UNIVERSITY OF CALIFORNIA SAN DIEGO

Family Favorites: Specific Roles of microRNA Sisters in *Caenorhabditis elegans* Aging

A dissertation submitted in partial satisfaction of the
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by

Laura Chipman

Committee in charge:

Professor Amy Pasquinelli, Chair
Professor Susan Ackerman
Professor Sreekanth Chalasani
Professor Jens Lykke-Andersen
Professor Miles Wilkinson

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The dissertation of Laura Chipman is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

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DEDICATION

To everyone who has supported me, big and small, thank you. I've been incredibly lucky and wouldn't be here without you.

EPIGRAPH

"If we knew what we were doing, it would not be called research. Would it?"
A. Einstein

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VITA

2014	Bachelor of Science in Biochemistry, Minor in Classical Studies University of Washington, Seattle, WA
2017- 2020	Graduate Research Program Fellow – National Science Foundation
2017-2020	Biological Sciences Graduate Student Representative – Graduate & Professional Student Association
2018- 2021	San Diego ARCS Foundation – ARCS Scholar
2020-2021	VP of Campus Affairs – Graduate & Professional Student Association
2021-2022	David V. Goeddel Endowed Graduate Fellowship – Division of Biological Sciences
2022	Doctor of Philosophy in Biology University of California San Diego

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ABSTRACT OF THE DISSERTATION

Family Favorites: Specific Roles of microRNA Sisters in *Caenorhabditis elegans* Aging

by

Laura Chipman

Doctor of Philosophy in Biology

University of California San Diego, 2022

Professor Amy Pasquinelli, Chair

MicroRNAs (miRNA) are critical regulators of many different biological processes, including cellular differentiation, development, response to stress, and aging. These small (~22 nucleotides) RNA molecules, post-transcriptionally regulate their targets in a sequence dependent manner but to exert their regulatory influence, a miRNA must be bound by an Argonaute (AGO) protein. A critical factor in miRNA targeting is base-pairing of nucleotides 2-7 of the miRNA, the seed, to the target RNA. Due to the importance of the seed sequence in

targeting, miRNAs who share their seed sequence are grouped into families. Yet, in Chapter 2, I review recent studies on non-canonical miRNA regulation, including miRNA targeting that is not seed dependent, such as the role of the miRNA's 3' end in targeting as well as miRNA overall expression. Thus, despite the importance of miRNAs, predicting the targets and biological roles is very challenging.

This outstanding problem in determining miRNAs targets and function, is especially acute for miRNAs that belong to the same family. MiRNAs in the same family, because they share a seed sequence, are often presumed to be redundant. As reviewed in Chapter 4, I explore how the miR-238, miR-239a, and miR-239b family regulate aging in *C. elegans*. I find that these miRNAs have distinct roles in aging, that loss of miR-238 leads to a reduced lifespan and many genes misergulated in adult, while loss of either miR-239a or miR-239b has no effect lifespan and leads to the misregultion of only a handful of non-overlapping genes. Interestingly, miR-239a or miR-239b can rescue the loss of miR-238 when they expressed from the miR-238 genetic locus suggesting that expression, not sequence, underlies the differential roles of this miRNA family in *C. elegans* aging.

In Chapter 3, I investigated how individual miRNAs contribute to the divergent roles of the somatic *C. elegans* miRNA AGOs, AGO-like-gene (ALG)-1 and ALG-2, in aging. I immunoprecipitated miRNAs bound by ALG-1 and ALG-2 and analyze miRNA misregulation upon the loss of these AGOs. Some of the miRNAs I identified as contributing to these phenotypes are already known regulators of aging while others may be novel aging regulators.

CHAPTER 1: Introduction

1.1 Discovery and importance of miRNAs

Around 1% of the human genome contains sequences that code for proteins (Harrow et al. 2009). Since proteins are considered the main workhorse of the cell, initially, the other 99% of genome was termed "junk DNA" as it produced no functional protein product (Ohno 1972). Rapidly though, it was found that this "junk" was a treasure trove. Many loci were in fact transcribed into RNA and determined to have vital biological functions despite producing no protein product. These non-coding genes were shown to have roles in basic cellular function, like translation, cellular differentiation, metabolism, immunity and more. Some participated directly in these processes, like ribosomal RNA (rRNA), which is a key component of the ribosome and, thus, critical to translation, while some regulated the expression of other genes. The vitality of these non-coding genes is underscored by the positive correlation between developmental complexity in higher eukaryotes and increasing amount of DNA attributed to non-coding RNA (G. Liu, Mattick, and Taft 2013). The spike in research into the role of these non-coding entities and the discoveries showing their essential functions in biology has been termed the RNA revolution (Cech and Steitz 2014; Amy E. Pasquinelli 2015).

A small but mighty member of this revolution is the microRNA (miRNA) (Amy E. Pasquinelli 2015). MiRNAs are ~22 nucleotide RNAs that post-transcriptionally repress target RNAs in a sequence dependent manner (Jonas and Izaurralde 2015; Bartel 2018). The first two miRNAs discovered, lin-4 and let-7, were found to control *Caenorhabditis elegans* developmental timing (R. C. Lee, Feinbaum, and Ambros 1993; Wightman, Ha, and Ruvkun 1993; Reinhart et al. 2000). Lin-4 and let-7 are required for proper transition from the first larval stage to the second, and for the transition from the fourth to last larval stage to adulthood,

respectively (R. C. Lee, Feinbaum, and Ambros 1993; Wightman, Ha, and Ruvkun 1993; Reinhart et al. 2000). To underscore its importance in *C. elegans*, the complete depletion of let-7 leads to a misregulation of the *lin-41* protein coding gene and, subsequently, a lethal vulval bursting phenotype (Reinhart et al. 2000; Slack et al. 2000).

After the initial studies characterizing these miRNAs, it was shown that let-7 was perfectly conserved in many bilaterian animals from *C. elegans* through humans (A E Pasquinelli et al. 2000). As well, targets of let-7 were conserved in addition to its function, in humans and in *C. elegans* the let-7 miRNA been shown to promote cellular differentiation (Slack et al. 2000; Johnson et al. 2005; Kanamoto et al. 2006). Given this role in promoting cellular differentiation, it is not surprising that let-7 has been characterized as a tumor-suppressor; in many cancers let-7 is down-regulated contributing to increased proliferation, a hallmark of cancer cells (Boyerinas et al. 2010). In addition to its role in cancer, misregulation of let-7 has been linked to cardiovascular and neurodegenerative diseases (Bernstein, Jiang, and Rom 2021). Because of these roles, let-7 is now being researched as a potential therapeutic target (Gilles and Slack 2018).

Today, there are over 2,600 mature miRNAs documented in humans and 437 in *C. elegans* (miRbase Release 22). While many miRNAs have been shown to have a role in development and cellular differentiation like lin-4 and let-7, miRNAs have also been implicated in many other processes. In *C. elegans*, miR-80 and the miR-229/miR-64 cluster promote survival during heat shock while miR-85 has been shown to regulate proper recovery from heat stress (Nehammer et al. 2015; Pagliuso, Bodas, and Pasquinelli 2021). Other *C. elegans* miRNAs, such as miR-355 promote survival upon *P. aeruginosa* infection (Zhi et al. 2017). Still there are many miRNAs with no annotated function (Alvarez-Saavedra and Horvitz 2010). Since

miRNAs are post-transcriptional gene regulators, their function is most likely linked to their direct targets and the pathways they regulate. With miRNAs being implicated in more and more biological pathways, understanding the methodology and mechanism of their regulation is crucial.

1.2 miRNA function in *C. elegans*

Most miRNAs in metazoan animals are transcribed by RNA polymerase II (Pol II) as part of a longer RNA called the primary miRNA (pri-miRNA) (Figure 1.1) (Bartel 2018). Each pri-miRNA will have a region that forms a stem-loop structure that is recognized by the heterotrimeric Microprocessor complex for further processing. The microprocessor complex is composed of an endonuclease, Drosha (DRSH-1 in *C. elegans*), which cuts each strand of the pri-miRNA hairpin with a 2 nucleotide (nt) offset, and two molecules of DGCR8/Pasha (PASH-1 in *C. elegans*), which provide fidelity to the processing (Nguyen et al. 2015). The ~60 nt stem-loop processed from the pri-miRNA is called the precursor miRNA (pre-miRNA) and is subsequently exported to the cytoplasm. After export, the pre-miRNA is further processed by the endonuclease Dicer (DCR-1 in *C. elegans*). Dicer cuts both strands of the pre-miRNA by the loop generating two ~22 nt miRNAs (Ha and Kim 2014). Of this miRNA duplex, one miRNA will be degraded and is termed the passenger or star strand. The other strand is called the guide strand and will be loaded into an Argonuate (AGO) protein. Strand selection can happen from either the 5' side of pre-miRNA (5p) or the 3' side (3p) but in most cases is largely biased to one end (Schwarz et al. 2003). Once loaded into an AGO protein, a miRNA can now interact with its targets.

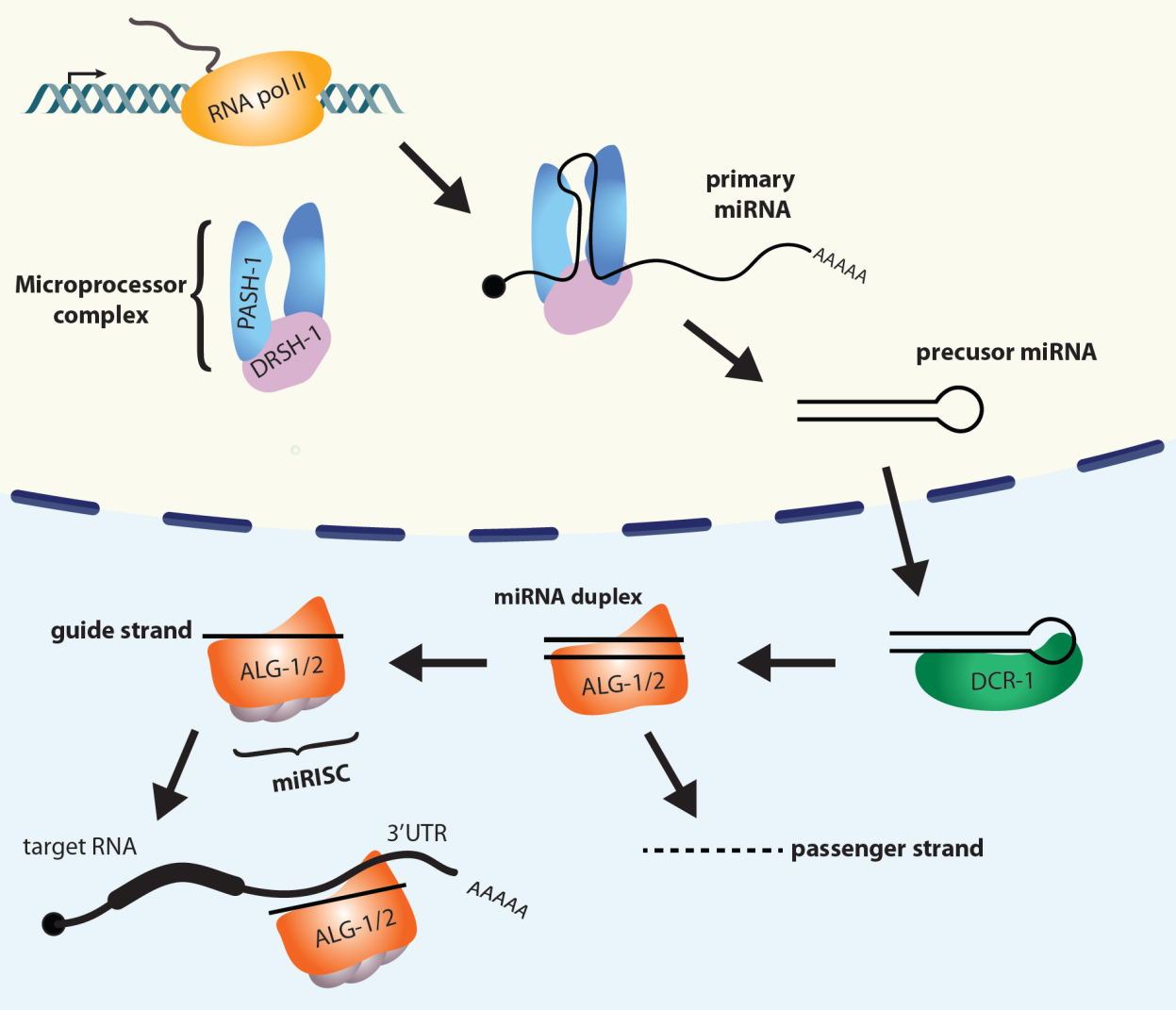


Figure 1.1: Overview of miRNA biogenesis.

AGOs are the companion proteins to various classes of small RNAs and necessary for their function. Made up of 4 protein domains: N-termini, Piwi-Argonaute-Zwille (PAZ), MID, and PIWI, AGO proteins cradle the guide and target nucleic acid molecules where the 5' phosphate of the guide interacts with the MID and PIWI domains while the PAZ binds the 3' end of the small RNA guide (Sheu-Gruttadauria and MacRae 2017). The PIWI domain also contains homology to RNase H enzymes and some AGOs can perform endonucleolytic cleavage of the target bound by the small RNA; this is referred to as slicer activity (J. Liu et al. 2004; Meister et

al. 2004). While humans have eight AGOs, *C. elegans* have over 25 AGOs (Höck and Meister 2008; Youngman and Claycomb 2014). This high number of AGOs is likely due to an explosion and sub-specialization of various small RNA pathways in *C. elegans*.

Of those many AGOs in *C. elegans*, there are three miRNA-associated AGO proteins, Argonaute-like-gene (ALG)-1, ALG-2 and ALG-5. ALG-1 and ALG-2 are broadly expressed AGO proteins that interact with most expressed miRNAs. Meanwhile ALG-5 has germline specific expression and binds a limited number of miRNAs (Brown 2017). While a loss of expression of ALG-1 leads to a developmental delay and global misregulation of miRNA biogenesis and target levels, loss of expression of ALG-2 leads to normal larval development (Grishok et al. 2001; Vasquez-Rifo et al. 2012; Tops, Plasterk, and Ketting 2006; Zinovyeva et al. 2015; Brown et al. 2017). Though *alg-2* is unable to compensate fully for *alg-1* during development, embryonic lethality is only observed upon the loss of *alg-1* and *alg-2* (Vasquez-Rifo et al. 2012; Grishok et al. 2001). Thus ALG-1 and ALG-2 are considered the primary miRNA AGOs in *C. elegans*.

Together, miRNAs and AGOs form the core of the miRNA induced silencing complex (miRISC). The guide strand miRNA directs the miRISC through imperfect base-pairing interactions to the target RNA (Bartel 2018). Once bound, miRISC co-factors such as ALG-1 interacting protein (AIN)-1 and AIN-2 direct the inhibition of translation and/or the destabilization of the target RNA. Several groups have shown evidence that translational inhibition proceeds target destabilization (Bazzini, Lee, and Giraldez 2012; Kobayashi and Singer 2022), but multiple studies show that destabilization of target RNA is the dominant outcome of miRNA regulation in somatic tissues (Eichhorn et al. 2014; Subtelny et al. 2014; Djuranovic, Nahvi, and Green 2012).

1.3 Rules of miRNA targeting

Canonically, miRNAs target transcripts for repression by imperfectly base pairing to the target RNA 3' untranslated region (UTR). Early on, it was found that sequences on the 5' end of the miRNA were important for miRNA-target interactions. The seed sequence, nucleotides (nts) 2-7 of the miRNA, was found to be both necessary and sufficient in some cases for proper miRNA-target regulation (Figure 1.2) (Bartel 2018). This is supported by structural studies that have shown the seed of AGO-bound miRNAs is available for base-pairing (Elkayam et al. 2012; Nakanishi et al. 2012; Schirle and MacRae 2012; Schirle, Sheu-Gruttadauria, and MacRae 2014; Salomon et al. 2015). Given the dominance of the seed region in determining functional miRNA targeting, we group together miRNAs that share the seed sequence, but differ to varying degrees in the other sequences, into families and call miRNAs that share their seed sequence sisters.

It is no surprise, then, that many of the miRNA target prediction algorithms heavily rely on seed sequence to predict targets. Yet, recent work to identify *in vivo* miRNA sites show that many of these canonical rules act more as guides lines. Studies using cross-linking and immunoprecipitation with sequencing (CLIP-seq) have shown that ~50% of *in vivo* miRNA targets do not contain a perfect seed site (Broughton et al. 2016). Furthermore, recent studies have shown that the 3' region of a miRNA is a large determinant in targeting, as it can lend target specificity and lead to functional miRNA-target RNA interactions especially in cases where the seed match is not perfect.

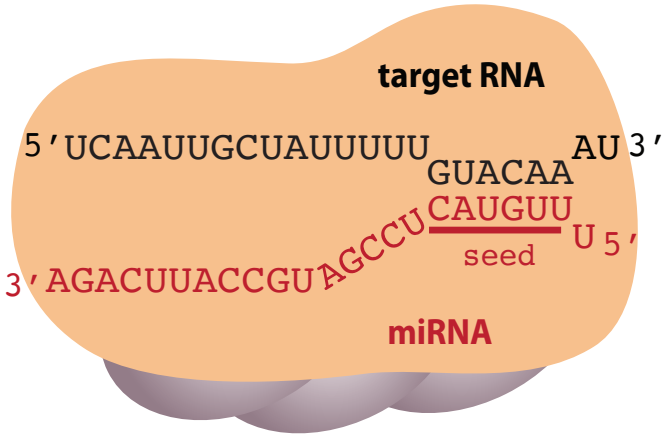


Figure 1.2: Diagram showing a perfect seed match between a miRNA (red) and its target RNA (black). The seed sequence, underlined, is nucleotides 2-7 on the 5' end of the miRNA. AGO protein in orange, other miRISC associated proteins in gray.

The importance of sequences beyond the seed in determining targets was shown in *C. elegans* where extensive 3' complementarity and a partial seed match between let-7 and *lin-41* leads to functional repression whereas let-7's miRNA sisters, who have similar expression patterns and share the seed sequence but differ in their 3' sequence, could not (Broughton et al. 2016; Brancati and Großhans 2018). As well, non-templated additions to the 3' end of a miRNA can add to its target repertoire. Uridylation of miR-27a in HEK cells leads to repression of non-canonical targets (partial seed matches) due to increased base pairing of the 3' end of the miRNA and the non-templated U to its target RNA (A. Yang et al. 2019). There have even been examples of miRNA recognition sites that are almost "seedless" yet have extensive base pairing between the miRNA's 3' end and its target. Interestingly, one such documented example, miR-20a, binds to its target RNA *DAPK* in its coding sequence (Zhang et al. 2018). I further explore the role of the 3' half of the miRNA and other non-canonical miRNA targeting examples in

Chapter 2 “miRNA Targeting: Growing beyond the Seed,” which is a re-print of a review I published.

While miRNA sequence determines its targets, its spatiotemporal expression and over all expression levels determine its availability for incorporation into miRISC. The first large scale effort to catalog spatiotemporal expression of miRNAs used miRNA promoters fused to GFP and found that less the 5% of miRNAs assayed were ubiquitously expressed (Martinez et al. 2008). Specific expression determines function, such as the neuronally expressed miRNA, lys-6. Despite being expressed at a low level globally, the specific expression of lys-6 in the left taste receptor neuron, ASE, leads to repression of *cog-1* and functionally differentiates it from the right ASE (Johnston and Hobert 2003). Also, miRNA abundance can affect the ability to regulate a potential target. It was demonstrated that overexpression of a miRNA can lead to repression of targets that have imperfect seed matches (Brancati and Großhans 2018).

These and other works show that while the seed sequence is indeed important for miRNA targeting, there are many nuances to targeting and regulation. This research has implications for how we determine a miRNA’s target and function, especially for when we study miRNA sisters.

1.4 miRNAs in Aging

Aging is a complex process that is characterized by the progressive loss of physiological integrity (López-Otín et al. 2013). In humans, aging is the primary risk factor for many diseases, including cancer, neurodegenerative diseases, and cardiovascular diseases. Research into the molecular, cellular, and genetic underpinnings of aging took off forty years ago when the first long lived *C. elegans* mutants were discovered (Klass 1983). Further study of these alleles of *age-1*, along with work on a loss-of-function (LOF) allele of *daf-2* in *C. elegans,* which also

extends lifespan, led to the identification of the first established aging pathway: the insulin/insulin-like growth factor-1 (IFG-1) signaling (IIS) pathway (Kenyon et al. 1993; Friedman and Johnson 1988). Ever since, *C. elegans* have been an important tool in understanding aging.

Unsurprisingly given the ubiquitous nature of miRNAs, the miRNA pathway has been implicated in the regulation of aging and linked to human aging related diseases (Jung and Suh 2012). In *C. elegans*, there are several cases that show losing a key component of the miRNA pathway leads to a reduced lifespan; loss of Pasha/DGCR6 which is required for miRNA maturation and processing, leads to a shortened lifespan phenotype (Lehrbach et al. 2012). Similarly, combined loss of *alg-1* and *alg-2* in adulthood by RNAi results in decreased lifespan phenotypes (Samuelson, Carr, and Ruvkun 2007; Kato et al. 2011).

As well, multiple miRNAs have been implicated in regulating normal aging (Kinser and Pincus 2020). Interestingly, individual miRNAs have been shown to both positively (lin-4, miR-71, miR-246, miR-238) and negatively (miR-34, miR-80, miR-83, miR-239a/b) regulate aging (Figure 1.3) (Boehm and Slack 2006; Boulias and Horvitz 2012; De Lencastre et al. 2010; Pincus, Smith-Vikos, and Slack 2011; Thalyana Smith-Vikos et al. 2014; J. Yang et al. 2013; Zhou et al. 2019). For some of these cases, this has been tied to the regulation of a specific target RNA. In the case of *lin-4*, that lifespan reduction upon loss of *lin-4* is due to the subsequent up-regulation of its target *lin-14* (Boehm and Slack 2006). For other miRNAs, they've been implicated in acting in aging pathways via genetic methodologies. The loss of miR-71 has been shown to shorten the extended *daf-2* lifespan, implicating it as required for the daf-2 extended lifespan and being involved with the IIS pathway (Boulias and Horvitz 2012).

Interestingly, many miRNAs that have aging phenotypes are often also implicated in other stress pathways (Kenyon 2005). Take for example the miR-238, miR-239a, miR-239b family which has been reported to regulate aging. Previous work by De Lencastre *et al*. showed that LOF of miR-238 leads to a reduced lifespan, while LOF of miR-239a/b leads to an extended lifespan (De Lencastre et al. 2010). In line with these lifespan results, loss of miR-239a/b led to increased survival during heat stress and oxidative stress while loss of miR-238 led to decreased survival during oxidative stress (De Lencastre et al. 2010; Nehammer et al. 2015). While phenotypic results often correlate across stresses, that is not always the case; loss of miR-238, while leading to an extended lifespan, has no effect on heat shock survival (De Lencastre et al. 2010; Nehammer et al. 2015).

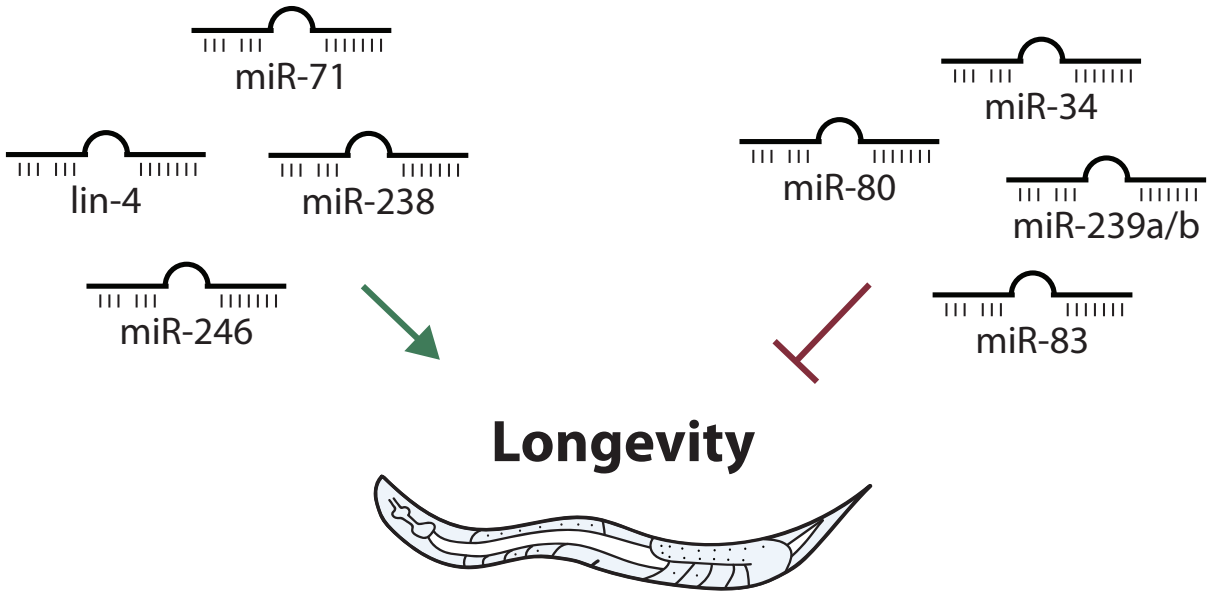


Figure 1.3: MiRNAs have previously been implicated in positively and negatively regulating *C. elegans* longevity.

The case of miR-238, miR-239a, and miR-239b is interesting not only for their differing phenotypes but because they all share the same seed sequence and belong to a miRNA family. Previous work showed that loss of miR-238 results in an reduced lifespan, while the deletion of both miR-239a, miR-239b and their intergenic space leads to an extended lifespan (De Lencastre et al. 2010). Using more precise tools, I reevaluated the role of miR-238/239a/239b family in aging and heat stress in Chapter 4. Surprisingly, I found that precise mutations of miR-239a and miR-239b do not exhibit the same phenotypes as previously reported and find that expression levels of these miRNAs greatly impact their role in aging. This work contributes to understanding how miRNA families, despite sharing a seed sequence, can play different roles in complex processes, like aging, and how expression is a large determinant of miRNA function.

CHAPTER 2: miRNA Targeting: Growing beyond the Seed

2.1 Highlights

- While canonical miRNA targeting involves pairing of the miRNA seed, nucleotides 2–7 of the miRNA, to target 3' UTR sequences, recent studies have revealed roles for miRNA sequences beyond this region in specifying target recognition and regulation.
- Auxiliary base pairing to sequences in the 3' half of the miRNA can overcome seed imperfections and confer specificity for individual members of a miRNA family that share identical seed sequences.
- Base pairing of 3'-end miRNA sequences enables targeting of protein-coding sequences that lack canonical seed-pairing interactions.
- Extensive pairing interactions between a miRNA and its target can lead to target-directed miRNA degradation.

2.2 Abstract

miRNAs are small RNAs that guide Argonaute proteins to specific target mRNAs to repress their translation and stability. Canonically, miRNA targeting is reliant on base pairing of the seed region, nucleotides 2-7, of the miRNA to sites in mRNA 3' untranslated region. Recently, the 3' half of the miRNA has gained attention for newly appreciated roles in regulating target specificity and regulation. Additionally, the extent of pairing to the miRNA 3'-end can influence the stability of the miRNA itself. These findings highlight the importance of sequences beyond the seed in controlling the function and existence of miRNAs.

2.3 Target Recognition and Regulation by miRNAs

Since their discovery in the early 1990s (R. C. Lee, Feinbaum, and Ambros 1993; Wightman, Ha, and Ruvkun 1993), thousands of microRNAs miRNAs have been identified across the plant and animal kingdoms (Kozomara, Birgaoanu, and Griffiths-Jones 2018). There is now evidence that miRNAs impact every major biological pathway by regulating the expression of substantial fractions of protein-coding genes (Bartel 2018; H. Liu et al. 2018). Given this omnipresent role in gene regulation, it is not surprising that misregulation of individual miRNAs can have dire consequences, contributing to a variety of diseases and afflictions in humans (Paul et al. 2018).

Within the miRNA induced silencing complex (**miRISC**) (see Glossary), the ~22 nucleotide (nt) miRNA recruits Argonaute (AGO) to specific target sites via base-pairing interactions (Bartel 2018; Gebert and MacRae 2018). Perfect base-pairing of the miRNA with its target site, which is common in plants but rare in animals, results in endonucleolytic cleavage by AGO of the target RNA. Animal miRNAs typically form a partial duplexes with their target site, which prevents cleavage and instead relies on AGO cofactors to regulate target expression through translational repression and mRNA destabilization (Bartel 2018; Gebert and MacRae 2018). Pairing of nucleotides 2-7 of the miRNA, called the **seed**, to its target site has generally been considered the minimal element needed to engage a target mRNA (Bartel 2018). Indeed, structural studies have shown that only sequences within the seed of the AGO-bound miRNA are available for initial pairing to a target site (Elkayam et al. 2012; Nakanishi et al. 2012; Schirle and MacRae 2012). In addition, single molecule studies have demonstrated the importance of the seed in stable target site engagement (Chandradoss et al. 2015; Jo et al. 2015; Salomon et al. 2015). Interestingly, once miRISC binds target RNA, AGO can undergo a conformational

change that allows for extended seed pairing and exposes part of the miRNA 3' region (nucleotides 13–16) for additional interactions with the target (Schirle, Sheu-Gruttadauria, and MacRae 2014). A recent systematic investigation of pairing interactions between a miRNA and target corroborates a sequential recognition model where the miRNA seed binds first and then nucleotides in the 3' half are able to bind the target site (Yan et al. 2018). The relevance of pairing to miRNA 3'-end sequences has been demonstrated in several new studies showing that it can impact the specificity of targeting, the regulatory mechanism, and the stability of the miRNA itself.

2.4 Same Seed, Different Target

Given its importance in many established miRNA-target interactions, seed-pairing potential is the foundation of popular target prediction programs (Bartel 2018). As such, members of a **miRNA family** that share seed sequences are typically assigned to the same target sites. However, there is mounting *in vivo* evidence that pairing interactions beyond the seed can lead to non-overlapping target profiles for individual miRNAs in a family. On a genome-wide scale, **crosslinking and immunoprecipitation with sequencing (CLIP-seq)** methods have been used to identify endogenous AGO-bound target sites. In some of these studies, the target sequence became ligated to the presumptive targeting miRNA, producing miRNA-target **chimeric reads** (Helwak et al. 2013; Grosswendt et al. 2014; Moore et al. 2015; Broughton et al. 2016). These types of sequencing reads allow for the unambiguous identification of which specific miRNA recruited AGO to a particular target site. Many chimeras contained target sequences that could pair to the seed of the attached miRNA, reaffirming the prevalence of this pairing motif in endogenous miRNA-target interactions (Helwak et al. 2013; Grosswendt et al.

2014; Moore et al. 2015; Broughton et al. 2016). Contrary to expectation, miRNAs within a family did not always produce chimeras with the same target sites, suggesting that sequences beyond the seed can contribute to target recognition *in vivo* (Moore et al. 2015; Broughton et al. 2016).

The examination of miRNA-target chimeras generated from CLIP-based studies in human cell culture, mouse brain, and whole larval-stage *Caenorhabiditis elegans* revealed that individual miRNA family members, often called **sisters**, can exhibit biased target interactions (Moore et al. 2015; Broughton et al. 2016). With identical seed sequences, miRNA sisters apparently rely on their potential for unique 3'-end pairing interactions to engage some targets. For sister-specific target sites in both the mammalian and worm studies, the chimera-forming miRNA had a more favorable predicted binding affinity than that of its sisters (Moore et al. 2015; Broughton et al. 2016). Consistent with non-identical target preferences, sister miRNAs were also shown to differ in their regulatory capacity for targets with 3'-pairing interactions that favored one of the family members. In mammalian cells, reporters with sister-specific target sites were generally repressed more potently by the chimera-forming miRNA versus other family members upon transfection into the culture system (Moore et al. 2015).

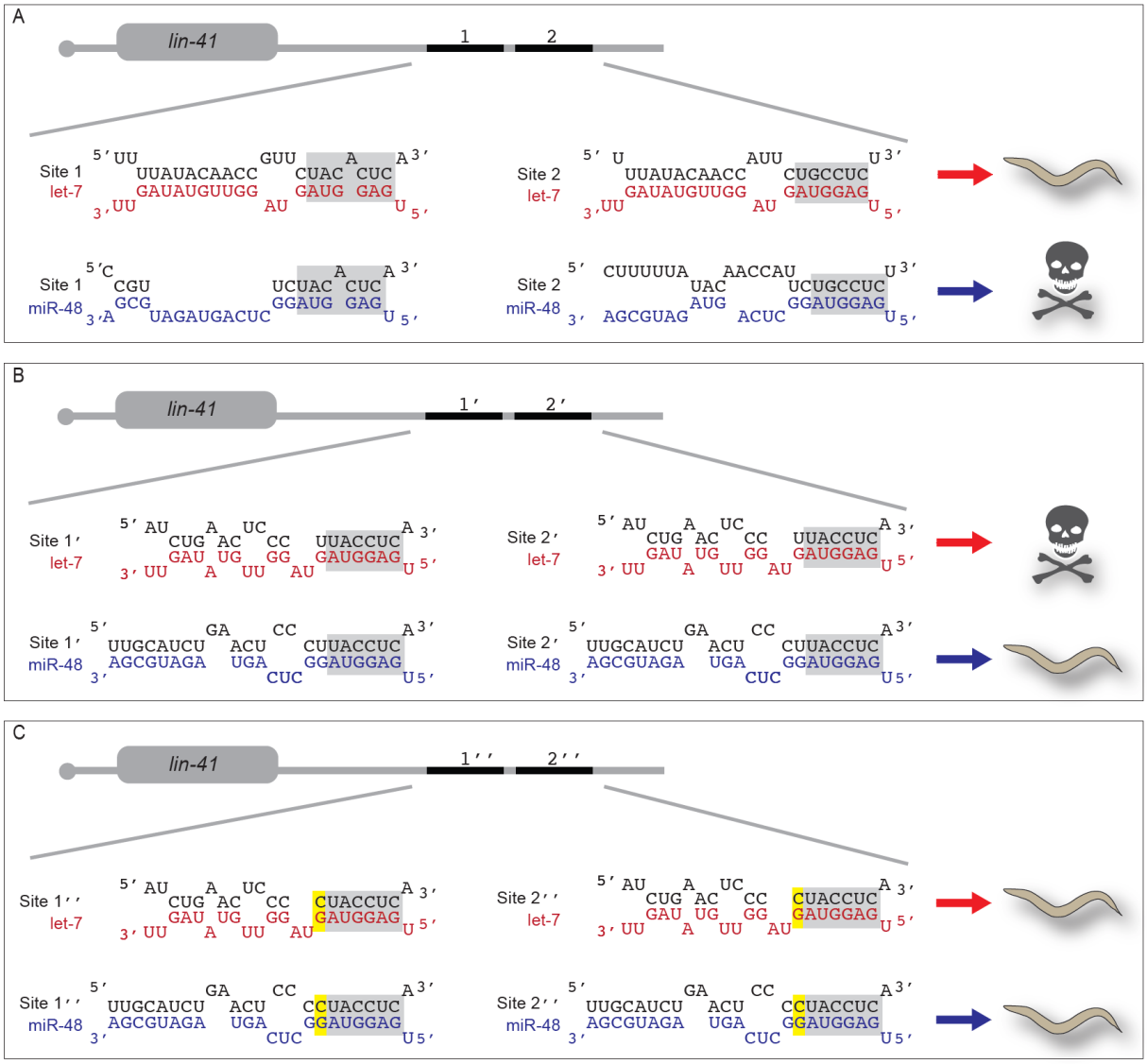
Taking advantage of a well-established let-7 miRNA target in *C. elegans,* Broughton *et al*. further demonstrated the importance of 3'-end pairing interactions for specific and functional targeting *in vivo* (Broughton et al. 2016). The *lin-41* 3'UTR contains two let-7 target sites that lack perfect seed pairing (one site involves a G-U pair and the other site forces a target nucleotide bulge) but support perfect pairing to nucleotides 11-19 of the let-7 miRNA (Figure 2.1A) (Reinhart et al. 2000; Slack et al. 2000). Loss of *let-7* results in misregulation of *lin-41* and lethality, despite the expression of sister miRNAs that apparently cannot compensate (Reinhart et

al. 2000; Slack et al. 2000; Broughton et al. 2016). The wild-type *lin-41* 3'UTR only formed chimeras with let-7 miRNA, but this specificity was found to be transferrable when 3'-end pairing was designed to favor a sister (Broughton et al. 2016). **CRISPR/Cas9**-based genome editing was used to swap each let-7 target site for a site that had formed chimeras exclusively with its sister miRNA, miR-48. Importantly, these new *lin-41* 3'UTR sites supported canonical seed pairing with any of the let-7 family members but were predicted to bind more extensively to the 3'-end of miR-48 (Figure 2.1B). Worms with the edited *lin-41* 3'UTR were found now to depend on miR-48, but not let-7, for regulation of this gene and, ultimately, viability (Figure 2.1B) (Broughton et al. 2016). This study confirmed that pairing to sequences beyond the seed can confer specific and functional miRNA-target interactions *in vivo.*

While imperfect pairing to the miRNA seed can be compensated by extended pairing interactions with the 3'-portion of the miRNA, even seemingly perfect seed matches can depend on additional pairing (Bartel 2018). One explanation for this phenomenon is that sequences immediately adjacent to the seed can influence targeting. In fact, there is a hierarchy of seed-pairing architecture wherein targets that pair to miRNA nucleotides 2-7 alone are generally less repressed than those that include pairing to the eighth position (Bartel 2018). New work from Brancati and Grosshans shows that the ability to pair with nucleotides 8 can also influence the specificity of miRNA-target interactions (Brancati and Großhans 2018). Using the same *C. elegans* model described above, these authors demonstrated that perfect pairing of the *lin-41* target sites to nucleotides 2-8 of let-7 permits regulation by other family members, regardless of differences in potential 3'-pairing interactions (Figure 2.1C). However, seed pairing at nucleotides 2-7 with a G:U wobble pair at position 8 was sufficient to reinstate dependence on pairing to miRNA 3'-end sequences. In some cases, bias for targeting by a specific sister was

sensitive to the expression levels of other family members (Brancati and Großhans 2018). This work highlights the importance of considering overall miRNA-target pairing architecture as well as miRNA abundance in understanding target recognition and regulation *in vivo*.

Figure 2.1: Auxiliary Pairing of miRNA 3'-End Sequences Can Overcome Seed Imperfections and Confer Target Specificity to miRNA Sisters. (A) In *Caenorhabditis elegans*, *the lin-41* 3' untranslated region (UTR) contains two let-7 miRNA target sites that each feature extensive complementarity to the 3' half of let-7 and imperfect seed-pairing potential: Site 1 forces a target nucleotide bulge and site 2 includes an unfavorable G:U base pair (pairing to the miRNA seed, nucleotides 2–7, is shaded gray). While let-7 family members, such as miR-48, can support the same seed-pairing architecture, only let-7 has sufficient 3'-end pairing capacity to regulate *lin-41*, allowing for normal worm development; loss of *lin-41* regulation by let-7 results in lethality (depicted by skull and crossbones) because the let-7 sisters cannot compensate. (B) Exchange of the let-7 sites for sequences predicted to correct the seed imperfections but pair more favorably to the 3' end of miR-48 transfers regulation of *lin-41* from let-7 to miR-48. Sites 1' and 2' are duplications of a sequence in the *dot-1.1* 3' UTR that only formed chimeras with miR-48 (Broughton et al. 2016). (C) The inclusion of pairing to nucleotide 8 (shaded yellow) in this context provides a seed architecture that allows regulation by let-7 or miR-48, regardless of 3'-pairing capacity (Brancati and Großhans 2018). Sites 1'' and 2'' are duplications of the sequence in (B) except for the substitution of U for C to enable canonical pairing to the G at the eighth position in let-7 and miR-48.


A
lin-41
1
2
Site 1
let-7
Site 2
let-7
Site 1
miR-48
Site 2
miR-48
B
lin-41
1′
2′
Site 1′
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C
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2.5 Target Sites within Coding Regions Supersede Canonical Recognition and Regulation

Many of the miRISC binding sites identified through CLIP-based studies include the expected features: seed-pairing capacity, 3'UTR residence, and an association with target mRNA destabilization (Figure 2.2) (Chi et al. 2009; Hafner et al. 2010; Zisoulis et al. 2010; Grosswendt et al. 2014; Helwak et al. 2013; Moore et al. 2015; Broughton et al. 2016; Xue et al. 2013). However, in all of these reports sizeable fractions of AGO-bound sites were also detected in protein-coding sequences (CDSs), often lacking in complementary seed motifs or clear effects on target mRNA regulation (Chi et al. 2009; Hafner et al. 2010; Zisoulis et al. 2010; Grosswendt et al. 2014; Helwak et al. 2013; Moore et al. 2015; Broughton et al. 2016; Xue et al. 2013). While specific examples of functional CDS-located miRNA target sites have emerged (Duursma et al. 2008; Tay et al. 2008; Hausser et al. 2013), in general this position in the mRNA has been regarded as suboptimal for eliciting a regulatory outcome because stable miRISC association would be thwarted by translating ribosomes (Gu et al. 2009). Thus, the relevance of the thousands of CDS miRNA target sites, including some that are highly reproducible across biological replicates, has been an outstanding question.

In recent work, Zhang *et al*. provide compelling evidence that some CDS miRNA target sites actually comprise a new category of recognition elements (Zhang et al. 2018). Multiple independent studies have identified a miR-20a target site in the second exon of *DAPK* (a p53-activating kinase) (Cai et al. 2015; Helwak et al. 2013; Chi et al. 2009; Xue et al. 2013). Curiously, this site lacks seed pairing and, except for a G:U wobble at position 6 and a bulged C at position 12, is perfectly complementary to miR-20a nucleotides 5-23 (Figure 2.2). Within the CDS context, this pairing architecture sufficed for target regulation, but it lost functionality when

inserted into in the 3'UTR (Zhang et al. 2018). Additional examples of CDS sites with weak seed and extensive 3'-end pairing interactions for a different miRNA were also shown to mediate target regulation when placed in coding, but not untranslated, regions, leading the authors to define these as a novel class of **miRNA recognition elements (MREs)** (Zhang et al. 2018).

In addition to the unusual, pairing architecture and location of MREs in this class, the regulatory mechanism is also atypical. These MREs apparently repress translation without triggering mRNA destabilization (Figure 2.2) (Zhang et al. 2018). In contrast to miRNA seed-dependent interactions in 3'UTRs, seedless sites in CDSs interfere with translation through a mechanism that does not rely on the AGO cofactor **GW182/TNRC6** (Figure 2.2) (Zhang et al. 2018). The GW182/TNRC6 protein is instrumental in recruiting deadenylation factors to initiate mRNA decay of canonical targets (Bartel 2018; Gebert and MacRae 2018). Its absence in complexes that regulate CDS targets may explain their lack of influence on mRNA levels. The stark contrasts between canonical 3'UTR target sites and the newly described CDS sites that depend more on 3'-pairing interactions highlight wide gaps in our understanding of functional miRNA targeting rules.

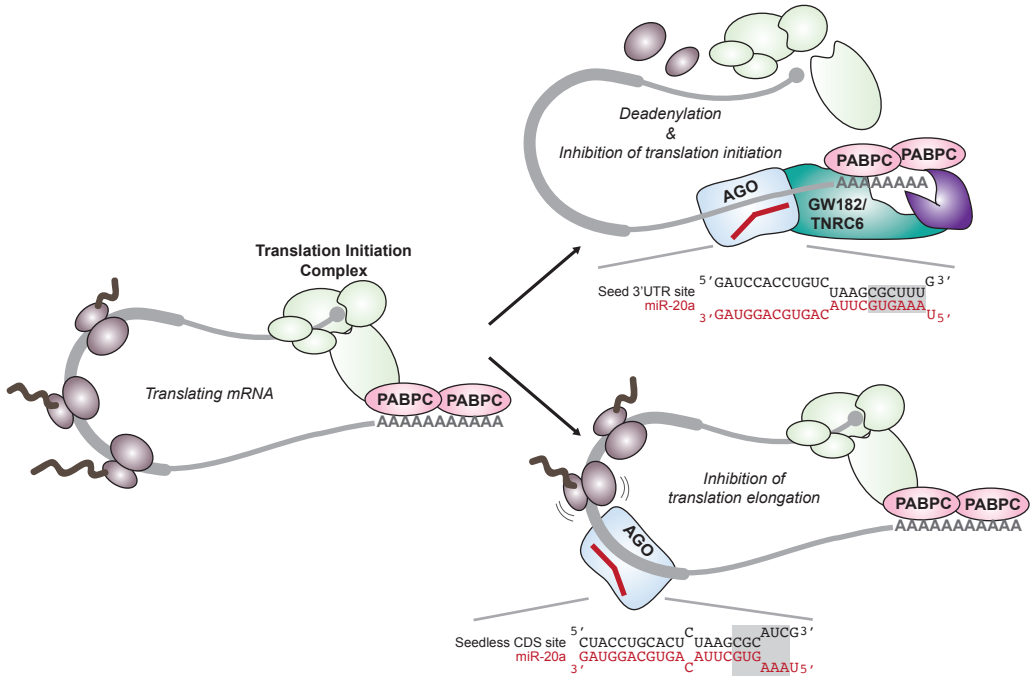

Figure 2.2: The Structure and Position of miRNA–Target Interactions Impose Different Regulatory Outcomes. Canonical miRNA target sites reside in 3' untranslated regions (UTRs), require seed pairing, and rely on the Argonaute (AGO) cofactor GW182/TNRC6 to recruit deadenylases and other factors that act to destabilize and repress translation initiation of the target mRNA (top) (Bartel 2018; Gebert and MacRae 2018). The example is a 3' UTR site engineered to limit pairing to the miR-20a seed region (Zhang et al. 2018). A new class of target sites are located in coding sequences (CDSs), lack seed complementarity, and instead offer extensive pairing to miRNA 3' ends; these types of sites seem to block translation elongation independently of GW182/TNRC6, resulting in reduced protein, but not mRNA, levels of the target (bottom) (Zhang et al. 2018). The example is the CDS site in exon 2 of *DAPK3* mRNA paired to miR-20a (Zhang et al. 2018). Abbreviation: PABPC, cytoplasmic poly(A)-binding protein.

2.6 Seeding miRNA Decay with 3'-End pairing

While miRNA pairing potential can greatly influence the specificity and mechanism of target regulation, it can also impact the fate of the miRNA. Following initial studies in *Drosophila* and mammalian cells (Ameres et al. 2010; Cazalla et al. 2010), numerous examples of **target RNA-Directed MicroRNA degradation (TDMD)** have emerged (Fuchs Wightman et al. 2018). In this pathway, seed along with extensive pairing to the 3' half of the miRNA to a target site can trigger decay of the miRNA itself. While the factors and mechanisms that sense this pairing structure and elicit destruction of the miRNA are yet to be fully revealed, TDMD often is associated with non-templated nucleotide additions, called **tailing**, and trimming at the miRNA 3'-end (Fuchs Wightman et al. 2018). Since efficient TDMD seems to require an unusually high degree of pairing to nucleotides in the 3' half of the miRNA (Ameres et al. 2010; Baccarini et al. 2011), how often this pathway functions *in vivo* has been an open question. Nonetheless, intriguing examples of viruses expressing transcripts that trigger TDMD of host miRNAs established that this is a biologically relevant mechanism for regulating gene expression (Cazalla et al. 2010; Libri et al. 2012; Marcinowski et al. 2012; S. Lee et al. 2013).

In just this past year, several examples of host-mediated TDMD of endogenous miRNAs have come to light (Bitetti et al. 2018; Kleaveland et al. 2018; Ghini et al. 2018). In one study, a conserved block of sequence containing a highly complementary site to miR-29b was shown to regulate spatial expression of this miRNA (Figure 2.3) (Bitetti et al. 2018). This element is present within a long non-coding RNA (lncRNA) called libra or the 3'UTR of the *Nrep* mRNA throughout vertebrate evolution. In mice and zebrafish, high expression of these transcripts in the cerebellum leads to TDMD of miR-29b in this brain region (Bitetti et al. 2018). Importantly, loss of this regulatory mechanism resulted in striking behavioral defects, including impaired motor

functions in mice and aberrant exploratory and anxiety-like behaviors in zebrafish (Bitetti et al. 2018).

One advantage of using TDMD to regulate miRNA expression is that it can be selective for miRNA family members with differing degrees of 3'-end pairing interactions. Indeed, the extended complementarity of miR-29b designates this miRNA, but not its sisters, for TDMD through the pathway described above (Bitetti et al. 2018). Likewise, the ability of a site in the *Serpine1* 3'UTR to form 10 contiguous pairs with miR-30b/c triggered TDMD of those sisters, but not the less complementary miR-30a/d/e family members in mouse fibroblasts (Figure 2.3) (Ghini et al. 2018). When this site was removed from the *Serpine1* 3'UTR by CRISPR/Cas9, levels of miR-30b/c increased, which impacted the degree and specificity of targeting by these miRNAs. Loss of *Serpine1*-mediated TDMD led to cell cycle and stress response defects, suggesting that this pathway plays a critical role in the regulation of miR-30b/c activity (Ghini et al. 2018).

While Ghini *et al.* suggested that *Serpine1* may be just one of more than a thousand endogenous TDMD trigger mRNAs, noncoding RNAs provide another source of potential targets for controlling miRNA stability through this pathway (Ghini et al. 2018). The Cyrano long noncoding RNA (lncRNA), which includes a highly complementary miR-7 site, is broadly conserved across vertebrates (Figure 2.3) (Ulitsky et al. 2011). While knockdown of Cyrano in zebrafish resulted in neurodevelopmental defects (Ulitsky et al. 2011), mouse knockouts of Cyrano appear normal (Kleaveland et al. 2018). However, loss of Cyrano in mouse brain tissue led to increased miR-7 levels, which was associated with a general derepression of its targets (Kleaveland et al. 2018). While the importance of miR-7 TDMD by Cyrano in mammals awaits

further studies, the conservation of this noncoding RNA and its potency in triggering miR-7 decay make it an intriguing model.

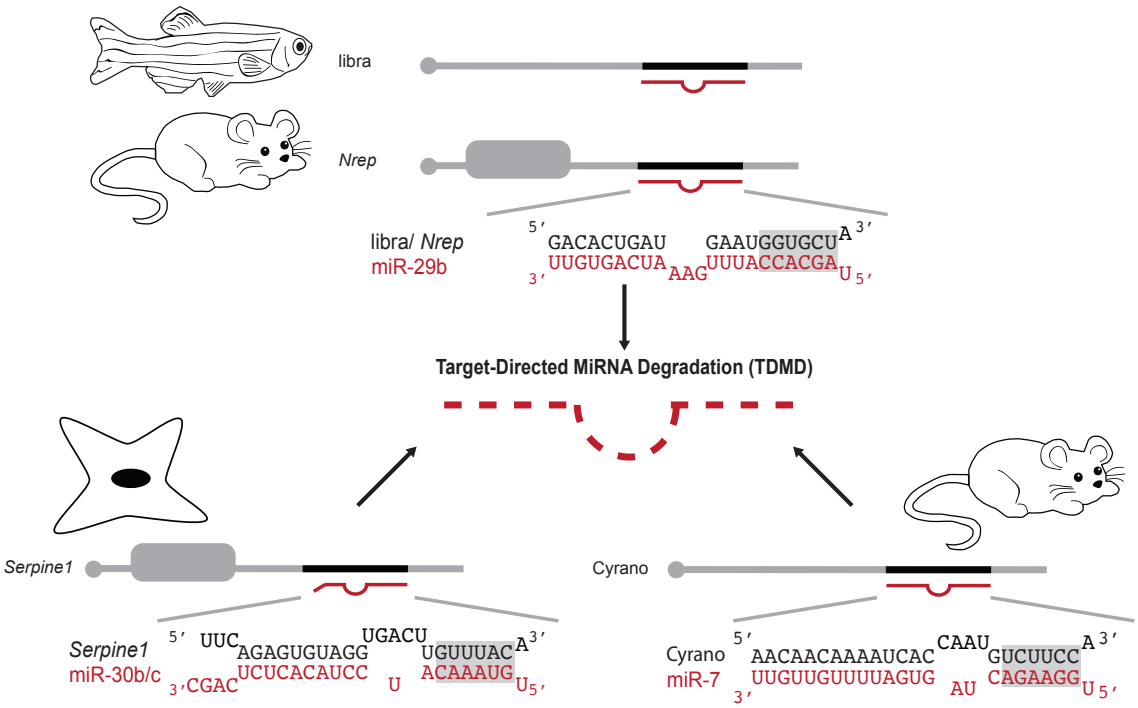

Figure 2.3: Extensive Pairing between a miRNA and Target Can Induce Target-Directed miRNA Degradation. Target-directed miRNA degradation (TDMD) of miR-29b can be triggered by pairing to a conserved region in the zebrafish long noncoding RNA (lncRNA), libra, or the mouse *Nrep* 3' untranslated region (UTR) (top) (Bitetti et al. 2018). A site in the 3' UTR of *Serpine1* induces TDMD of miR-30b/c in mouse fibroblasts (bottom left) (Ghini et al. 2018). In mice, pairing of miR-7 to a site in the lncRNA Cyrano results in rapid decay of the miRNA through TDMD (Kleaveland et al. 2018).

2.7 Concluding Remarks and Future Perspectives

While the seed is long recognized and well supported as a critical element in miRNA targeting (Bartel 2018; Gebert and MacRae 2018), there is a growing appreciation that sequences in the 3'-half of the miRNA have roles to play as well. Members of a miRNA family can

recognize unique targets depending on seed region architecture along with the potential for 3'-end pairing interactions. In parallel, specific sisters can be subjected to TDMD via differences in their 3'-end regions. Further elucidation of the pairing rules that govern selective targeting and TDMD will be needed to realize how widespread these events are (see Outstanding Questions). The demonstration of a new class of miRNA targets that depend on 3'-end interactions, but not seed pairing, suggests there is still much to be learned about how the miRNA complex engages targets *in vivo.* Furthermore, the existence of targets that lack seed complementarity, are located in CDSs, and exclusively undergo translational repression could mean that the extent of gene regulation by the miRNA pathway may be farther reaching than previously considered. With limited sequence content of only ~22 nts, it is now becoming clear that each nucleotide in a miRNA contributes to an overall pairing architecture that can influence recognition and regulation of the target, as well as stability of the miRNA.

2.8 Outstanding Questions

- How common is targeting by specific miRNA family members *in vivo*? Under what conditions can family members compensate upon the loss of the target-specific miRNA?

- How does miRISC recognize and stably associate with targets that lack seed complementarity? How do CDS target sites that lack seed-pairing recruit AGO devoid of its GW182/TNRC6 co-factor?

- What level of 3'-end complementarity is required to trigger TDMD *in vivo*? What is the extent and molecular mechanism of TDMD? How do differences in cell types and conditions affect this pathway?

2.9 Glossary

Chimeric Reads: contiguous sequences from CLIP-seq-based assays that contain two independently derived RNA sequences that became ligated during library preparation. The chimeric reads in AGO CLIP-seq datasets represent a miRNA associated with a specific target site. The name refers to different entities brought together into one being, as in the Chimera in Greek mythology composed of a lion's head, goat's body, and a serpent's tail.

CLIP-seq: crosslinking and immunoprecipitation with sequencing (CLIP-seq) is a technique used to isolate and sequence RNA bound to a specific protein. This method has been used to identify miRNAs and target sites bound by AGO.

CRISPR/Cas9: a genome editing method that uses guide RNAs to target CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) associated nuclease (Cas9) to specific DNA sequences for endonucleolytic cleavage. Repair of the cut DNA can be engineered to incorporate any new sequence of interest. This method is based on a natural Prokaryotic defense system against bacteriophage that involves the integration of foreign DNA sequences between CRISPR segments in a bacterial genome.

GW182/TNRC6: alternative names for a miRISC factor that bridges AGO to proteins that promote deadenylation and translational repression of bound targets. GW182 refers to the molecular weight and glycine/tryptophan repeats that characterize this protein. TNRC6 stands for Trinucleotide repeat containing gene 6.

miRISC: miRNA induced silencing complex (miRISC). A complex consisting of a mature miRNA, AGO, and potentially other proteins that function in target regulation.

miRNA family: a group of miRNAs that share a seed but differ to varying degrees in the rest of their sequence.

miRNA Recognition Element (MRE): miRNA binding site, the region in a target RNA that can base pair to a miRNA.

Seed: nucleotides (nts) 2-7 counting from the 5'end of a miRNA. Canonical targeting involves perfect pairing of the miRNA seed to target sequences.

Sisters: members of the same miRNA family.

Tailing: the addition of non-templated nucleotides to the 3'-end of an RNA, usually by addition of uridines and/or adenosines.

Target RNA-Directed miRNA Degradation (TDMD): degradation of a miRNA caused by binding to a highly complementary target RNA sequence.

2.10 Acknowledgments

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Chapter 2, in full, is a reprint of material as it occurs in *Trends in Genetics,* "miRNA Targeting: Growing Beyond the Seed," Chipman, L.B., Pasquinelli, A. E., 2019. I was the primary author.

CHAPTER 3: Role of Individual miRNAs in the divergent lifespan roles of *Caenorhabditis elegans* miRNA Argonaute proteins

3.1 Abstract

Argonaute (AGO) proteins partner with microRNAs (miRNAs) to post-transcriptionally regulate gene expression. In *C. elegans* there are two widely expressed Argonaute (AGO) proteins involved in the miRNA pathways, AGO-like-gene (ALG)-1 and ALG-2. Despite showing similar roles in development, we see that loss of *alg-1* leads to a shortened lifespan phenotype while loss of *alg-2* leads to an extended lifespan phenotype. These divergent lifespan phenotypes are reflected by the differential misregulation of protein coding genes and miRNAs as well as different associations with distinct miRNAs. Of the miRNAs differentially expressed and associated with *alg-1* and *alg-2*, some show promise as being involved in the differential lifespan phenotypes. As well, some may be unidentified regulators of lifespan on their own.

3.2 Introduction

Argonautes (AGOs) belong to a family of proteins that bind to various classes of small RNAs and are necessary for their ability to regulate their targets. One of these small RNA pathways is the microRNA (miRNA) pathway. MiRNAs are ~22 nucleotide non-coding RNAs with important roles in controlling gene expression from plants to animals. MiRNAs play roles in many different pathways and perturbations in their function or expression have been linked to many human diseases (Paul et al. 2018).

Canonically, a miRNA recognizes a target by partial base pairing between it and the 3' untranslated region (UTR) of the target messenger RNA (mRNA). This recognition is largely dependent on the miRNA seed sequence, nucleotides 2-7, matching the target site (Bartel 2018; Chipman and Pasquinelli 2019). In fact, structural and biochemical analyses of AGO bound miRNAs show that the seed sequence is readily available for binding and target recognition (Chandradoss et al. 2015; Schirle, Sheu-Gruttadauria, and MacRae 2014; Klum et al. 2017; Ruijtenberg et al. 2020; Parker et al. 2009). Upon recognition, miRNA targets are post-transcriptionally destabilized and degraded and/or translationally repressed (Bartel 2018). But, for a miRNA to exert this regulatory influence it must be bound by an AGO protein. The protein co-factors that associate with the AGO bound miRNA form the miRNA induced silencing complex (miRISC) and allow for negative regulation of its RNA target.

In *C. elegans* there are 27 AGOs, but only three are miRNA associated AGO proteins, Argonaute-like-gene (ALG)-1, ALG-2, and ALG-5. ALG-1 and ALG-2 are broadly expressed AGO proteins that interact with most expressed miRNAs (Youngman and Claycomb 2014; Grishok et al. 2001). Meanwhile ALG-5 has germline specific expression and binds a limited number of miRNAs (Brown et al. 2017). While a loss of expression of ALG-1 leads to a developmental delay and global misregulation of miRNA biogenesis and target levels, loss of expression of ALG-2 leads to normal larval development (Vasquez-Rifo et al. 2012). Though *alg-2* is unable to compensate fully for *alg-1* during development, embryonic lethality is only observed upon the loss of *alg-1* and *alg-2* (Grishok et al. 2001).

One of the many other biological processes that the miRNA pathway and AGOs have been implicated in regulating is aging. In *C. elegans*, loss of *alg-1* and *alg-2* by RNAi leads to decreased lifespan phenotypes (Samuelson, Carr, and Ruvkun 2007; Kato et al. 2011). Similarly,

loss of another component of the miRNA pathway, Pasha/DGCR6 which is required for miRNA maturation and processing, leads to a shortened lifespan phenotype (Lehrbach et al. 2012). As well, multiple miRNAs have been implicated in regulating normal aging. Interestingly, individual miRNAs have been shown to both positively (lin-4, let-7, miR-58, miR-61/miR-250, miR-63-66, miR-72, miR-80/miR-227/miR-81/miR-82, miR-238, miR-246) and negatively (miR-80, miR-239a/b) regulate aging (Boehm and Slack 2005; De Lencastre et al. 2010; Pincus, Smith-Vikos, and Slack 2011; Boulias and Horvitz 2012; Vora et al. 2013; Thalyana Smith-Vikos et al. 2014). For some of these cases, this has been tied to the regulation of a specific miRNA target. In the case of *lin-4*, that lifespan reduction upon loss of *lin-4* is due to the subsequent up-regulation of its target *lin-14* (Boehm and Slack 2005). Despite knowing the role of some individual miRNAs in aging we still have more to understand: How does the pathway interact with aging? What are the critical targets of this pathway that allow some miRNAs to negatively regulate aging and others positively?

The work I describe here is based on the surprising finding by the Pasquinelli lab that *alg-1* mutants are short-lived whereas *alg-2* mutants have the opposite aging phenotype and are long-lived (Aalto et al. 2018). To delve into the molecular explanations of the divergent role of ALG-1 and ALG-2, I, along with a master's student mentee, explored the specific miRNAs that might be contributing to these distinct aging phenotypes.

3.3 Results

3.3.1 – Divergent roles of miRNA Argonaute proteins in *C. elegans* aging

To further uncover the role of *alg-1* and *alg-2* in aging, the Pasquinelli lab used CRISPR/Cas9 genome editing to tag the endogenous protein coding sequence of *alg-1* and *alg-2*

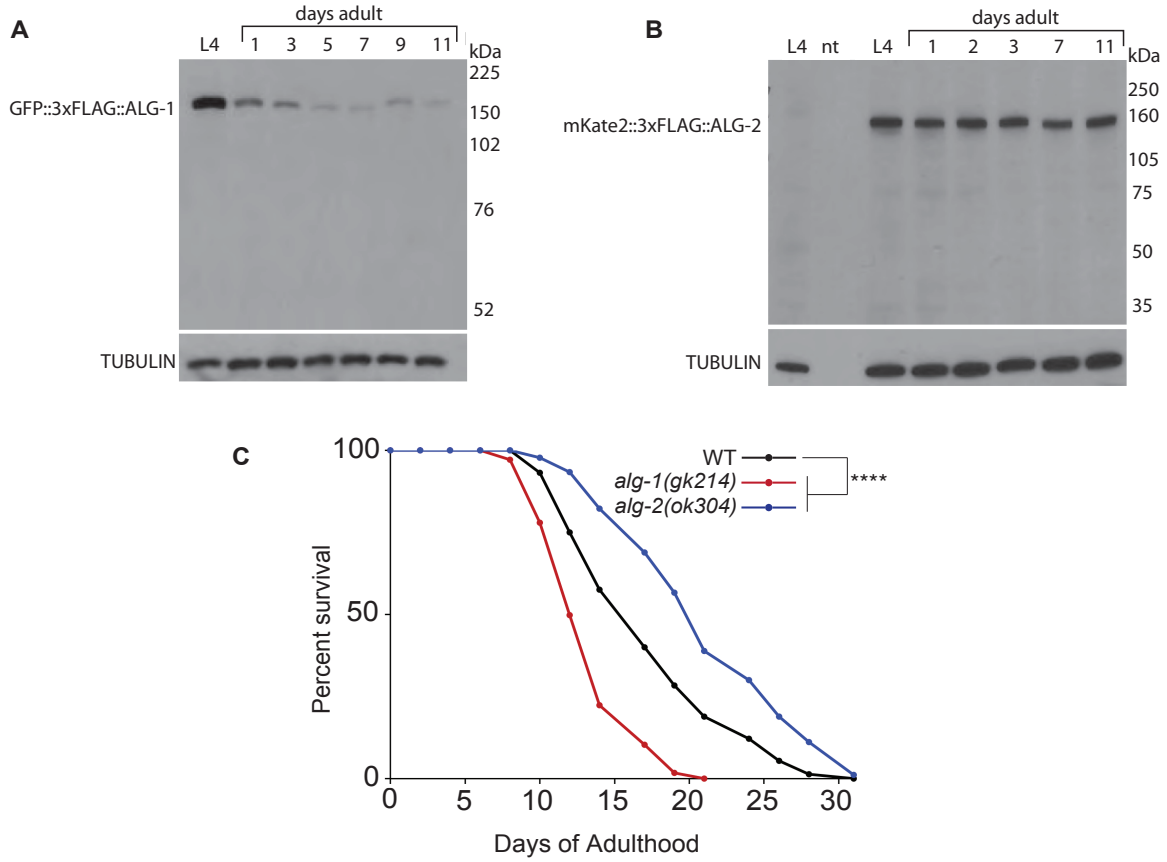
at the N-termini with 3xFLAG::GFP and 3xFLAG:: mKate2, respectively. Strains were confirmed to have retained their function and tagged ALG-1 and ALG-2 run at the expected protein size (Aalto et al. 2018) (Figure 3.1A-B). With these strains, we saw that while ALG-2 remained constant in its expression from larval stage 4 (L4) throughout adulthood, ALG-1 levels dramatically dropped as animals entered adulthood (Figure 3.1A-B).

Intrigued by the differential expression of these AGOs in adulthood, we explored the role of ALG-1 and ALG-2 in aging *C. elegans.* To do so, we performed lifespan analyses on genetic mutants for each AGO. Matching previous results (Kato et al. 2011), *alg-1(gk214)* loss-of-function mutants showed a reduced lifespan phenotype when compared to N2 wild-type (WT) strains (Figure 3.1C). Surprisingly, the *alg-2(ok304)* loss-of-function mutants showed the opposite lifespan effect, an extended lifespan phenotype. Previously it had been shown that RNAi of *alg-2* led to a decreased lifespan phenotype, but that RNAi targeted the coding sequence of *alg-2* which shares a high degree of similarity to *alg-1* (Samuelson 2007). To further explore the lifespan of *alg-2* in adulthood, we created a new RNAi vector that targeted the *alg-2* 3'UTR (which has a low degree of similarity to *alg-1)* and a new *alg-2* loss-of-function allele, *alg-2(ap426)*. Both the new *alg-2* 3' UTR RNAi strategy and *alg-2(ap426)* strain showed significantly extended lifespans when compared to WT animals (Aalto, Nicastro 2018).

Since the miRNA pathway post-transcriptionally regulates gene expression, we hypothesized that gene misregulation was leading to the *alg-1* and *alg-2* lifespan phenotypes. Thus, we performed transcriptome profiling to determine the gene expression in *alg-1(gk214)* and *alg-2(ok304)* animals at day 5 of adulthood. Day 5 was chosen because the animals were both viable and post reproductive. We detected significant up-regulation of 3,184 and down-regulation of 5,742 genes in the *alg-1(gk214)* mutants (Figure 3.1D). In contrast, only 81 genes

were up-regulated and 133 genes down-regulated in *alg-2(ok304)* mutants compared to WT animals (Figure 3.1E). As well, there was minimal overlap of the up and down-regulated genes, respectively, in the *alg-1(gk214)* and *alg-2(ok304)* loss-of-function mutants, further evidence that ALG-1 and ALG-2 are playing unique and opposing roles in *C. elegans* aging.

Figure 3.1: Opposite roles of ALG-1 and ALG-2 in adulthood. (A) Western blot of FLAG::GFP-tagged ALG-1 in protein samples from L4 and adult stage transgenic animals. Tubulin levels serve as a loading control. (B) Western blot of FLAG:: mKate2-tagged ALG-2 in protein samples from L4 and adult stage transgenic animals. A sample from L4 stage non-transgenic (nt) animals demonstrates the specificity of the anti-FLAG antibody. Tubulin levels serve as a loading control. (C) Survival curves showing reduced lifespan in *alg-1(gk214)* (red) and increased lifespan in *alg-2(ok304)* (blue) compared to WT (black). **** P<0.0001 (log-rank). (D-E) Volcano plots representing gene expression changes upon the loss of *alg-1(gk214)* (D) and *alg-2(ok304)* (E) compared to WT in day 5 adult *C. elegans* in three independent replicates. Colored dots (red for *alg-1* and blue for *alg-2)* represent all gene types with a P<.05. Western blot performed by A. Aalto, lifespan analyses by I. Nicastro, RNA-seq performed by A. Aalto.


A
days adult
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kDa
225
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76
52
GFP::3xFLAG::ALG-1
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L4 nt L4 1 2 3 7 11
kDa
250
160
105
75
50
35
mKate2::3xFLAG::ALG-2
TUBULIN
C
100
50
0
Percent survival
0 5 10 15 20 25 30
Days of Adulthood
WT
alg-1(gk214)
alg-2(ok304)
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D
alg-1(gk214) vs WT
DOWN in alg-1
UP in alg-1
120
80
40
0
-log10(P-value)
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E
alg-2(ok304) vs WT
DOWN in alg-2
UP in alg-2
120
80
40
0
-log10(P-value)
-5 0 5
log2(FoldChange)

3.3.2 – Identification of miRNAs involved in the *alg-1* and *alg-2* divergent lifespan phenotypes

Based on the differences in gene expression and the role of AGO proteins as the companion proteins to miRNAs, we wanted to identify individual miRNAs that might play roles in the *alg-1* and *alg-2* differential lifespan phenotypes. We predicted that since there is differential gene misregulation in the *alg-1* and *alg-2* mutants, that ALG-1 and ALG-2 are associating with distinct sets of miRNAs. To assess this, I sequenced the small RNAs that co-immunoprecipitated (co-IP) with ALG-1 and ALG-2 at day 5 of adulthood. Indeed, I saw 13 and 11 different miRNAs associated with ALG-1 and ALG-2, respectively (Figure 3.2A, Table 3.1). Of the 11 miRNAs that showed preferential association with ALG-2, five of them are members of the miR-35 family. The miR-35 family in strongly expressed in oocytes and during embryonic development while it is depleted from many somatic tissues, thus its preferential association is most likely due to expression of ALG-2 in embryos (Lau et al. 2001; Wu et al. 2010; Alvarez-Saavedra and Horvitz 2010; Alberti et al. 2018; Brown et al. 2017; Brosnan, Palmer, and Zuryn 2021). Interestingly, I saw enrichment of lin-4 and miR-71 with ALG-1 (Figure 3.2A). These miRNAs have previously been shown to be involved in regulating aging, as loss of either leads to a reduced lifespan whereas overexpression of lin-4 or miR-71 leads to an extended lifespan (Boehm and Slack 2005; De Lencastre et al. 2010). Notably, both miRNAs have lowered expression in the *alg-1(gk214)* mutant but not in the *alg-2(ok304)* mutant when assayed by Taqman qPCR (Figure 3.2B). The preferential association of these miRNAs with ALG-1 could explain the reduced expression in the *alg-1(gk214)* mutant when compared to WT since AGO association stabilizes miRNAs (Kai and Pasquinelli 2010). As well, the preferential association

of these lifespan promoting miRNAs, lin-4 and let-7*,* with ALG-1 is consistent with *alg-1* positively regulating lifespan.

To globally assess miRNA levels in the *alg-1* and *alg-2* mutants, a master's student and I performed global miRNA profiling in day 5 adults. We detected significant up-regulation of 34 and down-regulation of 41 miRNAs in the *alg-1(gk214)* mutants (Figure 3.2C). Meanwhile, 35 miRNAs were up-regulated and 24 miRNAs down-regulated in *alg-2(ok304)* mutants compared to WT animals (Figure 3.2D). Fitting with the evidence that AGOs stabilize associated miRNAs, there was a large overlap of miRNAs that were bound by ALG-1 and ALG-2 that had decreased expression in *alg-1*(*gk214)* and *alg-2(ok304),* respectively (Table 3.1).

To further explore the connection between a miRNA's misregulation upon the loss of a given AGO and that miRNA's preferentially association with that AGO, I performed a Pearson's correlation analyses on miRNAs with over 10 rpm detected in the co-IP sequencing data (n=154). Specifically, I ran a correlation analysis between a miRNA's fold change in *alg-1(gk214)* vs WT and its association with ALG-1 vs ALG-2. This analysis showed a modest but significant correlation of R = -.38 between misregulation in loss of *alg-1(gk214)* and association with ALG-1. I also ran a correlation analysis between a miRNA's fold change in *alg-2(ok304)* vs WT and its association with ALG-2 vs ALG-1. There was a significant correlation of R = -.61 between misregulation in *alg-2(gk214)* and association with ALG-2. The negative correlation values indicate that a miRNA's preferential association with a given AGO is inversely correlated with how it is misregulated upon loss of that AGO. These data suggest that preferential association with ALG-1 and ALG-2 is a driving factor in miRNA misregulation upon loss of either *alg-1* or *alg-2.*

To investigate which miRNAs are important for the opposing *alg-1* and *alg-2* loss-of-function phenotypes, we looked at the top misregulated miRNAs. The most down-regulated miRNA in the *alg-1(gk214)* vs WT dataset was miR-63, a miRNA that shows preferential association with ALG-1. While the top upregulated miRNAs in the *alg-2(gk214)* vs WT dataset were members of the miR-35 family and miR-61, all of which show preferential association with ALG-2. The most down-regulated miRNA in the *alg-2 (gk214)* vs WT dataset, miR-355, showed no preferential AGO association but previous work has shown its role in innate immunity and carbon nanotubule stress (Zhao, Yang, and Wang 2016; Zhi et al. 2017). Interestingly, the top up-regulated miRNA in the *alg-1(gk214)* vs WT dataset was miR-230 which co-IPs with ALG-2 suggesting that its preferential association with ALG-2 makes miR-230 less impacted by the loss of *alg-1*. Overall, this shows that in *C. elegans* adults, ALG-1 and ALG-2 associate with distinct sets of miRNAs that are misregulated upon their loss.

Table 3.1 The average enrichment values for miRNAs reproducibly enriched at least 1.5-fold with ALG-1 (top) or ALG-2 (bottom) in both replicates and their misregulation in *alg-1(gk214)* and *alg-2(ok304)* mutants. Specifically, the fold change values from smRNA sequencing of day 5 *C. elegans alg-1(gk214)* and *alg-2(ok304)* vs WT, bolded if padj<.05.

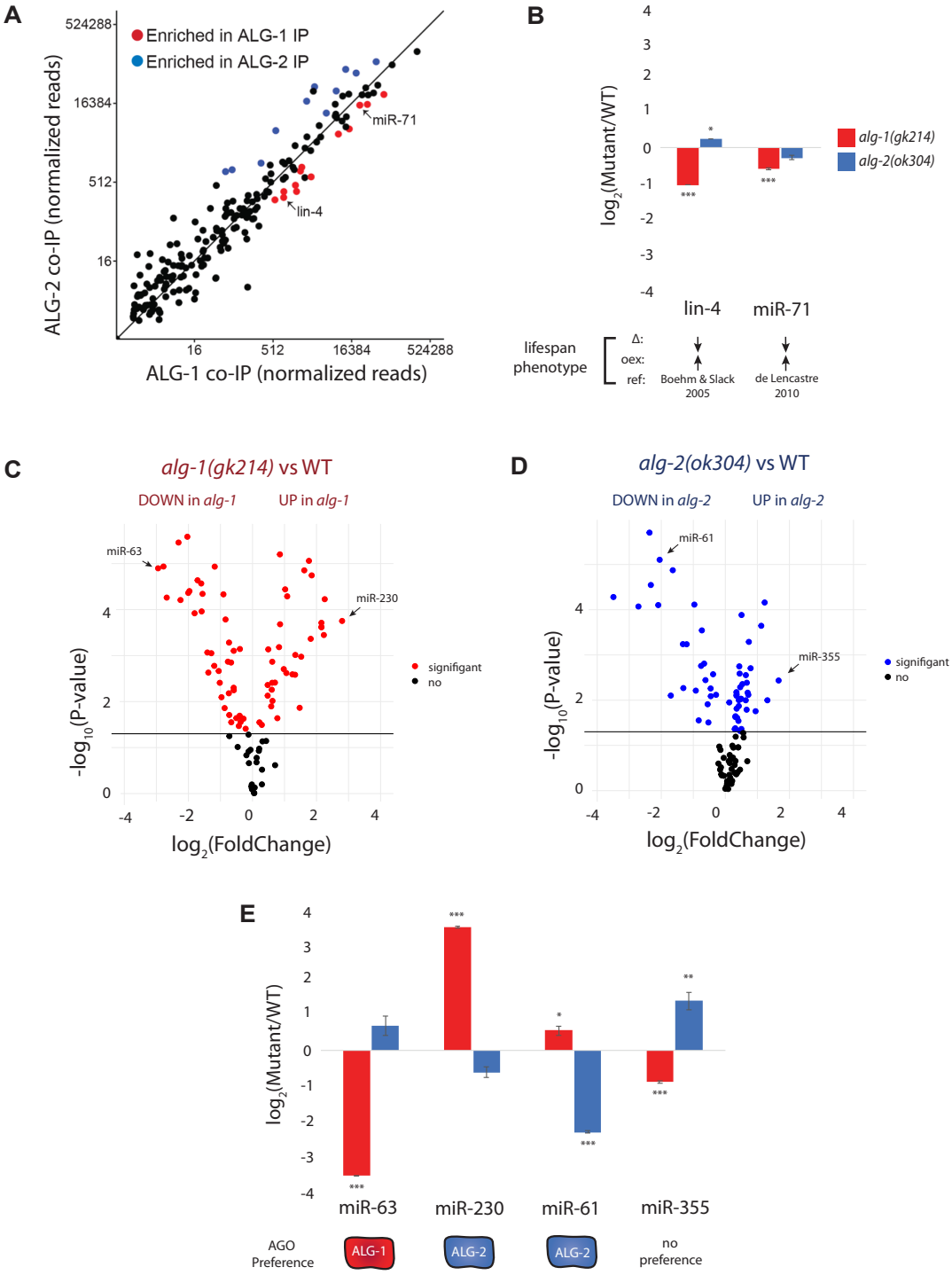
miRNAs reproducibly enriched in ALG-1 versus ALG-2 IPs

miRNA	ALG-1/ALG-2	*alg-1(gk214)*/N2	*alg-2(ok304)*/N2
cel-miR-60-3p	4.6	**0.29**	**1.62**
cel-miR-248	4.3	**0.34**	**1.37**
cel-lin-4-5p	3.3	**0.60**	**1.92**
cel-miR-63-3p	3.3	**0.13**	**1.38**
cel-miR-237-5p	2.8	0.99	**2.34**
cel-miR-240-3p	2.8	**0.44**	1.49
cel-miR-57-5p	2.8	**2.03**	**1.22**
cel-miR-54-3p	2.6	**3.53**	**1.58**
cel-miR-241-5p	2.4	0.92	1.10
cel-let-7-5p	2.3	**0.67**	**1.56**
cel-miR-48-5p	2.1	**1.16**	**1.29**
cel-miR-47-3p	1.8	1.00	1.27
cel-miR-71-5p	1.6	**0.67**	**1.42**

miRNAs reproducibly enriched in ALG-2 versus ALG-1 IPs

miRNA	ALG-2/ALG-1	*alg-1(gk214)*/N2	*alg-2(ok304)*/N2
cel-miR-250-3p	12.9	1.16	**0.63**
cel-miR-37-3p	10.6	**1.50**	**0.20**
cel-miR-230-3p	10.5	**6.96**	**0.56**
cel-miR-36-3p	9.8	1.05	**0.09**
cel-miR-61-3p	7.6	**2.37**	**0.31**
cel-miR-40-3p	5.6	**1.39**	**0.15**
cel-miR-253-3p	4.0	**1.81**	**0.43**
cel-miR-39-3p	3.4	1.24	**0.19**
cel-miR-35-3p	3.1	1.09	**0.23**
cel-miR-72-5p	2.2	**0.77**	**0.73**
cel-miR-66-5p	2.0	**0.41**	**0.73**

Figure 3.2: MiRNAs are differentially associated with ALG-1 and ALG-2 and differentially misregulated in *alg-1* and *alg-2* loss-of-function strains during adulthood. (A) Enrichment of specific miRNAs with ALG-1 (red) or ALG-2 (blue) detected by sequencing of small RNAs that co-immunoprecipitated (co-IP) with each AGO protein at day 5 of adulthood averaged from 2 independent experiments. See Table 3.1 for a complete list of miRNAs reproducibly enriched for association with ALG-1 or ALG-2. (B) Taqman qPCRs confirming the misregulation trends of known aging miRNAs in the *alg-1(gk214)* and *alg-2(ok304)* mutants. The error bars represent SEMs. *P<0.05, **P<0.01, ***P<0.001 (t-test). The published lifespan phenotypes observed with reduced expression (Δ), overexpression (OEX), and corresponding references (ref) are indicated. ↑ (increased lifespan), ↓ (decreased lifespan). (C & D) smRNA sequencing and analysis of miRNA gene expression of *alg-1(gk214)* (C) and *alg-2(ok304)* (D) *C. elegans* at day 5 of adulthood identifies 75 and 59 significantly ($p < 0.05$, N2 average reads > 100) misregulated miRNAs when compared to wildtype, n=5 biological replicates. (E) Taqman qPCRs confirming the misregulation trends of the top misregulated miRNAs in the *alg-1(gk214)* and *alg-2(ok304)* mutants. The error bars represent SEMs. *P<0.05, **P<0.01, ***P<0.001 (t-test). AGO-IP and smRNA-seq performed by Laura Chipman, smRNA-seq performed by Jesse Hulahan, Taqman performed by Jesse Hulahan and Laura Chipman.


A
Enriched in ALG-1 IP
Enriched in ALG-2 IP
miR-71
lin-4
ALG-2 co-IP (normalized reads)
ALG-1 co-IP (normalized reads)
524288
16384
512
16
B
log2(Mutant/WT)
alg-1(gk214)
alg-2(ok304)
lin-4
miR-71
lifespan phenotype
Δ:
oex:
ref:
Boehm & Slack 2005
de Lencastre 2010
C
alg-1(gk214) vs WT
DOWN in alg-1
UP in alg-1
miR-63
miR-230
-log10(P-value)
log2(FoldChange)
significant
no
D
alg-2(ok304) vs WT
DOWN in alg-2
UP in alg-2
miR-61
miR-355
E
miR-63
miR-230
miR-61
miR-355
AGO Preference
ALG-1
ALG-2
ALG-2
no preference

3.3.3 – Identifying potential targets of miRNAs differentially regulated in *alg-1* and *alg-2* mutants

Having identified the miRNAs preferentially associated with ALG-1 or ALG-2 and whose expression was most changed by the loss of either AGO, we asked if the altered gene expression was due to the altered miRNA expression. Unfortunately, too few genes were changed in *alg-2(ok304)* to test for enrichment or depletion of miRNA target sites, so we focused our analysis on the most down-regulated miRNA in *alg-1(gk214)*, miR-61, and the known aging miRNAs that showed preferential association with ALG-1 (Fig2A, C, Table 3.1). Having confirmed the expression pattern of these miRNAs with TaqMan qPCR in day 5 WT, *alg-1*(*gk214)* and *alg-2(ok304)* in day 5 adult animals (Figure 3.2B, E), we asked if the altered expression of miR-61, let-7, and lin-4 could be responsible for altered gene expression in the *alg-1(gk214)* mutants by performing seed enrichment analysis (Figure 3.3A). Seed enrichment analysis was performed by searching for reverse complementary of a miRNA's seed sequence, nucleotides 2-7, in the 3' UTR sequence of all genes.

I found that sequences capable of pairing to the seed region of miRNAs previously shown to promote longevity, lin-4 and miR-71, were enriched in the 3' UTR of genes up-regulated in *alg-1(gk214)* (Figure 3.3B). Strikingly, this was also the case for the most down-regulated miRNA in *alg-1(gk214)*, miR-63 (Figure 3.3B). These results suggest that up regulation of genes in the *alg-1(gk414)* is, at least in part, due to the down-regulation of lin-4, miR-71, and miR-63.

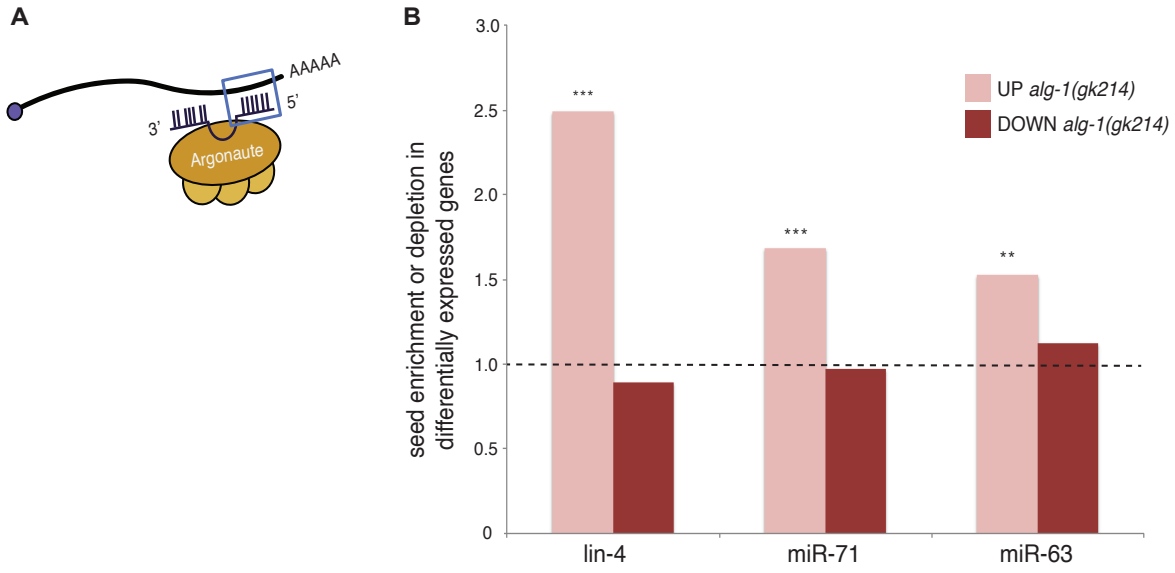


Figure 3.3: MiRNAs that are down regulated in *alg-1(gk214)* are enriched for targeting genes that are up-regulated in the *alg-1(gk214)*. (A) Schematic of a miRNA bound to AGO base pairing to the 3' UTR of a target RNA, seed base paring (nucleotide 2-7 of the miRNA) circled. (B) Enrichment and depletion of seed pairing for the indicated miRNAs with genes differentially expressed in *alg-1(gk214)* versus WT animals. The fold difference shown is in comparison to the fraction of seed-pairing sites detected in genes with unchanged expression patterns in the *alg-1* mutants. *P<0.05, **P<0.01, ***P<0.001 (Chi Squared with Yates Correction). Analysis performed by Laura Chipman.

3.4 Discussion

By identifying the miRNAs that are directly associated with ALG-1 and ALG-2 and misregulated in the respective loss-of-function backgrounds, I gained insight not only into which miRNAs could be playing a role in the divergent lifespan phenotypes of *alg-1* and *alg-2* mutants but also the role of individual miRNAs in aging. I found that two miRNAs known to promote longevity, lin-4 and miR-71, were enriched with ALG-1 protein and down-regulated upon loss of *alg-1*. To further implicate that their loss in the *alg-1* mutant could be contributing to the reduced lifespan phenotype of those animals, I showed that those miRNAs were enriched in targeting genes up-regulated in the *alg-1* loss-of-function animals. Interestingly, miR-63, the most down-

regulated miRNA in *alg-1(gk214)*, was preferentially bound to ALG-1 and its target site was enriched in the 3'UTR of genes up-regulated in *alg-1(gk214)* mutants. Yet, loss of miR-63 results in no lifespan phenotype on its own (Boulias and Horvitz 2012). This suggests that the loss of miR-63 is compensated for in WT animals.

In addition, some of the miRNAs with no lifespan phenotype in wildtype animals upon their loss could still have a role in the *alg-1* and *alg-2* aging phenotypes. The top miRNAs up-regulated in *alg-1*(gk214) and *alg-2(ok304)* loss-of-function animals were miR-230 and miR-355, respectively. Neither miRNA has a lifespan phenotype upon its loss but the effect of overexpression of these miRNAs on aging has not been explored (Boulias and Horvitz 2012; Zhi et al. 2017). MiRNA abundance is tied to its specificity and activity, especially its ability to repress a target site with an imperfect seed base-pairing (Brancati and Großhans 2018). To test if the overexpression of these miRNAs is contributing to the lifespan phenotypes, knock outs of *miR-230* and *miR-61* could be crossed to *alg-1* and *alg-2* loss-of-function animals, respectively, to see if there is a partial rescue of the lifespan phenotypes. Given that miR-355 has already been shown to be involved in pathogen defense and to regulate *daf-2*, an important member of the insulin signaling pathway, it is an especially attractive candidate for being involved in the *alg-2* extended lifespan (Zhi et al. 2017).

Interestingly, the predicted roles for one of the miRNAs that we identified in our data did not match with previously published data. The prediction for miR-61, as a miRNA down-regulated in *alg-2(ok304)* with preferential ALG-2 association, is that its loss is contributing to the extended lifespan of *alg-2(ok304)*. Yet *miR-61(nDf59)* loss-of-function mutants have a shortened lifespan phenotype (Boulias and Horvitz 2012). It is possible that loss of *miR-61* is not contributing to the *alg-2(ok304)* lifespan phenotype because another miRNA or other regulatory

mechanism is compensating for its loss. It is also possible that the deletion mutant used to study miR-61 is not precise enough to explore its specific role in aging; the *nDf59* deletion removes not only miR-61 but miR-250 and *sel-11*, a protein coding gene. Using CRIPSR/Cas9 technologies, a more precise mutant of *miR-61* could be made to assay its role in aging.

An outstanding question is how these two AGO proteins that are over 75% identical at the amino acid sequence have differing roles in regulating aging (Tops, Plasterk, and Ketting 2006). While the functional domains are largely conserved between ALG-1 and ALG-2, the N-terminal region is highly divergent and could serve as a site for protein interactions or protein modifications. In human AGO2, phosphorylation at different sites can affect its affinity for target and small RNA binding (Rüdel et al. 2011; Golden et al. 2017). While no known phosphorylation sites differ between ALG-1 and ALG-2, it is possible that the small coding differences could drive functional changes. Additionally, while the coding sequences are highly conserved, the regulatory sequences are not, exemplified by the distinct expression patterns of ALG-1 and ALG-2 (Aalto et al. 2018). The differing spatio-temporal expression of these AGOs could lead to different miRNA interactions and explain the differential association of miRNAs. To test these ideas, CRISPR/Cas9 can be used to swap coding and regulatory domains between ALG-1 and ALG-2 to see which are involved in the aging phenotypes. These experiments will strengthen our understanding of how the expression and function of these AGOs diverge and the contribution to *C. elegans* aging.

3.5 Experimental Procedures

RNA sequencing

Synchronized WT, *alg-1(gk214)* and *alg-2(ok304)* animals cultured at 20˚C were collected at adult day 5 after removing eggs and progeny larvae. Adult *C. elegans* were separated from eggs and progeny daily by washing plates with M9 into conical tubes and allowing the adults to settle by gravity for a few minutes on a bench top. The supernatant containing larvae and eggs was then removed, and this process was repeated 3–7 times until eggs and larvae were no longer visible. Three independent RNA samples of each strain were prepared for RNA sequencing with the TruSeq Stranded Total RNA Library Prep Kit (Illumina) according to the Low Sample Protocol. 50-bp single-end indexed RNA sequencing libraries were prepared from 1 µg of RNA of each sample and used for sequencing on an Illumina HiSeq platform. Subsequent mapping of sequencing reads to the *C. elegans* genome (ce10) was performed using RNA-STAR (Dobin et al. 2013). Total read counts for each gene were then quantified using HTSeq (Anders, Pyl, and Huber 2015). These read counts were then input into DESeq to determine log2-fold change and differential expression between the mutant and WT strains (Love, Huber, and Anders 2014).

Co-immunoprecipitation and small RNA sequencing

Synchronized FLAG::GFP::ALG-1 (PQ530) and FLAG::RFP::ALG-2 (PQ582) animals cultured at 20˚C were collected at adult day 5 after removing eggs and progeny larvae. Samples were collected and sonicated in 100 mM NaCl, 25 mM HEPES, 250 µM EDTA, 2 mM DTT, 0.1% (w/v) NP-40, 0.1% SDS, 1X Complete Mini Protease Inhibitor (Sigma Aldrich), and 25 U/mL rRNasin (N251A). Cell debris was removed by centrifugation and lysates were incubated with anti-FLAG (F1804) bound to Protein G Dynabeads (10004D) for 1 hour at 4˚C. Following

co- immunoprecipitation, beads were washed as previously described (Van Wynsberghe et al. 2011). RNA was isolated to use for small RNA library preparation. Two independent RNA samples of each strain were prepared for RNA sequencing with the TruSeq small RNA Library Prep Kit (Illumina). RNA sequencing libraries were prepared from 1 μg of RNA of each sample and sequenced on an Illumina HiSeq 4000. Adapter sequences were removed and, using miRDeep2, small RNA sequences were mapped to the *C. elegans* genome (WS261) and quantified based on miRNA annotations from miRBase release 21 (Friedländer et al. 2012; Kozomara and Griffiths-Jones 2014). To identify miRNAs that were enriched with ALG-1 or ALG-2, we first calculated the normalized reads (reads per million in the library). MiRNAs with more than 1.5-fold the number of normalized reads in one AGO co-IP versus the other, from independent replicates, were considered enriched. MiRNAs with less than 1000 reads across the libraries were not included in the enrichment analyses. The results are summarized in Table 3.1.

small RNA sequencing of LOF strains

Synchronized WT, *alg-1(gk214)* and *alg-2(ok304)* animals cultured at 20˚C were collected at adult day 5 after removing eggs and progeny larvae. Five independent RNA samples of each strain were prepared for RNA sequencing with the TruSeq small RNA Library Prep Kit (Illumina). RNA sequencing libraries were prepared from 1 μg of RNA of each sample and sent for single end sequencing on an Illumina HiSeq 4000. Adapter sequences were removed using Cutadapt, and smRNA reads were mapped to the annotated *C. elegans* genome (WS266) using Bowtie-build to first create indices and miRDeep2 to align and quantify reads (Friedländer et al. 2012). Differential expression analysis was performed by first normalizing reads to library size (read counts per million) and then measuring the log2foldchange of mutants to WT strains within

replicates. MiRNAs were called significantly misregulated if they had over 100 reads on average in WT samples and a p-value < 0.05.

MicroRNA Target Seed Analysis

Custom code was made to search for miRNA seed 7-mer complementary sequences in the 3' UTR of select gene datasets in R. Seed sequences were pulled from miRbase and 3' UTR sequences were pulled from WormBase (Kozomara, Birgaoanu, and Griffiths-Jones 2018). Fold difference in miRNA targeting was calculated by comparing the seed-pairing sites normalized by gene number present in a gene set to the seed-pairing sites normalized by gene number present in all detected genes minus those in the gene set of interest. MiRNA sites were determined enriched if p-value < 0.05 as calculated with Chi Squared with Yates Correction.

3.6 Acknowledgements

The lifespan assays, immunoblots, RNA-seq data, smRNA-seq in the LOF strains and some TaqMan qPCR assays were performed by A. Aalto, I. Nicastro, J. Hulahan, L. Chipman and A. Pasquinelli. Parts of the figures shown in this chapter are reproduced from a published manuscript with permission of the authors.

Chapter 3 contains material from the manuscript "Opposing Roles of the MicroRNA Argonautes during Aging in *Caenorhabditis elegans*" by Aalto, A.P., Nicastro, I.A., Broughton, J.P., Chipman, L.B., Schreiner, W.P., Chen, J.S., and Pasquinelli, A.E. Chipman, L.B. was a co-author on this manuscript.

CHAPTER 4: Re-evaluation of the role of the miR-238, miR-239a, and miR-239b microRNAs in *Caenorhabditis elegans* Aging

4.1 Abstract

MicroRNAs (miRNAs) are powerful post-transcriptional regulators that target RNAs in a sequence specific manner. A critical factor in miRNA targeting is the seed sequence, nucleotides 2-7 of the miRNA. Given the reliance of targeting on the seed sequence, it is often assumed that miRNA family members, miRNAs that share the same seed sequence, function redundantly. Here, we explore the miR-238, miR-239a, and miR-239b miRNA family's role in *C. elegans* aging. As previously reported, loss of miR-238 led to a reduced lifespan. Yet, we found that individual loss of miR-239a or miR-239b has no effect on *C. elegans*' longevity. Since a previously generated allele that deleted *miR-239a, miR-239b* as well as a snoRNA and ncRNA had an extended lifespan, we were surprised that a precise double mutant of *miR-239a* and *miR-239b* did not have a lifespan phenotype. Transcriptional profiling of adult animals revealed distinct sets of genes misregulated in the precise *miR-238*, *miR-239a* and *miR-239b* mutant strains, and was reflective of their lifespan phenotypes; *miR-238(-)* had many genes misregulated, while the *miR-239a(-)* and *miR-239b(-)* mutants only had a handful of genes misregulated. We further show that this miRNA family is differentially expressed in aging, and that the longevity role of miR-238 can be replaced by miR-239a or miR-239b. This works shows that the miR-238, miR-239a, miR-239b sisters differentially regulate *C. elegans* aging and underscores the importance of miRNA expression in determining function, even in a miRNA family where members share the same seed sequence.

4.2 Introduction

MicroRNAs (miRNAs) are small, ~22 nucleotide (nt), RNA regulators that post-transcriptionally repress target RNAs in a sequence dependent manner (Jonas and Izaurralde 2015; Bartel 2018). Most metazoan miRNAs are transcribed into long primary miRNAs (pri-miRNAs) by RNA Pol II with a stem-loop structure that is recognized and processed into a ~60nt precursor miRNAs (pre-miRNA) (Nguyen et al. 2015). Dicer cuts both strands of the pre-miRNA stem-loop structure, leaving a miRNA duplex where one strand will be degraded and the other loaded into a miRNA Arognaute (AGO) protein (Bartel 2018). Once miRNAs are loaded into AGO, it forms the core of the miRNA induced silencing complex (miRISC), which induces translational inhibition and decay of the target RNA (Bartel 2018). Early studies on miRNAs found that sequences on the 5' end the miRNA were important for miRNA-target interactions. The seed sequence, nts 2-7 of the miRNA, was found to be both necessary and sufficient in some cases for proper miRNA-target regulation (Bartel 2018). Due to the importance of the miRNA seed sequence in targeting, miRNAs who share a seed sequence are grouped into families. Given the reliance of targeting on the seed sequence, it is often assumed that miRNA family members function redundantly.

Yet, recent work has highlighted that sequence beyond the seed, as well as miRNA expression level, play big roles in determining miRNA-target interactions (Chipman and Pasquinelli 2019). High-throughput capture of miRNA/target complexes have revealed a high frequency of interactions with partial or poor seed matches between the miRNA and its target RNA, some with extensive base-pairing to the 3' end of the miRNA (Broughton et al. 2016; Grosswendt et al. 2014; Helwak et al. 2013; Moore et al. 2015). As well, careful *in vivo* studies

have shown that pairing of the 3' region of the miRNA can facilitate miRNA/target interactions on imperfect seed sites (a single mismatch) and give target specificity among miRNA family members who share their seed sequence but differ in their 3' sequences (Broughton et al. 2016; Brancati and Großhans 2018). Furthermore, some miRNA target sites require a higher miRNA concentration for silencing (Brancati and Großhans 2018). Since miRNA targeting and their biological roles are intertwined, it is important to understand the functional consequences of dynamic miRNA targeting.

One of the processes miRNAs have been implicated in regulating is aging (Elder and Pasquinelli 2022). In *C. elegans*, there are several cases that show losing a key component of the miRNA pathway leads to a reduced lifespan; loss of Pasha/DGCR6 which is required for miRNA maturation and processing, leads to a shortened lifespan phenotype (Lehrbach et al. 2012). Similarly, combined loss of the *C. elegans* somatic miRNA AGO proteins, ALG-1 and ALG-2, in adulthood by RNAi results in decreased lifespan phenotypes (Kato et al. 2011; Samuelson, Carr, and Ruvkun 2007). Also in *C. elegans*, individual miRNAs have been implicated in both positively and negatively regulating aging (Boehm and Slack 2006; Boulias and Horvitz 2012; De Lencastre et al. 2010; Pincus, Smith-Vikos, and Slack 2011; Thalyana Smith-Vikos et al. 2014; J. Yang et al. 2013). Interestingly, the miR-238, miR-239a, and miR-239b family has been implicated in regulating *C. elegans* longevity, with loss of miR-238 leading to a reduced lifespan while loss of miR-239a, miR-239b, and their surrounding genomic sequences resulting in an extended lifespan (De Lencastre et al. 2010).

In this study, we discovered that miR-238, miR-239a, and miR-239b miRNA family members have distinct roles in aging. Previously, it was shown that miR-238, miR-239a, and miR-239b all increase in expression over aging and have differential expression patterns (De

Lencastre et al. 2010). We further show, that the miR-238, miR-239a, and miR-239b miRNA family is differentially sensitive to the loss *alg-1*. As previously seen, we saw that loss of miR-238 led to a reduced lifespan (De Lencastre et al. 2010). But to understand the individual contributions of miR-239a and miR-239b, we used genomic editing to create clean, precise deletions of miR-239a, miR-239b, and both miR-239a and miR-239b without disrupting the genic sequences in between. The loss of just miR-239a, miR-239b, or miR-239a and miR-239b together had no effect on longevity. These lifespan results were paralleled by the distinct set of genes mis-regulated in the individual loss of function mutants for miR-238, miR-239a, and miR-239b; loss of miR-238 had a reduced lifespan and had many genes mis-regulated in adult *C. elegans*, while the loss of either miR-239a or miR-239b had no effect on lifespan and very few genes were mis-regulated. Rescue of the reduced lifespan caused by loss of miR-238 was achieved by inserting *miR-239a* or *miR-239b* into the endogenous *miR-238* locus. Altogether, our data reveal that the function of the miR-238/miR-239ab family in aging is primarily reliant on expression and not sequence differences.

4.3 Results

4.3.1 – Members of the miR-238/239ab miRNA family are differentially expressed in adult *C. elegans*

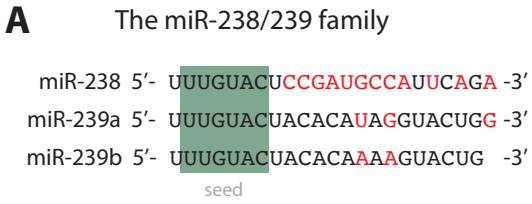
The miR-238, miR-239a, and miR-239b miRNAs are members of the same family, as they share their seed sequence but differ to varying to degrees in their 3' sequence (Figure 4.1A). All of these miRNAs were originally identified as potential regulators of aging due to their increase in expression over aging (De Lencastre et al. 2010). To further study the levels of these miRNAs in adult animals, we performed small RNA transcriptomics on adult day 5 wildtype

(WT) animals. Along with a previously generated small RNA-seq (smRNA-seq) dataset from L4 WT worms (Schreiner et al. 2019), we ranked miR-238, miR-239a and miR-239b expression out of all mature miRNAs at L4 and day 5 (Figure 4.1B). Although caution needs to be used when comparing levels of different miRNAs, as smRNA-seq can be subject to ligation bias thus leading to uneven quantification of miRNAs of different sequences (Kim et al. 2019), from these rankings, miR-238 is the most abundantly detected family member in L4 and day 5 (Figure 4.1B). Additionally, miR-238 has a slight increase in ranking from L4 to day 5 (Figure 4.1B). Meanwhile, miR-239a and miR-239b increase in ranking ~2 fold from L4 to day 5 but still are detected less frequently than miR-238 (Figure 4.1B). These data corroborate the finding that the miR-238/239 family increases in aging but also highlights potential differences in overall expression levels.

We also explored the relationship of the miR-238/239ab miRNAs to the main somatic miRNA AGO proteins, ALG-1 and ALG-2. It was previously reported that ALG-1 and ALG-2 have differing spatial expression in aging and play opposing roles in *C. elegans* longevity (Aalto et al. 2018). Thus, examining how miR-238, miR-239a, and miR-239b interact with ALG-1 and ALG-2 could inform on aging roles for these three miRNAs. As well, since AGO association stabilizes miRNAs and ALG-1 and ALG-2 have different spatial expression, it is possible that different levels of the miR-238/239ab miRNAs could be indicative of differing spatial expression and access to binding these AGOs (Kai and Pasquinelli 2010). Thus, we ranked miRNA association with ALG-1 and ALG-2 from day 5 ALG-1 and ALG-2 RNA immunoprecipitation data (Aalto et al. 2018), and in addition performed small RNA-seq in day 5 *alg-1(gk214)* and *alg-2(ok304)* mutant strains and analyzed the fold change in miRNA expression as compared to WT (Figure 4.1B). All three miRNAs, immunoprecipate with ALG-1 and ALG-2 at a level

relatively commensurate with their level of detection in total smRNA-seq at day 5 of adulthood (Figure 4.1B). Despite this proportionate association with AGOs, the miR-238, miR-239a, miR-239b family have different sensitivities to the depletion of ALG-1: miR-238 is 3x down in *alg-1(gk214)* compared to WT, while miR-239b is 2.4x up, and there is no significant change for miR-239a (Figure 4.1B.) The family had no significant change in *alg-2(ok304)* as compared to WT (Figure 4.1B). This differential sensitivity to loss of ALG-1 suggests that the expression and/or stability of miR-238, miR-239a, and miR-239b are subject to differential regulation.

Given the differences in levels of overall mature miRNA, and their differential relationship to the loss of ALG-1, we postulated that miR-238, miR-239a, miR-239b have differing spatial expression of their transcripts. Indeed, in previous studies using GFP-based transcriptional reporters fused to miRNA promoters it was shown that miR-238 has a different expression pattern than miR-239a/b (De Lencastre et al. 2010; Martinez et al. 2008). These authors observed that $_{p}$miR-238::GFP is expressed nearly ubiquitously in adult *C. elegans,* with highest levels detected in the intestine, hypodermis and rectal glands (De Lencastre et al. 2010; Martinez et al. 2008). Meanwhile, $_{p}$miR-239a/b::GFP was shown to be detectable in the neurons and intestine (De Lencastre et al. 2010). Overall, these results suggest that members of the miR-238/239ab family differ in their abundance, sensitivity to loss of ALG-1, and spatial expression patterns. These observations could underlie their previously reported opposing longevity roles (De Lencastre et al. 2010).



B

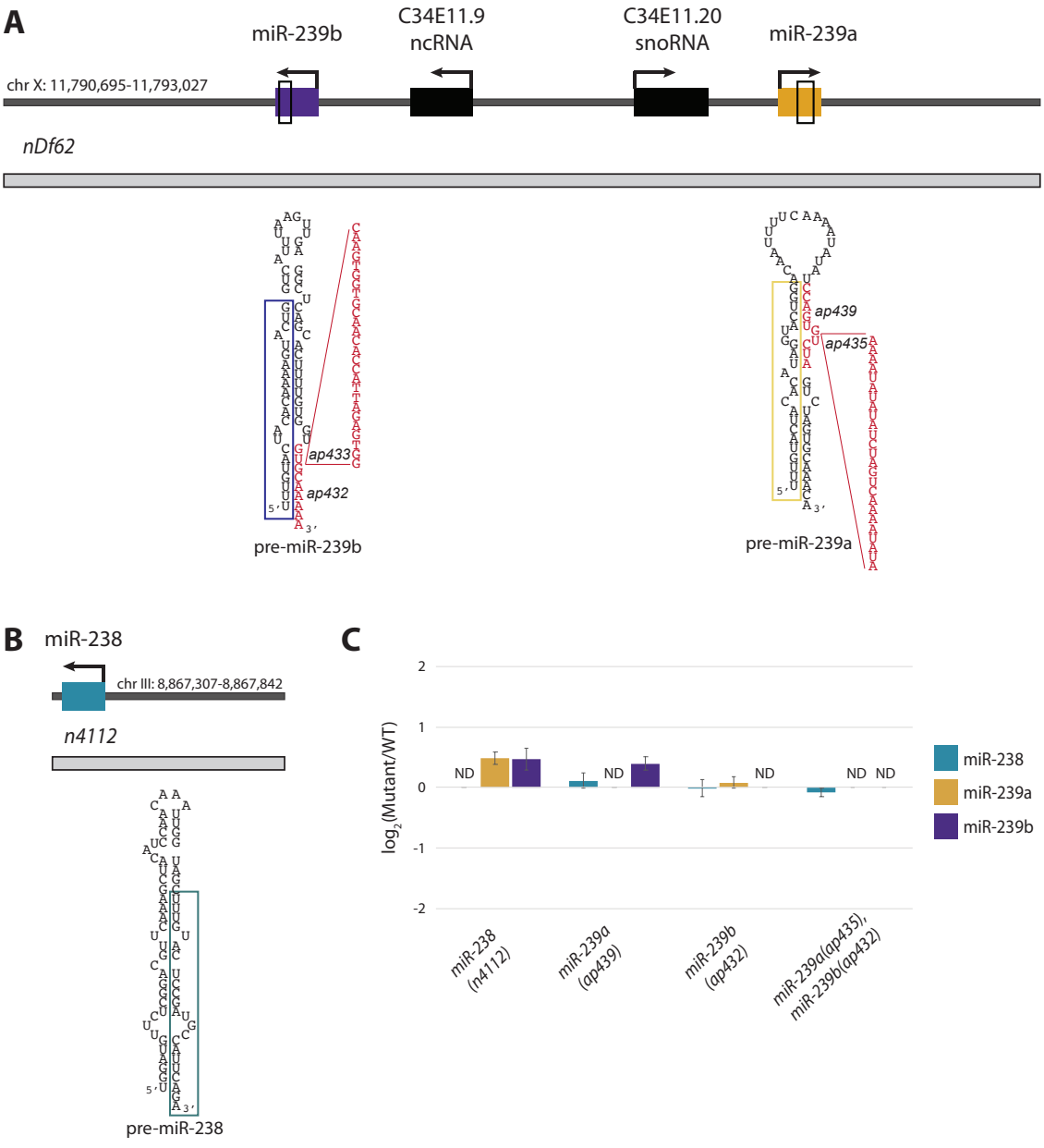
	RANK					
	TOTAL		ALG-1 RIP	ALG-2 RIP	FC in alg-1(-)	FC in alg-2(-)
	L4	d5	d5	d5	d5	d5
miR-238	20	17	20	29	↓3x	NC
miR-239a	91	37	35	49	NC	NC
miR-239b	83	41	48	54	↑2X	NC

Figure 4.1: Characterization of the expression of miR-238, miR-239a, miR-239b microRNA family members. (A) The miR-238-5p, miR-239a-3p and miR-239b-3p miRNAs share their seed sequence, nucleotides 2–7 (shaded green), but differ in other sequences, indicated in red. (B) The levels of miR-238, miR-239a, and miR-239b detected in total RNA from L4 (Schreiner et al. 2019) and day 5 adults and in ALG-1 and ALG-2 RNA immunoprecipitates (RIP) from day 5 adults (Aalto et al. 2018) are indicated by their rank compared to all other miRNAs detected with 1 being the most abundantly detected miRNA. Fold change (FC) in miRNA levels detected in day 5 *alg-1(gk214)* or *alg-2(ok304)* mutants compared to WT animals (n = 5 independent replicates, padj < 0.001 for significant FC).

4.3.2 – Levels of miR-238, miR-239a, or miR-239b are unperturbed by loss of other family members

The previous deletion (*nDf62*) used to characterize miR-239a and miR-239b deletes both miRNA sisters as well as a ncRNA and snoRNA (Figure 4.2A) (Miska et al. 2007). This is unlike the *miR-238(n4112)* allele, which disrupts the miR-238 gene and no other annotated genes in the vicinity (Figure 4.2B). To study the contribution of the individual miRNA sisters, miR-239a and miR-239b, to aging phenotypes, we used CRISPR/Cas9 to create new, precise loss of function (LOF) alleles. Due to the high sequence similarity within the mature miR-238, miR-239a, and miR-239b sequences, we targeted the pre-miRNA to disrupt miRNA processing and, thus, mature miRNA levels. Using this strategy, we made a new LOF allele for miR-239a, *miR-239a(ap439)*, two new LOF alleles for miR-239b, *miR-239b(ap432)* and *miR-239b(ap433)*, and a miR-239a and miR-239b dual LOF strain *miR-239a/b(ap435,ap432)* (Figure 4.2A). Indeed, these disruptions prevented the accumulation of mature miRNAs, as we were unable to detect miR-239a or miR-239b in their corresponding mutant backgrounds (Figure 4.2C). To test if these disruptions in miR-238, miR-239a, or miR-239b miRNA production led to compensatory expression of the other sisters, we examined the mature miRNA levels of each sister in *miR-238(n4112)*, *miR-239a(ap439)*, *miR-239b(ap432)* individual LOF strains as well as in the double mutant, *miR-239a/b(ap435,ap432)* (Figure 4.2C). Little if any change was detected for any of the miRNA sisters upon deletion of one or two members of its family, thus, confirming that we have the proper tools to assess how the loss of individual miR-238/239ab miRNA sisters contributes to aging (Figure 4.2C).

Figure 4.2: Loss of mature miR-238, miR-239a, miR-239b family members does not affect expression of sisters. (A-B) The genomic loci of miR-239a and miR-239b (A), and miR-238 (B) with surrounding genomic features. Gene directionality indicated with black arrows. The gray boxes indicate regions deleted in the *miR-239a/b (nDf62)* and *miR-238(n4112)* strains. New loss of function mutants generated in this study by CRISPR/Cas9 are indicated in red in the precursor structures; mature sequences are boxed. *miR-239a(ap439)* deletes 10 nucleotides in the 3' arm of the stem and *miR-239a(ap435)* inserts 25 nucleotides into this region. *miR-239b(ap432)* deletes 15 nt at the base of the 3' arm of the stem (not all nucleotides are shown) and *miR-239b(ap433).* deletes the GCAAAAA sequence and inserts 26 nt. (C) TaqMan RT-qPCR analysis of miR-238, miR-239a, miR-239b mature miRNA levels in WT, *miR-238(n4112)*, *miR-239a(ap439)*, *miR-239b(ap432)*, miR-*239a/b(ap435,ap432)*. The mean from 3 independent replicates is plotted; error bars represent SDs.


A
miR-239b
C34E11.9
ncRNA
C34E11.20
snoRNA
miR-239a
chr X: 11,790,695-11,793,027
nDf62
ap433
ap432
pre-miR-239b
ap439
ap435
pre-miR-239a
B
miR-238
chr III: 8,867,307-8,867,842
n4112
pre-miR-238
C
log2(Mutant/WT)
2
1
0
-1
-2
ND
ND
ND
ND ND
miR-238
miR-239a
miR-239b
miR-238 (n4112)
miR-239a (ap439)
miR-239b (ap432)
miR-239a(ap435), miR-239b(ap432)

4.3.3 – Loss of miR-238 leads to a reduced lifespan while loss of miR-239a or miR-239b has no effect on *C. elegans* lifespan

As seen in previous work, we confirmed that loss of *miR-238(n4112)* resulted in a reduced lifespan, implicating it as a positive regulator of longevity (Figure 4.3A) (De Lencastre et al. 2010). In contrast to the previously published extended lifespan attributed to loss of miR-239ab in the *nDf62* strain (De Lencastre et al. 2010), individual or coupled loss of miR-239a and miR-239b did not significantly alter lifespan compared to WT (Figure 4.3A and B). Furthermore, a strain lacking expression of the entire miR-238/239ab family had a similarly reduced lifespan as miR-238 (Figure 4.3A). Together, these data suggest that miR-238 plays an important role in aging adults, while miR-239a and miR-239b are dispensable.

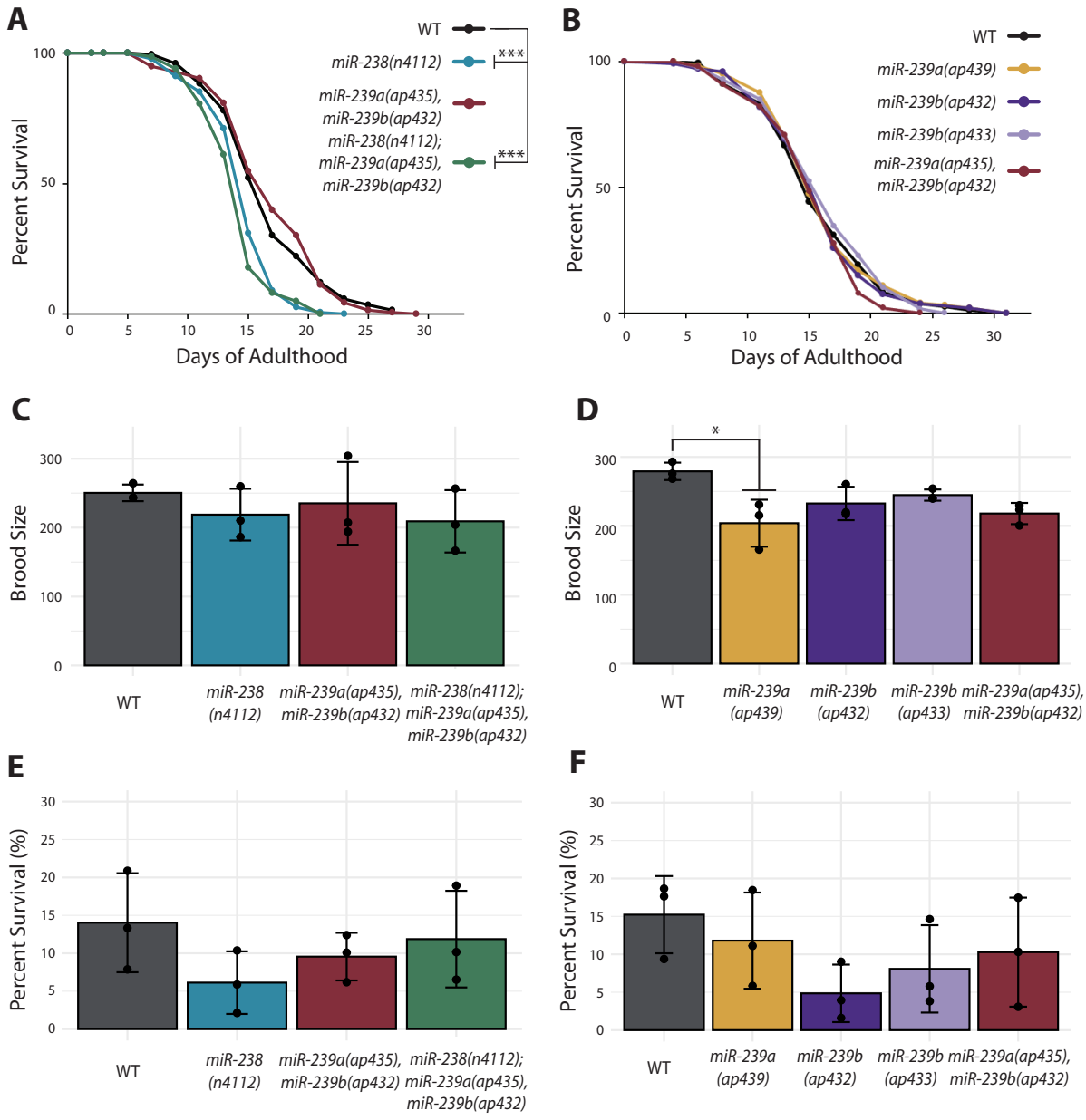
4.3.4 – The miR-238/239ab family is nonessential for fertility and heat stress recovery in early adulthood

The reduced lifespan of *miR-238(n4112)* is not apparently linked to any obvious developmental or other defects (Miska et al. 2007; De Lencastre et al. 2010). We found that the loss of miR-238 or miR-239b also had no significant effect on fertility, as judged by brood size analysis (Figure 4.3C-D). While the *miR-239a(ap439)* mutants produced slightly fewer progeny than WT animals, this difference was not observed in the double *miR-239(ap435), miR-239b(ap432)* or triple *miR-238(n4112); miR-239(ap435), miR-239b(ap432)* loss of function strains. (Figure 4.3D). Overall, the miR-238/239ab family seems to have a minor, if any, role in development and fertility under laboratory conditions.

The miR-238/239ab family has also been reported to differentially regulate stress responses. Previously, the *miR-239a/b(nDf62)* strain was shown to have increased thermotolerance and

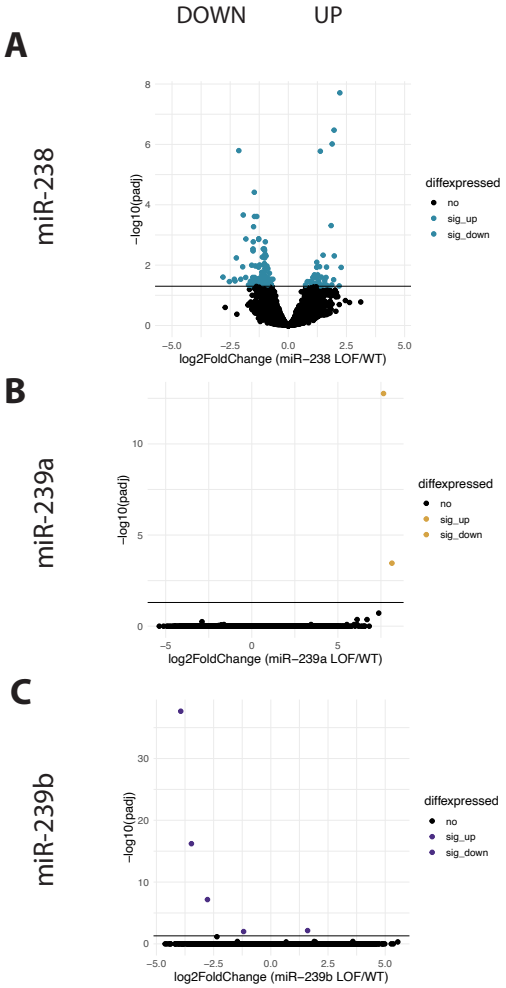
thermoresistance in adults (De Lencastre et al. 2010; Nehammer et al. 2015). While the *miR-238(n4112)* strain did not exhibit a heat shock phenotype, it was more sensitive to oxidative stress, and, conversely, *miR-239a/b(nDf62)* animals were more resistant to this stress than WT (De Lencastre et al. 2010). When we attempted to recapitulate the thermotolerance assay, which subjected day 2 adults to 12hr of heat shock at 35°C (De Lencastre et al. 2010), all animals died. However, the thermoresistance assay, where day 2 adults were exposed to 15hr of heat shock at 32°C and scored for survival after a 24 hr recovery period at 20°C, resulted in survival of WT animals at levels previously observed for this assay (Figure 4.3E and F) (Nehammer et al. 2015). While all the individual and combined mutant strains trended towards lower survival rates compared to WT, there was no statistically significant difference (Figure 4.3E and F). Taken together, the miR-238/239ab family does not obviously influence thermoresistance, as assayed here in adult *C. elegans*.

Figure 4.3: The miR-238/239a/239b sisters have distinct roles in adult *C. elegans*.
(A) Representative survival curves for WT (black), *miR-238(n4112)* (aqua), *miR-239a/b(ap435,ap432)* (maroon), and *miR-238(n4112);miR-239a/b(ap435,ap432)* (green) that show a reduced lifespan of *miR-238(n4112)* (aqua), and *miR-238(n4112);miR-239a/b(ap435,ap432)* (green) compared to WT (black). (n=5) *** P<0.0001 (log-rank).
(B) Representative survival curves for WT (black), *miR-239a(ap439)* (gold), *miR-239b(ap432)* (purple), *miR-239b(ap433)* (light purple), *miR-239a/b(ap435,ap432)* (maroon). (n=4-10) No significant difference in lifespan when compared to WT. (C-D) Results from heat shock on day 2 adults for 15 hours at 32°C followed by a 24hr 20°C recovery. Bar graph represents mean of three biological replicates, individual replicate data indicated with black dots. The error bars represent SDs. (C) *MiR-238(n4112)* (aqua), *miR-239a/b(ap435,ap432)* (maroon), and *miR-238(n4112);miR-239a/b(ap435,ap432)* (green) do not have a statistically significant difference when compared to WT (black). ANOVA and the post hoc test (Tukey's HSD). (D) *MiR-239a(ap439)* (gold), *miR-239b(ap432)* (purple), *miR-239b(ap433)* (light purple), *miR-239a/b(ap435,ap432)* (maroon) do not have a statistically significant difference when compared to WT (black). ANOVA and the post hoc test (Tukey's HSD). (E-F) Results from brood size analysis. Bar graph represents mean of three biological replicates, individual replicate data indicated with black dots. The error bars represent SDs. (C) *MiR-238(n4112)* (aqua), *miR-239a/b(ap435,ap432)* (maroon), and *miR-238(n4112);miR-239a/b(ap435,ap432)* (green) do not have a statistically significant difference when compared to WT (black). ANOVA and the post hoc test (Tukey's HSD). (D) *MiR-239a(ap439)* (gold), *miR-239b(ap432)* (purple), *miR-239b(ap433)* (light purple), *miR-239a/b(ap435,ap432)* (maroon). *P<0.05, ANOVA and the post hoc test (Tukey's HSD).


A
Percent Survival
Days of Adulthood
WT
miR-238(n4112)
miR-239a(ap435), miR-239b(ap432)
miR-238(n4112); miR-239a(ap435), miR-239b(ap432)
***
***
B
Percent Survival
Days of Adulthood
WT
miR-239a(ap439)
miR-239b(ap432)
miR-239b(ap433)
miR-239a(ap435), miR-239b(ap432)
C
Brood Size
WT
miR-238 (n4112)
miR-239a(ap435), miR-239b(ap432)
miR-238(n4112); miR-239a(ap435), miR-239b(ap432)
D
Brood Size
*
WT
miR-239a (ap439)
miR-239b (ap432)
miR-239b (ap433)
miR-239a(ap435), miR-239b(ap432)
E
Percent Survival (%)
WT
miR-238 (n4112)
miR-239a(ap435), miR-239b(ap432)
miR-238(n4112); miR-239a(ap435), miR-239b(ap432)
F
Percent Survival (%)
WT
miR-239a (ap439)
miR-239b (ap432)
miR-239b (ap433)
miR-239a(ap435), miR-239b(ap432)

4.3.5 – Non-overlapping sets of genes are mis-regulated upon loss of each miR-238/239ab family member

Given that miRNAs are post-transcriptional gene regulators that often induce degradation of their target mRNAs (Eichhorn et al. 2014; Subtelny et al. 2014; Djuranovic, Nahvi, and Green 2012), we asked if similar sets of genes would be mis-regulated upon loss of each miR-238/239ab family member. We performed transcriptomic analysis in day 5 adult animals of the individual *miR-238*, *miR-239a*, and *miR-239b* mutants, along with WT for comparison. In the *miR-238(n4112)* mutants, there was significant (padj > .05) up-regulation of 42 genes, and down-regulation of 110 genes (Figure 4.4A). In contrast, for the *miR-239(ap432)* and *miR-239b(ap435)* loss of function mutants, very few genes were found to be differentially expressed compared to WT (Figure 4.4B-D). Of the two genes mis-regulated in the miR-239a mutants, one is C34E11.20, a snoRNA adjacent to miR-239a (Figure 4.4B and D). While the *ap432* deletion does not span the annotated *C34E11.20* gene locus, most likely the genomic disruption, rather than the loss of miR-239a, affects the expression of this snoRNA. None of the mis-regulated genes in the three mutant backgrounds has a miR-238/239 binding site predicted by TargetScan (Lewis, Burge, and Bartel 2005), suggesting that the change in mRNA levels is an indirect consequence of loss of the miRNAs. While it is possible that these miRNAs primarily cause translational repression without substantial target mRNA degradation at this time point in adulthood, the lack of over-lapping downstream effects suggests that miR-238, miR-239a and miR-239b mostly regulate different genes. Furthermore, these data reflect the lifespan phenotypes with loss of miR-238 resulting in a greater extent of gene mis-regulation and a reduced lifespan and loss of miR-239a or miR-239b having almost no effect on gene expression and longevity.



Top 5 DOWN in *miR-238(n4112)*	Top 5 UP in *miR-238(n4112)*
ZC317.7	ilys-3
acbp-6	oac-54
grd-4	pqn-36
ugtp-1	kin-36
F23D12.11	clec-82

Top 5 DOWN in *miR-239a(ap439)*	Top 5 UP in *miR-239a(ap439)*
-	C34E11.20
-	spe-12
-	-
-	-
-	-

Top 5 DOWN in *miR-239b(ap432)*	Top 5 UP in *miR-239b(ap432)*
Y102A5C.6	nspc-18
Y102A5C.5	-
Y102A5C.36	-
ZC84.3	-
-	-

Figure 4.4: Non-overlapping sets of genes are mis-regulated upon loss of each miR-238/239a/b family member. Volcano plots representing gene expression changes upon the loss of *miR-238(n4112)* (A), *miR-239a(ap439)* (B), and *miR-239b(ap432)* (C) compared to WT in day 5 adult *C. elegans* in three independent replicates. Colored dots (aqua for *miR-238*, gold for *miR-239a*, and purple for *miR-239b*) represent genes with a padj < 0.05. Tables list the top genes up and down regulated in each background.

4.3.6 – The longevity role of miR-238 can be replaced by miR-239a or miR-239b

Despite belonging to the same miRNA family, the loss of miR-238 results in a reduced lifespan with many transcripts mis-regulated, while the loss of miR-239a or miR-239b causes no effect on lifespan and mis-regulation of very few genes (Figure 4.3A-B, Fig4.4A-C). The differences in the roles of the miRNA family could be due to differences in expression or in target RNA interactions due to differences in their 3' end sequences (Figure 4.1A), or a combination of both. To investigate these possibilities, we used CRISPR/Cas9 to replace the endogenous pre-miR-238 with the sequence for pre-miR-239a and miR-239b (Figure 4.5A). In these newly created miRNA family swap strains, we correspondingly saw no expression of miR-238, as in the miR-*238(n4112)* LOF strain (Figure 4.5B). As well, preliminary results show an increased level of miR-239a and miR-239b in the corresponding swapped strains, suggesting that they were expressed from the miR-238 locus in addition to the endogenous gene (Figure 4.5B).

With evidence that our new strains successfully replaced miR-238 with miR-239a or miR-239b, we asked if expression of these miRNAs from the miR-238 locus could compensate for the loss of miR-238. First, we looked at gene expression data for some of the top genes up-regulated in *miR-238(n4112)* in the $_{p}$*miR-238::miR-239a* and $_{p}$*miR-238::239b* strains (Figure 4.5A). While more replicates need to be analyzed, preliminary data show that *oac-54* and *pqn-36* trend towards up-regulation in *miR-238(n4112)* via RT-qPCR, validating the RNA-seq results (Figure 4.5C). However, in the $_{p}$*miR-238::miR-239a* and $_{p}$*miR-238::239b* strains, *oac-54* and *pqn-36* are no longer up-regulated and may even be down-regulated compared to WT. These results suggest that miR-239a or miR-239b expressed from the *miR-238* locus can reverse the up-regulation of these two genes caused by loss of miR-238 (Figure 4.5C).

Moreover, we asked if replacement of miR-238 with miR-239a or miR-239b would prevent the reduced lifespan caused by loss of miR-238. When we performed lifespan analyses, we found that the *$_p$miR-238::miR-239a* and *$_p$miR-238::239b* strains had survival curves indistinguishable from that of WT animals and were significantly longer lived than the *miR-238(n4112)* strain (Figure 4.5D). Overall, these data show that expression of miR-239a or miR-239b from the *miR-238* locus can rescue the reduced lifespan associated with loss of miR-238. This suggests that it is differences in expression, and not the 3' sequences, that drive the distinct roles in aging of the miR-238, miR-239a, and miR-239b miRNAs.

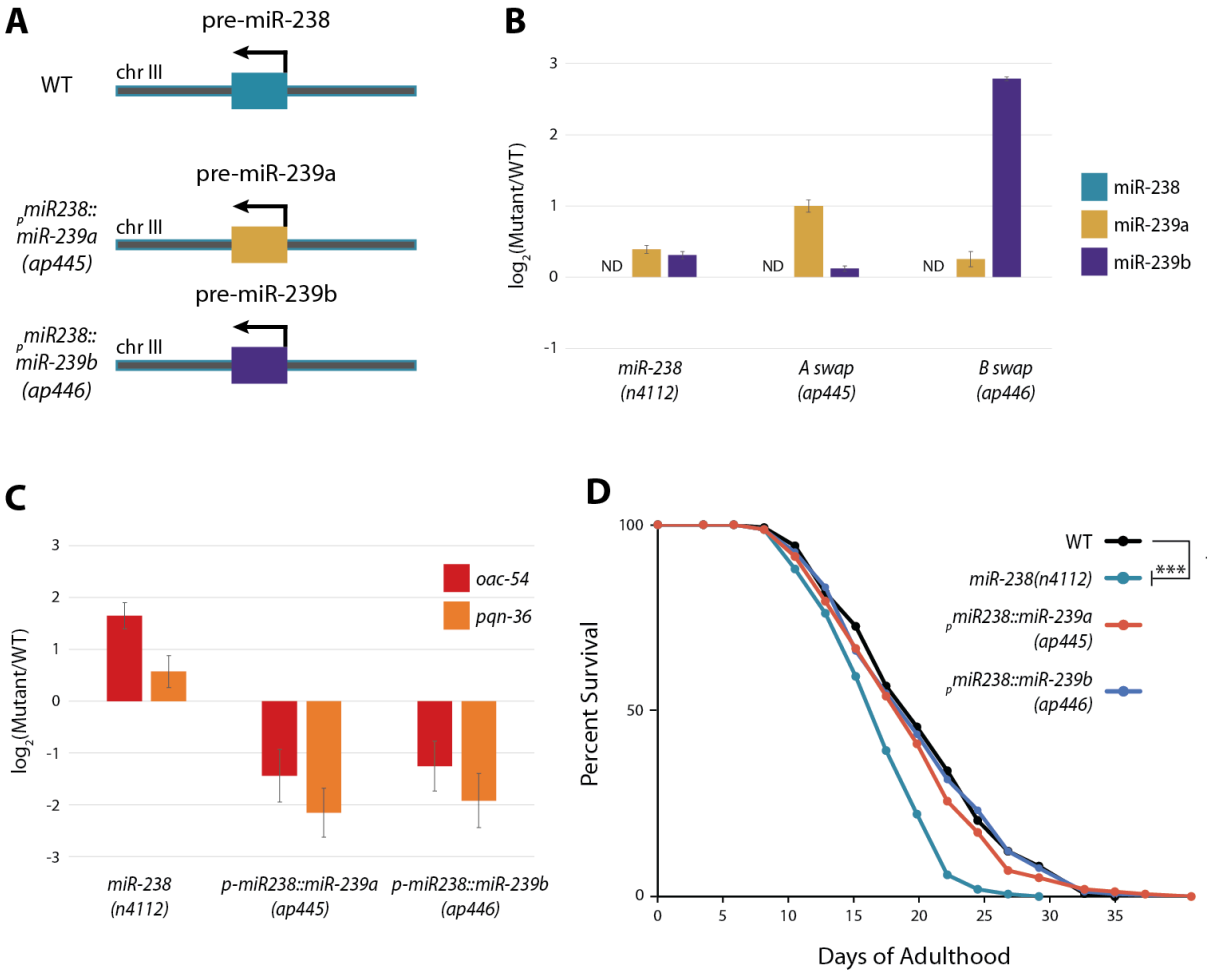


Figure 4.5: The longevity role of miR-238 can be replaced by miR-239a or miR-239b. (A) Schematic of the miR-238 locus in WT ($_p$miR-238::miR-238) (top), in the $_p$miR-238::miR-239a *(ap445)* strain (middle), and the $_p$miR-238::miR-239b *(ap446)* strain (bottom). (B) TaqMan RT-qPCRs of miR-238, miR-239a, miR-239b mature miRNA levels in WT, *miR-238(n4112)*, $_p$miR-238::miR-239a *(ap445)*, and $_p$miR-238::miR-239b *(ap446)*. The mean from 2 independent replicates is plotted; error bars represent SDs. (C) RT-qPCR for *oac-54* and *pqn-36* in *miR-238(n4112)*, $_p$miR-238::miR-239a *(ap445)*, and $_p$miR-238::miR-239b *(ap446)* compared to WT. The mean from 2 independent replicates is plotted; error bars represent SDs.
D) Representative survival curves for WT (black), *miR-238(n4112)* (aqua), $_p$miR-238::miR-239a *(ap445)* (blue), and $_p$miR-238::miR-239b *(ap446)* (coral) showing that loss of miR-238 is rescued by expression of miR-239a or miR-239b from the miR-238 locus. (n=2) *** P<0.0001 (log-rank).

4.4 - Discussion

Here we show that members of the miR-238, miR-239a, and miR-239b miRNA family have distinct roles in aging; the loss of miR-238 leads to a reduced lifespan, while the loss of miR-239a or miR-239b individually does not affect *C. elegans* longevity. Previously, miR-239a and miR-239b had been considered negative regulators of aging and heat shock response because a deletion of miR-239a, miR-239b, a ncRNA and a snoRNA resulted in an increased lifespan and heat shock survival (De Lencastre et al. 2010). With the precise mutants we created, we see that loss of just miR-239a and miR-239b has no effect on lifespan. As well, loss of miR-238, miR-239a, miR-239b has no effect on heat shock survival in adult *C. elegans*. Consistent with the lifespan phenotypic results, loss of miR-238 in adulthood leads to many misregulated genes, while loss of miR-239a or miR-239b leads to only a handful of non-overlapping genes being misregulated. We show that the loss of miR-238 can be rescued by inserting the miR-239a or miR-239b sequence into the endogenous miR-238 locus. Given miR-238, miR-239a, and miR-329b are differentially expressed in aging *C. elegans* (De Lencastre et al. 2010), this suggests that expression, not 3' sequence, drives the differences of miR-238, miR-239a, and miR-239b in aging.

4.4.1 Distinct expression of miR-238, miR-239a, miR-239b family in Aging

The miR-238, miR-239a, and miR-239b miRNAs were all initially implicated in aging because of their increase in expression over *C. elegans* adulthood (De Lencastre et al. 2010). Our work corroborates these results but also highlights the differences in the expression of miR-238/miR-239a/b during *C. elegans* adulthood (Figure 4.1). While the whole family increases in ranking of expression relative to other miRNAs, it is done to varying degrees. Additionally,

members of the miR-238/miR-239a/b family are differentially susceptible to the loss of the main miRNA AGO protein, ALG-1: miR-238 is down, miR-239a does not change, and miR-239b is up (Figure 4.1B). Differences in their adulthood expression is even further underlined by the differences in their spatial expression. Previous work showed that $_{p}$miR-238::GFP is expressed nearly ubiquitously in adult *C. elegans,* with highest levels detected in the intestine, hypodermis and rectal where $_{p}$miR-239a/b::GFP fluorescence was readily detectable in the intestine and the neurons (Martinez et al. 2008; De Lencastre et al. 2010). These differences in expression suggest distinct transcriptional control mechanisms. In fact, modENCODE data show differences in transcription factor biding profiles for this miRNA family (Gerstein et al. 2010). So, while miR-238, miR-239a, and miR-239b share their seed sequences, they hold distinct expression patterns in adult *C. elegans*.

4.4.2 Expression as a determinant of miRNA function

Since the miRNA seed region is so important for miRNA targeting, it is often assumed miRNAs that share a seed sequence function redundantly. Our precise loss of function mutants show that is not true for the miR-238/239a/b family in the context of aging. Indeed, this has been shown for other miRNA families in other contexts, such as the let-7/miR-48/84/241/795 miRNA family. Despite miRNA sisters with similar expression levels and spatial expression, loss of let-7 leads to a lethal vulval bursting phenotype during *C. elegans* development (Reinhart et al. 2000; Slack et al. 2000). This is due to additional base-pairing of the 3' end of the let-7 miRNA to specific target sites, giving let-7 target specificity (Broughton et al. 2016; Brancati and Großhans 2018). While the miR-238/239a/b family has 3' sequence differences, they also have extensive expression differences (Figure 4.1) (Martinez et al. 2008; De Lencastre et al. 2010). It has been

shown that miRNA expression pattern is also a key part of miRNA-target interaction, as a miRNA's spatial expression determines its target's spatial expression and higher expression of a miRNA can lead to repression of targets that have imperfect seed matches (Sood et al. 2006; Brancati and Großhans 2018).

Given the different expression patterns for miR-238, miR-239a, and miR-239b in aging, we explored if expression differences led to their distinct aging roles. Indeed, this study showed that either miR-239a or miR-239b expressed from the endogenous *miR-238* locus was able to rescue the *miR-238* mutant reduced lifespan phenotype (Figure 4.5D). Furthermore, we show that not only is the lifespan phenotype rescued but so is the repression of two genes up-regulated in the loss of *miR-238* mutants (Figure 4.5A,C). Together, these data show that differences in expression, not miRNA sequence, drive the different roles of the miR-238/239a/239b family in aging.

4.4.3 The role of non-coding RNAs in Aging

While many miRNAs have been implicated in aging, the role of other ncRNAs are not as well understood. While this study focused on the role of the miR-238/239a/b family in aging, the differences between the *nDf62* deletion allele (removes miR-239a, miR-239b, a ncRNA and a snoRNA) used in previous studies and the precise deletion mutants' phenotypes raises the question of what is causing the extended lifespan of the *nDf62* allele if not miR-239a and miR-239b? This implicates either the ncRNA (C34E11.9) or snoRNA (C34E11.20) in regulating lifespan. Individual snoRNAs have been implicated in lifespan regulation. Loss of the snoRNA *jouvance* in Drosophila leads to a reduced lifespan, while in *C. elegans* nucleolus size, the compartment in which snoRNAs reside and function, is correlated with lifespan (Soulé et al.

2020; Tiku et al. 2016). For long non-coding RNAs (lncRNAs), some have been implicated in many processes such as senescence, differentiation, and aging related disease across species. In *C. elegans* the lncRNA *tts-1* is required for the longevity conferred by the loss of the insulin/IGF-1 receptor, *daf-2* (Kour and Rath 2016; Essers et al. 2015). But, further studies are needed to study the aging role, if any, of C34E11.9 (ncRNA) and C34E11.20 (snoRNA).

While the role of miRNAs in *C. elegans* aging has been studied for decades, there is still more to learn. The complex nature of miRNA targeting makes target and miRNA function prediction difficult. Overall, this study highlights the role of expression in miRNA target determination and this study establishes that the distinct roles in aging for miR-238, miR-239a, and miR-239b are driven by the differences in their expression.

4.5 Experimental Procedures

Nematode culture and lifespan analyses

C. elegans strains were cultured under standard conditions and synchronized by hypochlorite treatment (Wood 1988). Lifespan analyses were conducted at 20˚C in the absence of FUdR, as previously described (Dillin, Crawford, and Kenyon 2002). Embryos were plated on NGM plates containing OP50 and the first day after the L4 stage was regarded as adult day 0. Worms were picked on fresh food every other day until reproduction ceased and scored for viability every 2 to 3 days. Animals that died by bagging, bursting, or crawling off the plates were censored. JMP IN 16 software was used for statistical analysis and P-values were calculated using the log-rank (Mantel-Cox) method.

Quantitative RT-PCR

RT-PCR analyses of mRNA (SYBR Green) and miRNA (TaqMan) levels were performed according to manufacturer's instructions with the StepOnePlus and QuantStudio 3 Real-Time PCR Systems (Applied Biosystems). Levels were normalized to Y45F10D.4 for mRNAs and U18 snoRNA for miRNAs.

RNA-seq

RNA was collected from day 5 wildtype (N2), and alg-1(gk214) animals grown at 20°C after L1 synchronization. An Illumina TruSeq mRNA Library Prep Kit was used to generate poly(A) selected RNA-seq libraries. Illumina NovaSeq Sequencing data was analyzed by mapping reads to WBcel282 assembly of the C. elegans genome using STAR (Dobin et al. 2013). Reads were then quantified using featureCounts using WBcel282 gene annotations (Liao, Smyth, and Shi 2014). Differential expression was calculated using DESeq2 (Love, Huber, and Anders 2014).

smRNA-seq

Small RNA sequencing was performed on five independent replicates of synchronized wildtype (N2), *alg-1(gk214)*, and a*lg-2(ok304)* strains collected on day 5 of adulthood. Strains were cultured at 20°C to day five of adulthood and collected for RNA isolation. Eggs and progeny were separated from adult worms through daily washes with M9 solution, followed by gravity separation of pelleted adult worms from the supernatant containing eggs and progeny. The supernatant was aspirated and M9 washed, this was repeated until the M9 remained clear. Total RNA was isolated and smRNA libraries were then prepared from 1 μg of total RNA from

samples of five independent replicates using the Illumina TruSeq Small RNA Library Prep Kit. Once prepared, smRNA libraries were sent for single-end sequencing on an Illumina HiSeq 4000. Adapter sequences were removed using Cutadapt, and smRNA reads were mapped to the annotated *C. elegans* genome (WS266) using Bowtie-build to first create indices and miRDeep2 to align and quantify reads (Friedländer et al. 2012; Langmead et al. 2009). Differential expression analysis was performed by first normalizing reads to library size (read counts per million) and then measuring the log2foldchange of mutants to WT strains within replicates. MiRNAs were called significantly misregulated if they exhibited an absolute mean log2foldchange greater than 1.5 and a padj less than 0.05.

Brood size assays

Between 5-9 individual L4 *C. elegans* of each genotype were moved to individual plates seeded the day prior with OP50. Every date post reaching adulthood, the parental adult *C. elegans* was moved to a new plate, and the eggs were counted. This was done until the end of the reproductive span of the individual *C. elegans*, for then N2 and the other genotypes we assayed, that was D4.

Nematode culture and heat shock viability assays

Adult head shock experiments were carried as described in De Lencastre *et al.* and Nehammer *et al.* with minor alterations, such as not using FuDr to stop progeny production. (Nehammer et al. 2015; De Lencastre et al. 2010). In more detail, for the de Lencastre *et al*. thermotolerance protocol: *C. elegans* strains were cultured under standard conditions and synchronized by hypochlorite treatment (Wood 1988). Heat shock viability assays were performed by plating bleach synchronized L1 worms rocked at 20°C overnight on UV treated

small NGM plates seeded with OP50 the day before. Worms were grown until L4, then for an additional 36 hours at 20°C before raising the temperature to 35°C for 12 hrs of heat shock. Assays were blinded before heat shock and were unblinded only after scoring viability. For the Nehammer *et al*. thermoresistance protocol: Gravid adults were allowed to egg lay for a 2-hour period to produce relatively synchronized populations of progeny at 20°C on UV treated NGM plates seeded with OP50 the day before. From the mid-point of the egg lay, worms were grown for 86 hours, during the first day of adulthood worms were moved to new UV treated small NGM plates seeded with OP50 the day before. Those adult worms were then incubated at 35°C for 12 hrs of heat shock. Worms recovered for 24hrs at 20°C before scoring.

For all heat shock experiments, at least 100 worms were subjected to heat shock and at least 100 worms were subjected to control conditions (kept at 20°C for entirety of experiment) per strain per replicate. No more than 20 worms were allowed on a single small NGM plate.

Strain Generation

To make PQ636 *miR-239a*(*ap439*), PQ592 *miR-239b*(*ap432*), PQ593 *miR-239b*(*ap433*) and PQ600 *miR-239b*(*ap432*),*miR-239a*(*ap435*) strains young adult wildtype worms (N2) were injected following methods described in Paix *et al.* with modifications suggested by the Dernburg lab (Paix et al. 2015). The injection mix included 0.5 uL of *dpy-10* crRNA (100uM), 1.0 uL of the appropriate crRNA, 2.5 uL of tracrRNA (100uM), and 7uL of Cas9 (40uM). Worms were grown at 25°C. 3 days later, *dpy + C. elegans* were singled onto new plates and PCR screened. Insertion was confirmed by Sanger sequencing. Successful deletions were backcrossed three times to N2 then homozygous.

To make the PQ679 - *miR-238(ap445[PmiR-238::pre-miR-239.1::miR-238 UTR] III);* and PQ680 - *miR-238(ap446[PmiR-238::pre-miR-239.2::miR-238 UTR] III);* strains, young adult wildtype worms (N2) were injected following methods for dsDNA asymmetric-hybrid donors as described in Dokshin *et al*. (Dokshin et al. 2018). The injection mix included 5µg Cas9 protein, 2mg tracrRNA, 1.12µg crRNA, 800ng pRF4::rol-6 plasmid and 4µg of a dsDNA donor cocktail. Homology arms were 120bp long.

Cas9 protein, tracrRNA, and crRNA were ordered from IDT. Worms were grown at 20°C, around 20 animals were injected per strain. 4-5 days later, F1 rollers were singled onto new plates as well as non-roller siblings from the same plate. After laying progeny, F1 were lysed and PCR screened for integration of the pre-miR-239a or pre-miR-239b sequence into the miR-238 locus. Insertion was additionally confirmed by Sanger sequencing. A successful integrant was backcrossed 5x to N2 to generate PQ679 and PQ680.

Sequences for crRNA guide strands ordered from IDT are listed below:

miR-238 crRNA (CD.Cas9.GBJV1265.AE)

- AUU CAG AUA GUU AUG AGC CA (UGG)

miR-239a crRNA (CD.Cas9.DYNF1483.AA)

- GUU UGC ACU AGA CUA GAC AC (UGG)

miR-239b crRNA (CD.Cas9.QTXM3347.AC)

- ACU UUU GUG GUG UGC AAA AA (UGG)

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Chapter 4, in full, is a reprint as it prepared for submission to PLoS Genetics, "Expression, not sequence, underlies the differential roles of a miRNA family in *Caenorhabditis elegans* aging" by Chipman, L.B., Luc, S.K., Nicastro, I.A., Hulahan, J.J., and Pasquinelli A.E., 2022. I was the primary author.

CHAPTER 5: Conclusions

5.1 – The role of the miRNA pathway in aging

The role of miRNA regulation in aging has been studied for nearly two decades since the very first miRNAs were discovered in *C. elegans* in the 1990s. The first known miRNA, lin-4, was later implicated as a positive regulator of aging (Boehm and Slack 2005). Since then, continued research has not only implicated other miRNAs as regulators of aging but also uncovered more of the mechanism of how these miRNAs are regulating aging. Through genetic work, miRNAs have been implicated in working in certain aging related pathways, the Insulin/IGF-2 signaling, dietary restriction, autophagy, proteostasis, and more (T. Smith-Vikos and Slack 2012; Elder and Pasquinelli 2022). In fewer cases, direct targets have been implicated in an individual miRNA's regulation of aging. Yet, there is still work to be done to connect the molecular mechanisms of the miRNA pathway to its phenotypic outcomes.

The more we continue to research into the molecular mechanisms of miRNAs in aging and in other contexts, the more we appreciate the breadth of miRNA regulation. It has become apparent that miRNA regulation is capable of base-pairing in a myriad of ways to target RNAs, including many interactions without perfect seed matches (Chi, Hannon, and Darnell 2012; Broughton et al. 2016). As well, miRNAs have been shown to have functional target sites within mRNA coding regions, in addition to targeting other ncRNAs (Zisoulis et al. 2012; Zhang et al. 2018). My work with the miR-238, miR-239a, and miR-239b miRNAs, underscores the importance of miRNA expression in miRNA target determination and function. While members of the miR-238/239a/b family share seed sequences, miR-238 is a regulator of aging (loss of miR-238 leads to a reduced lifespan) while miR-239a and miR-239b are largely dispensable

(Figure 4.3A-B). Yet miR-239a and miR-239b inserted into the miR-238 endogenous locus rescues the reduced lifespan caused by loss of miR-238 (Figure 4.5D).

While measuring the phenotypic effects of mutants and various other perturbations on aging is relatively simple, the tools used to study mutants and make molecular perturbations can greatly affect how we understand aging. As seen with miR-239a and miR-239b, the previously used deletion allele (*nDf62*) removed both miRNAs, as well an ncRNA and snoRNA (Figure 4.2A) (De Lencastre et al. 2010). This deletion had an extended lifespan and increased heat shock survival in adult *C. elegans* (De Lencastre et al. 2010; Nehammer et al. 2015), while precisely generated mutants of miR-239a and miR-239b did not (Figure 4.2A, Figure 4.3). Interestingly, this implicates the snoRNA and/or ncRNA as a regulator of aging. In addition, initial experiments characterizing the main somatic miRNA AGOs, ALG-1 and ALG-2, in aging used RNAi and concluded that loss of either AGO reduced lifespan (Samuelson, Carr, and Ruvkun 2007). Yet, loss of function mutants of *alg-2* had an extended lifespan, and it was found that the initial RNAi used, due to homology between *alg-1* and *alg-2*, was affecting expression of both AGOs (Aalto et al. 2018). It was found that these phenotypic differences of *alg-1* and *alg-2* were associated with underlying molecular differences (Aalto et al. 2018). I found that these AGOs have different associations with miRNAs and cause differential misregulation of miRNAs upon their loss, suggesting they affect their distinct roles in aging by differential association with and regulation of miRNAs (Figure 3.2) (Aalto et al. 2018).

Considering the growing factors underlying miRNA targeting and miRNA regulation of aging, more nuances are likely to be uncovered with further research. There are still many open questions remaining. How are miRNAs and the miRNA machinery in aging regulated? For example, circular RNAs (circRNAs) increase in *C. elegans* aging while miRNAs generally

decrease (Cortés-lópez et al. 2018; De Lencastre et al. 2010). Since circRNAs are also known to act at miRNA sponges (Hansen et al. 2013), could they be regulating miRNAs in aging? For some aging related miRNAs, the underlying direct targets, and genetic pathways important for the phenotypic outcome have been uncovered; what about the others? What is the contribution of translational inhibition to the miRNA pathway's regulation of aging?

5.2 – Further Studies

MiRNAs, though regulators themselves, are subject to many forms of regulation: transcription factors can regulate transcription of pri-miRNAs, let-7 autoregulates its own biogenesis, extensive base pairing of miRNAs to targets can lead to degradation of the miRNA through Target RNA-Direct miRNA Degradation (TDMD) and more (Finnegan and Pasquinelli 2013; Zisoulis et al. 2012; Chipman and Pasquinelli 2019). Given the importance of miRNA expression in miRNA targeting, understanding expression patterns and how miRNAs are regulated can give insight into the molecular mechanisms of miRNAs in aging.

For miR-238, miR-239a, and miR-239b, we see extensive differences in transcriptional reporters, as well as sensitivities to the loss of *alg-1* (Figure 4.1) (Martinez et al. 2008; De Lencastre et al. 2010). Additionally, modENCODE data shows differences in transcription factor biding profiles for this miRNA family (Gerstein et al. 2010). Investigation into how differential transcription factor binding affects miRNA expression, both overall and their spatial expression, could give insights into how these miRNAs are differentially regulated.

In addition to the my work looking at the expression of miR-238/239a/b in *C. elegans* adulthood, work during larval development also underscores the dynamic regulation of the miR-238/239a/b family (Alberti et al. 2018; Brosnan, Palmer, and Zuryn 2021). Alberti *et al*. used *in*

vivo enzymatic cell-type specific labeling with high-throughput sequencing in L1 *C. elegans* to assay for enrichment of mature miRNAs in neurons, pharynx, intestine, and body wall muscle, and saw the miR-238/239a/b family enriched in all tissues assayed (Alberti et al. 2018). Meanwhile, Brosnan *et al*. analyzed the *in vivo* cell-type specific loading of miRNAs in AGOs by performing cell-type specific immune-precipitation of ALG-1 and ALG-2 with high throughput sequencing in the neurons, intestine, and body wall muscle in L4 *C. elegans* (Brosnan, Palmer, and Zuryn 2021). Interestingly, miR-238 and miR-239a showed no tissue enrichment with ALG-2, but were enriched with ALG-1 in the intestine, while miR-239b was enriched with ALG-1 and ALG-2 (Brosnan, Palmer, and Zuryn 2021). For the miR-238/239a/b family, mature miRNA spatial expression and its association with AGO is uncoupled in larval development and differs from the transcriptional activity in adult *C. elegans* (Brosnan, Palmer, and Zuryn 2021). Using the techniques implemented by Alberti *et al.* and Brosnan *et al*. in aging *C. elegans* would give us data in the spatial expression of the mature miR-238/239a/b family as well as their association with AGO. This would allow us to better understand the importance and role of tissue specific expression vs. overall expression levels in the miR-238/239a/b family, as well as in other aging related miRNAs. Furthermore, paired with the larval data, we could see the dynamics of miRNA spatial expression over time.

Another area of future study is identifying the direct RNA targets and genetic pathways behind the miR-238 aging phenotype. De Lencastre *et al*. tested whether the loss of miR-238 would be affected by the loss of the insulin receptor, *daf-2* and found miR-238 was not necessary for longevity induced by loss of *daf-2* (De Lencastre et al. 2010). That the loss of miR-238 did not affect the long lifespan of loss if *daf-2* suggests that miR-238 functions upstream of *daf-2* or through an independent pathway thus further research is needed (De Lencastre et al. 2010).

Identification of key direct targets would greatly aid in understanding how loss of miR-238 leads to a reduced lifespan. Typically, this is accomplished by mutation of the miRNA binding site in a key target. For example, the reduced lifespan upon the loss of lin-4 is phenocopied by a mutation of its miRNA biding site in the 3'UTR of *lin-14,* which implicated it as a direct target (Boehm and Slack 2005). Identification of potential functionally important miRNA targets is varied: crosslinking and immunoprecipitation (CLIP)-seq is a high throughput method to identify RNA that directly interacts with AGOs but is technically challenging, CRIPSR-based 3'UTR mutagenesis screens have been proven to identify functionally relevant miRNA targets, and RNA-seq is used to see genes misregulated upon the loss of a given miRNA (Froehlich et al. 2021; Chi et al. 2009). Since miRNAs canonically destabilize their target RNAs, up-regulated genes in the RNA-seq dataset are implicated as potential direct targets, especially if they contain possible miRNA binding sites. But in RNA-seq, indirect targets, which can be up- and down-regulated, are also detected. While learning both direct and indirect targets is useful for identifying genetic pathways a miRNA may be working within, it also makes it ambiguous as to which are direct vs indirect targets. I used RNA-seq to study the roles of miR-238, miR-239a, and miR-239b and found dramatically different gene misregulation profiles in adulthood, which paralleled their lifespan data (Figure 4.4, Figure 4.3A-B).

One caveat of using RNA-seq, is that it only will show genes with expression changes upon the loss of a miRNA. While destabilization of target RNA is the dominant outcome of miRNA regulation in somatic tissues, this is not always the case in every context (Eichhorn et al. 2014; Subtelny et al. 2014; Djuranovic, Nahvi, and Green 2012). In zebrafish embryonic cells, it's been shown that miRNA repression leads to reduced translation but no target destabilization (Bazzini, Lee, and Giraldez 2012). Using techniques like ribosome profiling, or fluorescently tagged

reporters of direct miRNA targets could give insights into the role of target translational repression in aging *C. elegans*.

The *alg-2* extended lifespan phenotype is an interesting candidate where targets may be subject to translational decay but not destabilization (Aalto et al. 2018). Upon the loss of *alg-1,* at day 5 of adults there is significant up-regulation of 3,184 and down-regulation of 5,742 genes in the *alg-1(gk214)* mutant compared to WT. Meanwhile, in the loss of *alg-2* strain only 81 and 133 genes were up- or down-regulated, respectively, in *alg-2(ok304)* mutants compared to WT (Aalto et al. 2018). Given that ALG-1 and ALG-2 are expressed to similar levels at the day 5 timepoint and both have effects on lifespan, albeit divergent, it was surprising to see such a discrepancy (Aalto et al. 2018). Does ALG-2 just have less direct or indirect targets then ALG-1? Or are there some direct targets that are subject to translational repression? Alternatively, *alg-2* could be working in a tissue specific manner, repressing genes in certain tissues whose expression change may not be picked up in a global assay but are none the less important to the extended lifespan phenotype.

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