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# Identifying MicroRNAs Responsible for the Opposite Longevity Roles of *Caenorhabditis elegans* Argonautes

A thesis submitted in partial satisfaction of the requirements for the degree

Master of Science

in

Biology

by

Jesse Hulahan

Committee in charge:

Professor Amy Pasquinelli, Chair
Professor Sreekanth Chalasani
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Chair

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2019

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ABSTRACT OF THE THESIS

Identifying MicroRNAs Responsible for the Opposite Longevity Roles of *Caenorhabditis elegans* Argonautes

by

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Master of Science in Biology

University of California San Diego 2019

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MicroRNAs (miRNAs) are small non-coding RNAs that negatively regulate gene expression through the binding of an Argonaute (AGO) protein to facilitate the translational repression and degradation of a target messenger RNA (mRNA). In *Caenorhabditis elegans*, Argonaute-like Gene 1 (ALG-1) and ALG-2 act as the main AGOs in the miRNA-silencing pathway (He and Hannon, 2004; Jonas and Izaurralde, 2015). ALG-1 and ALG-2 were previously thought to function redundantly during development, but loss of these AGO proteins

in adulthood results in opposing lifespan phenotypes with *alg-1* and *alg-2* mutants reducing or increasing longevity, respectively (Aalto & Nicastro et al., 2017). The divergent lifespans observed in *alg-1* and *alg-2* mutants were shown to be associated with the misregulation of several aging-associated miRNAs, such as lin-4 and miR-239a/b, which differentially regulate longevity through the IGF-1/Insulin signaling (IIS) pathway (Boehm and Slack, 2005; de Lencastre et al., 2010; Aalto & Nicastro et al., 2017). Since there are several hundred annotated miRNAs in the *C. elegans* genome, I hypothesized that there are likely many more miRNAs that contribute to the differential lifespans of *alg-1* and *alg-2* mutants. — To identify these miRNAs, I performed Small RNA profiling on *alg-1* and *alg-2* mutants during adulthood. Through smRNA profiling, I was able to identify 51 and 19 significantly misregulated miRNAs in *alg-1* and *alg-2* backgrounds, respectively. Target prediction analysis of several misregulated miRNA was able to identify many gene targets known to regulate longevity though in the IIS pathway. For instance, *miR-355-5p* was found to be differentially expressed in *alg-1* and *alg-2* mutants and was predicted to target 102 longevity suppressing genes in the IIS pathway; these observations are consistent with the differential lifespans observed in *alg-1* and *alg-2* mutants. miRNA-mediated regulation of aging-associated pathways, such as the IIS pathway, is a highly conserved phenomenon in many eukaryotes, including humans (Lopez-Otin C, 2013; Guarente L & Kenyon C, 2000; Kenyon CJ, 2010). By understanding how perturbation of ALG-1 and ALG-2 differentially regulates aging in *C. elegans*, we can further our understanding of the molecular basis of aging as a whole.

# INTRODUCTION

## 1.1 miRNA Biogenesis, Function, & Importance

MicroRNAs (miRNAs) are small, ~22 nucleotides long, noncoding RNAs which post-transcriptionally regulate gene expression through the miRNA-induced silencing pathway (He and Hannon, 2004; Jonas and Izaurralde, 2015). miRNAs are first transcribed as long nascent transcripts in the nucleus called primary-miRNAs (pri-miRNAs) (He and Hannon, 2004). In the nucleus they are processed by the ribonuclease-III (RNase III) enzyme Drosha into a smaller, ~70 bps, stem-looped structure called the precursor-miRNA (pre-miRNA) (He and Hannon, 2004). pre-miRNAs are then shuttled from the nucleus into the cytoplasm by Exportin 5 (He and Hannon, 2004). In the cytoplasm they undergo a second round of processing by a secondary RNase III enzyme, Dicer, and are cleaved into a small dsRNA duplex (miRNA:miRNA*) consisting of the mature miRNA, and a complementary passenger strand (miRNA*) (He and Hannon, 2004). The mature miRNA is bound by an Argonaute (AGO) protein, which stabilizes the mature miRNA strand, whereas the miRNA* strand is unbound and degraded (He and Hannon, 2004; Winter and Diederichs, 2011; Khvorova et al. 2003; Schwarz et al, 2003). When a mature miRNA is bound by an AGO protein it forms the miRNA-induced silencing complex (miRISC) (He and Hannon, 2004; Jonas and Izaurralde, 2015). This complex can include other cofactors such the GW182 scaffold protein, the PAN2-PAN3 and CCR4-NOT deadenylase complexes, and DDX6 decapping factor (He and Hannon, 2004; Jonas and Izaurralde, 2015). When the miRNA incompletely base-pairs to the 3'-untranslated region (3'-UTR) of a target mRNA, the recruitment of this complex ultimately results in the inhibition of translation and/or target destabilization (He and Hannon, 2004; Jonas and Izaurralde, 2015).

Since the discovery of the first miRNA, *lin-4*, in the nematode *Caenorhabditis elegans*, miRNAs have been shown to regulate gene expression in many eukaryotes, including humans (He and Hannon, 2004; Jonas and Izaurralde, 2015). MiRNA-mediated gene regulation impacts

many pathways, such as development, cellular homeostasis, and aging (He and Hannon, 2004; Jonas and Izaurralde, 2015; Smith-Vikos and Slack, 2012). For instance, in *C. elegans,* mutation of *lin-4* causes larval defects in which cellular divisions from the first larval stage repeat later on in development leading to the absence of adult structures and egg laying defects (He and Hannon, 2004; Chalfie M, 1981; Rosalind CL, 1993; Grishok et al., 2001). Additionally, the loss of the heterochronic *let-7* miRNA in *C. elegans* leads to lethality largely due to vulva bursting (He and Hannon, 2004; Grishok et al., 2001). The let-7 miRNA sequence is highly conserved across species, including humans where let-7 also plays a pivotal role in regulating cell divisions and differentiation and has been shown to function as a tumor suppressor, inhibiting cancerous proliferation by oncogenic Ras (He and Hannon, 2004; Pasquinelli et al., 2000; Adams et al., 2006; Esquela-Kerscher and Slack, 2006).

### 1.2 The Role of Argonaute in the miRNA-induced Silencing Pathway

A crucial protein in facilitating miRNA-mediated gene silencing is the AGO protein of the miRISC (He and Hannon, 2004; Grishok et al., 2001; Topps et al., 2006; Vasquez-Rifo et al., 2012). There are 23 AGO proteins in *C. elegans*, but only Argonaute-like gene-1 (ALG-1) and Argonaute-like gene-2 (ALG-2) interact specifically with miRNAs (Grishok et al., 2001; Topps et al., 2006; Vasquez-Rifo et al., 2012). Underscoring the importance of these AGOs, and the miRNA pathway, is the finding that the dual loss of *alg-1* and *alg-2* is embryonic lethal (Grishok et al., 2001). During larval development, knock-down of *alg-1* by RNA interference (RNAi) causes a lethal vulva busting phenotype in a majority of treated worms, whereas knock-down of *alg-2* by RNAi does not produce as severe a phenotype (Grishok et al., 2001). Importantly, when *alg-1* RNAi was co-injected with other AGO RNAi homologues, only *alg-2* produced an enhanced lethal effect, suggesting overlapping functions between ALG-1 and ALG-2 during larval development (Grishok et al., 2001). Moreover, ALG-1 and ALG-2 are around 80% similar at the nucleotide and 77%-80% similar at the amino acid level; they also share similar spatiotemporal expression patterns (Grishok et al., 2001; Vasquez-Rifo et al., 2012). These

findings demonstrate the importance of ALG-1 and ALG-2 during larval development, while underscoring their similarities in both structure and function. From these observations made during *C. elegans* development, it was concluded that ALG-1 serves as the primary AGO with ALG-2 contributing somewhat redundant functions. Yet, contrary to their similarities during development, the Pasquinelli Lab has found that during adulthood *alg-1(gk214)* and *alg-2(ok304)* loss of function mutants exhibit opposing lifespan phenotypes, suppressing and promoting longevity, respectively, compared to wildtype (WT) N2 strains (Aalto & Nicastro et al., 2017). These findings suggest that the roles of ALG-1 and ALG-2 during adulthood are not entirely overlapping, and that ALG-1 and ALG-2 have opposing roles in regulating *C. elegans* aging.

### 1.3 Aging in *C. elegans*

There are many hallmarks of aging that are remarkably conserved across organisms including: genomic instability, telomere attrition, epigenetic alterations, disruption of proteostasis, deregulation of nutrition sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication (Lopez-Otin C, 2013). Many of these hallmarks of aging are regulated by disparate and intersecting pathways, including caloric restriction, gene silencing, hormone signaling, neuron sensing, reproductive signaling, oxidative stresses, and metabolism activation (Guarente L & Kenyon C, 2000; Kenyon CJ, 2010). One of the most highly conserved of these pathways across species and overlapping with other aging-pathways is the IGF-1/Insulin signaling (IIS) pathway.

The IIS pathway is controlled primarily through insulin signaling (Guarente L & Kenyon CJ, 2000; Kenyon CJ, 2010; Smith-Vikos and Slack, 2012; Murphy et al., 2003; Boehm and Slack, 2005). In the *C. elegans* nematode insulin binds the DAF-2 insulin receptor, causing a phosphorylation cascade, which ultimately phosphorylates the DAF-16/FOXO transcription factor (Smith-Vikos and Slack, 2012; de Lencastre et al., 2010; Murphy et al., 2003). The DAF-16/FOXO transcription factor is a highly conserved Forkhead Box transcription factor, that is

associated with promoting longevity through transcription of *Class I* genes (Murphy et al., 2003; Tepper et al., 2013). Once phosphorylated through insulin signaling, DAF-16 is sequestered from the nucleus, inhibiting its function as a transcription factor, leading to downregulated expression of *Class I* genes (Murphy et al., 2003; Tepper et al., 2013). *Class I* genes are defined as direct targets of DAF-16 that promote longevity primarily through the processes of oxidation, reduction, and carbohydrate metabolism (Murphy et al., 2003; Tepper et al., 2013. Disruption of insulin signaling through mutation of the *daf-2* insulin receptor has been shown to extend *C. elegans* longevity by as much as 2.3-fold, compared to WT, in a manner that is dependent on *daf-16* (Ramon Tabtlang, 1993; Riddle DL, 1995).

Once DAF-16 is phosphorylated and sequestered from the nucleus, the PQM-1 transcription factor, a DAF-16 antagonist, is then able to enter the nucleus and promote the expression of *Class II* genes (Murphy et al., 2003; Tepper et al., 2013). *Class II* genes are associated with reducing *C. elegans* longevity through activation of processes relating to metabolism, growth, reproduction, and development (Murphy et al., 2003; Tepper et al., 2013). In the absence of insulin, DAF-16 phosphorylation is alleviated, allowing DAF-16 to reenter the nucleus, and promote longevity through the expression of *Class I* genes (Murphy et al., 2003; Tepper et al., 2013). Although there are many other aging-related pathways independent of insulin signaling, the severe lifespan phenotypes observed with perturbation of the IIS pathway in *C. elegans*, coupled with the conservation of this pathway from yeast to humans make it an attractive pathway to study the molecular basis of aging.

### 1.4 The miRNA Pathway in *C. elegans* Aging

In *C. elegans*, the perturbation of several individual miRNAs alone has been shown to directly influence longevity in both positive and negative manners. For instance, *C. elegans* longevity is positively regulated by the miRNAs, lin-4, miR-71, miR-246, and miR-238 (Boehm and Slack, 2005; de Lencastre, 2010; Vora et al., 2013). Loss of these miRNAs has been shown to significantly reduce *C. elegans* longevity, whereas their overexpression, with the exception of

*miR-238*, has been shown to significantly increase longevity (Boehm and Slack, 2005; de Lencastre, 2010; Vora et al., 2013). Conversely, the miRNAs miR-239a, miR-239b, and miR-80 have all been shown to negatively regulate longevity. Loss of these miRNAs significantly increases longevity while the overexpression of miR-239a/b reduces it (Boehm and Slack, 2005; de Lencastre et al., 2010; Vora et al., 2013).

These miRNAs have also been shown to regulate longevity thought a variety of aging-related pathways. For example, the opposing lifespans observed with the loss and overexpression of lin-4, miR-71, miR-80, and miR-239a/b have all been shown to be dependent on DAF-16 signaling in the IIS pathway. *lin-4*, *miR-71*, *miR-80*, and *miR-239a/b* were all shown to act through the IIS pathway via RNA interference (RNAi) knock-down of *daf-2* and *daf-16,* whereas double LOF mutants were created to show the dependence of miR-80 on *daf-16* (de Lencastre et al., 2010; Vora et al., 2013). Conversely, the lifespan phenotypes observed in *miR-246* and *miR-238* mutants were found not to be dependent on *daf-2* signaling, suggesting that these miRNAs could be regulating aging through pathways other than the IIS pathway (de Lencastre et al., 2010; Vora et al., 2013).

Several of these miRNAs were also shown to function through pathways other than the IIS pathway, such as stress response, dietary restriction, and DNA damage pathways. The loss of *miR-71*, *miR-238*, *miR-246*, or *miR-239a/b* was found to have effects in stress response pathways mediated by heat and oxidative stress. The loss of *miR-71* and *miR-239a/b* conferred sensitivity or resistance to these pathways, respectively (de Lencastre et al., 2010). Furthermore, the loss of *miR-238* and *miR-246* sensitized strains to oxidative and heat stress, respectively (de Lencastre et al., 2010). *miR-80* was also implicated in the heat-shock pathway, as it's extended lifespan was shown to be dependent on heat-shock factor 1 (HSF-1), a major regulator of heat-shock response elements, which is also associated with promoting *C. elegans* longevity in an IIS independent manner (Vora et al., 2013; Kaeberlein et al., 2006; Kaeberlein et al., 2008; Sige Zou et al., 2006). The extension of lifespan caused by the loss of *miR-80* is

thought to be at least partially through the IIS independent dietary-restriction pathway (Vora et al, 2013). Moreover, the extension of longevity by the overexpression of *miR-71* was also shown through RNAi mediated knock-down of CDC-25.1 and CHK-1 to act through the DNA damage pathway (de Lencastre et al., 2010). These findings not only underscore the importance of miRNAs in the regulation of aging-related pathways, but also suggest inks between the IIS and other biologically relevant pathways, such as the DNA damage pathway.

### 1.5 *alg-1* and *alg-2* differentially regulate *C. elegans* longevity

The loss of *alg-1* and *alg-2* during adulthood causes opposing lifespan phenotypes that significantly suppress and promote *C. elegans* longevity, respectively as compared to WT (Aalto & Nicastro et al., 2017). Transcriptional profiling of *alg-1(gk214)* and *alg-2(ok304)* mutants during adulthood revealed the misregulation of thousands of genes compared to WT (Aalto & Nicastro et al., 2017). A significant portion of these differentially misregulated genes in the *alg-1* and *alg-2* mutants were identified as *Class I* and *Class II* genes, which are associated with promoting or suppressing *C. elegans* longevity, respectively, through the IIS pathway (Aalto & Nicastro et al., 2017; Murphy et al., 2003, Boehm and Slack, 2005). Double LOF mutants of *alg-1(gk214)* or *alg-2(ok304)* with key regulators of the IIS pathway, *daf-2* and *daf-16* showed that these mutants act at least partially through the IIS pathway (Aalto & Nicastro et al., 2017). Loss *alg-1* in *daf-2* mutants resulted in 30% shorter lifespan than loss of *daf-2* alone, suggesting that extension of longevity in *daf-2* is at least partially dependent on *alg-1* (Aalto & Nicastro et al., 2017). Additionally, loss of *daf-16* and *alg-1* together resulted in a shorter lifespan compared to single mutants alone (Aalto & Nicastro et al., 2017). These findings suggest that the short-lived phenotype of *alg-1* is not entirely dependent on the IIS pathway, and ALG-1 is likely acting through additional aging-related pathways (Aalto & Nicastro et al., 2017). Similar double mutant experiments were performed for the long-lived *alg-2* mutant. Loss of *daf-2* in *alg-2* mutant backgrounds had an additive effect in lifespan extension, which could be explained by the non-null mutation of *daf-2* (Aalto & Nicastro et al., 2017). Loss of *daf-16*, however, completely

suppressed the long-lived phenotype of *alg-2* to levels comparable to the short-live *daf-16* single-mutant. These findings show that the long-lived phenotype of *alg-2* is dependent on the IIS pathway, in a *daf-16* dependent manner (Aalto & Nicastro et al., 2017). Taken together, these studies illustrate an opposing relationship between the miRNA AGOs, ALG-1 and ALG-2, and the regulation of *C. elegans* longevity through the IIS pathway (Aalto & Nicastro et al., 2017).

Since the individual misregulation of certain miRNAs has already been shown to have a significant effect in modulating *C. elegans* longevity, it was asked whether candidate aging-associated miRNAs were misregulated in *alg-1* and *alg-2* mutants. Through TaqMan qRT-PCR lin-4, miR-71, miR-246, and miR-238 were all found to be downregulated in *alg-1* mutants when compared to WT. Loss of these miRNAs is associated with decreased longevity, which is consistent with the observed expression patterns in *alg-1* (Aalto & Nicastro et al., 2017). Conversely, the expression of miRNAs, miR-239a/b and miR-80, whose loss is associated with promoting longevity, were found to be significantly downregulated in the *alg-2* mutant, which is consistent with their published phenotypes (Aalto & Nicastro et al., 2017). Additionally, miR-239a and miR-239b were found to be upregulated in *alg-1* compared to WT, which is consistent with the longevity phenotypes observed in the overexpression of these miRNAs (Aalto & Nicastro et al., 2017).

To more directly answer the question of the relationship between these miRNAs and their association with ALG-1 and ALG-2, co-immunoprecipitation (Co-IP) small RNA sequencing (smRNA-seq) was performed during adulthood (Aalto & Nicastro et al., 2017). lin-4 and miR-71 were found to be enriched with ALG-1 Co-IP's when compared to ALG-2, which suggests that these miRNAs preferentially associate with ALG-1 (Aalto & Nicastro et al., 2017). Additionally, these data are consistent with the downregulation of lin-4 and miR-71 observed in *alg-1*, as the loss of ALG-1 likely results in destabilization of these miRNAs. Lastly, target analysis of lin-4

and miR-71 was able to show enrichment for targeting of genes upregulated in the *alg-1* mutants (Aalto & Nicastro et al., 2017).

Although several aging-associated miRNAs have already been implicated in the opposing lifespans on *alg-1* and *alg-2* mutants during adulthood, the full extent of miRNA involvement had yet to be investigated. I hypothesized that there were likely more miRNAs, outside those previously implicated, that are misregulated in *alg-1* and *alg-2* mutants. To identify additional miRNAs that could contribute to these divergent lifespan phenotypes, I performed smRNA profiling of *alg-1* and *alg-2* mutants during adulthood. I was able to identify 51 and 19 significantly misregulated miRNAs in *alg-1* and *alg-2* mutants, respectively, during adulthood when compared to WT (Fig 1). Several candidate miRNAs were selected from those identified as most highly misregulated in *alg-1* and *alg-2* mutants, and their expression levels were verified via TaqMan qRT-PCR (Fig 4). Through target prediction analysis, I was able to predict targets for my miRNA candidates that might explain the differential gene expression and possibly longevity phenotypes of *alg-1* and *alg-2* mutants. I found that the geneset upregulated in *alg-1* mutants was enriched for targeting by miR-44-3p, miR-52-5p, miR-57-5p, miR-61-3p, miR-355-5p, 236-3p, and miR-63-3p (Fig 5A). Similarly, genes downregulated with loss of *alg-1*, were enriched for targeting by miR-52-5p, miR-61-3p, miR-355-5p, and miR-70-3p (Fig 5B). Similar target prediction and seed enrichment analysis was performed for *Class I* and Class *II* genes in the IIS pathway (Fig 7). Although many *Class I* and *Class II* genes were predicted to be targets of my miRNA candidates, they were not found to be significantly enriched for targeting (Fig 7). By identifying those miRNAs misregulated in *alg-1* and *alg-2* mutants, and their predicted gene targets, a more comprehensive picture of the interactions within and beyond the IIS pathway can be created to elucidate the divergent lifespans observed in *alg-1* and *alg-2* mutants and the aging pathway as a whole.

## MATERIALS AND METHODS

### 2.1 Small RNA Sequencing

Small RNA sequencing was performed on five independent replicates of synchronized wildtype (N2), *alg-1(gk214)*, and *alg-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. 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 p-value less than 0.05.

### 2.2 Target Prediction Analysis

Gene targets were predicted for miRNAs candidates through seed analysis, in which the conserved 7mer seed sequence (nts 2-8 from the 5'-end) of a miRNA (Grosswendt et al., 2014) was overlaid with the sequence of a gene's 3'-UTR and sites of complementarity were counted as potential miRNA sites. Target sites were predicted base on the longest annotated 3'-UTR isoforms from release WS263 from WormBase, with sequences >20nt's.

### 2.3 Seed Enrichment Analysis

Seed analysis was then performed on miRNA candidates for enrichment or depletion of predicted gene targeting in those genes misregulated with loss of *alg-1* and *alg-2* (Aalto & Nicastro et al., 2017), and with *Class I* and *Class II* genes in the IIS pathway (Tepper et al, 2013). Targeting by a given miRNA of a respective gene set was compared to the predictive targeting of all other genes by that given miRNA, and significance was determined by a Chi Squared test with a Yates Correction (Aalto & Nicastro et al., 2017).

### 2.4 TaqMan qRT-qPCR

TaqMan qRT-PCR was performed to measure the expression of miRNA candidates in five independent replicates of *alg-1(gk214)* and *alg-2(ok304)* mutants relative to wildtype (N2) during day 5 of adulthood. TaqMan miRNA primers were obtained from Applied Biosystems and run on a QuantStudio 3 Real-Time PCR Systems (Applied Biosystems) as instructed. The U18 snoRNA was used as a control to normalize miRNA expression among samples.

## RESULTS

### 3.1 miRNAs are misregulated in *alg-1* and *alg-2* mutants during adulthood

The divergent lifespan phenotypes observed in *alg-1(gk214)* and *alg-2(ok304)* loss of function mutants are associated with the misregulation of distinct pools of miRNAs that preferentially bind ALG-1 or ALG-2 (Aalto & Nicastro et al., 2017). To assess the full extent of miRNA misregulation in *alg-1* and *alg-2* mutants, small RNA sequencing (smRNA-Seq) was performed on day five adults (Fig 1). This is a timepoint in which adult worms are mostly post-reproductive, still viable, and it allows for comparisons to be made with previous work in the lab (Aalto & Nicastro et al., 2017). SmRNA sequencing was performed on 5 independent replicates of *alg-1(gk214)*, *alg-2(ok304)*, and wildtype (N2) strains at day 5 of adulthood. Once samples were collected, prepared, and sequenced, analysis of smRNA libraries revealed 51 and 19 significantly misregulated miRNAs in *alg-1* and *alg-2* mutants, respectively, when compared to WT (N2) (Fig 1). In the case of *alg-1* mutants, smRNA profiling identified 24 and 27 up- and down-regulated miRNAs when compared to WT (Fig 1A). Similarly, smRNA profiling of *alg-2* mutants was able to identify 2 and 17 up- and down-regulated miRNAs when compared to WT (Fig 1B). Only the targeting guide-strands were considered for those miRNAs called significant, as these are the predominant strand in terms of read abundance, and are thought to be the main strand loaded into Argonaute to silence gene expression via the miRNA-induced silencing pathway (Khvorova et al. 2003; Schwarz et al, 2003) (Fig 1).

### 3.2 Down-regulation of miRNAs preferentially bound to ALG-1 or ALG-2

Previous work has shown that the binding of an Argonaute protein to a miRNA confers a stabilizing effect on the bound miRNA (Winter & Diederichs, 2011). Additionally, work has also shown that several miRNAs preferentially associate with ALG-1 or ALG-2 during adulthood (Aalto & Nicastro et al., 2017). Based on these studies, I hypothesized that the loss of *alg-1* or *alg-2* would lead to the specific destabilization of miRNAs that have been previously shown to preferentially bind ALG-1 or ALG-2, respectively (Aalto & Nicastro et al, 2017). The

destabilization of these miRNAs could then be measured as the downregulation of specific miRNA expression in their respective mutant background compared to WT. For *alg-1* mutants, 13 miRNAs (let-7-5p, lin-4-5p, miR-237-5p, miR-240-3p, miR-241-5p, miR-248, miR-47-3p, miR-48-5p, miR-54-3p, miR-57-5p, miR-60-3p, miR-63-3p, and miR-71-5p) have been implicated as preferentially binding ALG-1 compared to ALG-2 (Aalto & Nicastro et al., 2017). In my smRNA-seq data, the expression of all of those miRNAs that preferentially bound ALG-1 were also significantly downregulated with loss of a*lg-1*, with the exception of miR-48-5p, miR-54-3p, and miR-57-5p (Fig 2A). Similarly for ALG-2, 11 miRNAs (miR-230-3p, miR-250-3p, miR-253-3p, miR-35-3p, miR-36-3p, miR37-3p, miR-40-3p, miR-61-3p, miR-66-5p, and miR-72-5p) were preferentially bound to ALG-2 compared to ALG-1 (Aalto & Nicastro et al, 2017). In my smRNA-Seq data, the loss of *alg-2* resulted in the unanimous downregulation of miRNAs that preferentially bound ALG-2 (Fig 2B). Largely, my smRNA-Seq data was able to show that the expression of miRNAs that preferentially associate with ALG-1 or ALG-2 are downregulated with the loss of their associated AGO protein (Fig 2). These findings are consistent with previous publications which suggest that the binding of AGO has a stabilizing effect on miRNAs (Winter & Diederichs, 2011). Additionally, these analysis provide further evidence of specific association of miRNAs with ALG-1 or ALG-2 (Aalto & Nicastro et al., 2017). Taken together, these findings support my overall hypothesis that there could be more unidentified miRNAs that preferentially work with ALG-1 and ALG-2 to differentially regulate *C. elegans* longevity during adulthood.

### 3.3 Aging-associated miRNAs are misregulated in *alg-1* and *alg-2* during adulthood

Two of the miRNAs (lin-4 and miR-71) shown to preferentially associate with ALG-1, compared to ALG-2, were also miRNAs well characterized in regulating *C. elegans* longevity (Fig 2A) (Aalto & Nicastro et al, 2017; Boehm & Slack, 2005; de Lencastre et al., 2010). Because I am ultimately interested understanding how misregulation of miRNAs contributes the differential lifespans of *alg-1* and *alg-2* mutants, I asked whether other well characterized aging-associated miRNAs could be misregulated in these mutants (Fig 3).

Relative expression of aging-associated miRNAs was determined by comparing the reads of *alg-1* and *alg-2* mutants to N2 WT. To validate these smRNA-seq data, analysis compared the relative $Log_2$ fold change (LFC) of seven aging-associated miRNAs because of their well-established expression patterns during day five and their roles in regulating longevity through the IIS pathway (Fig 3) (Aalto & Nicastro et al., 2017; de Lencastre et al, 2010; Boehm and Slack, 2005). Those miRNAs that resulted in reduced longevity when deleted (lin-4-5p, miR-71-5p, miR-246-3p , and miR-238-3p) were observed to be significantly downregulated in *alg-1* mutants but remain unchanged in *alg-2* mutants (Fig 3) (Aalto & Nicastro et al., 2017; de Lencastre et al, 2010; Boehm and Slack, 2005). Conversely, those miRNAs that resulted in extended longevity when deleted (miR-239a/b, and miR-80) were significantly downregulated in *alg-2* mutants, with the exception of miR-239a-5p, whose expression was opposite of what was previously observed (Fig 3) (Aalto & Nicastro et al., 2017; de Lencastre et al, 2010; Boehm and Slack, 2005). Besides this discrepancy, these comparisons lend credence to the validity of these smRNA-seq data, by reproducibly demonstrating that expression of these miRNAs from the smRNA-seq data correlate with previously published TaqMan qRT-PCR data during similar conditions (Fig 3) (Aalto & Nicastro et al., 2017). These observations also support the hypothesis that *alg-1* or *alg-2* LOF causes the destabilization of aging-associated miRNAs, and that the downregulation of these miRNAs impacts *C. elegans* longevity (Fig 3) (Aalto & Nicastro et al., 2017).

### 3.4 Selection of miRNA candidates for possible involvement in the opposing lifespans of *alg-1* and *alg-2*

From those miRNAs identified as significantly misregulated in *alg-1(gk214)* or *alg-2(ok304)* mutants during adulthood, candidates were selected for their possible involvement in the divergent lifespan phenotypes of *alg-1* and *alg-2* mutants based on **1**) the extent of their up- or down-misregulation and **2**) their differential misregulation in *alg-1* and *alg-2* mutants (Fig 4). I focused on candidates that did not already exhibit published lifespan phenotypes under

conditions similar to those used to observe the divergent lifespans of *alg-1* and *alg-2* mutants (Aalto & Nicastro et al., 2017). From those miRNAs most upregulated in *alg-1*, miR-228-5p, miR-44-3p, miR-52-5p, and miR-57-5p were selected, and from those miRNAs most downregulated, miR-236-3p, miR-63-3p, and miR-70-3p were selected (Fig 4). Similarly, from those miRNAs most upregulated in *alg-2*, miR-237-5p and miR-355-5p were selected as candidates, and from those most downregulated, only miR-61-3p was selected (Fig 4). Notably, about twice as many miRNAs were identified as significantly misregulated with loss of *alg-1*, compared to the number of miRNAs misregulated with the loss of *alg-2* (Fig 1). These findings are consistent with the previous gene expression datasets in which many more genes were misregulated with loss of *alg-1* (8,822 genes) compared to *alg-2* (214 genes) during adulthood (Aalto & Nicastro et al., 2017) TaqMan™ quantitative RT-PCR (qRT-PCR) was then performed on these miRNA candidates and was able to recapitulate the expression patterns observed in the smRNA-Seq data of *alg-1* and *alg-2* mutants (Fig 4). These results give further confidence to these smRNA-seq data and allow for these miRNA candidates to be probed further for their contributions to divergent lifespans of *alg-1* and *alg-2* mutants.

### 3.5 miRNA candidates are predicted to target genes misregulated in *alg-1* and *alg-2* mutants

Previous gene expression profiling performed on *alg-1* or *alg-2* mutants has identified thousands of significantly misregulated genes in comparison to WT background (Aalto & Nicastro et al., 2017). To assess the impact these miRNA candidates could be having on the widespread misregulation of genes in *alg-1* and *alg-2* mutants, I performed target prediction analysis (Fig 5, 6) (Aalto & Nicastro et al., 2017). Target analysis predicts gene targets by looking at the complementarity between a miRNA's 7mer seed sequence (nucleotides 2-8 from the 5'-end) and the sequences within a gene's 3'-UTRs (Grosswendt et al, 2014).

I predicted that those miRNA candidates upregulated in *alg-1* and *alg-2* would target those genes downregulated in *alg-1* and *alg-2* mutants, respectively (Fig 5B, 6B) (Aalto &

Nicastro et al., 2017). All miRNA candidates upregulated in *alg-1* mutants (miR-228-5p, miR-44-3p, miR-52-5p, miR-57-5p, and miR-61-3p) were predicted to target at least 1 gene downregulated in *alg-1* mutants (Fig 5B) (Aalto & Nicastro et al., 2017). Seed enrichment analysis of these predicted targets found that only miRNAs miR-52-5p and miR-61-3p were enriched for targeting genes downregulated in *alg-1* mutants (Fig 5B). Unexpectedly, some miRNA candidates that were downregulated in *alg-1* mutants (miR-355-5p and miR-70-3p) were also found to be enriched for targeting genes downregulated in *alg-1* mutants (Fig 5B). The enrichment of targeting by those candidates downregulated in *alg-1* mutants is the opposite of what I expected and render these analyses less straightforward to interpret (Fig 5B). A possible explanation is that the high number of genes misregulated in *alg-1* mutants results in many secondary interactions between misregulated genes, and that the downregulation of genes in *alg-1* mutants is not entirely the result of direct misregulation between miRNAs and gene targets.

Similarly, for those miRNA candidates upregulated in *alg-2* mutants (miR-57-5p, miR-237-5p, and miR-355-5p), targets were predicted to assess whether candidates were enriched for targeting genes downregulated in *alg-2* mutants (Fig 6B). All miRNAs upregulated in *alg-2* mutants were predicted to target at least 1 gene downregulated in *alg-2* mutants, with the exception of miR-57-5p (Fig 6B). There was however no enrichment of targeting for genes downregulated in *alg-2* mutants by these candidates (Fig 6B). The lack of enrichment and scarcity of predicted targets for miR-57-5p is not entirely surprising, and could be explained by the limited number of total genes (n=81) that were downregulated in *alg-2* mutants (Fig 6B) (Aalto & Nicastro et al., 2017).

Conversely to upregulated candidates, I would expect that the downregulation of a miRNA candidate would alleviate silencing of gene targets and would result in an overexpression of predicted gene targets. Therefore, I would predict that those miRNA candidates downregulated in *alg-1* or *alg-2* would target genes upregulated in *alg-1* and *alg-2*

mutants, respectively (Fig 5A, 6A) (Aalto & Nicastro et al., 2017). Those miRNAs down-regulated in *alg-1* mutants (miR-355-5p, miR-236-3p, miR63-3p, and mR-70-3p) were all predicted to target at least 1 gene upregulated in *alg-1* mutants (Fig 5A) (Aalto & Nicastro et al., 2017). Furthermore, seed enrichment analysis of these predicted targets revealed that all miRNA candidates downregulated in *alg-1* mutants, except for miR-70-3p, were enriched for targeting genes downregulated in *alg-1* mutants (Fig 5A). Similar to previous enrichment analysis performed for those genes downregulated in *alg-1* mutants, some of the miRNAs upregulated in *alg-1* mutants (miR-44-3p, miR-52-5p, miR-57-5p, and miR-61-3p) were also found to be enriched for targeting genes upregulated in *alg-1* mutants in a manner opposite to what would be expected (Fig 5A). Again, these observations make interpretation of these data difficult, but the predicted gene targets are still useful in downstream analysis and could present possible gene candidates that could help elucidate the short-lived phenotype of *alg-1* mutants. For those miRNAs downregulated in *alg-2* mutants (miR-236-3p and miR-61-3p), only miR-61-3p was predicted to target genes upregulated in *alg-2* mutants (Fig 6A). Furthermore, only three downregulated genes were predicted to be targeted by miR-61-3p, further highlighting the limited number of total genes misregulated in *alg-2* mutants (Fig 6A) (Aalto & Nicastro et al., 2017).

Taken together, these analyses were able to implicate several miRNA candidates in the divergent lifespans of *alg-1* and *alg-2* mutants by predicting gene targets for those miRNAs misregulated in *alg-1* and *alg-2* mutants (Fig 5, 6) (Aalto & Nicastro et al., 2017). Enrichment analysis of these data were further able to highlight five miRNAs (miR-52-5p, miR-61-3p, miR-355-5p, miR-236-3p, and miR-63-3p) as having enriched targeting for genes misregulated in *alg-1* mutants, in a manner that is consistent with the observed expression patterns of these miRNAs. One caveat to these enrichment analyses is that several miRNAs which would not be expected to target genes up- and down-regulated in *alg-1* mutants, respectively, were found to be enriched (Fig 5). Moreover, despite the limited number of total genes misregulated in *alg-2*

mutants, several misregulated genes in *alg-2* mutants were predicted for those miRNAs misregulated in *alg-2* mutants, consistent with the expression patterns of these miRNA candidates (Fig 6). Although these findings paint a complicated picture between the interactions of misregulated genes and miRNAs, particularly with respect within *alg-1* mutants. These analyses are still valuable in identifying potential gene candidates and highlight the contributions of several miRNA candidates.

### 3.6 miRNA candidates are predicted to target *Class I* and *Class II* genes

Given that previous work has shown that the divergent lifespans of *alg-1* and *alg-2* mutants are associated with the misregulation of genes in the IIS pathway, I performed target prediction analysis to ask whether my miRNA candidates could be targeting genes in the IIS pathway (Aalto & Nicastro et al., 2017). Based on the observed lifespans of *alg-1* and *alg-2* mutants, I predicted that *Class I* genes, associated with promoting longevity, would be targeted by those miRNAs most up- and down-regulated in *alg-1* and *alg-2* mutants, respectively (Fig 4). Targeting of *Class I* genes by these miRNAs would be expected to result in the down- and up-regulation of longevity promoting *Class I* genes in *alg-1* and *alg-2* mutants, respectively, and would be consistent with their observed lifespan phenotypes. My miRNA candidates expected to target *Class I* genes would be miR-288-5p, miR-44-3p, miR-52-5p, and miR-57-5p for those miRNAs upregulated in *alg-1* mutants, and miR-61-3p and miR-236-3p for those miRNAs downregulated in *alg-2* mutants (Fig 7A). I found that all of these candidates were predicted to target at least one *Class I* gene (Fig 7A).

I also predicted that *Class II* genes, associated with suppressing longevity, would be targeted by those miRNAs most down- and up-regulated in *alg-1* and *alg-2* mutants, respectively. Targeting of *Class II* genes by these miRNAs would be expected to cause the up- and down-regulation of *Class II* genes in *alg-1* and *alg-2* mutants, respectively, in a manner consistent with their observed lifespan phenotypes. My miRNA candidates expected to target *Class II* genes are miR-63-3p, miR-70-3p, miR-236-3p, and miR-355-5p for those miRNAs

downregulated in *alg-1* mutants, and miR-57-5p and miR-237-5p for those miRNAs upregulated in *alg-2* mutants (Fig 7B). All of these miRNAs were predicted to target at least 1 *Class II* gene (Fig 7B).

Next I performed seed enrichment analysis to asked whether any of these miRNA candidates were enriched for targeting either *Class I* or *Class II* genes, compared to all other predicted gene targets (Fig 7). None of my miRNA candidates were found to be enriched for targeting *Class I* or *Class II* genes during my seed enrichment analysis (Fig 7). Although none of my candidates were enriched for targeting *Class I* or *Class II* genes, there was interesting overlap of *Class I* and *Class II* targeting by two miRNAs, miR-355-5p and miR-61-3p, as a result of their differential expression in *alg-1* and *alg-2* mutants (Fig 7). All five previously highlighted miRNAs (miR-52-5p, miR-61-3p, miR-355-5p, miR-236-3p, and miR-63-3), enriched for targeting genes misregulated in *alg-1* mutants, demonstrated targeting of *Class I* and *Class II* genes, respectively, in a manner consistent with their expression patterns and the observed lifespans of *alg-1* and *alg-2* mutants (Fig 7).

Finally, I asked whether any predicted *Class I* or *Class II* targets were also genes misregulated in *alg-1* or *alg-2* mutants, in a manner consistent with their respective lifespan phenotypes. Coinciding with their lifespan phenotypes, I expected that the expression of longevity promoting *Class I* genes would be downregulated in short-lived *alg-1* mutants and upregulated in the longer-lived *alg-2* mutants, respectively, compared to WT. So, I predicted that miRNA candidates upregulated in *alg-1* mutants would target *Class I* genes, and that the increased targeting by these miRNAs would result in the downregulation of predicted *Class I* targets. For those Candidates upregulated in *alg-1* mutants (miR-288-5p, miR-44-3p, miR-52-5p, miR-57-5p, and miR-61-3p), at least one of the predicted *Class I* targets was also a gene previously identified as downregulated in *alg-1* mutants (Fig 7A, data not shown) (Aalto & Nicastro et al., 2017). Conversely for *alg-2* mutants, I predicted that downregulated candidates (miR-61-3p and miR-236-3p) would target *Class I* genes, and that the alleviation of targeting by

these miRNAs would result in the upregulation of longevity promoting *Class I* genes in *alg-2* mutants. Unfortunately, none of the *Class I* genes, predicted to be targeted by these candidates overlapped with genes upregulated in *alg-2* mutants (Fig 7A).

Similar analysis was performed for *Class II* genes associated with suppressing longevity, asking if predicted *Class II* targets were in fact misregulated in *alg-1* and *alg-2* mutants in a manner consistent with their lifespans (Fig 7B). In short-lived *alg-1* mutants, it would be expected that these antagonistic *Class II* genes would be upregulated, so, I predicted that *Class II* genes would likely be targeted by candidates downregulated in *alg-1* mutants (miR-63-3p, miR-70-3p, miR-236-3p, and miR-355-5p). For these miRNAs, at least one of the predicted *Class II* targets was also a gene upregulated in *alg-1* mutants (Fig 7B, data not shown). For long-lived *alg-2* mutants, longevity suppressing *Class II* genes would be expected to be downregulated. So, I predicted that *Class II* genes would likely also be targeted by those miRNA candidates upregulated in *alg-2* mutants (miR-57-5p, miR-237-5p, and miR-355-5p). Despite the limited number of total genes downregulated in *alg-2* mutants, 2 predicted *Class II* targets of miR-355-5p were found to also be genes downregulated in alg-2 mutants (Fig 7B, data not shown).

Target prediction analysis was able to predict possible *Class I* and *Class II* targets of miRNA candidates, in a manner that was consistent with their expression and the opposing lifespans of *alg-1* and *alg-2* mutants (Fig 7). Most of my miRNA candidates (miR-44-3p, miR-52-5p, miR-57-5p, miR-61-3p, miR-63-3p, miR-70-3p, miR-236-3p, miR-288-5p, and miR-355-5p) were able to be further implicated in contributing to the divergent lifespans of *alg-1* and *alg-2* mutants by showing that the expression of predicted *Class I* and *Class II* targets was misregulated in *alg-1* and *alg-2* mutants consistent with previous predictions. Ultimately this analysis not only highlights many of my miRNA candidates, but also identifies several *Class I* and *Class II* genes as possible candidates that could help elucidate the opposing lifespan of *alg-1* and *alg-2* mutants.

## DISCUSSION

### 4.1 Specific miRNAs are misregulated in *alg-1* and *alg-2* mutants during adulthood

Small RNA profiling of *alg-1* and *alg-2* mutants during adulthood identified 51 and 19 significantly misregulated miRNAs in mutant backgrounds, respectively, compared to WT (N2) (Fig 1). Notably the number of misregulated miRNAs identified in *alg-1* was greater than *alg-2* mutants, which complements previous gene expression studies of *alg-1* and *alg-2* that also identified greater numbers of genes misregulated in *alg-1* compared to *alg-2* mutants during adulthood (Fig 1) (Aalto & Nicastro et al., 2017). Previous work has found that loss of Argonaute could have a destabilizing effect on miRNA stability, so it could be that loss of *alg-1* or *alg-2* causes misregulation of distinct populations of miRNAs associated with ALG-1 or ALG-2 proteins (Winter & Diederichs, 2011). This could explain why the numbers of misregulated genes identified in *alg-1* and *alg-2* mutants is proportional to the number of misregulated miRNAs identified in these same mutants (Fig 1) (Aalto & Nicastro et al., 2017). These findings could also help to elucidate the opposing lifespans of *alg-1* and *alg-2* mutants by allowing for targets of misregulated miRNAs to be predicted. It would be expected that some fraction of those miRNAs down- or up-regulated in *alg-1* mutants target genes that antagonize or promote longevity, and that under normal conditions, *alg-1* works to promote longevity. Similarly, for *alg-2* mutants, it would be expected that loss of *alg-2* results in the down- or up-regulated of miRNAs that target genes that promote or antagonize longevity, and that *alg-2* works to antagonize longevity under normal conditions. Based on previous gene expression data, it would be predicted that some of the misregulated genes are *Class I* or *Class II* genes which work to promote or antagonize longevity through the IIS pathway (Aalto & Nicastro et al., 2017; Murphy et al., 2003; Tepper et al., 2013). In future work it will be interesting to test whether predicted targets for misregulated miRNAs in *alg-1* or *alg-2* mutants target genes in aging-pathways independent of IIS such as caloric restriction or other stress response pathways (de Lencastre et al, 2010; Vora et al., 2013).

**4.2 Loss of a specific Argonaute affects the level of preferentially bound miRNAs**

Previous work has shown that the binding of an Argonaute to a miRNA confers a stabilizing effect on the bound miRNA (Winter & Diederichs, 2011). Additionally, work has also identified several miRNAs as preferentially binding to ALG-1 or ALG-2 during day 5 of adulthood (Aalto & Nicastro et al., 2017). Based on these studies I predicted that the loss of *alg-1* or *alg-2* would result in the downregulation of those miRNAs that are preferentially bound to ALG-1 or ALG-2, respectively. The majority of miRNAs identified as preferentially binding to ALG-1 (let-7-5p, lin-4-5p, miR-240-3p, miR-248, miR-60-3p, miR-63-3p, and miR-71-5p), compared to ALG-2, were found to be significantly downregulated in *alg-1* mutants (Fig 2A). Strikingly, the loss of *alg-2* led to the significant downregulation of all miRNAs which were shown to preferentially bind ALG-2, compared to ALG-1 (Fig 2B). Although these analyses do not identify new miRNAs as preferentially binding to either ALG-1 or ALG-2, these analyses are able to show that the loss of a particular Argonaute can result in the downregulation of miRNAs in a predictable manner; a concept that is used throughout my project to help identify miRNA candidates.

**4.3 Aging-associated miRNAs are misregulated in *alg-1* and *alg-2* during adulthood**

To assess the contributions that miRNA misregulation could be having in the differential lifespans of *alg-1* and *alg-2* mutants, I asked whether well-known aging-associated miRNAs were misregulated in a manner that was consistent with the observed lifespans of *alg-1* and *alg-2* mutants. Indeed, I found that those miRNAs that result in reduced longevity when deleted (lin-4-5p, miR-71-5p, miR-246-3p, and miR-238-3p) were observed to be significantly downregulated in *alg-1* mutants (Fig 3) (Aalto & Nicastro et al., 2017; de Lencastre et al., 2010; Boehm and Slack, 2005). Conversely, those miRNAs that result in extended longevity when deleted (miR-239a/b, and miR-80) were significantly downregulated in *alg-2* mutants, with the exception of miR-239a-5p, whose expression was opposite of what has been previously observed (Fig 3) (Aalto & Nicastro et al., 2017; de Lencastre et al., 2010; Boehm and Slack, 2005).

Many of these aging-associated miRNAs (lin-4, miR-71, miR-80, and miR-239a/b) misregulated in *alg-1* and *alg-2* mutants have been shown to regulate longevity in a *daf-16* dependent manner through the IIS pathway (Aalto & Nicastro et al., 2017; de Lencastre et al., 2010; Boehm and Slack, 2005). These findings were not entirely unexpected given that previous studies have already characterized the expression of these miRNAs in *alg-1* and *alg-2* mutants during adulthood, along with showing that the loss of *alg-1* and *alg-2* is associated with misregulation of *Class I* and *Class II* genes in the IIS pathway (Aalto & Nicastro et al., 2017). Several of these aging-associated miRNAs (miR-71, miR-80, miR-238, miR-239a/b, and miR-246) misregulated in *alg-1* and *alg-2* mutants are also implicated in regulating longevity through *daf-16* independent aging pathways such as the caloric restriction, oxidative/heat stress, and DNA damage pathways (de Lencastre et al., 2010; Boehm and Slack, 2005; Vora et al., 2013). A future direction of this project could be to reanalyze the small RNA profiles of *alg-1* and *alg-2* mutants during adulthood and ask whether miRNAs known to regulate *daf-16* independent aging pathways, such as the heat-shock pathway, are also misregulated in these mutants. If candidate pathways are identified, then gene expression profiles of *alg-1* and *alg-2* could be reanalyzed to identify those genes misregulated in candidate *daf-16* independent aging pathways.

### 4.4 Selection of miRNA Candidates for possible involvement in the opposing lifespans of *alg-1* and *alg-2*

In addition to analyzing the expression of known aging-associated miRNAs, I also selected several miRNA candidates which could play a role in the opposing lifespans of *alg-1* and *alg-2* mutants. Candidates were selected for their possible involvement in the divergent lifespan phenotypes of *alg-1* and *alg-2* mutants based on 1) the extent of up- or down-misregulation and 2) their differential misregulation in *alg-1* and *alg-2* mutants (Fig 1). I also focused on candidates that did not already exhibit published lifespan phenotypes under conditions similar to those used to observe the divergent lifespans of *alg-1* and *alg-2* mutants

(Aalto & Nicastro et al., 2017). Candidates were selected for all cases of up- (miR-228-5p, miR-44-3p, miR-52-5p, and miR-57-5p) and down-regulated (miR-236-3p, miR-63-3p, and miR-70-3p) miRNAs in *alg-1* mutants, along with up- (miR-237-5p and miR-355-5p) and down-regulated (miR-61-3p) miRNAs in *alg-2* mutants (Fig 4). TaqMan™ quantitative RT-PCR (qRT-PCR) was then performed on all miRNA candidates and was able to recapitulate the expression patterns observed in the smRNA-Seq data of *alg-1* and *alg-2* mutants (Fig 4). These results give further confidence to these smRNA-seq data and allow for these miRNA candidates to be probed further for their contributions to divergent lifespans of *alg-1* and *alg-2* mutants.

Several loss of function mutant strains for these miRNA candidates already exist. Surprisingly, lifespan curves for many of these strains are not published and could reveal interesting insight into the regulation of aging-pathways by these individual miRNAs. I have ordered, and am currently in possession of all existing LOF mutants available through the Caenorhabditis Genetics Center (CGC) for my miRNA candidates; ideally I would like to backcross all strains to the Pasquinelli Lab WT (N2) strain, and perform lifespan assays on all candidate mutants under conditions similar to those published for *alg-1* and *alg-2* mutants (Aalto & Nicastro et al., 2017). I would predict that the miRNA mutant strains for those miRNAs downregulated in *alg-1* (miR-63-3p, miR-70-3p, miR-236-3p, and miR-355-5p) or *alg-2* mutants (miR-61-3p and miR-236-3p) would exhibit similar short- and long-lived longevity phenotypes, respectively, based on the correlative expression of miRNA candidates and the subsequent overexpression of gene targets.

Additionally, overexpression mutants for miRNA candidates could be created by generating extrachromosomal arrays driven by miRNA promoters. I would expect that mutants for those miRNAs upregulated in *alg-1* (miR-228-5p, miR-44-3p, miR-52-5p, miR-57-5p, and miR-61-3p) and *alg-2* (miR-237-5p, miR-355-5p) mutants would exhibit opposite, long- and short-lived, phenotypes to what was originally observed in *alg-1* and *alg-2* mutants (Aalto & Nicastro et al., 2017). One caveat to these experiments is that the promoter sequences of

miRNAs are not always well characterized, and it would be important to capture enough upstream promoter sequence to drive endogenous expression of these transgenic miRNAs.

Once interesting lifespan phenotypes were observed for candidate mutants, the expression of predicted miRNA targets thought to impact relevant aging-related pathways, such as the IIS pathway, could be assessed via qRT-PCR. I would predict that for LOF mutants of miRNA candidates, predicted targets would be upregulated compared to WT. Conversely, the overexpression of a miRNA candidate in mutants should downregulate predicted gene targets.

**4.5 miRNA candidates are predicted to target genes misregulated in *alg-1***

Target prediction analysis was then performed for miRNA candidates to ask whether those genes misregulated in *alg-1* and *alg-2* mutants could be targeted by my miRNA candidates. Genes misregulated in *alg-1* mutants were predicted to be targeted for all miRNAs misregulated in *alg-1* mutants, in a manner consistent with the expression patterns observed for both misregulated genes and miRNA candidates (Fig 5). Similar target prediction performed for miRNA candidates and genes misregulated in *alg-2* mutants was able to predict expected targets for all miRNA candidates misregulated in *alg-2* mutants except for miR-57-5p and miR-236-3p (Fig 6). The lack of predicted targets for these miRNA candidates could simply be due to the limited number of total genes misregulated in *alg-2* mutants (Fig 6) (Aalto & Nicastro et al., 2017).

To further parse the contributions of these predicted targets in the opposing lifespans of *alg-1* and *alg-2* mutants, I performed subsequent enrichment analysis to determine whether particular miRNA candidates targeted genes misregulated in *alg-1* and *alg-2* mutants, respectively, compared to all other predicted gene targets. Those genes downregulated in *alg-1* mutants were enriched for targeting by two miRNA candidates (miR-52-5p and miR-61-3p) upregulated in *alg-1* mutants, consistent with previous predictions (Fig 5B). Two miRNA candidates (miR-70-3p and miR-355-5p) downregulated in *alg-1* mutants, however, were also found to be enriched for targeting genes downregulated in *alg-1* mutants (Fig 5B). The

prediction would have been that only those miRNAs upregulated in *alg-1* mutants would be enriched for targeting genes downregulated in *alg-1* mutants. The enrichment of targeting by miRNAs downregulated in *alg-1* mutants was unexpected and could be explained by the large set of total genes that are misregulated in *alg-1* mutants (Aalto & Nicastro et al., 2017). One possibility could be that key transcription factors are under the control of miRNA-mediated regulation by way of ALG-1-associated miRNAs, and that the loss of *alg-1* results in the misregulation of its associated miRNAs, and subsequently results in extensive transcriptional misregulation in these strains.

For those miRNAs down-regulated in *alg-1* mutants (miR-355-5p, miR-236-3p, miR-63-3p, and mR-70-3p), all candidates except for miR-70-3p were found to be enriched for targeting genes upregulated in *alg-1* mutants (Fig 5A). Similarly to previous enrichment analysis performed for miRNA candidates upregulated in *alg-1* mutants, unexpected candidates (miR-44-3p, miR-52-5p, miR57-5p, and miR-61-3p) upregulated in *alg-1* mutants were also found to be enriched for targeting genes upregulated in *alg-1* mutants (Fig 5A). The expectation would be that only miRNAs upregulated in *alg-1* mutants would preferentially target genes downregulated in *alg-1* mutants because of the increased miRNA-induced silencing of targets compared to WT. Finally, enrichment analysis was performed between those miRNAs and genes misregulated in *alg-2* mutants, however, due to the limited number of predicted targets, there was not enough statistical power to assess whether candidates were enriched for gene targeting (Fig 6). Nevertheless, the predicted gene targets of these miRNA candidates are still valuable in understanding the miRNA contributions to the opposing lifespan phenotypes of *alg-1* and *alg-2* mutants, particularly in downstream analysis when the contributions of gene candidates are assessed.

**4.6 miRNA candidates are predicted to target *Class I/II* genes**

Finally, similar target prediction and enrichment analyses were performed on miRNA candidates for longevity promoting (*Class I*) and antagonizing (*Class II*) genes in the IIS

pathway, which have been previously shown to be differentially misregulated in *alg-1* and *alg-2* mutants (Aalto & Nicastro et al., 2017). I predicted that *Class I* (longevity promoting) genes would be targeted by miRNAs upregulated in *alg-1* mutants (miR-288-5p, miR-44-3p, miR-52-5p, and miR-57-5p) because the increased expression levels of these miRNAs would correlate with a decreased expression of *Class I* genes and a shorter-lived phenotype, which is what is observed in *alg-1* mutants (Aalto & Nicastro et al., 2017). I also predicted that miRNAs downregulated in *alg-2* mutants (miR-61-3p and miR-236-3p) would also target *Class I* genes, as the alleviated inhibition by these miRNAs would correlate with an upregulation of *Class I* genes and a longer-lived phenotype, which is what is observed in *alg-2* mutants (Aalto & Nicastro et al., 2017). The inverse is predicted for miRNAs targeting of *Class II* (longevity antagonizing) genes, in which miRNAs down- and up-regulated in *alg-1* (miR-63-3p, miR-70-3p, miR-236-3p, and miR-355-5p) and *alg-2* (miR-355-5p, miR-57-5p, and miR-237-5p) mutants, respectively, would be predicted to target *Class II* genes. miRNA candidates expected to target either *Class I* or *Class II* genes, respectively, were all predicted to have at least 1 gene target. Similar target enrichment analysis to what was performed for genes misregulated in *alg-1* mutants did not, however, find any statistically significant targeting of *Class I* or *Class II* genes by these miRNA candidates. Nevertheless, the predictive targeting of *Class I* and *Class II* genes, respectively, by these miRNAs correlates with the observed longevity phenotypes of *alg-1* and *alg-2* mutants and gives further insight into specific genes which could be contributing to the differential regulation of longevity by *alg-1* and *alg-2*.

To further implicate miRNA candidates and genes in these differential lifespan phenotypes, I asked whether predicted *Class I* or *Class II* targets overlapped with those genes misregulated in *alg-1* or *alg-2* mutants in a manner that would be consistent with the observed lifespans of *alg-1* and *alg-2* mutants. For *Class I* genes, at least one of the predicted targets for miRNA candidates upregulated in *alg-1* mutants (miR-288-5p, miR-44-3p, miR-52-5p, miR-57-5p, and miR-61-3p) were also genes identified as downregulated in *alg-1* mutants (Fig 7A).

Similarly, *Class II* genes targeted by miRNA candidates downregulated in *alg-1* mutants (miR-63-3p, miR-70-3p, miR-236-3p, and miR-355-5p) were found to also be genes upregulated in *alg-1* mutants. Unfortunately, no predicted *Class I* or *Class II* targets for miRNA candidates misregulated in *alg-2* mutants overlapped with genes misregulated in *alg-2* mutants, likely due to the limited number of total genes misregulated in these mutant backgrounds. These analyses not only highlight most candidates in the short-lived lifespan of *alg-1* mutants, but also identify specific *Class I* and *Class II* genes misregulated in *alg-1* mutants, suggesting that the misregulation of these genes could play a role in short-live phenotype of *alg-1* mutants.

One limitation of these gene prediction/enrichment analyses is that targeting was only determined by complete complementarity of a miRNA's 7mer sequence (nts 2-8 from the 5'-end) with the sequence of a gene's 3'-UTR, potentially excluding bonified gene targets. A more robust picture of gene targeting could be gleamed by increasing the possible interactions between miRNAs and genes (i.e. looking at incomplete complementarity, all miRNA seeds (6mers, 7mers, etc.)). Although miRNA-mediated regulation is primarily thought to occur at a gene's 3'-UTR, new work continues to illustrate the importance of miRNA binding beyond the canonical seed and 3'-UTR binding (Grimson et al, 2007; Broughton et al, 2016; Sheu-Gruttadauria et al, 2019).

In summary, smRNA profiling of *alg-1(gk214)* and *alg-2(ok304)* mutants identified 51 and 19 significantly misregulated miRNAs, respectively, compared to WT (Fig 1). Aging-associated miRNAs were then shown to be misregulated in these mutant backgrounds in a manner consistent with their divergent lifespans (Fig 3). New miRNA candidates were selected based on their high levels of misregulation in *alg-1* and *alg-2* mutants and were subsequently shown though target prediction analysis to target genes misregulated in *alg-1* mutants and the IIS pathway (Fig 5, 7). By further characterizing the contributions of these miRNAs and their gene targets to the divergent lifespans of *alg-1* and *alg-2* mutants, I hope to uncover novel regulators of aging in order to more broadly elucidate the molecular basis of aging.

## FIGURES

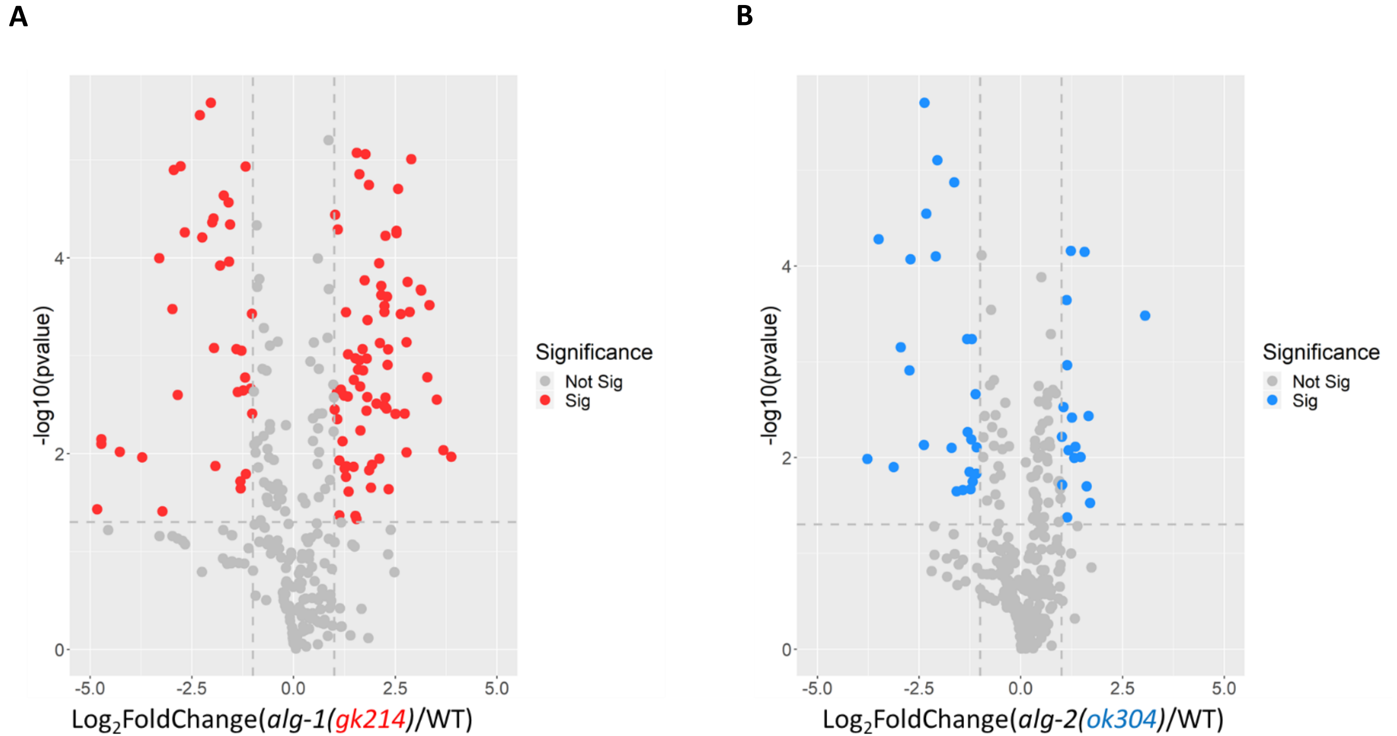


**Figure 1. Loss of *alg-1* or *alg-2* results in widespread misregulation of specific miRNAs.** smRNA sequencing of (**A**) *alg-1(gk214)* and (**B**) *alg-2(ok304) C. elegans* during day 5 of adulthood identifies 51 and 19 significantly ($p < 0.05$) misregulated miRNAs (> |1.0| $log_2$foldchange (mutant/wildtype)), respectively, when compared to N2 (wildtype), (n=5 biological replicates).

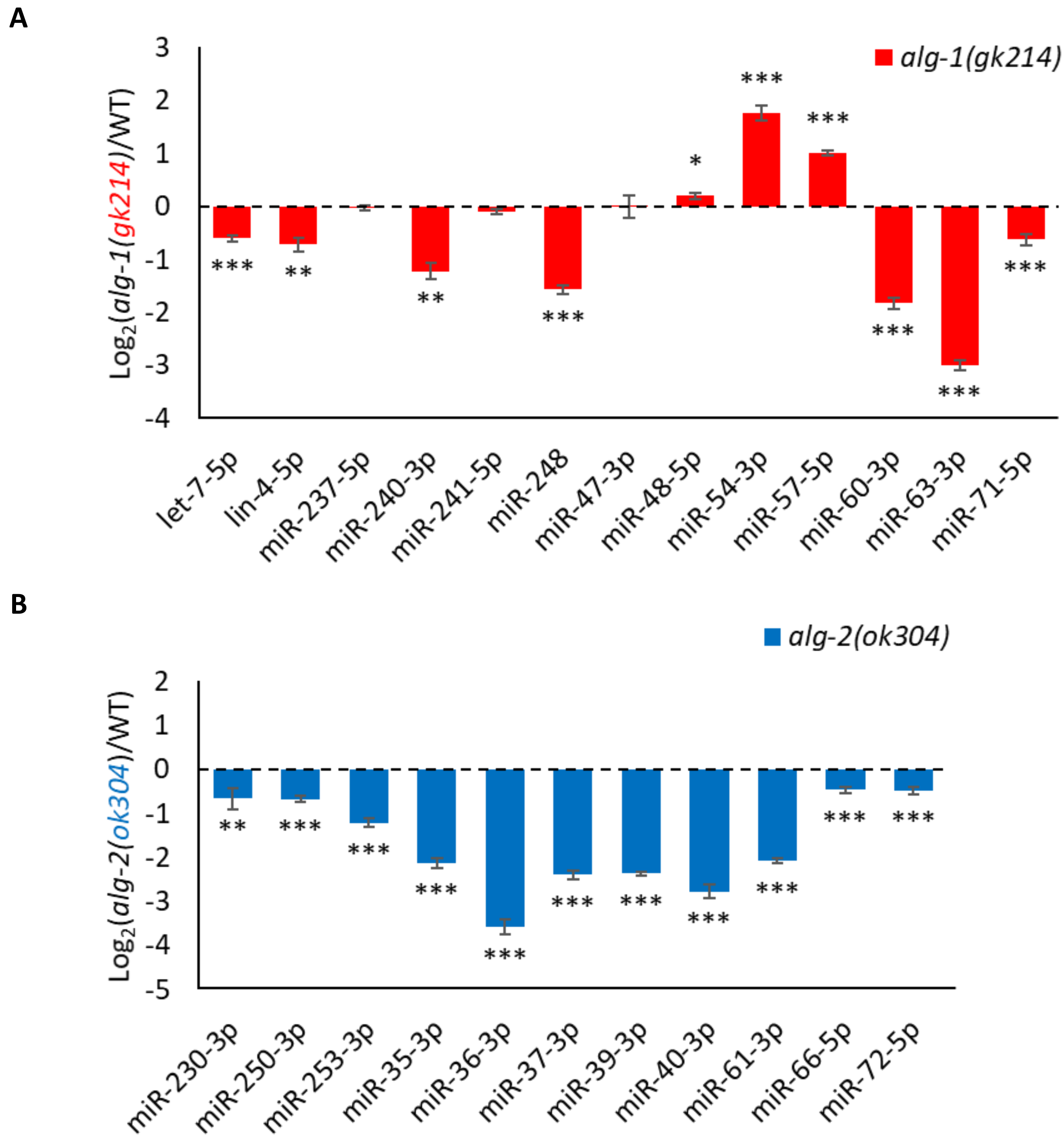


**Figure 2. Loss of *alg-1* or *alg-2* affects the expression of miRNAs preferentially bound to each Argonaute.** Loss of (**A**) *alg-1* or (**B**) *alg*-2 results in reduced expression levels of most preferentially associated miRNAs. The $\log_2$foldchange (mutant/wildtype) for miRNAs known to preferentially bind ALG-1 or ALG-2 are represented with error bars corresponding to SEMs, and significance (paired-end t-test, *p<0.05, **p<0.01, ***p<0.001).

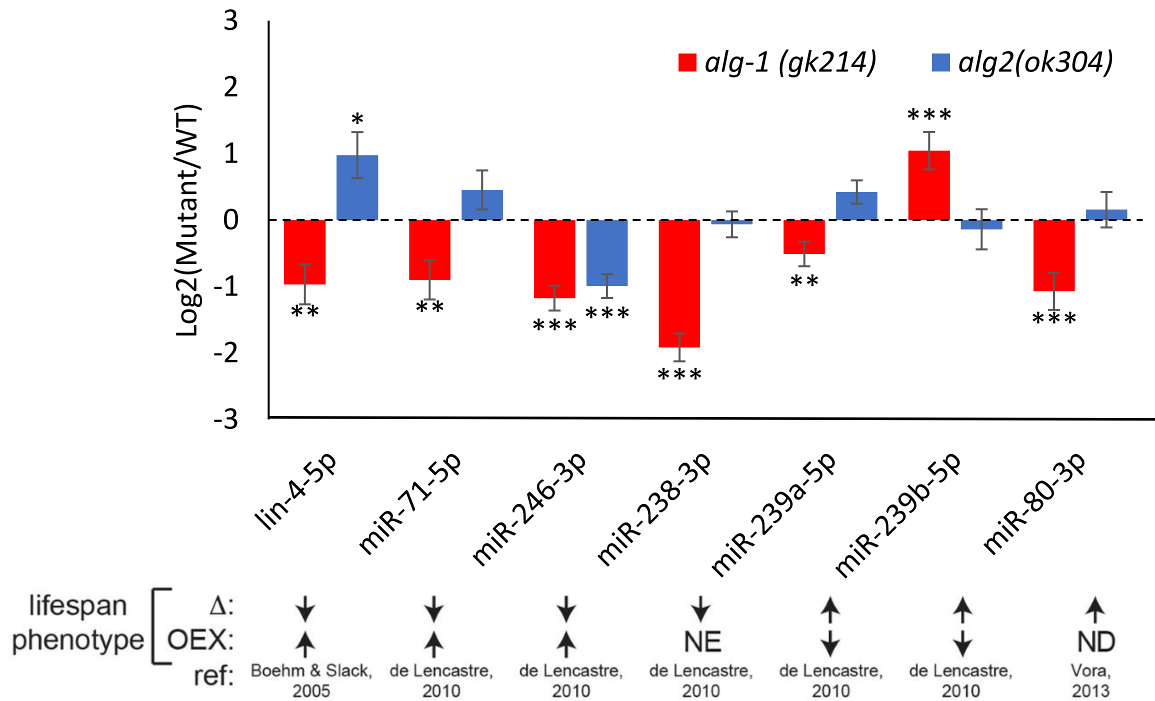


**Figure 3. Aging-associated miRNAs are differentially misregulated in *alg-1* and *alg-2* mutants during adulthood.** Small RNA sequencing reveals the differential expression of known aging-associated miRNAs with published longevity phenotypes, (Boehm & Slack, 2005; de Lencastre et al., 2010; Vora et al., 2013) indicated above, caused by either miRNA deletion (**Δ**) or overexpression (**OEX**). Expression of miRNA from 5 independent replicates (n = 5 biological replicates) based on $log_2$foldchange (mutant/wildtype) and significance (paired-end t-test, *p<0.05, **p<0.01, ***P<0.001) from small RNA sequencing of *alg-1* and *alg-2* LOF mutants during day 5 of adulthood.

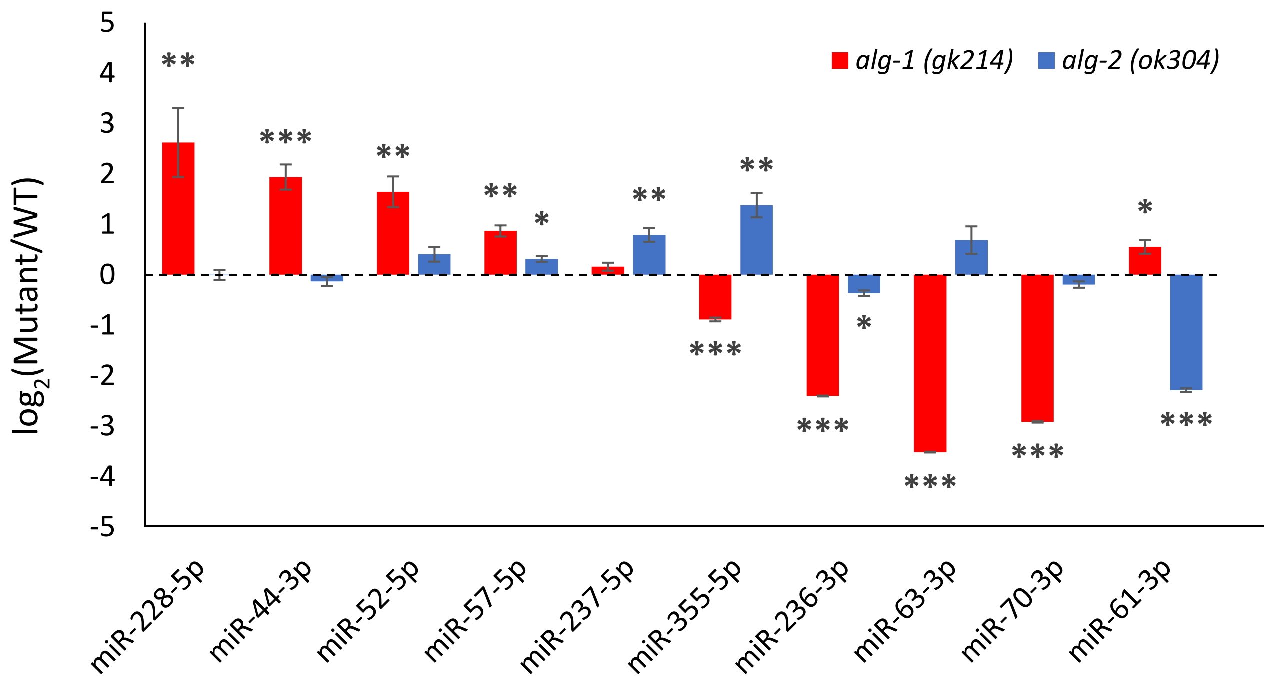


**Figure 4. miRNA candidates were selected for their possible involvement in the opposing lifespans of *alg-1* and *alg-2* mutants.** TaqMan RT-qPCR was performed to verify the expression of miRNA candidates, which were selected based to their top log2foldchange(mutant/wildtype) and significance (*p < 0.05) from smRNA-Seq data in *alg-1* and *alg-2* LOF mutants during day 5 of adulthood, (n=5 biological replicates and n=3 technical replicates were used, paired-end t-test, *p<0.05, **p<0.01, ***p<0.001).

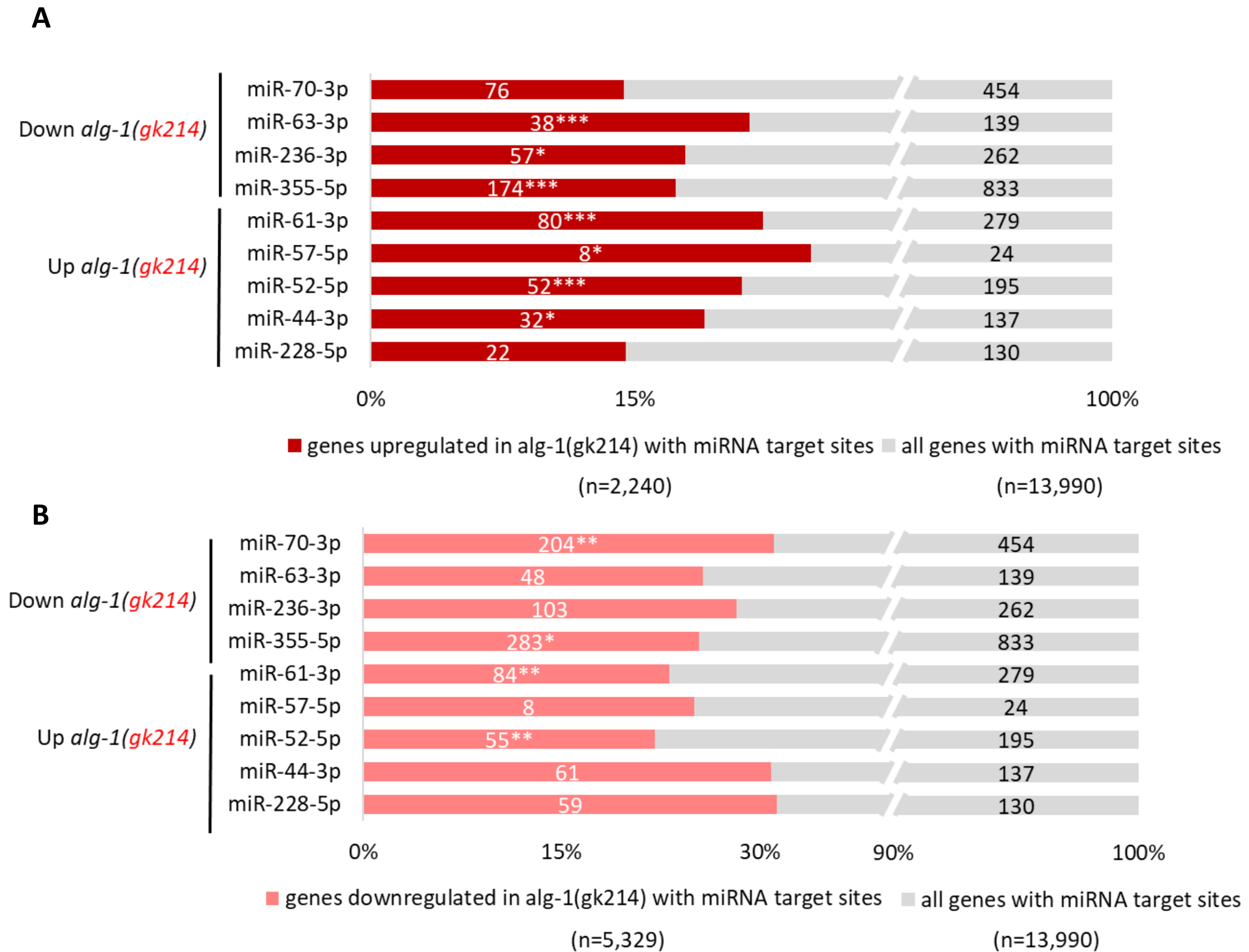


**Figure 5. miRNA candidates are predicted to target genes misregulated in *alg-1* mutants during adulthood.** Seed enrichment analysis was performed on candidate miRNAs to assess the enrichment of gene targets for those genes (**A**) up- (n=2,240) and (**B**) down-regulated (n=5,329) in *alg-1(gk214)* mutants during day five of adulthood (Aalto & Nicastro et al., 2017). (Chi-Squared Test with Yate's Correction, *p < 0.05, **p<0.01, ***p<0.001). The number in the colored bars to the left corresponds to the number of genes (**A**) up- and (**B**) down-regulated in *alg-1* mutants with predicted miRNA sites. The numbers in the gray bars to the far right correspond to the total number of genes (n=13,990) identified through RNA-seq of *alg-1* mutants with predicted miRNA sites.

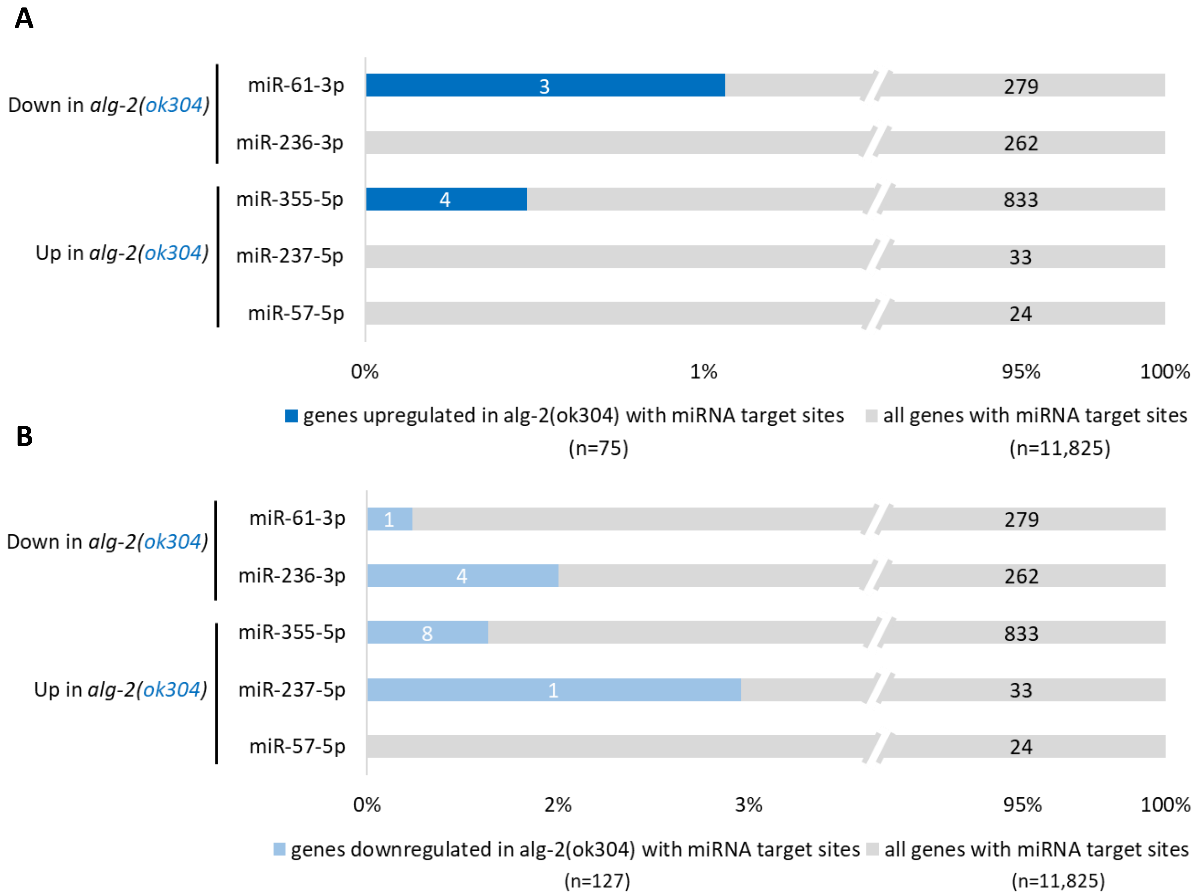


**Figure 6. miRNA candidates are predicted to target genes misregulated in *alg-2* mutants during adulthood.** Seed enrichment analysis was performed on candidate miRNAs to assess the enrichment of gene targets for those genes (**A**) up- (n=75) and (**B**) down-regulated (n=127) in *alg-2(ok304)* mutants during day five of adulthood (Aalto & Nicastro et al., 2017), (Chi-Squared Test with Yate's Correction, *p < 0.05, **p<0.01, ***p<0.001). The number in the colored bars to the left corresponds to the number of genes (**A**) up- and (**B**) down-regulated in *alg-1* mutants, respectively, with predicted miRNA sites. The number in gray to the far right corresponds to the total number of genes (n=13,990) identified through RNA-seq of *alg-1* mutants with predicted miRNA sites.

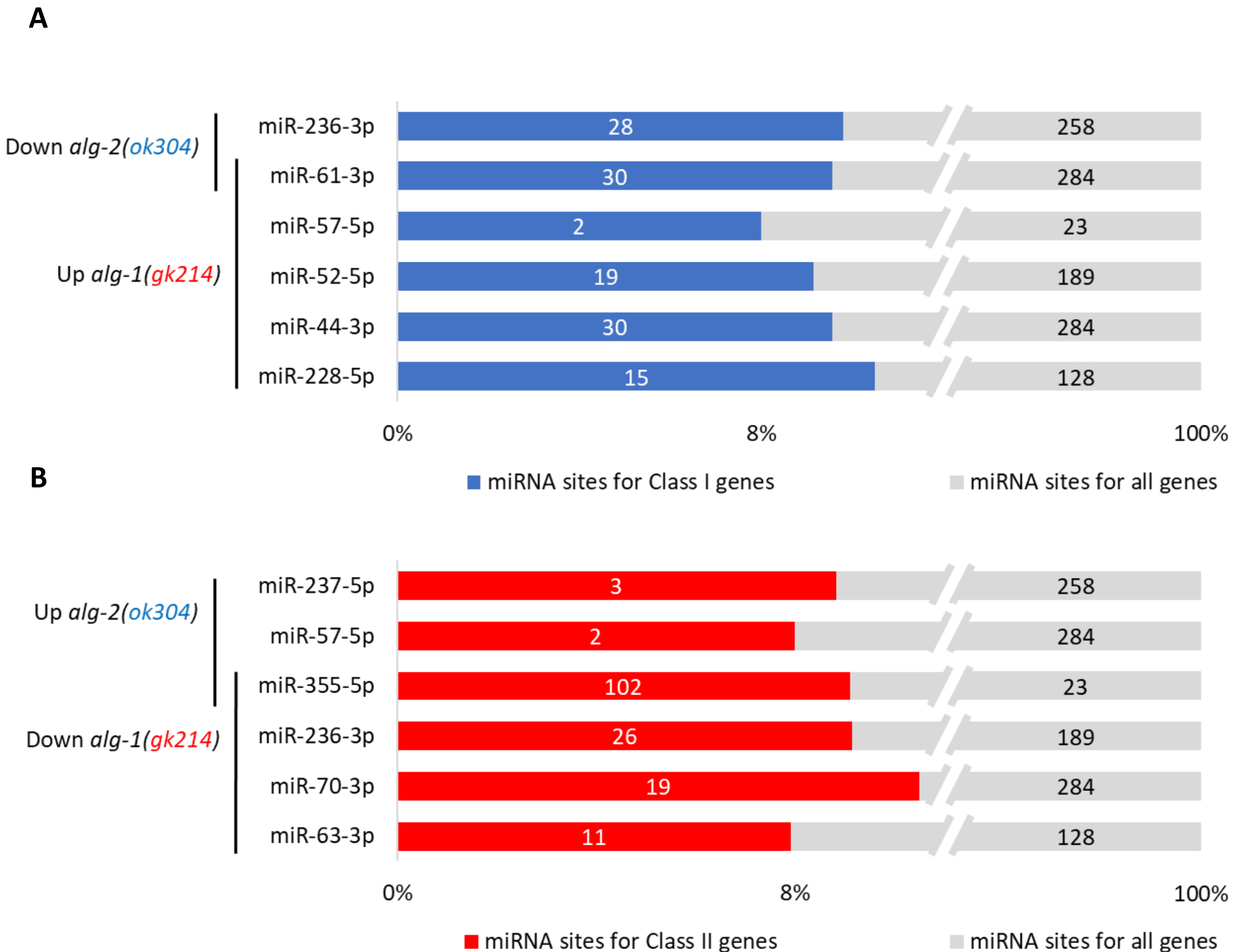


**Figure 7. miRNA candidates are predicted to target *Class I* and *Class II* genes of the Insulin/IGF-1 Signaling pathway.** (**A**) Seed target prediction analysis was performed on miRNA candidates to predict the number of Class II genes (Tepper et al., 2013) targeted by candidate miRNAs. The number in the colored bars (Red) corresponds to the number of predicted *Class II* targets and the number in gray on the far right corresponds to the total number of predicted gene targets (Chi-Squared Test with Yate's Correction, *p < 0.05). (**B**) Enrichment or depletion of *Class I* genes targeted by miRNA candidates (Chi Squared test with Yates Correction, *p < 0.05).

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