microRNA REGULATION OF ZEBRAFISH RETINAL DEVELOPMENT

By
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I dedicate this dissertation to my family, chosen family, and to James Andrew Hardaway III, Ph.D.
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<tr>
<td>7meG</td>
<td>7-methylguanosine</td>
</tr>
<tr>
<td>Ago</td>
<td>Argonaute</td>
</tr>
<tr>
<td>ath5</td>
<td>Atonal Homologue 5</td>
</tr>
<tr>
<td>BAR</td>
<td>Bin, Amphipysin, Rvs</td>
</tr>
<tr>
<td>BP</td>
<td>Base Pair</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CGZ</td>
<td>Circumferential Germinal Zone</td>
</tr>
<tr>
<td>CMZ</td>
<td>Ciliary Marginal Zone</td>
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<tr>
<td>DCP</td>
<td>Decapping Enzyme</td>
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<tr>
<td>DIC</td>
<td>Dye-injected Control</td>
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<tr>
<td>DIG</td>
<td>Digoxigenin</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyriboonucleic Acid</td>
</tr>
<tr>
<td>dpf</td>
<td>Days Post Fertilization</td>
</tr>
<tr>
<td>dRFP</td>
<td>Destabilized Red Fluorescent Protein</td>
</tr>
<tr>
<td>dsRBD</td>
<td>dsRNA Binding Domain</td>
</tr>
<tr>
<td>dsRNA</td>
<td>Double-stranded RNA</td>
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<td>Exp5</td>
<td>Exportin 5</td>
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<tr>
<td>GFAP</td>
<td>Glial Fibrillary Acid Protein</td>
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<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
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<tr>
<td>GTP</td>
<td>Guanosine-5'-triphosphate</td>
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<tr>
<td>hpf</td>
<td>Hours Post Fertilization</td>
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<td>HRP</td>
<td>Horseradish Peroxidase</td>
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x
Jag  Jagged
LNA  Locked Nucleic Acid
MG  Müller glia
Mib  Mind bomb
MID  Middle
miRNA  microRNA
miRNP  miRNA Ribonucleoprotein Complexes
MO  Morpholino
MRE  miRNA Recognition Element
mRNA  Messenger RNA
N  N-terminal
NECD  Notch Extracellular Domain
NICD  Notch Intracellular Domain
NT  Neural Tube
nt  Nucleotide
OP  Optic Primordium
ORF  Open Reading Frame
OV  Optic Vesicle
PAB  Poly(A) Binding Protein
PAZ  Piwi/Argonaute/Zwille
PBS  Phosphate Buffered Saline
PFA  Paraformaldehyde
PI3P  Phosphatidylinositol 3-monophosphate
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Full Form</th>
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<tr>
<td>PIP2</td>
<td>Phosphatidylinositol 4,5-bisphosphate</td>
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<tr>
<td>pre-miRNA</td>
<td>Precursor miRNA</td>
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<tr>
<td>pri-miRNA</td>
<td>Primary miRNA</td>
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<tr>
<td>PX</td>
<td>Phox-homology</td>
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<td>RISC</td>
<td>RNA Induced Silencing Complex</td>
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<tr>
<td>RLC</td>
<td>RISC-loading Complex</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
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<tr>
<td>RNAi</td>
<td>RNA Interference</td>
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<tr>
<td>RNase</td>
<td>Ribonuclease</td>
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<tr>
<td>RT-PCR</td>
<td>Reverse Transcription Polymerase Chain Reaction</td>
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<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
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<tr>
<td>siRNA</td>
<td>Small Interfering or Silencing RNA</td>
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<td>SNX</td>
<td>Sorting nexin</td>
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<tr>
<td>ssRNA</td>
<td>Single-stranded RNA</td>
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<tr>
<td>Tg</td>
<td>Transgenic</td>
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<td>UTR</td>
<td>Untranslated Region</td>
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microRNAs (miRNAs) are small noncoding RNAs that bind the 3' untranslated regions (UTRs) of mRNA targets and, acting with associated proteins, facilitate translation repression and degradation of target mRNAs. Since their discovery in *C. elegans*, miRNAs and their accessory proteins have been shown to be conserved throughout phylogeny. miRNAs exert their regulatory functions in myriad biological settings, from development and growth to disease. What follows is a general overview of miRNA discovery, organization, and function, and of the use of the powerful vertebrate developmental biology model organism, zebrafish (*Danio rerio*), zebrafish retinal development, and an overview of Notch-Delta signaling. The purpose of this dissertation is to examine the role of a specific miRNA, *miR-216a*, in regulation of the Notch-Delta signaling pathway during zebrafish retinal development.

**THE DISCOVERY OF miRNAs**

The first evidence that small RNAs play a regulatory role was published in 1993. Rosalind C. Lee and Rhonda L. Feinbaum, working in the lab of Victor Ambros, and Bruce Wightman and Ilho Ha, working in Gary Ruvkun’s lab, showed in concurrent publications that *lin-4*, known to be necessary for regulation of the heterochronic gene, *lin-14*, in *Caenorhabditis elegans* was not a...
protein coding gene, but a small RNA with complementarity to the \textit{lin-14} 3' UTR (Lee et al., 1993; Wightman et al., 1993).

\textit{lin-14} is expressed in a temporal gradient and regulates the normal sequence of cell lineage during \textit{C. elegans} development (Ambros, 1989; Ruvkun and Giusto, 1989). Before the seminal work from the Ambros and Ruvkun labs, it was known that \textit{lin-4} mutants affect cell lineage decisions with reiteration of larval cell fates during later stages (as do \textit{lin-14} gain of function mutants) and that \textit{lin-4} is a negative regulator of \textit{lin-14} (Ambros and Horvitz, 1987; Arasu et al., 1991). The Ambros group cloned the \textit{lin-4} locus and created mutants to disrupt potential open reading frames. However, even when the putative ORFs were disrupted, the constructs were still able to rescue \textit{lin-4} mutants. Coupled to the fact that \textit{lin-4} sequences are conserved in other \textit{Caenorhabditis} species, it was concluded that the \textit{lin-4} product does not function as a protein. The \textit{lin-4} product was then shown by northern blot and RNAase protection assays to encode two small RNAs of \~61 and \~22 nucleotides (nt) in length. Finally, the Ambros group showed that sequences in the 3' UTR of \textit{lin-14}, known from previous work to be necessary for its negative temporal regulation (Wightman et al., 1991), are complementary to sequences in \textit{lin-4} (Lee et al., 1993).

Ruvkun’s group showed that the 3' UTR of \textit{lin-14} is sufficient to confer temporal regulation to a \textit{lacZ} reporter and that regulation of the reporter was not recapitulated in \textit{lin-4} mutants. They also identified nucleotides in the 3' end of \textit{lin-4} complementary to seven sites in the \textit{lin-14} 3' UTR, which were found to be conserved in \textit{C. briggsae}. Based on these findings, the group hypothesized that
the seven binding sites in the lin-14 3' UTR are bound cooperatively by lin-4 to control downregulation of lin-14 posttranscriptionally at precise developmental time points (Wightman et al., 1993).

The discovery of lin-4 presented a fascinating new possibility for posttranscriptional regulation of gene expression but because lin-4 is not conserved in higher eukaryotes, it was unclear whether such regulation was restricted to worms. In 2000, the Ruvkun group published evidence for the existence of another small RNA, let-7, which also plays a role in developmental timing by regulating the expression of several heterochronic genes, including lin-14 and lin-41 (Reinhart et al., 2000). In contrast to lin-4, let-7 is conserved from arthropods to vertebrates and the developmental timing of let-7 expression is also conserved in Drosophila, zebrafish and mollusks (Pasquinelli et al., 2000). These RNAs were dubbed small temporal RNAs (stRNAs), based upon the expectation that discovery of other small RNAs would play a similar role in developmental timing (Pasquinelli et al., 2000; Reinhart et al., 2000; Slack et al., 2000). Subsequent work has since revealed multiple small RNAs in mammalian genomes, as well as in the genomes of fish, flies, worms, and plants. These small RNAs do not always facilitate developmental timing events so the name stRNAs gave way to the more general term, microRNAs (miRNAs). It is now apparent that miRNAs function in multiple biological processes, from growth and maintenance to apoptosis (Ambros, 2003; Aravin et al., 2003; Houbaviy et al., 2003; Lagos-Quintana et al., 2001; Lagos-Quintana et al., 2003; Lagos-Quintana
et al., 2002; Lau et al., 2001; Lee and Ambros, 2001; Lim et al., 2003a; Lim et al., 2003b; Reinhart et al., 2002).

UNDERSTANDING miRNA BIOGENESIS

Canonical miRNA Biogenesis: Dicer

Work to understand how miRNAs are transcribed and processed has been extensive. The discovery of RNA Interference (RNAi) mediated by short, double stranded RNAs by Fire et al in 1998 provided unexpected insight into miRNA biogenesis (Fire et al., 1998). Small RNAs with regulatory functions were studied in plants (Hamilton and Baulcombe, 1999), and the mechanism of mRNA degradation by small dsRNAs was further explored in other systems, namely Drosophila (Hammond et al., 2000; Parrish et al., 2000; Yang et al., 2000; Zamore et al., 2000). In work published in January 2001, Bernstein et al identified an enzyme that they termed Dicer, an RNase III superfamily member containing a PAZ domain (later shown to be an RNA-binding domain), two RNase III domains, and an amino-terminal helicase domain (Bernstein et al., 2001). They showed that Dicer is capable of producing 22 nucleotide small interfering RNA (siRNA) fragments from long dsRNA (the initial step of RNAi) and that transfecting cells with long dsRNAs complementary to Dicer mRNA in S2 cells abrogates the ability to silence a number of genes. They suggested, therefore, that Dicer is the enzyme responsible for the cleavage of long dsRNAs into 22 nt “guide strands” necessary for RNAi.
Because the initial discoveries of miRNAs detected RNAs of ~70 and ~22 nt on northern blots (Lee et al., 1993; Pasquinelli et al., 2000; Reinhart et al., 2000), several groups hypothesized that the 70 nt form is a precursor to the 22 nt RNA. The discovery of Dicer as the enzyme responsible for cleavage of long dsRNA into 22 nt siRNAs provided an excellent candidate for the enzyme that creates 22 nt mature miRNAs from the longer 70 nt precursors. Grishok et al showed in 2001 that dsRNAs targeting the C. elegans Dicer homologue dcr-1 caused a loss of the 22 nt RNAs and accumulation of the 70 nt forms for both lin-4 and let-7, suggesting that the 70 nt RNA is indeed a precursor to the 22 nt form and that Dicer is necessary for its processing (Grishok et al., 2001). The same year, Hutvágner et al demonstrated in HeLa cells, Drosophila pupae, and Drosophila extracts, that Dicer is necessary for let-7 maturation from a precursor stem loop structure into the 22 nt mature RNA (Hutvágner et al., 2001). Ketting et al also showed that year in C. elegans dcr-1 mutants, that let-7 22 nt RNA levels are reduced, while the 70 nt form of let-7 accumulates (Ketting et al., 2001). Recombinant human Dicer was later shown to generate both ~21-23 nt products from long dsRNA and mature let-7 from pre-let-7 transcripts (Provost et al., 2002). All of this evidence indicated that Dicer is the conserved RNase III family member responsible for generating the 22 nt mature miRNA duplexes from ~70 nt precursors (pre-miRNA).

Insight into Dicer’s structure revealed how it generates mature miRNAs. In 2004, Zhang et al generated mutations in putative catalytic residues in human Dicer and concluded that Dicer has a single dsRNA processing center containing
two RNase III sites for cleavage of phosphodiester bonds on opposite RNA strands, thereby yielding mature dsRNAs with two nt 3’ overhangs (Zhang et al., 2004). By solving the crystal structure of *Giardia intestinalis* Dicer in 2006, MacRae *et al* showed that the dsRNA binding PAZ domain of Dicer is ~65 Å from the processing center, which is the distance needed to accommodate ~25 basepairs of dsRNA, the length of *Giardia* small RNAs (MacRae et al., 2006). They therefore concluded that Dicer acts as a molecular ruler that measures a specified distance from the end of the dsRNA contained in the PAZ domain to position the RNase III domains for cleavage and generation of ~25 nt small RNAs. Their data suggested that the vertebrate Dicer processing site is positioned so as to allow cleavage of the precursor stem loop to generate mature miRNA duplexes of approximately 22 bp. This hypothesis was confirmed by V. Narry Kim’s lab using immunopurified human Dicer and radiolabeled synthetic pre-miRNAs (Park et al., 2011). Kim’s group further showed that the 5’ and 3’ ends of precursor miRNAs are anchored by the PAZ domain and regions surrounding it, and that Dicer measures ~22 nt from the 5’ end in order to determine the cleavage site. In 2012, Gu *et al* suggested that Dicer recognizes the ends of the miRNA as well as the loop, facilitating precise precursor cleavage and avoidance of off-target effects (Gu et al., 2012).

Dicer cleavage of pre-miRNAs has since been shown to require the action of cofactors. In two studies using human cell lines, the human immunodeficiency virus transactivating response dsRNA-binding protein (TRBP or TARBP2) was shown to associate with Dicer and to be required for miRNA biogenesis and post-
transcriptional gene silencing (Chendrimada et al., 2005; Haase et al., 2005). Three groups independently showed that the protein Loquacious (Loqs), the Drosophila homologue of TRBP, is found in complex with Dicer-1 (the Drosophila Dicer that is responsible for pre-miRNA cleavage). Loqs deficiency in S2 cells leads to the accumulation of precursor miRNAs, while loss of Loqs in the germline of male and female flies causes miRNA processing defects, as well as sterility (Fürstemann et al., 2005; Jiang et al., 2005; Saito et al., 2005).

**Canonical miRNA Biogenesis: Drosha**

Early cloning and genomic studies provided additional insight into the genomic organization of miRNAs and thus their biogenesis (also reviewed in (Olena and Patton, 2009)). Lau et al (in C. elegans), Lagos-Quintana et al (in Drosophila and human cells), and Mourelatos et al (in human cells) showed that some miRNAs are found in clusters in the genome (Lagos-Quintana et al., 2001; Lau et al., 2001; Mourelatos et al., 2002). The Drosophila miR-3/miR-6 cluster, which contains miRs-3, -4, -5, and three copies of miR-6, is not only encoded in close proximity in the genome, but expression of the these miRNAs is also temporally coordinated (Lagos-Quintana et al., 2001). In C. elegans, miRNAs in four clusters, including those encoded by the miR-35-miR-41 cluster, are also expressed in a temporally coordinated fashion during embryo and young adult stages (Lau et al., 2001). This suggested that miRNAs might be transcribed as polycistronic primary transcripts. Genomic data also showed that miRNAs are sometimes found antisense to protein coding genes and in intergenic regions,
which further indicated that they must (at least in some cases) be individual transcriptional units (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001; Mourelatos et al., 2002).

In 2002, V. Narry Kim’s group used HeLa cell total RNA to perform RT-PCR with primers outside the precursor sequences for two miRNA clusters (miR-23~27~24-2 and miR-17~18~19a~20~19b-1) and one individual miRNA (miR-30) and observed bands larger than the single precursors (Lee et al., 2002). This evidence suggested that both clustered and individual miRNAs are transcribed as longer units containing multiple stem-loop secondary structures, which Kim’s group termed primary miRNAs (pri-miRNAs). Kim’s group developed an in vitro processing assay, which showed that pri-miRNAs up to several kilobases long are the forerunners of miRNAs and are processed in at least two sequential steps: first to create ~70 nt precursors, a step which they demonstrated most likely happens in the nucleus, and then again in the cytoplasm by Dicer to create ~22 nt miRNAs. Just one year later, Kim’s group identified another RNase III family member that is responsible for nuclear cleavage of pri-miRNAs and termed this enzyme Drosha (Lee et al., 2003). They showed that immunopurified Drosha cleaves pri-miRNA into pre-miRNA in vitro, resulting in hairpin dsRNAs with 2 nt overhangs at the 3’ ends, characteristic of RNase III enzymes. They also showed that RNAi against Drosha in HeLa cells abrogates pri-miRNA processing, resulting in increased detection of pri-miRNA transcripts and a corresponding decrease in precursor and mature miRNAs.
Interestingly, similarly to Dicer, Drosha requires a dsRNA-binding protein cofactor. This factor, called DiGeorge syndrome critical region gene 8 (DGCR8) in humans and Pasha in Drosophila and C. elegans, was shown in human cells, S2 cells, and C. elegans to be in complex with Drosha. Depletion or mutation of DGCR8/Pasha in vitro and in vivo leads to an accumulation of pri-miRNA transcripts and a loss of mature miRNAs. The Drosha-DGCR8/Pasha complex is called the Microprocessor (Denli et al., 2004; Gregory et al., 2004; Han et al., 2004; Landthaler et al., 2004). The processing of pri-miRNAs by the Microprocessor occurs co-transcriptionally (Kim and Kim, 2007; Morlando et al., 2008; Pawlicki and Steitz, 2008).

In order to understand how the Microprocessor recognizes its substrate, Bryan Cullen’s group used a cell-free system to show that RNA hairpins with loops ≥10 nt and stems of ~30 bp are preferentially processed (Zeng et al., 2005). Cullen’s group demonstrated in vitro that the Microprocessor requires its pri-miRNA substrates to have a hairpin of at least 80 nt and flanking ssRNA regions of at least 10 nt, though in cells, the requirement for the length of flanking ssRNA is at least 40 nt. They also showed that the Drosha dsRNA binding domain (dsRBD) has a very low affinity for RNA (Zeng and Cullen, 2005). To clearly ascertain the molecular basis for Microprocessor recognition of its pri-miRNA substrate, V. Narry Kim’s lab examined the predicted primary structures of known miRNAs to determine a generalized structure for pri-miRNAs: hairpins containing a loop, approximately three helical turns, and 5’ and 3’ ssRNA elements. They then used an in vitro processing assay to show that the pri-
miRNA loop can be replaced by two ssRNA segments, implying that it is the instability of the loop and not the loop itself that is essential for processing. Kim’s group next confirmed that the ssRNA elements at both the 5’ and 3’ ends of the hairpin are essential and showed that deletion of basepairs from the helix nearest the ssRNA elements changed the site of cleavage. This data suggested that the ssRNA elements are used as a landmark in processing. Finally, they showed that it is DGCR8 that preferentially binds to pri-miRNAs to facilitate cleavage by Drosha within the Microprocessor (Han et al., 2006).

**Canonical miRNA Biogenesis: Nuclear Export**

Since miRNA processing had been demonstrated to have both nuclear and cytoplasmic localization, the question of how pre-miRNAs leave the nucleus was the next to be answered. Yi *et al* showed using RNAi in 293T cells against the nucleocytoplasmic transport factor Exportin5 (Exp5), which is Ran-GTP-dependent, that pre- and mature miRNA presence and function in the cytoplasm is decreased as compared to mock-transfected cells (Yi et al., 2003). They also showed *in vitro* that Exp5 binds the miR-30 precursor in the presence of Ran-GTP. Bohnsack *et al* showed independently that pre-miRNA export is sensitive to deficits of Ran-GTP in the nucleus and used affinity columns with bound precursor miRNAs to recover Exp5 (Bohnsack et al., 2004). Additionally, they demonstrated that they could block pre-miRNA export from *Xenopus* oocyte nuclei using antibodies raised against *Xenopus* Exp5. Lund *et al* added to the weight of evidence for the role of Exp5 in pre-miRNA export by showing a
decrease in cytoplasmic *let-7a* upon treatment of HeLa cells with siRNAs against Exp5 (Lund et al., 2004). They also showed in HeLa cell extract that the binding of pre-miRNAs to Exp5 is highly specific and resistant to competition by small RNAs with similar secondary structures to pre-miRNAs. All these data taken together confirmed that Exp5 is the nucleocytoplasmic transport factor responsible for pre-miRNA export from the nucleus, and that its role in the process is Ran-GTP dependent.

**Canonical miRNA Biogenesis: Transcription**

With the knowledge of compartmentalized, stepwise processing of miRNAs in hand, the field next turned to the question of how miRNAs are transcribed. Both the Kim and Cullen labs used the efficient m7G cap-binding properties of eIF4E to isolate capped RNAs and analyzed the content of the resulting RNA pool using RT-PCR with pri-miRNA specific primers (Cai et al., 2004; Lee et al., 2004). Both groups showed that pri-miRNAs were present in capped RNA fractions. Each group followed a similar principle to show the presence of pri-miRNAs in a HeLa cell cDNA library generated using an oligo-dT primer (Cai et al., 2004) and in a group of RNAs enriched with oligo-dT beads (Lee et al., 2004). These experiments provided strong evidence that pri-miRNAs are polyadenylated. Because 5’ capping and 3’ polyadenylation are hallmarks of RNA polymerase II transcription, the Kim lab used the Pol II specific inhibitor α-amanatin on HeLa cells and compared the transcription levels of several pri-miRNAs to their transcription levels in untreated cells. In all of the pri-miRNAs
they tested, a decrease in pri-miRNA transcription was observed upon α-amanatin treatment. The final piece of evidence that Pol II is responsible for pri-miRNA transcription came with the analysis of the promoter of the miR-23a~27a~24-2 cluster, which was shown to be bound by Pol II in vivo (Lee et al., 2004). Overall, miRNAs are transcribed as long primary transcripts that are then processed in two steps, one in the nucleus and the second in the cytoplasm (Figure 1.1).

Figure 1.1. Canonical miRNA Biogenesis
miRNAs are transcribed as primary transcripts by RNA Polymerase II. Pri-miRNAs contain one or more stem loops, which are recognized and cleaved by Drosha and its cofactor DGCR8. The resulting pre-miRNA is exported from the nucleus by Exportin 5, a process dependent upon Ran-GTP. In the cytoplasm, the pre-miRNA is processed by Dicer and its cofactor TRBP, resulting in a mature miRNA duplex containing two 21-22 nt long strands. This miRNA duplex is then incorporated into the RNA induced silencing complex (RISC).
Noncanonical miRNA Biogenesis

The large majority of miRNAs follow the biogenesis pathway described above but two noncanonical pathways have also been discovered, a Microprocessor-independent pathway and a Dicer-independent pathway. For an in depth review of these topics please see the excellent review from Jr-Shiuan Yang and Eric C. Lai (Yang and Lai, 2011). For an overview of noncanonical miRNA biogenesis, please see Figure 1.2.

![Figure 1.2. Noncanonical miRNA Biogenesis](image)

**Figure 1.2. Noncanonical miRNA Biogenesis**
Top, mirtron biogenesis. Mirtrons are transcribed as introns of protein coding genes by RNA Pol II. The spliceosome splices these mirtrons, which are debranched and undergo exosomal trimming (if needed). After these initial processing steps, mirtrons are treated as canonical miRNAs and transported to the cytoplasm by Exp5 to be processed by Dicer and TRBP and incorporated into the RISC. Bottom, Dicer-independent miRNA biogenesis. miR-451 is transcribed by RNA Pol II, processed by Drosha/DGCR8, and exported from the nucleus by Exp5. Once in the cytoplasm, miR-451 is bound by Argonaute 2 (Ago 2) and is cleaved to yield the functional, mature, single stranded miRNA.
Microprocessor-Independent miRNA Biogenesis

Genomically, miRNAs have been found in exons, introns, between genes (intergenic), and as part of either mono- or polycistronic RNAs (Griffiths-Jones et al., 2008; Thatcher et al., 2008a). In the special case of mirtrons, discovered in 2007 by Okamura et al and Ruby et al in Drosophila and C. elegans, mature miRNAs are derived from short intronic hairpins with splice sites on each end so that the sequence of the pre-miRNA corresponds exactly to the sequence of the intron (Okamura et al., 2007; Ruby et al., 2007). Instead of being dependent upon Drosha processing, these short intronic hairpins are excised by the spliceosome. After processing by the lariat-debranching enzyme, they fold into hairpin secondary structures resembling pre-miRNAs that are recognized by Exp5 for nuclear export, thus rejoining the canonical miRNA biogenesis pathway.

Since their initial discovery, mirtrons have also been identified in mammals by computational prediction and by high-throughput sequencing in RNA from human and rhesus macaque brains (Berezikov et al., 2007), from DGCR8 null mouse embryonic stem cells (Babiarz et al., 2008), from Drosha null murine T cells (Chong et al., 2010), and in the chick (Glazov et al., 2008). Chong et al showed that the majority of Drosha-independent miRNAs are not canonical mirtrons and appear to come from long introns or independent transcriptional units, suggesting that splicing is not solely responsible for the generation of this class of Drosha-independent miRNAs (Chong et al., 2010). Ruby et al, Babiarz et al, and Glazov et al also found evidence for tailed mirtrons, in which one of the two ends of the putative pre-miRNA does not correspond to a splice site,
confirming that another processing event must be necessary for pre-miRNA generation (Babiarz et al., 2008; Glazov et al., 2008; Ruby et al., 2007).

One such tailed mirtron, \(\text{miR-1017}\), was discovered by Ruby et al in \(C.\) \(\text{elegans}\) (Ruby et al., 2007) and more closely examined by Flynt et al in \(Drosophila\) (Flynt et al., 2010). High-throughput sequencing data from male \(Drosophila\) heads (Chung et al., 2008) contained approximately 6000 reads for \(\text{miR-1017}\), but also 14 reads for \(\text{miR-1017}^*\) (the strand paired with \(\text{miR-1017}\) in the precursor), whose sequence aligns exactly with the 5’ splice donor sequence, consistent with splicing being involved in \(\text{miR-1017}/\text{1017}^*\) biogenesis. In examining various \(Drosophila\) genomes, extensive variability was detected in the tail sequence (the sequence preceding the 3’ splice acceptor) of the \(\text{miR-1017}\) mirtron suggesting that the tail sequence is not important when generating a functional \(\text{miR-1017}\). Flynt and colleagues then used RNAi in S2 cells to show that the RNA exosome, a 3’ to 5’ exonuclease complex with multiple subunits, is responsible for the removal of this mirtron’s tail. The group also found genomic evidence for five more 3’ tailed mirtrons and suggested that these are processed by the RNA exosome as well (Flynt et al., 2010).

**Dicer-independent miRNA Biogenesis**

2010 brought the discovery of a Drosha-dependent, Dicer-independent miRNA, \(\text{miR-451}\) (Cheloufi et al., 2010; Cifuentes et al., 2010; Yang et al., 2010). Cleavage of the loop of pre-miRNAs by vertebrate Dicer generally yields mature miRNA duplexes with two paired RNA strands of ~22 nt, corresponding to either
side of the pre-miRNA stem. In the case of miR-451, the miRNA comes from a portion of the 5’ stem and the loop of the putative hairpin. miR-451 is conserved across phylogeny and was specifically examined in mouse (Cheloufi et al., 2010), zebrafish (Cifuentes et al., 2010), and Drosophila (Yang et al., 2010). These three groups showed independently that Argonaute 2 (Ago2), initially shown to be involved in miRNA function, is responsible for cleaving the hairpin and releasing a version of miR-451, which must then be trimmed by an as yet unknown exonuclease to generate the functional, mature miRNA (Cheloufi et al., 2010; Cifuentes et al., 2010; Yang et al., 2010).

MECHANISM OF miRNA FUNCTION

The discovery of the regulation of lin-14 by lin-4 coupled with the dependence of that regulation on elements in the 3’ UTR of lin-14 that are complementary to lin-4 provided initial insight into the mechanism of miRNA function (Lee et al., 1993; Wightman et al., 1993). What follows is an overview of the proteins that are involved in this mechanism, as well as information on how miRNA structure informs miRNA strand selection and mRNA target recognition, and how miRNAs and their associated proteins affect post-transcriptional gene silencing. For an overview, see Figure 1.3.
Dicer, TRBP, and Argonaute form the RISC Loading Complex (RLC), which facilitates assembly of the RISC and incorporation of the mature miRNA duplex. Once incorporated into the RISC, the nonfunctional strand of the miRNA duplex is degraded and the functional strand is guided to its target, generally in the 3’ UTR of a messenger RNA. If the miRNA pairs perfectly with its target (pictured top right) the miRNA-mRNA duplex is cleaved by Ago2 between nucleotides 10 and 11 of the miRNA strand, which leads to the target’s rapid degradation. If the miRNA pairs imperfectly with its target (depicted bottom right), the interaction can lead to translation repression, potentially occurring at the initiation step by interference with the mRNA cap, but still poorly understood, and to deadenylation; both outcomes may lead to eventual target degradation.

Assembly of the RNA-Induced Silencing Complex

The discovery of proteins that allow miRNAs to alter target gene expression was buoyed by work from the siRNA field. Studies using cultured mammalian and Drosophila cells and cell/embryo lysates showed that one strand of the siRNA duplex is the effector in RNAi and that it cooperates with a (then unknown) nuclease(s), that cleaves mRNAs at sites complementary to the
siRNA. The complex in which the siRNA and nuclease act was termed the RNA-induced silencing complex (RISC) (Elbashir et al., 2001a; Elbashir et al., 2001b; Hammond et al., 2000; Tuschl et al., 1999; Zamore et al., 2000). By purifying RISC from Drosophila cells, Hammond et al showed that one of the components of the RISC that copurifies with siRNA is Argonaute 2 (Ago2), a member of the Argonaute family (Hammond, 2001).

Argonaute family members have a PIWI and PAZ (Piwi/Argonaute/Zwille) domain (Cerutti et al., 2000), whose functions were elucidated with structural studies. Three groups solved the structures of the Drosophila Ago1 and Ago2 PAZ domains and demonstrated that the PAZ domains interact with the 3’ overhangs of dsRNA and prefer to interact with RNA rather than DNA. The structural data also suggested that the human Ago and Dicer PAZ domains are structurally similar and form a cleft for binding RNA (Lingel et al., 2003; Song et al., 2003; Yan, 2003). When structures of the PAZ domain bound to RNA and DNA were solved, it was shown that PAZ binding stabilizes dsRNA duplexes by securing the 2 nt 3’ overhangs in a conserved binding cleft and by binding the phosphodiester backbone of the overhang-containing strand. Also, the data indicated that the PAZ domain contributes to specific recognition of dsRNA duplexes within the RISC (Lingel et al., 2004; Ma et al., 2004). The first structure of full length Ago2 (from Pyrococcus furiosus) indicated that Ago2 is composed of four domains, previously unknown N-terminal (N) and MIDdle (MID) domains, as well as the PIWI and PAZ domains(Song et al., 2004). The structure showed that the N, PIWI, and MID domains form a crescent over which the PAZ domain
is held by a stalk-like region. Most importantly, the PIWI domain structure is similar to RNase H and is therefore responsible for the cleavage of mRNAs complementary to siRNAs, the catalytic component of RISC. Further analysis of the four mammalian Argonautes (Ago1-4) demonstrated that only Ago2 is able to cleave mRNA substrates (Liu, 2004).

While the structural work was done using siRNA-Ago pairs, miRNAs were also found to assemble in complexes containing Ago2 along with a helicase, Gemin3, and Gemin4 (Mourelatos et al., 2002). Though these complexes are highly similar to the RISC, they were initially termed miRNPs. In human cell extracts, let-7 was detected in RISCs but imperfect miRNA:mRNA pairing leads to translation repression rather than mRNA cleavage (Hutvágner and Zamore, 2002). siRNAs that do not pair perfectly with mRNA targets were also shown to translationally repress their targets, as opposed to cleave them (Doench et al., 2003; Saxena et al., 2003; Zeng et al., 2003). To assess whether Ago facilitates translation repression, the human protein was tethered to mRNAs in HeLa cells in the absence of miRNAs. Tethering human Ago1-4 to mRNAs mimics their miRNA-mediated repression (Pillai et al., 2004). Two groups published compelling data that TRBP recruits Dicer to Ago, thus coupling miRNA biogenesis to RISC function (Chendrimada et al., 2005; Gregory et al., 2005; Maniataki and Mourelatos, 2005). More recently, the association of Dicer, TRBP and Ago was named the RISC-loading complex (RLC) and shown to assemble spontaneously in vitro. Ago dissociates from Dicer and TRBP once it is loaded with a miRNA (MacRae et al., 2008). All this evidence confirms that siRNAs and
miRNAs enter the same complex, the RISC, of which Ago proteins serve as the main effectors.

In addition to Ago proteins, GW182, an RNA-recognition motif containing protein, has been shown to be a component of RISC and to be important for miRNA function. GW182, named for its multiple glycine (G)-tryptophan (W) repeats, was initially shown in cell culture to co-localize with exogenous Ago2 protein in distinct cytoplasmic foci, called processing or P-bodies (Sen and Blau, 2005). Further work demonstrated that transfected siRNAs and endogenous Ago proteins also co-localize with GW182 in P-bodies, and that when P-bodies are disrupted, siRNA regulation of targets is abrogated (Jakymiw et al., 2005). Rehwinkel et al used S2 cells transfected with dsRNA to show that GW182 is necessary for miRNA mediated target regulation (Rehwinkel et al., 2005). Liu et al showed that Argonaute proteins physically interact with GW182 and that silencing GW182 perturbs miRNA function (Liu et al., 2005). Consistent with this, Ding et al showed that a C. elegans protein sharing homology with GW182 is responsible for targeting the worm Ago homologue to P-bodies (Ding et al., 2005). The GW182-Ago interaction has since been established to be crucial for miRNA target repression (Eulalio et al., 2008). It is likely that Ago proteins interact in the RISC with other partners. Indeed, in a proteomics screen, an RNA helicase, MOV10, and another RNA-recognition motif containing protein, TNCR6B, were found to interact with Ago proteins (Meister et al., 2005).
Selection of the Functional miRNA Strand

In 2002, Martinez et al. showed that only one of the two strands of the siRNA duplex is retained in the RISC and works with the RISC components to effect sequence specific silencing (Martinez et al., 2002). This finding raised the question as to how one strand is chosen over the other. Direct cloning of miRNAs readily detected the presence of mature miRNA strands but in some cases, the other strand (denoted miRNA*) was also detected albeit at low levels (Lim et al., 2003b). The presence of the star strand was a good first indication that there is a preference for choosing which side of the pre-miRNA is incorporated into RISC. By designing a wide variety of synthetic siRNA duplexes, Schwarz et al. showed that for the majority of cases, the strand with the less stably paired 5’ end is the one incorporated into the RISC (Schwarz et al., 2003). To extend this paradigm to miRNAs, putative precursors of known miRNAs were analyzed to determine whether predictions based on 5’ stability agree with strand selection in vivo. The data are consistent with the model that the strand with lower internal thermodynamic stability at the 5’ terminus is generally the functional strand (Khvorova et al., 2003).

Canonical miRNA Target Recognition

The majority of early work on miRNA-target interactions showed that miRNAs do not bind perfectly with their targets (Lee et al., 1993; Reinhart et al., 2000; Vella et al., 2004; Wightman et al., 1993). Furthermore, miRNAs can bind combinatorially, so more than one miRNA usually regulates a target, and
miRNAs may target several mRNAs (Enright et al., 2003). In order to facilitate target prediction, several groups undertook to find guidelines for miRNA-target pairing. Lewis et al found pairing between nucleotides 2-8 (from the 5’ end) of the miRNA and the mRNA 3’ UTR to be the most reliable predictor of a verifiable miRNA-mRNA interaction and referred to this region as the "seed" of the miRNA, calling a Watson-Crick base paired interaction between the miRNA seed and an mRNA a “seed match” (Lewis et al., 2003). The seed rule was extended by analyzing miRNA seed sequences and finding an overrepresentation of adenosines on either side of seed sequences (Lewis et al., 2005).

Using reporter assays, Doench et al confirmed that miRNAs cooperatively bind to targets and that the 5’ region of the miRNA is extremely important for miRNA-target pairing (Doench and Sharp, 2004). They also showed that miRNA 3’ end pairing with the 3’ UTR of the mRNA is only important if the interaction at the 5’ end is less stable. Kiriakidou et al used mutagenesis of luciferase reporter constructs containing single MREs to show the requirement for a bulge in the center of miRNAs bound to targets (whose maximum length is context dependent) (Kiriakidou et al., 2004). Brennecke et al then showed in vivo that a single 8mer at the 5’ end of the miRNA pairing perfectly with the mRNA 3’ UTR is able to confer target repression, with a 7mer conferring slightly less repression (Brennecke et al., 2005). When two 8 or 7mers were present, target regulation increased. They also distinguished between canonical miRNA recognition sites (MREs), which have strong base pairing at both the 5’ and 3’ ends of the miRNA, seed sites, which have strong base pairing at the 5’ end with minimal 3’ end
pairing, and 3’ compensatory sites, which have mismatches or G:U wobbles in the seed but strong base pairing at the 3’ end. They also found that the 3’ end of the miRNA is responsible for differences in mRNA target recognition by members of miRNA family members, which share identical seed sequences but with differences at their 3’ ends.

To attempt to further refine target prediction, the Bartel group looked carefully in silico and in vivo at miRNA-target interactions (Grimson et al., 2007). They concluded that miRNA repression can be context dependent with several factors increasing the likelihood of target regulation. MREs positioned near AU-rich sequences or close to MREs for other miRNAs increase the efficiency of miRNA-mRNA pairing. Also, positioning within the 3’ UTR can also affect pairing: stronger sites are usually non-centrally located MREs in long UTRs and those at least 15 nt from the end of the coding sequence. They also corroborated the data from other labs that showed a role for the 3’ end of the miRNA, narrowing it down to pairing in nucleotides 13-16. The potential for cooperation between MREs was highlighted experimentally in other systems (Saetrom et al., 2007). In addition to confirming that MREs are found close to the beginning or end of long human 3’ UTRs, Gaidatzis et al observed that MRE location is evolutionarily conserved within 3’ UTRs (Gaidatzis et al., 2007). They suggested that this conservation of location could provide greater MRE accessibility for RISC and therefore have implications for miRNA function. Nielsen et al refined the importance of the context of the MRE within the UTR further by showing that an adenosine is generally opposite miRNA nt 1 in the
3'UTR and an adenosine or uridine is generally opposite miRNA nt 9 (Nielsen et al., 2007). Moreover, they showed that increased sequence conservation approximately 50 nt up and downstream of the seed match in the 3’ UTR predicts increased target repression.

Noncanonical miRNA Target Recognition

Perhaps not surprisingly, as previous work showed the ability of noncanonical MREs to regulate targets (Brennecke et al., 2005), several examples of functional noncanonical MREs have emerged. Li et al demonstrated that dispatched homolog 2 is regulated by miR-214 during zebrafish development through the action of three noncanonical MREs in its 3' UTR that act combinatorially to confer repression (Li et al., 2008). The Bartel group identified a new class of miRNA-target binding, in which the binding interaction does not feature strong seed pairing, but instead features at least 11 contiguous Watson-Crick base pairs starting at nt 4 of the miRNA (Shin et al., 2010). In vitro under certain buffer conditions, these sites can lead to mRNA cleavage, but in vivo they mostly work to repress translation. In the same work, more miRNAs were identified that facilitate target cleavage (as siRNAs do) than was originally thought, though the number of miRNAs acting in this fashion is still low compared to how the vast majority of miRNAs function. Chi et al used data from RNA libraries to show that miR-124, a common neuronal miRNA, binds its targets with a G-bulge in the 3’ UTR, between nt 5-6 of the miRNA (Chi et al., 2012). They further showed that nucleation bulges are evolutionarily conserved, functional in
the murine brain, and present in *C. elegans* as well as in mice. Finally, Loeb *et al* used a whole transcriptome approach to identify all the targets of *miR-155* in murine T cells (Loeb *et al.*, 2012). They found that 40% of miRNA-target interactions are noncanonical, with the majority of these interactions having one mismatch in the seed region. Interestingly, *miR-155* is still able to regulate gene expression using a noncanonical site. The discovery of noncanonical sites is necessary for facilitating understanding of miRNA function. Perhaps more importantly, the discovery of noncanonical sites highlights the difficulty in predicting miRNA targets and the necessity for experimental validation of predicted targets. See Figure 1.4 for a summary of the ways miRNAs recognize their targets.

**Mechanisms of miRNA Mediated Gene Silencing**

While direct miRNA mediated cleavage of mRNA targets is rare in vertebrates (Yekta *et al.*, 2004), examples abound of miRNA regulation of gene expression through both translation repression and mRNA deadenylation leading to degradation. Early evidence suggested that the levels of mRNA targets are unaffected by miRNA repression (Olsen and Ambros, 1999), consistent with translation repression models. Initial work used polysome profiling in *C. elegans* to show that mRNAs and miRNAs are sometimes associated with polyribosomes (Olsen and Ambros, 1999; Seggerson *et al.*, 2002). This work was corroborated in human cell lines and suggested that translation repression can occur after initiation (Maroney *et al.*, 2006; Nottrott *et al.*, 2006; Petersen *et al.*, 2006)
Figure 1.4. miRNA Target Recognition
The pairing of miR-203 and its target during zebrafish fin regeneration, *lef1*, is shown at top. Straight lines represent Watson-Crick base pairs, while colons represent G:U wobble pairing. The 3'UTR of *lef1* contains two miRNA target sites, both of which are good examples of canonical miRNA-target pairing, with perfect matches in the seed region (nt 2-8 of the miRNA, boxed in red, adapted from (Thatcher et al., 2008b). Second from top, the *disp2* 3'UTR contains three miR-214 target sites, all of which would be considered weak based on lack of perfect pairing in the seed region (boxed in red), but the three weak sites have been shown to act collaboratively to facilitate miR-214 regulation of *disp2* (Li et al., 2008). Second from bottom is an example of centered pairing between miR-1 and its target ZNF586 (Shin et al., 2010). At bottom, one example of a miR-155 target (Hif1a) with mismatches in the seed region (boxed in red); Hif1a also contains a canonical pairing site for miR-155 (not pictured) (Loeb et al., 2012).
Evidence also emerged suggesting that miRNAs can regulate their targets at the level of translation initiation. The Filipowicz lab showed in HeLa cells that a 5’ 7meG cap is required for miRNA mediated mRNA repression, and that artificially tethering the translation initiation factor eIF-4E to an uncapped mRNA allows repression by endogenous let-7 (Pillai et al., 2005). Further studies in HeLa cells confirmed that the 7meG cap and the 3’ poly(A) tail are necessary, but not sufficient, for miRNA mediated translation repression (Humphreys et al., 2005). Work from the Izarraulde lab showed involvement of the decapping enzymes, DCP1 and DCP2 in miRNA mediated target repression (Rehwinkel et al., 2005). Several groups used in vitro systems from Drosophila and human cells to confirm that translation repression can be facilitated by interference with cap-binding proteins at the translation initiation step (Mathonnet et al., 2007; Thermann and Hentze, 2007; Wakiyama et al., 2007; Wang et al., 2006). Recent evidence from zebrafish showed that miR-430 initially represses its targets in the developing embryo at the level of translation, likely by reducing the rate of initiation (Bazzini et al., 2012).

The mechanism by which repression might occur at the translation initiation step is still unclear, however. It was originally suggested that Ago2’s MID domain is significantly similar to the cap-binding domain of eIF-4E (Kiriakidou et al., 2007), but this domain has since been shown to bind GW182, as opposed to the cap (Eulalio et al., 2008; Kinch and Grishin, 2009). The data are somewhat confounding, though, as recently purified MID domains from several species have been shown to possess the ability to bind mimics of the
7meG cap in vitro (Djurano\v{c}ic et al., 2010). Another potential mechanistic explanation could be the involvement of e\text{IF}-6, a protein known to inhibit formation of the 80S ribosome. Chendrimada et al showed that e\text{IF}-6 is associated with human RISCs and that it plays a role in miRNA-mediated target repression in both human cells and in \textit{C. elegans} (Chendrimada et al., 2007). Other evidence points to the involvement of the poly(A) binding protein (PAB) (Walters et al., 2010), but the mode of miRNA repression of translation is still vague. A more in depth discussion of these issues can be found in a review from Shuo Gu and Mark Kay (Gu and Kay, 2010).

While some miRNAs repress targets during translation, evidence has also emerged that miRNAs can, in other cases, cause destabilization and degradation of targeted mRNAs (Bagga et al., 2005; Lim et al., 2005). The most common mechanism of mRNA destabilization seems to begin via deadenylation. In zebrafish, \textit{miR}-430 promotes rapid deadenylation and clearance of maternal mRNAs during the transition from maternal to zygotic transcription (Giralde\~{n}ez et al., 2006). Consistent with this, miRNA mediated mRNA degradation requires the CCR4:NOT deadenylase (Behm-Ansmant et al., 2006; Braun et al., 2011; Chekulaeva et al., 2011; Fabian et al., 2011). In mammalian cells, miRNAs are able to direct rapid deadenylation of targets (Wu et al., 2006), and in cell-free systems miRNAs can deadenylate mRNAs (Wakiyama et al., 2007). In \textit{Drosophila} cells, 60\% of targets of Ago are regulated by CAF1 or NOT1 deadenylases, indicating that deadenylation of miRNA targets is widespread (Eulalio et al., 2009).
It seems likely that the use of these two modes of target repression is context dependent, and in some cases both mechanisms are used in concert. Recent data support the idea that deadenylation and translational repression may not be mutually exclusive (Baek et al., 2008; Guo et al., 2010). In zebrafish for example, miR-430 first translationally represses and then destabilizes target mRNAs (Bazzini et al., 2012). A deeper discussion of these possibilities and a more thorough look at the evidence for all modes of miRNA mediated target repression can be found in excellent reviews from Fabian et al. and Huntzinger et al. (Fabian et al., 2010; Huntzinger and Izaurralde, 2011).

THE IMPORTANCE OF miRNA BIOGENESIS AND FUNCTION

Following their discovery in C. elegans, miRNAs have come to assume prominent roles in the regulation of gene expression in many biological contexts. Their unique discovery as functional, non-protein coding RNAs has spurred research into other classes of non-coding RNAs that are likely to have equally diverse and widespread functions (Mercer et al., 2009; Thomson and Lin, 2009). Research is ongoing in the areas of miRNA biogenesis and function, especially how these processes are regulated. As new work emerges, it is obvious there is much to learn. Moving forward, it will be important to recognize that the rapid progress that has been made needs to be tempered by the recognition that what seems like dogma based on initial studies might turn out to be much more complex as the complete story comes to light. Nevertheless, it seems clear that
complete understanding of miRNA biogenesis and function will provide further insight into the role of miRNAs during development and disease.

**miRNA FUNCTION DURING ZEBRAFISH DEVELOPMENT**

Zebrafish are widely used as a model organism based on many advantages, including large clutch sizes, external fertilization, relative ease of keeping adults, and embryo transparency during early development (Streisinger et al., 1981). Zebrafish have been used to answer myriad developmental biology questions in the nearly 30 years since gaining popularity as a model.

While various high throughput methods are now used to determine miRNA expression patterns during development, determining the targets of miRNAs is still a painstaking process, requiring *in vivo* validation of each miRNA-target pair. Because of the strengths outlined above and because tools are readily available to modulate the expression of miRNAs (and their mRNA targets) in zebrafish embryos, zebrafish have proved to be a powerful model system for the study of miRNA function during vertebrate development.

In work from the Patton lab alone, individual miRNAs have been shown to regulate hedgehog signaling and muscle cell fate (Flynt et al., 2007), neuronal development (Li et al., 2008), larval responses to stress (Flynt et al., 2009), endoderm formation and left-right asymmetry (Li et al., 2011), and synaptic transmission and neuronal function (Wei et al., 2013). Work from other groups has extended the role of miRNAs into virtually all realms of zebrafish development (Giraldez et al., 2005; Mishima et al., 2009; Stahlhut et al., 2012).
The zebrafish retina presents a powerful model for the study of retinal development. Like the retinas of most vertebrates, the zebrafish retina contains three highly organized cell layers, which perform conserved functions across phylogeny (Stenkamp, 2007). The outermost photoreceptor layer contains rods and cones, the light-sensitive cells of the retina. The inner nuclear layer contains the bipolar, horizontal, and amacrine cells, which integrate and transmit signals between the photoreceptors and the ganglion cells in the ganglion cell layer, nearest the lens. The ganglion cells convey visual information to the brain for processing. The inner nuclear layer also contains the Müller glia, which support the neurons (Stenkamp, 2007). See also Figure 1.5.

**Figure 1.5. Zebrafish Retina**

The zebrafish retina consists of the outer nuclear layer, which contains the photoreceptor cell nuclei, the inner nuclear layer, which contains the horizontal, amacrine, and bipolar cells, as well as the nuclei of Müller glia, and the ganglion cell layer. Light enters the retina through the lens at the bottom of the cartoon, where the majority of it is absorbed by the retinal pigmented epithelium at the back of the retina and detected by the photoreceptors. Figure adapted from (Stenkamp, 2007).
The zebrafish retina develops quickly: from the first appearance of optic tissue at about 11 hours post fertilization (hpf), it takes only three days for a measurable optokinetic response to emerge (Brockerhoff et al., 1995). The combined advantages of the zebrafish as a model system, as discussed above, and the conservation of vertebrate retinal development provide opportunities to investigate questions of retinal development.

Like other vertebrate eyes, the zebrafish eye begins as an optic primordium, which extends laterally from the developing neural tube on both the left and the right (Schmitt and Dowling, 1994). In zebrafish the optic primordium forms around 12 hpf (Schmitt and Dowling, 1994). The optic primordium continues to extend from the neural tube, and by 14 hpf, forms a protruding structure that resembles a wing and begins to separate from the developing forebrain to form the optic vesicle (Schmitt and Dowling, 1994). The optic vesicle, which is covered by the lens placode, rotates and invaginates to form an optic cup, a process that starts around 16 hpf and is completed by 24 hpf, with the obvious result of a visible eye cup in 1 day post fertilization (dpf) embryos (Schmitt and Dowling, 1994). See also Figure 1.6.

By 24 hpf, the eye cup has thickened to become the presumptive neural retina, a characteristic pseudostratified columnar epithelium, and the lens placode has separated from the adjacent ectoderm to become the lens (Li et al., 2000). Neurogenesis begins at 25 hpf, when proneural marker atonal homologue 5 (ath5) is expressed in the cells in the ventronasal region of the neural retina and then spreads in a fan-like gradient throughout the entire retina.
Figure 1.6. Zebrafish Eye Morphogenesis
The optic primordium (OP) extends from the neural tube (NT) starting at about 11 hpf. By 14 hpf, the OP has become the optic vesicle (OV), a wing-like protrusion, which begins to detach from the neural tube. By 24 hpf, the optic vesicle has invaginated and further separated from the neural tube and is recognizable as the eye cup. By 24 hpf, the lens has separated from the overlying ectoderm. Figure based on (Easter and Malicki, 2002).

by 36 hpf, leading to the differentiation of ganglion cells (Easter and Malicki, 2002). Around 38 hpf, inner nuclear layer neurons are born in the same ventronasal region where the initial ganglion cells appeared, and this second wave of neurogenesis proceeds until about 48 hpf (Hu and Easter, 1999). Outer nuclear layer neurons are born in a fan-like gradient, starting from the ventronasal area at about 48 hpf, with the third phase of this initial wave of neurogenesis complete by 60 hpf (Hu and Easter, 1999). Neurogenesis in the zebrafish retina is far from over at this point, however, and the ciliary marginal zone or circumferential germinal zone, the proliferative area at the lateral edges of the retina, continues to give rise to new neurons throughout the zebrafish’s life (Stenkamp, 2007).

Considerable work has been done to understand how the zebrafish retina is patterned. In 1996, evidence presented by Malicki et al showed that mutant
zebrafish with defects in tissue polarity and neuronal migration have abnormal retinas (Malicki et al., 1996). Multiple signaling pathways, including hedgehog signaling, wnt signaling, and FGF signaling, and other regulatory mechanisms have been shown to contribute to cell fate specification and retinal patterning in the zebrafish (reviewed in (Stenkamp, 2007)). Most relevant to this dissertation, Notch signaling has been shown to play an instructive role in the specification of Müller glia in the inner nuclear layer of the zebrafish retina (Scheer et al., 2001).

NOTCH SIGNALING

Notch signaling was named for Drosophila mutants that display a notched wing phenotype (Dexter, 1914; Mohr, 1919; Poulson, 1937). Notch is involved in multiple developmental processes in metazoans, from segmentation to cardiac development to neurogenesis (Louvi and Artavanis-Tsakonas, 2006; Ozbudak and Lewis, 2008; Rutenberg et al., 2006). Generally, a ligand, presented on one cell, and a single-pass transmembrane Notch receptor, presented on a nearby cell, interact, facilitating endocytosis of the ligand, cleavage of the Notch extracellular domain (NECD), and release of the Notch intracellular domain (NICD), which then translocates to the nucleus where it acts as a transcription factor and activates downstream genes (Le Borgne and Schweisguth, 2003). See also figure 2.5. The Notch signaling pathway is used by neighboring cells to communicate and orchestrate cell fate decisions throughout the embryo (Louvi and Artavanis-Tsakonas, 2006).
The best-known role for Notch signaling is in the regulation of neurogenesis. Notch signals act to maintain neural progenitor cells and inform the choice of neuronal or glial fates (Louvi and Artavanis-Tsakonas, 2006). In the zebrafish spinal cord, Delta-Notch signaling regulates cell fate decisions by coordinating lateral inhibition, or the specification of two different fates from one progenitor pool (Appel and Eisen, 1998; Appel et al., 2001). In the zebrafish retina, overexpression of NICD results in overproliferation of glial cells and large numbers of undifferentiated and apoptotic cells, suggesting that Notch signaling promotes gliogenesis (Scheer et al., 2001). Studies in Mib mutant zebrafish or those treated with an inhibitor of the enzyme that is necessary for cleavage of NECD, which do not develop Müller glia, also support the idea that Notch-Delta signaling is instructive for gliogenesis (Bernardos et al., 2005).

Here, I investigate the role of a miRNA, miR-216a, identified in a microarray of developing zebrafish eye fields. I determined that miR-216a regulates sorting nexin 5 (snx5) a novel component of the Notch signaling pathway.
CHAPTER II

miR-216a regulates snx5, a novel Notch signaling pathway component, during zebrafish retinal development

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ABSTRACT

Precise regulation of Notch signaling is essential for normal vertebrate development. Mind bomb (Mib) is a ubiquitin ligase that is required for activation of Notch by Notch’s ligand, Delta. Sorting Nexin 5 (SNX5) co-localizes with Mib and Delta complexes and has been shown to directly bind to Mib. We show that microRNA-216a (miR-216a) is expressed in the retina during early development and regulates snx5 to precisely regulate Notch signaling. miR-216a and snx5 have complementary expression patterns. Knocking down miR-216a and/or overexpression of snx5 resulted in increased Notch activation. Conversely, knocking down snx5 and/or miR-216a overexpression caused a decrease in Notch activation. We propose a model in which SNX5, precisely controlled by miR-216a, is a vital partner of Mib in promoting endocytosis of Delta and subsequent activation of Notch signaling.
INTRODUCTION

Since their discovery as regulators of *C. elegans* developmental timing in 1993 (Lee et al., 1993; Wightman et al., 1993), miRNAs have been shown to be involved in diverse aspects of development. miRNAs are 21-23 nucleotide (nt) non-coding RNAs that regulate gene expression by binding to complementary sequences in the 3'UTR of messenger RNAs (Bartel, 2004; Fabian et al., 2010; He and Hannon, 2004; Huntzinger and Izaurrealde, 2011; Liu et al., 2012). This results in the recruitment of the RNA Induced Silencing Complex (RISC), the effector complex that mediates translation repression, deadenylation, and decay of target mRNAs (Bazzini et al., 2012; Djuranovic et al., 2012; Giraldez et al., 2006). We and others have identified developmental roles for several individual miRNAs in zebrafish (Flynt et al., 2007; Flynt et al., 2009; Giraldez et al., 2005; Li et al., 2008; Li et al., 2011; Mishima et al., 2009; Stahlhut et al., 2012; Wei et al., 2013). However, the exact roles and mRNA targets for most miRNAs that function during development are still unknown.

Notch signaling regulates many processes during vertebrate development, from vasculogenesis to segmentation (Fortini, 2009; Lawson et al., 2001; Wright et al., 2011). It is especially important during neurogenesis (Louvi and Artavanis-Tsakonas, 2006), is instructive for gliogenesis in the zebrafish retina (Scheer et al., 2001), and has been shown to be essential for zebrafish retinal development (Bernardos et al., 2005). Notch is a transmembrane receptor that mediates interaction with adjacent cells through membrane bound ligands such as Delta that trigger proteolytic cleavage of Notch and release of an intracellular domain.
that travels to the nucleus to alter gene expression (Louvi and Artavanis-Tsakonas, 2006). Mind bomb is a ubiquitin ligase that ubiquitinates Delta thereby facilitating its endocytosis, which is essential for cleavage of Notch and subsequent activation of signaling (Itoh et al., 2003). Mutants in Mind bomb have disorganized retinal architecture and do not have Müller glia (Bernardos et al., 2005).

Sorting Nexin 5 (SNX5) is part of the large sorting nexin protein family, members of which have been previously shown to bind phosphoinositides through a specialized phox-homology (PX) domain (Cullen, 2008; Cullen and Korswagen, 2011). SNX5 is part of a select group of sorting nexins that also contain a carboxy-terminal BAR (Bin, amphipathin, Rvs) domain, thought to facilitate binding to and/or induce membrane curvature, possibly functioning in endocytosis or vesicle budding (Cullen, 2008). The sorting nexins function in diverse cellular trafficking processes, including developmental signaling cascades as in the case of SNX3, which has been shown to be required for Wnt secretion (Harterink et al., 2011) and SNX17, which functions in integrin recycling (Steinberg et al., 2012). SNX5 was previously shown to co-localize with Mib and Delta (Yoo et al., 2006). Knockdown of SNX5 using morpholinos in zebrafish causes defects in vascular development (Eckfeldt et al., 2005; Yoo et al., 2006). Accumulating evidence therefore suggests that SNX5 could play a role in modulating Notch signaling.

In this study, we show for the first time that *miR-216a*, a miRNA that is expressed in the developing zebrafish retina, regulates *snx5*. Results using
reporter fish show that miR-216a regulates snx5 to modulate Notch signaling during eye development.

MATERIALS AND METHODS
Zebrafish Lines and Maintenance
Wildtype (AB) (Walker, 1999), albino (University of Oregon, Eugene, OR), Tg(gfap:GFP) (Bernardos and Raymond, 2006), Tg(her4:dRFP) (Yeo et al., 2007), Tg(flk1:GFP) (Choi et al., 2007), and Tg(Tp1bglob:eGFP) (Parsons et al., 2009) lines were maintained at 28.5°C on a 14:10 hour light:dark cycle. Embryos were raised in egg water (0.03% Instant Ocean) at 28.5°C and staged according to morphology (Kimmel et al., 1995) and hours post fertilization (hpf). All experiments were performed with the approval of the Vanderbilt University Institutional Animal Care and Use Committee (M/09/398).

Microarrays of developing eyes
Developing eyes were dissected at 2 and 5 days post fertilization (dpf), homogenized in Trizol and total RNA was extracted. Small RNAs were enriched and arrays were performed and normalized as previously described (Thatcher et al., 2007). Fold changes were calculated compared to a negative controls consisting of probes for Pseudomonas aeruginosa dehydrogenase (Thatcher et al., 2007). Microarray data was analyzed using GeneSpring software, and paired t-tests were performed using Prism (GraphPad) to determine p values.
Molecular Cloning

Potential target mRNA 3'UTRs were amplified by RT-PCR using the primers below (Table 1). Each 3' UTR was cloned into pCS2+ downstream of the coding sequence of GFP (Flynt et al., 2007).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer (5’-3’)</th>
<th>Reverse Primer (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>snx5 (NM_214769)</td>
<td>ACCTGATCGAGATGACTGAG</td>
<td>TTATCTTCGCTGAGTTGCAC</td>
</tr>
<tr>
<td>her4.2 (NM_131090.3)</td>
<td>AGTCACATCTGGAGACCCCTG</td>
<td>GCTTCAACACACAAACAAGTC</td>
</tr>
<tr>
<td>notch1b (NM_131302.2)</td>
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<td>CACAAATCGTTTCATCGGATG</td>
</tr>
<tr>
<td>heyl (NM_181736.1)</td>
<td>GGGGTTTACGGTTCCCTCCAG</td>
<td>TCTCCTCAAGCACTTCAATCTC</td>
</tr>
<tr>
<td>numb (NM_001040406.1)</td>
<td>CGCTCCATCACCACAAACC</td>
<td>GACGAGTCGTTCCCTGTATGG</td>
</tr>
<tr>
<td>hey2 (NM_131622.2)</td>
<td>AGTAAACCATAACCGACCCGTG</td>
<td>GGTACATCCTTACAGGGGTTG</td>
</tr>
</tbody>
</table>

Table 1. Primers used to amplify predicted miR-216a targets

miRNA recognition elements (MREs) were deleted from the snx5 3'UTR with PCR. For MRE 1, forward (5’-TGCAGACACACAAAGTACCACATAG-3’) and reverse (5’-GCTAATATTTTCAATCGTGAATATG-3’) primers and for MRE 2, forward (5’-GTCCGAATGCATTCTGACATTACGAT-3’) and reverse (5’-TATTAGGGAAAGATATCTGGAACATGACAT-3’) primers were designed to exclude each MRE. snx5 mRNA was amplified by RT-PCR using forward (5’-GCCGAGGGATCCTGAGGAAACGACTTGCTGCTGGAA-3’) and reverse (5’-GCCGAGCTCGAGCAACTGGGGACATCAGTCTGCTT-3’) primers and cloned into pCS2+ (Rupp et al., 1994). snx5 mRNA without its 3'UTR was amplified by RT-PCR using forward (5’-GCCGAGGGATCCTGAGGAAACGACTTGCTGCTGGAA-3’) and reverse (5’-GCCGAGCTCGAGGTCATCATCGTGATGGGTC-3’) primers and cloned into
pCS2+. All clones and MRE deletions were verified by Sanger sequencing in the Vanderbilt DNA Sequencing Core.

**Microinjection**

All injections were performed in fertilized 1-cell zebrafish embryos. Phenol red dye (0.05%) was used in each injection solution and alone as an injection control. Capped *snx5* RNA (from the pCS2+ vector containing the *snx5* mRNA without 3'UTR) or GFP-*snx5* 3'UTR RNA (from the pCS2+ vector containing the coding sequence of GFP and either the full length *snx5* 3'UTR or the *snx5* 3'UTR with both MREs deleted) were prepared using an Sp6 mMessage Machine Kit (Ambion). *snx5* RNA was injected at 100 pg/embryo concentration for functional experiments and 50 pg/embryo for rescue experiments. GFP RNA was injected at 25 pg/embryo concentration. Synthetic *miR-216a* duplexes (Dharmacon) were injected at 50 pg/embryo concentration in functional experiments and 25 pg/embryo in GFP reporter experiments. Two different morpholinos against *miR-216a* (one against the mature *miR-216a*: 5’-TCACAGTTCCCAGCTGAGATTA-3’ and a second against the loop of pre-*miR-216a*: 5’-GCAGCGCCTGTGAGAGGGATGAAAA-3’), a morpholino against the *snx5* start site: 5’-ACGTCATGTTAGAGGAGATATTTCGC-3’ (Eckfeldt et al., 2005), an exon 4 splice donor morpholino: 5’-CAGAGTTAGACTCACGCCTCAAGTT-3’ (Yoo et al., 2006), and a *p53* morpholino (5’-GCGCCATTGCTTTGCAAGAATTG-3’) were from Gene Tools. Two different *miR-216a* morpholinos were injected together at 150 pg each/embryo for functional experiments and a morpholino targeting just
the mature form of miR-216a was used at 100 pg/embryo for GFP reporter experiments. sno5 morpholinos were injected together at 100 pg each/embryo for all experiments. The p53 morpholino was injected at 150 pg/embryo. All injection amounts were experimentally determined to be the lowest effective dose.

**In situ hybridization**

Staged albino zebrafish embryos were fixed in 4% paraformaldehyde (PFA) in 1X PBS (pH 7.4) at 4°C overnight on a 3D rocker. Whole-mount mRNA in situ hybridization was performed as described (Thisse and Thisse, 2008) using a digoxigenin (DIG)-labeled sno5 RNA probe generated with Roche Applied Science reagents and pCS2+ vector containing the full length sno5 mRNA sequence. Whole-mount miRNA in situ hybridization was performed as described (Lagendijk et al., 2012) using a miRCURY 5’- and 3’-DIG labeled hsa-miR-216a LNA probe (Exiqon).

**Immunoblotting**

Embryos were deyolked at 1 dpf (day post fertilization) and placed in lysis buffer [25 mM HEPES (pH 7.5), 5 mM MgCl₂, 300 mM NaCl, 1 mM EDTA, 0.2 mM EGTA, 1 mM DTT, 10% glycerol, 1.0% Triton X-100, 1 mM PMSF] for protein extraction. Total proteins were separated on 10% SDS-PAGE gels and transferred to PVDF-plus membranes (GE Osmonics). Membranes were incubated with rabbit polyclonal antibodies against SNX5 (1:2000, Aviva Systems
Biology) and a-tubulin (1:500, Abcam). Anti-rabbit HRP-conjugated secondary antibodies (1:5000, GE Healthcare) were used for visualization with ECL reagents (Perkin Elmer). Using ImageJ, SNX5 levels were normalized to a-tubulin control levels, after which the ratio of SNX5 under varying injection conditions was determined. One-way ANOVA using Bonferroni's correction to adjust for multiple comparisons was performed using StatPlus (AnalystSoft).

**Staining and Imaging**

Live embryos either *Tg(flk1:GFP)* at 3-4 dpf or those injected with GFP reporter transcripts were briefly anesthetized with 0.02% tricaine for imaging on a Zeiss Discovery V8 stereo microscope and photographed using an Axiocam MRM black and white camera and Axiovision software (Zeiss). Live embryos that were staged and fixed in 4% PFA in 1X PBS (pH 7.4) at room temperature for 2-3 hours or embryos upon which *in situ* hybridization had been performed were embedded in 1.5% agarose/5% sucrose in egg water. The resulting blocks were cryoprotected in 30% sucrose overnight, frozen, and sectioned on a Leica CM1850 cryostat (10-15µm sections). The resulting transverse sections were mounted on VistaVision Histobond slides (VWR). *Tg(her4:dRFP)* sections were stained with Alexa Fluor 488-conjugated phalloidin (1:100, Molecular Probes) and Hoescht (1:3000, Molecular Probes), and *Tg(gfap:GFP)* sections were stained with the mouse monoclonal antibody zpr-1 (1:1000, Zebrafish International Research Center), HuC/D (1:1000, Invitrogen), and TOPRO-3 (1:1000, Molecular Probes). TUNEL labeling was performed
using an in situ Cell Death Detection Kit, TMR red (Roche). Fluorescent sample slides were mounted with Vectashield (Vector Laboratories) and in situ sample slides were mounted in 100% glycerol. In situ and Tg(her4:dRFP) samples were imaged on a Leica DM6000B microscope or Leica LSM 510 confocal (inverted) microscope with a 40x objective. Tg(gfap:GFP) samples were imaged on a Leica LSM 510 confocal (inverted) microscope with a 20x or 40x objective in the Vanderbilt Cell Imaging Shared Resource. Images were processed using ImageJ and Adobe Photoshop, and one-way ANOVA was calculated as described for immunoblotting.

RESULTS

miRNA expression analysis in developing eyes

In order to examine the role of miRNAs during vertebrate eye development, we dissected developing eyes at 2 and 5 dpf zebrafish and isolated RNA for miRNA expression profiling. We detected 12 miRNAs expressed at levels above background at 2 dpf and 23 miRNAs detected at 5 dpf (Table 2). From in situ localization experiments, only three of these miRNAs (miR-9, miR-124, and miR-216a) are expressed specifically in the developing eye at these times, the remainder are expressed ubiquitously (Ason et al., 2006; Kapsimali et al., 2007; Wienholds, 2005; Wienholds and Plasterk, 2005). Because miR-9 and miR-124 have been extensively studied during neural development (Gao, 2010), we decided to focus on the role of miR-216a in zebrafish eye development.
Table 2. miRNA expression profiling in developing zebrafish eyes.
Microarrays containing probes for 346 zebrafish miRNAs were performed on tissue from developing retinas at 2 and 5 days post fertilization (dpf) zebrafish. Fold differences were calculated by dividing the normalized expression values by negative control signals derived from probes against a *Pseudomonas aeruginosa* dehydrogenase. All p-values were calculated based on paired t-tests.

<table>
<thead>
<tr>
<th>miRNA</th>
<th>2 dpf</th>
<th>Fold Difference</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-9</td>
<td>4.1791</td>
<td></td>
<td>0.0002</td>
</tr>
<tr>
<td>miR-17-5p</td>
<td>7.7904</td>
<td></td>
<td>0.0002</td>
</tr>
<tr>
<td>miR-19a</td>
<td>3.6866</td>
<td></td>
<td>0.0069</td>
</tr>
<tr>
<td>miR-20</td>
<td>4.6253</td>
<td></td>
<td>0.0018</td>
</tr>
<tr>
<td>miR-25</td>
<td>2.6127</td>
<td>&lt; 0.0001</td>
<td></td>
</tr>
<tr>
<td>miR-31</td>
<td>3.3801</td>
<td></td>
<td>0.0008</td>
</tr>
<tr>
<td>miR-93</td>
<td>3.7530</td>
<td></td>
<td>0.0002</td>
</tr>
<tr>
<td>miR-108</td>
<td>4.1121</td>
<td></td>
<td>0.0033</td>
</tr>
<tr>
<td>miR-124a</td>
<td>7.0932</td>
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<td></td>
</tr>
<tr>
<td>miR-152</td>
<td>4.9246</td>
<td></td>
<td>0.0017</td>
</tr>
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<td>miR-210</td>
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<td>miR-18</td>
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<td>6.6342</td>
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<tr>
<td>miR-19b</td>
<td>3.9508</td>
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<td>miR-213</td>
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<tr>
<td>miR-217</td>
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</table>
Expression of *miR-216a* in developing eyes is temporally and spatially specific

To determine the expression of *miR-216a* over the course of eye development, we performed whole mount LNA *in situ* hybridizations for *miR-216a* on zebrafish embryos, which were then sectioned and visualized (Fig. 2.1 and 2.2). *miR-216a* is robustly and widely expressed throughout the eye cup at 22 hpf and 24 hpf (Fig. 2.1), but its localization changes as development proceeds (Fig. 2.2A-C).

**Figure 2.1.** *miR-216a* and *snx5* are widely expressed in the developing eye at 22 and 24 hpf

In situ hybridizations were performed for *miR-216a* (A,C) and *snx5* (B,D) at 22 hpf (A,B) and 24 hpf (C,D). Embryos were sectioned and sections were imaged on an epifluorescence scope. At 22 hpf, both *miR-216a* and *snx5* are expressed throughout the developing eye. At 24 hpf, these expression patterns become slightly more restricted; however, expression of both *miR-216a* and *snx5* can still be found throughout the developing eye.
From 26 to 48 hpf, *miR-216a* expression shifts from the central retina to an increasingly restricted marginal region that will become the Circumferential Germinal Zone (CGZ) or Ciliary Marginal Zone (CMZ) (Hitchcock and Raymond, 2004). Given the role that miRNAs play in regulating the expression of target mRNAs, we conclude that the temporal and spatial specificity of the expression of *miR-216a* suggest that it plays a role in patterning the developing retina.

**Figure 2.2. miR-216a and snx5 have complementary expression patterns during development.**
Transverse sections of whole mount *in situ* hybridizations for *miR-216a* and *snx5* at 26 (A,D), 36 (B,E), and 48 (C,F) hours post fertilization (hpf). *miR-216a* expression spreads from the center of the developing retina toward the periphery. *snx5* is detected in a complementary pattern becoming increasingly restricted over time to a small number of cells at the far periphery of the developing retina. Arrowheads indicate the extent of signal, the red dashed line indicates the lateral edge of the optic cup. Scale bar: 20µm.
**miR-216a targets snx5**

MicroCosm and TargetScan online target prediction algorithms (Griffiths-Jones et al., 2008; Lewis et al., 2005) were used to identify potential targets of miR-216a. Concurrently, we conducted a series of miR-216a gain- and loss-of-function experiments in developing zebrafish embryos. We observed vascular defects upon altered expression of miR-216a that were remarkably similar to previous reports demonstrating an involvement of Notch signaling and a requirement for SNX5 in vascular development (Fig. 2.3 and 2.4) (Lawson et al., 2001; Yoo et al., 2006). Thus, we focused our target search on Notch pathway related genes and SNX5. Several Notch related genes contain one predicted miRNA recognition element (MRE) for miR-216a in their 3' UTRs, including her4.2, heyl, notch1b, hey2, and numb1. In contrast, snx5 contains two MREs in its 3' UTR (Fig. 2.2A). Based on the involvement of Notch signaling in retinogenesis (Bernardos et al., 2005; Scheer et al., 2001), we assessed whether these predicted targets of miR-216a were true targets using GFP reporter assays.

The full-length 3' UTR of each of these predicted targets was fused to the coding sequence of GFP. mRNA transcripts were then generated from these reporter constructs and injected into single cell zebrafish embryos in the presence or absence of co-injected, exogenous miR-216a. The effect of miR-216a was determined by measuring GFP fluorescence at 24 hpf. Fluorescence levels of the her4.2, heyl, notch1b, hey2, and numb 3'UTR reporters were comparable with or without co-injection of miR-216a, suggesting that these
Figure 2.3. Knockdown of snx5 and overexpression of miR-216a result in vascular patterning defects.

*Tg(flk1:eGFP)* embryos were injected at the 1-cell stage with dye control, snx5<sup>MOs</sup>, or miR-216a. Embryos were grown in egg water and vascular development was monitored. Intersegmental vessels were missing in the trunks of snx5<sup>MOs</sup>- and miR-216a-injected larvae at 4 dpf, but not from dye control-injected larvae. Left column contains representative images, and right column shows vessels outlined with dotted white lines in order to highlight the absence of some vessels.
Figure 2.4. Knockdown of **miR-216a** and overexpression of **snx5** result in vascular patterning defects.
*Tg(flk1:eGFP)* embryos were injected at the 1-cell stage with dye control, *miR-216a*MOs, or *snx5* mRNA. Embryos were grown in egg water and vascular development was carefully monitored. Branching defects in the intersegmental vessels were observed in the tails of 4 dpf larvae (indicated by white arrowheads) and imaged using a fluorescence stereoscope.

Genes are not targeted by *miR-216a* (Fig. 2.5). However, for *snx5*, we observed a robust decrease in GFP fluorescence upon co-injection with *miR-216a* (Fig. 2.6 B,C,E). Importantly, the effect of *miR-216a* could be partially suppressed by co-injection of a morpholino targeting the mature sequence of *miR-216a*, indicating specific suppression of *snx5* by *miR-216a* (Fig. 2.6 D,E). To further test for
specificity, we deleted each of the two predicted MREs in the snx5 3'UTR. No differences were observed in GFP fluorescence among fish injected with the mutated reporter transcripts compared to co-injection with miR-216a (Fig. 2.6F,G,I). As an additional test of specificity, co-injection of both miR-216a and miR-216a MO resulted in partial suppression of the silencing effect of miR-216a (Fig. 2.6H,I). These results indicate that miR-216a can regulate snx5 via two MREs located in its 3' UTR.

Figure 2.5. miR-216a does not target her4.2, notch1b, hey1, numb, or hey2. Embryos were injected at the 1-cell stage with GFP reporters containing the 3'UTRs of her4.2, notch1b, hey1, numb, or hey2 alone or in combination with miR-216a. Embryos were grown in egg water until 24 hpf, at which point they were photographed using a fluorescence dissecting scope. We did not detect changes in GFP fluorescence with co-injection of miR-216a.
To address whether endogenous snx5 is targeted by miR-216a, we isolated protein from 1 dpf embryos injected with either a dye control (DIC), miR-216a, or two morpholinos targeted to miR-216a, one complementary to the mature sequence of miR-216a and one targeted to the Dicer cleavage site of the miR-216a precursor (miR-216a MOs). We used two morpholinos because the combination allowed us to use a lower dose of each morpholino, which reduced the chances of off target effects. We then performed western blots using an antibody against SNX5 protein and α-tubulin as a control. Injection of miR-216a significantly decreased endogenous levels of SNX5, while injection of miR-216a MOs led to a significant increase in endogenous SNX5 (Fig. 2.2J,K). Taken together, these results indicate that miR-216a targets endogenous snx5 via two MREs in its 3’UTR.

**miR-216a spatially and temporally restricts expression of snx5 in the eye**

Because we observed specific spatial and temporal expression of miR-216a over the course of early eye development (Fig. 2.2A-C), we were interested to examine the expression of snx5 at corresponding time points. We thus performed *in situ* hybridization using snx5 riboprobes on whole mount zebrafish embryos, which were then sectioned and imaged (Fig. 2.2 D-F). Expression of miR-216a was largely complementary to that observed for snx5. As miR-216a expression moved toward the future CGZ at 36 and 48 hpf (Fig. 2.2 B,C), localization of snx5 became increasingly restricted (Fig. 2.2 E,F) until snx5 expression was virtually undetectable from all cells of the developing retina.
except for a limited number of cells at the very margins of the future CGZ. The complementary expression patterns of miR-216a and snx5 suggest that miR-216a restricts temporal and spatial expression of snx5 in the developing eye.

Figure 2.6. snx5 is a target of miR-216a.
The coding sequence of GFP was fused to the 3'UTR of snx5. (A) Predicted pairing between microRNA Recognition Elements (MREs) in the snx5 3' UTR (black) with miR-216a (red). (B) 1-cell stage embryos were injected with GFP-snx5 3' UTR reporter mRNAs alone, or co-injected with miR-216a (C), or the combination of miR-216a and miR-216aMO (D) and imaged at 1 dpf. (F-H) Silencing is MRE Dependent. 1 dpf embryos were injected with mRNAs lacking both MREs alone, or co-injected with miR-216a (G), or the combination of miR-216a and miR-216aMO (H). (E, I) Relative fluorescence levels from the representative embryos shown in B-H were quantified using ImageJ, and comparisons were made using one-way ANOVA with Bonferroni's correction. (J) Western blots for endogenous SNX5 and α-tubulin were performed on protein lysates from 1 dpf zebrafish injected with dye control (DIC), miR-216a, or miR-216aMOs. (K) Western signals were quantified using ImageJ, and comparisons were made using one-way ANOVA with Bonferroni’s correction. *, p<0.05; **, p<0.01; ***, p<0.001. Error bars show SEM.
Notch-Delta signaling and the \textit{miR-216a-snx5} interaction

Previous experiments have demonstrated interaction between SNX5 with MIB, co-localization with MIB and Delta (Yoo et al., 2006), and a role for MIB and Notch-Delta signaling in gliogenesis (Bernardos et al., 2005; Scheer et al., 2001). However, the exact effects of \textit{snx5} on Notch-Delta signaling have not been characterized nor has there been any previous work investigating the regulation of \textit{snx5} during early retina development. We therefore used a Notch reporter zebrafish line (\textit{Tg(her4:dRFP)}, which expresses dRFP under the control of the \textit{her4} Notch-responsive element (Takke et al., 1999; Yeo et al., 2007). We injected \textit{Tg(her4:dRFP)} single cell embryos with either dye control, synthetic \textit{miR-216a} duplexes, \textit{miR-216a}^{\text{MOs}}, \textit{snx5}^{\text{MOs}}, or \textit{snx5} mRNA, and then fixed the embryos at 30 hpf and sectioned to examine Notch activation in the developing retina. Strikingly, overexpression of \textit{miR-216a}, or knockdown of \textit{snx5}, resulted in a marked decrease in Notch activation compared to DICs, as reported by the loss of \textit{Tg(her4:dRFP)} fluorescent protein expression (Fig. 2.7 A,B,E).

Conversely, knockdown of \textit{miR-216a} or overexpression of \textit{snx5} mRNA resulted in expansion of the zone of \textit{Tg(her4:dRFP)} fluorescence and presumptive Notch activation compared to DICs (Fig. 2.7 C,F). Co-injection of \textit{snx5} lacking its 3'UTR with \textit{miR-216a} restored the zone of \textit{Tg(her4:dRFP)} activation (Fig. 2.7 D), as did co-injection of \textit{snx5}^{\text{MOs}} and \textit{miR-216a}^{\text{MOs}} (Fig. 2.7 G). These data indicate that \textit{snx5} is a positive regulator of Notch-Delta signaling and that \textit{miR-216a} negatively regulates Notch-Delta signaling via its interaction with \textit{snx5}. Consistent with this hypothesis, we used a second zebrafish Notch
reporter line \( (Tg(Tp1bglob:eGFP)) \) and observed repression of Notch activation by increasing amounts of \( miR-216a \) or knockdown of \( snx5 \) (Fig. 2.8) (Parsons et al., 2009).

**Figure 2.7. \( miR-216a \) and \( snx5 \) regulate Notch activation.**
Transverse sections of developing retinas from 30 hours post fertilization (hpf) \( Tg(her4:dRFP) \) embryos were injected with dye control (DIC; A), \( miR-216a \) (B), \( miR-216a^{MOs} \) (C), \( snx5^{MOs} \) (E), or \( snx5 \) mRNA (F). Reporter expression (white) indicates changes in the zone of Notch activation. Partial rescue of Notch activity is shown in (D) and (G) where embryos were co-injected with combinations of either \( snx5 \) and \( miR-216a \) (D) or \( snx5^{MOs} \) and \( miR-216a^{MOs} \) (G). Sections were stained with Alexa Fluor 488-conjugated phallolidin (green) to visualize cell boundaries. Scale bar: 20µm.
Figure 2.8. *Tg(Tp1:GFP)* reporter reveals changes in Notch signaling upon perturbation of *miR-216a* and *snx5*

Embryos (*Tg(Tp1:GFP)*)) were injected at the 1-cell stage with *miR-216a*, *miR-216a*<sup>MOs</sup>, *snx5*<sup>MOs</sup>, *snx5*, or dye control (DIC). Embryos were grown in egg water until 24 hpf, at which point they were photographed using a fluorescence dissecting scope. Top panel shows Tp1 reporter fluorescence in green superimposed onto images of fish; bottom panel shows Tp1 reporter fluorescence in white. We observed a decrease in reporter fluorescence in *miR-216a* and *snx5*<sup>MOs</sup> injected embryos, suggestive of lower Notch activation, and an increase in reporter fluorescence in *miR-216a*<sup>MOs</sup> and *snx5* injected embryos, suggestive of higher Notch activation, as compared to DIC.

Because it was formally possible that the effects we observed might be due to morpholino-induced apoptosis as opposed to regulation of *snx5* by *miR-216a*, we conducted TUNEL staining. Previous work has illustrated potential pitfalls with the use of morpholinos, including increased levels of apoptosis due to activation of p53 (Gerety and Wilkinson, 2011). To ensure that the effects we observed were specific to knockdown of *miR-216a* or *snx5*, we injected morpholinos in the presence and absence of p53 and found no change in the
levels of TdT-mediated incorporation of dUTP (Fig. 2.9). Combined with our suppression/rescue experiments, these results demonstrate that the effects of miR-216a and snx5 knockdown are specific and that the changes in Notch activation we observe are due to regulation of snx5 by miR-216a.

Figure 2.9. No differences in programmed cell death observed with morpholino injections. We injected morpholinos into one cell stage embryos in the presence and absence of p53 and found no change in the levels of TdT-mediated incorporation of dUTP (red) in sectioned eyes at 36-48 hpf, quantified on right.

Disruption of Müller glia

Notch signaling is required for gliogenesis (Bernardos et al., 2005; Scheer et al., 2001) and the prediction is that alteration of Notch signaling by miR-216a and snx5 should alter the number of Müller glia during retinal development. To assess the functional consequences of disrupting miR-216a and snx5
expression, we injected \textit{miR-216a, miR-216a}^{\text{MOs}}, \textit{snx5}^{\text{MOs}}, or \textit{snx5} mRNA into single cell \textit{Tg(gfap:GFP)} zebrafish embryos and examined fluorescence levels during early development. These animals express GFP under the control of the glial-specific GFAP promoter (Bernardos and Raymond, 2006). We initially examined retinas from embryos at 30 hpf to coincide with the \textit{her4} reporter experiments. Fluorescence was detectable at this time but the levels were not robust, consistent with the timing of Müller glia specification (Easter and Malicki, 2002). Since it has been reported that Müller glia are specified by 65 hpf (Bernardos et al., 2005) and because we observed Notch activation in Müller glia at 65 hours using the \textit{her4} reporter fish (Fig. 2.10), we counted GFP+ cells at this time. Upon overexpression of \textit{miR-216a}, a significant decrease in GFP+ cells was observed compared to DICs (Fig. 2.11). In contrast, knocking down \textit{miR-216a} with morpholinos resulted in an increase in GFP+ numbers (Fig. 2.11).

\textbf{Figure 2.10. Notch is activated in Müller glia at 65 hpf.}
In a cross section of \textit{Tg(her4:dRFP)} fish at 65 hpf, Notch activation (in red) was detected primarily in Müller glia. Cell membranes are labeled with phalloidin, here visualized in green.
Correspondingly, knockdown of snx5 resulted in significantly decreased numbers of GFP+ cells whereas overexpression of snx5 led to an increase in GFP+ cells (Fig. 2.11). These results are consistent with regulation of snx5 by miR-216a. To further test this hypothesis, we conducted co-injection rescue/suppression experiments. The prediction is that the decreased numbers of GFP+ cells caused by knockdown of snx5 should be suppressed by co-injection of miR-216a\(^{MOS}\). Similarly, the effects of overexpression of miR-216a should be suppressed by co-injection of snx5. In both cases, we observed rescue of GFP+ cell numbers indicating that Müller glia numbers were restored (Fig. 2.11). Taken together, these data are consistent with the hypothesis that miR-216a modulates gliogenesis via its interaction with snx5. Interestingly, injection of snx5\(^{MOS}\) did not result in as dramatic a decrease in Müller glia as miR-216a overexpression alone. This finding suggests a possible role for miR-216a in the regulation of Müller glia number beyond its interaction with snx5.

**Effects of Müller glia specification on cone photoreceptor differentiation**

A prediction of the effects of altered gliogenesis is that other retinal neuronal cell types would be altered after either loss or gain of Müller glia. For these experiments we used *Tg(gfap:GFP)* embryos fixed at 65 hpf and stained transverse retinal sections using antibodies that mark cone photoreceptors (Zpr-1). As shown in Fig. 2.12, alteration in Müller glia number was accompanied by complementary changes in the extent of Zpr-1 staining in the outer nuclear layer. Overexpression of snx5 or knockdown of miR-216a led to increased Müller glia...
and decreased Zpr-1 staining while overexpression of miR-216a or knockdown of snx5 led to decreased Müller glia and increased Zpr-1 staining. These results are consistent with the model that altered gliogenesis can in turn affect neuronal differentiation.

Figure 2.11. miR-216a and snx5 regulate Müller glia cell numbers. Tg(gfap:GFP) transgenic zebrafish were injected as indicated, grown to 65 hpf, and GFP+ cell numbers were counted. Compared to DICs, injection of miR-216a MOs or snx5 caused a significant increase in GFP+ cells (p<0.05). Injections with miR-216a or snx5 MOs caused a significant decrease in GFP+ cells (p<0.05). Partial rescue of GFP+ cell counts was observed in embryos co-injected with combinations of either snx5 and miR-216a, or snx5 MOs and miR-216a MOs. Error bars=SEM.
Figure 2.12. Inverse correlation between Müller glia numbers and cone photoreceptor staining.

*Tg(gfap:gfp)* embryos were injected with dye control (DIC; A), *miR-216a* (B), *miR-216aMOs* (C), *snx5MOs* (E), or *snx5* mRNA (F) at the 1-cell stage, fixed at 65 hpf, and transverse sections of developing retinas were obtained. Immunohistochemistry was performed using antibodies to identify cone photoreceptors in the outer nuclear layer (Zpr-1) or amacrine/ganglion cells in the inner nuclear layer and the ganglion cell layer (HuC). Changes in Müller glia cell numbers led to consistent changes in cone photoreceptor numbers. Zpr-1 staining increased in embryos injected with *miR-216a* or *snx5MOs* and decreased in embryos injected with *miR-216aMOs* or *snx5* compared to embryos injected with dye. Partial rescue of Zpr-1 levels is shown in (D) and (G) where embryos were co-injected with combinations of either *snx5* and *miR-216a* (D) or *snx5MOs* and *miR-216aMOs* (G). Amacrine and ganglion cell numbers demonstrated similar, though less striking and less consistent changes compared to cone photoreceptors. Nuclei were marked by staining with To-Pro.
DISCUSSION

We used expression profiling experiments to identify several candidate miRNA regulators of zebrafish eye development. As demonstrated by \textit{snx5} and \textit{miR-216a} expression, GFP reporter assays, and SNX5 immunoblotting, we show that \textit{miR-216a} regulates \textit{snx5}. Based on the expression of \textit{miR-216a} and \textit{snx5} in the retinal neuroepithelium, it appears that \textit{miR-216a} plays a role in both spatial and temporal control of \textit{snx5} expression, and, in turn, Notch signaling.

\textit{miR-216a} regulates Notch signaling via \textit{snx5}

SNX5 binds Mib and knocking down SNX5 leads to vascular defects (Eckfeldt et al., 2005; Yoo et al., 2006). The role of Notch signaling in vascular development is also well-established (Lawson et al., 2001). In addition to changes in fluorescent protein expression in \textit{Tg(her4:dRFP)} fish, we also observed defects in vascular patterning upon knockdown and overexpression of \textit{miR-216a} and \textit{snx5} (Fig. 2.3 and 2.4). This suggests that \textit{miR-216a} and \textit{snx5} also play a role in Notch signaling in zebrafish vascular development. We also show that perturbing expression of \textit{miR-216a} and \textit{snx5} causes changes in Notch activation, as reported by altered zones of fluorescent protein expression in the retinas of \textit{Tg(her4:dRFP)} embryos.

Based on prior work about SNX5 and Mib and our experiments, we propose a model where \textit{miR-216a} regulates Notch-Delta signaling via regulation of \textit{snx5} (Fig. 2.13). We hypothesize that SNX5 (bound to Mib) moves to the site of Delta activation where it binds to the membrane as Mib ubiquitylates Delta.
SNX5 then facilitates membrane curvature through its BAR domain with subsequent Delta endocytosis, which is required for Notch activation and neuronal development (Louvi and Artavanis-Tsakonas, 2006; Parks et al., 2000).

**Figure 2.13. Model for the role of SNX5 and miR-216a in Notch signaling**

SNX5 (bound to Mib) moves to the site of Delta activation, where it binds to the membrane as Mib ubiquitylates Delta. SNX5 then facilitates membrane curvature and Delta endocytosis, which is required for cleavage of the Notch extracellular domain (NECD). Cleavage of the NECD frees the Notch intracellular domain (NICD), which is translocated to the nucleus to co-activate downstream target genes with the CSL transcription factor.

While our experiments show a role for snx5/miR-216a in controlling Notch activity during retinal development, it is likely that overall control of Notch involves multiple factors and control points during cell fate specification and development. Focusing just on the retina, we show that early changes in Notch signaling manifest themselves at later time points by altering neuronal cell fate. However, several other Notch components, including Delta, are likely to be subject to additional temporal regulation as the wave of differentiation spreads...
from the central retinal to the periphery. Despite the fact that our morpholino knockdown experiments of miR-216a allow sufficient Notch activity to affect changes in cell fate, our experiments cannot preclude the role of additional Notch components and/or regulators during the dynamic processes occurring during retina development. This likely includes other miRNAs that might regulate other components of the Notch pathway.

**miR-216a and snx5 modulate Müller glia cell numbers**

The changes in Notch signaling in response to perturbation of snx5 and miR-216a expression that we observed are striking and consistent with previous experiments. Scheer et al. (2001) showed that expressing a constitutively active version of Notch1a resulted in a disruption of neurogenesis and an increase in gliogenesis (Scheer et al., 2001). Additionally, differentiation of Müller glia does not occur in mib mutant fish (Bernardos et al., 2005). These results suggest that Notch signaling is instructive for gliogenesis in the zebrafish retina. We observed that high Notch activation at 30 hpf, as reported by fluorescent protein expression in the Tg(her4:dRFP) zebrafish and induced by either miR-216a knockdown or snx5 overexpression, caused increased numbers of Müller glia at 65 dpf, as reported by Tg(gfap:GFP) fluorescence. Because high Notch signaling at 30 hpf, in the case of miR-216a knockdown or snx5 overexpression, translates to increased numbers of Müller glia, we hypothesize that the snx5-miR-216a interaction may directly impact Notch signaling, and therefore gliogenesis, in the developing retina. Of note, we observed Notch activation in Müller glia at 65hpf
(detected by *Tg(her4:dRFP; Fig. 4).

It has been suggested that SNX5 is localized to a distinct domain of the early endosome, a cellular location where it could be playing multiple, as yet unknown, roles in cellular trafficking (Yoo et al., 2006). Furthermore, *miR-216a and *snx5 are each expressed throughout the developing optic cup and retinal neuroepithelium in early development. By knocking down or overexpressing both *miR-216a and *snx5 globally at early stages, we have likely disrupted functions that manifest themselves later in development leading to a disruption in Notch activation and correspondingly, specification of Müller glia. It has been shown that the interaction of different Delta ligands with Notch can result in different outcomes for Delta activation in neural tissue (Matsuda and Chitnis, 2009).

We also found that altered gliogenesis impacts neuronal differentiation. We show that MG numbers show an inverse correlation with the staining of a marker of cone photoreceptor differentiation. This suggests that overall specification of cell types in the developing retina are coordinately regulated.

**miRNAs regulate developmental signaling**

We have previously shown that miRNAs play regulatory roles in Hedgehog signaling (Flynt et al., 2007), the development of endoderm and left-right asymmetry (Li et al., 2011), and synaptogenesis (Wei et al., 2013). miRNA regulation of Notch signaling is important during *Drosophila* follicle development (Poulton et al., 2011) and bone development in mice (Bae et al., 2012). Additionally, Notch signaling has been shown to regulate the expression of *miR-*
9, a miRNA that we detected in our eye-field microarray and is involved in multiple aspects of neural development (Coolen et al., 2012). The finding that miR-216a regulates snx5 adds to the mounting evidence for the importance of miRNAs in regulating developmental processes in vertebrates.
CHAPTER III
Discussion and Future Directions

The finding that \textit{miR-216a} regulates \textit{snx5} is not only informative based on its expansion of our understanding of zebrafish retinal development, but also has implications for increasing our understanding of Notch signaling, of the function of miRNAs, and of the possible roles for sorting nexins during vertebrate development.

\textbf{miRNA REGULATION OF NOTCH SIGNALING}

In 2004, Bartel and Chen proposed the analogy of miRNA function as a rheostat that modulates the production of proteins in a cell (Bartel and Chen, 2004). Whether it was intended or not, this analogy carried within it the idea that miRNAs do not have the ability to flip developmental switches, and that they function to merely “fine-tune” the expression of proteins in a cell. Evidence that has emerged since then has dispelled the impression that miRNAs do not play large roles in development, and one of the ways miRNAs can have the greatest impact is through the regulation of developmental signaling pathways. Flynt \textit{et al.} demonstrated that \textit{miR-214} can effectively regulate hedgehog signaling through the repression of Sufu, resulting in the loss of an entire class of muscle pioneers and effectively flipping a developmental switch (Flynt \textit{et al.}, 2007).

Previous work has also demonstrated myriad roles for miRNAs in regulation of Notch signaling during metazoan development, with diverse effects
on developmental events from differentiation in the developing heart to osteogenesis. In 2005, Lai et al. published evidence for extensive regulation of Notch signaling pathway genes by miRNAs in *Drosophila* (Lai et al., 2005). More specifically, *miR-1* has been shown to target the Delta ligand in *Drosophila*, and thus influences cardiac development (Kwon et al., 2005). During neuronal development of the ascidian, *Ciona intestinalis*, *miR-124* is both regulated by Notch signaling to promote epidermal fates and regulates the expression of Notch, Neuralized (a ubiquitin ligase that targets Delta), and several downstream effectors of the pathway (Chen et al., 2011). In *Xenopus laevis* embryos, *miR-449* regulates Delta in the epidermis to regulate the formation of cilia (Marcet et al., 2011). In mice, *miR-34c* has been shown to regulate Notch1 and Notch2 receptors and Jag1 ligand during bone development (Bae et al., 2012). In each of these cases, modulation of Notch signaling by miRNAs has far-reaching developmental effects, beyond the modulation of target protein levels.

Here, I have presented evidence for regulation of Notch signaling by *miR-216a* in the developing vertebrate eye, which results in changes in the numbers of Müller glia specified in the eye. Zebrafish injected with *miR-216a* or *snx5* morpholinos have fewer glial cells at 65 hpf, which is consistent with the role of Notch in gliogenesis, as discussed in Chapter I. To better understand the requirement of Notch signaling for gliogenesis and its relationship to both *miR-216a* and *snx5*, in the future it will be necessary to thoroughly examine the timeline of glial cell development and, as tools become available, restrict or
enhance Notch signaling, \textit{snx5}, and \textit{miR-216a} expression during different windows of time during retinogenesis.

The evidence generated using the \textit{Tg(her4:dRFP)} reporter fish and demonstrating changes in gliogenesis suggests that Notch signaling is perturbed by the gain and loss of function of both \textit{miR-216a} and \textit{snx5}. Though a tentative link between \textit{snx5} and Notch signaling was made by Yoo \textit{et al.} in 2006 when they demonstrated that SNX5 binds Mib (Yoo \textit{et al.}, 2006), this work is the first to use Notch reporters to examine the relationship between \textit{snx5} and Notch. This finding could be expanded upon using transgenic zebrafish expressing NICD under the control of a heat shock promoter (Scheer \textit{et al.}, 2001) or dominant negative Delta to suppress \textit{snx5} knock down and \textit{miR-216a} overexpression (Appel and Eisen, 1998). Because the deficits in glial cells present when knocking down \textit{snx5} or overexpressing \textit{miR-216a} are presumably due to a decrease in Notch activation, NICD overexpression or injection of mRNA encoding a dominant negative Delta isoform would give more insight into Notch’s role in the observed phenotypes. In these ways, we can hope gain a greater understanding of glial cell development and also of how miRNAs can create massive developmental effects by tweaking the levels of proteins that participate in cell-cell signaling, which will be applicable beyond just the relationship between \textit{miR-216a} and \textit{snx5}.

Notch signaling has been shown to play a role in arterial-venous differentiation in zebrafish (Lawson \textit{et al.}, 2001). Mib mutant embryos do not express artery-specific genes in the developing vasculature, and show ectopic
expression of venous markers (Lawson et al., 2001). Also, the vessels between the somites in the trunk of the fish (intersegmental vessels) show patterning defects in Mib mutants (Lawson et al., 2001). Others have reported expression of snx5 in hematopoietic precursor cells and defects in intersegmental vessel patterning after snx5 knockdown (Eckfeldt et al., 2005; Yoo et al., 2006), and I observed patterning defects in the vasculature of zebrafish larvae injected with snx5 morpholinos.

Interestingly, vascular defects were also apparent when both knocking down and overexpressing miR-216a, as well as in embryos in which I injected snx5 mRNA. It is not clear why this phenotype is observed since expression of miR-216a is not readily detectable in the developing vasculature. Because miR-216a is strongly expressed in the somites, it could potentially play an inhibitory role for snx5 or perhaps a different vascular target. While more work is needed to determine the relationship between miR-216a, snx5, and Notch signaling in the vasculature of the zebrafish, it is already known that miR-221 is required for angiogenesis in zebrafish (Nicoli et al., 2012). miR-221 regulates two mRNA targets, and its expression is inhibited by Notch signaling (Nicoli et al., 2012). Examination of arterial and venous specification and blood flow, and characterization of miR-216a expression in hematopoietic precursors and vascular tissues could reveal a novel mode of control of Notch signaling in the developing blood vessels.

This examination could also generate insight about the relationships between miRNAs and their targets in signaling pathways. For instance, the
relationship between miR-216a and snx5 presents a unique opportunity to understand how miRNA regulation of a target and signaling pathway works in different tissue types. The regulation of snx5 by miR-216a may function similarly in the vasculature as it does in the retina, but that is currently unknown. The differences in the environmental context of cells sending a receiving Notch-Delta signals in the developing retina and vasculature, as well as in the expression of miR-216a present interesting questions for future study. Examination of the role of miR-216a in the developing vasculature and comparisons to its role in the retina as examined here could reveal similarities and differences in general miRNA function in these two tissues and in how Notch signaling works to bias cells toward certain fates in distinctly different developmental environments.

**miRNA REGULATION OF RETINAL DEVELOPMENT**

In relatively few cases, clear roles for miRNAs expressed specifically in the retina have been well defined, but the identification and verification of miRNA-target pairs and their functions during retinal development remains painstakingly slow and rare. miR-24a was shown to regulate apoptosis in the developing *Xenopus* retina by targeting two proapoptotic genes, *caspase9* and *apaf1* (Walker and Harland, 2009). More evidence from *Xenopus* demonstrated the importance of a group of miRNAs that targeted two genes needed for bipolar cell differentiation in progenitor cells (Decembrini et al., 2009). Work from the Reh lab has demonstrated the requirement for miRNAs as a group during retinal development using Dicer knockout mice (Georgi and Reh, 2010; La Torre et al.,
2013). However when Jin et al. generated mouse knockouts of miR-182, which is expressed at high levels in the murine retina and also was detected in the microarrays performed as part of this work, they saw no retinal phenotype (Jin et al., 2009).

The identification of the miR-216a-snx5 interaction is novel and joins the ranks of confirmed miRNA-target interactions during eye development. The interaction should be explored during retinal regeneration, a process that happens easily in zebrafish, which is unusual in vertebrates, and recapitulates many developmental processes (Hitchcock and Raymond, 2004). It also adds to the growing list of confirmed miRNA-target interactions and could inform miRNA-target discovery going forward. By looking for miRNAs that function during eye development, I confirmed a role for a novel player in Notch signaling. Though it is not a traditional way to achieve greater understanding of signaling pathways, in the future, examination of tissue-specifics miRNA function could lead similar insights.

The miR-216a-snx5 interaction is convincing, based not only on reporter assays, but also on the complementary expression patterns in the developing retina. In the future, double in situ hybridization, in which both miR-216 and snx5 are detected in the same section of tissue could be helpful in confirming the finding. Furthermore, as new tools are now available (TALENs, CRISPRs, convergent transcription) to tissue specifically regulate expression of both miR-216a and snx5, exploring the phenotypes will likely give a more precise
understanding of how both the miRNA, the mRNA, and their interactions influence retinogenesis.

**A ROLE FOR SORTING NEXINS**

Sorting nexins are a large family of proteins defined by the presence of a phox-homology domain (PX), which is thought to bind membrane phosphoinositides (Cullen, 2008). 33 mammalian sorting nexins have been identified (Cullen, 2008), and the majority of these have homologues in other species. In zebrafish, homologues for each of the 33 mammalian sorting nexins have been identified (Flicek et al., 2014). Additionally, some of the zebrafish sorting nexins have both an a and b isoform, as in the case of snx10a and snx10b, which function in different ways during zebrafish development (Chen et al., 2012).

Most SNX proteins are thought to bind phosphatidylinositol 3-monophosphate (PI3P), a lipid found on the cytosolic face of endosomes (Cullen and Korswagen, 2011). Structural evidence has demonstrated that the PX domain of SNX5 instead preferentially binds phosphatidylinositol 4,5-bisphosphate (PIP2), which is the most abundant phosphoinositide in plasma membranes (Koharudin et al., 2009). In addition to the PX domain, SNX5 also contains a BAR (Bin/amphiphysin/Rvs) domain, which is also thought to aid in binding to curved membranes (Cullen, 2008). The affinity of SNX5 for PIP2 and the presence of the BAR domain suggests that the protein is likely to be localized at the membrane, which supports the idea that SNX5 is found there with Mib, as
has been previously demonstrated in cell culture (Yoo et al., 2006) and as I suggested in the proposed model in Chapter II (Figure 2.5).

Sorting nexins have been shown to be involved in multiple biological signaling events. In *Drosophila*, *Caenorhabditis elegans*, and human cell lines, SNX3 has been shown to be required for recycling of the Wnt-binding protein Wntless (Wls) (Harterink et al., 2011). In the absence of SNX3, Wls is not properly trafficked, resulting in defects in Wnt secretion (Harterink et al., 2011). SNX27 is required for β2 adrenergic receptor recycling and subsequent signaling (Temkin et al., 2011), and SNX17 is necessary for efficient sorting and recycling of integrins (Steinberg et al., 2012).

Especially relevant to the current work is the identified role of SNX17 in Notch signaling (Yin et al., 2012). Yin et al. showed in zebrafish that SNX17 binds Notch ligand Jagged 1a (Jag1a) and regulates Notch signaling by modulating ligand recycling and its presence on the plasma membrane (Yin et al., 2012). When the authors used a morpholino to knockdown SNX17 in zebrafish, they saw a reduction in exocrine pancreas cells, a defect that is also observed in Mind bomb (Mib) mutant fish (Yin et al., 2012). Interestingly, *miR-216a* is also expressed in the pancreas (Wienholds, 2005), and while it is not predicted to target *snx17* using available algorithms, potential miRNA interaction with any sorting nexin mRNA is worth exploring based on prospective regulatory implications.

Though my model proposes that SNX5 is necessary for endocytosis of Delta, Yin et al. did not find that SNX17 was necessary for the endocytosis of
Jag1a (Yin et al., 2012). It is feasible that, instead of being required for Delta endocytosis, SNX5 regulates the recycling of Delta during zebrafish development. It is known that SNX5 protein colocalizes with Mib and Delta in the early endosome HEK293 cells (Yoo et al., 2006), which is consistent with a role for SNX5 in Delta recycling.

In order to test whether SNX5 is necessary for Delta endocytosis, Delta recycling, or both, future experiments could include imaging Delta and SNX5 proteins in fixed tissues, which gave inconsistent results when attempted as part of the current work. Live imaging of Delta endocytosis, as developed by Maximilian Fürthauer (personal communication), would provide insight into both the role of SNX5 in Notch signaling and into the mechanism by which the protein is involved.

The role of SNX5 in Notch signaling revealed by this work also illustrates how little we still know about developmental signaling pathways. While the basic players in Notch signaling have been known for many years, discovery continually broadens the complexity of our understanding. In 2003, the role of Mib as a ubiquitinating agent was revealed (Itoh et al., 2003), and here a trafficking role for SNX5 has been proposed, with possibilities that the protein could play a role in both endocytosis and recycling of the Delta ligand. Further exploration of SNX5’s function could reveal information about how Notch signaling functions to generate specific cells and tissues during development and what happens when Notch signaling is perturbed.
As knowledge of sorting nexins has increased, it has become obvious that they play a role in myriad cellular trafficking events. By learning more about the ways this family of proteins influences trafficking, through exploration of the function of specific family members, we also stand to gain greater understanding of disease states in which sorting is perturbed. SNX9, for instance, is a target during *Escherichia coli* infection, which can lead to gastrointestinal disease (Alto et al., 2007). Exploring the role of SNX5 could have an impact on understanding endosomal sorting diseases in the future.

**CONCLUSIONS**

Zebrafish retinas develop in the same way that most vertebrate retinas, including human retinas, do. The conservation of developmental signaling pathways, as well as the conservation of miRNA sequences, including *miR*-216a, from teleost to human makes the zebrafish an effective model system in which to study retinal development, and particularly to explore the interaction between *miR*-216a and *snx5*.

*snx5* is a novel target of *miR*-216a. The miRNA and mRNA have spatially complementary, temporally specific expression patterns during early retinal development. Reporter assays and western blots of endogenous SNX5 suggest that the mRNA is a bonafide target of *miR*-216a. Overexpression of *miR*-216a or knockdown of *snx5* using morpholinos results in a decrease in Notch activation as reported by transgenic fish with a Notch-responsive element, *her4*, driving red fluorescent protein, and a decrease in gliogenesis as reported by Müller glia cell
numbers. Conversely, knockdown of miR-216a or overexpression of snx5 results in an increase in Notch activation and a slight increase in Müller glia cell numbers. Interestingly, the decrease in Müller glial cells was much more obvious than the Müller glial cell increase. One potential explanation for this observation is that there is an upper threshold of Müller glial cell specification that has been reached in both the miR-216a knockdown and snx5 overexpression.

I propose a novel model in which SNX5, a protein containing two membrane-binding domains, binds to Mib and facilitates membrane curvature and endocytosis of the ligand Delta, which is required for efficient Notch activation. The model is consistent with previous work demonstrating that Mib, Delta, and SNX5 colocalize in cell culture and that Mib is a binding partner of SNX5. The research and therefore the proposed model are limited in that SNX5 may function at the membrane, but it could also function in Delta ligand recycling. This work sets the stage to ask this question, as well as more general questions about the role of miRNAs in regulation of developmental signaling pathways, particularly Notch signaling, and the role of sorting nexin protein family members in vertebrate development. The ways in which protein trafficking regulates development are likely vast and yet to be uncovered.

While the introduction of SNX5 as a player in Delta trafficking is novel, its regulation by miR-216a is also of great interest. Depending on the tissue environment, developmental timing, and the widely varied proteins that participate in Notch signaling, the possibilities for extremely specific regulation of Notch signaling are extensive. As discussed above, many Notch signaling
components are repressed by miRNAs, and multiple different miRNAs play a role in the pathway’s regulation. When regulation of individual components by miRNAs is considered, the potential for developmental complexity seems almost infinite. In identifying the interaction between \textit{miR-216a} and \textit{snx5}, I have likely only scratched the surface of the regulation of Notch signaling and how it functions during development, but this work will now serve as another example of biological possibilities yet to be uncovered.
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