microRNA REGULATION OF ZEBRAFISH RETINAL DEVELOPMENT

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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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LIST OF ABBREVIATIONS

7meG	7-methylguanosine
Ago	Argonaute
ath5	Atonal Homologue 5
BAR	Bin, Amphiphysin, Rvs
BP	Base Pair
cDNA	Complementary DNA
CGZ	Circumferential Germinal Zone
CMZ	Ciliary Marginal Zone
DCP	Decapping Enzyme
DIC	Dye-injected Control
DIG	Digoxigenin
DNA	Deoxyribonucleic Acid
dpf	Days Post Fertilization
dRFP	Destabilized Red Fluorescent Protein
dsRBD	dsRNA Binding Domain
dsRNA	Double-stranded RNA
Exp5	Exportin 5
GFAP	Glial Fibrillary Acid Protein
GFP	Green Fluorescent Protein
GTP	Guanosine-5'-triphosphate
hpf	Hours Post Fertilization
HRP	Horseradish Peroxidase

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

PIP2	Phosphatidylinositol 4,5-bisphosphate
pre-miRNA	Precursor miRNA
pri-miRNA	Primary miRNA
PX	Phox-homology
RISC	RNA Induced Silencing Complex
RLC	RISC-loading Complex
RNA	Ribonucleic Acid
RNAi	RNA Interference
RNase	Ribonuclease
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SEM	Standard Error of the Mean
siRNA	Small Interfering or Silencing RNA
SNX	Sorting nexin
ssRNA	Single-stranded RNA
Tg	Transgenic
UTR	Untranslated Region

CHAPTER I

Introduction

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 *lin-14* 3' UTR (Lee et al., 1993; Wightman et al., 1993).

lin-14 is expressed in a temporal gradient and regulates the normal sequence of cell lineage during *C. elegans* development (Ambros, 1989; Ruvkun and Giusto, 1989). Before the seminal work from the Ambros and Ruvkun labs, it was known that *lin-4* mutants affect cell lineage decisions with reiteration of larval cell fates during later stages (as do *lin-14* gain of function mutants) and that *lin-4* is a negative regulator of *lin-14* (Ambros and Horvitz, 1987; Arasu et al., 1991). The Ambros group cloned the *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 *lin-4* mutants. Coupled to the fact that *lin-4* sequences are conserved in other *Caenorhabditis* species, it was concluded that the *lin-4* product does not function as a protein. The *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 *lin-14*, known from previous work to be necessary for its negative temporal regulation (Wightman et al., 1991), are complementary to sequences in *lin-4* (Lee et al., 1993).

Ruvkun's group showed that the 3' UTR of *lin-14* is sufficient to confer temporal regulation to a *lacZ* reporter and that regulation of the reporter was not recapitulated in *lin-4* mutants. They also identified nucleotides in the 3' end of *lin-4* complementary to seven sites in the *lin-14* 3' UTR, which were found to be conserved in *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 (Förstemann 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).

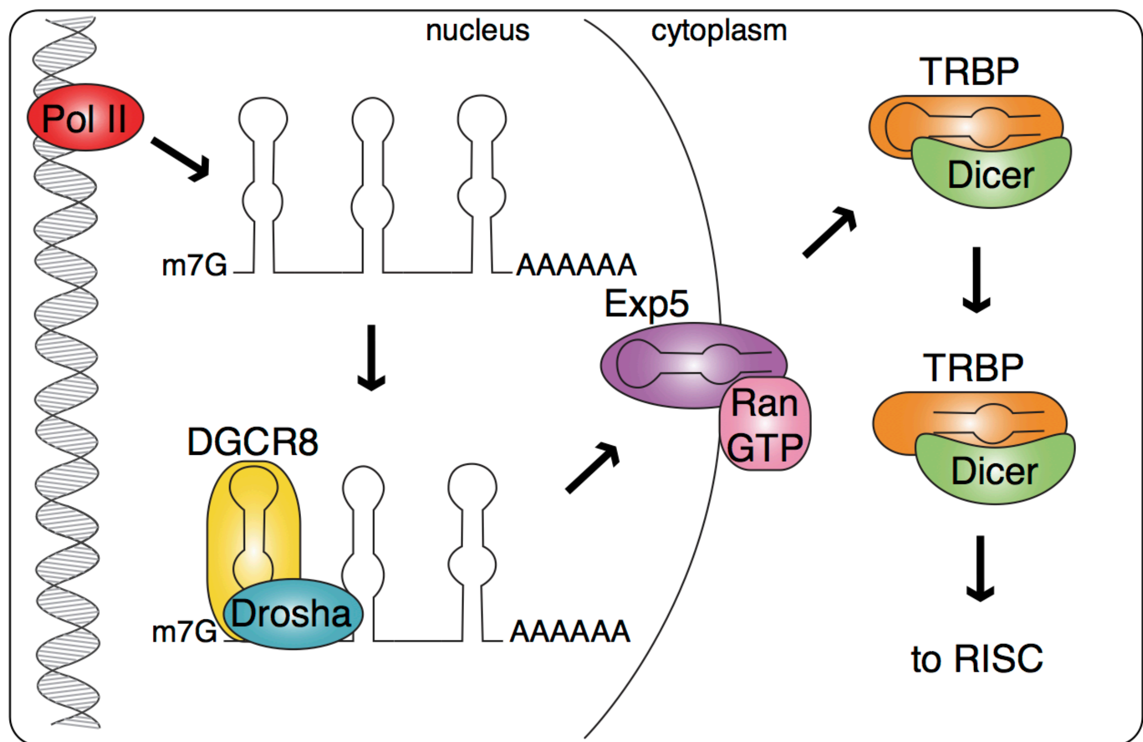


Figure 1.1. Canonical miRNA Biogenesis
miRNAs are transcribed as primary transcripts by RNA Polymerase II. Pri-miRNAs contain one or more stem loops, which are recognized and cleaved by Drosha and its cofactor DGCR8. The resulting pre-miRNA is exported from the nucleus by Exportin 5, a process dependent 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.

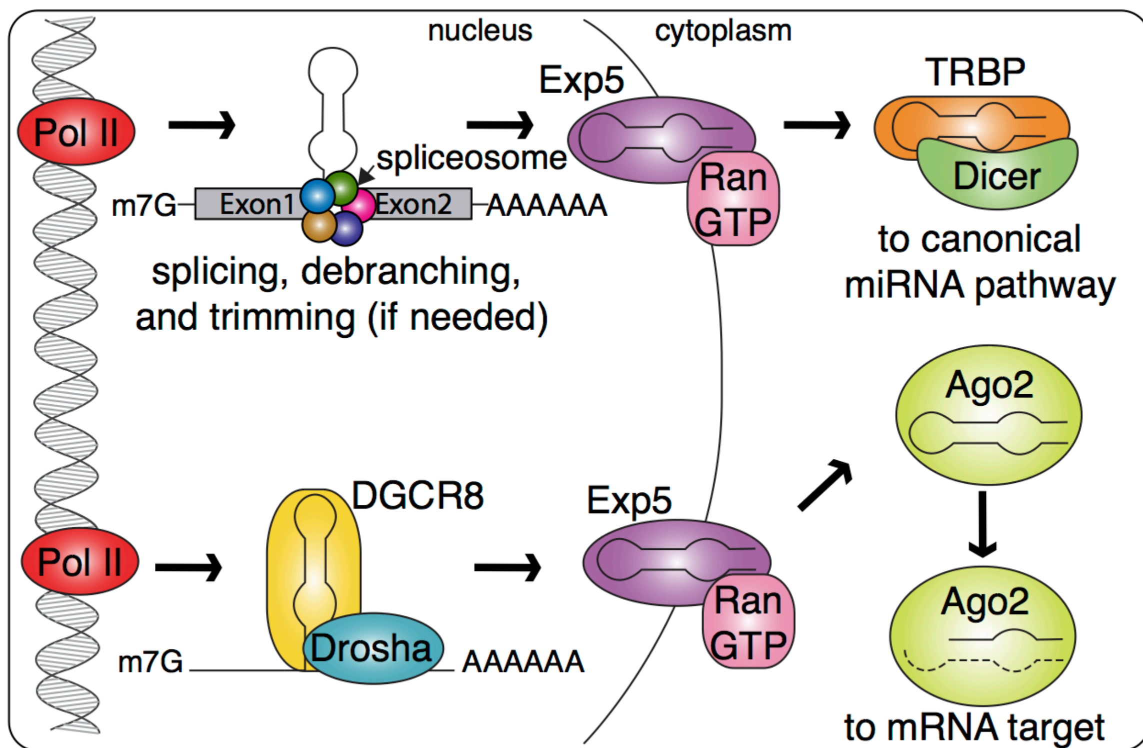


Figure 1.2. Noncanonical miRNA Biogenesis
Top, mirtron biogenesis. Mirtrons are transcribed as introns of protein coding genes by RNA Pol II. The spliceosome splices these mirtrons, which are debranched and undergo exosomal trimming (if necessary). After these initial processing steps, mirtrons are treated 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, *miR-1017*, was discovered by Ruby *et al* in *C. 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 *miR-1017*, but also 14 reads for *miR-1017** (the strand paired with *miR-1017* in the precursor), whose sequence aligns exactly with the 5' splice donor sequence, consistent with splicing being involved in *miR-1017/1017** biogenesis. In examining various *Drosophila* genomes, extensive variability was detected in the tail sequence (the sequence preceding the 3' splice acceptor) of the *miR-1017* mirtron suggesting that the tail sequence is not important when generating a functional *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, *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.

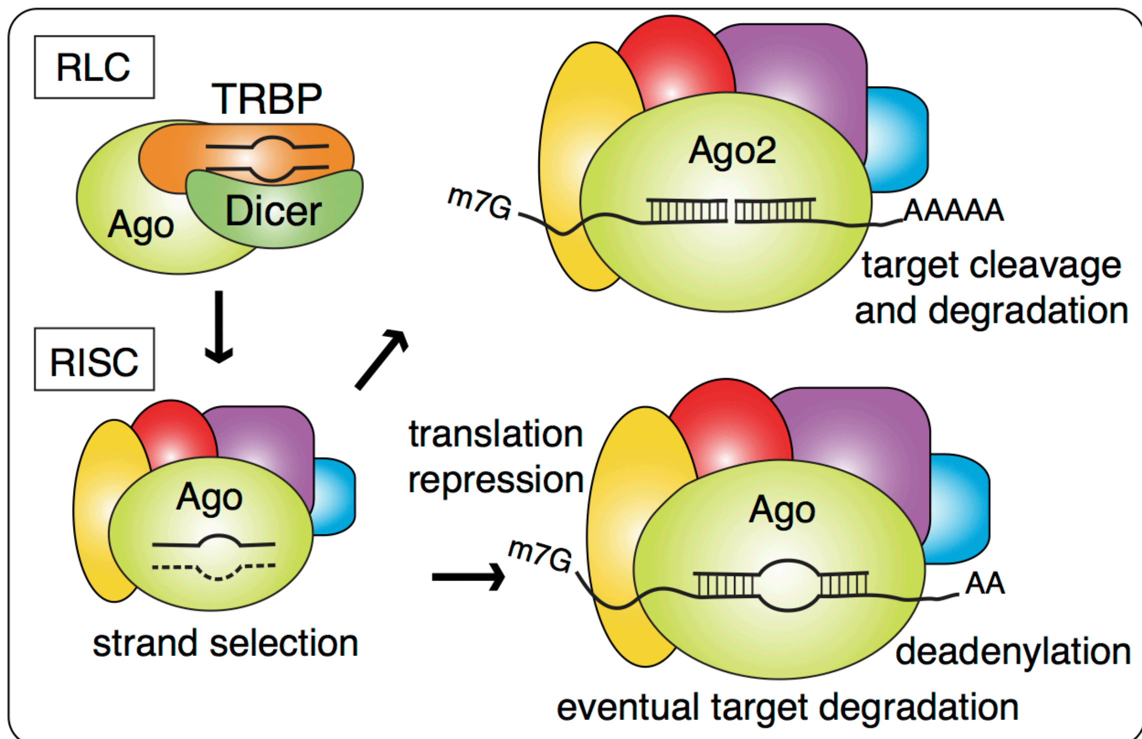


Figure 1.3. miRNA Function
Dicer, TRBP, and Argonaute form the RISC Loading Complex (RLC), which facilitates assembly of the RISC and incorporation of the mature miRNA duplex. Once incorporated into the RISC, the nonfunctional strand of the miRNA duplex is degraded and the functional strand is guided 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

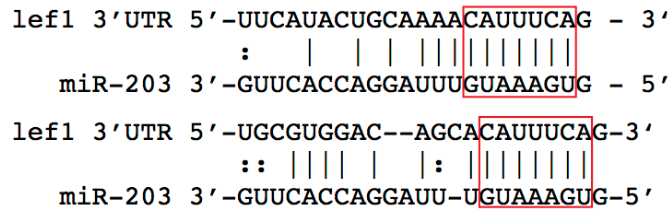
Perhaps not surprisingly, as previous work showed the ability of noncanonical MREs to regulate targets (Brennecke et al., 2005), several examples of functional noncanonical MREs have emerged. Li *et al* demonstrated that *dispatched homolog 2* is regulated by *miR-214* during zebrafish development through 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.

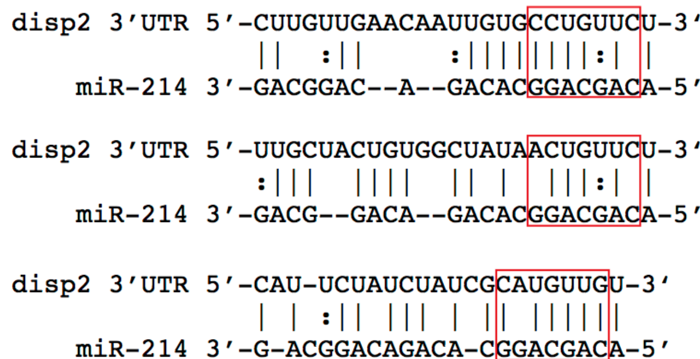
Mechanisms of miRNA Mediated Gene Silencing

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)

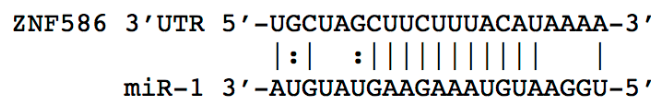
Canonical miRNA-Target Pairing



Combination of Weak Sites



Functional Sites with Centered Pairing



Seed Mismatch Sites

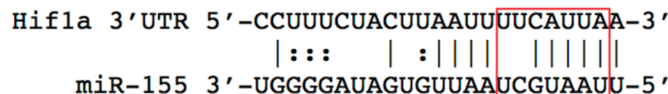


Figure 1.4. miRNA Target Recognition
The pairing of *miR-203* and its target during zebrafish fin regeneration, *lef1*, is shown at top. Straight lines represent Watson-Crick base pairs, while colons represent G:U wobble pairing. The 3'UTR of lef1 contains two miRNA target sites, both of which are good 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 (Djuranovic et al., 2010). Another potential mechanistic explanation could be the involvement of eIF-6, a protein known to inhibit formation of the 80S ribosome. Chendrimada *et al* showed that eIF-6 is associated with human RISCs and that it plays a role in miRNA-mediated target repression in both human cells and in *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, *miR-430* promotes rapid deadenylation and clearance of maternal mRNAs during the transition from maternal to zygotic transcription (Giraldez 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 *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 is 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).

ZEBRAFISH RETINAL DEVELOPMENT

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.

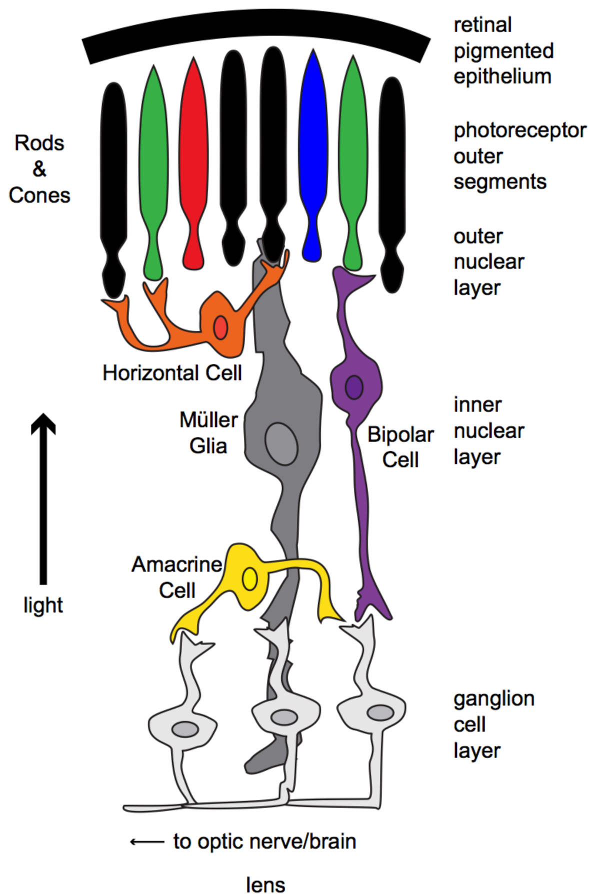


Figure 1.5. Zebrafish Retina The zebrafish retina consists of the outer nuclear layer, which contains the photoreceptor cell nuclei, the inner nuclear layer, which contains the horizontal, amacrine, and bipolar cells, as well as the nuclei of Müller glia, and the ganglion cell layer. Light enters the 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

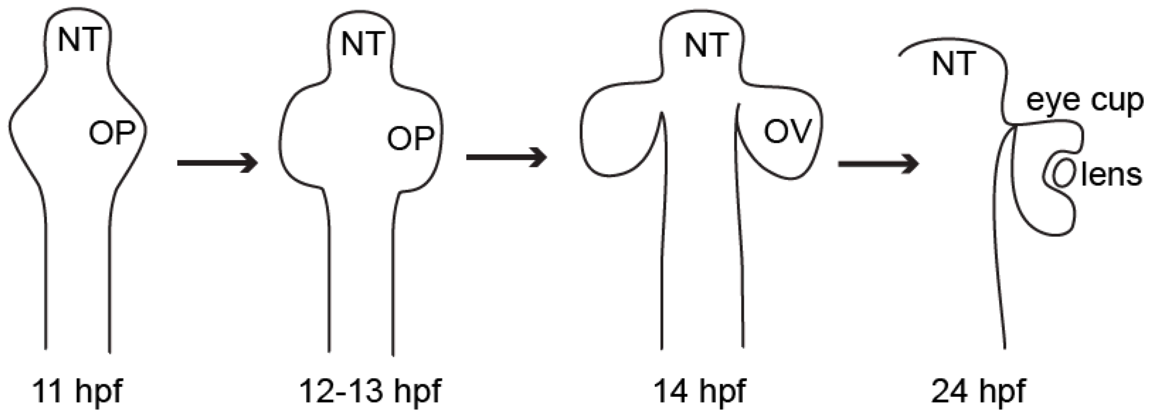


Figure 1.6. Zebrafish Eye Morphogenesis
The optic primordium (OP) extends from the neural tube (NT) starting at about 11 hpf. By 14 hpf, the OP has become the optic vesicle (OV), a wing-like protrusion, which begins to detatch from the neural tube. By 24 hpf, the optic vesicle has invaginated and further 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 Izaurralde, 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, amphiphysin, 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).

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
snx5 (NM_214769)	ACCTGATCGAGATGACTGAG	TTATCTTCGCTGAGTTGCAC
her4.2 (NM_131090.3)	AGTCACATCTGGAGACCCTG	GCTTCAACACACAAACAAGTCC
notch1b (NM_131302.2)	GTCACAAATCGGACACATGC	CACAAATCGTTTCAATCGGATG
heyl (NM_181736.1)	GGGCTTTGAGTTCCTCCAG	TCTCCTCAAGCACTTCAATCTC
numb (NM_001040406.1)	CGCTCCATCACCCACAAACC	GACGAGTCGTTCCCTGTATGG
hey2 (NM_131622.2)	AGTAAACCATACCGACCGTG	GGTTACATCTTACAGAGGGTGG

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'-TGCAGACACATAAAGTACCACTATG-3') and reverse (5'-GCTAATATTTGCATAACTTGGAATATG-3') primers and for MRE 2, forward (5'-GTCCGAATGCATTACTCTGCATTACAGAT-3') and reverse (5'-TATTAGGAGGAAAGATATCTGAAGCATTACA-3') primers were designed to exclude each MRE. *snx5* mRNA was amplified by RT-PCR using forward (5'-GCCGAGGGATCCTGAGGAACGAGCTTGCTGCTGGAA-3') and reverse (5'-GCCGAGCTCGAGCAACTGGGGACATCAGTCAGTCCTT-3') primers and cloned into pCS2+ (Rupp et al., 1994). *snx5* mRNA without its 3'UTR was amplified by RT-PCR using forward (5'-GCCGAGGGATCCTGAGGAACGAGCTTGCTGCTGGAA-3') and reverse (5'-GCCGAGCTCGAGGTCATCATCGTGTGGGTC-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'-ACGTCATGTTCAGGAGATATTTCGC-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. *snx5* 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 *snx5* RNA probe generated with Roche Applied Science reagents and pCS2+ vector containing the full length *snx5* 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 $MgCl_2$, 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 α-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 40× 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.

2 dpf	Fold Difference	p-value
miR-9	4.1791	0.0002
miR-17-5p	7.7904	0.0002
miR-19a	3.6866	0.0069
miR-20	4.6253	0.0018
miR-25	2.6127	< 0.0001
miR-31	3.3801	0.0008
miR-93	3.7530	0.0002
miR-108	4.1121	0.0033
miR-124a	7.0932	< 0.0001
miR-152	4.9246	0.0017
miR-210	2.8556	0.0076
miR-216	3.9684	0.0016
5 dpf	**Fold Difference**	**p-value**
miR-9	5.4529	< 0.0001
miR-17-5p	7.1188	< 0.0001
miR-18	3.8517	0.0002
miR-19a	6.6342	< 0.0001
miR-19b	3.9508	< 0.0001
miR-20	6.0296	< 0.0001
miR-22	5.6745	< 0.0001
miR-25	5.3233	< 0.0001
miR-31	3.4258	0.0001
miR-93	5.2311	< 0.0001
miR-108	5.1436	0.001
miR-124a	7.3811	< 0.0001
miR-125b	6.0221	< 0.0001
miR-152	4.0968	< 0.0001
miR-181a	4.4360	0.0001
miR-181b	4.6483	< 0.0001
miR-182	5.4448	< 0.0001
miR-183	6.3569	< 0.0001
miR-204	7.9874	< 0.0001
miR-210	8.0629	< 0.0001
miR-213	3.8558	< 0.0001
miR-216	8.8500	< 0.0001
miR-217	6.8886	0.0002

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 aeruginsa* dehydrogenase. All p-values were calculated based on paired t-tests.

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).

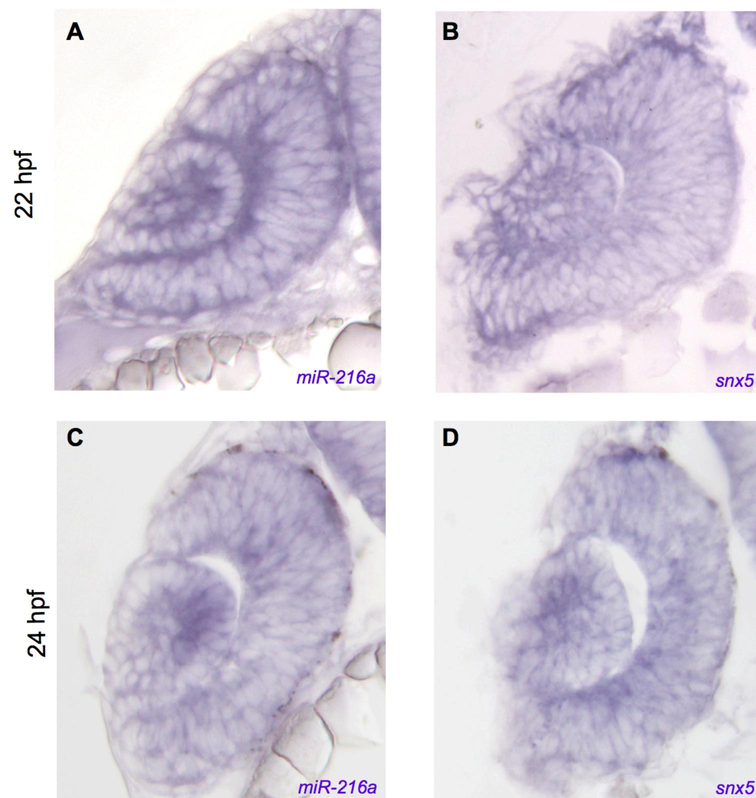

Figure 2.1. *miR-216a* and *snx5* are widely expressed in the developing eye at 22 and 24 hpf
In situ hybridizations were performed for *miR-216a* (A,C) and *snx5* (B,D) at 22 hpf (A,B) and 24 hpf (C,D). Embryos were sectioned and sections were imaged on an epifluorescence scope. At 22 hpf, both *miR-216a* and *snx5* are expressed throughout the developing eye. At 24 hpf, these expression patterns become slighty 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.

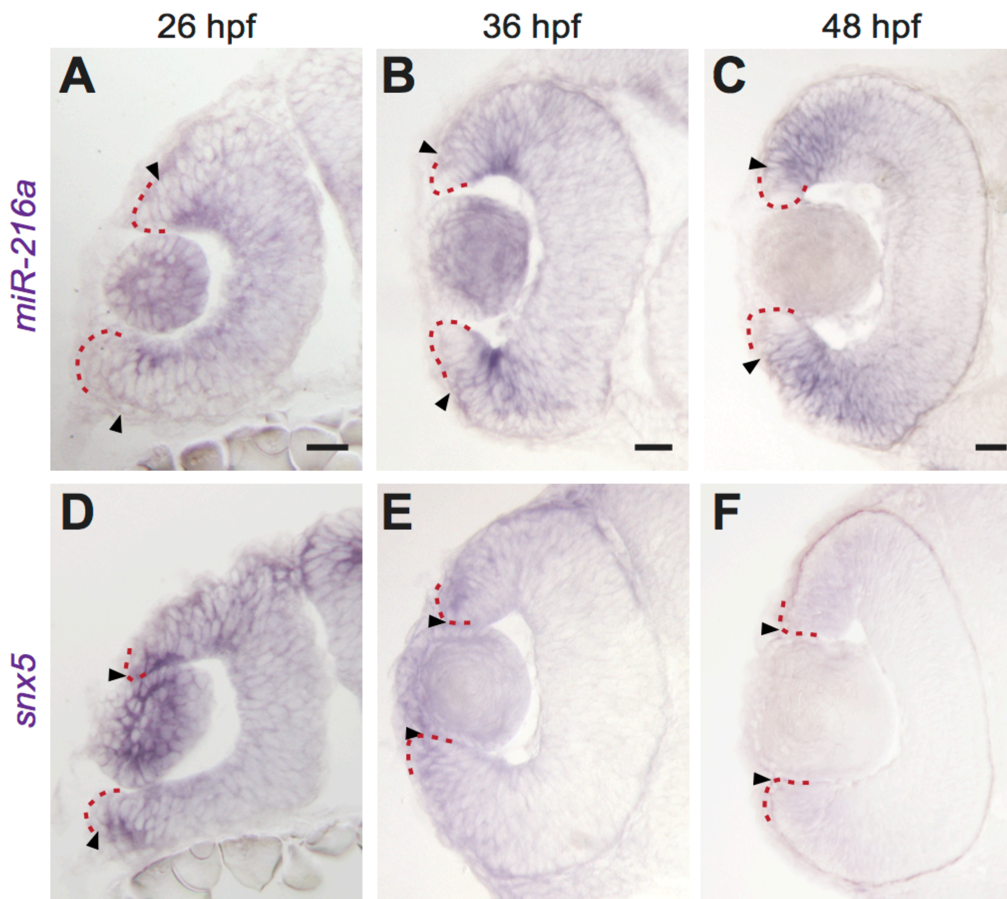

Figure 2.2. *miR-216a* and *snx5* have complementary expression patterns during development.
Transverse sections of whole mount *in situ* hybridizations for *miR-216a* and *snx5* at 26 (A,D), 36 (B,E), and 48 (C,F) hours post fertilization (hpf). *miR-216a* expression spreads from the center of the developing 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 *algorithm*s (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 *numbl*. 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

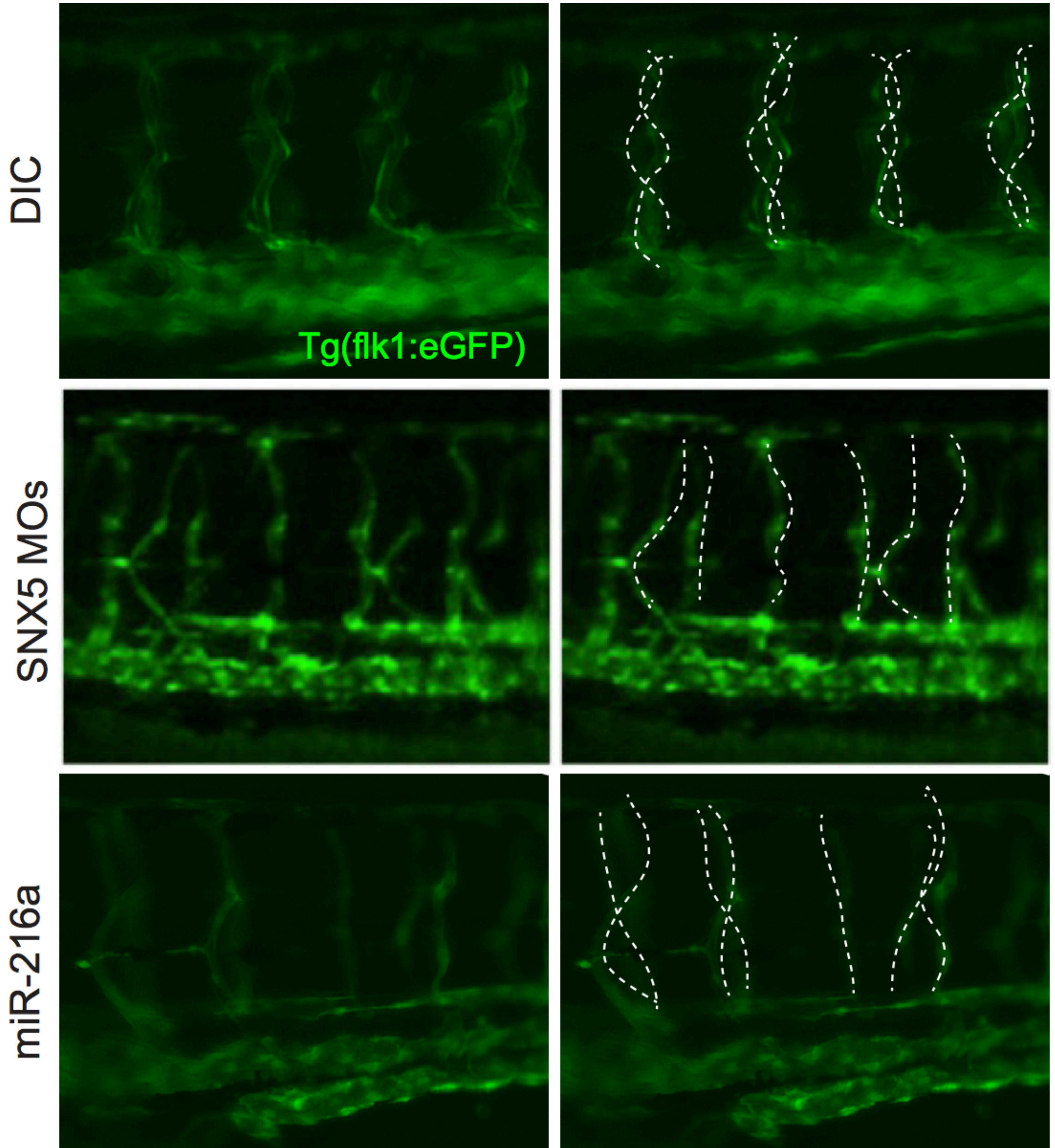

Figure 2.3. Knockdown of *snx5* and overexpression of *miR-216a* result in vascular patterning defects.
Tg(flk1:eGFP) embryos were injected at the 1-cell stage with dye control, *snx5*^{MOs}, or *miR-216a*. Embryos were grown in egg water and vascular development was monitored. Intersegmental vessels were missing in the trunks of *snx5*^{MOs}- 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.

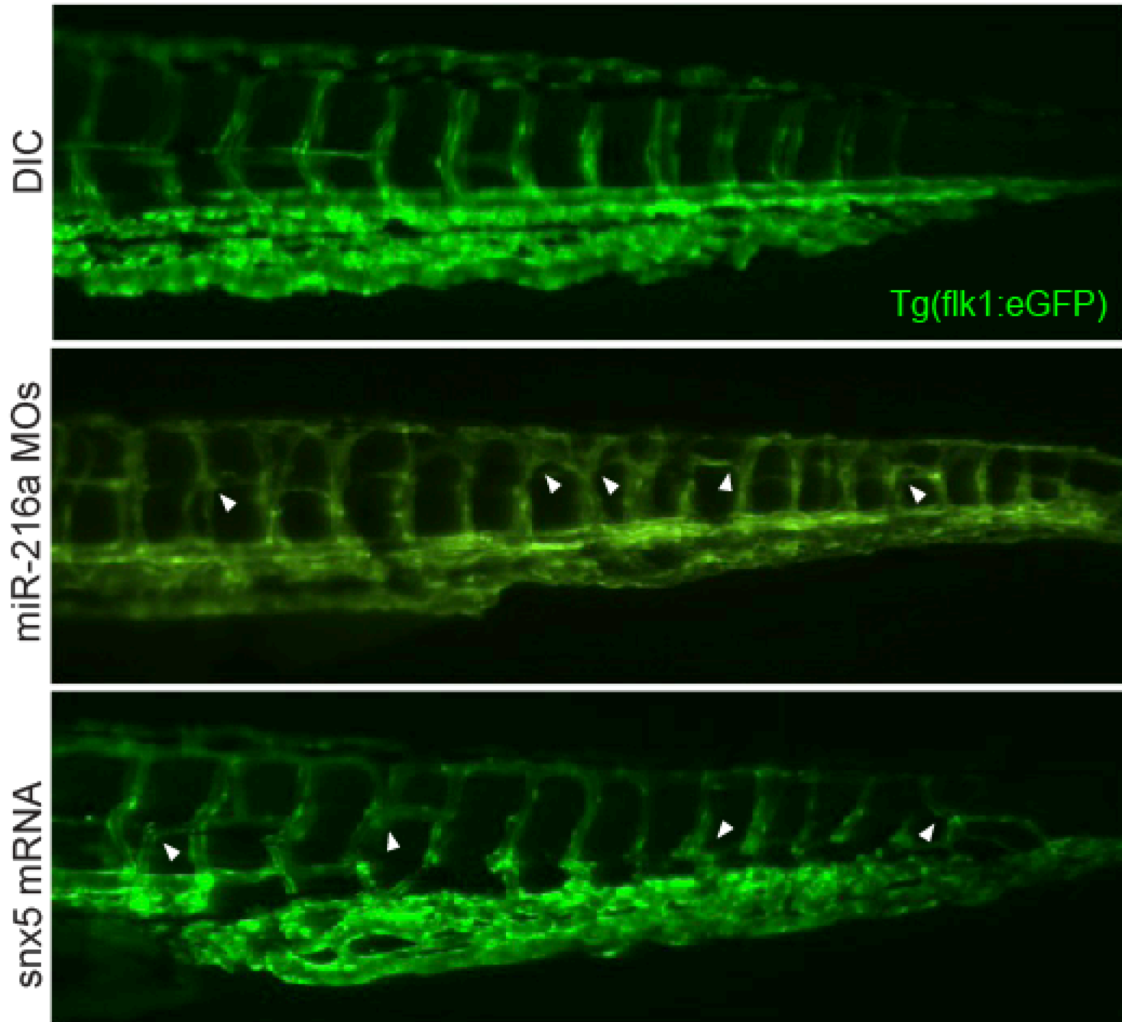

Figure 2.4. Knockdown of *miR-216a* and overexpression of *snx5* result in vascular patterning defects.
Tg(flk1:eGFP) embryos were injected at the 1-cell stage with dye control, *miR-216a*^{MOs}, or *snx5* mRNA. Embryos were grown in egg water and vascular development was carefully monitored. Branching 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 *snx*5 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.

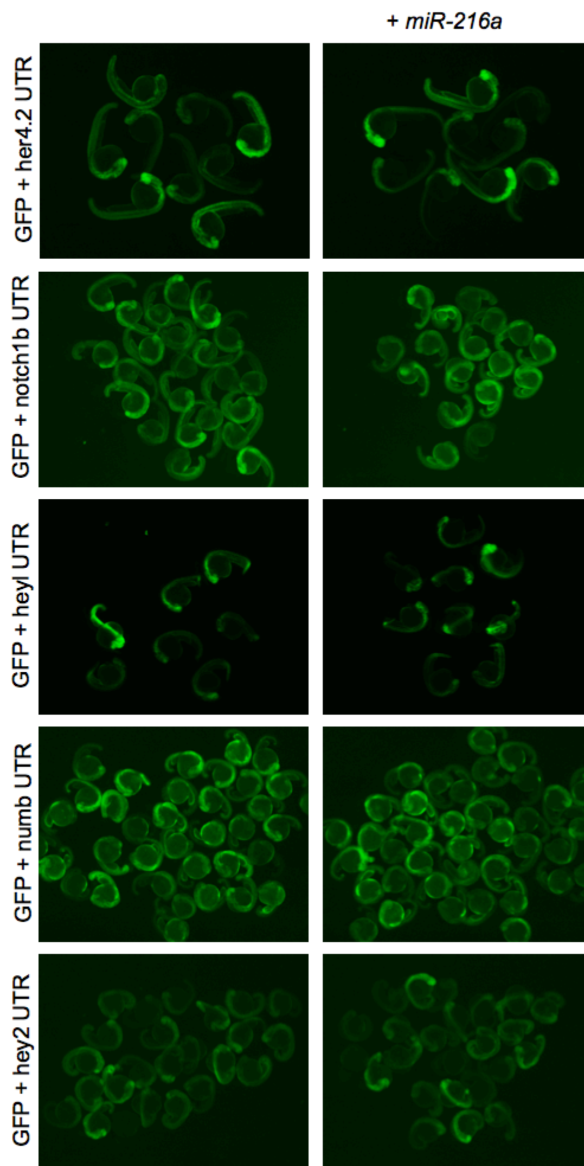

Figure 2.5. *miR-216a* does not target *her4.2, notch1b, heyl, numb,* or *hey2.*
Embryos were injected at the 1-cell stage with GFP reporters containing the 3'UTRs of *her4.2, notch1b, heyl, numb,* or *hey2* alone or in combination with *miR-216a*. Embryos were grown in egg water until 24 hpf, at which 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.

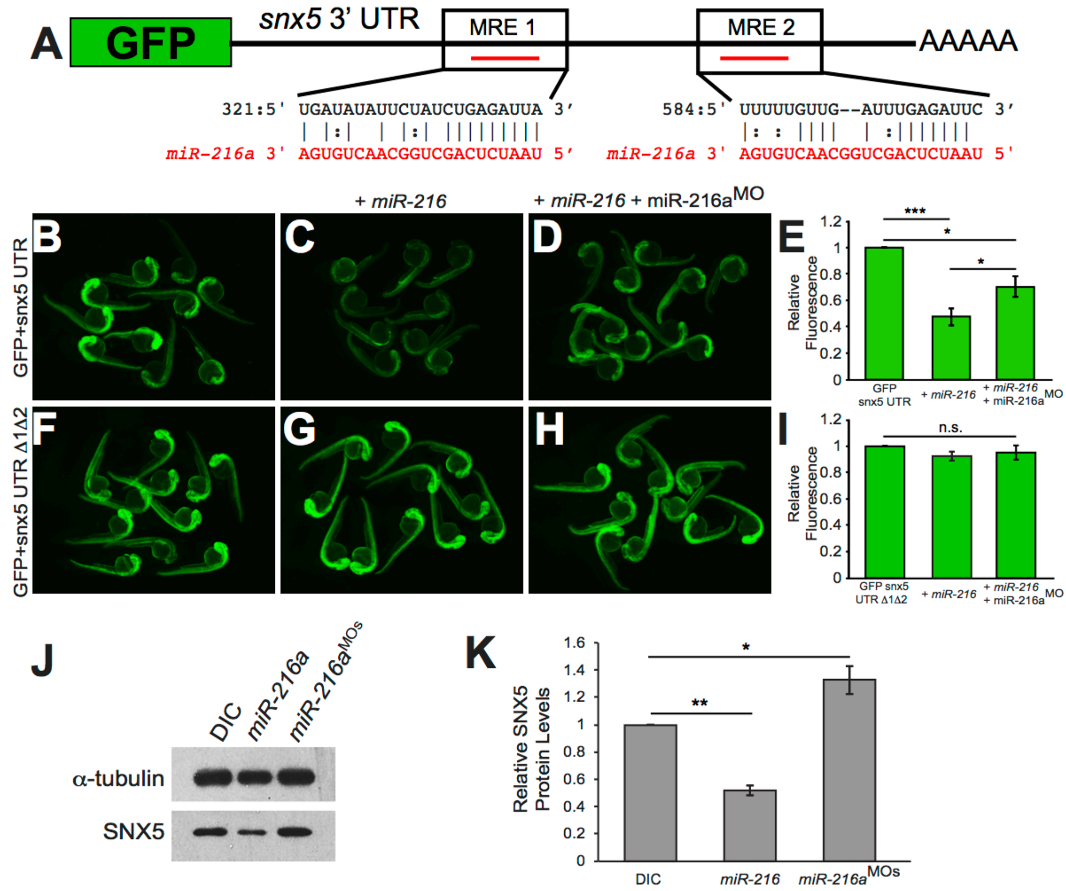

Figure 2.6. *snx5* is a target of *miR-216a.*
The coding sequence of GFP was fused to the 3'UTR of *snx5*. (A) Predicted pairing between microRNA Recognition Elements (MREs) in the *snx5* 3' UTR (black) with *miR-216a* (red). (B) 1-cell stage embryos were injected with GFP-*snx5* 3' UTR reporter mRNAs alone, or co-injected with *miR-216a* (C), or the combination of *miR-216a* and *miR-216a*^{MO} (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-216a*^{MO} (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 a-tubulin were performed on protein lysates from 1 dpf zebrafish injected with dye control (DIC), *miR-216a*, or *miR-216a*^{MOs}. (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 *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 *snx5* on Notch-Delta signaling have not been characterized nor has there been any previous work investigating the regulation of *snx5* during early retina development. We therefore used a Notch reporter zebrafish line (*Tg(her4:dRFP*), which expresses dRFP under the control of the *her4* Notch-responsive element (Takke et al., 1999; Yeo et al., 2007). We injected *Tg(her4:dRFP*) single cell embryos with either dye control, synthetic *miR-216a* duplexes, *miR-216a*^{MOs}, *snx5*^{MOs}, or *snx5* mRNA, and then fixed the embryos at 30 hpf and sectioned to examine Notch activation in the developing retina. Strikingly, overexpression of *miR-216a,* or knockdown of *snx5,* resulted in a marked decrease in Notch activation compared to DICs, as reported by the loss of *Tg(her4:dRFP*) fluorescent protein expression (Fig. 2.7 A,B,E).

Conversely, knockdown of *miR-216a* or overexpression of *snx5* mRNA resulted in expansion of the zone of *Tg(her4:dRFP*) fluorescence and presumptive Notch activation compared to DICs (Fig. 2.7 C,F). Co-injection of *snx5* lacking its 3'UTR with *miR-216a* restored the zone of *Tg(her4:dRFP*) activation (Fig. 2.7 D), as did co-injection of *snx5*^{MOs} and *miR-216a*^{MOs} (Fig. 2.7 G). These data indicate that *snx5* is a positive regulator of Notch-Delta signaling and that *miR-216a* negatively regulates Notch-Delta signaling via its interaction with *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).

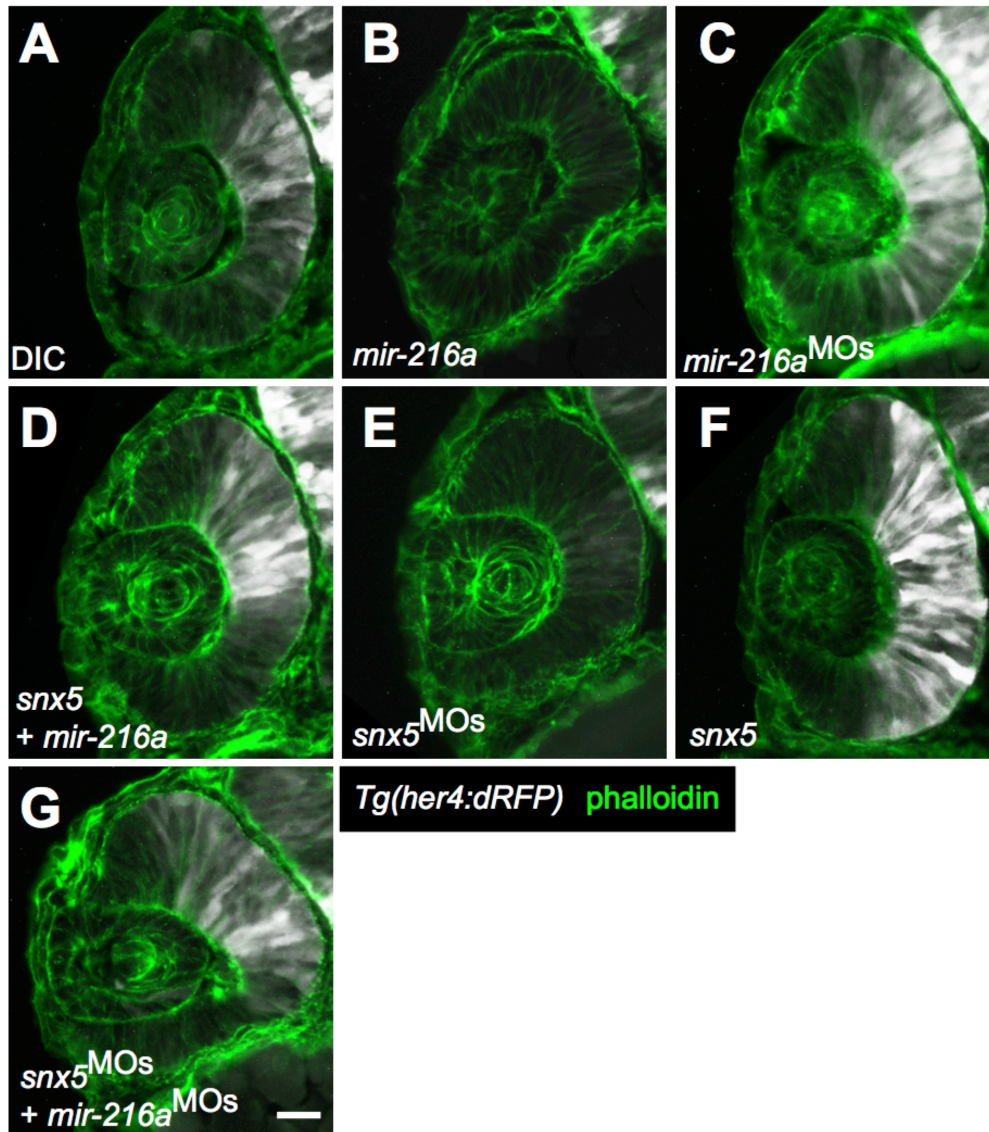

Figure 2.7. *miR-216a* and *snx5* regulate Notch activation.
Transverse sections of developing retinas from 30 hours post fertilization (hpf) *Tg(her4:dRFP)* embryos were injected with dye control (DIC; A), *miR-216a* (B), *miR-216a*^{MOs} (C), *snx5*^{MOs} (E), or *snx5* mRNA (F). Reporter expression (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 phalloidin (green) to visualize cell boundaries. Scale bar: 20μm.

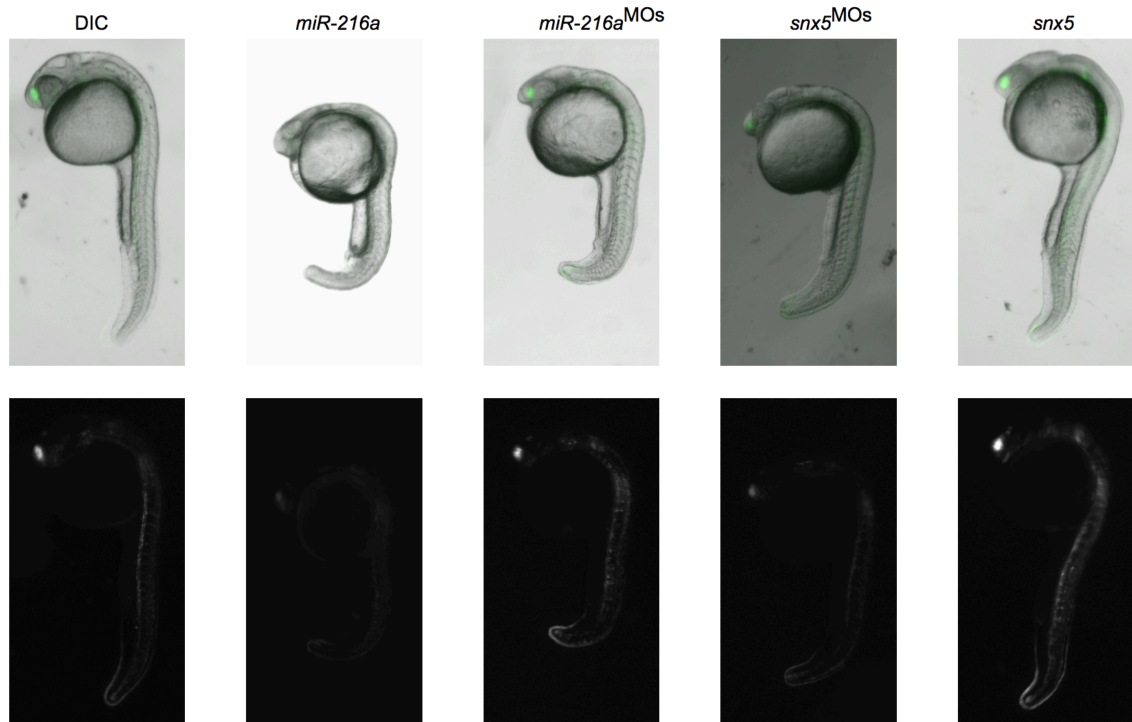

Figure 2.8. *Tg(Tp1:GFP)* reporter reveals changes in Notch signaling upon perturbation of *miR-216a* and *snx5*
Embryos *(Tg(Tp1:GFP))* were injected at the 1-cell stage with *miR-216a, miR-216a*^{MOs}, *snx5*^{MOs}, *snx5,* or dye control (DIC). Embryos were grown in egg water until 24 hpf, at which 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*^{MOs} injected embryos, suggestive of lower Notch activation, and an increase in reporter fluorescence in *miR-216a*^{MOs} 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*.

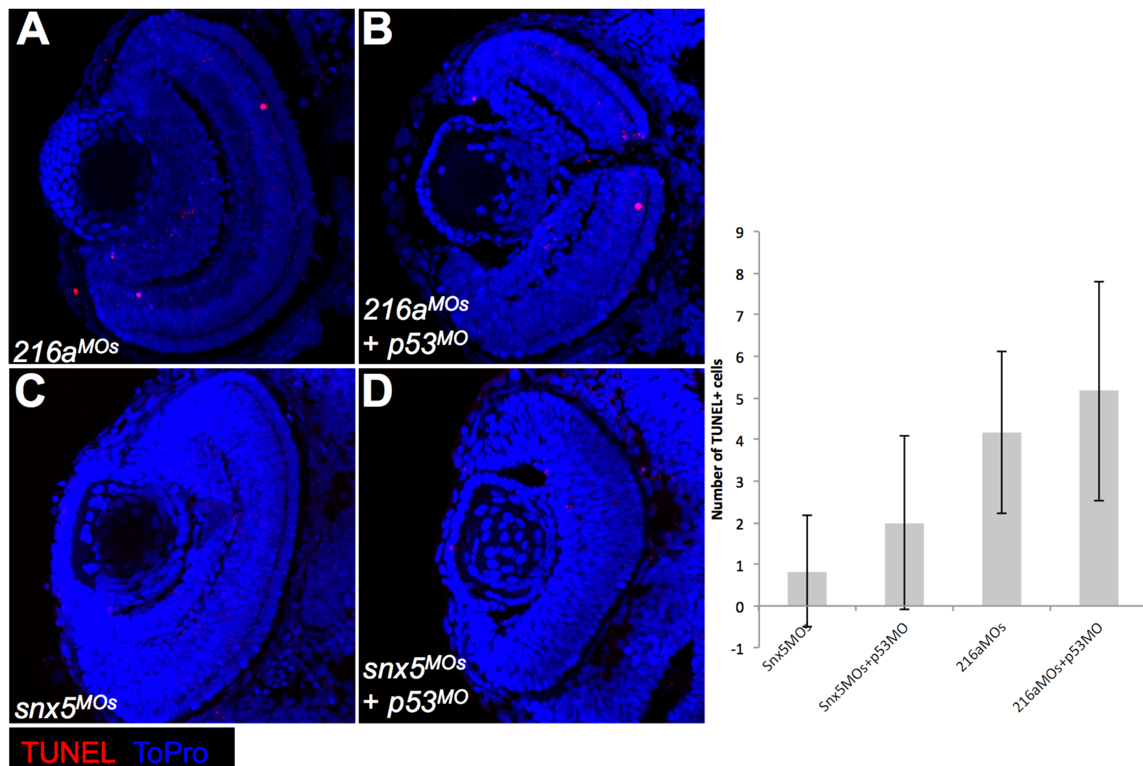

Figure 2.9. No differences in programmed cell death observed with morpholino injections.
We injected morpholinos into one cell stage embryos in the presence and absence of p53 and found no change in the levels of TdT-mediated incorporation of dUTP (red) in sectioned eyes at 36-48 hpf, quantified on right.

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 *miR-216a*, *miR-216a*^{MOs}, *snx5*^{MOs}, or *snx5* mRNA into single cell *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 *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 *her4* reporter fish (Fig. 2.10), we counted GFP+ cells at this time. Upon overexpression of *miR-216a*, a significant decrease in GFP+ cells was observed compared to DICs (Fig. 2.11). In contrast, knocking down *miR-216a* with morpholinos resulted in an increase in GFP+ numbers (Fig. 2.11).

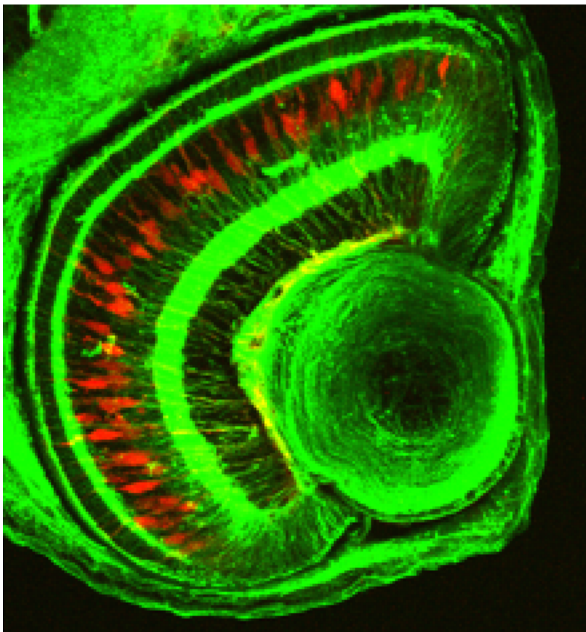

Figure 2.10. Notch is activated in Müller glia at 65 hpf.
In a cross section of *Tg(her4:dRFP)* fish at 65 hpf, Notch activation (in red) was detected primarily in Müller glia. Cell membranes are labeled with phalloidin, here visualized in green.

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*$^{\text{MOs}}$ 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.

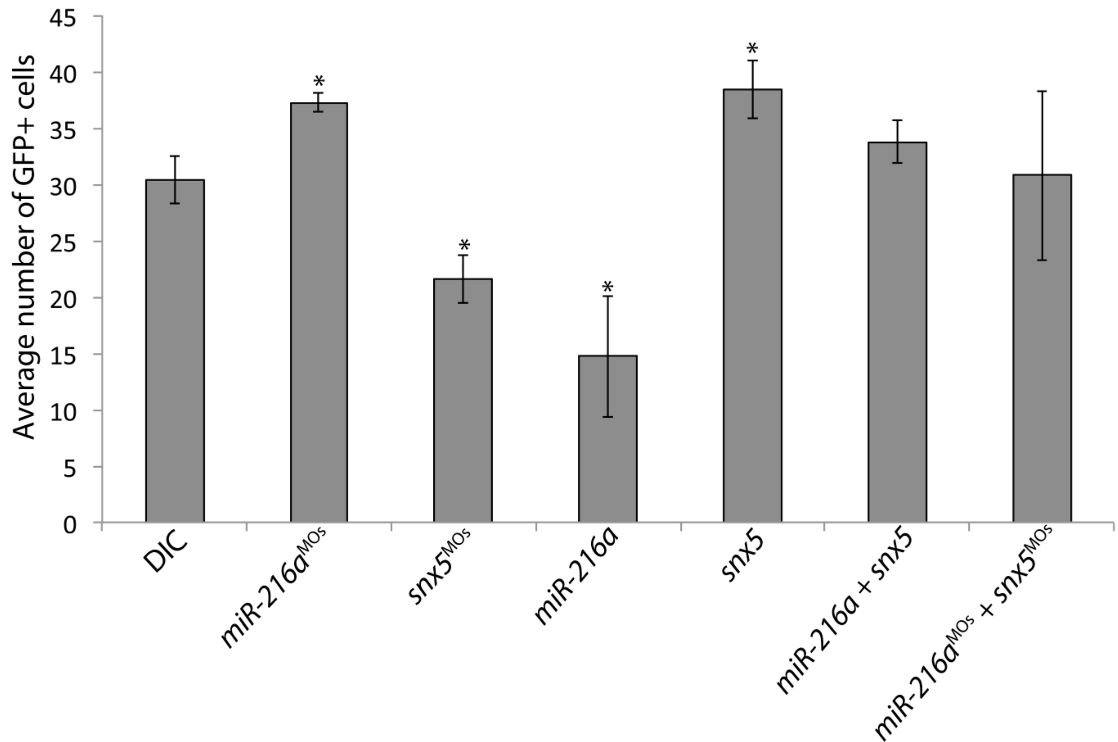

Figure 2.11. *miR-216a* and *snx5* regulate Müller glia cell numbers. *Tg(gfap:GFP)* transgenic zebrafish were injected as indicated, grown to 65 hpf, and GFP+ cell numbers were counted. Compared to DICs, injection of *miR-216a*^{MOs} or *snx5* caused a significant increase in GFP+ cells ($p<0.05$). 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.

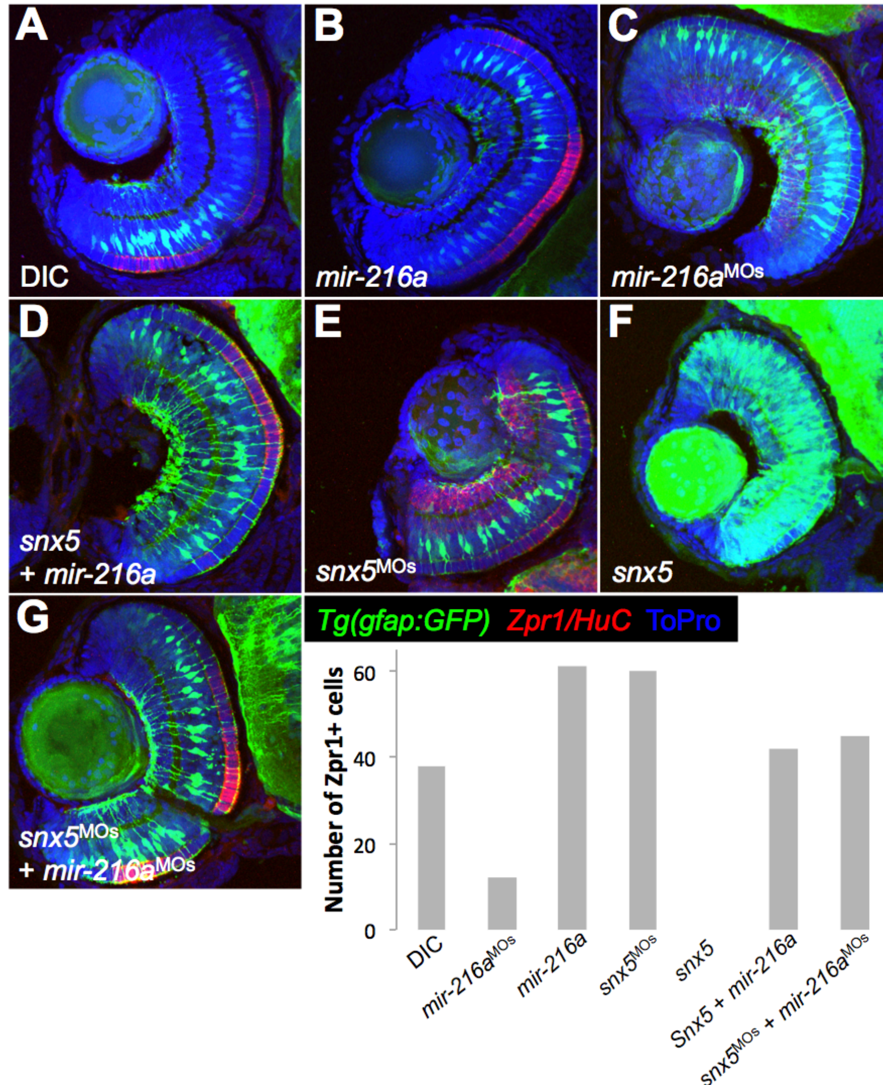

Figure 2.12. Inverse correlation between Müller glia numbers and cone photoreceptor staining.

Tg(gfap:gfp) embryos were injected with dye control (DIC; A), *miR-216a* (B), *miR-216a*^{MOs} (C), *snx5*^{MOs} (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 snx5^{MOs} and decreased in embryos injected with *miR-216a*^{MOs} 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 *snx5*^{MOs} and *miR-216a*^{MOs} (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 *snx5* and *miR-216a* expression, GFP reporter assays, and SNX5 immunoblotting, we show that *miR-216a* regulates *snx5*. Based on the expression of *miR-216a* and *snx5* in the retinal neuroepithelium, it appears that *miR-216a* plays a role in both spatial and temporal control of *snx5* expression, and, in turn, Notch signaling.

miR-216a* regulates Notch signaling via *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 *Tg(her4:dRFP*) fish, we also observed defects in vascular patterning upon knockdown and overexpression of *miR-216a* and *snx5* (Fig. 2.3 and 2.4). This suggests that *miR-216a* and *snx5* also play a role in Notch signaling in zebrafish vascular development. We also show that perturbing expression of *miR-216a* and *snx5* causes changes in Notch activation, as reported by altered zones of fluorescent protein expression in the retinas of *Tg(her4:dRFP*) embryos.

Based on prior work about SNX5 and Mib and our experiments, we propose a model where *miR-216a* regulates Notch-Delta signaling via regulation of *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).

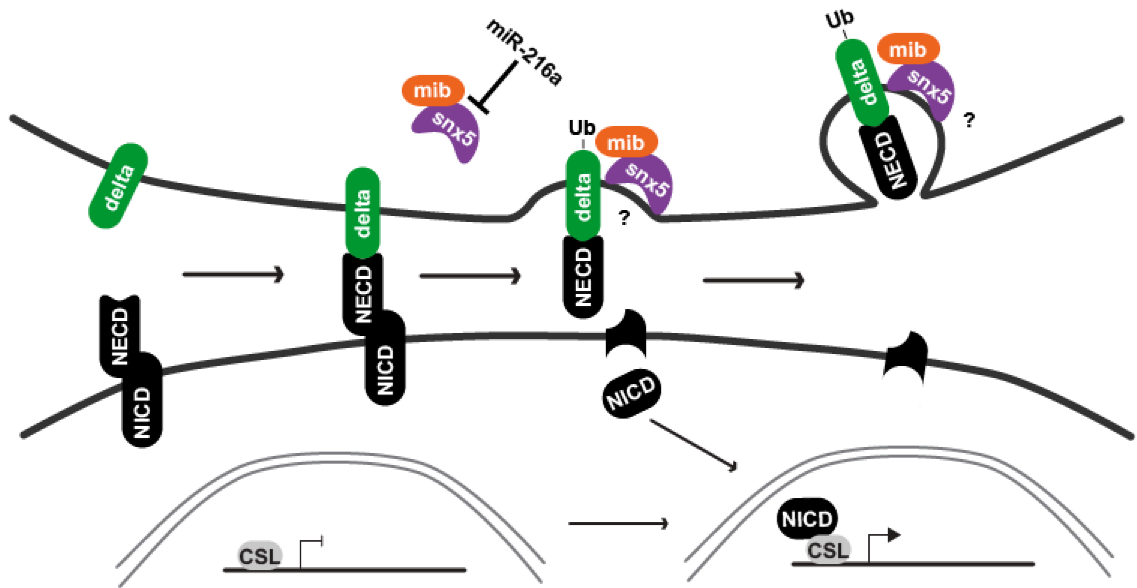

Figure 2.13. Model for the role of SNX5 and *miR-216a* in Notch signaling
SNX5 (bound to Mib) moves to the site of Delta activation, where it binds to the membrane as Mib ubiquitylates Delta. SNX5 then facilitates membrane curvature and Delta endocytosis, which is required for cleavage of the Notch extracellular 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 *miR-216a* regulates *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.

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 *et al.* demonstrated that *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 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 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, *snx5*, and *miR-216a* expression during different windows of time during retinogenesis.

The evidence generated using the *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 *miR-216a* and *snx5*. Though a tentative link between *snx5* and Notch signaling was made by Yoo *et al.* in 2006 when they demonstrated that SNX5 binds Mib (Yoo et al., 2006), this work is the first to use Notch reporters to examine the relationship between *snx5* and Notch. This finding could be expanded upon using transgenic zebrafish expressing NICD under the control of a heat shock promoter (Scheer et al., 2001) or dominant negative Delta to suppress *snx5* knock down and *miR-216a* overexpression (Appel and Eisen, 1998). Because the deficits in glial cells present when knocking down *snx5* or overexpressing *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 *miR-216a* and *snx5*.

Notch signaling has been shown to play a role in arterial-venous differentiation in zebrafish (Lawson 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*, *Caenorabditis 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 *miR-216a* and *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.

REFERENCES

Alto, N.M., Weflen, A.W., Rardin, M.J., Yarar, D., Lazar, C.S., Tonikian, R., Koller, A., Taylor, S.S., Boone, C., Sidhu, S.S., *et al.* (2007). The type III effector EspF coordinates membrane trafficking by the spatiotemporal activation of two eukaryotic signaling pathways. Journal of Cell Biology *178*, 1265-1278.

Ambros, V. (1989). A hierarchy of regulatory genes controls a larva-to-adult developmental switch in C. elegans. Cell *57*, 49-57.

Ambros, V. (2003). MicroRNA pathways in flies and worms: growth, death, fat, stress, and timing. Cell *113*, 673-676.

Ambros, V., and Horvitz, H.R. (1987). The lin-14 locus of Caenorhabditis elegans controls the time of expression of specific postembryonic developmental events. Genes & development *1*, 398-414.

Appel, B., and Eisen, J.S. (1998). Regulation of neuronal specification in the zebrafish spinal cord by Delta function. Development *125*, 371-380.

Appel, B., Givan, L.A., and Eisen, J.S. (2001). Delta-Notch signaling and lateral inhibition in zebrafish spinal cord development. BMC Developmental Biology *1*, 13.

Arasu, P., Wightman, B., and Ruvkun, G. (1991). Temporal regulation of lin-14 by the antagonistic action of two other heterochronic genes, lin-4 and lin-28. Genes & Development *5*, 1825-1833.

Aravin, A.A., Lagos-Quintana, M., Yalcin, A., Zavolan, M., Marks, D., Snyder, B., Gaasterland, T., Meyer, J., and Tuschl, T. (2003). The Small RNA Profile during Drosophila melanogaster Development. Developmental Cell *5*, 337.

Ason, B., Darnell, D.K., Wittbrodt, B., Berezikov, E., Kloosterman, W.P., Wittbrodt, J., Antin, P.B., and Plasterk, R.H.A. (2006). Differences in vertebrate microRNA expression. Proceedings of the National Academy of Sciences of the United States of America *103*, 14385-14389.

Babiarz, J.E., Ruby, J.G., Wang, Y., Bartel, D.P., and Blelloch, R. (2008). Mouse ES cells express endogenous shRNAs, siRNAs, and other Microprocessor-

independent, Dicer-dependent small RNAs. Genes & Development *22*, 2773-2785.

Bae, Y., Yang, T., Zeng, H.C., Campeau, P.M., Chen, Y., Bertin, T., Dawson, B.C., Munivez, E., Tao, J., and Lee, B.H. (2012). miRNA-34c regulates Notch signaling during bone development. Human Molecular Genetics *21*, 2991-3000.

Baek, D., Villén, J., Shin, C., Camargo, F., Gygi, S., and Bartel, D. (2008). The impact of microRNAs on protein output. Nature *455*, 64-71.

Bagga, S., Bracht, J., Hunter, S., Massirer, K., Holtz, J., Eachus, R., and Pasquinelli, A.E. (2005). Regulation by let-7 and lin-4 miRNAs results in target mRNA degradation. Cell *122*, 553-563.

Bartel, D. (2004). MicroRNAs: genomics, biogenesis, mechanism, and function. Cell *116*, 281-297.

Bartel, D.P., and Chen, C.-Z. (2004). Micromanagers of gene expression: the potentially widespread influence of metazoan microRNAs. Nature Reviews Genetics *5*, 396-400.

Bazzini, A.A., Lee, M.T., and Giraldez, A.J. (2012). Ribosome Profiling Shows That miR-430 Reduces Translation Before Causing mRNA Decay in Zebrafish. Science *336*, 233-237.

Behm-Ansmant, I., Rehwinkel, J., Doerks, T., Stark, A., Bork, P., and Izaurralde, E. (2006). mRNA degradation by miRNAs and GW182 requires both CCR4:NOT deadenylase and DCP1:DCP2 decapping complexes. Genes & Development *20*, 1885-1898.

Berezikov, E., Chung, W.J., Willis, J., Cuppen, E., and Lai, E.C. (2007). Mammalian mirtron genes. Molecular Cell *28*, 328-336.

Bernardos, R.L., Lentz, S.I., Wolfe, M.S., and Raymond, P.A. (2005). Notch-Delta signaling is required for spatial patterning and Müller glia differentiation in the zebrafish retina. Developmental Biology *278*, 381-395.

Bernardos, R.L., and Raymond, P.A. (2006). GFAP transgenic zebrafish. Gene Expression Patterns *6*, 1007-1013.

Bernstein, E., Caudy, A.A., Hammond, S.M., and Hannon, G.J. (2001). Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature *409*, 363-366.

Bohnsack, M.T., Czaplinski, K., and Gorlich, D. (2004). Exportin 5 is a RanGTP-dependent dsRNA-binding protein that mediates nuclear export of pre-miRNAs. RNA *10*, 185-191.

Braun, J.E., Huntzinger, E., Fauser, M., and Izaurralde, E. (2011). GW182 Proteins Directly Recruit Cytoplasmic Deadenylase Complexes to miRNA Targets. Molecular Cell *44*, 120-133.

Brennecke, J., Stark, A., Russell, R.B., and Cohen, S.M. (2005). Principles of microRNA-target recognition. PLoS Biology *3*, e85.

Brockerhoff, S.E., Hurley, J.B., Janssen-Bienhold, U., Neuhauss, S.C., Driever, W., and Dowling, J.E. (1995). A behavioral screen for isolating zebrafish mutants with visual system defects. Proceedings of the National Academy of Sciences of the United States of America *92*, 10545-10549.

Cai, X., Hagedorn, C.H., and Cullen, B.R. (2004). Human microRNAs are processed from capped, polyadenylated transcripts that can also function as mRNAs. RNA *10*, 1957-1966.

Cerutti, L., Mian, N., and Bateman, A. (2000). Domains in gene silencing and cell differentiation proteins: the novel PAZ domain and redefinition of the Piwi domain. Trends Biochem Sci *25*, 481-482.

Chekulaeva, M., Mathys, H., Zipprich, J.T., Attig, J., Colic, M., Parker, R., and Filipowicz, W. (2011). miRNA repression involves GW182-mediated recruitment of CCR4-NOT through conserved W-containing motifs. Nature Structural & Molecular Biology *18*, 1218-1226.

Cheloufi, S., Dos Santos, C., Chong, M., and Hannon, G. (2010). A dicer-independent miRNA biogenesis pathway that requires Ago catalysis. Nature *465*, 584-589.

Chen, J.S., Pedro, M.S., and Zeller, R.W. (2011). miR-124 function during Ciona intestinalis neuronal development includes extensive interaction with the Notch signaling pathway. Development *138*, 4943-4953.

Chen, Y., Wu, B., Xu, L., Li, H., Xia, J., Yin, W., Li, Z., Shi, D., Li, S., Lin, S., *et al.* (2012). A SNX10/V-ATPase pathway regulates ciliogenesis in vitro and in vivo. Cell Research *22*, 333-345.

Chendrimada, T., Gregory, R., Kumaraswamy, E., Norman, J., Cooch, N., Nishikura, K., and Shiekhattar, R. (2005). TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing. Nature *436*, 740-744.

Chendrimada, T.P., Finn, K.J., Ji, X., Baillat, D., Gregory, R.I., Liebhaber, S.A., Pasquinelli, A.E., and Shiekhattar, R. (2007). MicroRNA silencing through RISC recruitment of eIF6. Nature *447*, 823-828.

Chi, S.W., Hannon, G.J., and Darnell, R.B. (2012). An alternative mode of microRNA target recognition. Nature Structural & Molecular Biology *19*, 321-327.

Choi, J., Dong, L., Ahn, J., Dao, D., Hammerschmidt, M., Chen, J.N., 2007. FoxH1 negatively modulates flk1 gene expression and vascular formation in zebrafish. Developmental Biology 304, 735-744.

Chong, M.M., Zhang, G., Cheloufi, S., Neubert, T.A., Hannon, G.J., and Littman, D.R. (2010). Canonical and alternate functions of the microRNA biogenesis machinery. Genes & Development *24*, 1951-1960.

Chung, W.J., Okamura, K., Martin, R., and Lai, E.C. (2008). Endogenous RNA interference provides a somatic defense against Drosophila transposons. Current Biology *18*, 795-802.

Cifuentes, D., Xue, H., Taylor, D.W., Patnode, H., Mishima, Y., Cheloufi, S., Ma, E., Mane, S., Hannon, G.J., Lawson, N.D., *et al.* (2010). A novel miRNA processing pathway independent of Dicer requires Argonaute2 catalytic activity. Science *328*, 1694-1698.

Coolen, M., Thieffry, D., Drivenes, O., Becker, T.S., and Bally-Cuif, L. (2012). miR-9 controls the timing of neurogenesis through the direct inhibition of antagonistic factors. Developmental Cell *22*, 1052-1064.

Cullen, P.J. (2008). Endosomal sorting and signalling: an emerging role for sorting nexins. Nature Reviews Molecular Cell Biology *9*, 574-582.

Cullen, P.J., and Korswagen, H.C. (2011). Sorting nexins provide diversity for retromer-dependent trafficking events. Nature *14*, 29-37.

Decembrini, S., Bressan, D., Vignali, R., Pitto, L., Mariotti, S., Rainaldi, G., Wang, X., Evangelista, M., Barsacchi, G., and Cremisi, F. (2009). MicroRNAs couple cell fate and developmental timing in retina. Proceedings of the National Academy of Sciences of the United States of America *106*, 21179-21184.

Denli, A.M., Tops, B.B.J., Plasterk, R.H.A., Ketting, R.F., and Hannon, G.J. (2004). Processing of primary microRNAs by the Microprocessor complex. Nature *432*, 231-235.

Dexter, J.S. (1914). The Analysis of a Case of Continuous Variation in Drosophila by a Study of Its Linkage Relations. The American Naturalist *48*, 712-758.

Ding, L., Spencer, A., Morita, K., and Han, M. (2005). The developmental timing regulator AIN-1 interacts with miRISCs and may target the argonaute protein ALG-1 to cytoplasmic P bodies in C. elegans. Molecular Cell *19*, 437-447.

Djuranovic, S., Nahvi, A., and Green, R. (2012). miRNA-mediated gene silencing by translational repression followed by mRNA deadenylation and decay. Science *336*, 237-240.

Djuranovic, S., Zinchenko, M.K., Hur, J.K., Nahvi, A., Brunelle, J.L., Rogers, E.J., and Green, R. (2010). Allosteric regulation of Argonaute proteins by miRNAs. Nature Structural & Molecular Biology *17*, 144-150.

Doench, J.G., Petersen, C.P., and Sharp, P.A. (2003). siRNAs can function as miRNAs. Genes & Development *17*, 438-442.

Doench, J.G., and Sharp, P.A. (2004). Specificity of microRNA target selection in translational repression. Genes & Development *18*, 504-511.

Easter, S.S., and Malicki, J.J. (2002). The zebrafish eye: developmental and genetic analysis. Results and Problems in Cell Differentiation *40*, 346-370.

Eckfeldt, C.E., Mendenhall, E.M., Flynn, C.M., Wang, T.-F., Pickart, M.A., Grindle, S.M., Ekker, S.C., and Verfaillie, C.M. (2005). Functional analysis of

human hematopoietic stem cell gene expression using zebrafish. PLoS Biology *3*, e254.

Elbashir, S.M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., and Tuschl, T. (2001a). Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature *411*, 494-498.

Elbashir, S.M., Lendeckel, W., and Tuschl, T. (2001b). RNA interference is mediated by 21- and 22-nucleotide RNAs. Genes & Development *15*, 188-200.

Enright, A.J., John, B., Gaul, U., Tuschl, T., Sander, C., and Marks, D.S. (2003). MicroRNA targets in Drosophila. Genome Biology *5*, R1.

Eulalio, A., Huntzinger, E., and Izaurralde, E. (2008). GW182 interaction with Argonaute is essential for miRNA-mediated translational repression and mRNA decay. Nature Structural & Molecular Biology *15*, 346-353.

Eulalio, A., Huntzinger, E., Nishihara, T., Rehwinkel, J., Fauser, M., and Izaurralde, E. (2009). Deadenylation is a widespread effect of miRNA regulation. RNA *15*, 21-32.

Fabian, M.R., Cieplak, M.K., Frank, F., Morita, M., Green, J., Srikumar, T., Nagar, B., Yamamoto, T., Raught, B., Duchaine, T.F., *et al.* (2011). miRNA-mediated deadenylation is orchestrated by GW182 through two conserved motifs that interact with CCR4-NOT. Nature Structural & Molecular Biology *18*, 1211-1217.

Fabian, M.R., Sonenberg, N., and Filipowicz, W. (2010). Regulation of mRNA translation and stability by microRNAs. Annual Review of Biochemistry *79*, 351-379.

Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E., and Mello, C.C. (1998). Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature *391*, 806-811.

Flicek, P., Amode, M.R., Barrell, D., Beal, K., Billis, K., Brent, S., Carvalho-Silva, D., Clapham, P., Coates, G., Fitzgerald, S., *et al.* (2014). Ensembl 2014. Nucleic Acids Res *42*, D749-755.

Flynt, A., Li, N., Thatcher, E., Solnica-Krezel, L., and Patton, J. (2007). Zebrafish miR-214 modulates Hedgehog signaling to specify muscle cell fate. Nature Genetics *39*, 259-263.

Flynt, A., Thatcher, E., Burkewitz, K., Li, N., Liu, Y., and Patton, J. (2009). miR-8 microRNAs regulate the response to osmotic stress in zebrafish embryos. The Journal of Cell Biology *185*, 115-127.

Flynt, A.S., Greimann, J., Chung, W., Lima, C., and Lai, E.C. (2010). MicroRNA Biogenesis via Splicing and Exosome-Mediated Trimming in Drosophila. Molecular Cell.

Förstemann, K., Tomari, Y., Du, T., Vagin, V.V., Denli, A.M., Bratu, D.P., Klattenhoff, C., Theurkauf, W.E., and Zamore, P.D. (2005). Normal microRNA maturation and germ-line stem cell maintenance requires Loquacious, a double-stranded RNA-binding domain protein. PLoS Biology *3*, e236.

Fortini, M.E. (2009). Notch signaling: the core pathway and its posttranslational regulation. Developmental Cell *16*, 633-647.

Gaidatzis, D., van Nimwegen, E., Hausser, J., and Zavolan, M. (2007). Inference of miRNA targets using evolutionary conservation and pathway analysis. BMC Bioinformatics *8*, 69.

Gao, F.B. (2010). Context-dependent functions of specific microRNAs in neuronal development. Neural Development *5*, 25.

Georgi, S.A., and Reh, T.A. (2010). Dicer is required for the transition from early to late progenitor state in the developing mouse retina. The Journal of neuroscience : the official journal of the Society for Neuroscience *30*, 4048-4061.

Gerety, S.S., Wilkinson, D.G. (2011). Morpholino artifacts provide pitfalls and reveal a novel role for pro-apoptotic genes in hindbrain boundary development. Developmental Biology 350, 279-289.

Giraldez, A.J., Cinalli, R.M., Glasner, M.E., Enright, A.J., Thomson, J.M., Baskerville, S., Hammond, S.M., Bartel, D., and Schier, A.F. (2005). MicroRNAs regulate brain morphogenesis in zebrafish. Science *308*, 833-838.

Giraldez, A.J., Mishima, Y., Rihel, J., Grocock, R.J., van Dongen, S., Inoue, K., Enright, A.J., and Schier, A.F. (2006). Zebrafish MiR-430 promotes deadenylation and clearance of maternal mRNAs. Science *312*, 75-79.

Glazov, E.A., Cottee, P.A., Barris, W.C., Moore, R.J., Dalrymple, B.P., and Tizard, M.L. (2008). A microRNA catalog of the developing chicken embryo identified by a deep sequencing approach. Genome Research *18*, 957-964.

Gregory, R.I., Chendrimada, T.P., Cooch, N., and Shiekhattar, R. (2005). Human RISC couples microRNA biogenesis and posttranscriptional gene silencing. Cell *123*, 631-640.

Gregory, R.I., Yan, K.-P., Amuthan, G., Chendrimada, T., Doratotaj, B., Cooch, N., and Shiekhattar, R. (2004). The Microprocessor complex mediates the genesis of microRNAs. Nature *432*, 235-240.

Griffiths-Jones, S., Saini, H.K., van Dongen, S., and Enright, A.J. (2008). miRBase: tools for microRNA genomics. Nucleic Acids Res *36*, D154-158.

Grimson, A., Farh, K.K.-H., Johnston, W.K., Garrett-Engele, P., Lim, L.P., and Bartel, D.P. (2007). MicroRNA targeting specificity in mammals: determinants beyond seed pairing. Molecular Cell *27*, 91-105.

Grishok, A., Pasquinelli, A.E., Conte, D., Li, N., Parrish, S., Ha, I., Baillie, D.L., Fire, A., Ruvkun, G., and Mello, C.C. (2001). Genes and mechanisms related to RNA interference regulate expression of the small temporal RNAs that control C. elegans developmental timing. Cell *106*, 23-34.

Gu, S., Jin, L., Zhang, Y., Huang, Y., Zhang, F., Valdmanis, Paul N., and Kay, Mark A. (2012). The Loop Position of shRNAs and Pre-miRNAs Is Critical for the Accuracy of Dicer Processing In Vivo. Cell *151*, 900-911.

Gu, S., and Kay, M.A. (2010). How do miRNAs mediate translational repression? Silence *1*, 11.

Guo, H., Ingolia, N.T., Weissman, J.S., and Bartel, D.P. (2010). Mammalian microRNAs predominantly act to decrease target mRNA levels. Nature *466*, 835-840.

Haase, A.D., Jaskiewicz, L., Zhang, H., Lainé, S., Sack, R., Gatignol, A., and Filipowicz, W. (2005). TRBP, a regulator of cellular PKR and HIV-1 virus expression, interacts with Dicer and functions in RNA silencing. EMBO Rep *6*, 961-967.

Hamilton, A.J., and Baulcombe, D.C. (1999). A species of small antisense RNA in posttranscriptional gene silencing in plants. Science *286*, 950-952.

Hammond, S., Boettcher, S., Caudy, A., Kobayashi, R., Hannon, G. (2001). Argonaute2, a Link Between Genetic and Biochemical Analyses of RNAi. Science *293*, 1146-1150.

Hammond, S.M., Bernstein, E., Beach, D., and Hannon, G.J. (2000). An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells. Nature *404*, 293-296.

Han, J., Lee, Y., Yeom, K.-H., Nam, J.-W., Heo, I., Rhee, J.-K., Sohn, S.Y., Cho, Y., Zhang, B.-T., and Kim, V.N. (2006). Molecular basis for the recognition of primary microRNAs by the Drosha-DGCR8 complex. Cell *125*, 887-901.

Han, J., Lee, Y., Yeom, K.H., Kim, Y.K., Jin, H., and Kim, V.N. (2004). The Drosha-DGCR8 complex in primary microRNA processing. Genes & Development *18*, 3016-3027.

Harterink, M., Port, F., Lorenowicz, M.J., McGough, I.J., Silhankova, M., Betist, M.C., van Weering, J.R.T., van Heesbeen, R.G.H.P., Middelkoop, T.C., Basler, K*., et al.* (2011). A SNX3-dependent retromer pathway mediates retrograde transport of the Wnt sorting receptor Wntless and is required for Wnt secretion. Nature *13*, 914-923.

He, L., and Hannon, G.J. (2004). MicroRNAs: small RNAs with a big role in gene regulation. Nature Reviews Genetics *5*, 522-531.

Hitchcock, P.F., and Raymond, P.A. (2004). The teleost retina as a model for developmental and regeneration biology. Zebrafish *1*, 257-271.

Houbaviy, H.B., Murray, M.F., and Sharp, P.A. (2003). Embryonic stem cell-specific MicroRNAs. Developmental Cell *5*, 351-358.

Hu, M., and Easter, S.S. (1999). Retinal neurogenesis: the formation of the initial central patch of postmitotic cells. Developmental Biology *207*, 309-321.

Humphreys, D.T., Westman, B.J., Martin, D.I.K., and Preiss, T. (2005). MicroRNAs control translation initiation by inhibiting eukaryotic initiation factor 4E/cap and poly(A) tail function. Proceedings of the National Academy of Sciences of the United States of America *102*, 16961-16966.

Huntzinger, E., and Izaurralde, E. (2011). Gene silencing by microRNAs: contributions of translational repression and mRNA decay. Nature Reviews Genetics *12*, 99-110.

Hutvágner, G., McLachlan, J., Pasquinelli, A.E., Bálint, E., Tuschl, T., and Zamore, P.D. (2001). A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA. Science *293*, 834-838.

Hutvágner, G., and Zamore, P.D. (2002). A microRNA in a multiple-turnover RNAi enzyme complex. Science *297*, 2056-2060.

Itoh, M., Kim, C.-H., Palardy, G., Oda, T., Jiang, Y.-J., Maust, D., Yeo, S.-Y., Lorick, K., Wright, G.J., Ariza-McNaughton, L.*, et al.* (2003). Mind bomb is a ubiquitin ligase that is essential for efficient activation of Notch signaling by Delta. Developmental Cell *4*, 67-82.

Jakymiw, A., Lian, S., Eystathioy, T., Li, S., Satoh, M., Hamel, J.C., Fritzler, M.J., and Chan, E.K. (2005). Disruption of GW bodies impairs mammalian RNA interference. Nature Cell Biology *7*, 1267-1274.

Jiang, F., Ye, X., Liu, X., Fincher, L., McKearin, D., and Liu, Q. (2005). Dicer-1 and R3D1-L catalyze microRNA maturation in Drosophila. Genes & Development *19*, 1674-1679.

Jin, Z.-B., Hirokawa, G., Gui, L., Takahashi, R., Osakada, F., Hiura, Y., Takahashi, M., Yasuhara, O., and Iwai, N. (2009). Targeted deletion of miR-182, an abundant retinal microRNA. Molecular Vision *15*, 523-533.

Kapsimali, M., Kloosterman, W.P., de Bruijn, E., Rosa, F., Plasterk, R.H., and Wilson, S.W. (2007). MicroRNAs show a wide diversity of expression profiles in the developing and mature central nervous system. Genome Biology *8*, R173.

Ketting, R.F., Fischer, S.E., Bernstein, E., Sijen, T., Hannon, G.J., and Plasterk, R.H.A. (2001). Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in C. elegans. Genes & Development *15*, 2654-2659.

Khvorova, A., Reynolds, A., and Jayasena, S.D. (2003). Functional siRNAs and miRNAs exhibit strand bias. Cell *115*, 209-216.

Kim, Y.-K., and Kim, V.N. (2007). Processing of intronic microRNAs. EMBO J *26*, 775-783.

Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B., and Schilling, T.F. (1995). Stages of embryonic development of the zebrafish. Developmental Dynamics *203*, 253-310.

Kinch, L.N., and Grishin, N.V. (2009). The human Ago2 MC region does not contain an eIF4E-like mRNA cap binding motif. Biology Direct *4*, 2.

Kiriakidou, M., Nelson, P.T., Kouranov, A., Fitziev, P., Bouyioukos, C., Mourelatos, Z., and Hatzigeorgiou, A. (2004). A combined computational-experimental approach predicts human microRNA targets. Genes & Development *18*, 1165-1178.

Kiriakidou, M., Tan, G.S., Lamprinaki, S., De Planell-Saguer, M., Nelson, P.T., and Mourelatos, Z. (2007). An mRNA m7G cap binding-like motif within human Ago2 represses translation. Cell *129*, 1141-1151.

Koharudin, L.M.I., Furey, W., Liu, H., Liu, Y.-J., and Gronenborn, A.M. (2009). The phox domain of sorting nexin 5 lacks phosphatidylinositol 3-phosphate (PtdIns(3)P) specificity and preferentially binds to phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2). The Journal of Biological Chemistry *284*, 23697-23707.

Kwon, C., Han, Z., Olson, E.N., and Srivastava, D. (2005). MicroRNA1 influences cardiac differentiation in Drosophila and regulates Notch signaling. Proceedings of the National Academy of Sciences of the United States of America *102*, 18986-18991.

La Torre, A., Georgi, S., and Reh, T.A. (2013). Conserved microRNA pathway regulates developmental timing of retinal neurogenesis. Proceedings of the National Academy of Sciences of the United States of America *110*, E2362-2370.

Lagendijk, A.K., Moulton, J.D., and Bakkers, J. (2012). Revealing details: whole mount microRNA in situ hybridization protocol for zebrafish embryos and adult tissues. Biology Open *1*, 566-569.

Lagos-Quintana, M., Rauhut, R., Lendeckel, W., and Tuschl, T. (2001). Identification of novel genes coding for small expressed RNAs. Science *294*, 853-858.

Lagos-Quintana, M., Rauhut, R., Meyer, J., Borkhardt, A., and Tuschl, T. (2003). New microRNAs from mouse and human. RNA *9*, 175-179.

Lagos-Quintana, M., Rauhut, R., Yalcin, A., Meyer, J., Lendeckel, W., and Tuschl, T. (2002). Identification of tissue-specific microRNAs from mouse. Current Biology *12*, 735-739.

Lai, E.C., Tam, B., and Rubin, G.M. (2005). Pervasive regulation of Drosophila Notch target genes by GY-box-, Brd-box-, and K-box-class microRNAs. Genes & Development *19*, 1067-1080.

Landthaler, M., Yalcin, A., and Tuschl, T. (2004). The human DiGeorge syndrome critical region gene 8 and Its D. melanogaster homolog are required for miRNA biogenesis. Current Biology *14*, 2162-2167.

Lau, N.C., Lim, L.P., Weinstein, E.G., and Bartel, D.P. (2001). An abundant class of tiny RNAs with probable regulatory roles in Caenorhabditis elegans. Science *294*, 858-862.

Lawson, N.D., Scheer, N., Pham, V.N., Kim, C.H., Chitnis, A.B., Campos-Ortega, J.A., and Weinstein, B.M. (2001). Notch signaling is required for arterial-venous differentiation during embryonic vascular development. Development *128*, 3675-3683.

Le Borgne, R., and Schweisguth, F. (2003). Notch signaling: endocytosis makes delta signal better. Current Biology *13*, R273-275.

Lee, R.C., and Ambros, V. (2001). An extensive class of small RNAs in Caenorhabditis elegans. Science *294*, 862-864.

Lee, R.C., Feinbaum, R.L., and Ambros, V. (1993). The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell *75*, 843-854.

Lee, Y., Ahn, C., Han, J., Choi, H., Kim, J., Yim, J., Lee, J., Provost, P., Rådmark, O., Kim, S.*, et al.* (2003). The nuclear RNase III Drosha initiates microRNA processing. Nature *425*, 415-419.

Lee, Y., Jeon, K., Lee, J.-T., Kim, S., and Kim, V.N. (2002). MicroRNA maturation: stepwise processing and subcellular localization. EMBO J *21*, 4663-4670.

Lee, Y., Kim, M., Han, J., Yeom, K.-H., Lee, S., Baek, S.H., and Kim, V.N. (2004). MicroRNA genes are transcribed by RNA polymerase II. EMBO J *23*, 4051-4060.

Lewis, B.P., Burge, C.B., and Bartel, D.P. (2005). Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell *120*, 15-20.

Lewis, B.P., Shih, I.-h., Jones-Rhoades, M.W., Bartel, D.P., and Burge, C.B. (2003). Prediction of mammalian microRNA targets. Cell *115*, 787-798.

Li, N., Flynt, A., Kim, H.R., Solnica-Krezel, L., and Patton, J. (2008). Dispatched Homolog 2 is targeted by miR-214 through a combination of three weak microRNA recognition sites. Nucleic Acids Research *36*, 4277-4285.

Li, N., Wei, C., Olena, A.F., and Patton, J.G. (2011). Regulation of endoderm formation and left-right asymmetry by miR-92 during early zebrafish development. Development *138*, 1817-1826.

Li, Z., Joseph, N.M., and Easter, S.S. (2000). The morphogenesis of the zebrafish eye, including a fate map of the optic vesicle. Developmental Dynamics *218*, 175-188.

Lim, L.P., Glasner, M.E., Yekta, S., Burge, C.B., and Bartel, D. (2003a). Vertebrate microRNA genes. Science *299*, 1540.

Lim, L.P., Lau, N.C., Garrett-Engele, P., Grimson, A., Schelter, J.M., Castle, J., Bartel, D.P., Linsley, P.S., and Johnson, J.M. (2005). Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. Nature *433*, 769-773.

Lim, L.P., Lau, N.C., Weinstein, E.G., Abdelhakim, A., Yekta, S., Rhoades, M.W., Burge, C.B., and Bartel, D.P. (2003b). The microRNAs of Caenorhabditis elegans. Genes & Development *17*, 991-1008.

Lingel, A., Simon, B., Izaurralde, E., and Sattler, M. (2003). Structure and nucleic-acid binding of the Drosophila Argonaute 2 PAZ domain. Nature *426*, 465-469.

Lingel, A., Simon, B., Izaurralde, E., and Sattler, M. (2004). Nucleic acid 3'-end recognition by the Argonaute2 PAZ domain. Nature Structural & Molecular Biology *11*, 576-577.

Liu, J. (2004). Argonaute2 Is the Catalytic Engine of Mammalian RNAi. Science *305*, 1437-1441.

Liu, J., Rivas, F.V., Wohlschlegel, J., Yates, J.R., 3rd, Parker, R., and Hannon, G.J. (2005). A role for the P-body component GW182 in microRNA function. Nature Cell Biology *7*, 1261-1266.

Liu, X., Jin, D.-Y., McManus, M.T., and Mourelatos, Z. (2012). Precursor MicroRNA-Programmed Silencing Complex Assembly Pathways in Mammals. Molecular Cell *46*, 507-517.

Loeb, Gabriel B., Khan, Aly A., Canner, D., Hiatt, Joseph B., Shendure, J., Darnell, Robert B., Leslie, Christina S., and Rudensky, Alexander Y. (2012). Transcriptome-wide miR-155 Binding Map Reveals Widespread Noncanonical MicroRNA Targeting. Molecular Cell *48*, 760-770.

Louvi, A., and Artavanis-Tsakonas, S. (2006). Notch signalling in vertebrate neural development. Nature Reviews Neuroscience *7*, 93-102.

Lund, E., Güttinger, S., Calado, A., Dahlberg, J.E., and Kutay, U. (2004). Nuclear export of microRNA precursors. Science *303*, 95-98.

Ma, J.B., Ye, K., and Patel, D.J. (2004). Structural basis for overhang-specific small interfering RNA recognition by the PAZ domain. Nature *429*, 318-322.

MacRae, I.J., Ma, E., Zhou, M., Robinson, C.V., and Doudna, J.A. (2008). In vitro reconstitution of the human RISC-loading complex. Proceedings of the National Academy of Sciences of the United States of America *105*, 512-517.

MacRae, I.J., Zhou, K., Li, F., Repic, A., Brooks, A.N., Cande, W.Z., Adams, P.D., and Doudna, J.A. (2006). Structural Basis for Double-Stranded RNA Processing by Dicer. Science *311*, 195-198.

Malicki, J., Neuhauss, S.C., Schier, A.F., Solnica-Krezel, L., Stemple, D.L., Stainier, D.Y., Abdelilah, S., Zwartkruis, F., Rangini, Z., and Driever, W. (1996). Mutations affecting development of the zebrafish retina. Development *123*, 263-273.

Maniataki, E., and Mourelatos, Z. (2005). A human, ATP-independent, RISC assembly machine fueled by pre-miRNA. Genes & Development *19*, 2979-2990.

Marcet, B., Chevalier, B., Coraux, C., Kodjabachian, L., and Barbry, P. (2011). MicroRNA-based silencing of Delta/Notch signaling promotes multiple cilia formation. Cell Cycle *10*, 2858-2864.

Maroney, P.A., Yu, Y., Fisher, J., and Nilsen, T.W. (2006). Evidence that microRNAs are associated with translating messenger RNAs in human cells. Nature Structural & Molecular Biology *13*, 1102-1107.

Martinez, J., Patkaniowska, A., Urlaub, H., Lührmann, R., and Tuschl, T. (2002). Single-stranded antisense siRNAs guide target RNA cleavage in RNAi. Cell *110*, 563-574.

Mathonnet, G., Fabian, M.R., Svitkin, Y.V., Parsyan, A., Huck, L., Murata, T., Biffo, S., Merrick, W.C., Darzynkiewicz, E., Pillai, R.S., *et al.* (2007). MicroRNA inhibition of translation initiation in vitro by targeting the cap-binding complex eIF4F. Science *317*, 1764-1767.

Matsuda, M., and Chitnis, A.B. (2009). Interaction with Notch determines endocytosis of specific Delta ligands in zebrafish neural tissue. Development *136*, 197-206.

Meister, G., Landthaler, M., Peters, L., Chen, P.Y., Urlaub, H., Luhrmann, R., and Tuschl, T. (2005). Identification of novel argonaute-associated proteins. Current Biology *15*, 2149-2155.

Mercer, T.R., Dinger, M.E., and Mattick, J.S. (2009). Long non-coding RNAs: insights into functions. Nature Reviews Genetics *10*, 155-159.

Mishima, Y., Abreu-Goodger, C., Staton, A.A., Stahlhut, C., Shou, C., Cheng, C., Gerstein, M., Enright, A.J., and Giraldez, A.J. (2009). Zebrafish miR-1 and miR-133 shape muscle gene expression and regulate sarcomeric actin organization. Genes & Development *23*, 619-632.

Mohr, O.L. (1919). Character Changes Caused by Mutation of an Entire Region of a Chromosome in Drosophila. Genetics *4*, 275-282.

Morlando, M., Ballarino, M., Gromak, N., Pagano, F., Bozzoni, I., and Proudfoot, N.J. (2008). Primary microRNA transcripts are processed co-transcriptionally. Nature Structural & Molecular Biology *15*, 902-909.

Mourelatos, Z., Dostie, J., Paushkin, S., Sharma, A., Charroux, B., Abel, L., Rappsilber, J., Mann, M., and Dreyfuss, G. (2002). miRNPs: a novel class of ribonucleoproteins containing numerous microRNAs. Genes & Development *16*, 720-728.

Nicoli, S., Knyphausen, C.P., Zhu, L.J., Lakshmanan, A., and Lawson, N.D. (2012). miR-221 is required for endothelial tip cell behaviors during vascular development. Developmental Cell *22*, 418-429.

Nielsen, C.B., Shomron, N., Sandberg, R., Hornstein, E., Kitzman, J., and Burge, C.B. (2007). Determinants of targeting by endogenous and exogenous microRNAs and siRNAs. RNA *13*, 1894-1910.

Nottrott, S., Simard, M.J., and Richter, J.D. (2006). Human let-7a miRNA blocks protein production on actively translating polyribosomes. Nature Structural & Molecular Biology *13*, 1108-1114.

Okamura, K., Hagen, J.W., Duan, H., Tyler, D.M., and Lai, E.C. (2007). The mirtron pathway generates microRNA-class regulatory RNAs in Drosophila. Cell *130*, 89-100.

Olena, A.F., and Patton, J.G. (2009). Genomic organization of microRNAs. Journal of Cellular Physiology *222*, 540-545.

Olsen, P.H., and Ambros, V. (1999). The lin-4 regulatory RNA controls developmental timing in Caenorhabditis elegans by blocking LIN-14 protein synthesis after the initiation of translation. Developmental Biology *216*, 671-680.

Ozbudak, E.M., and Lewis, J. (2008). Notch signalling synchronizes the zebrafish segmentation clock but is not needed to create somite boundaries. PLoS Genetics *4*, e15.

Park, J.E., Heo, I., Tian, Y., Simanshu, D.K., Chang, H., Jee, D., Patel, D.J., and Kim, V.N. (2011). Dicer recognizes the 5' end of RNA for efficient and accurate processing. Nature *475*, 201-205.

Parks, A.L., Klueg, K.M., Stout, J.R., and Muskavitch, M.A. (2000). Ligand endocytosis drives receptor dissociation and activation in the Notch pathway. Development *127*, 1373-1385.

Parrish, S., Fleenor, J., Xu, S., Mello, C., and Fire, A. (2000). Functional anatomy of a dsRNA trigger: differential requirement for the two trigger strands in RNA interference. Mol Cell *6*, 1077-1087.

Parsons, M.J., Pisharath, H., Yusuff, S., Moore, J.C., Siekmann, A.F., Lawson, N.D., and Leach, S.D. (2009). Notch-responsive cells initiate the secondary transition in larval zebrafish pancreas. Mechanisms of Development *126*, 898-912.

Pasquinelli, A.E., Reinhart, B.J., Slack, F.J., Martindale, M.Q., Kuroda, M.I., Maller, B., Hayward, D.C., Ball, E.E., Degnan, B., Müller, P*., et al.* (2000). Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA. Nature *408*, 86-89.

Pawlicki, J.M., and Steitz, J.A. (2008). Primary microRNA transcript retention at sites of transcription leads to enhanced microRNA production. Journal of Cell Biology *182*, 61-76.

Petersen, C.P., Bordeleau, M.-E., Pelletier, J., and Sharp, P.A. (2006). Short RNAs repress translation after initiation in mammalian cells. Molecular Cell *21*, 533-542.

Pillai, R.S., Artus, C.G., and Filipowicz, W. (2004). Tethering of human Ago proteins to mRNA mimics the miRNA-mediated repression of protein synthesis. RNA *10*, 1518-1525.

Pillai, R.S., Bhattacharyya, S.N., Artus, C.G., Zoller, T., Cougot, N., Basyuk, E., Bertrand, E., and Filipowicz, W. (2005). Inhibition of translational initiation by Let-7 MicroRNA in human cells. Science *309*, 1573-1576.

Poulson, D.F. (1937). Chromosomal Deficiencies and the Embryonic Development of Drosophila Melanogaster. tl Acad Sci USA *23*, 133-137.

Poulton, J.S., Huang, Y.C., Smith, L., Sun, J., Leake, N., Schleede, J., Stevens, L.M., and Deng, W.M. (2011). The microRNA pathway regulates the temporal pattern of Notch signaling in Drosophila follicle cells. Development *138*, 1737-1745.

Provost, P., Dishart, D., Doucet, J., Frendewey, D., Samuelsson, B., and Radmark, O. (2002). Ribonuclease activity and RNA binding of recombinant human Dicer. EMBO J *21*, 5864-5874.

Rehwinkel, J.A.N., Behm-Ansmant, I., Gatfield, D., and Izaurralde, E. (2005). A crucial role for GW182 and the DCP1:DCP2 decapping complex in miRNA-mediated gene silencing. RNA *11*, 1640-1647.

Reinhart, B.J., Slack, F.J., Basson, M., Pasquinelli, A.E., Bettinger, J.C., Rougvie, A.E., Horvitz, H.R., and Ruvkun, G. (2000). The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans. Nature *403*, 901-906.

Reinhart, B.J., Weinstein, E.G., Rhoades, M.W., Bartel, B., and Bartel, D.P. (2002). MicroRNAs in plants. Genes & Development *16*, 1616-1626.

Ruby, J.G., Jan, C.H., and Bartel, D.P. (2007). Intronic microRNA precursors that bypass Drosha processing. Nature *448*, 83-86.

Rupp, R.A., Snider, L., and Weintraub, H. (1994). Xenopus embryos regulate the nuclear localization of XMyoD. Genes & Development *8*, 1311-1323.

Rutenberg, J., Fischer, A., Jia, H., Gessler, M., Zhong, T., and Mercola, M. (2006). Developmental patterning of the cardiac atrioventricular canal by Notch and Hairy-related transcription factors. Development *133*, 4381-4390.

Ruvkun, G., and Giusto, J. (1989). The Caenorhabditis elegans heterochronic gene lin-14 encodes a nuclear protein that forms a temporal developmental switch. Nature *338*, 313-319.

Saetrom, P., Heale, B.S., Snove, O., Jr., Aagaard, L., Alluin, J., and Rossi, J.J. (2007). Distance constraints between microRNA target sites dictate efficacy and cooperativity. Nucleic Acids Res *35*, 2333-2342.

Saito, K., Ishizuka, A., Siomi, H., and Siomi, M.C. (2005). Processing of pre-microRNAs by the Dicer-1-Loquacious complex in Drosophila cells. PLoS Biology *3*, e235.

Saxena, S., Jonsson, Z.O., and Dutta, A. (2003). Small RNAs with imperfect match to endogenous mRNA repress translation. Implications for off-target activity of small inhibitory RNA in mammalian cells. The Journal of Biological Chemistry *278*, 44312-44319.

Scheer, N., Groth, A., Hans, S., and Campos-Ortega, J.A. (2001). An instructive function for Notch in promoting gliogenesis in the zebrafish retina. Development *128*, 1099-1107.

Schmitt, E.A., and Dowling, J.E. (1994). Early eye morphogenesis in the zebrafish, Brachydanio rerio. J Comp Neurol *344*, 532-542.

Schwarz, D.S., Hutvágner, G., Du, T., Xu, Z., Aronin, N., and Zamore, P.D. (2003). Asymmetry in the assembly of the RNAi enzyme complex. Cell *115*, 199-208.

Seggerson, K., Tang, L., and Moss, E.G. (2002). Two genetic circuits repress the Caenorhabditis elegans heterochronic gene lin-28 after translation initiation. Developmental Biology *243*, 215-225.

Sen, G.L., and Blau, H.M. (2005). Argonaute 2/RISC resides in sites of mammalian mRNA decay known as cytoplasmic bodies. Nature Cell Biology *7*, 633.

Shin, C., Nam, J.-W., Farh, K.K.-H., Chiang, H.R., Shkumatava, A., and Bartel, D.P. (2010). Expanding the MicroRNA Targeting Code: Functional Sites with Centered Pairing. Molecular Cell *38*, 789-802.

Slack, F.J., Basson, M., Liu, Z., Ambros, V., Horvitz, H.R., and Ruvkun, G. (2000). The lin-41 RBCC gene acts in the C. elegans heterochronic pathway between the let-7 regulatory RNA and the LIN-29 transcription factor. Mol Cell *5*, 659-669.

Song, J., Liu, J., Tolia, N., Schneiderman, J., Smith, S.K., Martienssen, R.A., Hannon, G., and Joshua-Tor, L. (2003). The crystal structure of the Argonaute2 PAZ domain reveals an RNA binding motif in RNAi effector complexes. Nature Structural & Molecular Biology *10*, 1026-1032.

Song, J., Smith, S.K., Hannon, G., and Joshua-Tor, L. (2004). Crystal structure of Argonaute and its implications for RISC slicer activity. Science *305*, 1434-1437.

Stahlhut, C., Suarez, Y., Lu, J., Mishima, Y., and Giraldez, A.J. (2012). miR-1 and miR-206 regulate angiogenesis by modulating VegfA expression in zebrafish. Development *139*, 4356-4364.

Steinberg, F., Heesom, K.J., Bass, M.D., and Cullen, P.J. (2012). SNX17 protects integrins from degradation by sorting between lysosomal and recycling pathways. Journal of Cell Biology *197*, 219-230.

Stenkamp, D. (2007). Neurogenesis in the fish retina. Int Rev Cytol *259*, 173-224.

Streisinger, G., Walker, C., Dower, N., Knauber, D., and Singer, F. (1981). Production of clones of homozygous diploid zebra fish (Brachydanio rerio). Nature *291*, 293-296.

Takke, C., Dornseifer, P., von Weizsäcker, E., and Campos-Ortega, J.A. (1999). her4, a zebrafish homologue of the Drosophila neurogenic gene E(spl), is a target of NOTCH signalling. Development *126*, 1811-1821.

Temkin, P., Lauffer, B., Jager, S., Cimermancic, P., Krogan, N.J., and von Zastrow, M. (2011). SNX27 mediates retromer tubule entry and endosome-to-plasma membrane trafficking of signalling receptors. Nature Cell Biology *13*, 715-721.

Thatcher, E.J., Bond, J., Paydar, I., and Patton, J.G. (2008a). Genomic organization of zebrafish microRNAs. BMC Genomics *9*, 253.

Thatcher, E.J., Flynt, A.S., Li, N., Patton, J.R., and Patton, J.G. (2007). MiRNA expression analysis during normal zebrafish development and following inhibition of the Hedgehog and Notch signaling pathways. Developmental Dynamics *236*, 2172-2180.

Thatcher, E.J., Paydar, I., Anderson, K.K., and Patton, J.G. (2008b). Regulation of zebrafish fin regeneration by microRNAs. Proceedings of the National Academy of Sciences of the United States of America *105*, 18384-18389.

Thermann, R., and Hentze, M.W. (2007). Drosophila miR2 induces pseudo-polysomes and inhibits translation initiation. Nature *447*, 875-878.

Thisse, C., and Thisse, B. (2008). High-resolution in situ hybridization to whole-mount zebrafish embryos. Nature Protocols *3*, 59-69.

Thomson, T., and Lin, H. (2009). The biogenesis and function of PIWI proteins and piRNAs: progress and prospect. Annu Rev Cell Dev Biol *25*, 355-376.

Tuschl, T., Zamore, P.D., Lehmann, R., Bartel, D.P., and Sharp, P.A. (1999). Targeted mRNA degradation by double-stranded RNA in vitro. Genes & Development *13*, 3191-3197.

Vella, M.C., Choi, E.-Y., Lin, S.-Y., Reinert, K., and Slack, F.J. (2004). The C. elegans microRNA let-7 binds to imperfect let-7 complementary sites from the lin-41 3'UTR. Genes & Development *18*, 132-137.

Wakiyama, M., Takimoto, K., Ohara, O., and Yokoyama, S. (2007). Let-7 microRNA-mediated mRNA deadenylation and translational repression in a mammalian cell-free system. Genes & Development *21*, 1857-1862.

Walker, C. (1999). Haploid screens and gamma-ray mutagenesis. Methods Cell Biology *60*, 43-70.

Walker, J.C., and Harland, R.M. (2009). microRNA-24a is required to repress apoptosis in the developing neural retina. Genes & Development *23*, 1046-1051.

Walters, R.W., Bradrick, S.S., and Gromeier, M. (2010). Poly(A)-binding protein modulates mRNA susceptibility to cap-dependent miRNA-mediated repression. RNA *16*, 239-250.

Wang, B., Love, T.M., Call, M.E., Doench, J.G., and Novina, C.D. (2006). Recapitulation of short RNA-directed translational gene silencing in vitro. Mol Cell *22*, 553-560.

Wei, C., Thatcher, E.J., Olena, A.F., Cha, D.J., Perdigoto, A.L., Marshall, A.F., Carter, B.D., Broadie, K., and Patton, J.G. (2013). miR-153 regulates SNAP-25, synaptic transmission, and neuronal development. PLoS ONE *8*, e57080.

Wienholds, E. (2005). MicroRNA Expression in Zebrafish Embryonic Development. Science *309*, 310-311.

Wienholds, E., and Plasterk, R.H. (2005). MicroRNA function in animal development. FEBS Letters *579*, 5911-5922.

Wightman, B., Burglin, T.R., Gatto, J., Arasu, P., and Ruvkun, G. (1991). Negative regulatory sequences in the lin-14 3'-untranslated region are necessary to generate a temporal switch during Caenorhabditis elegans development. Genes & Development *5*, 1813-1824.

Wightman, B., Ha, I., and Ruvkun, G. (1993). Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans. Cell *75*, 855-862.

Wright, G.J., Giudicelli, F., Soza-Ried, C., Hanisch, A., Ariza-McNaughton, L., and Lewis, J. (2011). DeltaC and DeltaD interact as Notch ligands in the zebrafish segmentation clock. Development *138*, 2947-2956.

Wu, L., Fan, J., and Belasco, J.G. (2006). MicroRNAs direct rapid deadenylation of mRNA. Proceedings of the National Academy of Sciences of the United States of America *103*, 4034-4039.

Yan, K.S., Yan, S., Farooq, A., Han, A., Zeng, L., Zhou, M. (2003). Stucture and conserved RNA binding of the PAZ domain. Nature *426*, 469-474.

Yang, D., Lu, H., and Erickson, J.W. (2000). Evidence that processed small dsRNAs may mediate sequence-specific mRNA degradation during RNAi in Drosophila embryos. Current Biology *10*, 1191-1200.

Yang, J.-S., and Lai, E.C. (2011). Alternative miRNA biogenesis pathways and the interpretation of core miRNA pathway mutants. Molecular Cell *43*, 892-903.

Yang, J.S., Maurin, T., Robine, N., Rasmussen, K.D., Jeffrey, K.L., Chandwani, R., Papapetrou, E.P., Sadelain, M., O'Carroll, D., and Lai, E.C. (2010). Conserved vertebrate mir-451 provides a platform for Dicer-independent, Ago2-mediated microRNA biogenesis. Proceedings of the National Academy of Sciences of the United States of America *107*, 15163-15168.

Yekta, S., Shih, I.-h., and Bartel, D.P. (2004). MicroRNA-directed cleavage of HOXB8 mRNA. Science (New York, NY) *304*, 594-596.

Yeo, S.-Y., Kim, M., Kim, H.-S., Huh, T.-L., and Chitnis, A.B. (2007). Fluorescent protein expression driven by her4 regulatory elements reveals the spatiotemporal pattern of Notch signaling in the nervous system of zebrafish embryos. Developmental Biology *301*, 555-567.

Yi, R., Qin, Y., Macara, I.G., and Cullen, B.R. (2003). Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. Genes & Development *17*, 3011-3016.

Yin, W., Liu, D., Liu, N., Xu, L., Li, S., Lin, S., Shu, X., and Pei, D. (2012). SNX17 regulates Notch pathway and pancreas development through the retromer-dependent recycling of Jag1. Cell Regeneration *1*, 4.

Yoo, K.-W., Kim, E.-H., Jung, S.-H., Rhee, M., Koo, B.-K., Yoon, K.-J., Kong, Y.-Y., and Kim, C.-H. (2006). Snx5, as a Mind bomb-binding protein, is expressed in hematopoietic and endothelial precursor cells in zebrafish. FEBS Letters *580*, 4409-4416.

Zamore, P.D., Tuschl, T., Sharp, P.A., and Bartel, D.P. (2000). RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. Cell *101*, 25-33.

Zeng, Y., and Cullen, B.R. (2005). Efficient processing of primary microRNA hairpins by Drosha requires flanking nonstructured RNA sequences. The Journal of Biological Chemistry *280*, 27595-27603.

Zeng, Y., Yi, R., and Cullen, B.R. (2003). MicroRNAs and small interfering RNAs can inhibit mRNA expression by similar mechanisms. Proceedings of the National Academy of Sciences of the United States of America *100*, 9779-9784.

Zeng, Y., Yi, R., and Cullen, B.R. (2005). Recognition and cleavage of primary microRNA precursors by the nuclear processing enzyme Drosha. EMBO J *24*, 138-148.

Zhang, H., Kolb, F.A., Jaskiewicz, L., Westhof, E., and Filipowicz, W. (2004). Single Processing Center Models for Human Dicer and Bacterial RNase III. Cell *118*, 57.