miRNA Expression and Strand Selection Throughout *C. elegans* Development

by

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of the Requirements for the Degree
Master of Science

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ABSTRACT

MicroRNAs (miRNAs) are 17-22 nucleotide non-coding RNAs that regulate gene expression by targeting non-complementary elements in the 3' untranslated regions (3'UTRs) of mRNAs. miRNAs, which form complex networks of interaction that differ by tissue and developmental stage, display conservation in their function across metazoan species. Yet much remains unknown regarding their biogenesis, localization, strand selection, and their absolute abundance due to the difficulty of detecting and amplifying such small molecules.

Here, I used an updated HT qPCR-based methodology to follow miRNA expression of 5p and 3p strands for all 190 C. elegans miRNAs described in miRBase throughout all six developmental stages in triplicates (total of 9,708 experiments), and studied their expression levels, tissue localization, and the rules underlying miRNA strand selection. My study validated previous findings and identified novel, conserved patterns of miRNA strand expression throughout C. elegans development, which at times correlate with previously observed developmental phenotypes. Additionally, my results highlighted novel structural principles underlying strand selection, which can be applied to higher metazoans.

Though optimized for use in C. elegans, this method can be easily adapted to other eukaryotic systems, allowing for more scalable quantitative investigation of miRNA biology and/or miRNA diagnostics.

DEDICATION

To my amazing friends, whose support and presence has been critical in navigating the emotional quagmire that is higher education. To my teachers, professors, and mentors, whose deep care and professional acuity taught me what it means to be a lifelong learner, teacher, and pursuant of truth. To all members of my family, whose endless support and bottomless patience has inspired me to achieve far more than I ever thought I could.

To my brother, you are my best friend and my ultimate role model. Nothing about college has been easy, but thanks to you it has never for a moment felt too difficult to overcome. Thank you.

To my father, whose words and acts every day continue to inspire me to look inward and to find the strength to carry on, even in the face of failure or adversity. When I needed to hunker down and just push through, you were always at the forefront of my mind. Thank you.

To my mother, whose sacrifices and selfless acts made me who I am today — I seriously cannot properly enumerate all the ways you have made me the man, the son, and the scientist I am today. No matter where I go or what I choose to be, I know without the barest hint of doubt that you will be right there beside me. Gratitude does not begin to do that justice, but thank you.

Thank you.

Thank you.

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I would like to acknowledge first and foremost my mentor, PI, and thesis committee chair Dr. Marco Mangone for your countless contributions to this project and to my personal and professional development. Through setback and success, you have been a steady guide and steadfast example of what I strive to one day emulate in this field. Under your tutelage I have gone from learning that microRNAs exist to performing exploratory research into their functions and mechanisms *in vivo*, a feat I never would have thought possible just three short years prior. I am proud to be a part of the Mangone lab, and prouder still to contribute to the rich history of research therein. As I continue my graduate education under Dr. Mangone's tutelage, I have no doubt that no matter what avenue of research I should choose next to pursue, you will have my back.

Of course, this work would not be possible without the guidance and support of the each and every other member of my thesis committee. I would like to thank Dr. Joshua LaBaer for always keeping me honest and providing insightful feedback as to the methods and future applications of this project. Moving forward, I anticipate a collaborative effort on the horizon for which I am incredibly excited. Additionally, I would like to thank Dr. Vel Murugan, without whom this project would simply not have been possible. Your generous provision of resources and machinery with which to carry out high-throughput screens allowed me to collect and analyze far more data than I ever thought possible in just a few short months. Additionally, your expertise in qPCR workflows also proved invaluable in designing my own research methods. Finally, I would like to thank Dr. Jeanne Wilson-Rawls for being in my corner and always offering support along the way. Attending research colloquiums and seeing the ease and comfort with which you carry yourself and present your work has been inspiring, and

if I can display even a modicum of that confidence as I mature as a researcher, I will be happy indeed.

Next, I want to acknowledge the critical contributions of my fellow lab members; first to Aleah Deptula, whose mentorship set me on the path to independence and confidence as a researcher. I would also like to thank my fellow graduate students Anna Shorr, Emma Murari, and Nicholas Cuda who each have taught me so much about research and about myself: to Anna, thank you for teaching me to always take the time to eke out little kindnesses and to take care of myself; to Emma, thank you for teaching me patience and to always do my due diligence; to Nick, thank you for teaching me curiosity in the midst of frustration and to always be on the lookout for new opportunities. To the other two undergraduates in the Mangone lab, Reagan Conrad and Sara Keane, I want to say thank you for making even the hardest days bearable and the best days better.

I would especially like to acknowledge my two undergraduate trainees Jillian Murray and Hailee Hargis, who have taught me this past year the critical importance of humility and mentorship in science. It is one thing to set up and perform experiments; it is another entirely to be able to explain and teach them to someone else. Both of you have taught me how to teach, but more so that I always have more to learn. Every day in lab is a new excitement thanks to you, and without your endless encouragement and assistance, this project would not have been possible. To Hailee especially for your hours in the cold room and hours more spent thawing plates and tubes, I salute you.

Finally, I would like to acknowledge Arizona State University for this opportunity and for setting me on the path to where I am today.

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PREFACE

This thesis is the collaborative unpublished work of the author Dalton A. Meadows and undergraduate research assistants Jillian Murray and Hailee Hargis. None of the data contained herein has been previously published, though much of this research was only possible due to previous research conducted at the Mangone lab and the international *C. elegans* community.

Method design contained within is an extension of the work done by the Qian lab, adapted and refined for use in high-throughput. Analytical methods, including those presented in Supplemental Figure S1, are adapted from current consensus on analysis of qPCR data and current consensus on principles underlying strand selection.

Figures 1 and 2 are adaptations of previous work in the miRNA field, and proper accreditation is included with each. Figures 3 through 7 are the original work of the author. Figure 7 includes images produced using design software BioRender. All data included in Supplemental Tables S1 through S4 are the right of Arizona State University.

CHAPTER 1

INTRODUCTION

Thirty years ago, researchers at the Ambros and Rutkun groups discovered *lin-4*, a short, non-coding RNA in *Caenorhabditis* elegans (*C. elegans*) which bound to the 3′ untranslated region (3′ UTR) of the gene *lin-14* and suppressed it post-transcriptionally (Lee, Feinbaum, & Ambros, 1993; Wightman, Ha, & Ruvkun, 1993). This was the first microRNA (miRNA), a class of RNA polymerase II transcripts about 21 nucleotides in length whose discovery sparked a revolution in the field of microbiology that continues to this day. Post-transcriptional regulation via miRNA is ubiquitous and highly conserved across eukaryotes and has been implicated in countless processes from embryogenesis to the complex functions of postmitotic cells (Fabian, Sundermeier, & Sonenberg, 2010; Gross, Kropp, & Khatib, 2017).

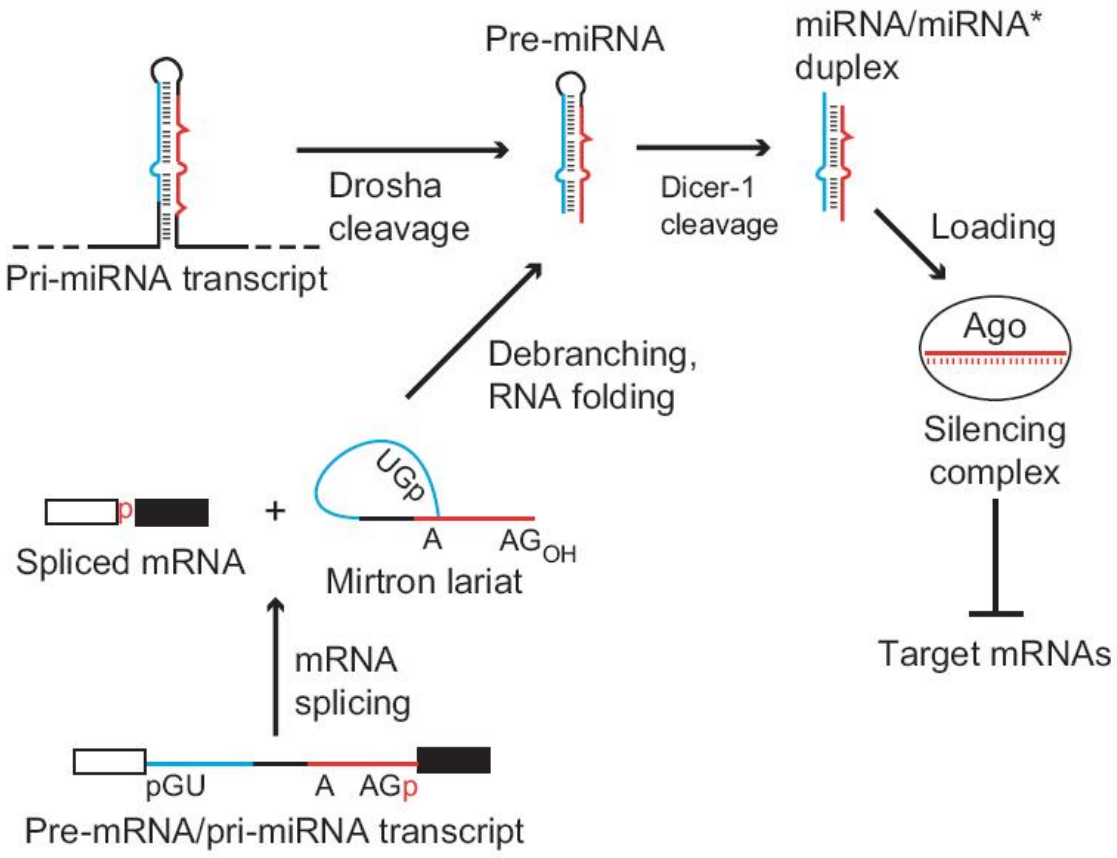
This regulation occurs as a result of the miRNA-induced silencing complex (miRISC), a protein-miRNA complex which permits semi-complimentary binding of the so-called 'seed region' of the miRNA to a target region on the 3′ UTR of mature mRNA transcripts (Medley, Panzade, & Zinovyeva, 2021). This seed region consists of the second through seventh nucleotides directly downstream of the 5′ end of the miRNA. Unlike silencing via short interfering RNA, miRISC silencing of mRNA targets does not require perfect complementarity, and thus a single miRNA can bind to numerous targets either on the 3′ UTR of one gene or the 3′ UTRs of multiple different genes (Lee et al., 1993). Before miRNAs can be loaded onto the miRISC, however, they must first undergo a series of maturation steps.

miRNAs derive as any other RNA pol II transcript from a complementary region of DNA (Lim et al., 2003). In *C. elegans*, intergenic pol II transcripts form hairpin structures called pri-miRNAs which must undergo an initial cleavage near the base of the stem by the endonuclease Drosha (Figure 1) (Medley et al., 2021). The result is a

short hairpin of around 60 nucleotides called pre-miRNA which can be exported from the nucleus via Exportin-5 (or the relevant eukaryotic homolog (Yi, Qin, Macara, & Cullen, 2003). Splicing of introns can also result in spontaneous generation of pre-miRNAs via folding, referred to as miRtrons (Ruby, Jan, & Bartel, 2007). Regardless of their means of generation, pre-miRNAs undergo one additional cleavage of the hairpin loop via the endonuclease Dicer, which acts as a molecular ruler to generate mature miRNA duplexes.

Figure 1

miRNA Maturation



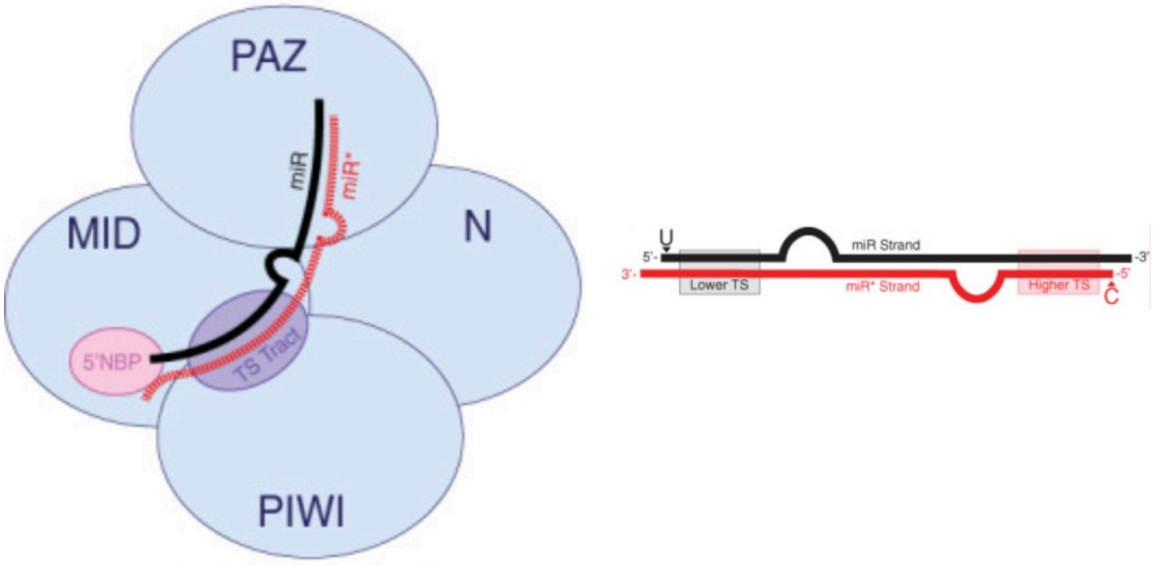
Note. miRNA maturation and silencing pathway for both intergenic and intronic miRNAs. Adapted from Ruby, Jan, & Bartel, 2007.

These duplexes consist of two near-complimentary strands of RNA around 21 nucleotides in length, often with 3′ overhangs of one to three nucleotides. One of these two strands — traditionally referred to as the miR strand or guide strand — is loaded onto the *C. elegans* Argonaute proteins Alg-1 and Alg-2 to form the RISC complex (Brosnan, Palmer, & Zuryn, 2021). It should be noted that traditional nomenclature distinguishes the two strands of a miRNA duplex as either miR/miR* or guide/passenger, reflecting which strand is preferentially loaded onto the miRISC. However, this fails to account for the independent roles each strand can fulfill. To better reflect the possible multifunctionality of both duplex strands, the remainder of this paper will refer to the strand distinction as 5p/3p: either the 5p strand proximal to the 5′ end of the pre-miRNA hairpin, or the 3p strand proximal to the 3′ end of the hairpin.

The mechanisms by which one strand is loaded onto the miRISC and the other discarded and digested are still not fully understood. However, all Argonaute proteins contain four domains: an N-terminal domain, a PAZ (Piwi/Argonaute/Zwille) domain, a MID (middle) domain, and a PIWI (P-element induced wimpy testis) domain (Muller, Fazi, & Ciaudo, 2019; Nakanishi, 2022; Niaz, 2018). During miRISC assembly, two regions have been proposed to be critical in strand selection: the 5′ nucleotide binding pocket (5′ NBP) within the MID domain and the thermostability (TS) tract at the interface of the MID and PIWI domains (Figure 2). Though the role each of these domains plays in miRNA strand selection remains uncertain, their binding affinities suggest two underlying principles: (1) that the strand contains a 5′ uracil, and (2) that the strand exhibits lower thermostability within the seed region. Taken together, these principles can predict with high accuracy (75-83%) which of either the 5p or 3p strand will be loaded onto the miRISC (Medley et al., 2021).

Figure 2

miRISC Complex Assembly



Note. Assembled miRISC complex with annotated 5′ nucleotide binding pocket and thermostability tract. The black miR/5p strand will be loaded, and the miR*/3p strand will be degraded. Adapted from Medley et al., 2021.

In *C. elegans*, certain miRNAs have been extensively profiled over the past thirty years. For instance, *let-7*, the second miRNA discovered, has been known to affect developmental timing for decades, however as recently as last year novel functions were elucidated regarding retinoid-related nuclear receptors and molting cycles (Ambros, 2001; Patel, Galagali, Kim, & Frand, 2022; Reinhart et al., 2000). However, many more *C. elegans* miRNAs remain understudied and poorly understood. This is partly due to the difficulty of predicting 3′ UTR binding sites, though the Mangone lab has published multiple versions of the *C. elegans* UTRome to assist in this endeavor (Mangone, Macmenamin, Zegar, Piano, & Gunsalus, 2008; Steber, Gallante, O'Brien, Chiu, & Mangone, 2019). The other major restriction in studying miRNA function and properties is the difficulty of accurately quantifying such small molecules in high-throughput. Currently, the most widespread approach to quantify

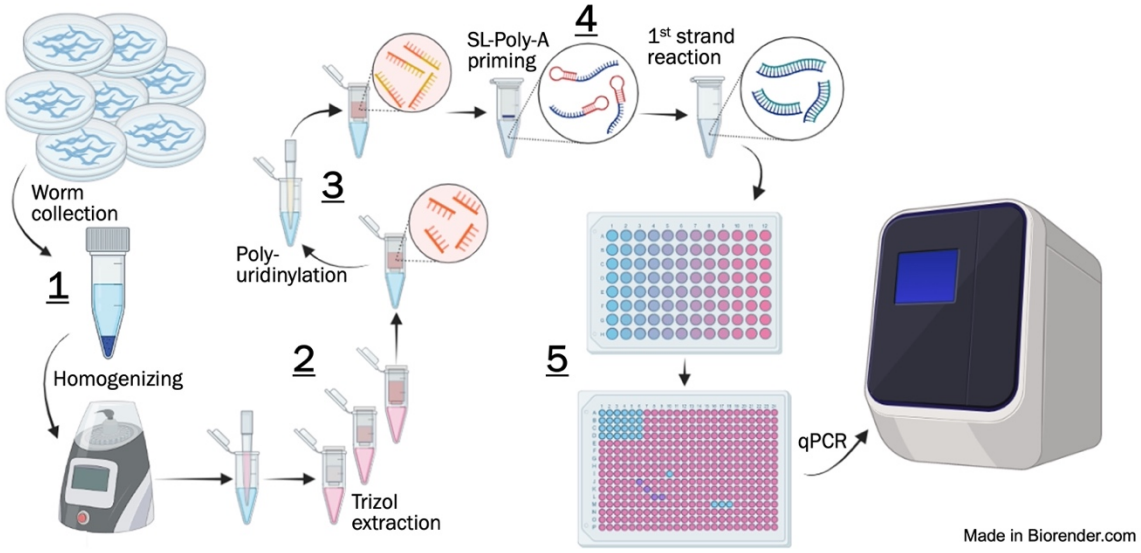
miRNAs is small RNA-seq, a genome-wide approach of which many variations exist, all of which exhibit their own strengths and weaknesses in bias, accuracy, complexity of workflow, and availability of reagents (Benesova, Kubista, & Valihrach, 2021).

Even as new and more refined variants of small RNA-seq continue to be developed, there remains the weakness of scalability. As a genome-wide approach, the researcher must conduct a whole-genome screen no matter how many or how few miRNAs are of interest. As such, there remains no current consensus as to the preferred method of analyzing miRNA levels (de Gonzalo-Calvo, Perez-Boza, Curado, Devaux, & CA, 2022; Moody, He, Pan, & Chen, 2017).

In order to address these issues and better characterize the role of understudied miRNAs in *C. elegans*, I have developed a scalable, high-throughput, qPCR-based workflow to quantify the expression of any number of miRNAs either in specific tissues or from whole worms (Figure 3). Using this method, I was able to

Figure 3

miRNA Extraction and Quantification by qPCR



Note. Total workflow of HT qPCR-based protocol for miRNA quantification.

quantify the levels of all 190 known miRNAs (both the 5p and 3p strands) in *C. elegans* through all six stages of development. With much already known about the developmental roles of certain miRNAs like *lin-4* and *let-7,* this provided both validation of my method and a novel investigation into the potential role of many less characterized miRNAs through analysis of their patterns of expression.

Furthermore, these developmental expression data provided a high fidelity look into the structural basis of miRNA strand selection. By analyzing the first seven 5′ nucleotides of miRNAs where one strand is predominant across all six stages of development, I was able to qualify the extant rules of strand selection. Additionally, I uncovered a potential novel principle which, though preliminary, may help explain cases in which miRNAs do not 'follow' the two previously described principles of strand selection.

CHAPTER 2

MATERIALS AND METHODS

Triton X-100 Lysis Buffer

Triton X-100 lysis buffer was prepared as advised by Cold Spring Harbor (150 mM NaCl, 1.0% Triton X-100, 50 mM Tris-HCl pH 8.0, 0.5 mM EDTA). 1 liter of buffer may be prepared as follows: 819 mL deionized H_2O, 100 mL 10% Triton X-100, 50 mL 1 M Tris-HCl pH 8.0, 30 mL 5 M NaCl, and 1 mL 0.5 M EDTA.

Stem-Loop Poly-A Primers

Stem-loop poly-A primers were ordered from IDT at a concentration of 50 ng/μL. The sequence is as follows: GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAAAAAAAAAAAAA.

High-Throughput miRNA Extraction and Quantification Protocol

To obtain sufficient lysate for RNA purification, *C. elegans* were washed into a Falcon 15 mL tube and spun down at 1500 rpm for 3 minutes to generate a pellet of approximately 1.0 mL (Figure 3.1). Following standard synchronization protocol, the *C. elegans* were bleached and plated onto 12 100x15 mm petri dishes seeded with nematode growth medium (17 g BactoAgar, 3 g NaCl, and 2.5 g peptone in 1 L H_2O) and OP50-1 *E. coli*. Once sufficient time had passed to allow growth to the desired stage of development (as per the stages of *C. elegans* development described within the Worm Atlas), the *C. elegans* were washed into a single Falcon 15 mL tube and spun down at 1500 rpm for 3 minutes. Following one additional wash to remove bacterial contamination and vacuum removal of the supernatant, the pellet was resuspended to a total volume of 1.5 mL in Triton X-100 lysis buffer and transferred into a 1.5 mL homogenization tube containing 0.5 mm zirconium beads. The *C. elegans*

were homogenized using a Beadbug benchtop homogenizer at 4000 rpm for 90 seconds, let chill on ice for 3 minutes, then homogenized again at 4000 rpm for 60 seconds.

After confirming success of homogenization under the microscope, the lysate was spun down and supernatant transferred to a 1.5 mL microcentrifuge tube. One additional spin down in a benchtop centrifuge was performed to pull down any remaining debris before transfer to a pre-chilled Falcon 15 mL tube. Under a fume hood, Trizol was added at a ratio with lysate of 3:1. After gentle inversion, an equal volume of 100% ethanol was added to the Trizol-lysate mixture. RNA was extracted in a 4° C cold room according to the instructions within the Zymo Research Directzol RNA Purification kit (Cat. No. R2070) (Figure 3.2). All lysate-Trizol-ethanol mixture was spun through a single column to maximize yield, and the optional DNaseI digestion was always performed. Total RNA was eluted in 30 μL nuclease-free H_2O.

Using the adapted protocol from Qian and colleagues, 3 replicates of the following reaction were prepared on ice in 50 μL flat-top microamp PCR tubes (Figure 3.3) (Qian, 2012): 10.25 μL H_2O, 9.0 μL total RNA, 2.5 μL 10x NEBuffer 2.0, 1.25 μL 10 mM UTP, 1.0 μL RNase inhibitor (murine was used; any should suffice), and 1.0 μL NEB poly-U polymerase. All reactions were incubated at 37 °C for 10 minutes followed by a 65 °C incubation for 20 minutes to deactivate the polymerase. Afterward, the reaction vessels were combined into a single 1.5 mL microcentrifuge tube and chilled on ice. 5 replicates of the following reaction were then prepared in 50 μL flat-top microamp PCR tubes (Figure 3.4): 12.0 μL previous reaction mixture, 1.0 μL dNTP mixture (10 mM each), and 1.0 μL stem-loop poly-A primers (as described above).

After a 5-minute incubation at 65 °C, the following reagents were added to each reaction vessel: 4.0 μL 5x first strand buffer (Thermofisher), 1.0 μL 0.1 M DTT, 1.0 μL superscript III reverse transcriptase (Thermofisher). All reactions were incubated at

50 °C for 50 minutes, then at 70 °C for 15 minutes. Resultant cDNA was recombined into a single 1.5 mL microcentrifuge tube. (If desired, cDNA can be frozen and stored at -20 °C). Following the steps above, 6 100 μL samples of cDNA corresponding to the total miRNA of each developmental stage in *C. elegans* were gathered and frozen.

Four 96-well plates were ordered containing unique forward primers each complimentary to a single mature strand of miRNA, totaling 380 primers — 190 complimentary to 5p strands and 190 complimentary to 3p strands — at a concentration of 50 ng/μL (Appendix A, Tables S1, S2). An additional 96-well plate was ordered containing a universal reverse primer complimentary to the stem-loop of the SL-poly-A primers (Appendix A, Table S3). Each forward primer plate and the universal reverse primer were diluted to a concentration of 10 ng/μL. Additionally, each developmental stage cDNA sample was thawed and quantified using a NanoDrop™ One microvolume UV spectrophotometer, then diluted to 75 ng/μL. Throughout all following quantifications, the following were added to each well of a 96-well plate: 20n μL PowerTrack™ SYBR Green Master Mix (Thermofisher, cat. no. A46109), 13n μL nuclease-free H_2O, 2n μL cDNA (75 ng/μL), 2 μL forward primer (10 ng/μL), 2n μL reverse primer (10 ng/μL), and 1n μL SYBR DNA dye (Thermofisher, included when purchasing PowerTrack™ SYBR Green Master Mix). In the preparation of high-throughput master mixes, all above reagents except the specific forward primer were mixed where n represents 4 + the desired number of samples to be quantified. For 96-well preparation, a master mix of n=100 was mixed and dispersed into the wells using a multichannel pipette. Afterward, 2 μL of unique forward primer were added to each well using a using a multichannel pipette. At least one well was set aside for an exogenous positive control using a pDONR 221 GFP plasmid at a concentration of 10 pg/μL and GFP forward and reverse primers at a concentration of 10 ng/μL. The resulting 96-well qPCR array was vortexed and spun down at 1000 rpm for 2 minutes,

keeping all reagents and plates on ice and protected from light as much as possible. Additional arrays were prepared as non-template controls for each unique forward primer, substituting 2 μL cDNA for 2 additional μL nuclease-free H_2O.

Each 96-well qPCR array was then stamped in 10 μL triplicate into a 384-well plate using a Biomek i7 automated workstation. The resulting 384-well array was sealed with Microamp adhesive optical film and quantified using a QuantStudio 7 Pro real-time PCR system.

miRNA Expression Data Analysis Workflow

Initial cycle threshold (Ct) values of each qPCR amplification were calculated using the QuantStudio 7 Pro design and analysis software and exported as a Microsoft Excel workbook. Using Excel, a ΔΔCt analysis was conducted to determine the relative quantity of each miRNA as compared to the exogenous control (Appendix B, Figure S1). The log of the resulting relative quantities, representing successive orders of magnitude increases in quantity of miRNA, was imported into a Mysql database grouped by miRNA and developmental stage. Sequences for the mature 5p and 3p strand of each miRNA duplex were added from data available through miRbase.org. Further analyses were conducted using PhpMyAdmin to query subsets of miRNAs fulfilling various criteria from the database and exporting the resulting expression values and/or sequences. Excel was used to graph trends of expression over developmental time using exponential smoothing (smoothing factor 0.6) of expression values through all six stages of development. Heatmapper.ca was used to visualize expression values as a heat map, following a ten-color logarithmic scale.

miRNA Strand Selection Data Analysis Workflow

Using PhpMyAdmin, the database was queried for all miRNAs which showed distinct preference for either the 5p or 3p strand throughout all six stages of development. The sequences of these subsets of miRNAs were then truncated to the first seven nucleotides, and graphed as sequence logo plots using WebLogo 3. To determine the combined effect of both guiding principles of strand selection, a coefficient of strand selection (C_{ss}) was developed to account for both the ratio of hydrogen bonds in the 5p and 3p strands and the adjustment for uracil (A_u), accounting for both guiding principles of strand selection (Figure 4). Thus, miRNAs which display a C_{ss} less than 1.0 are predicted to prefer the 5p strand, whereas miRNAs which display a C_{ss} greater than 1.0 are predicted to prefer the 3p strand.

Figure 4

Equation for Coefficient of Strand Selection

$$C_{ss} = \frac{\#\ 5p\ hydrogen\ bonds}{\#\ 3p\ hydrogen\ bonds} \pm A_U$$

CHAPTER 3

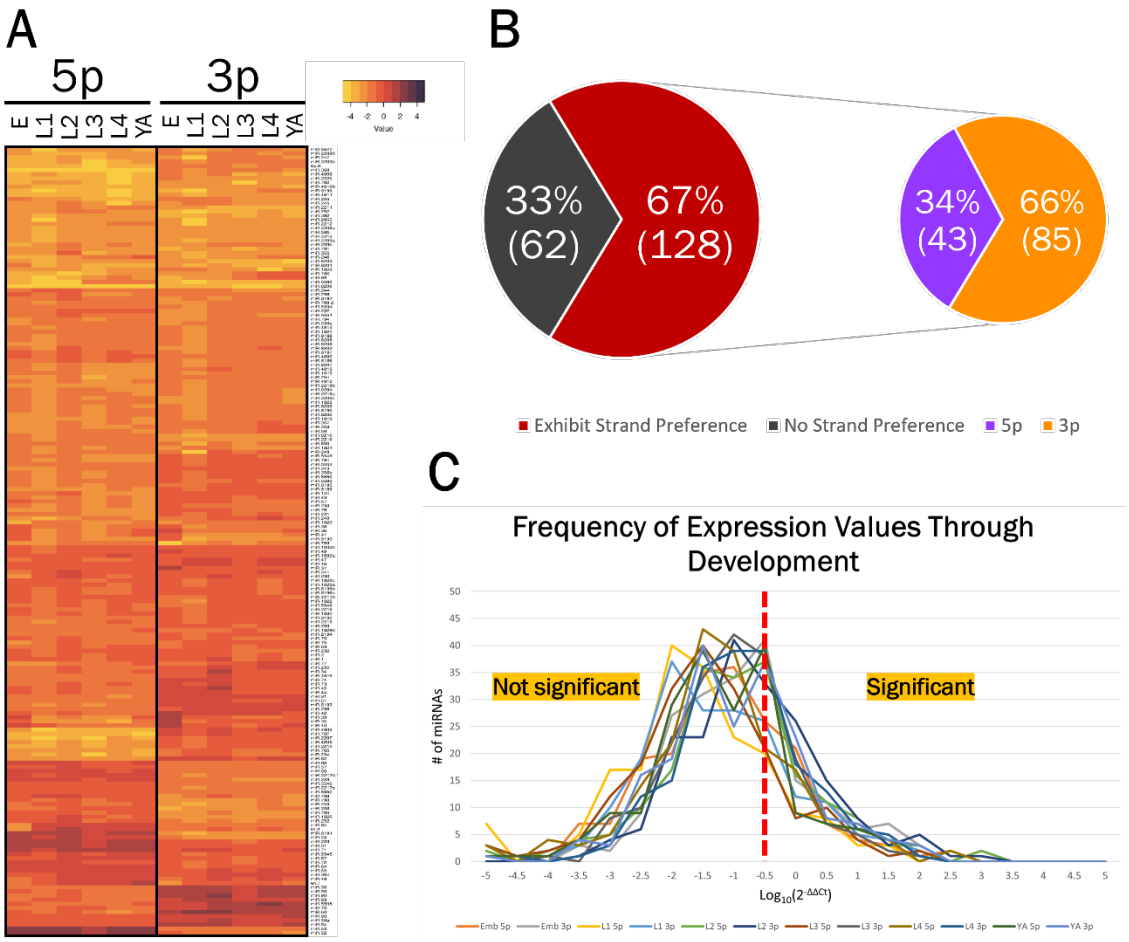
RESULTS

All Tested miRNAs Were Quantified during at Least One Developmental Stage.

All 190 *C. elegans* miRNAs were quantified using the method described above across all six stages of development (Figure 5A; Appendix A, Table S4). Euclidean

Figure 5

miRNA Quantification Through Development



Note. Heatmap of miRNA expression across all six *C. elegans* developmental stages (A). The majority of miRNAs displayed a strong preference for either the 5p or 3p strand across all developmental stages (B). Of the 190 miRNAs, 72 were selected for expression pattern analysis based on an expression value cutoff of -0.5 (C).

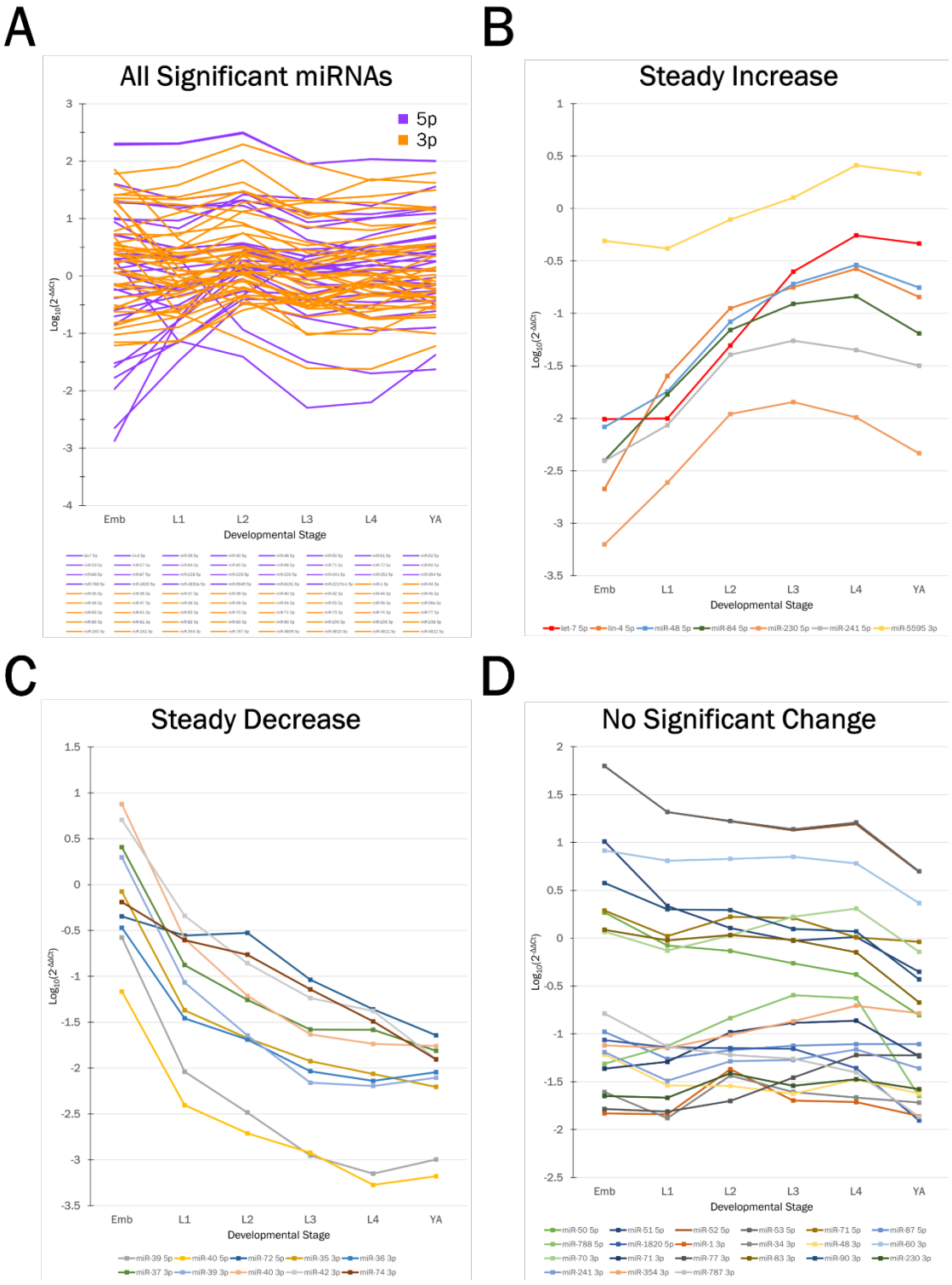
clustering revealed several distinctions: first, that all tested miRNAs displayed quantification to some degree during at least one stage of development; second, that the majority of miRNAs displayed a strong preference for either the 5p or 3p strand. By selecting for miRNAs for which one strand was more highly quantified across all six stages of development, it was determined that approximately two-thirds (67%) of all *C. elegans* miRNAs displayed a strong strand preference (Figure 5B). Of these miRNAs, approximately two-thirds (66%) displayed a preference for the 3p strand. Though all 190 miRNAs were quantified during at least one developmental stage in at least one strand, many were quantified at extremely low levels. Across all developmental stages, the average relative expression value of all miRNAs was -1.13. Thus, a filter was applied to screen any miRNAs which were never expressed above an expression value of -0.5 at any stage of development, corresponding to approximately four times greater expression than average (Figure 5C).

Highly Expressed miRNAs All Follow One of Three Distinct Patterns of Expression.

To determine whether strand selection bias was correlated with any specific patterns of expression, these 72 significant miRNAs were graphed according to expression value over time (Figure 6A). No such correlation was observed, and thus it did not appear that strand selection bias had any effect on trends in expression of miRNAs over time. To validate any expression patterns independent of strand selection, a representative subset of 38 of these significant miRNAs underwent quantification two additional times across all six stages of development to derive a more robust experimental triplicate of these data. These data were averaged, and trend analysis as described above revealed three distinct patterns.

Figure 6

Patterns in miRNA Expression Throughout Development



Note. No pattern was observed which defined significant miRNAs displaying 5p strand bias vs. 3p strand bias (A). However, all significant miRNAs could be sorted into three distinct patterns of expression over time — steady increase (B), steady decrease (C), and no significant change (D).

The first such pattern is a steady increase in overall expression over time (Figure 6B). The majority of miRNAs observed which follow this pattern, both 5p and 3p strands, fall under the *let-7* family (*let-7*, *miR-48, miR-84,* and *miR-241*). This family of miRNAs are known to regulate the transition from larval 4 stage to adult in *C. elegans*, controlling such genes as *let-60* to regulate adult structural development in the vulva (Hunter et al., 2013). Additionally, this pattern encapsulates the 5p strand of *lin-4*, which has been shown to be cyclically transcribed in short pulses corresponding to molting during each stage of *C. elegans* development (Kinney, 2022). *miR-230* has been implicated in the aging process of worms, though its mechanism of action remains unknown (de Lencastre et al., 2010; Lucanic et al., 2013). Taken together, all miRNAs which steadily increase over time regulate similar biological functions key to adult-stage-specific development in *C. elegans*.

The second pattern observed is just the opposite: a steady decrease in miRNA levels over time. Here too I observed consistent similarities between the miRNAs which followed this pattern and their functions *in vivo*. The vast majority of miRNAs of this pattern were members of the *miR-35* family (*miR-35, miR-36, miR-37, miR-39, miR-40,* and *miR-42*). This family functions to regulate genes implicated in the embryo to larva transition, and all share high sequence similarity in their seed region leading to rapid degradation in each subsequent larval stage (Donnelly et al., 2022). Such rapid degradation is also observed, though in a slightly different pattern, for members of the *miR-72* family (*miR-72* and *miR-74*), which have been shown to be downregulated during the aging process (Lucanic et al., 2013).

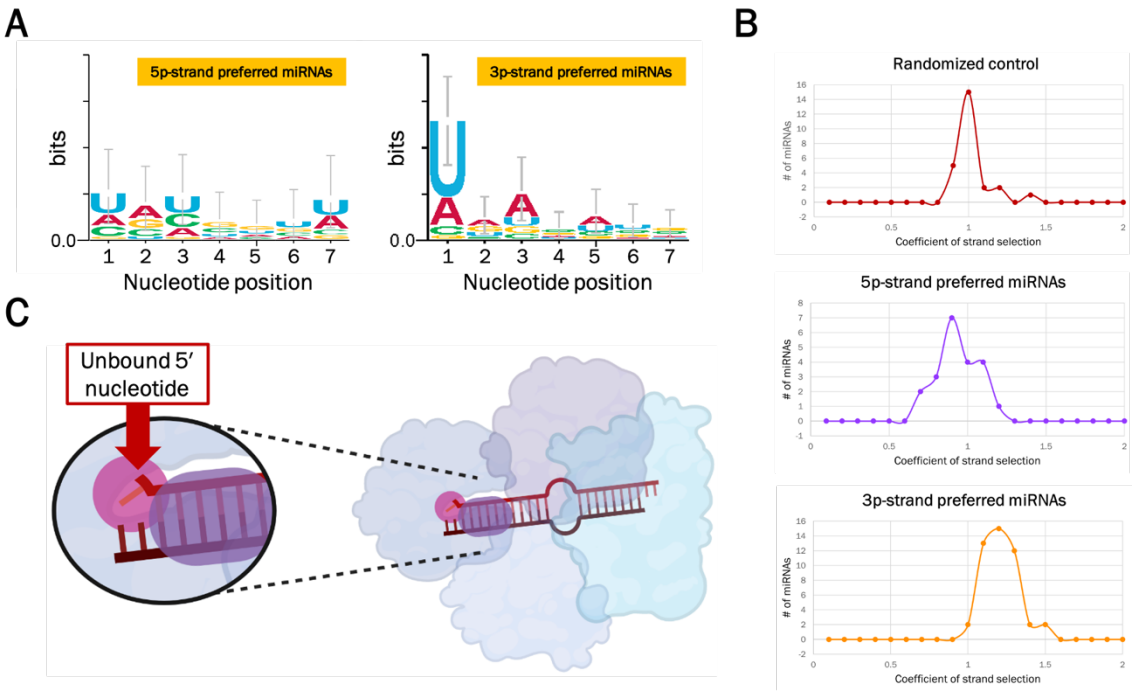
The third, final, and by far most numerous pattern of miRNA expression is that of no significant change. Two major miRNA families make up a significant portion of these miRNAs: the *miR-50* family (*miR-50, miR-90*), the *miR-51* family (*miR-51, miR-52, miR-53*). Not much is known regarding the function of the *miR-50* family besides the fact that the worm does not die if it is deleted (Miska et al., 2007). However, the *mIR-51* family is critical for embryonic development, though each miRNA seems to be redundant for achieving viability, and all but one can be knocked out to little effect (Alvarez-Saavedra & Horvitz, 2010). Other microRNAs that display this pattern of expression include *miR-1*, which regulates neuromuscular junctions, and *miR-34*, which mediates the DAF-16 stress network in *C. elegans* (Isik, Blackwell, & Berezikov, 2016; Schiffer et al., 2021). Numerous other miRNAs also follow this pattern, some members of miRNA families and others less well functionally characterized. Among these miRNAs which rarely fluctuate in their level of expression, diversity is the only constant.

Strand Selection in *C. elegans* Appears Far More Predictable in 3p-Strand Preferred miRNAs.

Using the data gathered above, I was able to further investigate the principles underlying miRNA strand selection in *C. elegans*. To do so, miRNAs were queried which either always showed a strong preference for the 5p strand or a strong preference for the 3p strand. These 5p-strand preferred and 3p-strand preferred miRNAs had their sequences truncated to seven nucleotides and plotted according to prevalence at each position (Figure 6A). While position 1 (the 5′ nucleotide of the selected strand) showed no particular nucleotide preference in 5p-strand preferred miRNAs, in 3p-strand preferred miRNAs this nucleotide was overwhelmingly a uracil. This supports the first

Figure 7

Principles of Strand Selection in 5p- or 3p-Strand Preferred miRNAs



Note. Nucleotide prevalence at the first seven 5′ nucleotides of 5p-strand preferred miRNAs vs. 3p-strand preferred miRNAs (A). Coefficient of strand selection graphed as frequency distribution of 5p-strand preferred miRNAs vs 3p-strand preferred miRNAs, as compared with a randomized control (B). Proposed third principle of strand selection — an unbound 5′ nucleotide — to account for aberrant strand selection seen in some 5p-strand preferred miRNAs which do not otherwise follow the two principles of strand selection (C). Figure 7C made using BioRender.

guiding principle of strand selection, but does not account for the second principle of thermostability. Accordingly, these miRNAs which displayed strong strand preference were graphed as a frequency distribution according to their coefficient of strand selection (see methods above), which accounts for both the presence of a 5′ uracil and the thermostability of the following 6 nucleotides (Figure 6B). This coefficient is a predictive calculation, and as such a randomized control showed a predictive peak at a coefficient of exactly 1.0. As expected, every 3p-strand preferred miRNA had a coefficient of greater than 1.0; however, not every 5p-strand preferred miRNA had a

coefficient of less than 1.0 — i.e. if these miRNAs were to follow the two guiding principles of strand selection, I would expect the 3p strand to be chosen, not the 5p strand. Yet these miRNAs displayed strong 5p strand preference.

To explain this disconnect, I analyzed the structure of the miRNA duplex for each such aberrant 5p-strand preferred miRNA. I found that in four out of six of these duplexes, the 5′ nucleotide of the preferred strand was not Watson-Crick paired to a complementary nucleotide (Figure 6C). This unbound nucleotide could therefore be more available for binding by the 5′ nucleotide binding pocket of an Argonaute homolog, leading to the selection of an otherwise thermodynamically unfavorable miRNA strand.

CHAPTER 4

DISCUSSION

Here I studied the changing dynamics of miRNA expression throughout *C. elegans* development. Among all 190 known miRNAs, 67% were found to exhibit strong preference for either the 5p or 3p strand across all six stages of development. This preponderance of strand preference supports existing literature surrounding the molecular machinery behind miRNA-induced repression of translation via the miRNA induced silencing complex, which across all eukaryotes seems to preferentially bind just one strand of any given miRNA duplex (Medley et al., 2021). This data further supported the idea that miRNAs for which both strands are loaded onto the miRISC and perform separate regulatory functions are examples of neofunctionalization. Further analysis is ongoing to determine whether any significant patterns emerge when tracking the expression of these multifunctional miRNAs throughout *C. elegans* development.

For those miRNAs which exhibit strong strand preference, 72 were found to be expressed significantly above the average during at least one developmental stage. Trend analysis of these miRNAs, however, revealed no significant patterns in their expression as related to the strand preferentially loaded onto the miRISC. Rather, three expression patterns were observed which relate to the biological role of any given highly expressed miRNA: a steady increase over time, a steady decrease over time, and a constant level of expression. It should be noted that due to time and material constraints, 38 of these miRNAs were quantified in triplicate and subjected to more rigorous trend analysis. Based on a preliminary trend analysis of all 72 highly expressed miRNAs, this subset was representative of the whole. Perhaps unsurprisingly, those miRNAs which increased in expression over developmental time exhibited functional roles in regulating the development of adult phenotypic traits in

worms, whereas those miRNAs which decreased in expression over time tended to regulate embryonic phenotypes and the transition to and between larval stages.

Separate from miRNAs classified under well-known families, though, several were far less well characterized. *miR-5595*, which followed the same expression trend as the very well-characterized *let-7* family, had not been the subject of any experimental functional analysis. Even so, the conservation of function across all other miRNAs that follow the same expression pattern suggests some regulatory function in adult phenotypic development. Similarly, several highly expressed yet functionally uncharacterized miRNAs — namely *miR-230*, *miR-787*, *miR-788* and *miR-1820* — were expressed at a steady level throughout development. Due to the diversity of regulatory function present among other miRNAs which exhibited a similar pattern of expression, the specific function of these miRNAs could not be predicted other than to say it is unlikely these miRNAs regulate developmental events specific to either embryonic or adult *C. elegans.*

Though replicable in triplicate, the findings of this assay were preliminary. Most of the observed patterns of expression were not novel, but rather served as validation for the methodology described herein (Isik, Korswagen, & Berezikov, 2010; Martinez et al., 2008). Nevertheless, several miRNAs detected by this assay had not been previously described nor detected. Further *in vitro* assays to confirm the presence of these miRNAs are planned.

Similarly, the assay presented herein did not account for the tissue specificity of many miRNAs. I quantified the miRNAs from whole worms, which did not account for the fact that certain miRNAs may have been under- or overrepresented due to being expressed in tissues with either few or many cells per organism. Therefore, though this method is RT-qPCR-based and can be used for absolute quantification, the data presented in this paper were relative quantities as normalized to an exogenous

positive control. Though precise quantities were not determined, any detected trends of expression were dependent only on relative quantification, and thus remain valid. Future implementation of this methodology using tissue-specific RNA extractions is ongoing.

Novel to this study, a significant difference between 5p-strand preferred miRNAs and 3p-strand preferred miRNAs was detected in both known principles of strand selection. While a 5′ uracil was overwhelmingly present in 3p-strand preferred miRNAs, 5p-strand preferred miRNAs displayed no such presence. Additionally, when comparing predicted strand preference to what was observed, 3p-strand preferred miRNAs were reliably less thermostable in their seed region when compared to the corresponding 5p strand. While most 5p-strand preferred miRNAs also followed this principle, a significant number deviated from what was predicted. The majority of miRNAs which did not follow the predicted pattern prescribed by either well-established strand selection principle were determined to contain a 5′ unbound nucleotide. Pending further experimental insight, I propose this unbound nucleotide as an additional principle of strand selection in *C. elegans*.

As discussed above, due to the nature of whole organism RNA harvested for these data, these findings could have been the result of under- or over-representation of tissues as described above. Likewise, the total number of miRNAs which did not follow the two well-known principles of strand selection were limited, and thus any conclusions drawn from them are at best preliminary.

Overall, I uncovered novel patterns of expression for miRNAs underrepresented in the literature and novel differences in the structural basis of strand selection between miRNAs which prefer either the 5p or 3p strand. Though not yet supported by additional experimentation, these data provide validation of the inexpensive, scalable, RT-qPCR-based method used to generate them. Such a method retains the

benefits of small-RNA sequencing while requiring far less technical expertise, time, and biased statistical interpretation. Though it cannot be used to discover previously undescribed miRNAs, so long as a library of miRNAs exist for any given eukaryote, this method can quantify them in high-throughput while also allowing for more targeted, smaller scale studies on a tissue-specific level. This resource provides the *C. elegans* community and the microbiology community at large a useful, practical tool for the furtherance of understanding miRNAs and the broad roles they play in both functional and diagnostic applications.

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APPENDIX A

SUPPLEMENTAL TABLES

Table S1

5p Primers and Sequences

Well	miRNA	Sequence
	Plate 1	
A1	let-7-5p	TGAGGTAGTAGGTTGTATAGTT
A2	lin-4-5p	TCCCTGAGACCTCAAGTGTGA
A3	miR-1-5p	CATACTTCCTTACATGCCCATA
A4	miR-2-5p	CATCAAAGCGGTGGTTGATGTG
A5	miR-34-5p	AGGCAGTGTGGTTAGCTGGTTG
A6	miR-35-5p	TGCTGGTTTCTTCCACAGTGGTA
A7	miR-36-5p	CGCCAATTTTCGCTTCAGTGCTA
A8	miR-37-5p	TGTGGGTGTCCGTTGCGGTG
A9	miR-38-5p	TCCGGTTTTTTCCGTGGTGATA
A10	miR-39-5p	AGCTGATTTCGTCTTGGTAATA
A11	miR-40-5p	AGTGGATGTATGCCATGATGATA
A12	miR-41-5p	GGTGGTTTTTCTCTGCAGTGATA
B1	miR-42-5p	GTGGGTGTTTGCTTTTTCGGTGAAG
B2	miR-43-5p	GACATCAAGAAACTAGTGATTATG
B3	miR-44-5p	CTGGATGTGCTCGTTGGTCATA
B4	miR-45-5p	CTGGATGTGCTCGTTAGTCATA
B5	miR-46-5p	AAGAGAGCCGTCTATTGACAGT
B6	miR-47-5p	AAGAGAGCAGTCTATTGACAGT
B7	miR-48-5p	TGAGGTAGGCTCAGTAGATGCGA
B8	miR-49-5p	CGCAGTTTGTTGTGATGTGCTCC
B9	miR-50-5p	TGATATGTCTGGTATTCTTGGGTT
B10	miR-51-5p	TACCCGTAGCTCCTATCCATGTT
B11	miR-52-5p	CACCCGTACATATGTTTCCGTGCT
B12	miR-53-5p	CACCCGTACATTTGTTTCCGTGCT
C1	miR-54-5p	AGGATATGAGACGACGAGAACA
C2	miR-55-5p	CGGCAGAAACCTATCGGTTATA
C3	miR-56-5p	TGGCGGATCCATTTTGGGTTGTACC
C4	miR-57-5p	TACCCTGTAGATCGAGCTGTGTGT
C5	miR-58a-5p	TGCCCTACTCTTCGCATCTCATC
C6	miR-59-5p	TCGTCCTGAAAACGAAACGGAA
C7	miR-60-5p	AACTGGAAGAGTGCCATAAAATC
C8	miR-61-5p	TGGGTTACGGGGCTTAGTCCTT
C9	miR-63-5p	TCTAACTCGTCGGTAGTCATCGT
C10	miR-64-5p	TATGACACTGAAGCGTTACCGAA
C11	miR-65-5p	TATGACACTGAAGCGTAACCGAA
C12	miR-66-5p	CATGACACTGATTAGGGATGTGA
D1	miR-67-5p	CGCTCATTCTGCCGGTTGTTATG
D2	miR-70-5p	CGAAATACTATCGACGAATAACA

D3	miR-71-5p	TGAAAGACATGGGTAGTGAGACG
D4	miR-72-5p	AGGCAAGATGTTGGCATAGCTGA
D5	miR-73-5p	TGGACTTCCATATCGAGCCACAG
D6	miR-74-5p	CGGGCTTCCATCTCTTTCCCA
D7	miR-75-5p	CAGTCGGTTGCAAGCTTAAATA
D8	miR-76-5p	TGGGCTTCACAATAGTCGAATA
D9	miR-77-5p	GATGGTTGTGCTCTGAGGAAAT
D10	miR-79-5p	CTTTGGTGATTCAGCTTCAATGA
D11	miR-80-5p	AGCTTTCGACATGATTCTGAAC
D12	miR-81-5p	CGGTTTTCACCGTGATCTGAGA
E1	miR-82-5p	CGGTTTTCTCTGTGATCTACAGA
E2	miR-83-5p	ACTGAATTTATGTGTGTACTTGA
E3	miR-84-5p	TGAGGTAGTATGTAATATTGTAGA
E4	miR-85-5p	CCGATTTTTCAATAGTTTGAAAC
E5	miR-86-5p	TAAGTGAATGCTTTGCCACAGTC
E6	miR-87-5p	CGCCTGATACTTTCGTCTCAACCT
E7	miR-90-5p	CGGCTTTCAACGACGATATCAAC
E8	miR-124-5p	GCATGCACCCTAGTGACTTTAGT
E9	miR-228-5p	AATGGCACTGCATGAATTCACGG
E10	miR-229-5p	AATGACACTGGTTATCTTTTCCATCG
E11	miR-230-5p	ACTTGGTCGGCGATTTAATATTA
E12	miR-231-5p	CTGACTGTTTCAAAAGCTTGTA
F1	miR-232-5p	CCTGCAGTTTCGATGATTTTATC
F2	miR-233-5p	TCGCCCATCCCGTTGCTCCAATA
F3	miR-234-5p	CGGTATTCCAGAGTTGATAATA
F4	miR-235-5p	AGGCCTTGGCTGATTGCAAAATT
F5	miR-236-5p	CGTCTTACCTGTTCAATATTTAGA
F6	miR-237-5p	TCCCTGAGAATTCTCGAACAGCT
F7	miR-238-5p	TGGATGTTCTCGGACGTTCAAAGC
F8	miR-239a-5p	TTTGTACTACACATAGGTACTGG
F9	miR-239b-5p	TTTGTACTACACAAAAGTACTG
F10	miR-240-5p	CGAGGATTTTGAGACTAGAATGC
F11	miR-241-5p	TGAGGTAGGTGCGAGAAATGA
F12	miR-243-5p	TATCTCGGTGCGATCGTAC
G1	miR-244-5p	TCTTTGGTTGTACAAAGTGGTATG
G2	miR-245-5p	GCTATTTGCAAGGTACCTAATTG
G3	miR-246-5p	CGCCTAACCGTTGTCATGTAATA
G4	miR-247-5p	TAGAGAAAAGTTTCTAATTACC
G5	miR-249-5p	AGCAACGCACAAACGTCTTCTGTG
G6	miR-250-5p	CCTTCAGTTGCCTCGTGATCC
G7	miR-252-5p	ATAAGTAGTAGTGCCGCAGGTAA
G8	miR-253-5p	CTTTTCACACACCTCACTAACA
G9	miR-254-5p	TACAGAAGCATAGATTTCCACA
G10	miR-255-5p	GTAAGAAATCTTTGTAGTTCTC
G11	miR-259-5p	AAATCTCATCCTAATCTGGTAGCA

G12	miR-354-5p	GGTGCGGCTGCAGACGGGTA
H1	miR-355-5p	TTTGTTTTAGCCTGAGCTATG
H2	miR-357-5p	CCCTACAACGCTGCGCATATG
H3	miR-358-5p	ACCTGGCCAGGCATTCCAACTG
H4	miR-360-5p	TTGTGACCGTTGTTACGGTCA
H5	lsy-6-5p	AAATGCGTCTAGTATCAAAAT
H6	miR-392-5p	AGCATTCGTGGTTGAGGATAT
H7	miR-784-5p	TGGCACAATCTGCGTACGTAGA
H8	miR-785-5p	AGCACAGAATTTTTCGCTAACA
H9	miR-786-5p	CGAATATCAGTTGGGGTATTTACA
H10	miR-787-5p	AAAGATACATACGATCTTACA
H11	miR-788-5p	TCCGCTTCTAACTTCCATTTGCAG
H12	miR-789-5p	AATTGATGACCCAGACAAGGAC

Plate 2

A1	miR-789-2-5p	AATTGATGACTCAGGCAGGGAC
A2	miR-790-5p	CTTGGCACTCGCGAACACCG
A3	miR-791-5p	ACCTTATCCGTTGTAGCCAAAGT
A4	miR-792-5p	TGAGAGTTCAAAAGATTTAGCAATT
A5	miR-794-5p	TGAGGTAATCATCGTTGTCACT
A6	miR-795-5p	TGAGGTAGATTGATCAGCGAGCTT
A7	miR-797-5p	TATCACAGCAATCACAATGAGAAGA
A8	miR-800-5p	TGACAATTTCCGAGTTAGGCCA
A9	miR-1019-5p	GTGAGCATTGTTCGAGTTTCATTTT
A10	miR-1020-5p	GTAAGTGTTACAGAATAATCTT
A11	miR-1022-5p	AAGATCATTGTTAGGACGCCATC
A12	miR-1819-5p	AATCATGCTCAAAACATTCGACA
B1	miR-1820-5p	TTTTGATTGTTTTTCGATGATGTTCG
B2	miR-1821-5p	TGCCCAACTTGCAGACTTTTCAAT
B3	miR-1822-5p	AGTTTCTCTGGGAAAGCTATCGGC
B4	miR-1823-5p	ACCCCTAACCCTATGCAGTATTT
B5	miR-1824-5p	TGGCAGTGTTTCTCCCCCAACTT
B6	miR-1829a-5p	AAGGGGACTTCTAATTGTTTGTA
B7	miR-1829b-5p	AAGCGATCTTCTAGATGGTTGTA
B8	miR-1829c-5p	AAGCGAAATTCAAGATGGTTGTA
B9	miR-1830-5p	CGAGGTTTCACGTTTTCTAGGCC
B10	miR-1832a-5p	CAGCGATTCGAACTCCGCCCA
B11	miR-58b-5p	GACTCGGTGTGTGATCTCTTA
B12	miR-2207-5p	TGTGAATTGAGACTGTGTATAAG
C1	miR-2208a-5p	AAGTGTACCCGAATCTGATATCC
C2	miR-2208b-5p	AAGTGTACCCGGATCTGATATCC
C3	miR-2209a-5p	GAGTGTAACCACTCTTCTCCTTC
C4	miR-2209c-5p	GAGTGTAACCGCACGTCTTGTTT
C5	miR-2210-5p	AGGCAGATCAATCAATTTTTAGG
C6	miR-2211-5p	CCTCCATCTATTCTCCATCTGACG

C7	miR-2212-5p	TGGCAGATCATAGGCTGACTTTG
C8	miR-2213-5p	TGGCGGACTCTTCACAGTTTGA
C9	miR-2215-5p	ACAGCACGTGTTACGATGCTCC
C10	miR-2216-5p	GCACATTTTAAGTCGGTAGGC
C11	miR-1832b-5p	CAGCGAATCGCTCGGCCCAC
C12	miR-2217a-5p	CAGAGTGGGCAGTCGGTGTC
D1	miR-2209b-5p	AGTGTAACAACTCTTCTCCTTC
D2	miR-2218a-5p	CAAACTACAAGTTTTAAGCCTCA
D3	miR-2218b-5p	AGACTACAAACTACATCATTTTC
D4	miR-2219-5p	ACAGCTTTCTCTCGCACATCGTC
D5	miR-2220-5p	GTAAGACCATAAACTATTTATC
D6	miR-2953-5p	TACAGAAGTGTTCGTGAAAAT
D7	miR-4805-5p	TGCGGCAAATTTGCCGAATTTGC
D8	miR-4806-5p	CACTTACCGGCTGAGCCATGC
D9	miR-4808-5p	GTAAGATAGATTAGTGCTTTC
D10	miR-4809-5p	GTAAGTTCAGAGTTGAATTATC
D11	miR-4811-5p	TGAACAATACCTGTGTTAAAAG
D12	miR-4812-5p	AAGAGCGCTTGTAGTGAGTTGT
E1	miR-4813-5p	AGACTATCTAGGAAATATTGAACT
E2	miR-4814-5p	TTCTCAACCAACTTTGGCCACT
E3	miR-4816-5p	GTAAGTGGTTTTTGTAGATCAA
E4	miR-5545-5p	GCCGGTTTGATCTACAAAAAATGC
E5	miR-5546-5p	ACCCTTTCGCCAATTTTTTTCGT
E6	miR-5547-5p	CAACTTTTAGACCAATAGGCAT
E7	miR-5548-5p	GCCTTCTCACTCTCCACGGC
E8	miR-5549-5p	CTTGTGAAATTAACGTGAGT
E9	miR-5550-5p	CCCCGCCCAAGATTTCATTTGC
E10	miR-5551-5p	TGTAAATGGTTGGAATCTGGTAT
E11	miR-5552-5p	TGTAGTTTGTAGTCTAGCAGACC
E12	miR-5553-5p	TTGCCACGCGCCATCCATTGATT
F1	miR-5592-5p	CGGCCCTTACCGTTTAATACATG
F2	miR-5593-5p	GATGGCTGGAATATCGGTATTC
F3	miR-5594-5p	AAGAGTACTGTAGTTTCAAAAT
F4	miR-356b-5p	TGGTGAGACACGTCGTAACGAAT
F5	miR-5595-5p	TCTCTTTTTTCTCGCATGCCATCT
F6	miR-8186-5p	ACTGCTCAAAGGACTTTGCTGC
F7	miR-8187-5p	TCGGGAATGCCTACGCCTGC
F8	miR-8188-5p	AGGCAAGATGTAGGCACGT
F9	miR-8189-5p	TCTCTTATCCACTAGGCCACGA
F10	miR-8190-5p	CGGGAAATCGCTTTGGAATCCAGGA
F11	miR-8191-5p	CCCCTGCCTGGGTCACCA
F12	miR-8192-5p	CGGTCGAGCGAGTCTCAGTC
G1	miR-8193-5p	CGCGGGACTAGTCAAGTGTC
G2	miR-8194-5p	ATGCGCCTTTAAAAAGGTACGG
G3	miR-8195-5p	GTCGAGCTGTCCGTACTCGT

G4	miR-8196a-5p	CCCCATAGAAATATTTTCTATGTC
G5	miR-8197-5p	AGTGCTTTGCTCCACCCAACTC
G6	miR-8198-5p	TTGAACAGTTTCAATTTGTT
G7	miR-8199-5p	TCGGACAATATCACTGGAT
G8	miR-8200-5p	TGGCTCAAATCTCCAGTCAGACC
G9	miR-8201-5p	TCTGGATCGATAATGTAAACCT
G10	miR-8202-5p	TGAACAGAAAAAGTCGTGTGT
G11	miR-8203-5p	TGAACATCATTCAGGGCATTAC
G12	miR-4810b-5p	GTAGGTTCATGAGTAGTCATAT
H1	miR-8204-5p	TGGTCTCACCACGCGTTACTCATT
H2	miR-8196b-5p	CCCATAGAAATATTTTCTATGTC
H3	miR-8205-5p	TGGCAGACTTCGTTGAGGCTA
H4	miR-8206-5p	TATATAATGTAATCTGAAATCA
H5	miR-8207-5p	TTGTCCTCTTTTCTCTCATTTGCA
H6	miR-8208-5p	TCCGCCCACAGTTGAACCAATT
H7	miR-8209-5p	AAAACGAAGAAAGAAGAAGAA
H8	miR-8210-5p	TGCCTTCTTTCCTTGTGTCGCC
H9	miR-2217b-5p	CAGAGTGGGCAGTCGGTGTC
H10	miR-8211-5p	ACTCGAGGCCGGTGAGCTGC
Total	190	

Table S2

3p Primers and Sequences

Well	miRNA	Sequence
	Plate 1	
A1	let-7-3p	CTATGCAATTTTCTACCTTACC
A2	lin-4-3p	ACACCTGGGCTCTCCGGGTAC
A3	miR-1-3p	TGGAATGTAAAGAAGTATGTA
A4	miR-2-3p	TATCACAGCCAGCTTTGATGTGC
A5	miR-34-3p	ACGGCTACCTTCACTGCCACCC
A6	miR-35-3p	TCACCGGGTGGAAACTAGCAGT
A7	miR-36-3p	TCACCGGGTGAAAATTCGCATG
A8	miR-37-3p	TCACCGGGTGAACACTTGCAGT
A9	miR-38-3p	TCACCGGGAGAAAAACTGGAGT
A10	miR-39-3p	TCACCGGGTGTAAATCAGCTTG
A11	miR-40-3p	TCACCGGGTGTACATCAGCTAA
A12	miR-41-3p	TCACCGGGTGAAAAATCACCTA
B1	miR-42-3p	TCACCGGGTTAACATCTACAGA
B2	miR-43-3p	TATCACAGTTTACTTGCTGTCGC
B3	miR-44-3p	TGACTAGAGACACATTCAGCT
B4	miR-45-3p	TGACTAGAGACACATTCAGCT

B5	miR-46-3p	TGTCATGGAGTCGCTCTCTTCA
B6	miR-47-3p	TGTCATGGAGGCGCTCTCTTCA
B7	miR-48-3p	ACATCCACCAGCCTAGCTCGCA
B8	miR-49-3p	AAGCACCACGAGAAGCTGCAGA
B9	miR-50-3p	CCCGCATATTAGACGTATCGAC
B10	miR-51-3p	CATGGAAGCAGGTACAGGTGCA
B11	miR-52-3p	CACGTTACAATGAAAGGGTAGC
B12	miR-53-3p	CACGGCACAATATATGGGTCGC
C1	miR-54-3p	TACCCGTAATCTTCATAATCCGAG
C2	miR-55-3p	TACCCGTATAAGTTTCTGCTGAG
C3	miR-56-3p	TACCCGTAATGTTTCCGCTGAG
C4	miR-57-3p	ACGAGCTAGACTACAAGGTGCA
C5	miR-58a-3p	TGAGATCGTTCAGTACGGCAAT
C6	miR-59-3p	TCGAATCGTTTATCAGGATGATG
C7	miR-60-3p	TATTATGCACATTTTCTAGTTCA
C8	miR-61-3p	TGACTAGAACCGTTACTCATC
C9	miR-63-3p	TATGACACTGAAGCGAGTTGGAAA
C10	miR-64-3p	GTGCAACGATCAGTGGCATGC
C11	miR-65-3p	CTGCTACGCGCAGTGCCATGC
C12	miR-66-3p	AAATTCCTAACGGTGTCAAAC
D1	miR-67-3p	TCACAACCTCCTAGAAAGAGTAGA
D2	miR-70-3p	TAATACGTCGTTGGTGTTTCCAT
D3	miR-71-3p	TATCACTATTCTGTTTTTCGC
D4	miR-72-3p	AGCTTCGCCACATTCTGCCACG
D5	miR-73-3p	TGGCAAGATGTAGGCAGTTCAGT
D6	miR-74-3p	TGGCAAGAAATGGCAGTCTACA
D7	miR-75-3p	TTAAAGCTACCAACCGGCTTCA
D8	miR-76-3p	TTCGTTGTTGATGAAGCCTTGA
D9	miR-77-3p	TTCATCAGGCCATAGCTGTCCA
D10	miR-79-3p	ATAAAGCTAGGTTACCAAAGCT
D11	miR-80-3p	TGAGATCATTAGTTGAAAGCCGA
D12	miR-81-3p	TGAGATCATCGTGAAAGCTAGT
E1	miR-82-3p	TGAGATCATCGTGAAAGCCAGT
E2	miR-83-3p	TAGCACCATATAAATTCAGTAA
E3	miR-84-3p	CACAATGTTTCAACTAACTCGGC
E4	miR-85-3p	TACAAAGTATTTGAAAAGTCGTGC
E5	miR-86-3p	CTGGGCTCAGATTCGCTTAGGC
E6	miR-87-3p	GTGAGCAAAGTTTCAGGTGTGC
E7	miR-90-3p	TGATATGTTGTTTGAATGCCCCT
E8	miR-124-3p	TAAGGCACGCGGTGAATGCCA
E9	miR-228-3p	GCGGATCATACGGTACCATAGC
E10	miR-229-3p	AGAAAGGTATCGGGTGTCATAG
E11	miR-230-3p	GTATTAGTTGTGCGACCAGGAGA
E12	miR-231-3p	TAAGCTCGTGATCAACAGGCAGAA
F1	miR-232-3p	TAAATGCATCTTAACTGCGGTGA

F2	miR-233-3p	TTGAGCAATGCGCATGTGCGGGA
F3	miR-234-3p	TTATTGCTCGAGAATACCCTT
F4	miR-235-3p	TATTGCACTCTCCCCGGCCTGA
F5	miR-236-3p	TAATACTGTCAGGTAATGACGCT
F6	miR-237-3p	CTGTCGAGTTTTGTCAAGGACC
F7	miR-238-3p	TTTGTACTCCGATGCCATTCAGA
F8	miR-239a-3p	AGTGTCTAGTCTAGTGCAAACA
F9	miR-239b-3p	GCACTTTTGTGGTGTGCAAAAA
F10	miR-240-3p	TACTGGCCCCCAAATCTTCGCT
F11	miR-241-3p	ATTGTCTCTCAGCTGCTTCATC
F12	miR-243-3p	CGGTACGATCGCGGCGGGATATC
G1	miR-244-3p	TACTGCTTTTCAGCTAAAGGA
G2	miR-245-3p	ATTGGTCCCCTCCAAGTAGCTC
G3	miR-246-3p	TTACATGTTTCGGGTAGGAGC
G4	miR-247-3p	TGACTAGAGCCTATTCTCTTCT
G5	miR-249-3p	TCACAGGACTTTTGAGCGTTGCC
G6	miR-250-3p	AATCACAGTCAACTGTTGGC
G7	miR-252-3p	CTTACCTACTGCCTTCTGC
G8	miR-253-3p	TTAGTAGGCGTTGTGGGAAGGG
G9	miR-254-3p	TGCAAATCTTTCGCGAC
G10	miR-255-3p	AAACTGAAGAGATTTTTTACAG
G11	miR-259-3p	CCACCGATTTGGCATGGGATTGAC
G12	miR-354-3p	ACCTTGTTTGTTGCTGCTCCT
H1	miR-355-3p	TAGCTTCTTGCTAAAACATGCC
H2	miR-357-3p	AAATGCCAGTCGTTGCAGGAGT
H3	miR-358-3p	ATTGGTATCCCTGTCAAGGTCT
H4	miR-360-3p	TGACCGTAATCCCGTTCACAA
H5	lsy-6-3p	TTTTGTATGAGACGCATTTCGA
H6	miR-392-3p	TATCATCGATCACGTGTGATGA
H7	miR-784-3p	TATGTACAAATGTTGCGCTGCC
H8	miR-785-3p	TAAGTGAATTGTTTTGTGTAGA
H9	miR-786-3p	TAATGCCCTGAATGATGTTCAAT
H10	miR-787-3p	TAAGCTCGTTTTAGTATCTTTCG
H11	miR-788-3p	GGAAATGGATTAGAATCGTGGAAA
H12	miR-789-3p	TCCCTGCCTGGGTCACCAATTGT

Plate 2

A1	miR-789-2-3p	TCCCTGCCTGGGTCACCAATTGT
A2	miR-790-3p	CGGCGTTAGCTCTGTGTCAAACC
A3	miR-791-3p	TTTGGCACTCCGCAGATAAGGCAA
A4	miR-792-3p	TTGAAATCTCTTCAACTTTCAGA
A5	miR-794-3p	TGAAAACGTTGTCTATCTCGAA
A6	miR-795-3p	AATCGTGATCAGTATACTTCGTC
A7	miR-797-3p	TTTTCATTGGTTTCTGTGAAAT
A8	miR-800-3p	GCCAAACTCGGAAATTGTCTGC

A9	miR-1019-3p	CTGTAATTCCACATTGCTTTCCAG
A10	miR-1020-3p	ATTATTCTGTGACACTTTCAG
A11	miR-1022-3p	ATGATAGTCCAATGATGATCCAGC
A12	miR-1819-3p	TGGAATGATTGAGCTTGATGGA
B1	miR-1820-3p	AACCATTGTAAACAATCAAAGA
B2	miR-1821-3p	TGAGGTCTTATAGTTAGGTAGA
B3	miR-1822-3p	GAGCTGCCCTCAGAAAAACTCT
B4	miR-1823-3p	TACTGGAAGTGTTTAGGAGTAA
B5	miR-1824-3p	GTTGGCCGTGGTGAACACTTCC
B6	miR-1829a-3p	CAACCATTGGAATTTCTCTATT
B7	miR-1829b-3p	CAACCACTGGAATTTCTCTATT
B8	miR-1829c-3p	CAACCACTGGAATTTCTCTATT
B9	miR-1830-3p	CCTAGGAAATGAGAAAACTCGGC
B10	miR-1832a-3p	TGGGCGGAGCGAATCGATGAT
B11	miR-58b-3p	AGAGATCAACCATTGAGATCCAA
B12	miR-2207-3p	ATGCACAGGCTCAATGCACACA
C1	miR-2208a-3p	ATGCAGTTTCTGGTATACTTCA
C2	miR-2208b-3p	ATGCAGATTTTGGTACACTTCA
C3	miR-2209a-3p	AGAGATCAGCGGTTACACTACA
C4	miR-2209c-3p	AAAAGACCACCGGTTACACTACA
C5	miR-2210-3p	TAAAGTCGATTGCTCTACCCAC
C6	miR-2211-3p	TCAGGTAGAATTTAGAGGAGAAA
C7	miR-2212-3p	AAGTGGCATTTGATAAGCCATC
C8	miR-2213-3p	AAGCTGTAAGAGGACTGCCTAA
C9	miR-2215-3p	AGAATCGTAGCGCGTGTTGTTT
C10	miR-2216-3p	CTATCTACTTAAAATGTGCCT
C11	miR-1832b-3p	AGTGGGCAGAGCGATTCGCTGAT
C12	miR-2217a-3p	TCGACCCTTGTGCCTGTTTCGGT
D1	miR-2209b-3p	AGAGATGAGCGGTTGTGCTTCA
D2	miR-2218a-3p	AGGCCAGAATAGTGTAGTTTGTA
D3	miR-2218b-3p	AAATTTGTAGTTTGTAGTGAGA
D4	miR-2219-3p	CTGACGAAGTGCGAGGGAAAGC
D5	miR-2220-3p	TCAATTGTTTGTGGACTTACAG
D6	miR-2953-3p	GATCACTAGCTCTTCTGTAGC
D7	miR-4805-3p	AAATTTTGAGATTTTCCGCACA
D8	miR-4806-3p	ATAGCTCAGCCGGTAAGAGGC
D9	miR-4808-3p	AAGCTCTATTCTCTCTTACAG
D10	miR-4809-3p	TAATACAACTTCTGGGCTTCCAG
D11	miR-4811-3p	TATAACACTCGTATTGTTCGCT
D12	miR-4812-3p	CAACCACTGCAATTTCTCTAT
E1	miR-4813-3p	TTCAATATACCGGATGGTCTGG
E2	miR-4814-3p	TGGGCAAAGTTGGTTGAGAAGT
E3	miR-4816-3p	ATCTACAATTTTCGCACTTACAG
E4	miR-5545-3p	ACTTTTGTAGATCAAACCGACAT
E5	miR-5546-3p	GAAAAATTGCGCGCATGGGTTG

E6	miR-5547-3p	GCCTATCGGCCTAAAAGTTGTC
E7	miR-5548-3p	AGCCCTGTGGATTGGAGAAGGAGA
E8	miR-5549-3p	TCATGTTGGTTTTTTGTTGGT
E9	miR-5550-3p	CAAATGAAATCGTGGGCGGG
E10	miR-5551-3p	ACCAATTTCTTACCATTAGCATC
E11	miR-5552-3p	TCTGCCAGACTACAAACCACACA
E12	miR-5553-3p	TCAATGGGTAGCACGTGGCAAGA
F1	miR-5592-3p	TGTATTAAACGGTAAGGGCCGGC
F2	miR-5593-3p	ATACCGCATTCCAACCAACAA
F3	miR-5594-3p	TTTGAACTACAGTACTCTTCA
F4	miR-356b-3p	TTTGTTCGCGTTGCTCAACCACA
F5	miR-5595-3p	AGAGCGTGTGGAGAAGAGAGACG
F6	miR-8186-3p	CAGCAAAGTCCTTTGAGCAGTT
F7	miR-8187-3p	AGGCAAGATGTAGGCACGATG
F8	miR-8188-3p	CAGTAGACACTTGCGAAATATGC
F9	miR-8189-3p	ATGGCCTAGTGGATAAGAGGGA
F10	miR-8190-3p	ATTCCTACCGATTTTCAGCG
F11	miR-8191-3p	GTATCCAAGTAGTCCAAGGGAC
F12	miR-8192-3p	CGAAAGAGACTCATTGGGCCTGC
G1	miR-8193-3p	CGACACTTTCCGGTTTTGCGAA
G2	miR-8194-3p	GTACACTCTTAAAGGCGCATGC
G3	miR-8195-3p	GTAGAGGTACGAGCTGTGACGG
G4	miR-8196a-3p	GTAGTTAATTTTTCTTTGGGAAT
G5	miR-8197-3p	GTTGGCAAAGCAAGGTTCTGA
G6	miR-8198-3p	TAATTACGGATACTGTTCAAA
G7	miR-8199-3p	GTAGAAGGATGTTGGAAAATA
G8	miR-8200-3p	TCTGACGGGAGATTTGAGCCATC
G9	miR-8201-3p	AATTACATTATTGATCCAGAGA
G10	miR-8202-3p	AGAGACATTTTGGACAATTGC
G11	miR-8203-3p	TAAATACCCCAACTGATATTCGAT
G12	miR-4810b-3p	TGACTATCTCATCAACTTACAG
H1	miR-8204-3p	TGGGTTGCGCGTAGCGAGGCCATTC
H2	miR-8196b-3p	TGTAGTTAATTTTTTCTTTGGGAA
H3	miR-8205-3p	TTAGCCTCACCCAGTTTTCCACG
H4	miR-8206-3p	TTTCAGATTACATTCTATAGG
H5	miR-8207-3p	CATTGGAAGAAAAGAGAACGAGA
H6	miR-8208-3p	TTGGTTCAGGAATGGGCGGAACT
H7	miR-8209-3p	TCTTCTTCTCCCTTCGTTTCCC
H8	miR-8210-3p	CGACGAAAAAAAGAAAGAAAGAAA
H9	miR-2217b-3p	TTGACCCTTGTGCTTGTTTCGGC
H10	miR-8211-3p	ACGGCTCGCCGAGCTTCGAGAAC
Total	190	

Table S3

Universal Reverse Primer

Well	miRNA	Sequence
	Plate 1	
A1	Universal Reverse	GCAGGGTCCGAGGTATTC
A2	Universal Reverse	GCAGGGTCCGAGGTATTC
A3	Universal Reverse	GCAGGGTCCGAGGTATTC
A4	Universal Reverse	GCAGGGTCCGAGGTATTC
A5	Universal Reverse	GCAGGGTCCGAGGTATTC
A6	Universal Reverse	GCAGGGTCCGAGGTATTC
A7	Universal Reverse	GCAGGGTCCGAGGTATTC
A8	Universal Reverse	GCAGGGTCCGAGGTATTC
A9	Universal Reverse	GCAGGGTCCGAGGTATTC
A10	Universal Reverse	GCAGGGTCCGAGGTATTC
A11	Universal Reverse	GCAGGGTCCGAGGTATTC
A12	Universal Reverse	GCAGGGTCCGAGGTATTC
B1	Universal Reverse	GCAGGGTCCGAGGTATTC
B2	Universal Reverse	GCAGGGTCCGAGGTATTC
B3	Universal Reverse	GCAGGGTCCGAGGTATTC
B4	Universal Reverse	GCAGGGTCCGAGGTATTC
B5	Universal Reverse	GCAGGGTCCGAGGTATTC
B6	Universal Reverse	GCAGGGTCCGAGGTATTC
B7	Universal Reverse	GCAGGGTCCGAGGTATTC
B8	Universal Reverse	GCAGGGTCCGAGGTATTC
B9	Universal Reverse	GCAGGGTCCGAGGTATTC
B10	Universal Reverse	GCAGGGTCCGAGGTATTC
B11	Universal Reverse	GCAGGGTCCGAGGTATTC
B12	Universal Reverse	GCAGGGTCCGAGGTATTC
Total	190	

Table S4

Subset of Significant miRNAs Used in Trend Analysis

miRNA	Sequence	Log(2^-ΔΔC$_t$) (avg. 3 replicates)					
		E	L1	L2	L3	L4	YA
let-7 5p	UGAGGUAGUAGGUUGUAUAGUU	-2.0080	-1.9980	-0.8421	-0.1330	-0.0260	-0.3858

lin-4 5p	UCCCUGAGACCUCAAGUGUGA	-2.6729	-0.8801	-0.5207	-0.6157	-0.4571	-1.0239
miR-39 5p	AGCUGAUUUCGUCUUGGUAAUA	-0.5772	-3.0138	-2.7825	-3.2659	-3.2827	-2.8941
miR-40 5p	AGUGGAUGUAUGCCAUGAUGAUA	-1.1650	-3.2316	-2.9147	-3.0640	-3.5082	-3.1151
miR-48 5p	UGAGGUAGGCUCAGUAGAUGCGA	-2.0824	-1.5196	-0.6362	-0.4781	-0.4170	-0.8982
miR-50 5p	UGAUAUGUCUGGUAUUCUUGGGUU	0.2676	-0.3056	-0.1701	-0.3488	-0.4575	-1.0866
miR-51 5p	UACCCGUAGCUCCUAUCCAUGUU	1.0103	-0.1110	-0.0476	-0.1141	0.0382	-0.5934
miR-52 5p	CACCCGUACAUAUGUUUCCGUGCU	1.7974	0.9970	1.1566	1.0634	1.2342	0.3607
miR-53 5p	CACCCGUACAUUUGUUUCCGUGCU	1.7991	0.9982	1.1619	1.0802	1.2560	0.3581
miR-71 5p	UGAAAGACAUGGGUAGUGAGACG	0.2880	-0.1554	0.3581	0.2045	-0.1276	-0.0676
miR-72 5p	AGGCAAGAUGUUGGCAUAGCUGA	-0.3469	-0.6956	-0.5078	-1.3775	-1.5735	-1.8319
miR-84 5p	UGAGGUAGUAUGUAAUAUUGUAGA	-2.4029	-1.3516	-0.7485	-0.7435	-0.7893	-1.4274
miR-87 5p	CGCCUGAUACUUUCGUCUCAACCU	-0.9793	-1.4474	-1.1081	-1.0930	-1.0954	-1.1074
miR-230 5p	ACUUGGUCGGCGAUUUAAUAUUA	-3.2018	-2.2203	-1.5244	-1.7680	-2.0885	-2.5631
miR-241 5p	UGAGGUAGGUGCGAGAAAUGA	-2.4052	-1.8403	-0.9454	-1.1712	-1.4078	-1.5982
miR-252 5p	AUAAGUAGUAGUGCCGCAGGUAA	-1.0641	-1.3702	-1.1211	-1.8502	-1.6168	-2.0139
miR-354 5p	GGUGCGGCUGCAGACGGGUAU	-1.4179	-1.3021	-1.0643	-0.8982	-0.6639	-0.9149
miR-788 5p	UCCGCUUCUAACUUCCAUUUGCAG	-1.3116	-1.0034	-0.6409	-0.4359	-0.6481	-2.3300
miR-1820 5p	UUUUGAUUGUUUUUCGAUGAUGUUCG	-1.0642	-1.1899	-1.1571	-1.1583	-1.4933	-2.2691
miR-1 3p	UGGAAUGUAAAGAAGUAUGUA	-1.8302	-1.8478	-1.0549	-1.9141	-1.7235	-1.9567
miR-34 3p	ACGGCUACCUUCACUGCCACCC	-1.6070	-2.0618	-1.1399	-1.7205	-1.7029	-1.7519
miR-35 3p	UCACCGGGUGGAAACUAGCAGU	-0.0767	-2.2324	-1.8756	-2.0926	-2.1558	-2.3002
miR-36 3p	UCACCGGGUGAAAAUUCGCAUG	-0.4702	-2.1156	-1.8437	-2.2649	-2.2108	-1.9820
miR-37 3p	UCACCGGGUGAACACUUGCAGU	0.4076	-1.7333	-1.5098	-1.7954	-1.5853	-1.9640
miR-39 3p	UCACCGGGUGUAAAUCAGCUUG	0.2974	-1.9766	-2.0314	-2.5020	-2.2190	-2.0463
miR-40 3p	UCACCGGGUGUACAUCAGCUAA	0.8766	-1.5696	-1.6255	-1.9156	-1.8049	-1.7753
miR-42 3p	UCACCGGGUUAACAUCUACAGA	0.7058	-1.0380	-1.2025	-1.4894	-1.4716	-2.2488
miR-48 3p	ACAUCCACCAGCCUAGCUCGCA	-1.2144	-1.7542	-1.5480	-1.6790	-1.3792	-1.7207
miR-60 3p	UAUUAUGCACAUUUUCUAGUUCA	0.9156	0.7373	0.8422	0.8641	0.7362	0.0892
miR-70 3p	UAAUACGUCGUUGGUGUUUCCAU	0.0687	-0.2617	0.1339	0.3554	0.3657	-0.4423
miR-71 3p	UAUCACUAUUCUGUUUUUCGC	-1.3623	-1.2444	-0.7781	-0.8188	-0.8469	-1.4811
miR-74 3p	UGGCAAGAAAUGGCAGUCUACA	-0.1906	-0.8818	-0.8699	-1.3930	-1.7253	-2.1806
miR-77 3p	UUCAUCAGGCCAUAGCUGUCCA	-1.7844	-1.8286	-1.6261	-1.2937	-1.0639	-1.2260
miR-83 3p	UAGCACCAUAUAAAUUCAGUAA	0.0882	-0.0980	0.0702	-0.0530	-0.2347	-1.0187
miR-90 3p	UGAUAUGUUGUUUGAAUGCCCCU	0.5769	0.1186	0.2869	-0.0362	0.0526	-0.7626
miR-230 3p	GUAUUAGUUGUGCGACCAGGAGA	-1.6488	-1.6795	-1.2416	-1.6276	-1.4285	-1.6473
miR-241 3p	AUUGUCUCUCAGCUGCUUCAUC	-1.1900	-1.6894	-1.1494	-1.2640	-1.0893	-1.4919
miR-354 3p	ACCUUGUUUGUUGCUGCUCCU	-1.1202	-1.1663	-0.9206	-0.7735	-0.5958	-0.8377
miR-787 3p	UAAGCUCGUUUUAGUAUCUUUCG	-0.7867	-1.3639	-1.2748	-1.2864	-1.4987	-2.1737
miR-5595 3p	AGAGCGUGUGGAGAAGAGAGACG	-0.3088	-0.4283	0.0783	0.2437	0.6172	0.2814

APPENDIX B

SUPPLEMENTAL FIGURES

Figure S1

Equation for the Relative Quantification of qPCR Data

$$Relative\ Quantity = 2^{-\Delta\Delta C_t}$$

where C_t = cycle threshold and

$$\Delta\Delta C_t = (\Delta C_t\ Gene\ of\ Interest) - (C_t\ Positive\ Control)$$

$$\Delta C_t\ Gene\ of\ Interest = (Average\ C_t\ Gene\ of\ Interest) - (Average\ C_t\ Nontemplate\ Control)$$

$$\Delta C_t\ Positive\ Control = (Average\ C_t\ Positive\ Control) - (Average\ C_t\ Nontemplate\ Control)$$