DISSECTING MICRORNA-MEDIATED CONTROL MECHANISMS REGULATING DENDRITE DEVELOPMENT

by

Atit A. Patel
A Thesis
Submitted to the
Graduate Faculty
of
George Mason University
in Partial Fulfillment of
The Requirements for the Degree
of
Master of Science
Biology

Committee:

[Signatures]

Dr. Daniel N. Cox, Thesis Director
Dr. Geraldine M. Grant, Committee Member
Dr. R. Edward Otto, Committee Member
Dr. James D. Willett, Director, School of Systems Biology
Dr. Donna M. Fox, Associate Dean, Office of Student Affairs & Special Programs, College of Science
Dr. Peggy Agouris, Dean, College of Science

Date: 04/15/2015

Spring Semester 2015
George Mason University
Fairfax, VA
Dissecting microRNA-Mediated Control Mechanisms Regulating Dendrite Development

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at George Mason University

by

Atit A. Patel
Bachelor of Science
George Mason University, 2013

Director: Daniel N. Cox, Associate Professor
Neuroscience Institute, Georgia State University, Affiliate Faculty, School of Systems and Krasnow Institute for Advanced Study, George Mason University

Spring Semester 2015
George Mason University
Fairfax, VA
DEDICATION

This is dedicated to my loving family.
ACKNOWLEDGEMENTS

First and foremost I would like to thank Dr. Cox for being an amazing mentor and for being extremely understanding and supporting in the past few months. I would also like to thank members of the Cox Lab for helping and guiding me through my thesis work. I am likewise grateful to my committee members, Dr. Otto and Dr. Grant for their support and encouragement. Lastly, I would like to thank my parents and my brother for supporting me in achieving my dreams and helping me build a better future.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>List of Figures</td>
<td>vi</td>
</tr>
<tr>
<td>List of Abbreviations and/or Symbols</td>
<td>vii</td>
</tr>
<tr>
<td>Abstract</td>
<td>viii</td>
</tr>
<tr>
<td>Chapter One</td>
<td>1</td>
</tr>
<tr>
<td>Preliminary Studies</td>
<td>6</td>
</tr>
<tr>
<td>Chapter Two</td>
<td>11</td>
</tr>
<tr>
<td>Specific Aims</td>
<td>11</td>
</tr>
<tr>
<td>Chapter Three</td>
<td>23</td>
</tr>
<tr>
<td>Discussion</td>
<td>23</td>
</tr>
<tr>
<td>Methods and Materials</td>
<td>27</td>
</tr>
<tr>
<td>References</td>
<td>33</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1 miRNA Pathway</td>
<td>2</td>
</tr>
<tr>
<td>Figure 2 <em>Drosophila</em> da neurons</td>
<td>5</td>
</tr>
<tr>
<td>Figure 3 miRNA-microarray analysis</td>
<td>8</td>
</tr>
<tr>
<td>Figure 4 Ectopic over-expression analysis of k box miRNAs</td>
<td>9</td>
</tr>
<tr>
<td>Figure 5 Ectopic over-expression analysis of <em>miR-279</em></td>
<td>10</td>
</tr>
<tr>
<td>Figure 6 K box miRNA target validation</td>
<td>15</td>
</tr>
<tr>
<td>Figure 7 Schematic of MARCM system</td>
<td>17</td>
</tr>
<tr>
<td>Figure 8 <em>miR-279</em> MARCM loss of function analysis</td>
<td>20</td>
</tr>
<tr>
<td>Figure 9: Schematic diagram of the proposed role of <em>miR-279</em> in mediating dendrite development</td>
<td>22</td>
</tr>
</tbody>
</table>
LIST OF ABBREVIATIONS

dendritic arborization neurons...........................................da neurons
Green fluorescent protein..................................................GFP
Gain of function............................................................GOF
Loss of function.............................................................LOF
microRNA...................................................................miRNA
no ocelli........................................................................noc
Quantitative real time polymerase chain reaction......................qRT-PCR
Ribonucleic acid..............................................................RNA
Ribonucleic acid interference.............................................RNAi
spastin...........................................................................spas
TAK1-associated binding protein 2.....................................tab2
MicroRNAs (miRNAs) are a class of short, non-coding RNAs (~22 nucleotides) that function as critical post-transcriptional regulators of gene expression. miRNA-mediated gene regulation has been implicated across numerous species and in a wide variety of tissues with functional role in diverse biological processes including embryonic development, stem cell division, germline specification, neuronal morphogenesis and cancer. *Drosophila melanogaster* dendritic arborization (da) neurons have emerged as an exceptional model for miRNA studies. We have previously demonstrated that the K box miRNAs, including *miR-2b/miR-13b* regulate the expression of genes required to restrict dendritic branching. To elucidate the molecular bases of K box miRNA-mediated regulation of dendritic development, we employed bioinformatics strategies to identify putative mRNA targets of *miR-2b/miR-13b* based on inverse correlation expression analyses. Candidate K-box target mRNAs were phenotypically validated using the
*GAL4-UAS* system and live confocal microscopy to analyze dendritic morphology. Dendritic architecture was quantitatively evaluated based on three criteria including total dendritic length, number of branches and branch density as a function of length. To further quantitatively assess miR-based regulation of putative target genes that produced significant increases in dendritic branching morphology, we conducted target mRNA expression validation studies and confirmed that these K box miRNAs regulate the expression of *noc*, *Tab2*, *spas* and *CG4911* in modulating class-specific dendritic homeostasis. In contrast to the K box miRNAs, which regulate the expression of genes required to restrict dendritic branching, we identified *miR-279* as one miRNA that functions by regulating the expression of genes required to promote dendritic growth and branching via gain-of-function analyses. Overexpression of *miR-279* in multiple da neuron subclasses results in overall reductions in dendritic growth and branching, whereas MARCM loss-of-function analyses reveal a cell autonomous role for *miR-279* in promoting dendritic branching via gain-of-function analyses. To further characterize the role of *miR-279*, we conducted cell autonomous MARCM clonal analyses on *miR-279* mutants which revealed concomitant reductions in overall growth and branching, but significant increases in branch density. These analyses suggest that *miR-279* may play a role in coordinating class-specific dendritic outgrowth and branching to achieve homeostatic branch density in neurons with complex dendritic arbors.
CHAPTER ONE

MicroRNAs (miRNAs) constitute a class of small (~22nt) RNAs that have emerged in recent years as potent post-transcriptional regulators that play critical functional roles in controlling gene expression during animal development (Bushati & Cohen, 2007; Carthew, 2006). This is accomplished when the miRNA binds to a messenger RNA (mRNA) at the three prime untranslated region (3'UTR), which can either inhibit the initiation of protein translation and cause the degradation of the mRNA via the RNA-induced silencing complex (RISC) (Figure 1). Moreover, miRNA-mediated gene regulation has been implicated across numerous species and in a wide variety of tissues with functional roles in diverse biological processes including embryonic development, stem cell division, germline specification, neuronal morphogenesis and cancer to name a few (Hatfield et al., 2005; Kosik & Krichevsky, 2005; Leaman et al., 2005; Megosh, Cox, Campbell, & Lin, 2006; O’Donnell, Wentzel, Zeller, Dang, & Mendell, 2005) Several recent studies in Drosophila have implicated individual miRNAs, as well as, RISC components, essential for miRNA biogenesis, in various aspect of neuronal development including, local translation at synapses to regulate synaptic strength, and growth of dendritic spines, in addition to specification of neuronal identities, neuronal asymmetry and brain morphogenesis (Chang, Johnston, Frøkjaer-Jensen, Lockery, & Hobert, 2004; Giraldez et al., 2005; Jin, Alisch, & Warren, 2004;
Despite these advances, the precise role of miRNAs in neuronal morphogenesis and, in particular, dendrite development, remains largely unknown.

Figure 1: miRNA Pathway. Schematic diagram of miRNA biogenesis pathway. Adapted from [www.microrna.ic.cz](http://www.microrna.ic.cz).
The binding of the miRNA to its target gene does not require perfect base pairing, which allows a single miRNA to target a wide variety of mRNAs (~200 on average). Currently, several computational approaches have been taken to address this issue through development of target prediction algorithms, e.g. miRanda, TargetScan, PicTar (Burgler & Macdonald, 2005; Enright et al., 2003; Grün, Wang, Langenberger, Gunsalus, & Rajewsky, 2005; Robins, Li, & Padgett, 2005; Sethupathy, Corda, & Hatzigeorgiou, 2006; Thadani & Tammi, 2006). These methods rely primarily on identification of the seed region between the miRNA and the corresponding target genes, which although suggestive, is not a completely reliable method to identify functional miRNA targets. These methods for miRNA target prediction have some significant limitations and thus developing novel strategies that reduce the false positive rate are important in uncovering the molecular mechanisms via which miRNAs regulate gene expression to control biologically important processes such as dendrite morphogenesis. Recent studies suggest that the simultaneous expression profiling of miRNAs and mRNAs could be an effective platform for identification of miRNA targets (Chen & Rajewsky, 2006; Gennarino et al., 2009, 2011). The Cox Lab has developed novel bioinformatic mining tools for specific use in the Drosophila genome that allow for integrated analyses of genomic microarray and miRnome microarray expression profiles in order identify high confidence, statistically significant miRNA-target mRNA predictions to aide in the dissection of miRNA-mediated regulation of dendrite development. This strategy is based on analysis of inverse-correlative expression relationships between the miRNAs and the putative target mRNAs in transcriptome based data sets, as miRNAs function by degrading the
target transcripts.

*Drosophila* dendritic arborization (da) sensory neurons have emerged as an exceptional model for dissecting the molecular mechanisms regulating class-specific dendrite development including dendritic outgrowth, branching, maintenance, and tiling (Corty, Matthews, & Grueber, 2009; Grueber, Jan, & Jan, 2002; E. P. R. Iyer, Iyer, Sulkowski, & Cox, 2009; Jan & Jan, 2010; Parrish, Emoto, Kim, & Jan, 2007). Furthermore, da neurons can be grouped into four distinct morphological classes (Classes I-IV) based upon their unique dendritic arborization profiles which are so denoted as a reflection of their increasing orders of dendritic complexity (Figure 2) (Grueber et al., 2002). Class I da neurons are the simplest of da sensory neurons with only primary and secondary branches and class II neurons have more symmetrical branching (Grueber et al., 2002). Class III dendritic arborization neurons have a greater area and are more complex than either class I or II, also class III da neurons have dendritic spikes, which are rich in actin, but lack stable microtubules (Grueber et al., 2002). Class IV da neurons have the most complex branching pattern, lack dendritic spikes and are the only da neurons to achieve tiling (Grueber et al., 2002). Class I and II da neurons have partial coverage of body wall in each hemi-segment, meanwhile class III and IV da neurons have complete body wall coverage (Grueber et al., 2002). The presence of a wide array of morphologies from simple to complex dendritic arbors makes da neurons well suited for molecular dissection of regulatory programs, e.g. miRNA control mechanisms that dictate the acquisition of their unique shapes.
Figure 2: Drosophila da neurons. (A) Dorsal view of larva with da neurons labeled with GFP. (B) Schematic of a single abdominal hemisegment in which da neurons are represented as diamonds and color-coded according to morphological classification (Class I-IV) with numbers corresponding to increasing orders of dendritic complexity. (C) Dendritic territories (field coverage) of different da neuron subclasses. Representative images of class I neuron (ddaD (D), class II neuron (ddaB) (E), class III neuron (ddaA) (F), and class IV neuron (ddaC) (G). Note (D-G) images are taken at different magnifications. Panels (A-C) adapted from (Jan & Jan, 2010).

Dissecting the Role of miR-279 in Drosophila melanogaster

miR-279 has been studied in various systems including the insect CO2 sensory system, Drosophila ovary, the endogenous clock, which generates circadian rhythms, and the Drosophila wing (Bejarano et al., 2012; Cayirlioglu et al., 2008; Luo & Sehgal, 2012;
Drosophila melanogaster’s CO2 receptors are located in the antenna; however, in mutant miR-279 flies CO2 receptors are ectopically formed in the neurons of maxillary palp olfactory receptor neurons (Cayirlioglu et al., 2008; Hartl et al., 2011). miR-279 is also required for normal development of the Drosophila embryo, where miR-279 inhibits STAT, Signal Transducer and Activator of Transcription, in both migratory border cells and non-migratory follicle cells (Yoon et al., 2011). miR-279 regulates the JAK/STAT pathway via unpaired to control circadian rhythm’s behavioral output in Drosophila. While miR-279 is not required for normal wing patterning, miR-279 negatively impacts wing margin sensory bristles (Bejarano et al., 2012). The gcm/glide gene, a transcription factor expressed in glial cells, is negatively regulated by miR-279 (Laneve et al., 2013). Moreover, Ef1gamma, nefrin-1, escargot, gcm, and STAT have been shown to be in vivo targets of miR-279 (Cayirlioglu et al., 2008; Hartl et al., 2011; Laneve et al., 2013; Marco, 2014; Monahan & Starz-Gaiano, 2013; Yoon et al., 2011). Despite these previous studies, little is known regarding the putative role(s) of miR-279 in mediating dendritic architecture.

**Preliminary Studies**

The central question under investigation centers on the mechanistic role(s) by which miRNAs regulate class specific dendrite morphogenesis in Drosophila sensory neurons. To address this question, class specific mRNA and miRNA expression profiling was previously conducted in three distinct da neuron subclasses in our lab. Moreover, a gain-of-function overexpression screen of miRNAs expressed in these neurons has been
conducted to identify miRNAs that function in regulating dendrite morphogenesis. In addition loss-of-function phenotypic analyses have also been performed on select miRNAs. Of particular interest for this proposal, we have identified members of the K box miRNA family including miR-2b and miR-13b, as well as miR-279, as important regulators of class-specific dendrite morphogenesis. Our miRnome analyses revealed that both miR-2 and miR-13 family miRNAs are downregulated in class I da neurons, but upregulated in class IV neurons (Figure 3). Furthermore, miRnome microarray analysis revealed that miR-279 is highest in class IV da neurons, followed by class III da neurons, and is lowest in class I neurons (Figure 3) suggesting that differential expression of this miRNA may contribute to class-specific dendritic architecture. We found that ectopic overexpression of miR-2a, miR-2b and miR-13b (Figure 4) miRNAs in both class I and class IV da neurons leads to a significant increase in dendritic branching complexity in these neurons. Conversely, loss-of-function studies with these miRNAs revealed reductions in both class I and IV da neuron dendritic branching (Figure 4). Collectively, these analyses indicate that these K box miRNAs (miR-2a/b and miR-13b) normally act to repress the expression of genes required for restricting dendritic branching. In contrast, ectopic overexpression of miR-279 led to an overall reduction in dendritic complexity (growth/branching) among class I, III, and IV da neurons (Figure 5), which is indicative of a potential role in regulating expression of genes that serve to promote normal dendritic growth, and branching. However, the putative target genes that these miRNAs regulate to achieve this effect on dendrite development are currently unknown as is the native role of miR-279 in this process.
Figure 3: miRNA microarray analysis. Heat map showing results from triplicate microRNA microarray expression analysis, where green represents upregulation and red represents down regulation. The microarray data represented are quantile normalized. Fold-change values are scaled to z-scores for data visualization where red was selected to represent down-regulation (negative values) and green was selected to represent up-regulation (positive values). The z-score method scales the values of the fold change to -1 to +1 thereby facilitating visualization of changes in gene expression on the heat maps. The K box miRNAs, miR-2a, miR-2b and miR-13b exhibit similar patterns of miRNA expression with highest expression levels in CIV, followed by CIII and lowest levels in CI neurons. miR-279 exhibits a similar pattern of gene expression to that of the K box miRNAs.
Figure 4: Members of the K-box miRNA family regulate dendrite patterning. (A) WT ddaE neuron. (B-D) ddaE neurons overexpressing miR-2a (B) miR-2b (C) miR-13b (D) displaying an overall increase in branching density (E) as compared to WT ddaE neuron (A). (F) WT ddaC neuron. (G-I) ddaC neurons overexpressing miR-2a (G), miR-2b (H), miR-13b (I) show an overall increase in branching density (J) as compared to WT ddaC neuron (F), qualitatively seen as a higher number of shorter branches closely spaced to each other. (K-N) Live confocal micrographs of ddaE neurons expressing miR-2b-SP (M), miR-13b-SP (N), show an overall decrease in total dendritic length (O) and an increase in average branch length (P) as compared to miR-scrambled-SP (L) and WT ddaE (K). (Q-T) Images of ddaE neurons expressing miR-2b-SP (S) miR-13b-SP (T), show an overall decrease in total dendritic length (U) and an increase in average branch length (V) as compared to miR-scrambled-SP and WT ddaC (Q). Dotted boxes are examples of representative phenotypic regions. Scale bar represents 100 microns and n=10 for each genotype quantitatively analyzed. * p<0.05, ** p<0.01
Figure 5: Ectopic overexpression of *mir-279* leads to reduced dendritic branching complexity in da neurons. Representative live confocal micrographs of selected UAS-miRNA-induced phenotypes observed in CI, CIII and CIV da neurons, labeled using UAS-mCD8::GFP driven by respective class-specific GAL4 drivers. Relative to wild-type (A,C,E), ectopic overexpression of *miR-279* in class I (B), III (D), and IV (F) leads to decrease in branching complexity, as highlight by the red boxes. The scale bars represent 100 microns.
CHAPTER TWO

Functional characterization of miRNAs in da neurons reveals that miRNAs play an important role in fine-tuning dendritic morphologies of da neurons. miR-2b/miR-13b function by regulating genes that normally restrict dendrite complexity. Meanwhile, miR-279 functions by restricting downstream effectors, which normally promote dendrite complexity.

Specific Aims

AIM I. Phenotypically characterize and molecularly validate bioinformatically predicted target mRNAs for miR-2b/miR-13b.

Hypothesis: We hypothesize that miR-2b/miR-13b function by regulating the expression of genes that are required to restrict dendritic branching complexity.

Approach: To identify and characterize putative target mRNAs of miR-2b or miR-13b, we used bioinformatic strategies developed in our lab in order to arrive at high confidence, statistically significant predicted target mRNAs. Using this list of potential targets, we performed phenotypic analyses whereby the GAL4/UAS system was used to systematically knockdown candidate target genes in specific da neuron subclasses via in vivo RNA interference (RNAi) and phenotypes observed by live image confocal microscopy were compared with those previously observed with miR-2b or miR-13b.
overexpression. Target gene-specific RNAi transgenic strains were obtained from the Bloomington and Vienna Drosophila stock centers and a minimum of two independent RNAi lines were tested for each candidate gene to control for potential variability and transgene expression and variable knockdown effects of the RNAi. We predicted that if the target gene is regulated in these neurons by a given miRNA, then the dendrite defect observed in the miRNA overexpression and mRNA target gene knockout would be phenotypically similar since the role of the miRNA is to downregulate or degrade its target mRNAs. Both qualitative and quantitative phenotypic analyses were performed, including neuronal reconstructions, to measure the degree of phenotypic similarity between miRNA and prospective target genes.

Once, we identified target genes that shared phenotypically similar dendrite morphogenesis defects, we verified target gene knockdown in vivo under normal and miRNA knockdown conditions using miR-sponge constructs. For these validation studies, we used magnetic bead based cell sorting to isolate class I or class IV da neurons and extract mRNA for qPCR analyses on target genes(Iyer et al., 2009).

Results

To understand the mechanistic bases for how miR-2b and miR-13b may regulate class-specific dendrite patterning, we performed bioinformatic analyses to identify putative targets showing anti-correlated expression trends to the miR-2b and miR-13b miRNAs (see Materials and Methods). Since miR-2b and miR-13b share similar seed sequences (Lai, Wiel, & Rubin, 2004), target analyses revealed overlapping sets of
putative target genes for these K box miRNAs (Fig. 6A). Gene ontology (GO) analyses for common anti-correlated target genes revealed a number of highly relevant and significantly enriched biological categories and cellular processes, including transcriptional control, neuronal development, and cell morphogenesis among others (Fig. 6B). Among the list of target genes that exhibit anti-correlative expression patterns to both miR-13b and miR-2b, we selected noc, spas, Tab2 and CG4911 to further characterize as putative functional downstream targets of K box-mediated regulation of dendrite morphogenesis. Given that miR-2b and miR-13b overexpression in CIV neurons leads to an increased branching density and formation of short clustered branching, we reasoned that knockdown of putative target genes for these miRNAs may phenotypically mimic these changes in dendritic morphology (Fig. 4H,I,J). Consistent with our hypothesis, ddaC and vdaB CIV neurons expressing RNAi-mediated knockdown of tab2 (Fig. 6D,I), noc (Fig. 6E,J), spas (Fig. 6F,K) and CG4911 (Fig. 6G,L) show a mild to moderate increase in branch density (Fig. 6M) and reduction in average branch length (Fig. 6N), compared to WT control neurons (Fig. 6C,H) which is phenotypically consistent with the miR-2b and miR-13b GOF phenotype in these neurons. To verify the miRNA-target relationship, we isolated CIV neurons expressing miR-2b-SP or miR-13b-SP transgenes and examined the normalized expression levels of noc, CG4911, tab2 and spas, in comparison to WT CIV neurons. These analyses demonstrate that knockdown of miR-2b results in an upregulation of tab2, and CG4911 mRNA levels (Fig. 6O). Knockdown of mature miR-13b levels causes an increase in the spas, and CG4911 mRNA levels (Fig. 6O), indicating that in addition to common targets, K-box family
members may preferentially regulate certain targets, to modulate dendrite patterning.
Thus, in this study, we present a logical strategy that allows us to capitalize on miRNA GOF phenotypic screens followed by secondary target RNAi screening, for validating miRNA-target relationships that are mechanistically relevant to dendrite patterning.
Figure 6: Characterization of K box miRNA target gene regulation in mediating dendrite development. (A) Venn diagram shows the overlap between the predicted target genes of that exhibit anti-correlative expression patterns to host genes of miR-13b and miR-2b. (B) Gene ontology fold enrichment analyses of biological categories for common putative target genes which exhibit anti-correlated gene expression patterns to miR-2b and miR-13b K-box miRNAs. Diamonds indicate the modified Fisher’s exact p-
value for each biological category. (C-L) Live confocal images of dorsal (ddaC) and ventral (vdaB) class IV da neurons labeled with GFP. vdaB and ddaC neurons expressing RNAi constructs for Tab2(D,I), noc (E,J), spas (F,K) and CG4911(G,L) show a mild to moderate increase in branch density (M) and reduction in the average branch length (N), compared to WT vdaB (C), and ddaC (H) neurons which is consistent with increased branching complexity that is characteristic of miR-2b and miR-13b GOF phenotype in these neurons. (O) qRT-PCR (n=4) results of class IV neurons expressing miR-2b-SP and miR-13b-SP reveals upregulation of predicted gene targets as compared to WT neurons. Dotted boxes are examples of representative phenotypic regions Pairwise statistical comparisons were performed using Student's t-test. Numbers in the graph represent N number for neurons used for statistical analyses. Quantitative data are expressed as standard error of the mean and *P≤0.05.

AIM II: Further dissection of the regulatory function of miR-279 in directing class specific dendrite morphogenesis via MARCM analyses.

**Hypothesis:** We hypothesize that miR-279 functions to restrict the expression of genes that promote dendritic growth and branching complexity. Conducting miR-279 MARCM analyses will help elucidate whether or not miR-279 functions cell autonomously or cell non-autonomously to confer its regulation over class specific dendrite morphogenesis.

**Approach:** We conducted miR-279 loss of function analyses via miR-279 null mutants and used the MARCM system to generate single cell miR-279 mutants da neurons tagged with GFP for assessing function in class specific dendrite morphogenesis. Phenotypic analyses were conducted via live confocal microscopy and quantitative analyses were conducted via semi-automated digital reconstructions. miR-279 MARCM live confocal images were compared to wild-type MARCM clones as controls.
Figure 7: Schematic illustration of the loss-of-function (LOF) MARCM system. (a) In cells containing the GAL80 protein, GAL4-dependent expression of a UAS–gene (GFP) is repressed. By contrast, cells containing GAL4 but lacking GAL80 will express the UAS–gene (GFP). In this schematic, genes are denoted by colored boxes whereas proteins are denoted by colored ovals. (b) LOF MARCM requires two FRT sites located at the same position on homologous chromosomes; GAL80 located distal to one of the FRT sites; FLP recombinase located anywhere in the genome; GAL4 located anywhere in the genome except distal to the FRT site on the FRT, GAL80 recombinant chromosome arm; UAS–marker located anywhere in the genome except distal to the FRT site on the FRT, GAL80 recombinant chromosome arm; and optionally a mutation distal to FRT, in trans to but not on the FRT, GAL80 recombinant chromosome arm. Site-specific mitotic recombination at FRT sites (black arrowheads) gives rise to two daughter cells, each of which is homozygous for the chromosome arm distal to the FRT sites. Ubiquitous expression of GAL80 represses GAL4-dependent expression of a UAS–marker (GFP) gene. Loss of GAL80 expression in homozygous mutant cells results in specific expression of GFP. Adapted directly from (Wu & Luo, 2006).
Results

Analyses of the miR-279 single cell null mutant MARCM clones tagged with GFP for visualization of the dendritic arbor in da neurons revealed that miR-279 plays a minor role in determining the dendrite morphology (Figure 8). miRNA-microarray analysis revealed that miR-279 expression is positively correlated with an increase in dendritic complexity, where lowest levels of miR-279 were observed in class I da neurons followed by class III and highest in class IV da neurons (Figure 3). Similarly, we observed the greatest differences in dendrite morphology with respect to total dendritic length and number of branches in class III miR-279 mutants clones, where there is an intermediate level of miR-279, then followed by class IV and lastly class I da neurons when compared to wild-type MARCM clones (Figure 8, class I data not shown). However, when changes in dendrite complexity were assessed as number of branches over total dendritic length (branch density), then only class III miR-279 mutant clones showed a significant divergence from the wild-type MARCM clones (Figure 8). There was a significant decrease in number of branches in both class III and class IV da neurons and only a significant decrease in total dendritic length in class III da neurons, which suggests that with a loss of miR-279 in class III da neurons, there is a significant increase in branch density of the dendritic arbor, which is largely due to an increase in the presence of short dendritic filopodia emanating from the primary branches of class miR-279 mutant neurons. There was not a significant change in dendrite morphology with a loss of miR-279 in class I da neurons, which display the lowest levels of miR-279 expression (Figure 3). When miR-279 is ectopically over-expressed in class III da neurons, there is an overall
reduction in dendrite complexity, when looking at total dendritic length, number of branches and branch density (Figure 5, quantitative analysis not shown). Meanwhile, there is a reduction in number of branches and total dendritic length, but an increase in branch density with a knockout of miR-279 in class III da neurons. This suggests that miR-279 in, at least, class III da neurons, functions by maintaining the appropriate levels of downstream effectors, some of which function in increasing and others that decrease dendrite complexity, thus playing a homeostatic role in maintaining dendrite morphology. Another interpretation of these data is that in miR-279 mutant class III neurons, there is a stronger reduction in growth, than in branching which leads to an overall increase in branch density, whereas in the miR-279 overexpression analyses, both growth and branching are coordinately reduced, leading to an overall reduction in branch density (Figure 9).
Figure 8: *miR-279* loss of function analysis. (A-D, A’-D’, C’’,D’’) Representative live confocal micrographs of MARCM clones, where (A,A’) and (B, B’) are class III da neurons and (C,C’,C’’) and (D,D’,D’’) are class IV da neurons. (A,A’,C’,C’’) Wild-type MARCM clones of class III and class IV da neurons, respectively, and (B,B’,D’,D’’) *miR-279* null mutant MARCM clones of class III and class IV da neurons, respectively. Images (A’-D’,C’’,D’’) are zoomed in areas of interest marked with dashed box in respective (A-D) images. (E-J) Shows neurometric analysis comparing wild-type MARCM clones versus *miR-279* null mutant MARCM clones. Quantitative data are expressed as means with standard error of mean (SEM) error bars and means were compared by Student’s t-tests, where *P*≤0.05 and the N number for each genotype represented on the histogram bars.
Figure 9: Schematic diagram of the proposed role of miR-279 in mediating dendrite development. Gain of function analyses of miR-279 in multiple da neuron subclasses results in overall reductions in dendritic growth and branching. Meanwhile, loss of function analyses miR-279, in which we conducted cell autonomous MARCM clonal analyses on miR-279 mutants, revealed concomitant reductions in overall growth and branching, but significant increases in branch complexity. These analyses suggest that miR-279 may play a role in coordinating class-specific dendritic outgrowth and branching to achieve homeostatic branch density in neurons with complex dendritic arbors.
CHAPTER THREE

Discussion

Our results show that several members of the K box miRNAs, including \textit{miR-2b} and \textit{miR-13b} are expressed at levels proportional to dendritic complexity in da neurons, with the highest levels in class IV da neurons and the lowest levels in class I da neurons. Co-transcription of these complementary miRNAs in a given context may have a functional role in duplex formation, thereby possibly sequestering them from their corresponding mRNA targets (Lai, Tam, & Rubin, 2005). We previously found that \textit{miR-2b} and \textit{miR-13b} function by regulating the actin and microtubule cytoskeleton. Consistent with this, recent studies in other cellular contexts have shown similar capacity for miRNAs to effect changes in the cytoskeleton composition. For example, \textit{miR-221} overexpression was associated with reproducible increases in cortical actin in mast cells, and with altered cellular shape and cell cycle in murine fibroblasts (Mayoral et al., 2011). Similarly, a recent study also found that the heterochronic microRNA \textit{let-7} inhibits cell motility by regulating the genes in the actin cytoskeleton pathway in breast cancer (Hu et al., 2013) indicating the potential role of miRNAs in regulating the cytoskeleton to mediate change in cell shape or patterning in disease conditions. In addition to using miRNA GOF as an approach to studying miRNA biology, we have adapted it to further identify relevant miRNA targets that can affect dendrite patterning. Based on a secondary, follow-up
RNAi based loss-of-function approach, we tested candidate target genes whose LOF can phenocopy the miRNA GOF. GO analyses show that K box miRNAs may mediate effects on dendrite patterning by targeting genes in many diverse processes. Knockdown of *Drosophila noc*, a putative transcription factor that is involved in brain development (Cheah et al., 1994), causes an increase in class IV da neurons dendritic branching. In addition, we find that miR-2b regulates the endogenous levels of Tab2, the *Drosophila* homolog of human TAB2. TAB2 is a component of the IL-1 signaling component TAK1 binding proteins and plays an important role in modulating the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) and c-Jun N-terminal kinases (JNK) signaling pathways (Zhuang et al., 2006). NF-kB modulation is associated with the pathophysiology of ischemic/traumatic injuries and neurodegenerative disease and recent studies have shed light on the mechanism for controlling the growth of neural processes in developing peripheral and central neurons in cortical sensory, involving NF-kB (Gutierrez, Hale, Dolcet, & Davies, 2005; Teng & Tang, 2010). While all members of the K box family share the UGUGAU nucleotide seed sequence and as such likely have similar target genes (Lai, Burks, & Posakony, 1998; Lai et al., 2005) our results indicate that different members of this family may preferentially regulate certain targets in a given cellular context. For instance, loss of miR-13b causes an upregulation in the levels of the cytoskeletal microtubule severing protein Spastin. Microtubule severing proteins such as Spastin and Katanin are important for axon and dendrite development (Jinushi-Nakao et al., 2007; Lee, Jan, & Jan, 2009; Yu, Ahmad, & Baas, 1994), possibly by keeping microtubules sufficiently short to be efficiently transported into developing neurites.
(Ahmad, Yu, McNally, & Baas, 1999) or by creating more free ends of microtubules to interacts with other proteins in developing processes. In addition, we find that miR-2b and miR-13b regulate the endogenous levels of CG4911, a novel gene predicted to have proteosomal ubiquitin-protein transferase activity. Elements of the ubiquitin proteasome (UPS) have been found to regulate such diverse neuronal functions as synaptic strength, homeostatic plasticity, axon guidance, and neurite outgrowth (Hamilton & Zito, 2013). Given that miRNAs may exert modest effects, yet on several hundred targets, thereby ‘fine-tuning’ the expression levels of target genes, their profound effects on cell fate and patterning may be due to the level of regulatory networks in which they participate (Shalgi, Lieber, Oren, & Pilpel, 2007; Tsang, Zhu, & van Oudenaarden, 2007).

Our methodology for elucidating how miRNAs mediate dendrite morphogenesis in da neurons revealed that ectopic over-expression of miRNA is great starting point. However, further analysis is required before conclusively determining what roles a particular miRNA plays in dendrite morphogenesis. In the case of K box miRNA functional analysis, both ectopic over-expression and loss of function via knockdown analyses were extremely useful in dissecting how K box miRNAs impact dendrite morphogenesis. We employed a slightly different method, but a more rigorous technique, for evaluating how loss of miR-279 impacts da neuron dendritic architecture. Instead of using miR-sponges for knocking down particular miRNAs, we used MARCM analysis to evaluate how complete knockout of miR-279 impacts dendrite development in da neurons. Ectopic over-expression of miR-279 in class III and class IV da neurons resulted in an overall reduction in dendrite complexity across multiple parameters (number of
branches, total dendritic length, and branch density) (data not shown) and in the complementary miR-279 knockout analysis, there was a modest reduction in number of branches and a greater reduction in total dendritic length, resulting in an overall increase of branch density. One possibility for miR-279 null mutant clones not having statistically significant phenotypic changes is that miR-279 targets genes, may have opposing function (i.e. a set of genes that control reduction of complexity and another set of genes, which are responsible for increasing the dendritic complexity). Thus, miR-279 functions in fine tuning dendritic branching pattern by down-regulating or degrading target mRNA transcripts.

Using GAL4\textsuperscript{miR-279}, where promoter region of miR-279 gene has been placed upstream of the GAL4, it was discovered that miR-279 is highly expressed in oenocyte cells (data not shown). Oenocytes have been implicated in larval tracheal water-proofing, where oenocytes produce long fatty acids, and in adult Drosophila melanogaster oenocytes produce cuticular hydrocarbons (Makki, Cinnamon, & Gould, 2014). Oenocyte cells are located in relative close proximity to da neurons, which greatly increases the chances of having cell-cell signaling communications between oenocytes and da neurons (data not shown). Further studies are needed to potentially connect non-cell autonomous functions of miR-279 in terms of it’s role in regulating dendritic architecture. To investigate this question, it would be interesting to examine how overexpression or LOF of miR-279 in oenocytes may impact da neuron dendritic architecture in a non-autonomous manner. In addition, performing miR-279 target validation, for da neurons,
via RNAi knockdown screen could shed light on how dendritic phenotypes for miR-279 disruption (LOF/GOF) are mediated at a molecular level.

Cumulatively, we present a two pronged method for functional validating bioinformatically predicted targets for miRNAs, first, phenotypically by knocking down putative targets and, secondly, by molecularly knocking down or knocking out miRNAs and evaluating amount of putative target gene upregulation. This method of analysis for two-part validation of molecular interactions can be useful for various kinds of molecular interactions present in nature.

Materials and Methods

Drosophila genetics:

Drosophila strains used in these studies are as follows: GAL4/GAL80 driver stocks include: (1) yw; +; GAL4221, UAS-mCD8::GFP (Grueber, Ye, Moore, Jan, & Jan, 2003); (2) w; GAL4477, UAS-mCD8::GFP/CyO-GAL80 (E. P. R. Iyer et al., 2013; S. C. Iyer et al., 2013); (3) GAL419-12, UAS-mCD4-tdGFP; (4) FRT82B, miR-279962-7 (Cayirlioglu et al., 2008); (5) hsFLP, elav-GAL4, UASmCD8::GFP; +; FRT82B, tubP-GAL80. MARCM analyses were conducted as previously described (Sulkowski et al., 2011). The sequences of all K box miR-SP (miR-2bSP, miR-13bSP and SCRAMBLE-SP constructs are as described in Bejarano et. al, 2012). The following UAS-RNAi transgenic stocks were used for phenotypic validation of putative miR-2b and miR-13b target mRNAs UAS-noc-RNAi (B29370, B50659), UAS-Tab2-RNAi (B29417), UAS-CG4911 (JF01511/B01511), UAS- spas-RNAi (B27570). Oregon-R was used as a wild-type
strain. Flies were maintained at 25°C on standard cornmeal molasses media, and for miRNA overexpression, miR-SP, and target RNAi screen, the crosses were performed at 29°C to enhance GAL4 expression which produced no significant effects on dendrite morphology relative to 25°C.

Confocal microscopy

For each line screened, 6-10 third instar larvae were analyzed and representative image data was collected. For live confocal analyses, larvae were mounted in 1:5 (v/v) diethyl ether to halocarbon oil and visualized on a Nikon C1 Plus. Images were collected as z-stacks using a 20X oil immersion lens at a step-size of 2 µm and 1024 x 1024 resolution. In this study, an alteration in dendritic complexity is characterized by a statistically significant change in at least one of three phenotypic parameters, including total dendritic length, and/or dendritic branching and/or branching density, as compared to controls. p-value greater than or equal to 0.05 was used to evaluate statistical significance.

Cell isolation and purification

The isolation and purification of CIV da neurons was performed as previously described (E. P. R. Iyer et al., 2013, 2009; S. C. Iyer et al., 2013). Briefly, 150-200 age-matched third instar larvae expressing UAS-mCD8::GFP under the control of the GAL4477 (class IV) driver were collected and washed several times in ddH_2O and RNase Away. The tissue was then dissociated using a combination of enzymatic and mechanical perturbations to yield single cell suspensions that were filtered using a 30μm membrane. The filtrate is then incubated with superparamagnetic beads (DynabeadsMyOne
Streptavidin T1, Invitrogen) coupled with biotinylated mouse anti-CD8a antibody (eBioscience) for 45 minutes. Finally, the da neurons attached to the magnetic beads were then separated using a powerful magnetic field. The isolated neurons were washed at least five times with PBS to remove any potential non-specific cells and the quality and purity of isolated neurons was assessed under a stereo-fluorescent microscope equipped with phase contrast for examining the number of fluorescent vs. non-fluorescent cells. Only if the isolated cells were free of cellular debris and non-specific (i.e. non-fluorescing) contaminants were they retained. The purified class IV da neuron populations were then lysed in miRcury™ (Exiqon) RNA lysis buffer followed by storage at -80°C.

Quantitative analyses of dendritic morphology

Semi-automated digital neuronal reconstruction and quantitative analyses were performed as previously described (E. P. R. Iyer et al., 2013; S. C. Iyer et al., 2013). Briefly, raw confocal images were manually curated to eliminate non-specific auto-fluorescent spots like the larval denticle belts. The raw pixel intensity for each image was globally thresholded and converted to a binary file format. Background noise was filtered and eliminated using the analyze particles plugin in ImageJ (radius ≤50 microns), following which the images were skeletonized and analyzed in ImageJ (Arganda-Carreras et al., 2010). Images with low fluorescence or high background were eliminated from analysis. Quantitative information including total dendritic length and total dendritic branches were extracted and compiled using custom Python algorithms. Data was
analyzed in Microsoft Excel 2010 (Microsoft) and statistical tests were performed and plotted in SigmaPlot 11.0 (Systat Software).

**Bioinformatic target prediction analyses of intragenic miRNA genes**

For the K-box miRNAs (miR-13b and miR-2b), bioinformatic analyses were performed for target prediction and gene ontology analyses for significantly enriched biological categories of putative target genes for this cluster. Briefly, we employed the HOCTAR algorithm ([http://hoctar.tigem.it/](http://hoctar.tigem.it/)) (Gennarino et al., 2009, 2011) which is based upon the principle that the expression value of an intragenic miRNA can be defined by, and is tightly correlated with, the expression value of the host mRNA in which the intragenic miRNA is located. miR-13b is located within the CG7033 host gene and miR-2b within the host gene spi. These were used as proxy for the expression of the respectively intragenic miRNAs. For these analyses, raw microarray data was obtained from the ArrayExpress database which contains the GEO datasets (244 experiments, each experiments containing a minimum of (5) microarray assays) ([http://www.ebi.ac.uk/arrayexpress/experiments/browse.html](http://www.ebi.ac.uk/arrayexpress/experiments/browse.html)) (Parkinson et al., 2007). These microarray data are normalized using the rma function of the Affymetrix package (Gautier et al., 2004). For each experiment, microarray probe ids and expression values related to the host gene (i.e. CG7033/spi) are obtained as proxy expression data for the miR-2b or miR-13b miRNAs across assays from all experiments. These data are stored in the form of a hash map together with the data for all other mRNAs (probe id and expression values). Using these data, we performed both parametric (Pearson) and non-parametric (Distance) anti-correlation analyses between host geneexpression values and
all other mRNAs using the R function cor.test (Pearson) and dcor.ttest (Distance) in order to calculate $p$ values as well as correlation coefficients. These statistical comparisons are repeated across all experiments. From these analyses, a false discovery rate calculation (Benjamini-Hochberg) is done on the $p$ values obtained for each miRNA-mRNA pair (using the host gene as the miRNA proxy) using the p.adjust function in R. To identify statistically significant anti-correlated mRNA targets for a particular miRNA host gene, all mRNAs with a q-value of less than 0.01 are selected across all experiments. From these analyses, the top 25% most frequently occurring mRNAs are then compared with the targets predicted for a given miRNA in a variety of target databases (TargetScan, PITA, and Miranda). Target mRNAs which are in common with the target databases can then be identified and a union of the statistically significant Pearson and Distance correlation targets is retained as putative high confidence targets for a given miRNA. This list of putative target mRNAs is then uploaded to the DAVID server (http://david.abcc.ncifcrf.gov) (Huang et al., 2007) for functional annotation and gene ontology (GO) analyses for Biological Processes. The non-redundant functional annotations for each gene list is plotted against the enrichment values and p-values using a modified Fisher’s exact test (EASE score).

**Quantitative real time-PCR analysis**

To determine changes in target gene expression in a miRNA sponge background, CIV da neuronal subpopulations were isolated from $miR$-$2b$-$SP$ or $miR$-$13b$-$SP$ and compared to wild-type controls using magnetic bead based sorting. Following RNA purification using Exiqon miRCURY™ RNA Isolation kit, qRT-PCR was conducted as
previously described in (Sulkowski et al., 2011; Iyer et al., 2012). Results from three technical replicate were averaged to generate $C_t$ values for each biological replicate. Then, analysis of differential fold change based on $C_t$ results was performed using the Livak ($\Delta\Delta C_t$) method (Livak et al., 2001).

The genes tested and the primer details from Qiagen are as follows: Tab2 (QT00950341), noc (QT00936530), spas (QT00981239), CG4911 (QT00961107). Expression data were normalized to Rpl32 (5′-AGG GTA TCG ACA ACA GAG TG-3′ and 5′-CAC CAG GAA CTT CTT GAA TC-3′); In addition, using the Refgenes other reference genes were selected that showed highly stable expression across microarray experiments (Hruz et.al, 2011) and include CG2200 (QT00921235) and CG14130 (QT00962808). These genes also did not contain a binding site for miR-2b or miR-13b (accounting for direct miR-mediated effects on expression). miScript Primer Assays (Qiagen) used in this study include: miR-2b (MS00018256) and miR-13b (MS00017976). miRNA amplification and measurement were performed as described in the miScript PCR kit (Qiagen). Expression values for the miRNAs were normalized to internal U1 small nuclear RNAs (snRNA) expression [Nesler et. al, 2013, Pfaffl et. al, 2004]. Custom oligo primers (Integrated DNA Technologies) for these analyses include U1 snRNA-F1 [5′- GGC GTA GAG GTT AAC CGT GAT -3′], U1 snRNA-R1 [5′- ACA CGC ACG AGT TAT TCA CA -3′].
REFERENCES


Atit A. Patel graduated valedictorian from Fairfax High School, Fairfax, Virginia, in 2009. He graduated with Summa Cum Laude in Bachelor of Science in Biology and minors in German and Chemistry from George Mason University in 2013. As an undergraduate, he investigated the effectiveness of decompressive craniectomy after middle cerebral artery infarct with respect to timing, risk factors, neurological tests, demographics, and outcome at Inova Hospital System and GMU Biology department under the mentorship of Dr. James Ecklund in 2012. He later shifted his research interests to molecular neuroscience under the guidance of Dr. Daniel N. Cox, where he has dissected mechanistic roles of miRNAs in determining dendrite morphology in *Drosophila melanogaster* dendritic arborization neurons. His current interests include investigation of larval behavioral response to noxious cold and how cytoskeletal elements function in determining the dendritic arbor.