenesis of endo-siRNAs varies from species to species. In many organisms their biogenesis is dependent upon the RNase III helicase Dicer. In fungi, plants, and *C. elegans*, siRNAs are generated from target RNA transcripts via RNA-dependent RNA polymerases (RdRPs). Endo-siRNAs associate with Argonautes from the AGO clade and regulate expression of their targets co-transcriptionally and post-transcriptionally through effects on transcription, chromatin structure and mRNA stability.

A third class of conserved small RNAs called piRNAs primarily functions in the germline to repress deleterious nucleic acids such as repeats and transposable elements. piRNAs are approximately 19-30nt in length, have a 5' monophosphate and a bias for a 5'U. The biogenesis of piRNAs differs between animals, and the entire sequence of biogenesis events is not entirely clear in each organism. In *C. elegans*, piRNAs are genomically encoded and predominantly transcribed by RNA polymerase II from large clusters called piRNA clusters, located on chromosome IV. They have also been shown to be generated by transcription of promoter regions of protein coding genes, with subsequent additional processing, but this is not the main means of biogenesis. Across organisms, piRNAs are defined by their association with
PIWI Argonautes and can negatively regulate their targets post-transcriptionally through endonucleolytic cleavage or transcriptionally through chromatin modifications.\textsuperscript{77,78}

1.1.3 Small RNA regulation in \textit{C. elegans}

The nematode \textit{C. elegans} possesses at least 27 Argonaute-like genes, of which 21 likely encode functional proteins (Uri Seroussi, Claycomb lab, unpublished). In recent years, studies have begun to elucidate the functions of some of these Argonautes, but the role of many of these proteins in gene regulation remains unclear. The \textit{C. elegans} Argonautes fall into three clades: the previously mentioned AGO and PIWI clades, and a Worm-specific Argonaute clade (WAGO), which is an expanded Argonaute clade found only in nematodes. These 21 functional Argonautes associate with miRNAs, piRNAs and siRNAs to regulate expression in somatic and germline tissues.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{phylogenetic_tree.png}
\caption{Phylogenetic tree of \textit{C. elegans} Argonautes.} The Argonautes are divided into three clades: the AGO clade (shown in black), the PIWI clade (green) and the WAGO clade (red). Tree provided by Uri Seroussi.
\end{figure}
1.1.3.1 Worm-specific small RNA pathways

The *C. elegans* genome encodes 13 functional worm-specific Argonautes, which bind to a unique class of endo-siRNAs called 22G-RNAs (so named for their bias for a 5' guanine residue and 22-nucleotide length). Unlike miRNAs and piRNAs, the worm-specific 22G-RNAs are synthesized by RdRPs. *C. elegans* possesses four RdRPs, EGO-1, RRF-1, RRF-2 and RRF-3, that differ in their tissue localization patterns and the small RNA pathways in which they function. RdRP-mediated small RNA synthesis is dependent upon the Dicer-related helicase DRH-3 and the Tudor-domain-containing protein EKL-1. DRH-3 and EKL-1, together with the RdRPs EGO-1 or RRF-1, form a biogenesis complex that synthesizes 22G-RNAs antisense to a target transcript utilizing the target as a template. The worm-specific Argonautes function in the germline and soma to target protein-coding genes, repetitive sequences, pseudogenes and transposable elements.

26G-RNAs are a second class of endo-siRNAs that are specific to *C. elegans*. 26G-RNAs are important for germline development and are bound by three Argonaute proteins: ALG-3, ALG-4 and ERGO-1. ALG-3/4 associated 26G-RNAs function in sperm and play an important role in sperm development. The ERGO-1 26G-RNA pathway functions in oocytes and embryos to direct silencing of targets (including many gene duplications), and can stimulate the production of secondary 22G-RNAs. The RdRP RRF-3, Dicer and the dsRNA-binding protein RDE-4 are required for the biogenesis of 26G-RNAs.

1.1.3.2 Cytoplasmic small RNA pathways

Most of the *C. elegans* Argonautes function in the cytoplasm of the germline and soma via post-transcriptional gene silencing (PTGS). Across animals, PTGS has been shown to elicit gene regulatory effects through mRNA decay, mRNA degradation, and translational inhibition.

Perhaps one of the best-characterized examples of cytoplasmic small RNA regulation in the worm germline and soma is siRNA-mediated gene silencing during exogenous RNA interference (RNAi). Following exposure to an exogenous double-stranded RNA molecule, the Dicer complex (including Dicer, the dsRNA binding protein RDE-4, and the Argonaute RDE-1) processes the exogenous RNA into primary single-stranded siRNAs. These primary siRNAs
guides the catalytically active Argonaute RDE-1 to its target, leading to endonucleolytic destruction of the RNA. In parallel, the endo-siRNA pathway leads to the production of secondary siRNAs via RdRP activity, amplifying the silencing signal. These secondary siRNAs biochemically resemble 22G-RNAs and are loaded into worm-specific Argonautes that primarily function in the cytoplasm to negatively regulate expression of their targets. The precise mechanism by which these worm-specific Argonautes/secondary siRNA complexes regulate expression is unclear. It is important to note that most of these secondary worm-specific Argonautes lack key catalytic residues within their PIWI domains and therefore are unlikely to regulate their targets via endonucleolytic cleavage.

1.1.3.3 Nuclear small RNA pathways

In addition to cytoplasmic activity, Argonautes in several organisms can influence expression of their targets in the nucleus by impacting the transcriptional machinery or influencing histone modifications. Of the 14 worm-specific Argonautes in *C. elegans*, 3 show some degree of nuclear localization during development: HRDE-1 (heritable RNAi defective-1), CSR-1 (chromosome segregation and RNAi deficient-1), and NRDE-3 (nuclear RNAi deficient-3). HRDE-1 and NRDE-3 function in the germline and soma respectively to modulate nuclear silencing. Specifically, both Argonautes associate with nascent pre-mRNAs, recruit factors that aid in the deposition of H3K9me2 (a histone modification associated with heterochromatin) and inhibit the activity of RNA polymerase II. Furthermore, HRDE-1 and NRDE-3 have both been implicated in the trans-generational inheritance of silencing initiated by primary siRNAs during exogenous RNAi.

Small RNA pathways are largely known as negative regulators of gene expression, yet compelling evidence suggests the Argonaute CSR-1 is likely not involved in repression of its target genes. CSR-1 is enriched in the germline and localizes to the nucleus in embryos (specifically during cell division) and in oocytes of the adult germline (Amanda Charlesworth, Claycomb lab, unpublished). CSR-1 plays a critical role in germline development and fertility as csr-1 null mutants have under-proliferated germlines and are sterile. The role of CSR-1 in nuclear gene regulation is complex. CSR-1 targets approximately 50% of germline-expressed protein coding genes and associates with chromatin in a siRNA-dependent manner. It has been
hypothesized that one way in which CSR-1 regulates its targets is by impacting histone post-translational modifications. Unpublished data from our lab shows a decrease in genome-wide distribution of euchromatin-associated histone modifications H3K36me3 and H3K4me2 at CSR-1 target loci in csr-1 mutants. Furthermore, immunoprecipitation experiments indicate that CSR-1 interacts with methyltransferases; therefore, CSR-1 may recruit these factors to its target loci. In addition to its suggested role in chromatin structure, Global Run-On sequencing (GRO-seq) data points to a role for CSR-1 in promoting sense-oriented RNA polymerase II transcription. In csr-1 mutants, antisense transcription accumulates at CSR-1 targets and ectopic transcription is present at normally silent regions\(^{94}\).

Throughout much of the adult germline CSR-1 shows cytoplasmic localization\(^{83,95}\). CSR-1 also harbors a functional catalytic tetrad, which has been shown to be required for endonucleolytic cleavage of targets\(^{79}\). Interestingly, in csr-1 null mutants the expression levels of most genes (targets and non-targets) appear unchanged, while a proportion increase (~7%) or decrease (~22%) in expression more than 2-fold relative to wildtype\(^{83}\). Therefore, it has been suggested that CSR-1 plays a role in ‘fine-tuning’ germline gene expression both positively and negatively\(^{95}\). Although the mechanisms by which CSR-1 regulates its targets remain to be fully elucidated, it is likely that CSR-1 acts both co-transcriptionally and post-transcriptionally to regulate gene expression through more than one mechanism.

1.1.4 Small RNA regulation in other organisms

1.1.4.1 *Schizosaccharomyces pombe*

The mechanism of transcriptional gene silencing (TGS) has been extensively studied in yeast. As mentioned above, the fission yeast genome encodes a single Argonaute protein, Ago1, which associates with siRNAs and guides heterochromatin formation at pericentromeric regions in the nucleus. An RNA-induced initiation of transcriptional silencing (RITS) complex, which includes the siRNA, Ago1, and the chromodomain-containing protein CHP-1, is directed to centromeres through the recognition of nascent mRNA transcripts and the heterochromatin-associated histone modification H3K9me\(^{69,96–98}\). The RITS complex recruits both a histone methyl transferase that will deposit new H3K9me marks and an RdRP to synthesize siRNAs against the transcript to amplify the silencing signal\(^{99,100}\). Therefore, in *S. pombe*, both siRNAs
and H3K9me are necessary for heterochromatin formation through a self-reinforced feedback loop.

1.1.4.2 *Drosophila melanogaster*

The *Drosophila* genome encodes 5 Argonaute proteins, which fall into the AGO and PIWI clades, and have been reported to bind siRNAs, miRNAs and piRNAs\(^{20,101,102}\). Much of what we know about piRNA biogenesis and regulation comes from studies carried out in *Drosophila*. The fly genome encodes 3 PIWI Argonautes Piwi, Aubergine (Aub), Argonaute (Ago3), that differ in their subcellular localization in the germline and soma\(^{103-105}\). piRNA pathways direct silencing of deleterious elements, including repeats and transposable elements, and in the fly, piRNAs recognize their targets through perfect or near-perfect sequence complementarity. Most piRNAs are produced from discrete loci in the genome called piRNA clusters\(^{106,107}\). Although the biogenesis of piRNAs is very complex and many details need to be further elucidated, recent studies have begun to tease apart the molecular mechanisms underlying piRNA biogenesis. Primary piRNA biogenesis begins in the nucleus with transcription of piRNA clusters and export to processing sites in the cytoplasm where they are cleaved and processed into mature piRNAs\(^{108-112}\). The mature piRNA/Argonaute complex can enter the nucleus to direct TGS through chromatin modifications or can localize to perinuclear cytoplasmic sites and enter an amplification loop called the ping-pong cycle\(^{106,113,114}\). The ping-pong cycle is used to simultaneously amplify the silencing triggers and destroy target transcripts through PTGS via endonucleolytic cleavage. Briefly, the ping-pong cycle starts with the recognition of a target transcript by an Argonaute/piRNA complex (either the Aub or Ago3) and cleavage to produce a piRNA intermediate that is loaded into the reciprocal Argonaute (Aub or Ago3), where piRNA maturation occurs. This process is repeated, leading to the amplification of the silencing signal together with PTGS\(^{115-117}\).

1.1.4.3 *Arabidopsis thaliana*

In *A. thaliana*, diverse small RNA pathways function together to silence expression of targets in the nucleus and cytoplasm through several mechanisms\(^{118-120}\). One example is the miRNA pathway, which is essential for regulating transcription factors and other genes required for plant development\(^{121,122}\). In both plants and animals, miRNA pathways can lead to translational
repression of target mRNAs. In animals, miRNA-directed target silencing largely occurs via Argonaute-dependent recruitment of deadenylase complexes, such as CCR4-NOT, and only rarely occurs via endonucleolytic cleavage\textsuperscript{23,123–125}. In contrast, in plants, Argonaute/miRNA complexes largely direct slicing of their target mRNAs. The slicing of targets by the Argonaute protein requires a high degree of complementarity between the miRNA and target mRNA\textsuperscript{126–128}. It remains unclear whether slicer-independent mechanisms of Argonaute-mediated RNA degradation exist in plants.

1.1.4.4  \textit{Mus musculus}

Mammalian cells produce multiple small RNA species including siRNAs, miRNAs and piRNAs. Three murine PIWI proteins (Miwi, Mili, Miwi2) are expressed almost exclusively in the germline and are required for spermatogenesis; loss of mouse PIWI proteins results in spermatogenic cell arrest\textsuperscript{129–131}. Four AGO clade members (Ago1-4) are ubiquitously expressed and required for miRNA-mediated translational repression\textsuperscript{132–134}. Mouse embryonic stem cells and germ cells also express siRNAs, which interact with Argonautes from the AGO clade and have been recently shown to play an essential role in female fertility\textsuperscript{135}.

1.1.5  Germ granules: hypothesized sites of small RNA regulation

1.1.5.1  Germ granule structure and function

A characteristic feature of germ cells across the animal kingdom is the presence of cytoplasmic, non-membrane bound organelles called germ granules\textsuperscript{136–138}. In several organisms, including \textit{Drosophila}, \textit{C. elegans} and \textit{Xenopus}, germ granules are maternally deposited into the embryo and segregate with the germ lineage. In these animals, germ granules are thought to play a role in germ cell specification. Germ granules have been observed in numerous animal species to date; however, their precise molecular functions are still largely unknown.

Germ granules are enriched in RNA and RNA-binding proteins and are predicted to function in various stages of RNA metabolism. Many of these proteins are conserved across animals. For example, decapping proteins (Dcp1 and Dcp2 in flies; DCP-2 in worm) and the eukaryotic initiation factor 4F (eIF4F) are present in germ granules, implicating these structures in translation and mRNA decay\textsuperscript{139–146}. mRNA processing and export factors are also enriched in
germ granules including the conserved export factor DDX19 (Dbp80 in fly) and the spliceosomal Sm proteins\textsuperscript{147–150}. Germ granules share some protein components with other cytoplasmic RNA-protein granules (including P-bodies or stress granules); however, numerous proteins specifically associate with germ granules, making these structures unique mRNA metabolism sites.

During much of development, germ granules localize to the nuclear periphery\textsuperscript{151–154}. In \textit{C. elegans}, germ granules associate with the majority of nuclear pores in the transcriptionally active regions of the germline and are sites of mRNA export from the nucleus\textsuperscript{149,155}. The conserved nuclear export factor 1 (NXF1), which is required for mRNA export, localizes to germ granule-associated nuclear pores in \textit{C. elegans}. Furthermore, a study monitoring the export of GFP transcripts expressed under the control of a heat shock promoter using RNA fluorescence \textit{in situ} hybridization (FISH) found that GFP mRNA is primarily exported through germ granule-associated nuclear pores post-heat shock\textsuperscript{149}. Interestingly, not all nuclear pores associate with germ granules, and non-germ granule associated nuclear pores may have distinct functions in nucleocytoplasmic transport and possibly RNA metabolism.

\subsection{1.1.5.2 Germ granules and small RNA regulation in \textit{C. elegans}}

In \textit{C. elegans}, 3 germ granule structures have been identified to date: P granules, Z granules and \textit{Mutator} foci. In the adult germline, all three granules localize to the nuclear periphery, adjacent to nuclear pores\textsuperscript{149,156,157}. Experimental evidence suggests that P granules, Z granules and \textit{Mutator} foci are independent structures. For example, disruption of P granules does not alter the structure and localization of Z granules or \textit{Mutator} foci\textsuperscript{25,156,157}. Furthermore, microscopy data suggest Z granules localize between P granules and \textit{Mutator} foci, with P granules being most closely associated with the nuclear periphery\textsuperscript{156}. Together, the three structures form a large perinuclear compartment through which newly synthesized transcripts pass and can interact with appropriate regulatory factors.

P granules, Z granules and \textit{Mutator} foci have all been implicated in small RNA pathway activity. Key mediators of small RNA regulation, Argonautes, RdRPs and Dicer, localize to germ granules including \textsuperscript{83,104,145,158–160}. Unpublished work from our lab in conjunction with
several published studies has shown that 9 germline Argonaute proteins localize to perinuclear granules\textsuperscript{83,156}. Some Argonautes show granule-specific localization patterns, suggesting possible compartmentalization of small RNA regulation. For example CSR-1 localization appears to be restricted to P granules, while the Argonaute WAGO-4 localizes to Z granules\textsuperscript{83,156}. The granule-specific localization patterns of several other small RNA pathway factors, including some germ granule Argonautes, have yet to be carefully examined.

Core structural germ granule proteins appear to play direct and/or indirect roles in small RNA regulation. The P granule nucleation factors PGL-1 and DEPS-1 are required for exogenous RNAi while another conserved P granule component GLH-1 (Vasa in fly) has been shown to interact with Dicer\textsuperscript{160–162}. Furthermore, several core components of Z granules and Mutator foci interact with Argonautes and RdRPs and have been implicated in proper siRNA amplification\textsuperscript{25,156,157,163}.

1.1.5.3 Germ granules and small RNA regulation in other organisms

Numerous studies in \textit{Drosophila} have highlighted the role for germ granules in piRNA regulation\textsuperscript{164}. Two \textit{Drosophila} PIWI Argonautes, Aub and Ago3, localize to perinuclear germ granules (termed nuage) in the nurse cells of the fly ovary\textsuperscript{165–167}. The nuage is a predicted amplification site of the piRNA silencing signal via the ping-pong cycle. The core nuage protein Vasa (GLH-1 in worm P granules) is required for the nuage localization of PIWI Argonautes and is necessary for piRNA processing and amplification\textsuperscript{28,168,169}.

The chromatoid body (CB) in the male germ cells of mice is believed to be the mammalian counterpart of the fly nuage. A relationship has emerged between CBs and miRNA pathways. Dicer and other components of the miRNA machinery localize to CBs\textsuperscript{145,170}. Furthermore, FISH experiments using probes against specific miRNAs also show miRNA enrichment in CBs\textsuperscript{145}. Recently, the CB has also been implicated in piRNA regulation: following isolation of germ granules and RNA sequencing, a study found that piRNA transcripts accumulate in CBs\textsuperscript{171,172}.

Despite the progress made in understanding the relationship between germ granules and small RNA pathways in the worm and other animals, the precise role for germ granules in small RNA regulation is still unclear. Are newly synthesized transcripts sorted into small RNA pathways in
germ granules? Are germ granules sites of siRNA or piRNA amplification? Is small RNA mediated post-transcriptional regulation of transcripts occurring in germ granules or are they simply sorting stations? Further genetic, biochemical and live-imagining studies are required to answer these questions.

1.2 Intersection between RNA processing and export factors, and small RNA regulation

Small RNA regulation of gene expression in the nucleus begins with an Argonaute/small RNA complex binding a nascent mRNA to influence transcriptional machinery and the surrounding chromatin landscape. The relationship between small RNA regulation of gene expression and transcription has been carefully investigated over the years. Importantly, transcription is only the first step in gene expression. Transcription is integrated spatiotemporally with mRNA processing events, which in turn are coupled to mRNA export and expression in the cytoplasm. Notably, numerous studies in recent years have implicated mRNA processing and export factors in small RNA-mediated gene regulation16,19,26,27,173.

1.2.1 Nuclear RNA processing and export

Prior to their nuclear export, mRNA molecules must be processed and packaged into mature messenger ribonucleoprotein (mRNP) complexes. An mRNA molecule will undergo three major processing events: 5' end processing, splicing and 3' end processing. These processing steps lead to the deposition of RNA-binding proteins (RBP) onto the nascent transcript that are necessary for the efficient export of the mRNA, and its subsequent localization and expression in the cytoplasm. Transcription and mRNA processing are biochemically distinct and require different cellular machineries; therefore, it was traditionally thought that they occur independently of one another. Research in several animals, however, has shown that these processing events occur co-transcriptionally and can influence one another’s specificity and efficiency174,175.

1.2.1.1 Nuclear life cycle of an RNA transcript

The first processing event to take place is the acquisition of the 7-methylguanosine-cap structure at the 5' end of the mRNA. Capping occurs very early in transcription after the first 25 or so nucleotides have been transcribed. After the successful addition of a 5' cap structure, the mRNA
is protected from 5'-3' exonuclease-mediated decay\textsuperscript{176–178}. Following capping, the mRNA undergoes ATP-dependent removal of introns and splicing of exon sequences. The proper removal of introns from mRNA molecules is necessary for export and protein expression in the cytoplasm. Splicing reactions are catalyzed by a large multiprotein complex called the spliceosome. At the core of the spliceosome are five small nuclear ribonucleoproteins (snRNPs) that direct spliceosome binding to the pre-mRNA and catalyze the removal of introns. In addition to the snRNPs, over 100 other proteins dynamically associate with the spliceosomal machinery and are required for specific steps in splicing\textsuperscript{179,180}. The third and final processing step involves endonucleolytic cleavage of the mRNA followed by addition of a poly(A) tail to the 3' end. As with the 5' cap structure, the 3' poly(A) tail plays a role in the stability of the mRNA\textsuperscript{181,182}. In many species, transcripts can be alternatively polyadenylated, leading to changes in stability, export and cytoplasmic localization\textsuperscript{183–185}.

A plethora of evidence exists to suggest transcription, capping, splicing and polyadenylation are functionally coupled. The catalytic subunit of RNA polymerase II contains an unstructured carboxyl terminal domain (CTD), which plays roles in both transcription and mRNA processing. The CTD can be dynamically phosphorylated and these marks are critical for subsequent interactions with mRNA processing factors throughout transcription\textsuperscript{186–190}. In yeast for example, a phosphorylated CTD has been shown to interact with at least 100 different proteins\textsuperscript{191}. The CTD domain of RNA polymerase plays an important role in processing through interactions with key capping, splicing and polyadenylation factors. For example, CTD truncations in mammalian cells result in inefficient splicing and reduced localization of splicing factors to transcription sites\textsuperscript{192,193}. Furthermore, in both yeast and mammals, inhibition of transcription elongation results in changes to the splicing patterns of certain transcripts\textsuperscript{194,195}. Processing events also appear to be functionally dependent on one another. For example, capping has been shown to play a role in splicing in mammalian cells, where the presence of the cap structure increases the efficiency of splicing\textsuperscript{196–198}.

1.2.1.2 Role of RNA-binding proteins in the fate of an mRNA transcript

During transcription and mRNA processing, RBPs dynamically associate with nascent transcripts and influence their fate in several ways\textsuperscript{175,199–201}. Many of these proteins are
deposited onto the mRNA co-transcriptionally in response to processing, and provide a “code” associated with the mRNA that is critical for subsequent expression of the transcript. For example, following the excision of an intron and ligation of flanking exons, the multiprotein exon-junction complex (EJC) is deposited approximately 24 nucleotides upstream of the exon-exon junctions. The EJC is composed of 4 core proteins (MAGOH, eIF4A3, Y14 and MLN51) and numerous peripheral proteins that dynamically associate with the core complex. The EJC remains associated with the mRNA until the first round of translation in the cytoplasm, and appears to play several roles in mRNA metabolism, including nuclear export, mRNA surveillance, cytoplasmic localization and post-transcriptional processing. Another example of splicing-related mRNP components are serine/arginine rich (SR) proteins. SR proteins associate with mRNAs co-transcriptionally and are involved in the recruitment and assembly of splicing machinery. Similar to the EJC, SR proteins can remain associated with the mRNA during export and can influence the translation of the mRNA.

Many RBPs that associate with an mRNA co-transcriptionally mediate nuclear export of the transcript. The transcription-export complex (TREX) illustrates the coupling between transcription and export. The TREX complex is composed of the THO subcomplex, the mRNA splicing and export factor UAP56 (yeast Sub2, worm HEL-1) and the export factor REF/Aly (yeast Yra). During transcription, the TREX complex associates with the nascent transcript and influences transcriptional elongation (through associations with the elongating RNA polymerase II), splicing (through association with key splicing factors), and mRNA export (through recruitment of the conserved nuclear export factor 1 (NXF1)).

1.2.1.3 Nuclear export of mRNPs and subsequent cytoplasmic localization

Once a nascent transcript is processed and packaged into a mature mRNP, it is exported out of the nucleus through the NPC. The NPC is one of the largest protein complexes in the cell, composed of ~30 different proteins called nucleoporins (Nups). A scaffold anchors the NPC to the nuclear envelope and a central channel allows for selective nucleocytoplasmic transport. Many nucleoporins contain intrinsically disordered domains rich in phenylalanine-glycine repeats (called FG-domains). These FG-domains have several roles in mRNP export. The FG-domains form a permeability barrier across the central channel, preventing the passive
transport of larger molecules (over \(\sim 40\text{kDa}\)) through the NPC\textsuperscript{218–220}. Furthermore, they interact with key export factors that mediate the movement of an mRNP through the nuclear pore\textsuperscript{219,221–224}. For example, the export factor NXF1 binds to FG-Nups and is required to facilitate the translocation of mRNPs through the nuclear pore\textsuperscript{223,224}. Notably, several germ granule proteins in various animals also contain disordered FG-domains, or other similarly disordered repeat domains. In \textit{C. elegans}, these FG-domains in P granule proteins form a size-exclusion barrier similar to that of the pore central channel and serve to extend the selective environment of the nuclear pore\textsuperscript{155}.

Following export through the nuclear pores, many mRNPs will be shuttled to particular sites in the cytoplasm. In \textit{Drosophila} it was shown that approximately 71% of the 3370 mRNAs analyzed exhibit distinct localization patterns in the cytoplasm\textsuperscript{225}. This pattern of restricted localization is common across organisms and across cell types, and is a mechanism cells can use to regulate gene expression\textsuperscript{226–228}. Studies have uncovered several factors that influence cytoplasmic mRNP localization; these include \textit{cis}-acting regulatory elements in the UTRs, selective degradation/stabilization of a transcript, and motor-based active transport of an mRNP\textsuperscript{185,229–234}. Notably, factors deposited onto transcripts co-transcriptionally have also been implicated in cytoplasmic localization. For example, in \textit{Drosophila}, the EJC plays a role in the cytoplasmic localization of \textit{oskar} mRNA to the posterior pole of the oocyte\textsuperscript{4}. Furthermore, a recent study in \textit{Drosophila} showed that members of the EJC and the splicing/export factor UAP56 are involved in both intra-nuclear trafficking of a piRNA transcript and its export to perinuclear granules\textsuperscript{29}. Details of this study will be discussed below.

1.2.2 Small RNA pathways and splicing

In the past decade, studies across model organisms have pointed to a relationship between splicing factors and small RNA-mediated gene regulation. In \textit{C. elegans}, a genome-wide RNAi screen utilizing a \textit{gfp} reporter identified numerous proteins implicated in small RNA regulation, including several proteins with predicted roles in mRNA splicing\textsuperscript{17}. A phylogenetic analysis performed by Tabach \textit{et al.} also pointed to a role for splicing factors in small RNA activity. The authors performed phylogenetic clustering to search for proteins with similar sequence conservation to proteins with known roles in small RNA regulation and identified numerous
RBPs not previously implicated in small RNA pathways. Interestingly, several of these proteins are known members of the spliceosome complex. A recent study published from our lab along with a companion study implicated the conserved splicing factor EMB-4 (human AQR/IBP-60) in small RNA pathway activity. EMB-4 distinctly interacts with the targets of CSR-1 and HRDE-1 and is required for their proper expression in the germline. Specifically, EMB-4 appears to bind both intronic and exonic sequences of HRDE-1 targets, while remaining strictly enriched at intronic sequences of CSR-1 targets. We hypothesize EMB-4 is part of a broader molecular signature that marks a transcript as a HRDE-1 or CSR-1 target for further regulation in perinuclear germ granules.

Studies in yeast have also examined the role for splicing factors in small RNA regulation. In S. pombe, small RNA pathways mediate heterochromatin formation at centromeres. A study reported that loss of specific splicing factors led to defects in siRNA regulation. Loss of two spliceosome proteins, Cwf10 and Prp39, resulted in aberrant siRNA accumulation and consequently improper heterochromatin formation. Importantly, the authors confirmed that the defects in siRNA production were not simply due to defects in splicing of mRNA encoding RNAi components, implying that the silencing roles of Cwf10 and Prp39 are likely unrelated to their roles as splicing factors. Furthermore, the authors showed that Cwf10 co-immunoprecipitated with RdRPs, further implicating splicing factors in small RNA mediated silencing.

A study by Zhang et al. uncovered that the splicing and export factor UAP56 is required for piRNA-mediated regulation in perinuclear germ granules in Drosophila. The authors showed that UAP56 is required for the nuage localization of PIWI proteins Aub and Ago3, and of Vasa, a nuage protein that has a previously established role in piRNA activity. Interestingly, nuclear UAP56 localizes to pores across from Vasa, and piRNA cluster transcripts immunoprecipitate with both UAP56 and Vasa. Therefore, the authors hypothesize that UAP56 binds to nascent cluster transcripts and is involved in their trafficking to the perinuclear nuage, where Vasa and other members of the piRNA biogenesis machinery are concentrated.
1.2.3 Small RNA pathways and nuclear export

The TREX complex has been implicated in small RNA regulation across species. In *Arabidopsis*, loss of *tho2*, the largest subunit of the TREX complex, leads to aberrant siRNA and miRNA production, and consequently leads to loss of gene silencing\(^{237}\). In *Drosophila*, the TREX complex is required for the biogenesis of piRNAs. A component of the TREX complex, Thoc5, localizes to sites of piRNA transcription and loss of *thoc5* result in defects in piRNA biogenesis and derepression of transposable elements\(^{26}\).

The link between small RNA pathways and nuclear export is largely dependent on the EJC. In recent years, UAP56 and several members of the EJC have been implicated in the nuclear trafficking and export of *flamenco (flam)* piRNA transcripts in *Drosophila*\(^{29}\). The *flam* transcripts first localize to a single focus within the nucleus, in close proximity to the nuclear periphery, suggesting the *flam* transcripts gather together in the nucleus prior to their export\(^{29,238}\). Notably, the nuclear site of *flam* accumulation is distinct from the *flam* locus\(^{29}\). Following translocation through the nuclear pore, *flam* transcripts accumulate in perinuclear granules, called Yb-bodies, where piRNA biogenesis and regulation are thought to occur. Members of the EJC, UAP56 and the export factor NXF1 are required for either the intranuclear trafficking of the *flam* transcripts and/or their subsequent export to Yb-bodies\(^{29}\).

1.3 Thesis Rationale

In the *C. elegans* germline, 9 Argonaute proteins, representing every type of small RNA pathway, localize to germ granules: CSR-1, WAGO-1, WAGO-4, WAGO-10, ALG-3, ALG-4, ALG-5, PRG-1, PPW-2. Unpublished work from our lab alongside several published studies has shown that these Argonautes can target unique subsets of transcripts. An important question that arises is: how do transcripts get sorted into a specific small RNA pathway as they exit the NPC and enter germ granules? Intrinsic sequence features of transcripts, compartmentalization of small RNA pathways within germ granules, and RNA binding proteins associated with transcripts could all contribute to mRNA sorting. To begin to interrogate this broad question, however, we first needed to establish a system to track transcripts of interest in their export and subsequent localization, as this has not previously been achieved in *C. elegans*. In this thesis, I
will outline the single molecule FISH (smFISH) pipeline that I established in our lab. I will also discuss data derived from this pipeline in interrogating the role of a panel of mRNA processing/export, germ granule, nuclear pore and small RNA pathway factors that may contribute to the proper export and cytoplasmic localization of CSR-1 target transcripts. Finally, I will summarize the work I did to establish a system to visualize mRNA trafficking and localization live in *C. elegans*. 
Chapter 2

2 Materials and Methods

2.1 Nematode strains and culture

2.1.1  *C. elegans* and growth conditions

All strains were derived from the Bristol N2 strain (wild type) and cultured as previously described\textsuperscript{239}. Worms were grown at 20°C on Nematode Growth Media (NGM) plates seeded with *Escherichia coli* OP50, unless otherwise stated. Details of strains used are provided in Table 1.

**Table 1. List of worms strains used in this study.**

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<thead>
<tr>
<th>Strain Name</th>
<th>Text Name</th>
<th>Genotype</th>
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<td>Wild type</td>
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### 2.2 RNAi, quantitative real-time PCR (qRT-PCR) RNAi conditions

#### 2.2.1 RNAi conditions

RNAi experiments were carried out as previously described\(^\text{240}\). *npp-7*, *hel-1* and L4440 (control) RNAi clones were obtained from the Ahringer RNAi library\(^\text{240}\). 15mL cultures of RNAi vector were grown 6-10 hours in LB media supplemented with 50µg/ml carbenicillin at 37°C. 2mL of bacteria was then plated on 10cm NGM plates supplemented with 50µg/ml carbenicillin and 2mM IPTG and allowed to grow overnight. For *npp-7* RNAi, ~200 L1 worms were plated and left to grow to gravid adulthood at 20°C. For *hel-1* RNAi, ~200 L4 worms were plated and left to grow for ~24 hours at 20°C. Gravid adults were then harvested for single molecule fluorescence *in situ* hybridization and RNA extraction. RNAi knockdown was validated by qRT-PCR and by phenotypic analysis.

#### 2.2.2 RNA extraction, cDNA synthesis and quantitative real-time PCR

Total RNA was extracted from ~100 gravid adult worms. Worms were picked into M9 buffer (22mM KH\(_2\)PO\(_4\), 42mM Na\(_2\)HPO\(_4\), 85mM NaCl, 1mM MgSO\(_4\)), washed 5 times with M9 buffer and 2 times with UltraPure DNase/RNase-Free distilled water to remove any bacteria. Worms were then resuspended in 100µL UltraPure DNase/RNase-Free distilled water and 400µL of TRI reagent (Molecular Research Centre Inc.) was added. To freeze-crack the worms, pellets were placed at -80°C for at least 15 minutes, then at room-temperature shaking for 15 minutes.
The freeze-cracking step was repeated 2 additional times. RNA was then isolated according to the TRI reagent protocol.

Up to 3µg of total RNA was treated with DNase I (Life Technologies) for 1 hour at 37°C. RNA was then extracted with phenol:chloroform and precipitated overnight with 20µg of glycogen, 10% of the volume of 3M sodium acetate and 4x the volume of ethanol. Reverse transcription was performed on 500ng of RNA using SuperScript® VILO™Mix (Life Technologies).

qRT-PCR was performed on the StepOnePlus™ Real-time PCR system (Life Technologies) using default thermocycling conditions. cDNA standards were initially diluted 1:10 and serial 10-fold dilutions were made up to 1:10000 and cDNA samples were diluted 1:50. Mastermixes were composed of 7.5µL Fast SYBR® Green Master Mix (Life Technologies), 0.6µL of 10uM for each primer, 4µL of diluted cDNA and 2.3µL of water. Each biological replicate was performed in triplicate with primers outlined in Table 2. mRNA levels for each gene were determined relative to an internal control (gpd-2).

2.3 Protein extraction, Immunoblotting and RNA-immunoprecipitation

2.3.1 Protein extraction

Synchronous worm populations were grown on 15cm Petri dishes until ~24 hours post L4. Worms were harvested by washing 3 times for 10 minutes with M9 buffer (22mM KH$_2$PO$_4$, 42mM Na$_2$HPO$_4$, 85mM NaCl, 1mM MgSO$_4$). Worm pellets were then washed 3 times with water and snap frozen in a dry ice/ethanol bath and stored at -80°C. To extract protein, pellets were thawed on ice in 1:2 pellet/buffer volume of DROSO complete buffer (30mM HEPES, 100mM CH$_3$CO$_2$K, 2mM Mg(CH$_3$C00)$_2$, 0.1% v/v np40, 2mM DTT, 1% v/v Phosphatase Inhibitor Cocktail 2(Sigma), 1% v/v Phosphatase Inhibitor Cocktail 3 (Sigma), 1 tablet/5mL buffer Mini Complete Protease Inhibitor Cocktail (Roche)). Samples were homogenized using a pre-chilled Dura-Grind® Stainless Steel Dounce (Wheaton) with approximately 50 strokes. Samples were centrifuged at 10,000 x g for 15 minutes at 4°C and the supernatant was transferred to a fresh pre-chilled 1.5mL tube and the protein concentration was quantified using a Lowry assay (BioRad).
2.3.2 Immunoblotting

Samples were resolved on a 4-12% Bis-Tris gradient gel (Life Technologies) and transferred onto a nitrocellulose blotting membrane (Amersham Protran, GE Healthcare) using a semi-dry transfer. Membranes were blocked for 1 hour at room temperature with blocking solution (5% w/v powdered milk in PBST (137 mM NaCl, 2.7 mM KCl, 10 mM phosphate pH 7.4, 0.1% Tween-20)) and incubated with primary antibody against GFP (ab290, Torrey Pines Scientific) resuspended in blocking solution at a 1:1000 concentration overnight at 4°C. Membranes were washed 3 times for 10 minutes with PBST, incubated in blocking solution for 1 hour and then in secondary antibody resuspended in blocking solution at a 1:1000 concentration for 1 hour at room temperature. Membranes were washed 3 times with PBST for 10 minutes and visualized using Luminata HRP Classico/Forte Substrate (Millipore) and Clonex Bioflex Econofilm.

2.3.3 RNA immunoprecipitation (RIP)

For MS2 RIP experiments, protein was extracted as described above. Each IP was preformed using 3mg of protein lysate and 10% of lysate was taken for input samples. GFP-Trap® beads (Chromotek) were washed 3X with DROSO buffer and lysates were incubated with 25µL of GFP-Trap® beads for one hour at 4°C with rotation. Lysates were then washed 6x for 10 minutes with DROSO buffer. Two separate IPs were performed for each sample and on the final wash the IP samples were combined. 10% of the IP was removed for western blot validation and protein was denatured by incubation with 2x Bolt Sample Buffer (Thermofisher) for 10 minutes at 70°C. To the remaining IP, 5x volumes of Tri-Reagent was added and RNA was extracted as described above. Subsequently, cDNA was made and input samples were diluted 1/50 and IP samples were diluted 1/10 for qRT-PCR.

2.4 Single molecule fluorescence in situ hybridization (smFISH) and microscopy

2.4.1 Single molecule fluorescence in situ hybridization

Custom Stellaris FISH probes (Biosearch Technologies Inc.) were designed using the Stellaris FISH Probe Designer available online. The probe set targeting the pgl-3 transcript included 48
unique oligonucleotides antisense to exons of the \textit{pgl-3} transcript labelled with the Quasar® 570 dye. As a negative control, 48 unique oligonucleotides sense to the \textit{pgl-3} transcript were used. Probes were dissolved in UltraPure DNase/RNase-Free distilled water to create a 100µM probe stock.

Populations of worms were bleached and the following day ~200 L1 worms were placed at 20°C or 25°C on OP50 or RNAi food where specified. Animals were grown until ~24 hours post L4 stage. 40 gravid adults were dissected to extrude gonads in 1x PBS (137 mM NaCl, 2.7 mM KCl, 10 mM phosphate pH 7.4) buffer and dissected animals were pipetted into 1.5mL eppendorf tubes. Samples were washed 3 times with 1xPBS/0.1% Tween-20 (PBST) and fixed with 4% (v/v) cold paraformaldehyde in PBST at room temperature, rotating for 30 minutes. All spins were done at 2000 rpm for 1 minute. After fixation, samples were washed 3 times with PBST and permeabilized with 300µL of 100% methanol at -20°C for a minimum of 10 minutes (or up to a week). Methanol was removed and samples were washed 3 times with PBST and once with wash buffer (2X saline-sodium citrate (SSC), 10% deionized formamide in RNase-free water). Gonads were hybridized with 0.25µM of \textit{pgl-3} probe in hybridization solution (228 mM Dextran sulfate, 2X SSC, 10% deionized formamide in nuclease-free water) for 24-48 hours at 37°C shaking. After the addition of probes, samples were kept in the dark for all incubations using aluminum foil. After hybridization, samples were washed 3 times with wash buffer and incubated with wash buffer containing 1µg/mL diamidinophenylindole (DAPI) at room temperature, rotating for 25 minutes. Samples were washed twice with wash buffer and mounted onto slides (FisherBrand Superfrost™ Plus) using ProLong® Gold Antifade Mountant (ThermoFisher). All images were acquired using a Nikon TiE inverted microscope with a C2 confocal unit and NIS elements software. Images were collected at 60x magnification and Z sections were taken at 0.4µm.

\textbf{2.4.2 Quantification of smFISH images using Imaris}

Single molecule fluorescence \textit{\textit{in situ}} hybridization (smFISH) results were quantified using the ImarisCell module of Imaris. Figure 1 details the workflow and specific parameters used. Briefly, Z-stacks were reconstructed and the cell/vesicle option was selected to define nuclei and RNA foci (nuclei were defined as cells and RNA foci were defined as vesicles). Nuclei were
defined using a minimum size threshold of 4.27µm. Partially cut-off nuclei, or closely clustered nuclei that were difficult to split into two objects, were manually deleted from the data. RNA foci were filtered based on size (< 0.35µm or < 0.7µm) and distance to the nuclear periphery. For each reconstructed germline, the average number of pgp-3 foci per nucleus was calculated. For each genotype, at each temperature, 5 germlines (20-40 nuclei per germline) were reconstructed and quantified.
Figure 2: Schematic illustrating foci quantification workflow using ImarisCell. i) Z-stacks were imported into the Imaris software and the cell/vesicle function was applied. In this application, nuclei are designated as ‘cells’ and RNA foci as ‘vesicles’. ii) Nuclear boundaries were defined by a minimum size threshold of 4.27 µm. A 0.2 – 0.5 µm boundary around DAPI-stained bodies was applied by the Imaris software and is illustrated as a red dashed circle. Fused or cut-off nuclei were manually removed images prior to further processing. iii) RNA foci were defined by a minimum size threshold of 0.35 µm or 0.7 µm. Localization designations were defined based on the distance a focus is from the nuclear boundary moving inward in the nucleus. Foci between 0 – 0.5 µm from the nuclear boundary are defined as perinuclear; foci further than 0.5 µm are defined as nuclear.
2.5 Molecular cloning methods and strain generation

2.5.1 Generation of strains expressing MS2 protein using the Mos1-Mediated Single Copy Insertion (MosSCI) method

The ms2::gfp fusion construct under mex-5 and tbb-2 regulatory elements was generated using Gateway® cloning technology (Invitrogen). The ms2 coding sequence was amplified from a plasmid (MS2-HB, Addgene #35573) using the Q5® High-Fidelity DNA Polymerase (New England Biolabs) and the PCR product was recombined into the Gateway entry plasmid pDONR221 using the BP clonase enzyme mix (Invitrogen). To generate the expression clone, the entry clone containing the ms2 coding sequence, together with entry clones containing the mex-5 promoter and GFP::tbb-2 3’UTR (pJA252, Addgene #21512 and pJA256, #21509), were recombined into the destination vector pCFJ150 using the LR clonase enzyme mix (Invitrogen). Resulting plasmids were verified by sequencing. The ms2::mNeonGreen fusion constructs were similarly generated. mNeonGreen was amplified from a plasmid (kindly provided by the Derry lab) and the Gibson Assembly® protocol (New England Biolabs) was used to replace gfp with mNeonGreen in the entry plasmid (pJA256). The three entry clones containing the mex-5 promoter, ms2 coding sequencing and mNeonGreen::tbb-3 3’UTR were recombined using the LR clonase enzyme mix. Resulting plasmids were verified by sequencing. All primer sequences used are outlined in Table 2.

Using the previously described MosSCI technique, the ms2::gfp and ms2::mNeonGreen transgenes were inserted onto chromosome I in the strain EG8078 using the plasmids pJC0042 and pJA0043, respectively241. An injection mix containing the following concentrations of plasmids was made: 22.5ng/µL ms2::gfp or ms2::mNeonGreen transgene, 50ng/µL pCFJ150 (peft-3::transposase), 5ng/µL pCFJ104 (pmyo-3::mCherry, co-injection maker) and 10ng/µL pMA122 (phsp::peel-1, co-injection maker for heat-shock selection). Adult EG8078 hermaphrodites ~24hr post the L4 stage were injected and recovered in 1x PBS (137 mM NaCl, 2.7 mM KCl, 10 mM phosphate pH 7.4) for 15 minutes, before being transferred to a new plate. 3 injected animals were placed on a given plate and left at 25°C ~ 1 week (until plates were completely starved). Starved plates were then placed in an 34°C incubated for 4 hours and
screened for MosSCI insertions by picking wildtype, non-red movers 4-6 hours post heat-shock. Putative MosSCI insertions were validated by PCR.

2.5.2 Generation of strains expressing MS2 stem-loops in the 3'UTRs of target genes using the CRISPR/Cas9-mediated integration method

MS2 stem-loops were integrated into the 3'UTRs of genes using CRISPR-Cas9 technology. sgRNA sequences were determined using an online tool (available at: http://crispor.tefor.net/). Plasmids containing sgRNA sequences were generated as previously described\(^242\). For long-range homology directed repair (HDR), repair constructs consisted of long homology arms (500-700bp) spanning a repetitive stem-loop cassette. Homology arms were amplified from wildtype genomic DNA using Q5\(^\text{®}\) High-Fidelity DNA Polymerase (New England Biolabs) and 12 MS2 stem-loops were amplified from a plasmid (pSL-MS2-12X, Addgene #27119) using PrimeSTAR\(^\text{®}\) HS DNA polymerase (Takara Bio USA). Homology arms and the stem-loop cassette were combined using the Gibson Assembly\(^\text{®}\) protocol and the assembled product was PCR amplified using PrimeSTAR\(^\text{®}\) HS DNA polymerase and cloned into pCR\(^\text{®}\)-Blunt II-TOPO\(^\text{®}\) vector (Life Technologies). The resulting plasmids were verified by sequencing. A previously described co-CRISPR strategy was used to screen for transgenic animals harbouring the stem-loop cassette\(^243\). Injection mixes containing the following concentrations of plasmids were made: 25ng/µL repair plasmid (stem-loop cassette flanked by homology arms), 50ng/µL each sgRNA plasmid, 20ng/µL co-CRISPR repair oligo (dpy-10 ssODN) and 50ng/µL co-CRISPR sgRNA plasmid (dpy-10 sgRNA). Adult wildtype hermaphrodites ~24h post L4 stage were injected, allowed to recover for 15 minutes in 1x PBS and transferred to a fresh plate. A single animal was transferred per plate and plates were placed at 20°C for 5 days. After 5 days, F1 progeny with a roller phenotype were single picked onto fresh plates, allowed to lay for 1-2 days and total gDNA was extracted from the F1’s. PCR screening was used to select for animals with an integrated stem-loop cassette. All primers sequences are outlined in Table 2.

For short-range HDR primers with a 30bp overhang containing the homology arm sequence were used to amplify 12 or 24 synonymous, non-repetitive MS2 stem-loops from a plasmid (HaloTag-bActinCDS-bActinUTR-MS2V5, Addgene #102718). Two 50µL PCRs done using KAPA HiFi DNA polymerase (KAPA biosystems) were combined, gel-extracted using the
Monarch® DNA Gel Extraction Kit (New England Biolabs), cleaned with phenol:chloroform and precipitated overnight with 20 µg of glycogen, 10% of the volume of 3M sodium acetate and 4X the volume of ethanol. Injection mixes containing the following concentrations of plasmids were made: 50 ng/µL PCR product (stem-loop cassette flanked by homology arms), 50 ng/µL each sgRNA plasmid, 20 ng/µL co-CRISPR repair oligo (dpy-10 ssODN) and 50 ng/µL co-CRISPR sgRNA plasmid (dpy-10 sgRNA). Injections and screening were carried out as described above.

Table 2. Primers used in this study.

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Chapter 3

3 Results

3.1 Identification of genetic requirements for the trafficking and localization of an mRNA transcript to perinuclear foci

To determine the localization pattern of mRNA transcripts targeted by the CSR-1 small RNA pathway, I utilized a cutting edge technique called single molecule fluorescence in situ hybridization (smFISH)\(^\text{244}\). I have successfully optimized and established the smFISH technique in the lab. The smFISH method differs from more conventional RNA FISH techniques by using many short oligonucleotide probes (around 20 nucleotides in length) that are each bound to a single fluorophore to detect transcripts. The smFISH technique is a powerful tool to study mRNA localization and there are several advantages to using this method. Multiple probes are required to bind the transcript to detect clear foci and binding of a single probe is faintly visible on its own, limiting the number of foci detected due to non-specific binding. Furthermore, this technique can be used to detect both high- and low-abundance transcripts, as the small oligonucleotide size allows the probes to penetrate tissue efficiently. Previous studies utilizing more conventional RNA FISH techniques did not see a clear enrichment of transcripts to germ granules over the rachis of the worm in the adult germline\(^\text{245,246}\). Therefore the high sensitivity of the smFISH technique and its ability to detect mRNA transcripts at a single molecule resolution is critical to assess transcript localization relative to germ granules.

To examine the factors required for the appropriate export and localization of CSR-1 target mRNA transcripts I performed a candidate screen using mutants or RNAi for 17 mRNA processing/export, nuclear pore, germ granule and small RNA pathway factors (Fig 3). I decided to test certain factors based on their mutant phenotypes and their known roles in small RNA regulation and/or mRNA export functions in worms and other species. I performed smFISH experiments at 20°C and 25°C and quantified changes in mRNA localization. Although *C. elegans* can comfortably grow at 20°C or 25°C, the higher temperature introduces a slight heat stress to the worm and could uncover factors that play a role in mRNA trafficking and
localization under stressful conditions. Furthermore, higher temperatures begin to perturb the liquid-like properties of germ granules. Some of the factors I am examining show more severe phenotypes at 25°C; therefore analyzing transcript localization at 25°C may uncover a phenotype that is not present or difficult to detect at 20°C. I simultaneously measured mRNA levels with qRT-PCR to assess if changes in mRNA export and/or localization are coupled with changes in expression. *pgl*-3 localization patterns are summarized in Table 3.

![Figure 3. Schematic illustrating the factors examined for their role in mRNA trafficking and localization to germ granules. Several small RNA pathway proteins (CSR-1, DRH-3, HRDE-1), mRNA processing/export proteins (UAF-1, RNP-3, EMB-4, HEL-1, DDX-19), nuclear pore proteins (NPP-1, NPP-11, NPP-14, NPP-16) and germ granule proteins (PGL-1, DEPS-1, GLH-1, MUT-17) were examined in this study.](image)

**Table 3.** Table summarizing *pgl*-3 mRNA localization patterns in mutants or following RNAi of certain factors. Subcellular localization and broad role of each factor is stated. 10-20 independent germlines were scored and the penetrance of the phenotypes is indicated. Factors depleted by RNAi are indicated with *. Shaded boxes signify that animas lacking a factor did not survive at 25°C (*hel*-1, *npp*-7, *uaf*-1) or was not checked (*csr*-1). WT = phenotype resembles wildtype.
3.1.1 The CSR-1 target transcript pgl-3 localizes to perinuclear germ granules in wildtype animals

I have performed smFISH experiments with a probe set that recognizes the transcript pgl-3 (Fig 4). The pgl-3 gene is targeted by the CSR-1 pathway and is robustly expressed in the germline. The PGL-3 protein is germline specific, localized to P granules and plays a role in P granule nucleation\textsuperscript{162}. To ensure my probe sets recognize specific transcripts and not just background
RNA, I used a set of probes that are sense to the *pgl-3* transcript as a negative control and was unable to detect non-specific foci (Fig 4b). The *pgl-3* transcript appears to be most abundant in the pachytene region of the germline (where a burst of transcription is known to occur) and I observed foci of different sizes both at the nuclear periphery and in the rachis of the pachytene germline (Fig 4b). Larger foci are likely attributed to the co-localization of multiple *pgl-3* transcripts.

To visualize the localization of *pgl-3* transcripts relative to germ granules I attempted to combine smFISH with Immunofluorescence (IF) to stain for the P granule marker PGL-1. I was unable to combine the two techniques without a substantial loss of either the smFISH signal or the IF signal. This could be due to several factors. The reagents used during smFISH and IF are quite different making it difficult to combine the two techniques. The smFISH conditions are very harsh, including long formaldehyde and formamide incubations, and may have an effect on the PGL-1 antibody-antigen interaction. Furthermore, many of the IF reagents, including the antibody and blocking agent, are not RNAse free which could affect the smFISH signal.

An alternative way to visualize *pgl-3* transcripts simultaneously with germ granule proteins is to use a worm strain that has a P granule or Mutator foci protein tagged with GFP. I used a worm strain that has CSR-1 tagged with GFP. CSR-1 protein co-localizes with the P granule marker PGL-1 and therefore I decided to use this strain as a proxy for P granule location^{83}.

I preformed *pgl-3* smFISH in the GFP-tagged CSR-1 strain. A proportion of the *pgl-3* transcript is perinuclear (Fig. 5a,b). I counted manually how many perinuclear *pgl-3* foci overlap or are adjacent to GFP tagged CSR-1 (Fig. 5c). Of the 308 *pgl-3* foci I scored, 62% (191 of 308) either completely or partially overlap with CSR-1, 29% (89 of 308) are directly adjacent to CSR-1 and 9% (28 of 308) are not near CSR-1 foci. The *pgl-3* foci overlapping with CSR-1 are likely localizing to P granules. The *pgl-3* transcript adjacent to CSR-1 could localize to one of the other germ granules that have been recently identified as sitting adjacent to P granules (*Mutator* foci or Z granules)^{156,157}. I performed *pgl-3* smFISH in a worm strain that has a *Mutator* foci protein tagged with GFP. However the smFISH protocol completely disrupted the GFP signal and therefore I was not able to determine if *pgl-3* localizes to *Mutator* foci. In conclusion, these
data demonstrate that a proportion of the \textit{pgl-3} transcript localizes to perinuclear germ granules and the smFISH technique is sensitive enough to visualize this localization.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure4.png}
\caption{Single molecule fluorescence \textit{in situ} hybridization (smFISH) in the \textit{C. elegans} germline. (a) Schematic of the \textit{C. elegans} germline. The adult germline is a syncytium in which germ cells surround a cytoplasmic core called a rachis. (b) Control smFISH using probes sense to the \textit{pgl-3} transcript. (c) Gonads dissected from wildtype adults hybridized with antisense probes targeting the \textit{pgl-3} transcript. Red foci represent the \textit{pgl-3} transcript. Large, bright foci in the cytoplasm are likely the co-localization of multiple transcripts (indicated with a yellow arrowhead). All probe sets consisted of a pool of forty-eight-20-nt oligonucleotide probes. Worms were counterstained with DAPI to visualize DNA.}
\end{figure}
3.1.2 Specific germ granule proteins are required for the localization of \( pgl-3 \) transcript to perinuclear foci

To examine the role for specific germ granule proteins in \( pgl-3 \) transcript localization, I focused on three worm-specific proteins, DEPS-1, PGL-1 and MUT-16, and the conserved factor GLH-1. I used three null mutants for \( deps-1 \), \( glh-1 \) and \( pgl-1 \), and a hypomorphic mutant for \( mut-16 \). DEPS-1, GLH-1 and PGL-1 are structural P granule factors important for P granule nucleation.
at the nuclear periphery. The P granule assembly pathway places DEPS-1 most upstream, followed by GLH-1 then PGL-1\textsuperscript{161}. In addition to their role in P granule structure, GLH-1 and PGL-1 likely have other roles in RNA metabolism due to the presence of RNA binding domains. Furthermore, GLH-1 has been shown to interact with small RNA factors in other organisms\textsuperscript{28,168}. MUT-16 is a \textit{Mutator} foci protein and is required for the localization of other key \textit{Mutator} foci proteins to the nuclear periphery\textsuperscript{157}. Notably, all four germ granule proteins have been implicated in small RNA regulation; however, in most cases it is unclear whether these factors play a direct or indirect role in small RNA pathways\textsuperscript{157,161–163}.

smFISH experiments in \textit{mut-16} mutants revealed no changes in \textit{pgl-3} transcript localization at 20°C or 25°C compared to wildtype. \textit{pgl-3} smFISH in the P granule mutants revealed a specific role for GLH-1 and PGL-1 in perinuclear \textit{pgl-3} localization at 20°C (Fig 6). When quantifying perinuclear \textit{pgl-3} foci, I binned the foci based on size (either foci < 0.35µm or foci <0.7µm) to more carefully examine the amount of \textit{pgl-3} at the nuclear periphery.

In both \textit{glh-1} and \textit{pgl-1} mutants, there was a reduction in the number and size of \textit{pgl-3} foci at the nuclear periphery (Fig 6a,b). Interestingly, DEPS-1 is hypothesized to act upstream of GLH-1 and PGL-1 in the P granule nucleation pathway, yet \textit{pgl-3} localization appeared wildtype in \textit{deps-1} mutants (Fig 6a,b). Transcript levels of \textit{pgl-3} at 20°C were comparable to wildtype levels in \textit{glh-1} and \textit{deps-1} mutants, and only slightly reduced in \textit{pgl-1} mutants, as measured by qRT-PCR (Fig 6c). Furthermore, \textit{pgl-3} smFISH in \textit{deps-1} and \textit{glh-1} mutants did not reveal an obvious depletion of \textit{pgl-3} transcripts in the rachis of the germline, corroborating the qRT-PCR results (Fig 6a). Therefore reduction in perinuclear \textit{pgl-3} mRNA in germ granule mutants was unlikely due to reduced expression and export of the transcript.

At 25°C there was a reduction in the number and size of perinuclear \textit{pgl-3} foci in all three P granule mutants (Fig 7a,b). The reduction in perinuclear \textit{pgl-3} mRNA in \textit{glh-1} and \textit{pgl-1} mutants at 20°C was exacerbated at the higher temperature. Interestingly, in wildtype animals at 25°C there was nuclear accumulation of \textit{pgl-3} transcript coupled with a 20-30% increase in expression (Fig 7c,d). The nuclear accumulation of \textit{pgl-3} was not seen in wildtype animals at 20°C.
Notably, the nuclear accumulation of *pgl-3* in wildtype animals at 25°C was not seen in *deps-1* or *glh-1* mutants at 25°C. Reduction in *pgl-3* mRNA levels in *deps-1* and *glh-1* mutants at 25°C could in part explain the absence of nuclear *pgl-3* (Fig 7e). Interestingly, *pgl-1* mutants also showed a reduction in *pgl-3* mRNA levels at 25°C together with nuclear accumulation of *pgl-3* transcript. Defects in mRNA export may be more severe in *pgl-1* mutants at 25°C than in the other P granule mutants, resulting in nuclear accumulation of mRNA.

The smFISH experiments in germ granule mutants reveal a role for specific P granule proteins in the localization of *pgl-3* mRNA to perinuclear foci. The reduction in number and size of perinuclear *pgl-3* foci points to a defect in transcript localization to germ granules, though future smFISH experiments using germ granule markers are needed to validate this. The wildtype localization of *pgl-3* mRNA in *mut-16* mutants could indicate that the *pgl-3* transcript does not localize to Mutator foci, and its localization might be restricted to P granules in wildtype animals. Alternatively, the *mut-16* hypomorphic allele utilized in these experiments may not result in enough of a perturbation to Mutator foci structure to affect *pgl-3* localization.
**Figure 6. pgli-3 smFISH in germ granule protein mutants at 20°C.** (a) Single molecule fluorescence *in situ* hybridization (smFISH) shows pgli-3 transcript localization in the pachytene germline. Red foci represent pgli-3 transcripts and DNA is stained with DAPI in blue. Surface and core slices of the germline are shown and a single nucleus within the dashed box is magnified for each genotype. A pgli-3 focus of 0.7µm in size is indicated for reference. (b) Quantification of the number and size of pgli-3 foci around the nuclear periphery. Bars represent the average number of perinuclear pgli-3 foci per nucleus. Five pachytene germlines were reconstructed per genotype and approximately 20-40 nuclei were quantified per germline. pgli-3 foci were binned into two categories: perinuclear foci greater than 0.35µm and perinuclear foci greater than 0.7µm. The average number of pgli-3 foci per nucleus was measured for each germline and error bars represent the standard deviation between germlines. (c) qRT-PCR experiments were conducted to determine pgli-3 mRNA levels in wildtype worms and in germ granule mutants. Bar graph indicates levels of pgli-3 mRNA in germ granule mutants relative to wildtype at 20°C. Error bars represent the standard deviation between independent biological replicates, n=2.
a. Surface  Core  Zoom

GT

deps-1  (bn124)  

glh-1  (gk100)  

pgl-1  (bn101)  

mut-16  (mg461)  

DAPI / pgl-3 transcript
Figure 7. *pgl-3* smFISH in germ granule protein mutants at 25°C. (a) Single molecule fluorescence in situ hybridization (smFISH) shows *pgl-3* transcript localization in the pachytene germline. Red foci represent *pgl-3* transcript and DNA is stained with DAPI in blue. Surface and core slices of the germline are shown and a single nucleus within the dashed box is magnified for each genotype. Quantifications of perinuclear (b) or nuclear (c) *pgl-3* foci are shown. Five pachytene germlines were reconstructed per genotype and approximately 20-40 nuclei were quantified per germline. For perinuclear *pgl-3* quantification, *pgl-3* foci were binned into two categories:
perinuclear foci greater than 0.35µm and perinuclear foci greater than 0.7µm. For nuclear pgl-3 quantification, all foci over 0.35µm were scored. The average number of perinuclear or nuclear pgl-3 foci per nucleus was measured for each germline and error bars represent the standard deviation between germlines. qRT-PCR experiments were conducted to determine pgl-3 mRNA levels in wildtype worms at 25°C relative to 20°C (d) and mRNA levels in germ granule mutants at 25°C relative to wildtype at 25°C (e). Error bars represent the standard deviation between independent biological replicates, n=2.

3.1.3 Perturbations in the CSR-1 pathway results in reduced levels of pgl-3 transcript at perinuclear foci

To determine if a functional CSR-1 pathway is required for the perinuclear localization of the pgl-3 transcript, I performed pgl-3 smFISH experiments in mutants of two key CSR-1 pathway proteins: the Argonaute CSR-1 and the helicase DRH-3. DRH-3 is a core component of the RNA dependent RNA polymerase (RdRP) complex, which is essential for the biogenesis of 22G-RNAs that are destined for several germline small RNA pathways, including the CSR-1 pathway\textsuperscript{84}.

I used a null mutant of csr-1 and a temperature sensitive drh-3 mutant, which results in a partial loss-of-function phenotype at 25°C. The smFISH experiments revealed a reduction in number and size of perinuclear pgl-3 foci in csr-1 mutants at 20°C (Fig 8a,b) and in dhr-3 mutants at 25°C (Fig 9a,b). The mRNA levels of pgl-3 in csr-1 mutants were comparable to wildtype, as measured by qRT-PCR (Fig 8c). Therefore the reduction in perinuclear pgl-3 transcript in csr-1 mutants was not coupled with a reduction in pgl-3 expression. A reduction in pgl-3 mRNA levels was seen in drh-3 mutants at 25°C, as is evidenced by qRT-PCR and pgl-3 smFISH (Fig 9a,c).

To investigate if the mislocalization of the pgl-3 transcript is specific to a disrupted CSR-1 pathway, I also examined pgl-3 mRNA localization in HRDE-1 null mutants. HRDE-1 is a germline specific Argonaute protein, and acts downstream of the piRNA pathway to repress the expression of deleterious nucleic acids\textsuperscript{22,247}. HRDE-1 and CSR-1 are to date the only germline Argonaute proteins in the worm that have been shown to localize to the nucleus. The smFISH experiments did not reveal any changes in the abundance of perinuclear pgl-3 transcript in hrde-1 null mutants at 20°C compared to wildtype (Fig 8a,b). At 25°C there was a subtle reduction in the size of perinuclear pgl-3 foci in hrde-1 mutants coupled with an increase in nuclear pgl-3 foci (Fig 9). At 25°C hrde-1 mutants show a mortal germline phenotype (progressive sterility
over generations). Therefore, a reduction in perinuclear *pgl-3* mRNA specifically at higher temperatures is not surprising, and points to a direct or indirect role for the *hrde-1* pathway in transcript export and cytoplasmic localization at higher temperatures.

The smFISH experiments in three small RNA pathway mutants, CSR-1, DRH-3 and HRDE-1, point to a role for small RNA pathways in the localization of mRNAs to perinuclear foci. Additional smFISH experiments in other small RNA pathway mutants would further elucidate the specificity of the role for certain small RNA pathways in transcript localization.
Figure 8. *pgl-3* smFISH in small RNA pathway protein mutants at 20°C. (a) Single molecule fluorescence *in situ* hybridization (smFISH) shows *pgl-3* transcript localization in the pachytene germline. Red foci represent *pgl-3* transcripts and DNA is stained with DAPI in blue. Surface and core slices of the germline are shown and a single nucleus within the dashed box is magnified for each genotype. A *pgl-3* focus of 0.7µm in size is indicated for reference. (b) Quantification of the number and size of *pgl-3* foci around the nuclear periphery. Bars represent the average number of perinuclear *pgl-3* foci per nucleus. Five pachytene germlines were reconstructed per genotype and approximately 20-40 nuclei were quantified per germline. *pgl-3* foci were binned into two categories: perinuclear foci greater than 0.35µm and perinuclear foci greater than 0.7µm. The average number of *pgl-3* foci per nucleus was measured for each germline and error bars represent the standard deviation between germlines. (c) qRT-PCR experiments were conducted to determine *pgl-3* mRNA levels in wildtype worms and small RNA pathway mutants. Bar graph indicates levels of *pgl-3* mRNA in mutants relative to wildtype 20°C. Error bars represent the standard deviation between independent biological replicates, n=2.
a. Surface Core Zoom

WT

hrde-1 (tm1200)

drh-3 (ne4253)

DAPI / pgf-3 transcript
Figure 9. *pgl-3* smFISH in small RNA pathway protein mutants at 25°C. (a) Single molecule fluorescence in situ hybridization (smFISH) shows *pgl-3* transcript localization in the pachytene germline. Red foci represent *pgl-3* transcript and DNA is stained with DAPI in blue. Surface and core slices of the germline are shown and a single nucleus within the dashed box is magnified for each genotype. Quantification of perinuclear (b) or nuclear (c) *pgl-3* foci is shown. Five pachytene germlines were reconstructed per genotype and approximately 20-40 nuclei were quantified per germline. For perinuclear *pgl-3* quantification, *pgl-3* foci were binned into two categories: perinuclear foci greater than 0.35µm and perinuclear foci greater than 0.7µm. For nuclear *pgl-3* quantification, all foci over 0.35µm were scored. The average number of perinuclear or nuclear *pgl-3* foci per nucleus was measured for each germline and error bars represent the standard deviation between germlines. (d) qRT-PCR experiments were conducted to determine *pgl-3* mRNA levels in wildtype worms and small RNA pathway mutants. Bar graph indicates levels of *pgl-3* mRNA in mutants relative to wildtype 25°C. Error bars represent the standard deviation between independent biological replicates, n=2.
3.1.4 Mutations in mRNA processing and export factors lead to defects in *pgl-3* mRNA export and cytoplasmic localization

To gain a better understanding of the mRNA processing and export factors important for *pgl-3* trafficking out of the nucleus and localization to germ granules, I examined 5 mRNA processing/export factors using mutants or RNAi. Numerous studies across several model organisms have revealed that the processing of transcripts is tightly linked to their export\textsuperscript{174,175}. In recent years, studies in worm and other animals have highlighted a role for mRNA splicing factors in small RNA regulation, independent of their roles in splicing\textsuperscript{18,19,28,240}. I chose to examine certain splicing and export factors based on their previously established role in small RNA regulation (HEL-1, EMB-4), localization patterns (for instance, DDX-19 localizes to germ granules) and availability of mutants\textsuperscript{28,149,235}.

At 20°C there were defects in *pgl-3* mRNA export in *ddx-19* mutants and following *hel-1* RNAi (Fig 10a). The *ddx-19* mutant has a 1kb deletion spanning the second and third exon of the gene, which likely results in either a null protein product or a protein with severely reduced function. DDX-19 is a conserved mRNA export protein and plays a role in mRNP remodeling. In *C.elegans* DDX-19 localizes to the cytoplasmic face of nuclear pores and associates with P granules\textsuperscript{149}. HEL-1 is a conserved mRNA splicing and export factor and has been implicated in small RNA regulation\textsuperscript{28}. In both *ddx-19* mutants and following *hel-1* RNAi, I saw nuclear accumulation of the *pgl-3* transcript (Fig 10c). In addition to the nuclear localization of *pgl-3*, I saw a reduction in number and size of perinuclear *pgl-3* foci, suggesting a defect in *pgl-3* trafficking and accumulation in germ granules (Fig 10b). *pgl-3* smFISH and qRT-PCR data revealed a reduction in *pgl-3* mRNA levels following *hel-1* RNAi and no change in mRNA levels in *ddx-19* mutants (Fig 10d).

At 25°C I saw a reduction in the number and size of perinuclear *pgl-3* foci in two additional mutants, *emb-4* and *rnp-3* (Fig 11a,b). The reduction in perinuclear *pgl-3* was coupled with a reduction in *pgl-3* mRNA levels in both mutants at 25°C, implicating these proteins in proper expression of the *pgl-3* gene (Fig 11d). The *emb-4* mutant is a characterized null mutant. EMB-4 is a conserved intron binding protein and is thought to recruit EJC components to the splicesome. EMB-4 has been previously implicated in small RNA pathways\textsuperscript{235,236}. The *rnp-3*...
mutant has a 1kb deletion in the *rpn-3* locus and likely results in either a null protein product or a protein with severely reduced function. RNP-3 is a conserved splicesome-associated protein.

The absence of nuclear *pgl-3* in *emb-4, rnp-3* and *uaf-1* mutants at 20°C or 25°C, suggests that these mRNA processing factors may not be required for shuttling the *pgl-3* transcript out of the nucleus (Fig 10d, 11c). However, the reduction in perinuclear *pgl-3* in the *emb-4* and *rnp-3* mutants at 25°C, suggests these proteins may play a direct or indirect role in localization of the *pgl-3* transcript to perinuclear granules. In conclusion, the smFISH experiments uncovered roles for specific mRNA processing/export factors in nuclear export of the *pgl-3* transcript and its perinuclear localization.
**ddx-19 (ok783)**

**DAPI / pgl-3 transcript**

**b.**

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Figure 10. \textit{pgl-3} smFISH in mRNA processing/export mutants at 20°C. (a) Single molecule fluorescence \textit{in situ} hybridization (smFISH) shows \textit{pgl-3} transcript localization in the pachytene germline. Red foci represent \textit{pgl-3} transcript and DNA is stained with DAPI in blue. Surface and core slices of the germline are shown and a single nucleus within the dashed box is magnified for each genotype. A \textit{pgl-3} focus of 0.7µm in size is indicated for reference. Quantification of perinuclear (b) or nuclear (c) \textit{pgl-3} mRNA is shown. Five pachytene germlines were reconstructed per genotype and approximately 20-40 nuclei were quantified per germline. For perinuclear \textit{pgl-3} quantification, \textit{pgl-3} foci were binned into two categories: perinuclear foci greater than 0.35µm and perinuclear foci greater than 0.7µm. For nuclear \textit{pgl-3} quantification, all foci over 0.35µm were scored. The average number of perinuclear or nuclear \textit{pgl-3} foci per nucleus was measured for each germline and error bars represent the standard deviation between germlines. (d) qRT-PCR experiments were conducted to determine \textit{pgl-3} mRNA levels in wildtype worms and mRNA processing/export mutants. Bar graph indicates levels of \textit{pgl-3} mRNA in mutants relative to wildtype 20°C. Error bars represent the standard deviation between independent biological replicates, n=2.
a.

WT

rnp-3
(ok1424)

emb-4
(hc60)

ddx-19
(ok783)

DAPI / pgI-3 transcript
Figure 11. *pgl-3* smFISH in mRNA processing/export mutants at 25°C. (a) Single molecule fluorescence in situ hybridization (smFISH) shows *pgl-3* transcript localization in the pachytene germline. Red foci represent *pgl-3* transcript and DNA is stained with DAPI in blue. Surface and core slices of the germline are shown and a single nucleus within the dashed box is magnified for each genotype. Quantification of perinuclear (b) or nuclear (c) *pgl-3* mRNA is shown. Five pachytene germlines were reconstructed per genotype and approximately 20-40 nuclei were quantified per germline. For perinuclear *pgl-3* quantification, *pgl-3* foci were binned into two categories: perinuclear foci greater than 0.35µm and perinuclear foci greater than 0.7µm. For nuclear *pgl-3* quantification, all foci over 0.35µm were scored. The average number of perinuclear or nuclear *pgl-3* foci per nucleus was measured for each germline and error bars represent the standard deviation between germlines. (d) qRT-PCR experiments were conducted to determine *pgl-3* mRNA levels in wildtype worms and mRNA processing/export mutants. Bar graph indicates levels of *pgl-3* mRNA in mutants relative to wildtype 25°C. Error bars represent the standard deviation between independent biological replicates, n=2.
3.1.5 Specific nuclear pore proteins are required for the proper expression and localization of \textit{plg-3} transcripts

Transcripts are exported out of the nucleus via nuclear pore complexes. Cytoplasmic and nuclear faces of the nuclear pore interact with various mRNA processing and export factors to facilitate efficient export. Nuclear pore complexes in worms associate with germ granules and have been implicated in germ granule structure\textsuperscript{248}. Interestingly, genome-wide screens in several animals have implicated nuclear pore proteins in small RNA regulation; however their specific roles in small RNA pathways have yet to be determined\textsuperscript{16,17,240}. I examined the role of 4 nuclear pore proteins in \textit{pgl-3} mRNA export and perinuclear localization using mutants or RNAi (Fig 12, 13).

Following \textit{npp-7} RNAi at 20°C I saw a reduction in the number and size of perinuclear \textit{pgl-3} foci (Fig 12a,b). NPP-7 is part of the nuclear basket, which protrudes into the nucleus and interacts with mRNA export factors\textsuperscript{215,216,223}. qRT-PCR results showed no change in \textit{pgl-3} mRNA levels following \textit{npp-7} RNAi (Fig 12c). Interestingly, \textit{pgl-3} mRNA localization appeared wildtype in null mutants of another nuclear basket protein, NPP-16 (Fig 12a,b). Both NPP-16 and NPP-7 are enriched in the germline. The different \textit{pgl-3} mRNA localization patterns in \textit{npp-16} mutants and following \textit{npp-7} RNAi point to a role for a specific nuclear basket protein in \textit{pgl-3} mRNA localization.

At 20°C and 25°C, I saw a reduction in perinuclear \textit{pgl-3} localization in \textit{npp-14} mutants (Fig 12,13). This reduction in perinuclear \textit{pgl-3} was coupled with reduced mRNA levels as measured by qRT-PCR at 25°C but not at 20°C (Fig 12c, 13d). NPP-14 is part of the cytoplasmic face of the nuclear pore, which projects into the cytoplasm. In the \textit{C. elegans} germline, cytoplasmic nucleoporins extend into P granules and may be important for proper perinuclear localization of several P granule proteins.

Interestingly, I did not see accumulation of \textit{pgl-3} transcript in the nucleus of any nuclear pore mutants I examined at 20°C. Furthermore, the \textit{pgl-3} transcript accumulation in certain nuclear pore mutants at 25°C was comparable to wildtype (Fig 13c). These results indicate that the nuclear pore proteins I examined may not be required for the export of \textit{pgl-3} mRNA out of the nucleus. However certain nuclear pore proteins appear to be required for perinuclear localization.
of *pgl*-3 mRNA. The nuclear pore mutants all contain at least a 1kb deletion spanning the open reading frame. It is important to note these mutants have not been characterized and may not be null mutants. Therefore the absence of an export defect in the mutants may be due to residual activity of the proteins in the mutants.
a. Surface  Core  Zoom

WT

npp-7 RNAi

npp-11 (ok1599)

npp-14 (ok1389)

npp-16 (ok1839)

DAPI / pgl-3 transcript
Figure 12. *pgl-3* smFISH in nuclear pore protein mutants at 20°C. (a) Single molecule fluorescence in situ hybridization (smFISH) shows *pgl-3* transcript localization in the pachytene germline. Red foci represent *pgl-3* transcripts and DNA is stained with DAPI in blue. Surface and core slices of the germline are shown and a single nucleus within the dashed box is magnified for each genotype. A *pgl-3* focus of 0.7µm in size is indicated for reference. (b) Quantification of the number and size of *pgl-3* foci around the nuclear periphery. Bars represent the average number of perinuclear *pgl-3* foci per nucleus. Five pachytene germlines were reconstructed per genotype and approximately 20-40 nuclei were quantified per germline. *pgl-3* foci were binned into two categories: perinuclear foci greater than 0.35µm and perinuclear foci greater than 0.7µm. The average number of *pgl-3* foci per nucleus was measured for each germline and error bars represent the standard deviation between germlines. (c) qRT-PCR experiments were conducted to determine *pgl-3* mRNA levels in wild-type worms and nuclear pore mutants. Bar graph indicates levels of *pgl-3* mRNA in mutants relative to wild-type 20°C. Error bars represent the standard deviation between independent biological replicates, n=2.
a. 

WT

npp-11 (ok1599)

npp-14 (ok1389)

npp-16 (ok1839)

DAPI / pgl-3 transcript
Figure 13. *pgl-3* smFISH in nuclear pore protein mutants at 25°C. (a) Single molecule fluorescence *in situ* hybridization (smFISH) shows *pgl-3* transcript localization in the pachytene germline. Red foci represent *pgl-3* transcript and DNA is stained with DAPI in blue. Surface and core slices of the germline are shown and a single nucleus within the dashed box is magnified for each genotype. Quantification of perinuclear (b) or nuclear (c) *pgl-3* foci is shown. Five pachytene germlines were reconstructed per genotype and approximately 20-40 nuclei were quantified per germline. For perinuclear *pgl-3* quantification, *pgl-3* foci were binned into two categories: perinuclear foci greater than 0.35µm and perinuclear foci greater than 0.7µm. For nuclear *pgl-3* quantification, all foci over 0.35µm were scored. The average number of perinuclear or nuclear *pgl-3* foci per nucleus was measured for each germline and error bars represent the standard deviation between germlines. (d) qRT-PCR experiments were conducted to determine *pgl-3* mRNA levels in wildtype worms and nuclear pore mutants. Bar graph indicates levels of *pgl-3* mRNA in mutants relative to wildtype 25°C. Error bars represent the standard deviation between independent biological replicates, n=2.
3.2 A toolkit for the visualization of mRNA trafficking in real-time using the MS2 stem-loop system

To better understand the dynamics of mRNA trafficking to germ granules in wildtype animals and how the dynamics change in the mutants examined in the smFISH experiments, I worked to build a system to visualize mRNA trafficking in real time\textsuperscript{159,249–253}. I followed a similar approach to what has been previously done in other animals and used the MS2 stem-loops system. MS2 is a bacteriophage coat protein that binds to a specific stem-loop structure\textsuperscript{254}. This technique involves the insertion of stem-loops into a gene of interest and simultaneous expression of the stem-loop binding protein MS2 fused to a fluorescent marker (Fig 14a). Studies have inserted from 6 to 24 individual MS2 stem-loops into UTRs of genes and together with a GFP-tagged MS2 protein, have been successful in visualizing RNA live.

3.2.1 A single copy of MS2 fused to GFP is expressed in the C. elegans germline

Using the Mos1-mediated single copy insertion method (mosSCI) I generated a strain of worms that expresses MS2 protein fused to GFP from a single copy transgene under germline specific regulatory elements (Fig 14b). I attempted to create transgenic worms expressing a single copy of MS2 fused to mNeonGreen, a fluorescence protein approximately 5 times brighter than GFP\textsuperscript{255}. Using mNeonGreen could allow for greater sensitivity and minimize the number of stem-loops required to detect a clear signal. In total I made 7 independent strains that contain a single copy of MS2 (3 of the 7 contain a nuclear export signal to minimize unbound MS2::GFP in the nucleus) fused to mNeonGreen. I was unable to detect any mNeonGreen signal under a confocal microscope in any of the MS2::mNeonGreen strains. In the C. elegans germline foreign sequences (such as mNeonGreen) are prone to silencing by small RNA pathways and it is possible the mNeonGreen construct fused to MS2 is being silenced by these surveillance mechanisms.

3.2.2 Insertion of 12 and 24 MS2 stem-loops into the 3'UTR is insufficient to visualize mRNA

Using CRISPR-Cas9 technology, I successfully integrated MS2 stem-loops into the 3'UTRs of germline expressed CSR-1 target genes at their endogenous loci. Initially, I integrated a highly
repetitive stem-loop cassette that contained identical stem-loops, flanked by repetitive linker sequences. Using long-range homology-directed repair, where the homology arms in the repair construct ranged from 500-600bp, I successfully integrated 6 stem-loops into the 3'UTR of car-1. However, these repetitive stem-loop cassettes are very prone to recombination and following a single generation these stem-loops recombined and were deleted from the genome.

To overcome stem-loop recombination and deletion, I used a new MS2 stem-loop cassette recently developed by Dr. Robert Singer’s lab. This cassette contains 24 non-repetitive, but still functional, stem-loops flanked by unique sequences. The authors showed a drastic reduction in deletion through recombination of the stem-loops in cell culture models. In addition to using this new non-repetitive cassette, I switched from using long homology arms in the repair template to short ones (30-50bp). C. elegans data suggests that using short homology arms is more efficient at inserting sequences up to 2kb into the genome. The integration of 6 stem-loops into the 3'UTR of car-1 using long homology arms was a rare event and required PCR screening approximately 300 worms to identify a single integration event. Therefore by switching to short homology arms, I hoped to increase homologous recombination frequency and minimize PCR screening.

Utilizing the non-repetitive stem-loop cassette together with short homology arms, I stably integrated 12 or 24 MS2 stem-loops into the 3'UTRs of 6 CSR-1 target genes. I integrated the stem-loops close to the 5' end of the 3'UTRs, upstream of any putative polyadenylation sites. Stem-loops in 5 of the 6 3'UTRs resulted in a phenotype mimicking that of either a loss-of-function mutant or the RNAi induced phenotype (phenotypes summarized in Table 4). In most cases the animals were inviable and often exhibited phenotypes that mimicked those of null mutants, or following RNAi knockdown. Prior to crossing the viable stem-loop strains into the ms2::gfp strain, I measured mRNA levels of the transcripts into which I inserted stem-loops to ensure the mRNA is expressed to sufficient levels and could be detected by MS2::GFP. I measured mRNA levels of car-1, mel-26 and ubc-20 in the transgenic animals. I saw a 20-30% reduction in car-1 and mel-26 mRNA levels in the transgenic strains and a 2-fold increase in ubc-20 mRNA levels in the transgenic strains (Fig 14c). Due to the abundant expression of car-
and *mel-26* in wildtype worms, even with a 30% reduction in expression, there should be a sufficient amount of transcript expressed to be detected by MS2::GFP.

I crossed the viable stem-loop strains with the transgenic strain expressing the *ms2::gfp* construct. Using a confocal microscope I was unable to detect any distinct GFP foci over the background signal (unbound MS2::GFP molecules) in strains with either 12 or 24 MS2 stem-loops integrated into the 3’UTRs (Fig 14d). To determine if MS2::GFP is binding the stem-loops in my transgenic strain I performed an RNA immunoprecipitation (RIP) experiment. I pulled down MS2::GFP in a strain expressing both MS2::GFP and 12 stem-loops integrated into the 3’UTR of *ubc-20*, and in a strain expressing MS2::GFP alone. The enrichment of *ubc-20* mRNA in the IP sample relative to the input sample is comparable in both RIP experiments, suggesting that MS2::GFP is not binding the stem-loops in the 3’UTR of *ubc-20*, or is binding at a rate too low to detect using the RIP method (Fig 14e). This result is consistent with the absence of visible RNA foci under a confocal microscope.
Figure 14. Live visualization of mRNA trafficking in the C. elegans germline using the MS2 system. (a) Schematic illustrating the MS2 system. MS2 protein fused to GFP binds stem loops in the 3'UTRs of CSR-1 targets, allowing for the visualization of transcript trafficking in real time. (b) Confocal image were taken using a 20x objective lens of the transgenic strain expressing a single copy of GFP::MS2 under the control of a germline specific promoter and 3'UTR. The germline (indicated with the red arrow) shows a clear green haze in the transgenic strain compared to wildtype. Schematic of the construct is show above. (c) Confocal image were taken with a 60x objective lens of a transgenic worm expressing MS2::GFP (left) and a transgenic worm expressing MS2::GFP crossed with a worm homozygous for 12 MS2 stem-loops in the 3'UTR of car-1 (right). (d) qRT-PCR experiments conducted to determine the effect of MS2 stem-loops on mRNA levels. Bar graph shows the levels of the indicated mRNA in stem-loop transgenic strains relative to wildtype. Error bars represent the SD between biological replicates, n=2. (e) MS2::GFP RIP followed by qRT-PCR was performed to determine whether MS2 binds stem-loops in the 3'UTR of ubc-20. A control RIP was performed in a strain expressing MS2::GFP without its corresponding stem-loops. The enrichment of ubc-20 mRNA in both the experimental and control IPs compared to input is similar, and is not greater than the enrichment of two non-targets (gpd-2 and pgl-3). This result suggests that MS2::GFP is not binding the stem-loops in the 3'UTR of ubc-20. Error bars represent SD of technical replicates, n=3
Table 4. Summary of phenotypes exhibited by the stem-loop transgenic strains following integration of 12x or 24x MS2 stem-loops. Phenotypes of null mutants or RNAi knockdowns are stated.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Stem-loop insertion phenotype</th>
<th>Mutant phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>car-1</em></td>
<td>12x: ~50% sterile, severely reduced brood size</td>
<td>Null: embryonic lethal, sterile</td>
</tr>
<tr>
<td></td>
<td>24x: 100% sterile</td>
<td></td>
</tr>
<tr>
<td><em>cdk-1</em></td>
<td>12x: L2/L3 larval arrest</td>
<td>Null: embryonic arrest</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RNAi: embryonic arrest, larval arrest</td>
</tr>
<tr>
<td><em>cyb-3</em></td>
<td>12x: ~90% sterile, embryos laid by non-sterile</td>
<td>RNAi: embryonic lethal</td>
</tr>
<tr>
<td></td>
<td>adults are 100% inviable</td>
<td></td>
</tr>
<tr>
<td></td>
<td>24x: 100% sterile</td>
<td></td>
</tr>
<tr>
<td><em>snr-4</em></td>
<td>12x: embryonic lethal, L2/L3 larval arrest</td>
<td>Null: embryonic lethal</td>
</tr>
<tr>
<td><em>ubc-20</em></td>
<td>12x: slow growing</td>
<td>RNAi: low frequency late larval arrest</td>
</tr>
<tr>
<td></td>
<td>24x: slow growing</td>
<td></td>
</tr>
<tr>
<td><em>mel-26</em></td>
<td>12x: no obvious phenotype</td>
<td>Null: no reported phenotype</td>
</tr>
</tbody>
</table>
Chapter 4

4 Discussion and Future Directions

4.1 Discussion

In C. elegans, several Argonaute proteins localize to perinuclear germ granules and target unique subsets of the transcriptome. When a newly synthesized transcript is exported out of the nucleus, it is likely sorted into a particular small RNA pathway as it enters germ granules. Intrinsic sequence features of transcripts, compartmentalization of small RNA pathways within germ granules, and RNA binding proteins associated with transcripts could all contribute to mRNA sorting. To begin to interrogate how transcripts are sorted, in this thesis I established a way to track transcript export and subsequent germ granule localization of mRNA. I examined a target of a single small RNA pathway, the CSR-1 pathway, and determined the factors that are necessary for its proper export and cytoplasmic localization.

Once we have a thorough understanding of the localization patterns of CSR-1 targets and the factors that are required for their proper export, we can apply this same pipeline to other small RNA pathways and begin to identify potential differences between each of these pathways to address the broad question of how mRNAs are sorted.

4.1.1 Examining the wildtype localization of CSR-1 target pgl-3 using smFISH

Using a very sensitive RNA in situ technique called smFISH, I was able to visualize the localization of pgl-3 transcripts relative to germ granules. As expected, the majority of pgl-3 mRNA localized to the rachis of the worm where translation occurs, and a proportion localized to the nuclear periphery. Using a P granule marker strain, I determined that the majority of perinuclear pgl-3 transcript localized to P granules. We currently hypothesize that transcripts are sorted into their respective small RNA pathways within germ granules because numerous small RNA pathway components localize to germ granules, and newly synthesized mRNAs are trafficked through germ granules during their export out of the nucleus. As such, the P granule...
localization of \textit{pgl-3} mRNA was expected and follows the enrichment of CSR-1 in P granules throughout most of the adult germline.

Interestingly, a subset of perinuclear \textit{pgl-3} appeared to localize adjacent to P granules. This localization could be due to the technical drawbacks of the smFISH technique: the smFISH protocol utilizes harsh denaturants and fixatives which likely result in the denaturation of a fraction of GFP molecules. Consequently, P granules appear reduced in size. The \textit{pgl-3} transcript that shows P granule-adjacent localization in reality may localize to P granules. Alternatively, a proportion of the perinuclear \textit{pgl-3} transcript could localize to Z granules and/or \textit{Mutator} foci, structures that are directly adjacent to P granules. Unfortunately, examining \textit{pgl-3} transcript localization in \textit{Mutator} foci and Z granule marker strains proved to be challenging as the harsh conditions of smFISH rendered many GFP-tagged \textit{Mutator} foci and Z granule markers invisible. Another aspect to consider is the fact that Argonautes can be restricted to a specific germ granule; for instance, CSR-1 primarily localizes to P granules while WAGO-4 is enriched in Z granules. Determining whether the target transcripts of Argonautes show granule-specific localization patterns that perhaps coincide with the localization of their corresponding Argonaute would provide valuable insight into the compartmentalization of small RNA pathways within germ granules.

4.1.2 Investigating the nuclear export of \textit{pgl-3}

4.1.2.1 The mRNA processing and export factors DDX-19 and HEL-1 are required for nuclear export of \textit{pgl-3}

I examined 5 mRNA processing/export factors for their role in nuclear export of \textit{pgl-3}: HEL-1, DDX-19, EMB-4, UAF-1 and RNP-3. Following the loss of two export factors, \textit{ddx-19} and \textit{hel-1}, \textit{pgl-3} transcript accumulated in the nucleus. DDX-19 localizes predominantly to the boundary between germ granules and nuclear pores, and is required for the remodeling of mRNP complexes as they exit the nuclear pore\textsuperscript{149,258}. HEL-1 is a member of the TREX complex which couples mRNA processing to export\textsuperscript{211}. Both factors have been previously implicated in mRNA export and therefore nuclear accumulation of \textit{pgl-3} mRNA in \textit{ddx-19} mutants and following \textit{hel-1} RNAi was not unexpected. A previous study in \textit{C. elegans} showed that loss of \textit{hel-1} results in the accumulation of poly(A)$^+$ mRNAs in gut nuclei and studies in yeast have shown a
requirement for DBP5 (yeast homolog of DDX-19) in the export of poly(A)$^+$ mRNAs$^{259,260}$. However, the present study is the first time a requirement for both factors in mRNA export has been shown in the germline nuclei of *C. elegans*.

Due to the tightly coupling of mRNA processing and export, we hypothesized that loss of certain splicing factors could result in export defects of mRNA. The absence of nuclear *pgl-3* in the mutants of three splicing factors *emb-4*, *uaf-1* and *rnp-3* suggests that these factors are not required for export of the *pgl-3* transcript. Notably, the subsequent localization of *pgl-3* mRNA to germ granules is perturbed in *emb-4* and *rnp-3* mutants and will be further elaborated on below.

### 4.1.2.2 *pgl-3* mRNA accumulates in the nuclei of wildtype animals at higher temperatures

Nuclear *pgl-3* foci were infrequently detected in wildtype animals at 20°C. Using probe sets targeting other transcripts, I often saw 1-2 bright foci in the nuclei of wildtype worms, likely reflecting the active transcription site (ATSs). Other studies utilizing the smFISH technique in *C. elegans* have also reported the detection of ATSs, though not for every target$^{261}$. The dynamics of *pgl-3* mRNA export, coupled with the nuclear positioning of the *pgl-3* locus, could contribute to the absence of visible *pgl-3* ATSs in wildtype worms. Furthermore, just as transcripts can be actively translated by more than one ribosome at a time, gene bodies can be processed by multiple polymerases at once, and differences in the transcriptional activity of a gene likely contribute to whether ATSs can be detected *in situ*. In *C. elegans* pachytene nuclei, DNA is positioned around the nuclear periphery in close proximity to the nuclear envelope. It is plausible that the *pgl-3* locus is positioned near NPCs, and newly synthesized *pgl-3* mRNAs may be immediately exported through nearby pores, resulting in very transient nuclear localization of the transcript.

Interestingly, nuclear *pgl-3* foci were detected in wildtype worms at 25°C. There are several possibilities that could contribute to nuclear accumulation of *pgl-3* transcript at higher temperatures. qRT-PCR data show that *pgl-3* mRNA levels increase about 30% at 25°C, possibly reflecting higher transcriptional activity of the *pgl-3* locus. Additionally, changes to germ granule structure may lead to defects in *pgl-3* mRNA export. Due to the liquid-like
properties of germ granules, higher temperatures begin to perturb their structure and consequently, germ granule localization of mRNA export factors (e.g. DDX-19) may be disrupted\textsuperscript{262}. Increased transcription of the \textit{pgl}-3 locus, coupled with defects in mRNA export as a result of structural changes to germ granules, could contribute to nuclear accumulation of \textit{pgl}-3 transcript. It is important to note however, that in several mutants, including some core structural P granule mutants (\textit{deps}-1 and \textit{glh}-1) I did not see nuclear \textit{pgl}-3 mRNA at 25°C. In most of these mutants qRT-PCT data show a depletion of \textit{pgl}-3 mRNA. It is possible that a reduction in the transcriptional activity of the \textit{pgl}-3 locus results in fewer \textit{pgl}-3 molecules to be exported.

Another possibility for the nuclear accumulation of \textit{pgl}-3 mRNA at higher temperatures could be the active nuclear retention of transcripts due to exposure to stress. In yeast, exposure to stress (including heat stress) leads to the retention of poly(A)$^+$ RNAs in the nucleus\textsuperscript{263}. Studies in yeast have identified several factors that lead to the nuclear retention of mRNAs. For example, following stress, certain mRNA processing factors relocalize from the nucleus to the cytoplasm, resulting in changes to mRNP composition\textsuperscript{264,265}. Furthermore, alternations in components of NPCs and changes in NPC-associated proteins have also been identified in heat-stressed yeast\textsuperscript{266,267}. The changes in mRNA metabolism during stress result in the preferential export of specific mRNAs and the translation of specific proteins that aid in the recovery following stress. The nuclear accumulation of \textit{pgl}-3 mRNA in wildtype worms at 25°C may be part of an overall cellular strategy to ensure proper recovery following stress. Simple experiments examining the localization of specific processing/export factors that have been shown previously to relocalize following heat stress in yeast, would reveal if the nuclear accumulation of \textit{pgl}-3 mRNA in wildtype worms 25°C is at least in part due to the active retention of mRNA as a response to stress.

4.1.2.3 Perturbations to the nuclear pore do not effect \textit{pgl}-3 export

Unexpectedly, I did not see nuclear accumulation of \textit{pgl}-3 mRNA following perturbations to the nuclear pore. Loss of 4 nuclear pore proteins from different substructures of the NPC did not affect export of \textit{pgl}-3. The nuclear basket is a prominent structure on the nucleoplasmic side of the NPC, and comprises protein filaments that protrude into the nucleus. The nuclear basket
plays several roles in the cell, including mRNA biogenesis and transport. Specific nuclear basket proteins have been implicated in mRNA export. For example, in yeast, Nup153 (worm NPP-7) is thought to anchor the nuclear basket-associated protein TPR (worm NPP-21), whose loss results in accumulation of poly(A)$^+$ mRNAs in the nucleus. Furthermore, vertebrate Nup153 contains an RNA-binding domain that preferentially interacts with mRNAs, suggesting that Nup153 may directly contact mRNAs during export. Interestingly, following RNAi of npp-7, I did not observe nuclear accumulation of pgl-3 mRNA. A mundane explanation for this result is that the RNAi knockdown of npp-7 was insufficient: qRT-PCR data show only a 30% reduction in npp-7 mRNA (although 100% sterility was observed). Alternatively, NPP-7 may be required for the export of a subset of transcripts. In vitro experiments showed that binding of RNA by vertebrate Nup153 partly depends on specific nucleotide distributions within the transcript. Interactions between motifs in an mRNA and nuclear pore proteins could influence what mRNAs are exported and could subsequently affect downstream mRNA fate.

Several nuclear pore proteins form a cytoplasmically-oriented subcomplex, shown to be required for both protein and mRNA transport. Nucleoporins on the cytoplasmic side can act as terminal binding sites for mRNA export complexes, and depletion of specific cytoplasmic nucleoporins results in defects in mRNA export. For instance, in Xenopus, the cytoplasmic nucleoporin Nup214 (worm NPP-14) interacts with the export factor DDX-19, and loss of Nup214 in mouse embryos leads to nuclear accumulation of poly(A)$^+$ mRNAs. I did not observe nuclear accumulation of pgl-3 transcript following loss of npp-14. Although the mRNA export pathway is highly conserved, it is possible that in worms NPP-14 may predominantly play a role in protein transport. Alternatively, NPP-14 could be involved in mRNP export in the worm; however, its role in export may not be essential. A large set of proteins function in mRNA processing/export, and some have redundant roles. For instance, in C. elegans Ref/Aly proteins function as adaptors to recruit the export receptor NXF-1 to mRNPs. However, studies in worm have shown that the Ref/Aly adaptor proteins are dispensable for mRNA export, suggesting that additional adaptors can recruit NXF1. Similarly, in npp-14 mutants other cytoplasmic filaments may compensate for NPP-14 in mRNA export.
4.1.3 Investigating the germ granule localization of pgl-3

4.1.3.1 pgl-3 mRNA levels do not correlate with perinuclear localization

Following the loss of several mRNA processing/export, germ granule, nuclear pore and small RNA pathway factors, there was a reduction in the number and size of perinuclear pgl-3 foci. I initially showed in wildtype animals that the majority of pgl-3 transcript at the nuclear periphery localized to P granules; therefore reduced levels of perinuclear pgl-3 transcript in mutants likely correlate with reduced localization to P granules. In parallel with smFISH experiments, I assessed pgl-3 transcript levels via qRT-PCR. I used primers spanning exon-exon junctions of pgl-3 thus only providing information regarding steady state levels of the transcript. Reduced perinuclear localization of transcript in mutants was sometimes coupled with a reduction in pgl-3 mRNA levels at 20°C and/or 25°C (e.g. emb-4, hel-1, rnp-3, pgl-1, npp-14). In these instances, possible decreases in transcriptional activity of the pgl-3 locus, subsequently leading to lower levels of pgl-3 export, could account for reductions in perinuclear localization of the transcript. An alternative possibility is that changes in post-transcriptional regulation lead to higher rates of pgl-3 mRNA turnover in the cytoplasm, resulting in less pgl-3 transcript in germ granules. Importantly, these proteins may also be directly required for the proper trafficking and localization of pgl-3 to germ granules, for example through interactions with nuclear pore or germ granule proteins.

In several mutants at 20°C and/or 25°C, no changes in mRNA levels of pgl-3 were observed despite changes to the perinuclear localization of the transcript (e.g. csr-1, ddx-19, glh-1, and npp-7). These proteins may be directly required for perinuclear pgl-3 mRNA localization, for example through interactions with certain nuclear pore or germ granule proteins during pgl-3 export from the nucleus. Another possibility is that loss of these proteins results in changes to nuclear pore or germ granule structure, consequently leading to changes in pgl-3 localization.

4.1.3.2 DEPS-1 is not required for perinuclear localization of pgl-3 at 20°C.

An interesting and unanticipated result was the wildtype localization of pgl-3 transcript in deps-1 null mutants at 20°C. Even more interesting, loss of pgl-1 and glh-1 resulted in reduced perinuclear localization of pgl-3 mRNA. DEPS-1 is a central component of the P granule
assembly pathway and is required for proper expression and localization of other structural P granule proteins, including PGL-1 and GLH-1. *deps-1* mutants show reduced accumulation of *glh-1* mRNA and reduced perinuclear localization of PGL-1 protein\(^\text{161}\). DEPS-1 is hypothesized to act upstream of GLH-1 and PGL-1 in the P granule nucleation pathway and therefore, I expected to see the most drastic changes in perinuclear *pgl-3* localization in *deps-1* mutants.

The changes to *pgl-3* mRNA localization in *glh-1* and *pgl-1* mutants, together with the wildtype localization in *deps-1* mutants, point to a role for specific germ granule proteins in *pgl-3* transcript localization. Both *pgl-1* and *glh-1* have RNA-binding domains and in addition to their structural roles, may play a more direct role in regulating RNA metabolism. For example, using a protein-RNA tethering assay, a recent study uploaded onto bioRxiv showed that tethering PGL-1 to an mRNA results in repression of the transcript. In this example, PGL-1 may be directly regulating mRNA expression through its slicer activity or PGL-1 may be recruiting other proteins that affect mRNA stability\(^272,273\). Furthermore, in *Drosophila*, the GLH-1 homolog Vasa forms a complex with PIWI type Argonaute proteins, piRNA transcripts, and other small RNA pathway components in the nuage, and is necessary for piRNA biogenesis\(^168\). In P granules, GLH-1 and/or PGL-1 could interact with Argonautes, like CSR-1, and their target transcripts, aiding in small RNA-mediated regulation. Another possible explanation for the reduction in perinuclear *pgl-3* mRNA in *pgl-1* and *glh-1* mutants is that these proteins are required for the recruitment of other RNA regulatory proteins to germ granules, which in turn interact with the *pgl-3* transcript. As an example, PGL-1 is necessary for the P granule localization of the PUF-domain RNA binding proteins FBF-1 and FBF-2 (*Drosophila* Pumilio homologs) and their silencing of mRNA transcripts\(^274\). Therefore, structural P granule proteins may facilitate mRNA localization through the recruitment of key RNA regulatory proteins to the nuclear pore, so they can bind their target transcripts upon exit from the nucleus.

The wildtype localization of *pgl-3* mRNA in *deps-1* mutants at 20°C suggests that DEPS-1 may not be the initiator of P granule nucleation. Several lines of evidence hint at a non-essential role for DEPS-1 in P granule assembly, particularly at 20°C. First, at lower temperatures, GLH-1 protein levels are reduced only 2 to 3-fold in *deps-1* mutants and it is not known whether the remaining GLH-1 protein localizes to perinuclear granules\(^161\). Therefore GLH-1 localization in
_deps-1_ mutants may still be perinuclear. Second, in _deps-1_ mutants there is only a partial loss of PGL-1 protein from the nuclear periphery, and the remaining perinuclear PGL-1 may be sufficient for transcript localization. Third, loss of _deps-1_ does not drastically affect mRNA levels in germ cells, suggesting that P granules in _deps-1_ mutants may be largely functional\textsuperscript{161}. Finally, both _in vitro_ and _in vivo_ experiments have shown that other germ granule proteins, namely the PGL family of proteins, can phase separate to form granules independent of DEPS-1 presence, and can recruit other proteins and mRNA to the granules\textsuperscript{275,276}.

In P granule mutants at 25°C, _pgl-3_ localization defects are exacerbated. Notably, in _deps-1_ mutants at higher temperatures there is a reduction in size of perinuclear _pgl-3_ foci compared to wildtype. Larger _pgl-3_ foci are likely the accumulation of multiple transcripts to germ granules, and their reduction in mutants suggests DEPS-1 may be required for _pgl-3_ accumulation in P granules at higher temperatures. Due to the effects of temperature on P granule structure, high temperatures coupled with the removal of structural proteins may produce more severe perturbations in P granule structure, leading to exacerbated _pgl-3_ localization changes. Interestingly, in _deps-1_ mutants at higher temperatures there is a more severe loss of GLH-1 protein compared to in _deps-1_ mutants at lower temperatures\textsuperscript{161}. This further decrease in GLH-1 levels could contribute to the _pgl-3_ localization defects I observed in _deps-1_ mutants at higher temperatures.

4.1.3.3 A functional CSR-1 pathway is required for perinuclear localization of _pgl-3_

Loss of two members of the CSR-1 small RNA pathway, CSR-1 and DRH-3, resulted in reduced levels of perinuclear _pgl-3_ mRNA. No CSR-1 protein is produced in the _csr-1_ mutant used in this study and a missense DRH-3 protein is made. Importantly, it is not known whether the mutated DRH-3 protein localizes to P granules. In wildtype animals CSR-1 and DRH-3 may play a direct or indirect role in _pgl-3_ localization. Both wildtype CSR-1 and DRH-3 localize to P granules, and likely interact with their targets, including _pgl-3_, within these perinuclear structures as mRNAs exit the nuclear pore. A complex consisting of the mRNA, the Argonaute and other small RNA pathway proteins can function to regulate the stability of the target mRNA and/or biogenesis of additional small RNAs using the mRNA as a template. Regulation of the
mRNA or small RNA biogenesis may occur within P granules, or the complex may be shuttled to the cytoplasmic core of the worm where regulation will occur.

In addition to a direct role for CSR-1 and DRH-3 in \( pgl-3 \) localization, these proteins might play an indirect role through their effects on germ granule structure. Several studies using mutants or RNAi have shown changes in P granule structure following perturbations to the CSR-1 pathway. Importantly, all studies have used PGL-1 as a marker for P granules. In wildtype animals, P granules associate with the nuclear periphery, and are fairly uniform in their shape and size.

RNAi of \( csr-1 \), \( drh-3 \) and \( ego-1 \) (the RdRP necessary for synthesis of CSR-1 22G RNAs) results in enlarged and disorganized P granules\(^{248} \). Similarly, worms harboring a mutation in the slicer region of CSR-1 also show enlarged and disorganized P granules\(^{95} \). Other studies using \( csr-1 \) null mutants show detachment of P granules from the nuclear periphery\(^{83} \). Therefore the changes to P granule structure and localization following loss of functional CSR-1 pathway proteins may contribute to the reductions in perinuclear \( pgl-3 \) that I see in CSR-1 and DRH-3 mutants.

4.1.3.4 Loss of splicing factors effects the cytoplasmic localization of \( pgl-3 \)

Studies across organisms have implicated mRNA processing/export factors in nuclear and cytoplasmic localization of transcripts. In \( Drosophila \), splicing factors influence both the localization of piRNA cluster transcripts to perinuclear granules as well as the proper assembly of granules\(^{28,29} \). A link between mRNA processing factors and germ granules has also been shown in \( C. elegans \): an RNAi screen looking at factors necessary for the perinuclear localization of PGL-1 identified several known or putative splicing factors\(^{248} \). Additionally, Sm proteins (core components of the spliceosome) localize to P granules\(^{148} \).

Together with the nuclear retention of \( pgl-3 \) mRNA following loss of the splicing and export factor \( hel-1 \), I observed a reduction in perinuclear \( pgl-3 \) levels suggesting that HEL-1 may be required for the proper localization of \( pgl-3 \) mRNA to germ granules. The fly homologue of HEL-1, UAP56, localizes to the nuclear face of the nuclear pore, and is found directly across the nuclear membrane from Vasa (fly homologue of GLH-1)\(^{28} \). In this position, UAP56 is hypothesized to hand off piRNA cluster transcripts to Vasa in the nuage, where they can undergo further processing. It is interesting to speculate what role, if any, HEL-1 plays in small
RNA regulation in *C. elegans*. Is HEL-1 directly involved in trafficking of target mRNAs to germ granules, for example through recruitment of specific proteins to the mRNP? Is HEL-1 enriched at targets of a particular small RNA pathway? Is HEL-1 required for proper localization of small RNA pathway components to P granules? Understanding both the relationship between HEL-1 and small RNA pathway proteins, and the role HEL-1 plays in the localization of small RNA pathway targets would provide valuable insight into whether processing factors contribute to transcript sorting between small RNA pathways in germ granules.

In addition to HEL-1, the two splicing factors EMB-4 and RNP-3 also appear to play a role in the perinuclear localization of *pgl-3*. Loss of *emb-4* and *rnp-3* resulted in reduced levels of *pgl-3* mRNA at the nuclear periphery, suggesting these splicing factors influence the trafficking and localization of *pgl-3* to germ granules. Our lab has previously shown that EMB-4 immunoprecipitates with DDX-19 and possibly recruits DDX-19 to mRNPs for their export into germ granules (Amy Nabih, Claycomb lab, unpublished). Notably, in *ddx-19* mutants, there is also a reduction in amount of *pgl-3* at the nuclear periphery. As discussed above with HEL-1, EMB-4 and RNP-3 may likewise play a role in the trafficking of targets to particular small RNA pathways. A previous study from our lab showed that EMB-4 interacts with CSR-1 and HRDE-1 target transcripts in distinct ways, and hypothesized that EMB-4 is part of a molecular signature that guides transcripts to their respective Argonaute regulators. My experiments focused on one target of a single small RNA pathway, however future experiments with other targets of the CSR-1 pathway as well as targets of other small RNA pathways would help elucidate the possible role for EMB-4 in mRNA sorting.

The mRNA localization defects in *emb-4* and *rnp-3* are seen strictly at higher temperatures. The conditional nature of this phenotype could be in part due to functional redundancies with other splicing proteins at 20°C. *emb-4* presumptive null mutants show a temperature-sensitive brood size defect, with lower brood sizes present at high temperatures, suggesting a critical role for this factor in fertility at higher temperatures. Another possibility for the temperature-dependent phenotype is that the overall perturbation to germ granule structure at higher temperatures may exacerbate a *pgl-3* localization defect that is very subtle at 20°C.
4.1.3.5  The nuclear and cytoplasmic faces of the nuclear pore are required for perinuclear *pgl-3* localization

The RNAi screen discussed above uncovered a small subset of nuclear pore proteins that are required for tethering PGL-1 to the nuclear periphery, including the nuclear basket protein NPP-7 (yeast Nup153). In this study I showed that RNAi of *npp-7* resulted in reduced levels of *pgl-3* transcript at the nuclear periphery. Interestingly, loss of a different nuclear basket protein, *npp-16* (yeast Nup50), did not affect *pgl-3* mRNA localization. Notably, *npp-16* is not required for the perinuclear localization of PGL-1. In yeast, Nup153 is more broadly required for nuclear basket assembly, and recruits Nup50 to the nuclear pore. Therefore, loss of *npp-7* in worm likely leads to a more severe defect in nuclear basket structure, and consequently has a greater effect on P granule structure. It is possible that the changes in *pgl-3* mRNA localization following RNAi specifically is a result of changes to germ granule structure. PGL-1 is necessary to recruit various proteins to P granules, and loss of PGL-1 from the nuclear periphery may lead to localization changes of numerous RNA metabolism proteins that bind to and regulate *pgl-3* mRNA.

The cytoplasmic face of the nuclear pore also appears to be required for proper *pgl-3* localization. Loss of *npp-14* (yeast Nup214), a cytoplasmic nucleoporin, resulted in lower levels of perinuclear *pgl-3* transcript. The cytoplasmic nups extend into P granules, and likely interact with numerous P granule proteins. RNAi of *npp-14* does not disrupt PGL-1 localization, but the localization of other P granule proteins may be affected in *npp-14* mutants, resulting in changes to *pgl-3* mRNA localization.

An interesting question to consider is what role nuclear pore proteins play in small RNA regulation. Genome-wide screens have identified specific nuclear pore proteins (*npp-1* and *npp-9*) as necessary for small RNA regulation; however, it is unclear if they do so directly, for example by interacting with small RNA machinery, or indirectly, through their effects on germ granule structure. My smFISH experiments showed that specific nuclear pore proteins are required for the proper perinuclear localization of *pgl-3*. Therefore, nuclear pore proteins may be implicated in small RNA regulation due to their effects on target localization.
4.1.4 Visualizing trafficking of the plg-3 transcript in real time

4.1.4.1 Alternative ways to visualize RNA trafficking live

To better understand the dynamics of mRNA transcription, export, and localization, I worked to build a system to visualize mRNA trafficking in real time. Previous studies in various cell types and organisms have utilized the MS2-stem-loop system to track RNA, and therefore I decided to try and implement this system in C. elegans. Following the insertion of 12 or 24 stem-loops into the 3'UTRs of CSR-1 target genes, I was not able to detect clear RNA foci. Furthermore, RIP experiments revealed MS2 not to be binding the 12X stem-loop cassette integrated into the 3'UTR of ubc-20. In this study, I used an MS2 protein that harbors a mutation in the stem-loop binding site that increases the affinity of MS2 for its stem-loops. This specific MS2 variant has never before been used together with the recently developed synonymous stem-loop cassette, and it is possible that they are incompatible. It would be worthwhile in future experiments to use a different MS2 variant.

Another possible explanation for the absence of detectable RNA foci in the transgenic strains is the inefficient dimerization rate exhibited by MS2. MS2 protein binds to stem-loops as a dimer, and previous studies have shown that the rate of MS2 dimerization in cells is low, resulting in high levels of background fluorescence\(^{252}\). A way to overcome this technical issue is to generate a worm strain that expresses a tandem dimer of MS2. A previous study showed that expressing MS2 as a dimer greatly reduces background fluorescence and increases sensitivity of RNA labeling\(^{252}\). The MS2::GFP strain I generated shows robust GFP expression throughout the germline, therefore if MS2 is binding stem-loops at low rates, it will be very difficult to detect over background GFP. Expressing MS2 as a dimer may result in a better signal-to-noise ratio.

In addition to using the MS2 system to target mRNA, recent studies have exploited the CRISPR-associated Cas13 nuclease to target and edit RNAs\(^{278,279}\). Studies have primarily focused on RNA editing by fusing Cas13 to various editing enzymes (e.g. ADAR proteins), and have successfully highlighted the RNA association of Cas13. Several groups have proposed the use of a catalytically inactive Cas13 (dCas13) for live imaging of mRNA by fusing dCas13 to a fluorescent marker, and providing gRNAs targeting an mRNA of interest\(^{278,280}\). I generated plasmids that contain a single copy of dCas13 from Prevotella sp. P5-125 (PspCas13b) fused to
GFP under the control of germline regulatory elements. I successfully integrated a single copy of this transgene into the genome of *C. elegans*, but I was unable to detect GFP under the microscope. The *C. elegans* germline is prone to silencing of foreign nucleic acid sequences through the piRNA pathway. The worm community has struggled in the past with expressing CRISPR-associated Cas9 protein in the germline. Recently, however, a group re-encoded the Cas9 gene, eliminating all predicted piRNA-targeting sites, and successfully expressed it in the germline\(^\text{281}\). By eliminating piRNA-targeting sites in dCas13 we might be successful in expressing it in the germline, and subsequently we may be able to use this system to visualize mRNA live as an alternative to the MS2 system.

4.1.4.2 Insertion of MS2 stem-loops into the 3'UTR disrupts gene expression

The integration of MS2 stem-loops into the 3’UTRs of endogenous genes led to changes in gene expression. Given the importance of the 3’UTR in post-transcriptional regulation, the disruptions to gene expression were not completely unexpected. Groups that have employed the MS2 live imaging system in other organisms often inserted stem-loops into transgenes to avoid disruptions in endogenous gene expression.

There are several possibilities that can be contributing to gene expression changes following stem-loop integration. The stem-loops may be recognized as foreign sequences in the germline, resulting in targeting by small RNA pathways. Alternatively, the stem-loops may disrupt the binding of various RNA binding proteins necessary for proper expression and localization of the mRNA. A previous study found that in the *C. elegans* germline 3'UTRs are sufficient for wildtype tissue-specific gene expression and therefore integrating stem-loops into the 3'UTR may be considerably disrupting gene expression\(^\text{282}\). Another possibility is that integration of stem-loops may cause the mRNAs to enter the nonsense-mediated decay (NMD) pathway. Studies in yeast and human cells have shown that mRNAs with aberrantly extended 3'UTRs, and even wildtype mRNAs with long 3'UTRs, are targeted by NMD\(^\text{283–285}\). *C. elegans* have short 3'UTRs relative to other organisms and inserting 12 stem-loops (~600bp) or 24 stem-loops (~1200bp) more than doubles the length of most wildtype 3'UTRs in the worm, and this insertion could target these transcripts for NMD. Finally, the position of the integration site along the 3'UTR, coupled with the secondary structure of stem-loops, may contribute to defects
in translation, particularly translation termination. When deciding on the insertion position of the stem-loops, I chose sites upstream of any putative poly(A) sites. Due to the short length of worm 3'UTRs, in most cases I inserted the stem-loops within 100bp of the stop codon. The close proximity of the stem-loops to the stop codon could interfere with translation.

The mRNA levels in transgenic stem-loop strains do not show a consistent trend in gene expression changes following stem-loop insertion. Insertion of stem-loops into mel-26 and car-1 resulted in a down regulation of mRNA, while insertion into ubc-20 led to a 50% increase in mRNA levels. Therefore, more than one of the possibilities discussed above could contribute to gene expression changes. There are several ways to test these possibilities: checking the presence of small RNAs antisense to the stem-loop cassette sequence (to test targeting by small RNA pathways), examining mRNA levels in NMD mutants (to test whether NMD causes mRNA turnover), inserting the stem-loops at positions closer to the 3' end of the 3'UTR (to test whether the location of stem loops is deleterious), and inserting sequences that do not form secondary structures (to test if the stem loop structure disrupts expression/stability). A better understanding of how the stem-loops interfere with gene expression could provide useful information about C. elegans 3'UTR biology.

4.2 Summary & Future Directions

4.2.1 Summary

Studies from a wide range of organisms have demonstrated a link between mRNA processing/export factors, and small RNA mediated regulation; however, the relationship between the two remains largely unexplored. In the C. elegans germline, several Argonaute proteins localize to perinuclear sites of small RNA regulation and target unique subsets of the transcriptome. An important question that arises is: how do transcripts get sorted into a specific small RNA pathway as they exit the nuclear pore and enter germ granules? We hypothesize that specific processing and export factors bind to mRNAs in the nucleus and aid in the trafficking of target transcripts to their respective small RNA pathways in germ granules. Furthermore, the compartmentalization of small RNA pathways within germ granules also likely plays a role in mRNA sorting. To begin to interrogate how transcripts are sorted, we first need to understand...
how transcripts are trafficked out of the nucleus, what cytoplasmic compartments they localize to, and what factors influence their cytoplasmic localization.

In this thesis I examined the trafficking and localization of a target of the CSR-1 pathway, *pgl-3*. Components of the CSR-1 pathway are enriched in P granules, including CSR-1 and the RdRP EGO-1. I determined that *pgl-3* mRNA localizes to P granules where it likely engages with members of the CSR-1 pathway. The routing of CSR-1 targets to P granules may facilitate the biogenesis of 22G-RNAs and consequently reinforce proper regulation of CSR-1 targets by the 22G-RNAs. Furthermore, in this study I uncovered a role for specific mRNA processing/export, nuclear pore, germ granule and small RNA pathway proteins in the export and subsequent cytoplasmic localization of *pgl-3* mRNA. We hypothesize that some of these proteins may be specific to the CSR-1 pathway and may mark CSR-1 targets for appropriate export into P granules. Future experiments examining the localization of other CSR-1 targets, in addition to targets of other small RNA pathways, will be important to understand how various RNA-binding proteins contribute to mRNA sorting.

4.2.2 Future Directions

4.2.2.1 Compartmentalization of small RNA pathways

We hypothesize that the compartmentalization of small RNA pathways within germ granules aids in the sorting of mRNAs. Of the 8 Argonautes that are enriched at perinuclear sites, 2 have been shown to localize to specific germ granules: CSR-1 localizes to P granules and WAGO-4 is enriched in Z granules. A current PhD student in our lab has utilized CRISPR-Cas9 technology to GFP-tag all the *C. elegans* Argonautes at their endogenous loci. Furthermore, Z granule and Mutator foci marker strains are readily available. Therefore, determining the germ granule-specific localization pattern of the remaining 6 perinuclear Argonautes is imminently feasible by crossing the GFP-tagged Argonaute strains with the RFP-tagged germ granule marker strains.

In addition to determining the localization of Argonaute proteins, we can do smFISH experiments to examine the localization of their target transcripts. We predict that transcripts will localize to the same perinuclear compartments as their Argonaute regulators. There are two
important caveats to these experiments. The targets of most germ granule Argonautes are very weakly expressed in the germline; therefore, it may be difficult to visualize their localization. Furthermore, due to the harsh conditions of the smFISH protocol, the fluorescent signal in most GFP-tagged germ granule marker strains does not withstand the protocol, resulting in a very faint GFP signal. There are a couple of experimental modifications that would help us address these caveats. First, as most Argonautes negatively regulate their targets, to increase mRNA levels we could assess mRNA localization in Argonaute mutants. Second, using CRISPR-Cas9 technology we could tag MUT-16 (a maker of Mutator foci) and ZNFX-1 (a Z granule marker) with mNeonGreen, a fluorescent protein approximately 5 times brighter than GFP. Using mNeonGreen will hopefully enable us to visualize mRNA localization relative to Mutator foci and Z granule using smFISH.

4.2.2.1 Identifying factors that ‘mark’ transcripts for regulation by specific small RNA pathways

When a transcript is exported out of the nucleus it is coated in various RBPs. We hypothesize that specific RBPs associated with transcripts during export and subsequent cytoplasmic localization can function as part of a ‘code’ to route transcripts into their respective small RNA pathways within germ granules. We can test this hypothesis by examining the localization of small RNA pathway targets in situ in wildtype animals and assess how the localization changes following loss of certain RBPs. In this thesis, I utilized the smFISH technique to identify factors that are required for the perinuclear localization of a CSR-1 target. Expanding the smFISH experiments to targets of other small RNA pathway could uncover potential differences between pathways, and may identify proteins that influence the sorting of transcripts into specific small RNA pathways.

Importantly, there is an extensive list of mRNA processing/export, nuclear pore and germ granule factors that may potentially play a role in the trafficking and localization of transcripts to their sites of small RNA regulation. Due to the labor-intensive nature of the smFISH technique, in this study I tested only a small subset of possible factors. To narrow down the list of proteins that may contribute to transcript sorting, we could perform BioID experiments to determine the protein interactors of Argonautes (or other small RNA pathway components). We
suspect that many of the interactions between Argonautes and proteins associated with newly exported mRNAs are transient, making the BioID technique particularly suitable. Once we have identified candidate factors for each small RNA pathway, we would subsequently test how loss of these factors affects transcript localization using smFISH.

4.2.2.2 Visualizing the export and localization of mRNA in real time

smFISH experiments provide only a snapshot of mRNA export and localization. Visualizing mRNA live could provide valuable insight into the reasons for both export and localization changes of mRNA in the various mutants examined in this thesis. For example, in wildtype animals we hypothesize that targets of small RNA pathways are trafficked through germ granule-associated nuclear pores, resulting in the perinuclear accumulation of transcripts in germ granules. In several mutants examined in this study, there is reduction in the amount of transcript localizing to the nuclear periphery. The reduction in transcript could be due to increased export of mRNA through non-germ granule-associated nuclear pores. Alternatively, mRNA may be trafficked through germ granule-associated nuclear pores, but loss of certain RBPs may disrupt the retention of transcripts in germ granules.

In this study, I worked to establish an mRNA live imagining protocol in the worm using the MS2 system. I was not able to visualize mRNA following integration of 12 or 24 stem-loops into the 3'UTR of 6 CSR-1 targets. There are several steps we could take to help us tackle this problem. Due to the possible incompatibility between the MS2 variant used and the synonymous stem-loop cassette, we could use an alternative MS2 variant. Furthermore, we could express MS2 as a dimer to increase its stem-loop binding efficiency. To avoid disrupting endogenous gene expression, we can insert stem-loops into a transgene, as is commonly done in other organisms. Finally, the dCas13 system can be used as an alternate approach to the MS2 stem-loop system to visualize RNA live.
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