DIVERSE ROLES FOR MICRORNAS IN ZEBRAFISH

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Dissertation

Submitted to the Faculty of the

Graduate School of Vanderbilt University

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

in

Biological Sciences

December, 2007

Nashville, Tennessee

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To my cherished wife J. Erin Flynt,

Who always reminded me what is most important in life.

Having her by my side made this work possible.

ACKNOWLEDGEMENTS

This work was supported by grants from the Vanderbilt Zebrafish Initiative and the NIH (GM 075790). I was supported in part by T32 GM 008554. First I'd like to thank Jim Patton and Lilianna Solnica-Krezel for mentoring during graduate school. I am grateful for how much freedom I was given to pursue experiments of my own design. I'd also like to thank them for their sage advice that shaped my work and my thinking in essential ways. You both have made positive, lasting improvements to my capacity as a scientist. Next I am grateful to the members of the Patton lab and Solnica-Krezel lab not only for their comradeship, but also their opinions concerning my experiments. Graduate school would have been decidedly more difficult if not for the excellent and helpful people in both labs.

I would also like to acknowledge all of the wonderful friends that have made my time at Vanderbilt bearable. There are too many to list here-you know who you are. I have also been very fortunate to have my family close by, they have given me the absolutely necessary emotional and financial support that allowed me to flourish while I've been in graduate school. Finally, I would like to thank my wife, Erin, for all her help and support. It has been wonderful to experience the whole of grad school with you. Marrying a fellow scientist has also been a fantastic thing. You truly understand when I rant and rave about how asinine or unreasonable mine (and your) colleagues could be.

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LIST OF ABBREVIATIONS

RNAi ..... RNA interference

siRNA ..... Short-interfering RNAs

HSGS ..... Homology Dependent Gene Silencing

dsRNA ..... Double-stranded RNA

RISC ..... RNA Induced Silencing Complex

miRNA ..... microRNA

MBT ..... Mid Blastula Transition

YSL ..... Yolk Syncytial Layer

shRNAs ..... small hairpin RNAs

pri-miRNAs ..... primary microRNAs

pre-miRNAs ..... precursor micoRNAs

piRNAs ..... Piwi RNAs

MO ..... Morpholino

MZdicer ..... Maternal Zygotic Dicer Mutant

Hh ..... Hedgehog ligand

Su(fu) ..... suppressor of fused

nherf ..... Sodium Hydrogen exchange regulatory factor

NHE ..... Sodium Hydrogen Exchanger

SG ..... Stress Granules

CHAPTER I

INTRODUCTION

RNAi and microRNAs

Discovery of RNAi

During RNA interference (RNAi), ~22nt short interfering RNAs (siRNA) inhibit expression of mRNAs due to sequence complementarity between a given siRNA and a specific mRNA. In recent years RNAi has become a common method for generating loss of gene function. This technique has been applied successfully in a broad spectrum of model organisms from worms and flies to mice. RNAi is a form of homology dependent gene silencing (HDGS), a phenomenon that relies on nucleic acid sequence similarities between different loci or transcripts to create unusual nucleic acid structures and thereby affect gene expression. HDGS generated by introduction of exogenous genes was initially described as co-suppression in petunias (Napoli et al., 1990). Additional copies of the Chalcone Synthase gene were inserted into the petunia genome to increase pigmentation in flowers. The result of this manipulation, however, produced loss of flower pigmentation. It was assumed that the cause of gene silencing was likely a result of transcriptional inactivity, but in retrospect the mechanism behind the silencing of this gene was likely through RNAi. The first clear example of RNAi came from *C. elegans*. Injection of double-stranded RNA (dsRNA) produced substantially more effective knockdown of homologous mRNAs as opposed to injection of either sense or anti-sense single strands. The degree of silencing achieved by dsRNA was shown to be effective at

extraordinarily low concentrations, a few molecules per cell, and to be heritable (Fire, 1998). At first this seemed paradoxical because dsRNA is already paired with a perfectly complementary sequence and therefore seemingly less likely to affect stability by binding to mRNAs. Further studies, however, illuminated the biological mechanism surrounding RNAi and its intracellular processing (Bartel, 2004). Long dsRNAs are processed in the cell producing smaller RNAs termed short-interfering RNAs that are the effectors of gene silencing. Experiments in *D. melanogaster* embryo lysates revealed that both strands of a dsRNA are cleaved in an ATP-dependent manner yielding small duplexes of RNA characterized by 2 nucleotide (nt) overhangs, 5'phosphates, and 3' hydroxyl groups (Zamore et al., 2000). This process was shown to be independent of a complementary mRNA and ribosome function. mRNAs targeted by siRNAs are cleaved at 21-23nt intervals indicating that each siRNA can direct sequence specific cleavage. The RNAse III enzyme Dicer is responsible for production of siRNAs from dsRNA. Dicer was originally identified through a candidate gene approach (Ketting, 2001). When recombinant Dicer was incubated with dsRNA it was able to generate siRNAs (Bernstein, 2001b). These RNAs were then shown to incorporate into a large complex termed the RNA Induced Silencing Complex (RISC). The RISC was first isolated from ribosomal fractions, and was shown to be a multicomponent ~500kDa complex capable of identifying and targeting cognate mRNAs (Hammond, 2000). The discovery of RNAi has ushered in a new era of genetic manipulation that has been applied in most model organisms, and has become an exciting potential therapeutic approach. Initially the role of RNAi was assumed to be a defense mechanism recognizing viruses and mobile genetic elements (Bernstein, 2001a). It has become apparent, however, that RNAi has another

role: regulating development through the action of endogenous, siRNA-like genes termed microRNAs (miRNAs) (Ambros, 2001).

Discovery of microRNAs

While the ability to use RNAi in reverse genetic analysis of gene function has produced great opportunity for researchers, equally important has been the discovery that the processing machinery and effector complexes that carry out RNAi have largely been co-opted from an endogenous family of RNAs known as miRNAs. The first characterized miRNA, *lin-4*, was discovered in 1993 by positional cloning of a gene regulating developmental timing in *C. elegans* (Lee et al., 1993). Interestingly, the *lin-4* gene was found not to code for protein. It was not until 1999 that a second *C. elegans* miRNA, *let-7*, which also affected development in worms, was discovered (Reinhart et al., 2000). Both *lin-4* and *let-7* were shown to produce small RNAs from hairpin precursors that inhibit gene expression through translational inhibition by imprecisely pairing with the 3' untranslated region of their respective target mRNAs. *lin-4* triggers the early timer that allows proper progression through larval stages, whereas *let-7* triggers the late timer that controls appropriate progression into the adult stage of *C. elegans* (Palatnik et al., 2003). Research describing *let-7* and *lin-4* created the paradigm for understanding miRNAs, where small ~22nt RNAs derived from hairpin precursors are generated from endogenous loci. These molecules function in manner similar to exogenously supplied dsRNAs used to trigger RNAi (Elbashir, 2001; Hamilton and Baulcombe, 1999; Hammond, 2000; Zamore et al., 2000). These findings spurred three groups to clone and sequence small RNAs from worms, flies, and humans leading to the

identification of hundreds of new miRNAs (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001). Continued cloning and bioinformatic approaches have identified more and more miRNAs (Grad et al., 2003; Lai, 2004; Lai et al., 2003; Lim et al., 2003)

miRNA Diversity

miRNAs are thought to constitute roughly 1% of the genes encoded by a typical vertebrate genome (Griffiths-Jones, 2004). These genes also show significant conservation between species (Griffiths-Jones, 2004). Efforts to directly clone miRNAs have been designed to exploit specific characteristics of these molecules such as 5' phosphates and 3' hydroxyl groups, dsRNA hairpin precursors, and conservation between species (Lagos-Quintana et al., 2001; Lagos-Quintana et al., 2003; Lagos-Quintana et al., 2002; Lim et al., 2003). Bioinformatics have also uncovered that most miRNAs are predicted to target >200 mRNAs based on sequence similarity to miRNAs (Kiriakidou et al., 2004; Lewis et al., 2003). Because of the lack of perfect complementarity, bioinformatic approaches to identify target genes has been difficult meaning that all targets will likely need to be experimentally validated (Enright et al., 2003; Kiriakidou et al., 2004; Lai, 2004; Lewis et al., 2003; Miska et al., 2004; Stark et al., 2003).

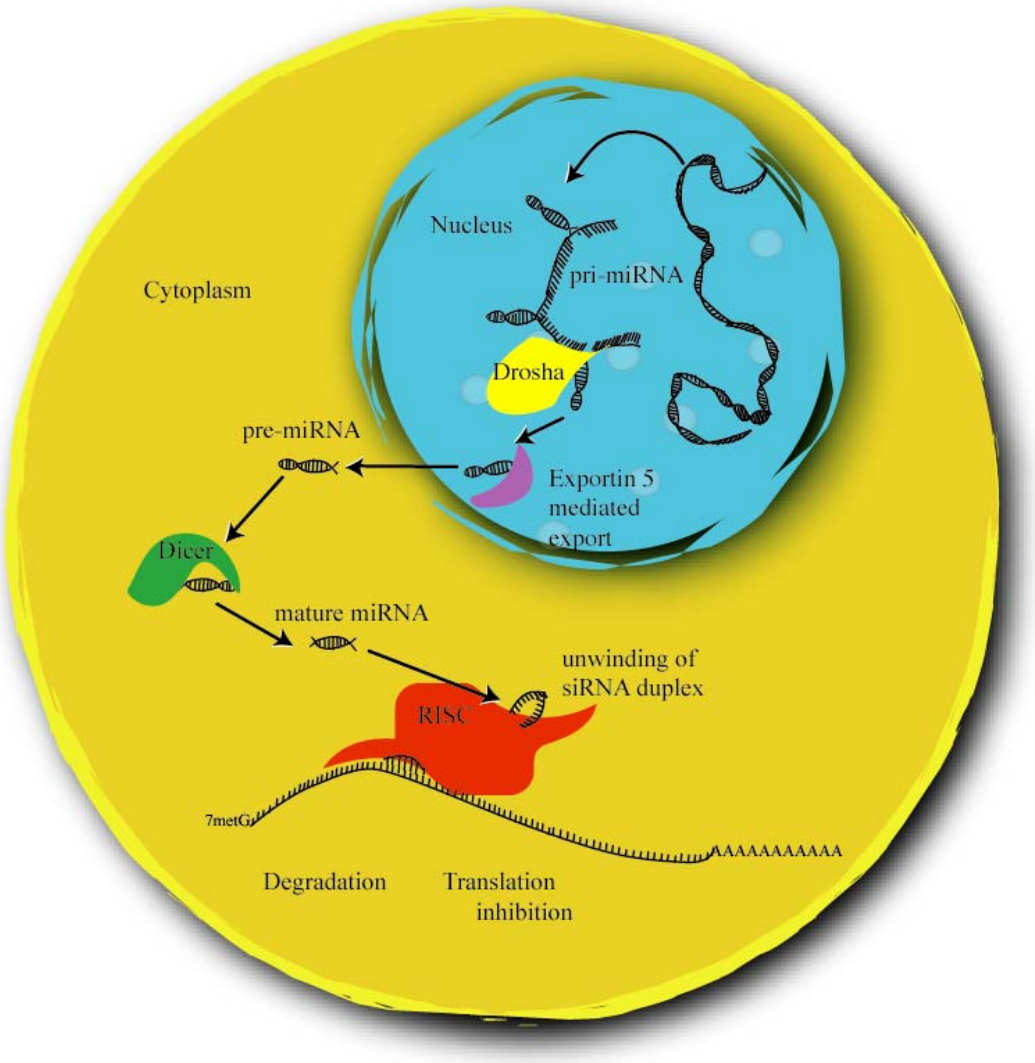


Figure 1. **Synthesis and Processing of miRNAs**. Precursor miRNA transcripts are processed by Drosha in the nucleus and by Dicer in the cytoplasm to generate 21-23nt miRAs. Exogenous dsRNAs are processed in a similar manner before both RNAs are incorporated into RISC complexes leading to specific cleavage or translation arrest of target mRNAs**.**

Biogenesis and action of microRNAs

Production of miRNAs is characterized by progressive processing of nascent, primary miRNA transcripts (pri-miRNAs) (Lee et al., 2002) (Figure 1). miRNAs are

transcribed from loci typically under the control of Pol II promoters (Lee et al., 2004a). The majority (~70%) are transcribed from intergenic regions. The remainders are located within introns in either the sense or anti-sense orientation (Rodriguez et al., 2004). The first step in miRNA biogenesis is excision of ~70nt hairpin precursor-miRNAs (pre-miRNA) by action of the RNase type III enzyme, Drosha (Lee et al., 2003). Removal of pre-miRNAs defines one terminus of the mature miRNA (Bartel, 2004). Pre-miRNAs are exported by binding to Exportin5 in a RanGTP-dependent fashion (Bohnsack et al., 2004; Yi et al., 2003). Dicer, another RNase III enzyme originally implicated in siRNA production, is responsible for the final step in miRNA processing, which produces a small RNA duplex (Ketting, 2001). Dicer, in conjunction with other factors, loads nascent mature miRNAs into the RISC (Liu, 2003). Selection of the strand of the duplex is based on RNA duplex stability. The strand that exhibits lower free energy at the 3' end is loaded into the RISC (Khvorova et al., 2003; Schwarz et al., 2003).

RISC is a multifunctional complex that exhibits "slicer" nuclease activity. This function of RISC degrades targeted mRNAs that show perfect complementarity to a siRNA (Caudy et al., 2002; Hammond, 2001; Liu et al., 2004). miRNAs, in contrast to siRNAs, do not typically induce cleavage of target mRNAs and simply inhibit translation. miRNAs function in this manner presumably due to only partial complementarity to their targets (Lai, 2002). However if RISC loaded with a miRNA is presented with mRNAs exhibiting perfect complementarity to that miRNA, cleavage does occur (Zeng et al., 2003). In rare cases miRNAs exhibit perfect complementarity to their endogenous targets, which results in cleavage of the target (Yekta et al., 2004). This suggests that miRNAs and siRNAs act through similar pathways.

RNAi in Zebrafish

Understanding of RNAi has been made possible through a variety of experimental approaches using different model organisms (Bernstein, 2001a). Some of these systems are amenable to mutational screening, facilitating forward genetic approaches and the discovery of novel genes involved in the RNAi pathway. Others permit biochemical approaches producing mechanistic information. Over the last eight years, observations from different groups using these techniques in different systems have yielded a clear outline of the basic mechanisms behind RNAi and the fuction of miRNAs (Bartel, 2004). We have chosen to use Zebrafish as a model organism.

Characteristics of zebrafish: Embryogenesis and Manipulation

Zebrafish (*Brachydanio rerio*) are a small cyprinid fish marked by a series of horizontal blue and yellow stripes that give this fish the zebra moniker. Sexual dimorphism is somewhat subtle--females have a white to silver, round-shaped belly while males have an yellow colored, flat belly. Experimentally, zebrafish are an extremely accessible embryonic system (Kimmel et al., 1995). Mating pairs can be placed in small tanks with a mesh for separating newly laid eggs. Zebrafish lay freely drifting eggs that, like most fish, are fertilized externally. Zebrafish zygotes possess a large, vegetal yolk while the embryo proper forms on the animal pole of the yolk cell.

After fertilization, six synchronous divisions occur at a regular orientation but the cells remain continuous with the yolk via cytoplasmic bridges. Blastula stages are marked by a loss of the regular distribution of cells and the beginning of metasynchronous division. The mid blastula transition (MBT) signals the onset of

zygotic transcription and happens at the tenth cell cycle whereas prior to this point, gene expression is derived exclusively from maternally contributed RNAs (Francisco, 2003). At the same time MBT occurs, an important signaling center for mesendoderm induction called the yolk syncytial layer (YSL) forms from marginal cells (Erter et al., 1998).

Similar to other metazoans, gastrulation entails internalization of cells that give rise to mesoderm and endoderm. This process begins at the dorsal blastoderm margin in a conspicuous region referred to as the embryonic shield and is comprised of several distinct cell movements: convergence, extension, and continuation of epiboly (Myers et al., 2002). After gastrulation, cells have migrated to envelop the yolk and have accumulated along the anterior-posterior axis on the dorsal side of the embryo. Subsequently, neurulation and somitogenesis occur and are completed by 24 hours post fertilization (Kimmel et al., 1995). The remainder of embryonic development occurs over a period of four days. Embryos hatch from their chorions during the second day after which they are free swimming but still reliant on yolk for nutrients. On the fifth day after fertilization, a protruding jaw is visible and larvae are ready to hunt prey.

Due to rapid, external development it is easy to observe cellular behavior and other phenomena without disrupting zebrafish embryos. Before pigmentation occurs, after approximately the first 24 hours of development, zebrafish embryos are transparent, further facilitating observation of embryonic structures. Healthy adult fish typically lay hundreds of eggs allowing for easy access to numerous embryos. Initial interest in zebrafish arose from its potential as a vertebrate model that was amenable to forward mutagenesis screens (Kimmel et al., 1989). These screens are made possible because of the capacity to breed and are useful for discovering genes involved in embryogenesis due

to transparent and accessible embryos. Many mutants have been recovered from such screens (Driever et al., 1996; Haffter and Nusslein-Volhard, 1996).

Reverse genetic methodologies and the creation of transgenic animals are also becoming well-established technologies in zebrafish (Culp et al., 1991). Due to the connections between the yolk cell and early dividing animal cells, it is relatively easy to deliver molecules, typically nucleic acid, through microinjection. Overexpression of specific genes can be accomplished through injection of synthetic RNAs. A standard method for producing gene knockdown is through the use of morpholino oligonucleotides, a form of anti-sense technology (Nasevicius and Ekker, 2000). These oligonucleotides are approximately 25 bases long and posses a modified backbone, which makes them resistant to degradation thus prolonging the interaction between the morpholino and its target sequence. Typically, attenuation of mRNA expression is accomplished by designing morpholinos complementary to start codons and surrounding sequences. This prevents assembly of ribosomes, inhibiting translation, and ultimately expression of the gene. Likewise, morpholinos can be designed to hybridize to pre-mRNA splice sites causing skipping of exons and generation of truncated proteins or destabilized mRNA (Schmajuk et al., 1999).

In addition to the relative ease of antisense based reverse genetics in zebrafish, methodologies for generating transgenic embryos are available. Transgenes are introduced through microinjection of DNA constructs (Culp et al., 1991). Transgene incorporation is random and mosaic but genetic outcrossing can result in stable transgenic lines. Successful integration into the germline causes transmission to progeny and equal

distribution throughout the animal. Additionally, the rate of transgenesis can be enhanced by transposition (Kawakami et al., 2000).

Based on the above characteristics, zebrafish are an excellent system to study vertebrate embryonic development. However, it is not yet possible to create mutants through homologous recombination. This is due to a lack of zebrafish embryonic stem cells. Methodologies have been developed to circumvent this limitation, one of which is called TILLING (Targeting Induced Local Lesions IN Genomes), which will be discussed below (Wienholds et al., 2003b). Additionally, the zebrafish genome is currently being sequenced, but is not yet complete as of this writing. These obstacles will likely be surmounted, especially the latter, in the coming years. The strengths of the zebrafish system greatly facilitate analysis of miRNA function though simple introduction of RNAs directly into embryos via microinjection and the ability to observe phenotypes in gain-of-function and loss-of-function experiments.

Application of RNAi in zebrafish and other model organisms

The ability to use long dsRNA as an effective agent of knocking down gene expression has been effective in plants and invertebrates despite the fact that a different composition of RNAi pathway components exists in these different organisms (Bernstein, 2001a). In lower animals and plants, triggering RNAi with long dsRNA is thought to have initially evolved to be an immune response to viruses and parasitic genetic elements (Hammond et al., 2001; Tabara et al., 1999). Plants seem particularly reliant on RNAi as a viral defense because siRNAs efficiently diffuse through plant tissue ahead of spreading viral infection (Aaronson and Horvath, 2002). Invertebrates also use RNAi for anti-viral

purposes but primarily employ it for silencing transposable elements. In *D. melanogaster* a class of repeat associated siRNAs (rasiRNAs) have been discovered that correspond to repetitive genetic elements (Aravin et al., 2003a).

For vertebrate cells, long dsRNA is cytotoxic and does not enter the RNAi pathway, instead, triggering an interferon mediated anti-viral response through a factor called Protein Kinase RNA-activated (PKR). PKR binds dsRNA and phosphorylates the translation intiation factor eIF-2α (Wu and Kaufman, 1997). This results in global inhibition of mRNA translation, causing cell death. Also activation of RNase L/Oligo(A) Synthetase, and Toll-like Receptor 3, recognize long dsRNA molecules and trigger cell death (Castelli et al., 1998; Fukuda et al., 2006). The usefulness of RNAi as a defense against viruses in vertebrates has perhaps become reduced due to the evolution of sophisticated cell-mediated immune systems. As far as is known, vertebrates do not use RNAi as an antiviral response or to control repetitive element multiplication. Instead, the pathway is used for the production and function of miRNAs (Bartel and Chen, 2004).

Shortly after the discovery of RNAi by Fire and Mello, long dsRNA was successfully used to knockdown genes in *C. elegans*, fruit flies, planarians, hydra, and plants (Bernstein, 2001a). Initial efforts seeking to attenuate gene expression in zebrafish through injection of long dsRNA claimed the method to be effective (Wargelius et al., 1999). These experiments sought to target *no tail* (*ntl*), *floating head* (*flh*), and *pax2.1*, genes that are essential for early development. *ntl* is essential for mesoderm formation, and is one of the first genes expressed in the germ ring being stimulated by signals from the YSL. Mutations in *ntl* result in a loss of notochord and severe truncation of the tail. Likewise, *flh* results in loss of the same structures. While injections of dsRNA in

zebrafish resulted in nonspecific effects at a high rate, some of the expected phenotypic effects were observed. Also, *in situ* staining of *ntl* mRNA suggested measurable knockdown of the gene. These results, as well as others (Li et al., 2000; Oates et al., 2000), suggested that RNAi could be successfully used in zebrafish to specifically knockdown homologous genes although the success of knockdown varied greatly.

Despite early reports, later work using long dsRNAs strongly argued that all defects in zebrafish were due to nonspecific effects (Zhao et al., 2001). Regardless of the sequence of injected dsRNA, dose dependent toxicity was observed. Zhao et al. tested the ability of dsRNA injection to eliminate protein expression using transgenic embryos expressing GFP under the influence of the GATA-1 promoter, which drives expression in blood cells. Despite severe morphological defects induced by injection of dsRNA homologous to GFP, no silencing of the GFP was observed. Additionally, injection of dsRNA, regardless of sequence, resulted in a global decrease of mRNA expression. Attempts to silence expression of *pou2* expression through injection of dsRNA homolgous to GFP or to *pou2* both resulted in a similar decrease in *pou2* expression. In retrospect, nonspecific effects induced by long dsRNA were predictable considering the interferon response induced by these molecules in vertebrates. Translation arrest induced by PKR activation followed by degradation of RNAs by RNase L could easily explain these results.

These conflicting reports have for the most part resulted in reluctance among zebrafish researchers to use RNAi to knockdown gene expression. Currently, antisense morpholino oligonucleotides are the preferred method to inhibit gene function. At low doses they are generally non-toxic, and stable. Despite this preference and because long

dsRNAs do not lead to specific gene knockdown, RNAi may still function in zebrafish, provided the dsRNAs are short. Introduction of RNAs less than thirty nucleotides can apparently circumvent most PKR activation (Stein et al., 2005). Small RNA molecules have been shown to be effective in producing an RNAi response in vertebrates. These molecules behave like miRNAs and are processed accordingly. Injection of small RNAs, when introduced as small heteroduplex RNAs, produces specific RNAi in zebrafish (Kloosterman et al., 2004). Thus, there may be applications for RNAi technology in zebrafish, particularly through the production of transgenic lines inactivating specific genes by synthesis of short dsRNAs encoded on the transgene.

In flies and worms a typical RNAi strategy is to create transgenic animals that express long dsRNAs by transcription of an inverted repeat (IR) construct derived from target mRNA sequence (Piccin et al., 2001). This IR dsRNA can be driven tissue specifically, either with a specific promoter or from a GAL4 promoter in the GAL4 system where GAL4 protein is expressed in a tissue specific manner (Inbal et al., 2006; Perrimon, 1998). Similar to invertebrate IR expressing systems, many transgenic mice have been created that express short-hairpin RNAs (shRNAs) (Hemann et al., 2003). The dsRNA portion of the hairpin must be small enough to avoid PKR activation. This is also a viable option for cultured mammalian cells. Plasmids bearing an shRNA cassette can be either transiently or stably expressed. As of this writing there have been no published reports using shRNA cassettes in zebrafish. Employing these constructs may be the most productive application of RNAi in zebrafish.

Currently, analysis of loss of gene function in zebrafish is accomplished using mutants, antisense morpholinos, or more infrequently, dominant negatives. While each

of these strategies is well established there are several limitations that could be overcome using shRNA technology. For example, mutant lines are perhaps the most desirable tools for loss of function analysis but mutant alleles can be variable as to the degree of attenuation of gene function and little control can be exerted over when and where loss of function occurs. If a specific gene is required for both early and late stages of development, it is difficult to study later requirements for that gene with such a mutant. RNAi technologies may be useful for such purposes (Figure 2). shRNA constructs can be made that drive shRNA under heat shock promoters to induce expression at specific times (Figure 2A). Likewise, expression using tissue specific promoters such as the GAL4 system could be used to generate tissue specific knockdown of target gene expression (Figure 2B).

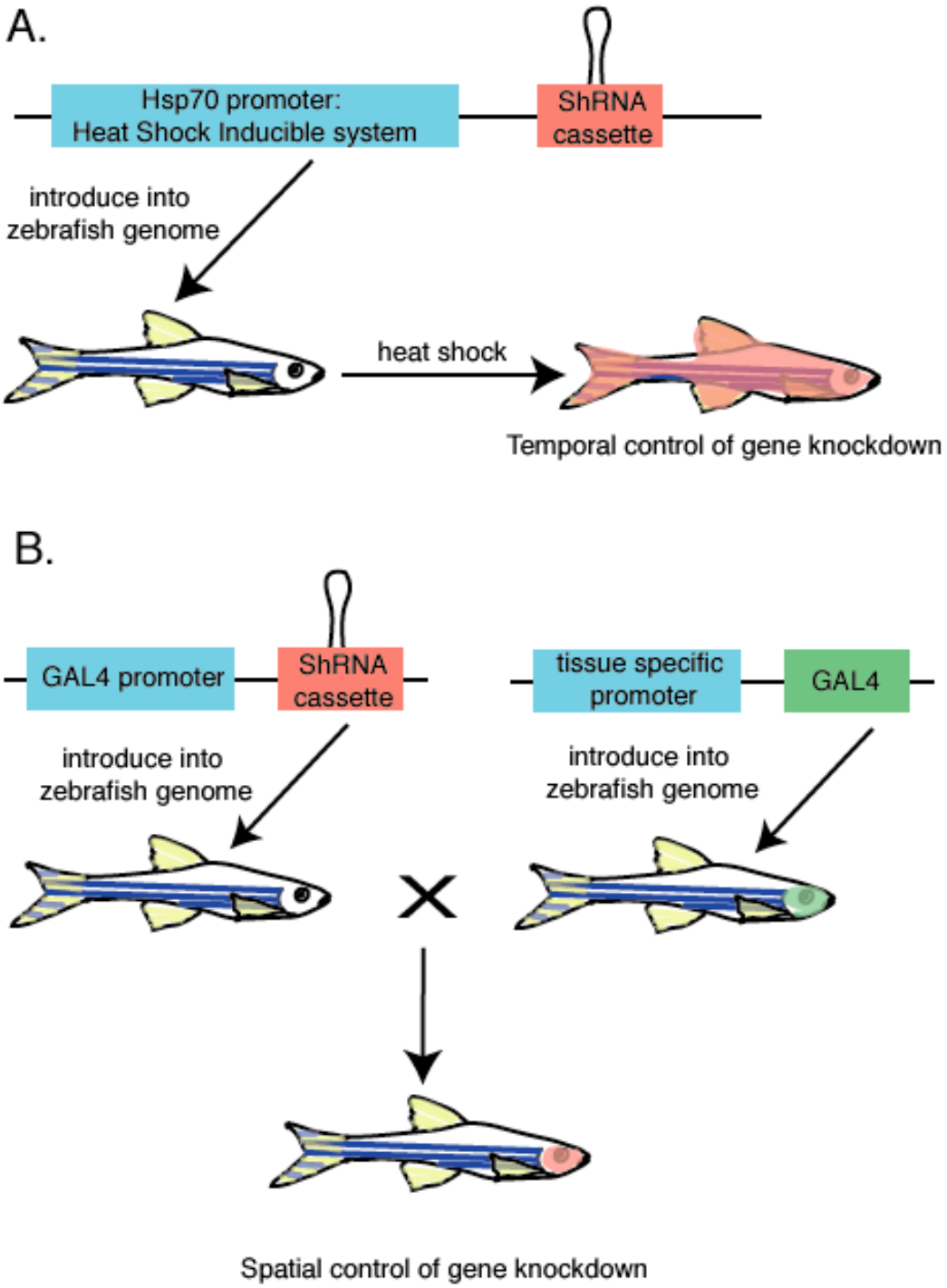


Figure 2. Use of transgenic animals to deliver shRNAs under the control of conditional promoters. (A) An shRNA under the control of a heat shock promoter. (B) An shRNA under the control of the GAL4 promoter is used to create transgenic lines that are crossed with lines expressing GAL4 protein in a tissue specific manner.

The vertebrate siRNA/miRNA pathway

In their seminal article Fire and Mello (1998) proposed that the likely mechanism behind RNAi was due to RNA molecules acting as a component of nuclear feedback loops silencing transcription of homologous genes through binding to genomic DNA (Fire, 1998). As an aside they admitted that RNAi could be through a mechanism based on RNA-RNA interaction. It is now clear that RNA-RNA interaction is central to RNAi.

Argonaute proteins are core members of RISC, possessing PAZ and PIWI domains, that physically interact with RNA molecules (Carmell et al., 2002). Argonautes bind the small RNAs and use them as specificity factors to identify mRNAs with complementary sequence. The fate of mRNAs targeted by RISC is dependent on the degree of base-pairing between the small RNA and the mRNA (Doench et al., 2003; Lai, 2002; Olsen and Ambros, 1999; Zeng et al., 2003). Perfect pairing results in cleavage of the mRNA at the 9th nucleotide of interaction counting from the 5' end of the siRNA (Lewis et al., 2003). Cleavage of mRNAs is specifically carried out by Argonaute 2 (Liu et al., 2004). The precise mechanism of translation inhibition by imperfect pairing is unknown but it seems that relocalization of mRNAs from polysomes to processing-bodies (P-bodies) is an important aspect of this activity (Liu et al., 2005). Immunostaining of Argonautes reveals localization to P-bodies, which are cytoplasmic foci that contain decapping and deadenylating enzymes and are thought to be sites of mRNA decay (Rehwinkel et al., 2005; Sen and Blau, 2005).

Zebrafish RNAi components

RNAi component composition is highly variable between phyla. For example *D. melanogaster* have 2 Dicers and 4 Argonaute homologs, *C. elegans* have a single Dicer and 27 Argonautes, and humans encode 1 Drosha, 1 Dicer, and 4 Argonautes. Zebrafish contain 2 genes possessing RNAse III domains, 8 genes with PAZ domains that correspond to 1 Drosha, 1 Dicer, 5 Argonautes, and two Piwi-like genes. There are clear homologs of each one of the human Argonautes AGO1, AGO2, AGO3, and AGO4. The fifth zebrafish argonaute is most similar to AGO3. Interestingly, the Ago1 and Ago3-2 genomic loci are juxtaposed, similar to that seen in the human genome with AGO1 and AGO3. Beyond the core Argonautes, the zebrafish genome encodes 2 Piwi-like (piwil) proteins called Piwil1 and Piwil2. Zebrafish Piwil1 is most similar to human Piwil1 but is also similar to human Piwil3 and Piwil4. Zebrafish Piwil2 is similar to human Piwil2. Piwi proteins do not appear to be associated strictly with the RNAi pathway with apparent roles in mitotic spindle formation and also association with unique small RNAs expressed in testis called piwiRNAs (piRNAs) (Kennerdell et al., 2002; Lau et al., 2006). Given evolutionary conservation of RNAi components in mammals and zebrafish, the basic role of RNAi is likely shared, and indicates that major questions best approached in zebrafish will likely translate accurately to mammalian systems and ultimately humans.

Zebrafish Dicer mutants

Analysis of the role of RNAi during zebrafish development has been facilitated by the creation of strains carrying mutations in *dicer1*. These mutations were isolated using TILLING methodology (Weinholds, 2003). TILLING is a PCR based strategy that

involves PCR amplification of genomic sequence to identify point mutations. For *dicer1*, three mutations were uncovered that produce premature stop codons in the *dicer1* open reading frame. Embryos homozygous for any of the mutations exhibit a growth arrest phenotype. Most embryos do not survive for more than two weeks, show lethargic behavior, and fail to grow at normal rates beginning after the first week. In contrast to mutants identified by TILLING, injection of morpholinos complementary to *dicer1* result in earlier phenotypic consequences (Weinholds, 2003). These results reflect the essential nature of Dicer1 function with survival out to two weeks due to contributions from maternal sources. Morpholino mediated inhibition of *dicer1* targets mRNA and is able to down-regulate not only *dicer1* transcripts from zygotic sources but also maternally contributed message. The survival of morpholino injected embryos can be attributed to the presence of maternal protein or to the incomplete elimination of *dicer1* expression.

The loss of Dicer prevents miRNA maturation (Wienholds et al., 2003a). Consistent with this, embryos failed to produce mature miRNAs, and show accumulation of pre-miRNA intermediates. In addition to demonstrating an essential role for miRNAs in zebrafish development, these results indicate that zebrafish *dicer1* is not redundant and is required for final maturation of miRNAs in zebrafish.

The *dicer1* null embryos exhibit a general growth arrest phenotype, which is a result of attenuated miRNA expression but the exact physiological defects that result in this phenotype remain to be determined. miRNAs are typically highly tissue specific and if loss of specific miRNAs in several tissues leads to cell death it would be expected that these tissues would exhibit greater deficiencies than others. This is not observed in *dicer1-/-* mutant embryos implying that either different miRNAs are needed in all cells or

that a specific miRNA or a set of miRNAs is required to the same degree in all cells. Requirement of a singular cellular process that is necessary to the same extent in all cell types seems unlikely. Even processes as intrinsic as metabolism would likely affect faster dividing cells more dramatically. A general requirement for miRNAs in normal development was established through analysis of *dicer1* mutants, but understanding a requirement for specific miRNAs during zebrafish development remains.

To address the issue of maternal contribution of *dicer1* and to dissect the requirement for miRNA function during the earliest stages of embryogenesis, the Schier group generated a maternal-zygotic *dicer* (*MZdicer*) mutant (Giraldez et al., 2005). Mutant phenotypes that are embryonic lethal can be rescued by supplying wild-type message, typically through micro-injection. Examples of mutants that can be rescued are *one-eyed pinhead* (EGF-CFC) or *swirl* (Bmp2) (Gritsman et al., 1999; Kishimoto et al., 1997). Rescued embryos that do not have an essential requirement for the specific gene during later development can be grown to adulthood but these individuals still retain mutations and pass them on to progeny. Due to the lack of functional gene loci in the female's germline, embryos laid by a rescued zebrafish female receive no maternal contribution of that gene.

Maternal-zygotic mutants can show drastically different phenotype from conventional null mutants, which is well illustrated by the example of *one-eyed pinhead* (*oep*) (Gritsman et al., 1999). Zygotic *oep* mutants have midline defects, resulting in mild cyclopia. MZ*oep*, on the other hand, have a much more dramatic phenotype, lacking mesendoderm entirely. The same result was obtained when MZ*dicer1* embryos were created (Giraldez et al., 2005). The approach required to generate MZ*dicer1*

mutants varied significantly from creation of MZ*oep*. The primary difference between dicer and oep mutants is that Dicer function is required throughout zebrafish development and into adulthood. *oep* is only essential for mesoderm induction and dorsal ventral patterning, both early events. Rescuing *dicer1* mutant defects would only be temporary due to the transient nature of gene expression delivered by mRNA injection. To surmount this obstacle, the Schier group employed a specialized technique called germ-line replacement (Ciruna et al., 2002). Zebrafish germ-line cells can be visualized with a GFP marker and transplanted into wild type embryos. The germ-line cells in the wild type embryos are eliminated via injection of a MO that targets *miles apart*, a gene required for germ-line cell formation. The resulting chimeric embryo lacks the ability to contribute functional gene products to offspring, both from maternal sources or through inheritance of non-mutant loci.

MZdicer1 embryos exhibit a much more severe phenotype compared to zygotic dicer mutants due to a complete lack of mature miRNA production (Giraldez et al., 2005). Development in *MZdicer1* embryos is severely perturbed. By the end of embryogenesis, most organ systems have failed to form resulting in embryonic lethality. *MZdicer1* embryos are able to develop a variety of structures such as eyes, heart, brain, and notochord but, in all cases, the tissues are dysmorphic. Morphogenesis defects and developmental delay are observable from early gastrulation stages. *MZdicer1* embryos fail to coordinate gastrulation movements, specifically internalization of mesendoderm proceeds more rapidly relative to epiboly movements resulting in inappropriate placement of the prechordal plate while envelopment of yolk lags. The inability to fully extend the embryonic axis results in a failure to complete yolk plug closure and

ultimately, an accumulation of cells in the forming head. Neurulation is particularly disrupted in *MZdicer1* embryos. The neural plate forms and develops into the neural rod but fails to form the neurocoel, an evacuation that occurs along the entire anterior-posterior axis forming ventricles in the brain and the central canal in the spinal cord. Many neuronal projections were found to be misplaced or in a state of degeneration. Accompanying this, embryos exhibited little capacity to respond to physical stimuli, consistent with a dysfunctional nervous system. In addition to defects in nervous system development, *MZdicer1* embryos exhibit deficiencies in many peripherial tissues. *MZdicer1* embryos develop U-shaped somites, blood circulation is disrupted, and have abnormal hearts. However, despite these dramatic effects on morphogenesis, many cell types are surprisingly appropriately specified. Dorsal-ventral patterning and regionalization remain intact. Marker analysis of neuronal compartments revealed that despite defects in neural development, patterning is largely unaffected. Likewise, although many organs showed abnormal development, marker analysis demonstrated that specification had occurred appropriately.

Some of the most illuminating insights concerning the role of miRNAs in zebrafish development afforded by analysis of *MZdicer1* embryos are not the finding of which tissues are perturbed but rather those that are unaffected. In *MZdicer1* embryos, axis formation and patterning of organ system primoridia is relatively intact. Additionally, germ-line cells develop without Dicer1 function. The lack of a requirement for miRNAs during these events indicates that miRNAs are not essential components of many major early signaling pathways. Dorso-ventral patterning requires signals from Wnt, Fgf, and Bmp pathways (Schier and Talbot, 2005). These are master regulatory

signaling pathways that participate in numerous morphogenesis events in both embryos and adult animals. The importance of these pathways to animal physiology cannot be understated, mutations in these pathways typically yield lethal birth defects or diseases such as cancer. The absence of a role for miRNAs in these pathways may indicate that miRNA function appeared after these pathways evolved or that the function of a specific miRNA is redundant or divergent, perhaps affecting pathways in other species. While loss of miRNAs does not dramatically affect these pathways in zebrafish, it does not eliminate the possibility that miRNAs may modulate these signaling pathways, or be important agents of regulation in adult tissues.

Comparison of *MZdicer1* zebrafish mutants to other Dicer null animals reveals widely divergent functions of miRNAs. Elimination of Dicer function in mice by homologous recombination resulted in extremely early embryonic lethality (Bernstein, 2003). Embryos fail to develop embryonic stem cells and do not progress into gastrulation stages. Similarly, *D. melanogaster dicer1* mutants display early defects such as ventralization (Lee et al., 2004b). It is unclear how these discrepancies have arisen. miRNA-mediated regulation may be more susceptible to the forces of evolution than protein coding genes. One reason behind this could be the targeting of 3'UTR sequences by miRNAs. UTR sequences show poor conservation and a change in sequence could disrupt miRNA binding.

Another interesting aspect of the *MZdicer1* analysis is the viability of *dicer1-/-* germ cells. Fish that possess a germ line comprised of Dicer mutant cells are fertile. This indicates that miRNAs are not required for maintenance of germ cells at least for metazoans. Both *C. elegans* and *Drosophila* Dicer mutants display defects in germ cell

function (Hatfield et al., 2005; Ketting, 2001). *C. elegans* mutants are sterile displaying both a burst vulva phenotype and improper maturation of oocytes. Fly *dicer1* mutants are embryonic lethal, however, generation of mosaic Dicer mutants revealed that Dicer is required for proper oocyte formation. While lower organisms have a requirement for Dicer function in germ cell formation, vertebrates apparently do not. Creation of the *dicer1* knockout mouse required generation of Dicer null embryonic stem cells, which are viable suggesting that miRNA function is not absolutely required for maintenance of ES cell pluripotency. Following the typical procedure of generating knockout mice, chimeric individuals were made that possessed Dicer deficient germ cells. These mice were successfully bred to generate heterozygotes. Thus, the requirement for Dicer function between different species reveals a dramatic divergence. While the Dicer null phenotypes can all be in part attributed to attenuation of miRNA function, it is unclear whether Dicer participates in other cellular process and whether some of the observed defects are a result of the loss of an unknown role for Dicer.

Zebrafish miRNAs

Substrate requirements for miRNA function

miRNAs typically exhibit near perfect pairing between their 5' end and a target mRNA (Brennecke et al., 2005). This segment of the miRNA is called the "seed" and is considered to be nucleotides 2-8 from the 5' end. Analysis of sequence requirements in zebrafish shows that, similar to other metazoans, zebrafish miRNAs also rely heavily on seed pairing to identify targets (Kloosterman et al., 2004).

Role of specific miRNAs in zebrafish development

The analysis of *dicer1* mutants revealed a general requirement for miRNAs in zebrafish development but did not reveal a role for specific miRNAs. To examine specific miRNAs, direct cloning of miRNAs was performed during early development revealing a highly expressed group of miRNAs, the *miR-430* family (Giraldez et al., 2005). These miRNAs begin to be expressed in stages immediately preceding gastrulation. Surprisingly, supplying individual mature members of this family through microinjection into *MZdicer1* embryos led to rescue of many of the defects observed in such embryos including restoration of ventricle formation. The *miR-430* family is unique both in genomic organization and conservation between species. Family members are encoded in a repetitive manner from a single locus duplicated over 90 times. This locus seems to be unique to fish species being found in the genomes of *F. rubripes* and *T. nigroviridis*. Other vertebrates encode *miR-430* family members or miRNAs with similar sequence but these miRNAs are not encoded in such a highly repetitive manner, indicating a potentially unique role for *miR-430* in fish.

It is now known that *miR-430* family members target maternally contributed mRNAs (Giraldez et al., 2006). The inability to fully clear these transcripts yields many of the defects associated with *MZdicer1* embryos. The composition of mRNAs expressed in embryos revealed inappropriate accumulation of maternally contributed mRNAs. Normally, these messages are targeted for degradation but are stabilized in the absence of *miR-430* due to a lack of poly(A) tail shortening. Subsequent experiments demonstrated that mRNAs targeted by *miR-430* family members show decreased poly(A) tail lengths. Such destabilization of mRNAs by miRNA targeting is also seen in mammalian cells

(Wu et al., 2006). Mechanistically, a decrease of mRNA steady state levels is thought to occur through localization of mRNAs to processing bodies.

miR-430 is known to be expressed broadly in early zebrafish embryos. Injection of reporter mRNAs that bear sequence complementary to *miR-430* results in down-regulation of the reporter throughout the entire animal. However, not all predicted targets of *miR-430* behave in this manner. Nanos mRNA, a determinant of primordial germ cell fate, is a target of *miR-430* but is not downregulated in PGCs (Mishima et al., 2006). The mechanism that is responsible for this behavior is not understood, and highlights the sophistication of miRNA mediated gene regulation.

miRNA diversity in zebrafish

As of this writing there are 337 miRNAs currently listed in the miRNA registry for zebrafish (Griffiths-Jones, 2004). This number is derived from sequence comparisons between species and efforts to directly clone small RNAs (Kloosterman et al., 2006a; Lagos-Quintana et al., 2001). Cloning of miRNAs in zebrafish revealed that very few miRNAs are expressed during early stages but the number of miRNAs expressed increases dramatically as development progresses (Chen et al., 2005). Initial miRNA cloning strategies also identified 250 rasiRNAs. rasiRNAs are derived from repetitive genomic elements but the significance of these RNAs is not known in zebrafish. miRNA expression analysis is particularly amenable to microarray technology. Small RNAs can be isolated and hybridized to oligonucleotide arrays and such experiments have confirmed the finding that miRNA repetoir complexity increases with development (Babak et al., 2004; Neely et al., 2006; Schmittgen et al., 2004; Wienholds et al., 2005).

While it is essential towards understanding the function of miRNAs to first know their temporal expression patterns, one of the most useful breakthroughs offered by zebrafish is *in situ* RNA hybridization detection of miRNA expression. The Plasterk group developed miRNA *in situ* technology based on LNA (locked nucleic acid) probes (Wienholds et al., 2005). LNA is a high-affinity RNA analogue with a bicyclic furanose unit locked in a sugar mimicking conformation (Jepsen et al., 2004). Tissue specific expression of many zebrafish miRNAs has been determined using LNA probes (Wienholds et al., 2005).

Functional Analysis of miRNAs in Zebrafish

Essential components of phenotypic analysis are loss and gain of function experiments. While use of the MZdicer mutant provided great insight into the role of *miR-430*, it will be difficult to use this mutant to dissect the function of other specific miRNAs. Mutational approaches also present extreme challenges to understanding individual miRNA function due to their small size. Currently, antisense technology is the standard methodology for inhibiting miRNA expression. Pairing between miRNAs and antisense oligonucleotides can effectively ablate miRNA function, especially using modified, stable antisense oligonucleotides (Kloosterman et al., 2004; Meister et al., 2004). The use of antisense oligonucleotides called antagomirs have been used in mice to inhibit miRNA expression (Krutzfeldt et al., 2005). These techniques have proved quite effective, resulting in complete elimination of miRNA expression. For zebrafish, antisense morpholino oligonucleotides can be used to block miRNA function (Flynt et al., 2007; Kloosterman et al., 2007). Zebrafish, which allows analysis of the phenotypic

consequences due to gain or loss of function of specific miRNAs in a developmental context, will provide a powerful system to describe miRNA function.

Subsequent chapters will outline our efforts to apply expression analysis in combination with loss-of-function and gain-of-function experiments to describe the function of miRNAs in zebrafish development. We applied this strategy to study the miRNAs, *miR-214* and the *miR-8* family. In chapter II we demonstrate that *miR-214* is expressed beginning during segmentation stages in zebrafish embryos and is expressed in somites. This miRNA functions to regulate cell specification by modulating signaling mediated by the morphogen Hedgehog (Hh). *miR-214* affects Hh signaling by targeting an intercellular effector of this pathway, *su(fu)*. Su(fu) binds both repressor and activator forms of the Gli transcription factors. By down-regulating Su(fu) expression, *miR-214* ensures the precise exposure of developing somite cells to stimulation by Hh signaling. In chapter III we explore the role of the *miR-8* family in the physiology of a specialized cell type called ionocytes. These cells particpate in ion balance and acid/base regulation in zebrafish embryos. These cells have been previously shown to express members of the miR-8 family (Wienholds et. al., 2005). This family of miRNAs regulates the factor Nherf1, which affects the localization of apical transmembrane proteins. By down-regulating *nherf1*, the *miR-8* family ensures effective uptake of ions during osmotic stress. Experiments described in Chapter II represent the first reports of using morpholinos to analyze miRNA function. This discovery represents a major development in miRNA research in zebrafish by demonstrating the feasibility of functional analysis in this organism. The efforts presented in Chapter III are the first report of miRNAs functioning outside developmental processes in zebrafish embryos.

This finding highlights the similarity of the general function of miRNAs in fish and mammals, and suggests one of the great advantages of using the zebrafish system to study miRNAs is strong correlation of miRNA function between fish and mammals.

CHAPTER II

MICROARRAY ANALYSIS OF MICRORNA EXPRESSION

Portions of the data presented in this chapter have been published: Elizabeth J. Thatcher, Alex S. Flynt Nan Li Jonathan R. Patton James G. Patton. (2007). MiRNA expression analysis during normal zebrafish development and following inhibition of the Hedgehog and Notch signaling pathways. Developmental Dynamics. 236(8), 2172-2180.

Introduction

A central problem toward complete understanding of miRNA function is identifying the target genes regulated by individual miRNAs (Brennecke et al., 2005; Enright et al., 2003; Krek et al., 2005). Most miRNAs do not pair with perfect complementarity to their targets such that bioinformatic prediction is difficult and experimental validation is required. As a first step toward target identification, global miRNA expression patterns are needed, both temporal and spatial (Wienholds et al., 2005). Integrating miRNA and target expression data at specific stages of development will help to refine lists of possible targets for specific miRNAs. Here, we designed a sensitive (~0.1-0.7 fmols) microarray to expand expression analysis to 346 vertebrate miRNAs. We utilized this array to study miRNA expression during normal zebrafish development (Thatcher et al., 2007).

Results

Using sequences from the miRNA Registry as well as published reports (Griffiths-Jones, 2004; Lim et al., 2003), we designed DNA oligonucleotides containing 2 complete regions of complementary to 346 known and predicted vertebrate miRNAs. DNAs were synthesized containing C6 amine modified amino termini for coupling to slides. As controls, 6 positive and negative control oligonucleotides were included, complementary to zebrafish mRNAs (APC, β-actin, and β-catenin), zebrafish 18S rRNA, tomato lycopene synthase, and a bacterial dehydrogenase (PsA-NAD-dehyd). All oligonucleotides were spotted in duplicate or triplicate on glass mirror slides (Genexprex) by a 16-bit spotter in the Vanderbilt Microarray Shared Resource facility.

miRNAs are estimated to account for approximately 1-2% of cellular RNA (Lau et al., 2001). To enrich for microRNAs from zebrafish embryos, total RNA was isolated using TRI reagent and then passed over columns from mirVana miRNA isolation kits (Ambion) or sucrose gradients (Fig. 3C). Small RNAs were fluorescently labeled by attachment of Cy5 to the N7 position on G residues using LabelIT (Mirus) (Fig. 4). This leaves all base pairs that participate in hydrogen bonding unaffected and since only three vertebrate miRNAs are known to lack even a single G residue (*miR-197, miR-467a, and miR-467b*), the majority of miRNAs should be labeled. Fluorescently labeled RNAs were then hybridized to array slides. For analysis of miRNA expression, local background fluorescence levels were subtracted as well as signals from negative control spots containing oligonucleotides complementary to a bacterial dehydrogenase (PsA-NAD-dehyd). Subsequently, signal intensities for individual miRNAs were determined at multiple RNA concentrations. Signals were too close to background to provide

reliable data until 50-100ng of purified RNA were used for labeling whereas signal intensities appeared to plateau at concentrations above 4µg (Fig. 4C). Based on these results, we used 2µg of labeled purified small RNA for all subsequent arrays.

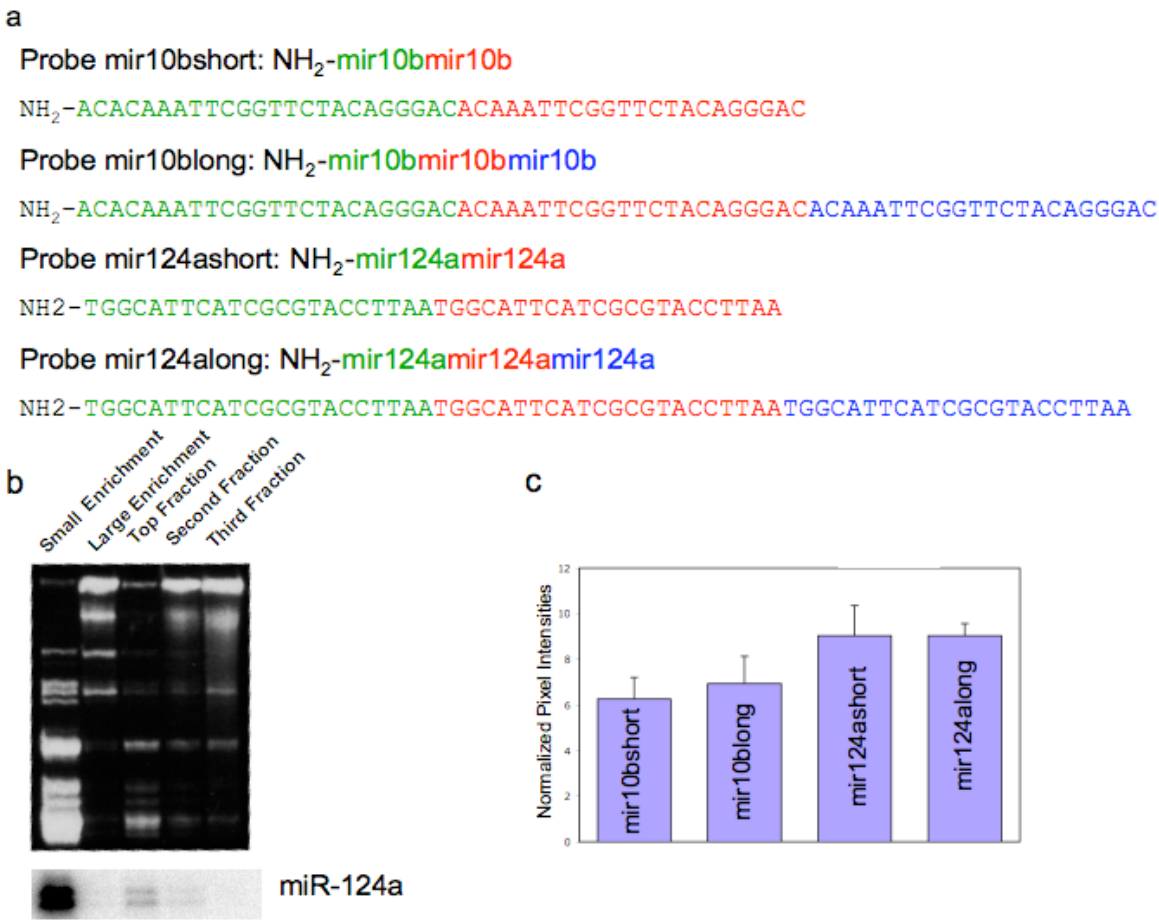


Figure 3. Microarray Probe Design and RNA Purification A) Oligonucleotide probes containing 2 (44 nucleotides) or 3 (66 nucleotides) regions of complementarity to either *miR-10b*, or *miR-124a* were coupled to mirror slides for initial microarray hybridization experiments. Individual antisense regions are indicated in green, red, or blue. B) Total RNA from zebrafish embryos was fractionated using either MirVana isolation kits or sucrose gradients. RNAs were separated on 12% 19:1 polyacrylamide gels and stained with ethidium bromide. MirVana fractions (Lanes 1, 2) and the top three sucrose gradient fractions (Lanes 3-5) are shown. C) Small RNAs isolated from 2 days post fertilization (2 dpf) zebrafish embryos were fluorescently labeled and hybridized to small scale arrays containing the probes described in A and pixel intensities were measured. Despite the increase in base pairing, no significant difference in fluorescence.

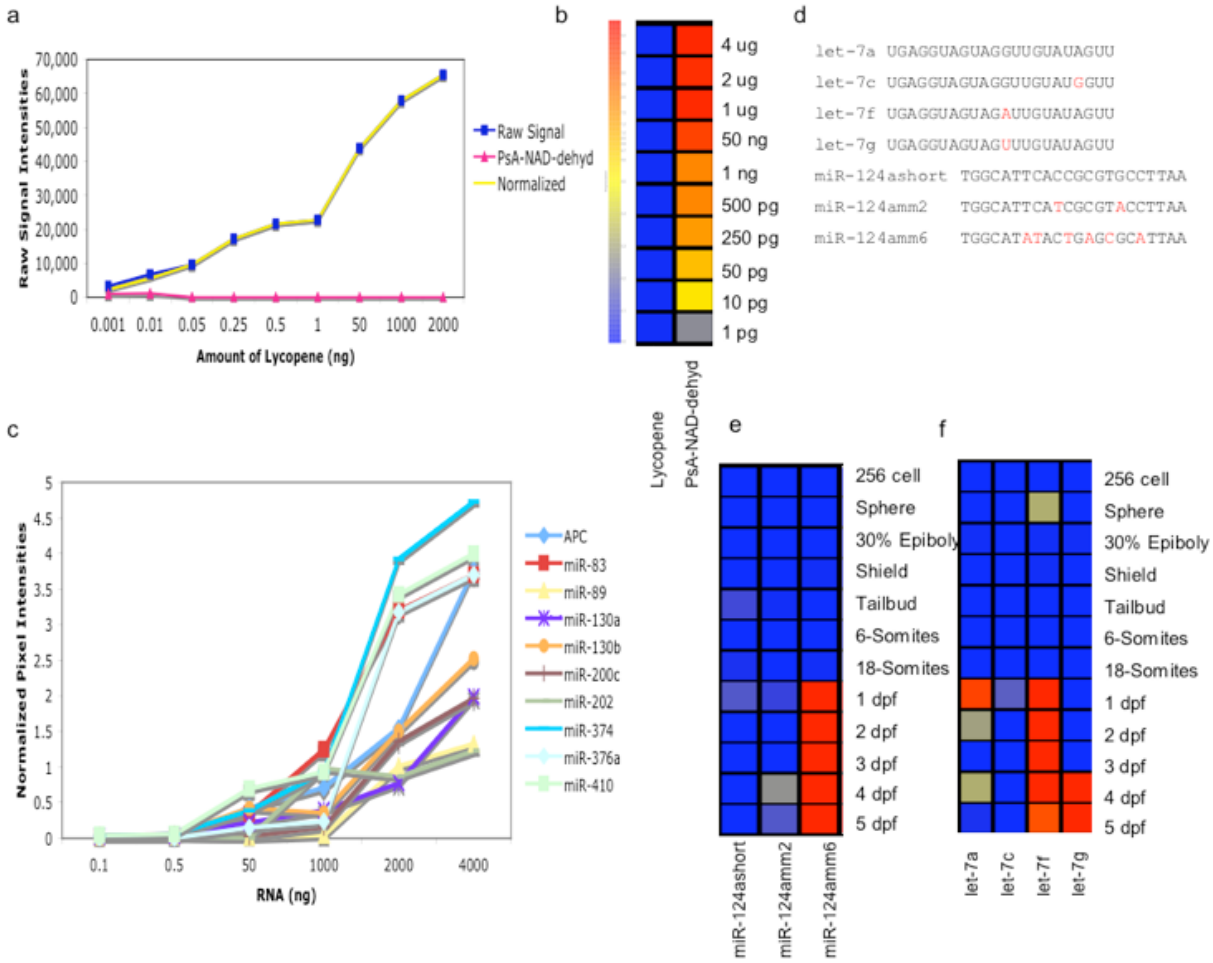


Figure 4. Microarray Sensitivity and Specificity. A,B) Sensitivity. Increasing amounts of a 22 nucleotide RNA encoding tomato lycopene synthase were spiked into microarray hybridizations and signal intensities were determined at each concentration after background and negative control subtraction. Values are shown in graphical (a) and heat map (b) format. As little as 1pg could be detected well above background. C) RNA Labeling. To optimize for the levels of RNA needed in each hybridization, signal intensities from 9 different miRNAs were determined after microarrays performed using from 0.1 to 4000ng labeled RNA. APC is a probe complementary to the zebrafish adenomatous polyposis coli mRNA. Based on these results, we chose to use 2ug for all subsequent arrays. D) Specificity. Single nucleotide specificity using Let-7 family members and mismatch controls. Four members of the *let-7* miRNA family are shown with indicated single nucleotide differences. Two and six mismatches were also incorporated into *miR-124a* short oligonucleotides. E, F) Heat map representation of signal intensities for the miRNAs shown in D. Specificity is demonstrated by the lack of cross-hybridization between probes (Red: high expression values; Blue: low to zero expression values).

Microarray sensitivity was determined by spiking hybridizations with exogenously added, fluorescently labeled 22nt RNAs complementary to tomato lycopene synthase mRNA. Background and negative control fluorescence intensities were again subtracted and the resulting signals were quantified and plotted (Fig. 4a,b). Dose dependent increases in fluorescent signals were observed but, importantly, as little as 1pg of lycopene synthase RNA could be detected at levels sufficiently above background**.** This suggests that the array is able to detect individual miRNAs within small RNA pools down to 0.1-0.7 fmols (1-5pg).

To analyze specificity, we utilized oligonucleotide probes that contained 1, 2, or 6 nucleotide mismatches. When probes containing 2 or 6 mismatches were tested, little or no hybridization could be detected due to the altered sequence (Fig. 4D-F). For single nucleotide changes, expression patterns from different members of the *let-7* family were compared where *let-7a, c, f,* and *g* all differ by just one nucleotide (Fig. 4D). Completely different patterns of expression between these *let-7* members were detected during zebrafish development (Fig. 4D,F). If cross-hybridization was occurring, similar, if not identical, expression patterns would have been observed.

Using the methodology described above, we analyzed global miRNA expression patterns by isolating total RNA from 12 specific stages of zebrafish development and carrying out hybridizations at each time point (Fig. 5). Local background and negative control signals were subtracted as above and the data from each stage were obtained from 3 independent hybridizations. As shown in Figures 5, 6, the overall pattern of miRNA expression increased during development. Such changes are indicated by an increase in red color as development proceeds. This agrees with the hypothesis that miRNAs play an

important role in differentiation and development (Chen et al., 2005; Giraldez et al., 2006; Naguibneva et al., 2006; Schratt et al., 2006; Voorhoeve et al., 2006; Wienholds et al., 2005). The one notable exception to relatively limited expression of miRNAs during early development is the pattern observed at sphere stage embryos where there appears to be significantly greater expression compared to other early time points (Fig. 5). Sphere stage coincides with the mid-blastula transition signaling the beginning of zygotic transcription and coincides with silencing or degradation of maternal RNAs in the zygote. The *miR-430* family has been shown to be involved in this process (Giraldez et al., 2006).

The array shown in Figs. 5, 6 analyzed 346 known miRNAs. To verify the array results, northern blots were performed using probes against 3 different miRNAs at 5 different stages of development (Fig. 7). As expected, the array was more sensitive but, importantly, the two different techniques showed very similar results.

Discussion

Previous analyses of miRNA expression during zebrafish development examined 154 different miRNAs (Wienholds et al., 2005). Here we expand the analysis to 346 miRNAs and analyze expression at specific developmental stages. A consistent difference between the data shown in Figs. 5, 6 and previous work is our finding that a significant number of miRNAs were detected at sphere stage. One potential explanation could be related to developmental timing as the previous reports harvested RNA based on hours post fertilization whereas we specifically isolated RNA from sphere stage embryos.

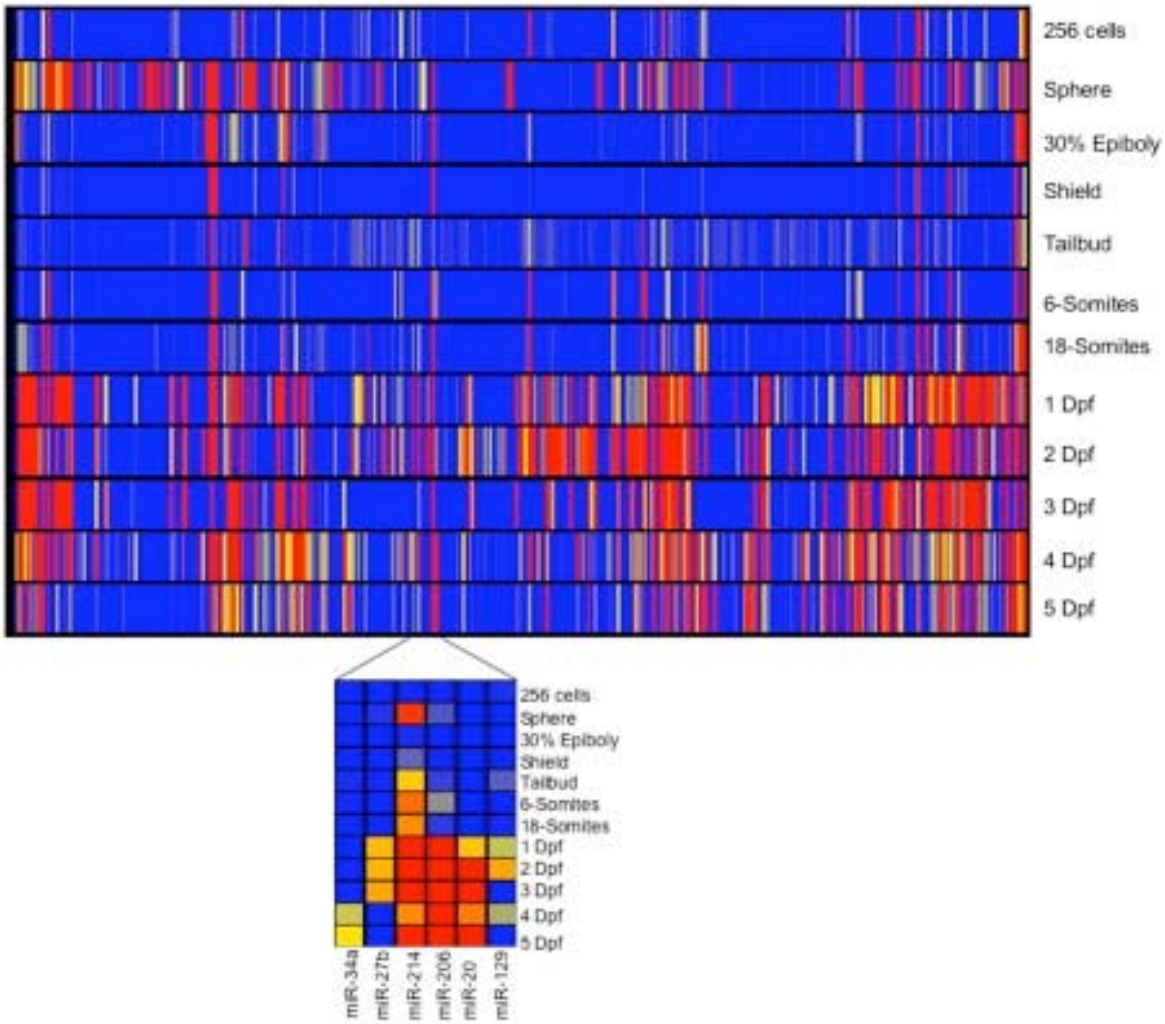


Figure 5. Wild-type Zebrafish Developmental Array. Small RNAs were isolated from 12 different stages during zebrafish embryonic development, fluorescently labeled, and hybridized to an array containing 346 miRNAs. The increase in expression across developmental stages is shown with a blow up of the region containing *miRs-34a, -27b, -129a, -20, -206* to illustrate individual miRNAs. Blue indicates background with red indicating high expression.

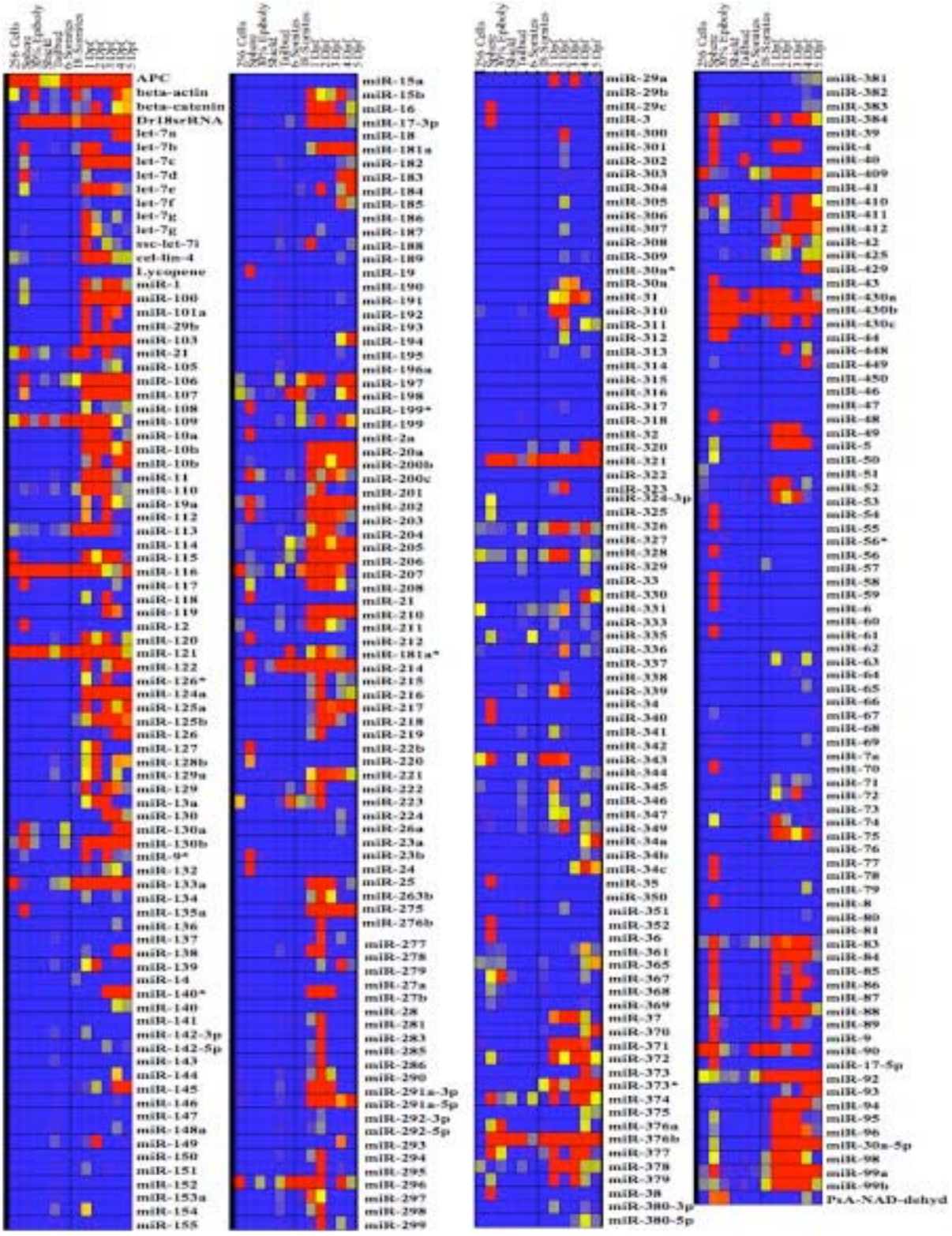


Figure 6. miRNA Expression Patterns During Zebrafish Development.
Heat maps as in Fig. 1 illustrate the changes in expression for individual miRNAs, denoted at the right.

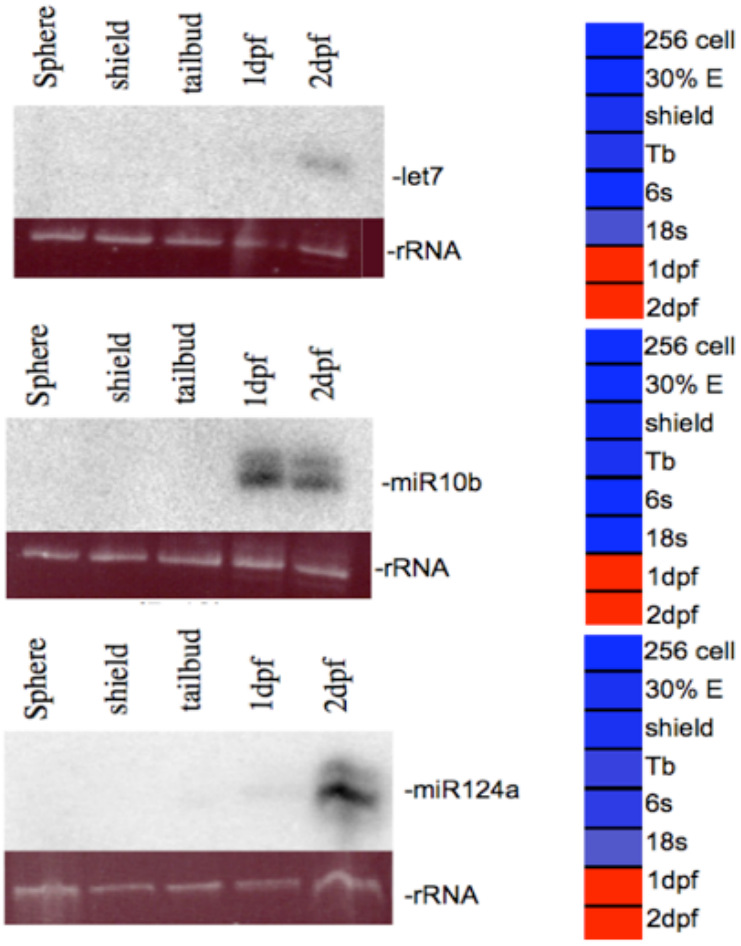


Figure 7. Northern blot Verification of Microarray Results.
Microarray results were verified for three microRNAs using Northern blots.

Deciphering global miRNA expression patterns during development is a necessary first step toward identifying functional miRNA targets. Temporal expression patterns can rapidly be determined using microarray approaches and coupling such assays with the ability to analyze spatial patterns using *in situ* hybridization (Wienholds et al., 2005) makes zebrafish a powerful model system to examine miRNA expression. Here,

we used direct labeling of small RNAs to examine the expression of 346 miRNAs during zebrafish development. Our array was found to be sensitive to as little as 1 pg of miRNA and was able to distinguish between single base mismatches. This provides a powerful approach to examine overall patterns of miRNA expression in wild type or mutant embryos as well as embryos exposed to small molecules, drugs, or other environmental stimuli. While the approach has proven quite sensitive, microRNAs that are expressed in single cells, such as *lsy-6* in *C. elegans* (Johnston and Hobert, 2003), will likely go undetected due to low abundance in whole embryo RNA preparations.

The overall profile of miRNA expression during normal development is shown in Figs. 5,6 and in general agrees with previous work showing that miRNA expression patterns become more complex as development proceeds (Chen et al., 2005; Wienholds et al., 2005). One difference over and above the fact that we expanded the analysis to 346 miRNAs, is that we observed significantly higher expression of miRNAs at sphere stage. In agreement with previous work, the *miR-430* family is highly expressed at this early stage where they function to mediate targeted clearance of maternal mRNAs at the mid-blastula transition when zygotic transcription begins (Giraldez et al., 2006). However, we also observed significant expression of many more microRNAs at this time. One plausible explanation is that we developmentally staged embryos prior to RNA isolation whereas previous analyses likely had a slightly asynchronous population since selection was based entirely on hours post fertilization. It is possible that a narrow window exists at exactly the midbastula transition when many genes are initially transcribed even if they then become downregulated until later development. Previous work has suggested that assembly of chromatin versus association of the basal

transcription machinery limits transcription prior to the midbastula transition but that specific transcription activators can still access the DNA before this time (Prioleau, 1995). After the midblastula transition, the basal transcription machinery joins to activate transcription but subsequent production of other regulators could then establish developmentally regulated transcriptional programs.

Besides the sphere stage, expression patterns detected using our array are mostly in agreement with previous cloning and microarray approaches showing limited early miRNA expression which increases upon organogenesis and development into adulthood with terminal differentiation of multiple cell types (Chen et al., 2005; Wienholds et al., 2005). For example, muscle specific *miR-206* is initially expressed during somite development and then persists through adulthood. Likewise, *miR-181a* begins expression early before localization to the nose and eyes in adult fish (Wienholds et al., 2005). However, notable differences were also observed between the approaches as exemplified by analysis of the *let-7* family of miRNA where timing differences were noted that are most likely due to differences in specificity since our array was able to detect single base mismatches.

Methods

Microarrays

All DNA oligonucleotides were purchased from MWG Biotech and printed on mirror slides (Genexprex) in duplicate or triplicate with two complete arrays per slide. Slides were blocked with a quick wash in 0.2% SDS, a 45 min. incubation at 55^0C in blocking buffer (5X SSC, 1% BSA, 1% SDS), rinsed with deionized water, and spin-

dried. Hybridizations were carried out in 25% deionized formamide, 5X SSC, 0.1% SDS for exactly 16 hrs. in ArrayIt© hybridization chambers followed by three successive washes (3 min. each) in 2X SSC, 0.1% SDS; 1X SSC; and 0.1X SSC. All microarrays were scanned with a GenePix4000B scanner and data was analyzed with GeneSpring 7.0 software. All time points were performed in triplicate. For normalization, hybridizations were spiked with identical amounts of tomato lycopene synthase RNA. Arrays with significant background and/or insufficient lycopene synthase levels were discarded.

miRNA Isolation

Total RNA was isolated from zebrafish embryos using TRI Reagent (Molecular Research Center). Small RNAs were isolated using 15-45% sucrose gradients or by passage over mirVana miRNA isolation kits (Ambion).

RNA Labeling

For Cy5 labeling, 2.4 mg of small RNA were labeled in a 1:1 (RNA:Cy5) ratio using LabelIT (Mirus) for 1 hr at 37^0C. Unincorporated Cy5 was removed by passage over nucleotide removal kits (Qiagen) and diluted to 2 μg per array after purification. Synthetic lycopene synthase RNA was labeled and purified using the above process and was diluted to 50 pg per array.

Northern Blots

RNAs were separated on 12% acrylamide gels and electroblotted to positively-charged nylon membranes. DNA oligonucleotides complementary to specific miRNAs

were labeled with α-^{32}P-dATP using StarFire labeling kits (IDT). Hybridizations were carried out in 7% SDS and 0.2 M $NaPO_4$, pH 7.2 for 12 hours followed by washes in 2XSSPE-0.1%SDS, 1XSSPE-0.1%SDS, and 0.5XSSPE-0.1%SDS.

CHAPTER III

FUNCTIONAL ANALYSIS OF MIR-214

The data presented in this chapter have been published: Alex S. Flynt, Nan Li, Elizabeth J. Thatcher, Lilianna Solnica-Krezel, and James G. Patton. (2007). Zebrafish *miR-214* Modulates Hedgehog Signaling to Specify Muscle Cell Fate. Nature Genetics. 39(2), 259.

Introduction

Numerous miRNAs have been discovered in the genomes of higher eukaryotes and functional studies indicate they play important roles during development. However, little is known concerning the function of individual miRNAs. We approached this problem in zebrafish by combining identification of miRNA expression, functional analyses, and experimental validation of potential targets. We show that *miR-214* is expressed during early segmentation stages in somites and that varying its levels alters the expression of genes regulated by Hedgehog (Hh) signaling. Inhibition of *miR-214* results in a reduction or loss of slow muscle cell types. We show that *su(fu)* mRNA, encoding a negative regulator of Hh signaling, is targeted by *miR-214.* Through regulation of *su(fu), miR-214* enables precise specification of muscle cell types by sharpening cellular responses to Hh.

Results

Multicellular organisms employ miRNAs to regulate gene expression in a tissue- or temporal-specific manner, guiding developmental decisions (Giraldez et al., 2005). To identify target genes regulated by miRNAs, we first developed a microarray to examine temporal miRNA expression patterns during the first 12 hours of zebrafish development (Thatcher et al., 2007).

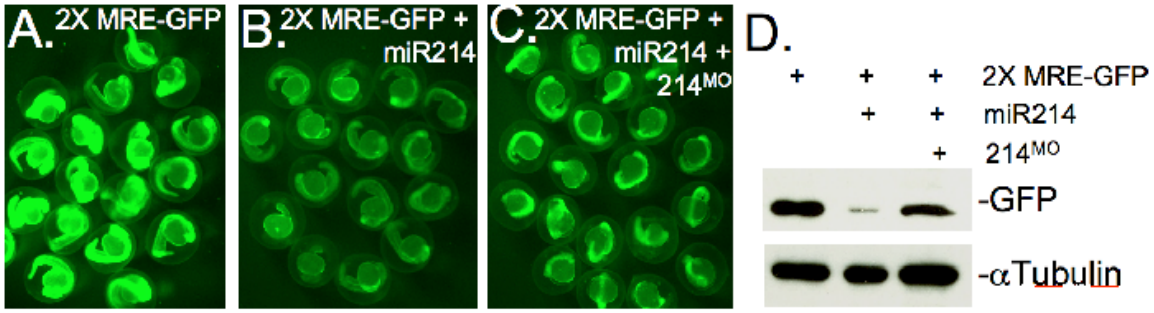


Figure 8. Morpholino Mediated Inhibition of *miR-214*.
(A) Injection of zebrafish embryos with synthetic mRNA encoding GFP fused to a 3'UTR containing tandem miR-214 recognition elements (2XMRE). (B) Co-injection of miR-214 with the GFP reporter mRNA as in A. (C) Co-injection of morpholinos targeted (214^{MO}) to miR-214 and miR-214 synthetic RNAs with the GFP reporter mRNA. (D) Western blots of embryos as in A-C with antibodies against GFP or α-tubulin.

To understand the function of a subset of these miRNAs, we performed loss-of-function experiments employing antisense morpholino oligonucleotides (MOs) complementary to mature miRNAs. MOs have been used extensively in zebrafish as antisense inhibitors of mRNA translation and splicing (Nasevicius and Ekker, 2000) but are also capable of interfering with miRNA function (Fig. 8A-D). Among the miRNAs tested, injection of MOs designed to block the function of *miR-214* (214^{MO}) yielded embryos exhibiting U-shaped somites at 1 day post fertilization (1dpf) (Fig. 9A-D). Expression of *miR-214* begins during early somitogenesis and continues throughout

embryogenesis (Fig 9E). *In situ* hybridization revealed that *miR-214* is expressed in somites at 1dpf (Fig 9F-G) (see also Weinholds et al 2005) (Wienholds et al., 2005).

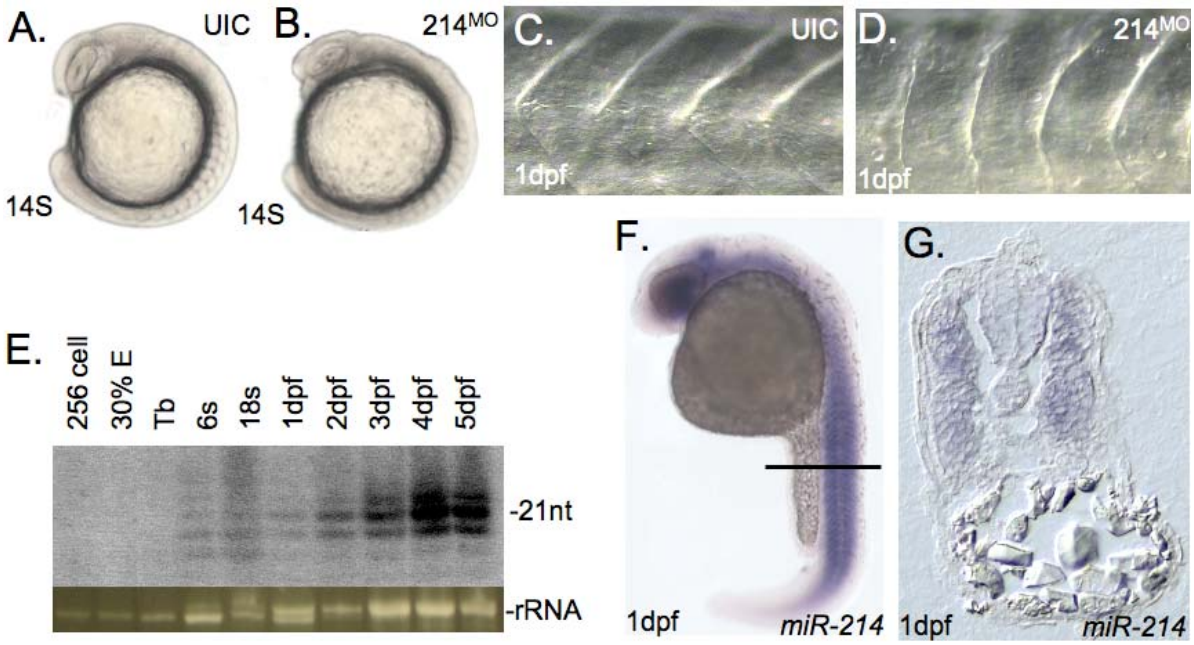


Figure 9. *miR-214* participates in somitogenesis. (A-B) Morphology of embryos injected with 214^{MO} at 14 somite stage. (C-D) Somite morphology in uninjected controls (UIC)(C) or embryos injected with antisense morpholino oligonucleotides complementary to miR-214 (214^{MO})(D). (E) Expression of *miR-214* determined by northern blotting. Embryonic stages are listed above and ethidium stained rRNA is shown for loading control. (F-G) Expression of *miR-214* in somites at 1dpf determined by *in situ* hybridization in whole mount embryos (F) and somite cross section through the trunk region as indicated (G)

***miR-214* Regulates Hedgehog signaling**

Somites are transient embryonic structures derived from paraxial mesoderm that give rise to muscle and skeleton (Lewis et al., 1999). Pre-somitic mesodermal cells immediately juxtaposed to the notochord (adaxial cells) are highly influenced by Hh and give rise to the slow twitch muscle lineage (Ingham and Kim, 2005; Wolff et al., 2003). Lateral presomitic cells give rise to fast twitch muscle fibers and experience little

stimulation Hh initially while later developing fast muscle fates are dependent on Hh signaling (Feng et al., 2006). There are two slow muscle cell types that require precise Hh signals for proper development: superficial slow fibers (SSFs) which migrate from the midline to populate the surface of the myotome, and slow muscle pioneers (MPs) that remain close to the midline (Henry and Amacher, 2004; Wolff et al., 2003). MPs require higher levels and longer exposure to Hh for proper specification than do SSFs and can be distinguished from slow muscle fibers by the expression of the transcription factor Engrailed (Eng) (Lewis et al., 1999; Wolff et al., 2003). *In situ* hybridization revealed that inhibition of *miR-214* function resulted in a loss of *eng2a* positive cells during early segmentation (Fig. 10A-B), consistent with the U-shaped somite defects observed in 214^{MO} injected embryos (Fig. 9A-D). Reduction of this marker suggested an overall decrease in Hh signaling in the adaxial cells of 214^{MO} injected embryos. To test this, we analyzed expression of *patched1* (*ptc1)*, which encodes a Hh ligand receptor whose transcription is activated by Hh (Goodrich et al., 1996). Embryos injected with the 214^{MO} showed decreased expression of *ptc1* in adaxial cells and upregulation in lateral cells. (Fig. 10C-F, 11A-B). This suggested that *miR-214* might regulate the level of Hh signaling during somite differentiation.

To further characterize defects associated with inhibition of *miR-214* function, we monitored several markers of adaxial cell derivatives at later stages. All slow fibers express the homeodomain protein Prox1 as well as a myosin heavy chain isoform specific to slow muscle (Slow Myosin HC) (Roy et al., 2001). Co-detection of Slow Myosin HC and Prox1 by immunohistochemistry revealed decreased numbers of slow muscles in *miR-214* morphants at 1dpf (Table 1) (Fig. 11C-D). Additionally, we monitored Eng

proteins which are expressed in both MPs and medial fast fibers (MFFs), a fast twitch muscle cell type that arises after SSF migration and is dependent on Hh signaling but does not express Prox1 (Wolff et al., 2003). Injection of 214^{MO} reduced the number of MPs during late somitogenesis (Fig. 10G)(Table 1). To verify that the altered somite differentiation in *miR-214* morphant embryos is due to perturbation of Hh signaling, we sought to rescue the 214^{MO} phenotype by co-injection of synthetic *sonic hedgehog* (*shh)* RNA (Fig. 10G-I, 11C-F)(Table 1). Significantly, *miR-214* morphant defects were completely suppressed by Shh misexpression.

Next, we analyzed the effect of *miR-214* misexpression on Hh-mediated cell fate specification (Fig. 10J-M, 11G-J). Unlike down-regulation of *miR-214* function, injection of synthetic *miR-214* into 1-cell embryos did not yield appreciable alterations of *ptc1* expression in somites (Fig. 11I,J). However, ectopic expression of excess *miR-214* did result in perturbation of Hh regulated markers in the ventral neural tube (Fig. 10J-M, 11G,H). *nkx2.2a* is expressed in the lateral floor plate of the neural tube and its expression is stimulated by the highest levels of Hh activity whereas *olig2* expression is adjacent and dorsal to the *nkx2.2a* domain (Park et al., 2004; Schafer et al., 2005; Schauerte et al., 1998). Misexpression of *miR-214* resulted in expanded expression of *nkx2.2a* and a dorsal shift of *olig2* expression, further supporting a role for *miR-214* in the modulation of Hh signaling.

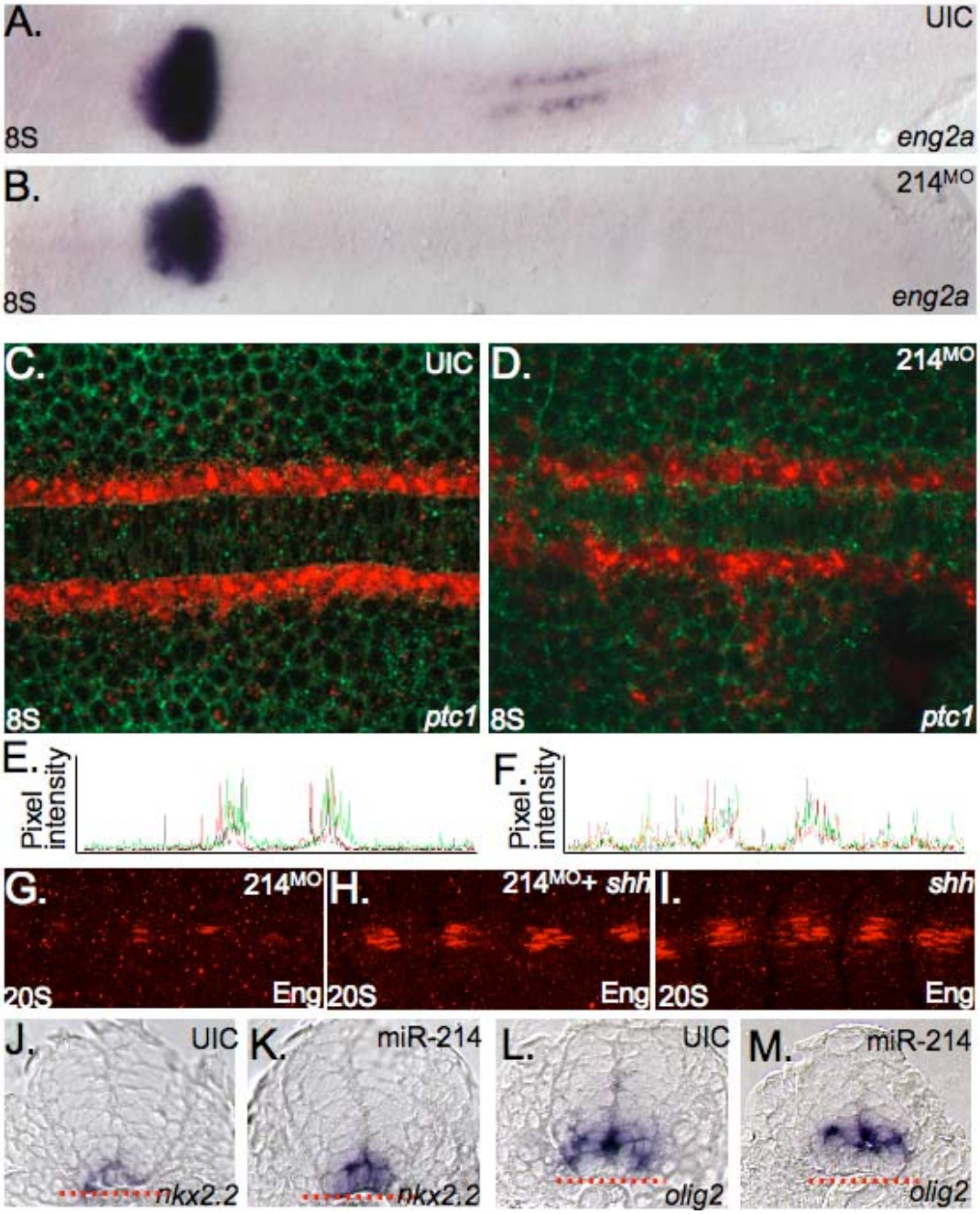
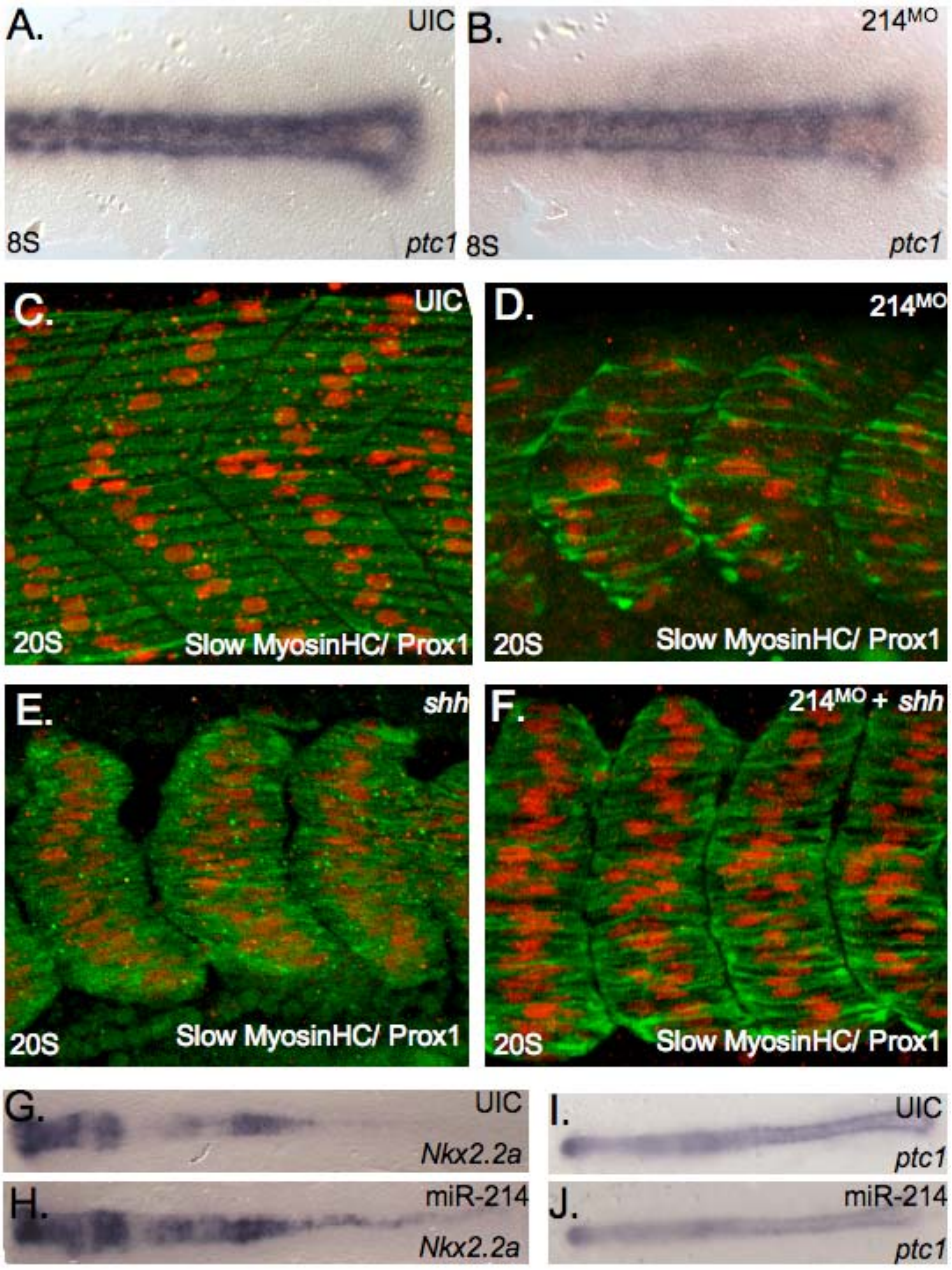


Figure 10. Loss of *miR-214* function prevents specification of muscle pioneer cells. (A-B) Loss of muscle pioneers revealed by *eng2a in situ* hybridization in 214^{MO} morphants. UIC (A) 214^{MO} injected embryo (B). (C-D) Aberrant *ptc1* expression in 214^{MO} injected embryos (D) compared to UIC (C). (E-F) Pixel intensities of *ptc1* stain in three embryos plotted from bottom to top of images in C,D (embryo's left on bottom); UIC (E) and 214^{MO} (F) (G-I) Eng positive nuclei in 214^{MO} injected (G) 214^{MO} and *shh* co-injected (H) and *shh* mRNA injected (I) embryos. (J-M) Expression of *nkx2.2a* and *olig2* in sections of spinal cord from UIC embryos (J,L) and embryos injected with *miR-214* (K,M)



Supplemental Figure 11. miR-214 regulates Hh signaling. (A-B) *ptc1* (blue) expression in UIC (A) and 214^{MO} injected (B) flat-mounted embryos. Images acquired with a Zeiss Axioplan microscope using a 10X objective, an AxioCam digital camera, and Axiovision Software (A-B) Detection of Slow MyosinHC (green) and Prox1 positive nuclei (red) in twenty somite stage embryos by immunohistochemistry using a Zeiss LSM510 Meta laser scanning confocal microscope. NIH ImageJ software was used to generate Z-projections with contrast adjusted in Photoshop. UIC (C), 214^{MO} morphants (D), *shh* mRNA injected (E), and 214^{MO} *shh* mRNA co-injected (F). **(**G-H) Expression of *nkx2.2a* in UIC (G) and *miR-214* injected (H). **(**I-J) Expression of *ptc1* in UIC (I) and *miR-214* injected embryos (J).

su(fu)* is a target of *miR-214

Similar to most miRNAs, *miR-214* has many predicted targets, one of which is *suppressor of fused* (*su(fu)* (Chen et al., 2005). Su(fu) is a well-characterized negative regulator of Hh signaling, essential for proper specification of muscle cell types during somitogenesis (Koudijs et al., 2005; Wolff et al., 2003). To test whether *miR-214* targets *su(fu)*, we monitored GFP fluorescence in embryos microinjected with mRNAs derived from the following reporter constructs: the 3'-UTR of *sufu* cloned downstream of the GFP ORF (GFP*su(fu)*), GFP fused to two perfect *miR-214* recognition elements (GFP 2XMRE), or GFP lacking heterologous 3'UTR sequences (GFP –UTR) (Fig 12B-H). Consistent with the hypothesis that sequences in the *su(fu)* 3'UTR contain *bona fide* recognition elements, GFP fluorescence levels of embryos co-injected with *miR-214* RNA and GFP*su(fu)* mRNA were significantly decreased (Fig. 12E). Western blots performed with antibodies against GFP on whole embryo lysates were used to verify the fluorescence experiments (Fig. 12H).

To confirm that the decrease of slow muscle cells caused by *miR-214* inhibition is directly due to *su(fu)* de-repression, we sought to rescue the defect through simultaneous down-regulation of both *su(fu)* and *miR-214* (Fig. 13A-H) (Table 1). Injection of two independent MOs targeted to *su(fu)* (*su(fu)*^{MO1,2}) caused an increase in the number of slow muscle cells but no significant change in the number of MPs (Fig. 13G-H)(Table 3) (Wolff et al., 2003). In contrast, injection of a mis-matched morpholino (*su(fu)* mm^{MO}) had no effect (Fig. 13A-B)(Table 1). Importantly, co-injection of 214MO along with *su(fu)*^{MO1,2} resulted in a restoration of slow muscle cells (Fig. 13E-F)(Table 1) compared

to embryos co-injected with 214^{MO} and *su(fu)*-mm^{MO} (Fig. 13C-D)(Table 1). Inhibition of *su(fu)* alone has been shown to increase the number of MFFs while not affecting specification of MPs (Wolff et al., 2003). Co-labeling of Eng and Prox1 revealed that the rescued Eng-expressing cells in 214^{MO} and $sufu^{MO1,2}$ co-injected embryos are MPs and not MFFs (Fig. 13A,C,E,G). Together, our results indicate that *su(fu)* inhibition suppresses the deficiency of MPs and SFFs associated with loss of *miR-214* function supporting the hypothesis that *miR-214* modulates Hh signaling largely by regulation of *su(fu)*.

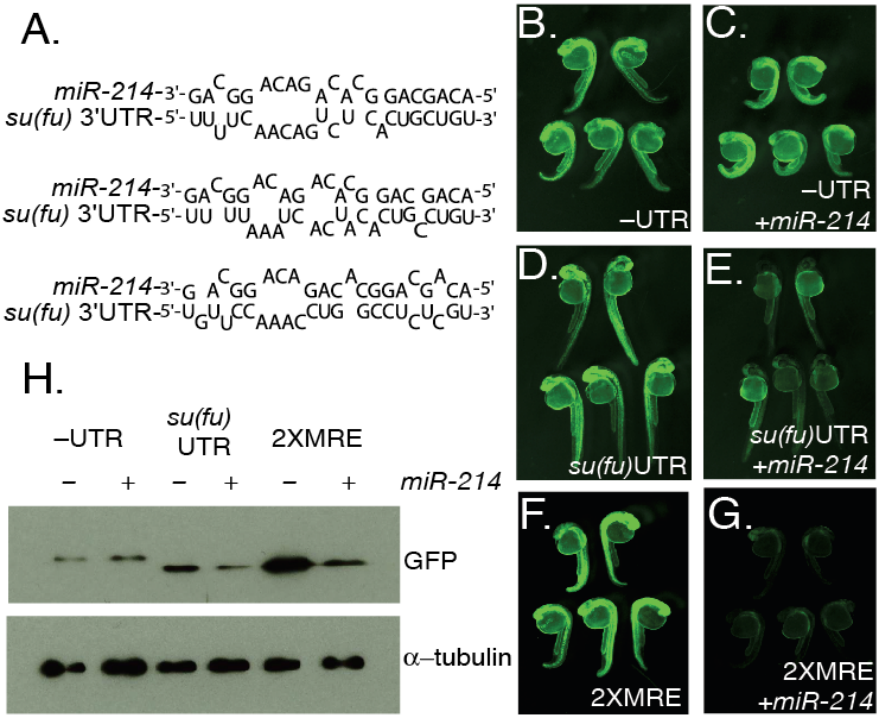


Figure 12. ***su(fu)* is a target of *miR-214*.** (A) *su(fu)* 3'UTR sequence elements possessing complementarity to *miR-214*. (B-G) Fluorescence in embryos injected with synthetic mRNAs encoding GFP with or without co-injected *miR-214* RNA. (B,D,F) Embryos injected with GFP without UTR sequence (–UTR)(B), GFP fused to the *su(fu)* 3' UTR (*su(fu)UTR*)(D), or GFP fused to two perfect *miR-214* sites (2XMRE)(F) alone. (C,E,G) Embryos co-injected with *miR-214* RNA and GFP reporter on left. (H) Western analysis of lysates prepared from embryos injected with –UTR, *su(fu)*UTR, or 2XMRE GFP with or with out miR-214 RNA co-injection. As control, blots were performed on the same lysates with α-tubulin antibodies.

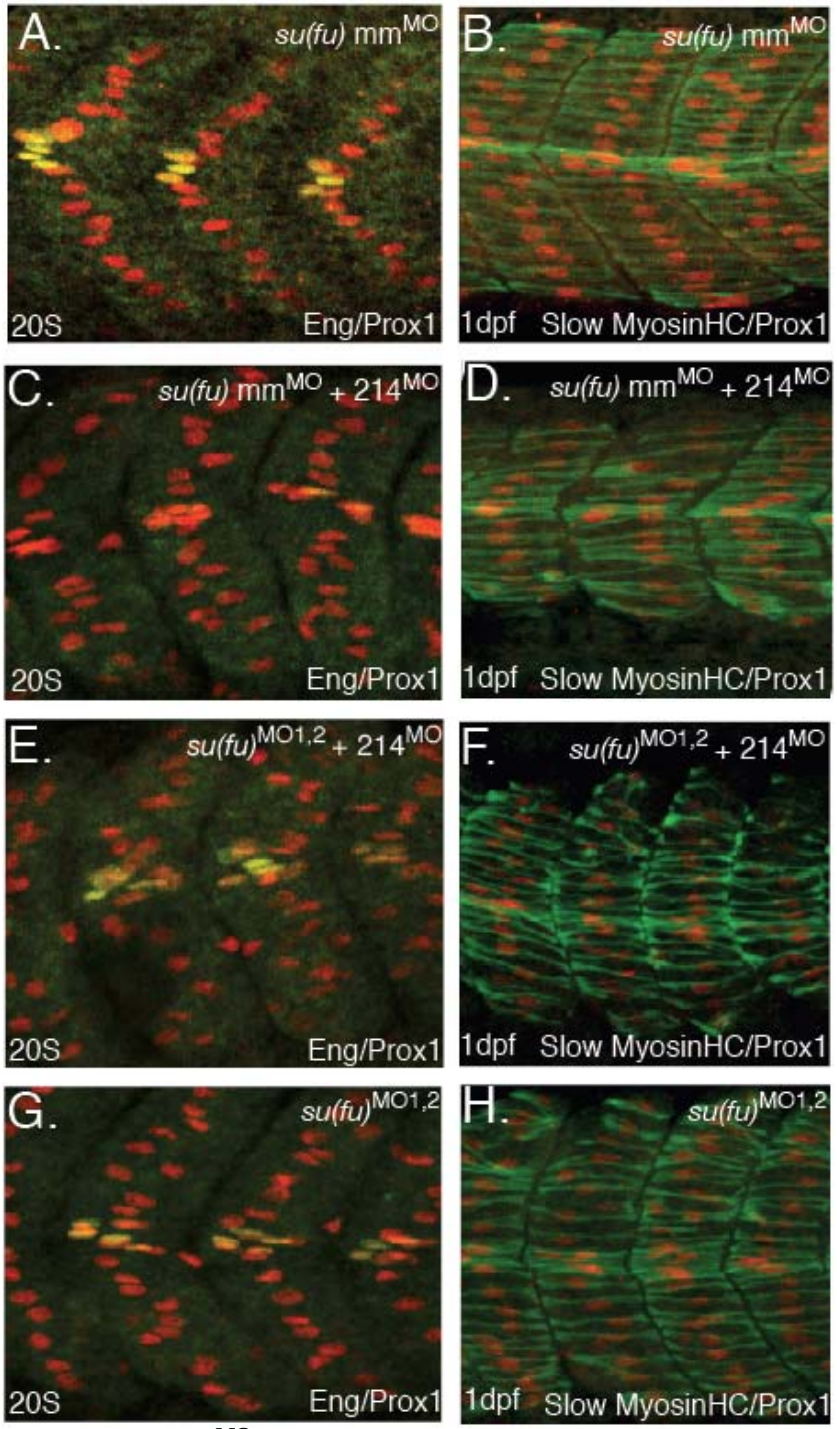


Figure 13. Rescue of miR-214MO phenotype by simultaneous inhibition of *su(fu)* expression. (A-H) Expression of Eng (green) and Prox1 (red) in 20 somite embryos (A,C,E,G), or expression of Prox1 (red) and slow muscle myosin (green) in 1dpf embryos (B,D,F,H). Embryos were injected with *su(fu)* mis-matched MOs (*su(fu)* mm^{MO})(A-B). Embryos injected with two MOs targeted to *su(fu)* (*su(fu)*^{MO1,2})(G-H). Embryos coinjected with either *su(fu)* mm^{MO} and 214MO (C-D) or with *su(fu)*^{MO1,2} and 214MO (E-F).

Treatment	Slow muscle cells	Muscle Pioneers
UIC	23.7±.0.28(47)[a]	3.4±0.11(47)[a]
214^{MO}	16.0±0.26(73)[b]	1.7±0.13(68)[b]
214^{MO} + *shh*	43.0±1.24(53)[c]	6.4±0.34(25)[c]
shh	42.6±1.68(34)[c]	6.2±0.31(39)[c]
sufu mm^{MO}	24.3±0.33(48)[a]	3.3±0.11(35)[a]
214^{MO} + *sufu* mm^{MO}	16.5±0.26(71)[b]	1.4±0.10(103)[b]
214^{MO} + $sufu^{MO1,2}$	22.5±0.34(62)[a]	3.6±0.10(108)[a]
$sufu^{MO1,2}$	28.0±0.44(43)[d]	3.8±0.09(127)[a]

Table 1. Numbers of muscle cell types in morphants. Embryos treatments are indicated on the left and analyzed cell types are indicated above. Slow muscle cells are positive for both Prox1 and Slow MyosinHC. Muscle Pioneers are positive for Eng and Prox1. Markers were visualized by fluorescent immunohistochemistry and confocal microscopy as in Fig. 2, Fig.4, and Supplemental Fig. 2. Values are the mean of the specified cell type per somite ± s.e.m. Number of somites analyzed are indicated in parentheses. ANOVA was performed to determine statistical significance within a 95% confidence interval. Comparisons are within individual columns where values labeled with different superscript letters indicate significant differences whereas those with the same superscript do not differ significantly from each other.

Disscussion

Su(fu) participates in Hh signaling by altering the function of the Gli family of transcription factors. Su(fu) tethers both the activator and repressor forms of Glis in the cytoplasm, resulting in down-regulation of both activities (Dunaeva et al., 2003; Wolff et al., 2003). Inhibition of Su(fu) by *miR-214* in adaxial cells, which experience high levels of Hh signaling, allows maximal activation of Gli mediated transcription. Regulation of *su(fu)* by *miR-214* in lateral muscle cells, which are exposed to lower levels of Hh

signaling, results in increased repressor activity ensuring a commitment to fast muscle cell fate. These distinct effects on Hh signaling are demonstrated by changes in the expression of *ptc1* in 214^{MO} injected embryos. Inhibition of *miR-214* function permits increased expression of Su(fu), resulting in decreased *ptc1* expression in adaxial cells and increased expression in lateral presomitic mesoderm (Fig. 10D). The decreased Hh response in adaxial cells causes cell types dependent on high levels of Hh signaling to be lost (MPs) or reduced (SSFs) triggering the formation of U-shaped somites. In contrast, increased expression of *ptc1* in lateral somites can be explained by the decreased activity of Gli repressor forms, which are also negatively regulated by Su(fu). Likewise, aberrant expression of Hh regulated genes in the neural tube caused by ectopic expression of *miR-214* can be explained by disruption of Su(fu) expression. In the presence of ectopic *miR-214*, neural tube cells become more sensitive to Hh signals causing them to acquire more ventral fates, as demonstrated by the increase in *nkx2.2a* expression and a dorsal shift in *olig2* expression. Together, these data support a role for *miR-214* mediated regulation of *su(fu)* that is essential for specification of muscle cell types during somitogenesis by sharpening the response to different levels of Hh signals.

Compared to invertebrates, fungi, and plants, the function of miRNAs in vertebrates has been proposed to be more limited, subtly modulating cell types or as a redundant mechanism for ensuring appropriate gene expression patterns (Bartel and Chen, 2004). We have shown a requirement for *miR-214* to specify muscle cell fate during somitogenesis. This finding suggests that some vertebrate miRNAs play decisive roles during development being required for the generation of specific cell types.

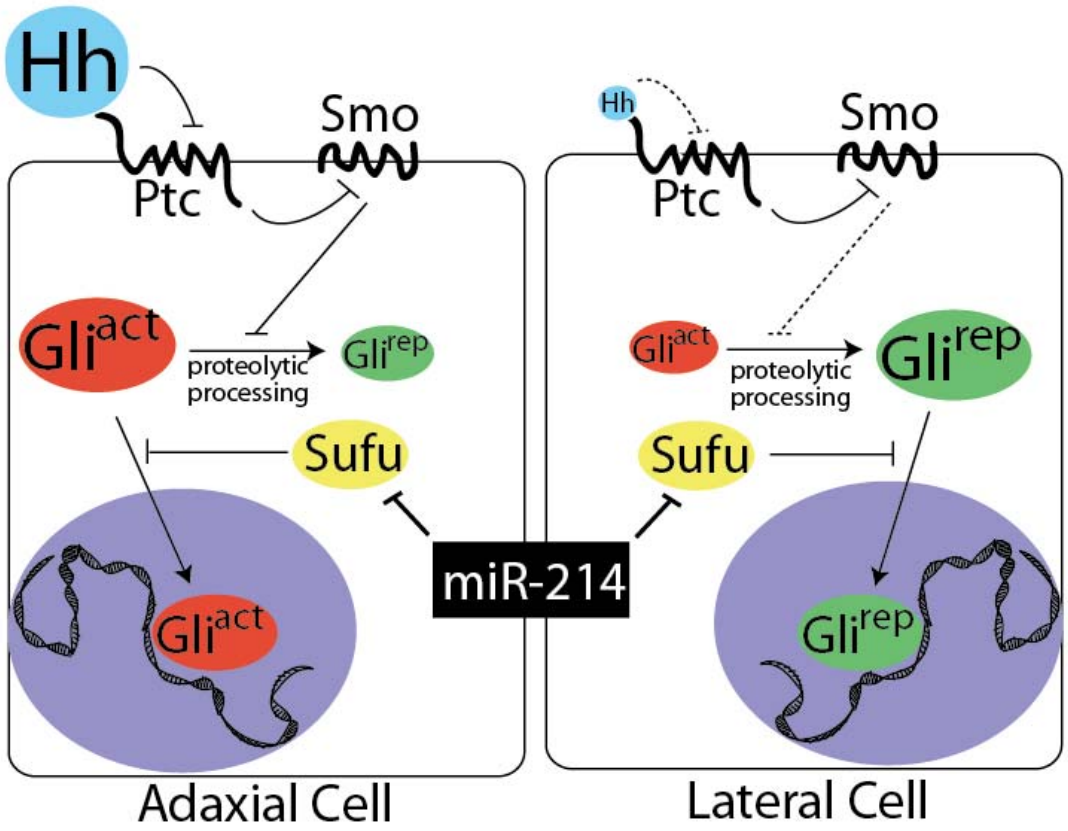


Figure 14. Modulation of Hh signaling in somite cells by miR-214. Su(fu) acts on both activator and repressor forms of Gli, inhbiting nuclear trafficking. *miR-214* down-regulates Su(fu) allowing both maximal activation in the presence of Hh and complete repression when Hh signaling is minimal.

Methods

Micro-injections

Fertilized 1-cell zebrafish embryos were injected with 1nl volumes at the following concentrations: 2ng of 214MO, 5'-CTGCCTGTCTGTGCCTGCTGT-3', 2ng of miR-214 identical siRNAs 5'-ACAGCAGGCACAGACAGGCAG-3', 1ng of sufu^{MO1} 5'-GCCGCATCTCATCCATCCCGCACGG-3' and 1ng of sufu^{MO2} 5'-CGCCAAACAGGGAAAAGTTCTCGAA-3' combined together (Wolff et al., 2003), 2ng of sufu mm^{MO} 5'-GCGGCTTCTCATGCATCCCCCAGG-3' (Wolff et al., 2003) 100pg of *in vitro*

transcribed, capped *Shh* mRNA, and 50ng of *in vitro* transcribed, capped GFP reporter. Zebrafish *sufu* 3'UTR sequences were amplified by RT-PCR and subcloned into pGEM. Synthetic *shh* mRNA was a gift from Chunyue Yin.

Nothern blots and *In situ* hybridization

Northerns were performed as previously described (Sempere et al., 2003). Detection of mRNAs and primary miRNAs was accomplished as previously described (Thisse et al., 1993) using digoxigenin (DIG) labeled antisense RNA probes and NBT/BCIP or fast red color development. Detection of mature *miR-214* RNAs was carried out using DIG-labeled Locked Nucleic Acid (LNA) probes (Exiqon) following previous reports (Wienholds et. al., 2005) and visualized using NBT/BCIP color development. Cryosectioning was performed as described (Barnfield et al., 2005).

Immunohistochemistry

Immunostaining was as described (Topczewska et al., 2001). Antibodies included rabbit polyclonal antibodies against Prox1 (Abcam), mouse monoclonal antibodies against SlowMHC (F59; a gift from F. Stockdale), rabbit polyclonal antibodies against chick Engrailed (a gift from A. Joyner), and rabbit polyclonal antibodies against GFP (Torry Pines Biolabs). The 4D9 monoclonal antibody against Engrailed was developed by Corey Goodman and obtained from the Developmental Studies Hybridoma Bank under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences. Secondary antibodies against mouse or rabbit IgG were Cy2 or Cy3 conjugated (Jackson Immuno Research).

Western Blotting

1 day post fertilization (1dpf) embryos were manually dechorionated and deyolked. Embryos were briefly sonicated in passive lysis buffer (25mM HEPES pH 7.5, 5mM MgCl2, 300mM Na, 1mM EDTA, 0.2mM EGTA, 1mM DTT, 10% glycerol, 1.0% Triton X100, 1mM PMSF). 20μg of total proteins were then separated on 10% polyacrylamide gels followed by transfer to nitrocellulose membranes. After blocking with 5% nonfat dry milk, blots were probed with antibodies against GFP (Santa Cruz) or α-tubulin (Abcam). Detection used HRP conjugated secondary antibodies against mouse and rabbit, respectively, followed by visualization with ECL.

Imaging

Live embryos were mounted in 2% methyl cellulose. NBT/BCIP developed *in situ* hybridizations were whole mounted or flat mounted in 100% glycerol. Both live and NBT/BCIP treated embryos were photographed using a Zeiss Axiophot compound microscope and an Axiocam digital camera. Images were acquired with use of Axiovision software and imported into photoshop for orientation. Embryos subjected to immunohistochemistry were mounted in 50% glycerol and imaged with a Zeiss LSM510 Meta Laser Scanning microscope. Stacks were acquired with LSM510 software, Z-projections and contrast adjustments were made with NIH ImageJ. Images were imported into photoshop for orientation.

Statistical Analysis

ANOVA was performed to determine statistical significance between the number of cells counted from confocal images. Differences were established at a 95% confidence interval. Skewness and kurtosis tests determined all data was normally distributed.

CHAPTER IV

MIR-8 MIRNAS REGULATE THE RESPONSE TO OSMOTIC STRESS IN ZEBRAFISH EMBRYOS

This data is currently submitted. Alex S. Flynt, Kristopher Burkewitz, and James G. Patton. miR-8 miRNAs Regulate the Response to Osmotic Stress in Zebrafish Embryos. Cell Metabolism.

Summary

miRNAs have been shown to have diverse roles in animal development and physiology. Here, we show a role for the *miR-8* family of miRNAs in osmoregulation in zebrafish embryos. These miRNAs are expressed in mitochondrial rich cells called ionocytes, a specialized cell type scattered throughout the epidermis but especially prevalent over the yolk sac of zebrafish embryos. Ionocytes are responsible for pH and ion homeostasis during early development, prior to gill formation. The highly conserved *miR-8* family enables precise control of ion transport by modulating the expression of Nherf1, a regulator of apical trafficking of transmembrane ion transporters. Disruption of the function of *miR-8* family members leads to an inability to respond to osmotic stress and blocks the ability to properly traffic and/or cluster transmembrane glycoproteins at the apical surface of ionocytes.

Introduction

miRNAs are a class of small, ~22nt non-coding RNAs that negatively regulate gene expression (Lagos-Quintana et al., 2001; Reinhart et al., 2000). Functional miRNAs are derived from larger precursors that mature through sequential nuclear and cytoplasmic cleavages carried out by the RNAse III enzymes Drosha and Dicer, respectively (Bernstein, 2001b; Ketting, 2001; Lee et al., 2003; Lee et al., 2002). The longer primary miRNA transcripts contain hairpin folds which are recognized and excised by a Drosha containing complex, and are required for nuclear export and final maturation by Dicer in the cytoplasm (Lee et al., 2003). Normally, one strand of the fully processed 22nt double stranded miRNA is incorporated into the RISC, a multi-subunit complex that associates with polyribosomes and is responsible for inhibiting translation of associated mRNAs (Ishizuka et al., 2002; Okamura et al., 2004; Tuschl et al., 1999; Zamore et al., 2000).

miRNAs target specific mRNAs for down-regulation, usually by pairing imperfectly to miRNA recognition elements (MRE) in 3' untranslated regions (UTR) (Brennecke et al., 2005; Enright et al., 2003; Lai, 2002; Lewis et al., 2003). Higher eukaryotic genomes encode anywhere from hundreds to thousands of miRNAs to enable precise control of gene expression (Kloosterman and Plasterk, 2006). Understanding and identifying the exact genes regulated by specific miRNAs remains a difficult problem. Prediction of miRNA targets through genome wide analysis of 3'UTR sequences is complicated by imperfect complementarity between most miRNAs and their targets. Reporter assays and direct functional tests are therefore required to verify prediction algorithms.

The expression patterns of multiple miRNAs have been described in different organisms, tissues, and developmental time points (Miska et al., 2004; Sempere et al.,

2004). In vertebrate embryos, particularly zebrafish, temporal expression patterns have been complemented with *in situ* localization utilizing LNA oligonucleotides to hybridize to mature miRNA sequences (Kloosterman et al., 2006a; Kloosterman et al., 2006b; Wienholds et al., 2005). These analyses have revealed a striking variety of expression patterns of different miRNAs during early vertebrate development. The sequences of many miRNAs are conserved showing similar expression patterns, genomic organization, and copy numbers, suggesting that the use of genetically tractable organisms such as zebrafish could yield insight into the role of a miRNAs in humans and their potential role in physiology and disease.

One such conserved family of miRNAs is the *miR-8* family which has five members in vertebrates. These miRNAs (*miR-200a, miR-200b, miR-200c, miR-141,* and *miR-429*) are very similar in sequence, particularly at their 5' ends and appear to have descended from *miR-8* in insects (Ambros et al., 2003; Griffiths-Jones, 2004; Griffiths-Jones et al., 2006). All vertebrates encode *miR-8* homologs arranged identically in two polycistrons, and at least in zebrafish, show identical tissue specificity in nasal epithelia, neuromasts, the pronephros, and a subset of epidermal cells (Wienholds et al., 2005).

While the above tissues may seem quite distinct, they all possess cells that are rich in mitochondria. Among these cell types, we focused on ionocytes that are interspersed among keratinocytes in the skin of early zebrafish embryos. Functionally, these cells mimic intercalated cells in the mammalian distal nephron and collecting duct that function to regulate ion flux (Hsiao et al., 2007; Janicke et al., 2007). In zebrafish, these cells express *miR-8* family miRNAs which participate in osmoregulation through the targeting of Nherf1 (Na^+,H^+ Exchanger Regulatory Factor 1), a protein that was

originally shown to regulate the activity of Na^+,H^+ Exchanger 3 (NHE3) in renal brush border cells (Weinman et al., 2000) but which also controls apical presentation and trafficking of membrane proteins such as ion transporters and receptors (Hsiao et al., 2007; Janicke et al., 2007; Lin et al., 2006). Disruption of *miR-8* results in zebrafish embryos deficient in responding to osmotic stress and incapable of properly maintaining ion and acid base homeostasis.

Results

***miR-8* family microRNAs are expressed in mitochondrial rich cells.**

In situ hybridization experiments using LNA probes complementary to *miR-200a* and *miR-200b* have shown that these miRNAs are expressed in a number of tissues in zebrafish embryos including nasal epithelium, neuromasts, the pronephros, and scattered epithelial cells (Wienholds et al., 2005). Interestingly, these same cells and structures can be stained with fluorescent dyes that target mitochondrial rich cells (MRC) (Figure 15A) (Jonz and Colin A., 2006). One of these stains is the styryl-dye DASPEI (Harris et al., 2003). This compound is cell permeable, accumulates in mitochondria, and allows staining and visualization of live embryos. We stained embryos with DASPEI followed by live cell imaging of mitochondrial rich cells and performed *in situ* hybridization of fixed embryos with an LNA probe against *miR-200b* (Figure 15). As shown in Figure 15 B-D', neuromasts, the pronephros, and epithelial cells can all be visualized by DASPEI staining and also show an accumulation of *miR-200b*. To determine whether the scattered epidermal cells positive for expression of *miR-8* family members are the same MRCs stained by DASPEI, we localized *miR-200b* in embryos stained with MitoTracker

red before fixation. MitoTracker behaves similar to DASPEI, accumulating as a fluorescent tracer of mitochondria that can be visualized in living embryos (Esaki et al., 2007). However, unlike DASPEI, the dye exhibits a much more narrow emission spectra and becomes covalently attached to mitochondrial proteins through thiol conjugation. Thus, MitoTracker staining persists after fixation of embryos allowing co-labeling experiments. Merging images of direct interference contrast (DIC) microscopy of the dark purple stain of *miR-200b* expression with images resulting from fluorescence emitted from fixed cells stained with MitoTracker revealed that the MRC epidermal cells stained by MitoTracker are positive for expression of the *miR-8* family (Figure 15E, E'). The cells shown by DIC (E) and in color (D), are clearly ionocytes based on their ovoid cell morphology, location in the epidermis (Jonz and Colin A., 2006; Lin et al., 2006). There are at least two different populations of mitochondrial rich ionocytes present in the skin of zebrafish embryos that can be differentiated based on the expression of H^+-ATPases (H^+ pump rich cells, HRCs) or Na^+-K^+ATPases (Na^+-K^+ pump rich cells, NRCs) (Esaki et al., 2007; Lin et al., 2006). HRCs can be differentiated from NRCs by their strong affinity to the lectin concanavalin A (conA). HRCs are responsible for the accumulation of Na^+ whereas NaRCs are thought to participate in regulating appropriate levels of K^+ and Na^+ with a subset responsible for uptake of Ca^{++} (Esaki et al., 2007; Janicke et al., 2007). Expression analysis of *miR-200b* in conjunction with MitoTracker staining demonstrates that both types of zebrafish ionocytes express members of the *miR-8* family.

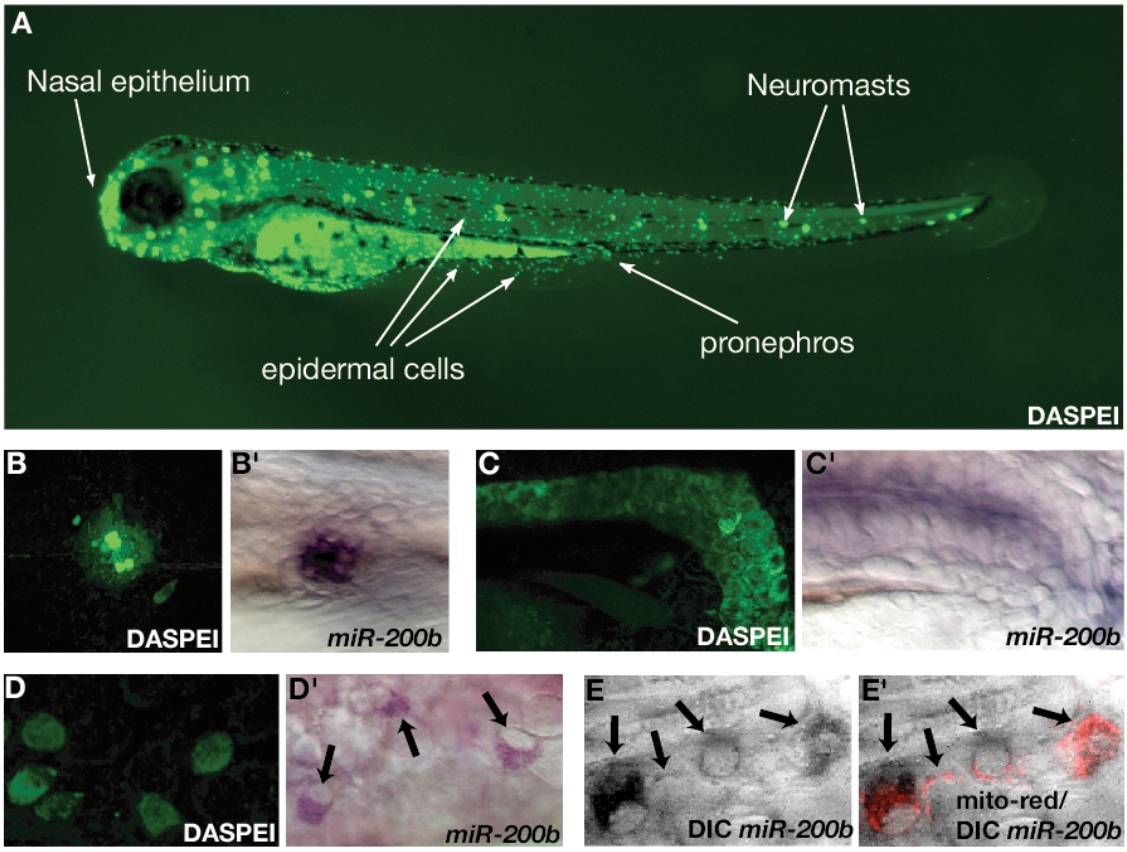


Figure 15. ***miR-200b*** **is expressed in mitochondrial rich cells (MRCs)**(A) Zebrafish embryos were incubated in the mitochondrial stain DASPEI and imaging was performed on live embryos at 36 hpf. DASPEI labels nasal epithelium, neuromasts, epidermal cells, and the pronephros, as indicated. (B) DASPEI staining of a single neuromast. B') *In situ* hybridization was performed on zebrafish embryos at 36 hpf with LNA antisense oligonucleotides complementary to *miR-200b*. Localization of *miR-200b* was performed using NBT/BCIP color development in single neuromasts. (C) DASPEI staining of the pronephros. C') *miR-200b in situ* localization shows expression in the pronephros.(D) DASPEI staining in epidermal cells overlying the yolk in 36 hpf zebrafish embryos. (D') *miR-200b in situ* localization in epidermal cells. (E) DIC microscopy of *miR-200b in situ* hybridization shows expression in epidermal cells. (E') Identical cells from E were co-stained with the mitochondrial stain MitoTracker red before fixing and *in situ* hybridization with LNA probes against *miR-200b*. E' shows the overlay of the DIC image from (E) with fluorescence emitted by the mitochondrial stain MitoTracker red.

Morpholino Knockdown of *miR-8* Family Members

miR-200b is a member of a larger family of miRNAs named for the founder miRNA in *Drosophila*, *miR-8* (Aravin et al., 2003b; Chen et al., 2005). While all members of the *miR-8* family share a high degree of sequence similarity, modest changes have occurred during the diversification of this miRNA family. Alignment of sequences from the hairpin precursor sequences shows the relatedness of the members (Figure 16A). Focusing on only the mature sequences, *miR-200b* and *miR-200c* are identical as are *miR-200a* and *miR-141* (Figure 16B). The 5' end of the founder miRNA, miR-8, is most similar to *miR-200b*, *miR-200c*, and *miR-429* (Figure 16B). This region is referred to as the seed sequence and is thought to play an important role in target pairing (Lewis et al., 2003).

Antisense technology has been widely used to interfere with miRNA function (Krutzfeldt et al., 2006). In zebrafish, morpholino oligonucleotides have been used to inhibit miRNA function for up to 72 hours post fertilization (hpf) (Flynt et al., 2007; Kloosterman et al., 2007). To target the *miR-8* family in zebrafish, we designed a series of antisense morpholinos complementary to the mature sequence of *miR-200b* (B^{MO1}), the mature sequence of *miR-200a* (A^{MO1}), and the loop sequence of the *miR-200b* precursor (B^{MO2}) (Figure 16C). In combination, these oligonucleotides should effectively target all members of the *miR-8* family with the possible exception of *miR-429* which contains 4 mismatches but since 3 of these are at the 3' end, hybridization is still likely.

To determine the effectiveness of the morpholinos alone and in combination, we performed northern blots using a *miR-200b* probe and RNA extracted from 36 hpf embryos that were injected at the single cell stage with a combination of morpholinos

(Figure 16D). The greatest knockdown of the *miR-8* family was achieved through injection of a combination of A^{MO1} and B^{MO1} morpholinos. Injection of B^{MO2} should result in targeting of *miR-200b* alone, leaving other *miR-8* family members unaffected. This is clearly the outcome of the B^{MO2} injection as no significant decreases in miRNA levels were observed.

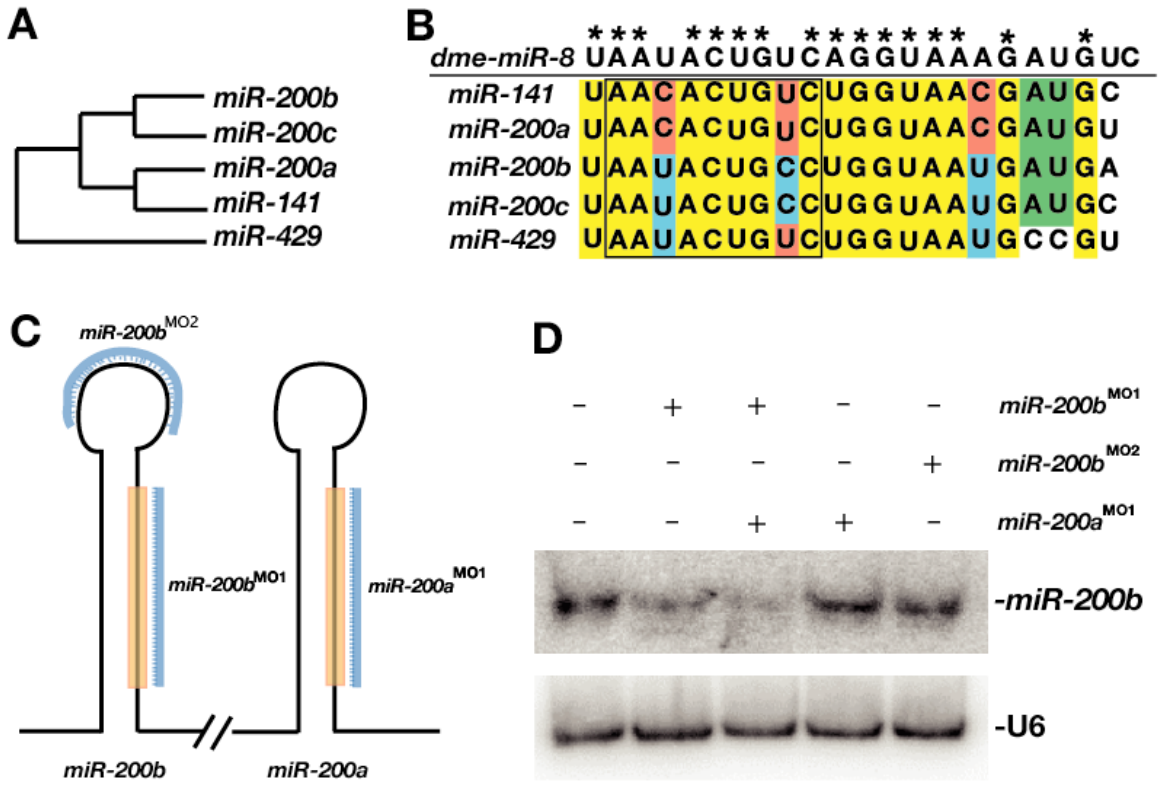


Figure 16. Knockdown of *miR-8* miRNAs by Morpholino Inhibition.
(A) Phylogeny of zebrafish *miR-8* family by alignment of miRNA precursor hairpin sequences. (B) Alignment of mature miRNA sequences from the *miR-8* family in zebrafish. Identical nucleotides are in yellow with those matching the founding member in *Drosophila* (dme-miR-8) indicated with an asterisk. (C) Design of targeting antisense morpholino oligonucleotides against mature *miR-200b*, (*miR-200b*^{MO1}), mature *miR-200a* (*miR-200a*^{MO1}), and the loop sequence from *miR-200b* (*miR-200b*^{MO2}).(D) Expression of *miR-200b* family members at 36 hpf after injection of morpholinos into single cell zebrafish embryos. RNA was isolated from embryos injected as indicated and northern blots were probed with an oligonucleotide against *miR-200b*.

Similarly, injection of A^{MO1} did not result in decreased levels due to expression of *miR-200b* and *miR-200c*. We did observe reduction in *miR-8* miRNA levels upon injection of B^{MO1} alone but the most significant knockdown occurred upon co-injection A^{MO1} and B^{MO1} (AB^{MO}). While detectable levels are still observed, the AB^{MO} combination of morpholinos is able to significantly decrease overall levels of *miR-8* family member expression sufficient to generate phenotypic effects on ionocyte function (see below).

***miR-8* Function and Osmotic Stress**

We next sought to determine the effects of knockdown of the *miR-8* family on early zebrafish development. Injection of the AB^{MO} combination did not result in any clear defects in gross zebrafish embryo morphology at 36 hpf. Uninjected embryos (UIC) and those injected with the AB^{MO} combination were virtually indistinguishable when examined under either light microscopy (data not shown) or after DASPEI staining (Figure 17 A,B). Thus, at this time point and with this level of knockdown, there was no apparent defect in the specification of MRCs. We hypothesized that because the *miR-8* family is not involved in the development of MRCs, it may have a function in regulating the physiology of MRCs.

To test whether the *miR-8* family functions to regulate the physiology of ionocytes, we subjected embryos injected with the AB^{MO} combination to osmotic stress. AB^{MO} morphants were transferred to high salt buffer (10x Danieau buffer) after being allowed to develop in 1x buffer for the first 24 hrs. of development. Neither UIC embryos or AB^{MO} morphants raised at this high salt concentration exhibited obvious developmental defects (data not shown). After 24 hrs. in high salt, the embryos were then transferred to

distilled water. The transition to dramatically different salt concentrations was designed to induce severe osmotic stress and the morphological effects of such stress were then documented 24 and 48 hrs. after the final transfer to distilled water (Figure 17C-E). Consistent with the idea that the *miR-8* family functions to regulate the physiology of ionocytes, zebrafish embryos injected with the AB^{MO} combination exhibited increased sensitivity to osmotic stress displaying significantly increased edema, both in severity and frequency, compared to UIC embyos at both 3 and 4 dpf (Figure 17C, D, E). Interestingly, when UIC and AB^{MO} injected embryos were transferred to distilled water after equilibrating for 24 hrs. in 1x buffer, no observable defects were detected (data not shown). This suggests that the AB^{MO} phenotype is exclusively a result of the inability to handle extreme osmotic stress and consistent with a role for *miR-8* family members in ionocyte physiology.

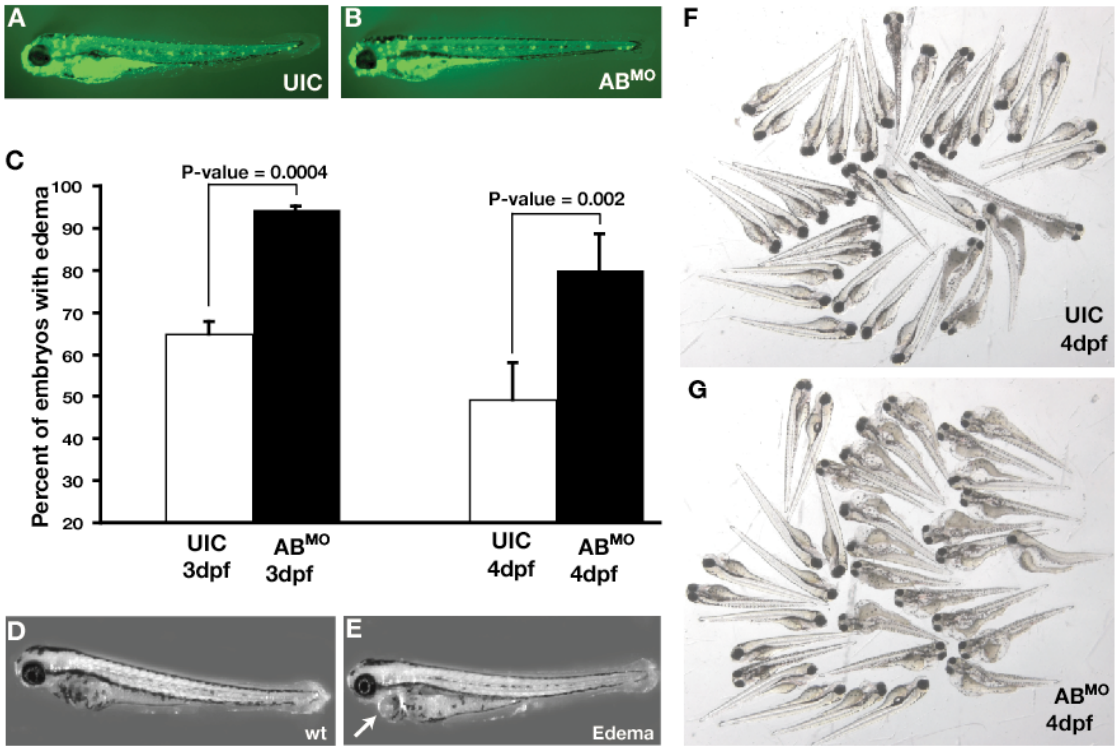


Figure 17. Loss of *miR-8* miRNAs Inhibits Osmotic Stress Response. (A-B) DASPEI staining to show morphology of zebrafish embryos in uninjected (UIC) or AB^{MO} injected embryos. (C) Percent of UIC and AB^{MO} injected embryos exhibiting edema. P-values from paired t-tests are as indicated, n=5, error bars show s.e.m. (D-E) 36hpf embryos subjected to osmotic stress, either unaffected (D) or exhibiting edema (E). Arrow indicates swelling of epicardium typical of edema.(F-G) Severity of edema in UIC and AB^{MO} injected 48hpf embryos.

The *miR-8* family participates in the regulation of Na^+ accumulation in ionocytes

Next we sought to determine if changes in ion homeostasis could be observed in control and morphant embryos. To examine the accumulation of Na^+ in HRC ionocytes we used Sodium Green which emits increased fluorescence in the presence of increasing Na^+ concentration (Esaki et al., 2007). As with DASPEI and MitoTracker red, Sodium Green is cell permeable and can be used to stain live embryos. After a 60 min. incubation of embryos in the presence of Sodium Green, Na^+ accumulation in ionocytes

was readily observed using fluorescence microscopy (Figure 18). We used a combination of Sodium Green and MitoTracker red to visualize ionocytes in normal zebrafish embryos at three different pHs in 1x buffer (Figure 21A-C). The combination of dyes also allowed verification that the Sodium Green fluorescence was indeed derived from ionocytes. As shown, the accumulation of Na^+ in zebrafish embryos was dependent on the pH of culture water with embryos raised at low pH exhibiting the greatest accumulation (Figure 18A). This is because Na^+ accumulation in HRCs is dependent on the function of Na^+/H^+ exchangers (NHEs) and therefore linked to H^+ efflux (Esaki et al., 2007). These antiporters are important to ion movement and pH homeostasis in a number of different organisms (Claiborne et al., 2002). Interestingly, acidosis increases localization of NHEs at the apical membranes of mammalian renal cells, which in turn leads to enhanced rates of Na^+/H^+ exchange (Claiborne et al., 2002). A similar phenomenon is apparently occurring in zebrafish HRCs where the need for increased acid secretion is balanced by Na^+ accumulation.

Next we sought to determine if a change in Na^+ accumulation could be observed in embryos injected with the AB^{MO} combination. Consistent with a role for these miRNAs in regulating ion homeostasis in ionocytes, we observed a decrease in Na^+ accumulation in AB^{MO} morphants (Figure 18D-F). The change in Na^+ accumulation was most pronounced when comparing the AB^{MO} morphants to uninjected controls at pH 5.0 (Figure 18A and D; for quantification, see Figure 21E). To focus on this pronounced difference, embryos were raised at this pH for subsequent experiments where ionocytes were then visualized in living embryos.

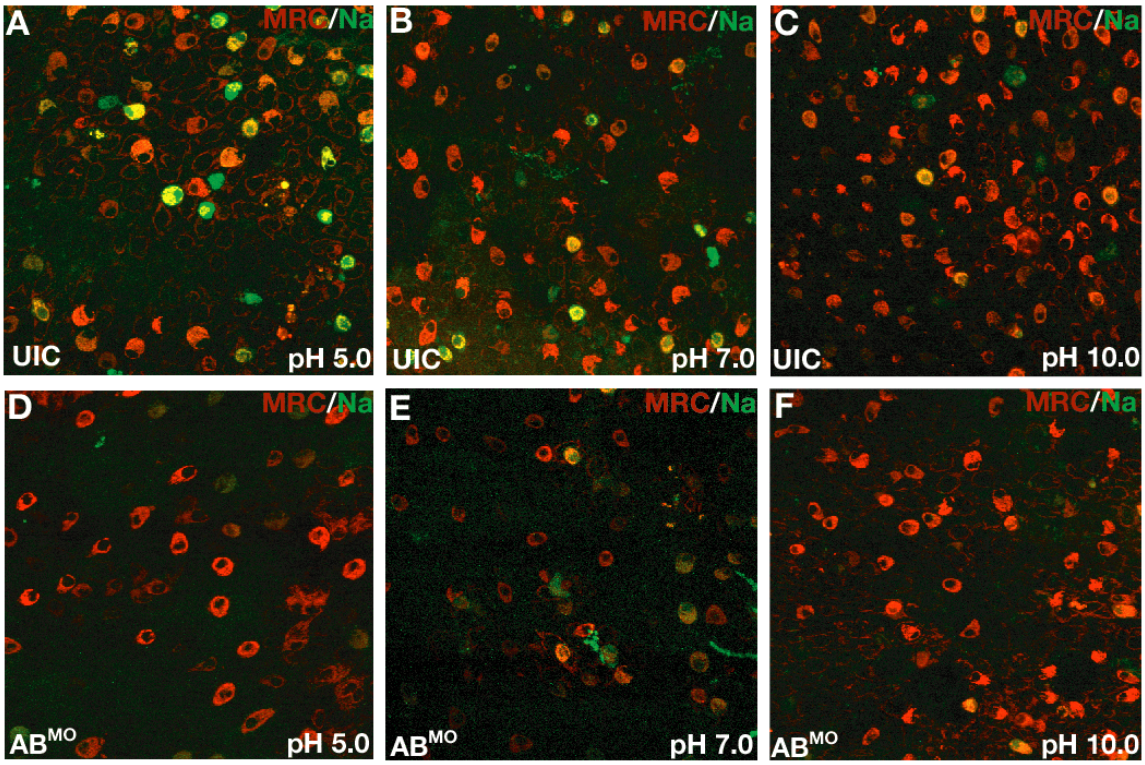


Figure 18. Loss of *miR-8* miRNAs blocks Na^+ accumulation in ionocytes. (A-C) Live, wild type zebrafish embryos were incubated with Sodium Green (green) and MitoTracker (red) at pH 5.0, pH 7.0, or pH 10.0. Na^+ accumulation is indicated by green stained cells. (D-F) Live embryos injected with AB^{MO} were visualized by Sodium Green and MitoTracker red at three pHs, as above.

Na^+/H^+ exchange regulatory factor 1 (*nherf1*) is a target of the *miR-8* family

To better understand how the *miR-8* miRNA family influences the physiology and function of ionocytes, we sought to identify *miR-8* target genes that could be responsible for regulating Na^+ accumulation. A variety of algorithms have been created to predict the targets of specific miRNAs based on sequence complementarity, sequence context, and conservation across species (Chen et al., 2005; Grimson et al., 2007; Lewis et al., 2003). One of the predicted targets for both *miR-200a* and *miR-200b* is *slc9a3r2* or Na^+/H^+ Exchange Regulatory Factor 1 (Nherf1) (Chen et al., 2005). This gene encodes a phosphoprotein containing two N-terminal PDZ domains that interact with a variety of

membrane-associated binding partners among which are NHEs and other ion transporters (Lederer et al., 2003; Morales et al., 2007; Murthy et al., 1998; Wheeler et al., 2007; Yun et al., 1997). The C-terminal domain of Nherf1 interacts with the cytoskeletal proteins Merlin, Ezrin, Radixin, and Moesin enabling Nherf1 to serve as an adaptor molecule linking membrane proteins to cytoskeletal actin filaments (Figure 19A) (Morales et al., 2007; Weinman et al., 2000). There are two Nherf isoforms that are similar in domain structure but they associate with different partners and their tissue specific expression patterns are distinct (Weinman et al., 2000; Yun et al., 1997). In addition to being an excellent candidate based on the regulation of Na^+ accumulation by Nherf1, the miRNA recognition elements (MREs) in the *nherf1* 3'UTR are exceptionally strong, matching the current criteria described for efficient targeting by miRNAs (Figure 19B). These criteria include nearby AU rich elements and targeting by tightly co-expressed miRNAs, consistent with the *nherf* 3' UTR structure and the polycistronic arrangement *miR-8* family members (Figure 20) (Grimson et al., 2007).

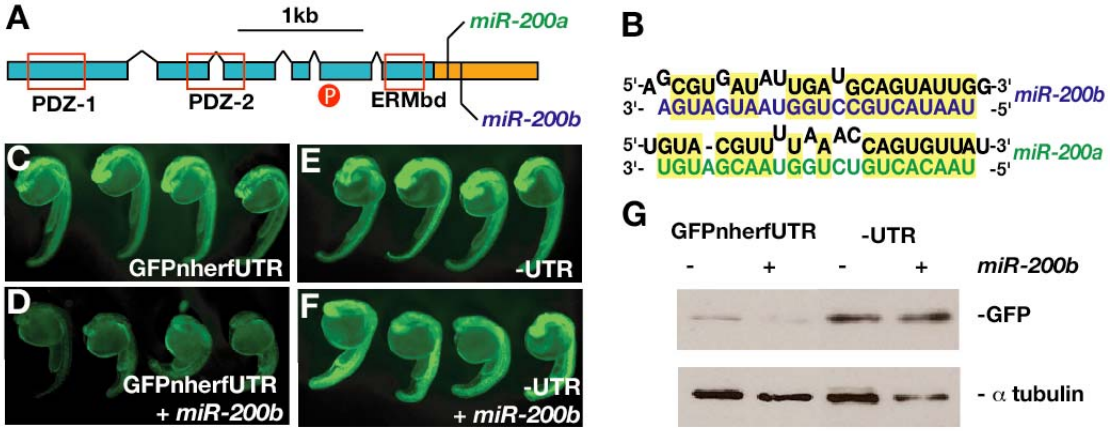


Figure 19. *nherf1* is a target of the *miR-8* family. (A) Diagram indicating the domain structure of Nherf1. The ORF is indicated by blue boxes and the 3'UTR in orange. Conserved protein domains, PDZ-1, PDZ-2, and ERMbd are indicated by red boxes. Location of a phosphorylated residue is indicated by the red circle. Positions of the *miR-8* family MREs are as indicated. (B) *miR-8* family MREs predicted by the miRanda algorithm. The *nherf1* mRNA is in black and miR-200a and miR-200b sequences are shown in green and blue, respectively. (C-F) Single cell embryos were injected with mRNAs derived from GFP*nherf1*UTR or a GFP construct lacking the *nherf1* 3' UTR (-UTR) in the presence or absence of exogenous *miR-200b.* Fluorescence levels were examined at 1dpf. (G) Embryo lysates were prepared from embryos treated as in C-F and GFP protein levels were determine by western blotting.

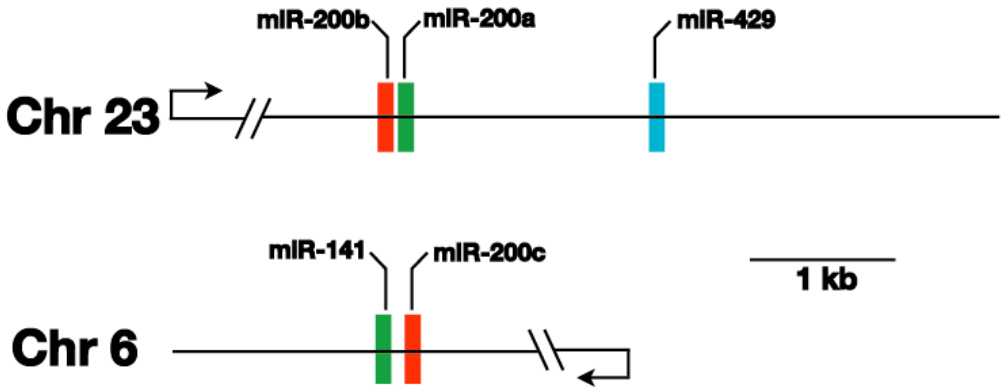


Figure 20. Genomic organization of the zebrafish miR-8 miRNAs. Family members exhibiting identical mature miRNA sequences are indicated by same color

As a first test of whether *nherf1* is a target of the *miR-8* family, we constructed a reporter in which the 3'UTR of *nherf1* was fused to GFP (GFPnherfUTR). Synthetic mRNAs prepared from this construct were then injected into single cell zebrafish embryos in the presence or absence of co-injection of *miR-200b.* By simple examination of GFP levels in injected embryos at 1 dpf, it is clear that co-injection of *miR-200b* resulted in down regulation of GFP levels compared to embryos injected with either GFPnherfUTR mRNA alone or mRNA lacking the *nherf1* 3' UTR (Figure 19C-F). Detection of GFP protein levels via western blotting of lysates from embryos injected as described above confirmed repression of GFP expression in embryos co-injected with *miR-200b* and GFPnherfUTR (Figure 19). These results are consistent with targeting of *nherf1* by *miR-8* family members.

If *nherf1* is indeed a target of *miR-8* family members, the defect in sodium accumulation in the AB^{MO} morphants should be rescued by direct repression of *nherf1*. Nherf1 has been shown to be a negative regulator of Na^+,H^+ Exchange (NHE) activity by promoting phosphorylation and subsequent internalization of NHEs (Murthy et al., 1998; Yun et al., 1997). To repress *nherf1,* we designed a morpholino complementary to the translation start site of *nherf1 (*$nherf1^{MO}$). Next, we monitored Na^+ accumulation in ionocytes with Sodium Green and MitoTracker red in embryos injected with the AB^{MO} combination, the $nherf1^{MO}$, or all three morpholinos (Figure 21A-D). Consistent with *nherf1* expression being up-regulated in AB^{MO} morphants due to lack of repression by the *miR-8* family, repression of *nherf1* by the $nherf1^{MO}$ allowed restoration of Na^+ accumulation in AB^{MO} morphants (Figure 21B and 21D). To verify this result, we quantified Sodium Green fluorescence (Figure 21E). The average pixel intensity at

488nm was determined for individual ionocytes and divided by the local background to determine the fold increase in Na^+ accumulation. This analysis showed no significant differences in Na^+ accumulation between UIC, *nherf1*^{MO}, and AB^{MO}+*nherf1*^{MO} injected embryos. In contrast, Na^+ accumulation in AB^{MO} injected embryos was significantly decreased. These results are consistent with targeting of *nherf1* by *miR-8* miRNAs in HRC ionocytes.

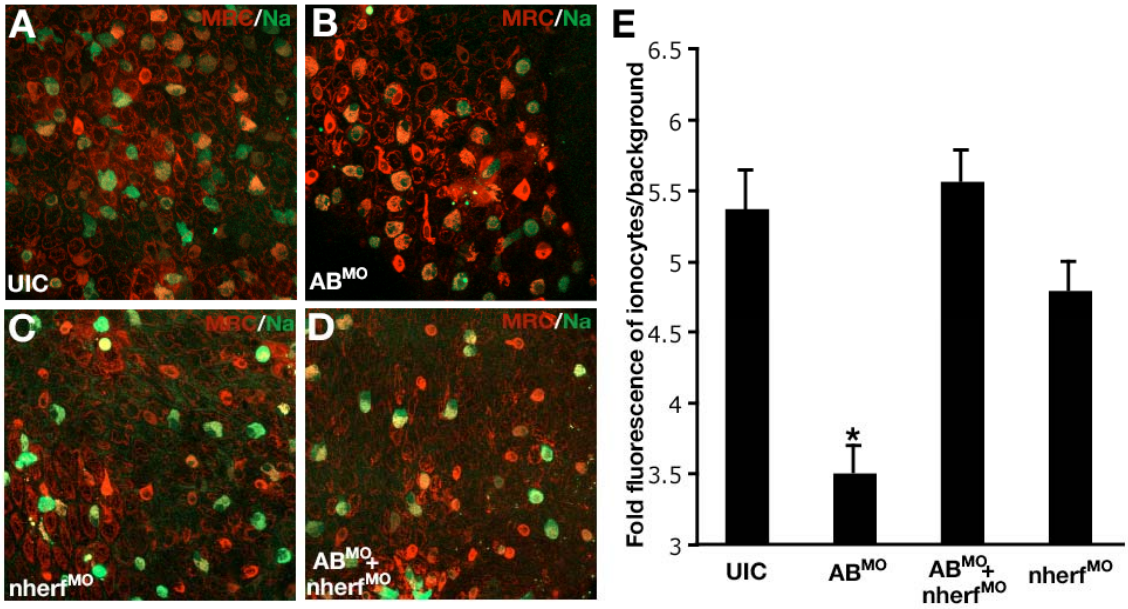


Figure 21. Rescue of Na^+ accumulation defects in AB^{MO} morphants by repression of *nherf1*. (A-D) Visualization of Sodium Green (green) and MitoTracker (red) in UIC, AB^{MO} injected embryos, *nherf1*^{MO} injected embryos, and AB^{MO} and *nherf1*^{MO} co-injected embryos was performed on live embryos incubated at pH 5. (E) Quantification of Sodium Green fluorescence levels from embryos injected in A-D. Average fluorescence was divided by local background. Statistical significance determined by ANOVA at $\alpha \leq 0.05$ is indicated by the asterisk; n=20 from five different embryos from three independent experiments.

Besides PKA regulation of sodium accumulation, Nherf1 has also been shown to regulate the trafficking and membrane localization of a variety of proteins including ion channels, G protein coupled receptors, and other glycosylated transmembrane proteins

(Morales et al., 2007; Theisen et al., 2007; Voltz et al., 2001). To test whether defects in membrane localization occur in AB^{MO} morphants, we examined ionocytes after staining with FITC conjugated concanavalin A (FITC-conA). The HRC subtype of ionocytes display numerous sugar groups that are components of glycoproteins localized to their apical membranes and can therefore be labeled with the lectin conA (Esaki et al., 2007). Embryos were incubated briefly with FITC-conA and apical membranes of HRCs were examined using fluorescent microscopy. Immediate visualization of ionocyte membranes after conA staining showed little difference between uninjected control embryos and AB^{MO} morphants (data not shown). However, allowing an hour to pass after FITC-conA staining revealed considerable differences in conA distribution (Figure 22A,B). In control embryos, conA distribution was mostly localized to apical membranes of HRC ionocytes in a dense, clustered structure. In contrast, a radical redistribution of conA labeled glycoproteins was observed in AB^{MO} morphants. In addition to a more punctate appearance, the apical character of these ionocytes was disrupted and increased levels of internalized FITC-conA signals could be observed along the z axis. This is consistent with a role for Nherf1 in controlling membrane trafficking and internalization of specific receptors (Yun et al., 1997). To ensure that the defect was specific, we again used the *nherf1*^{MO} to determine if repression of elevated *nherf1* expression in the AB^{MO} morphants could rescue the change in localization of conA labeled glycoproteins (Figure 22C,D). As above, repression of *nherf1* expression rescued the alteration of conA localization seen when *miR-8* function was blocked. To quantify the differences in distribution of conA localization, we counted the number of FITC-conA labeled foci using two criteria. The first was whether the staining resulted in foci that were either clustered or ungrouped

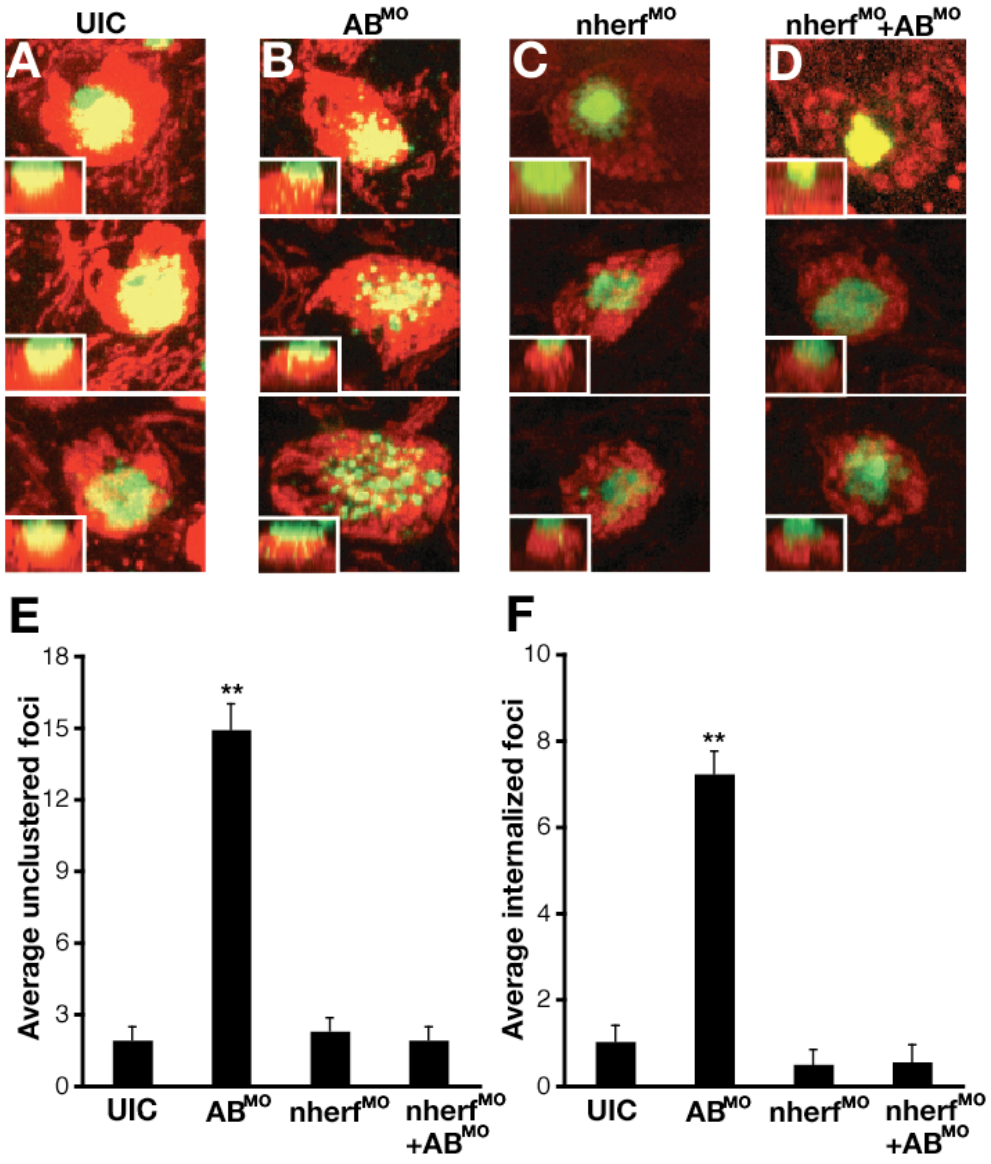


Figure 22. Loss of *miR-8* miRNAs alters apical membrane trafficking. FITC-conconavalin A (conA; green) and MitoTracker (red) staining was performed to examine the apical membranes of H^+-pump rich ionocytes. (A-D) ConA staining in UIC, AB^{MO} injected embryos, *nherf1*^{MO} injected embryos, and AB^{MO} *nherf1*^{MO} co-injected embryos. Live embryos were imaged with each panel showing a view of cells on the surface of the yolk sac with the inset showing a side view of the z-stack with apical basal localization of conA staining. (E-F) Average number of ungrouped (E) or internalized (F) foci of conA staining from embryos in A-D. Error bars represent s.e.m., statistical significance was determined by ANOVA, n=14 for UIC, n=11 for AB^{MO}, n=13 for *nherf1*^{MO}, and n=10 for *nherf1*^{MO}+AB^{MO}. Data was gathered from three independent experiments.

and the second whether there was increased number of internalized conA labeled foci along the z axis toward the basolateral surface. As shown, the AB^{MO} morphants showed statistically significant increases in both measurements (Figure 22E-F). This is consistent with a role for the *miR-8* family in regulating membrane dynamics and trafficking of transmembrane proteins through regulation of Nherf1.

Discussion

miR-8 family miRNAs regulate *nherf1* in zebrafish ionocytes

Here, we demonstrate a role for the *miR-8* family of miRNAs in zebrafish osmoregulation. These miRNAs modulate the expression of *nherf1* which plays a critical role in regulating Na^+/H^+ exchange activity. Nherf1 negatively regulates $Na^{+/}H^+$ Exchanger 3 (NHE3) in a cAMP dependent manner by recruiting activated PKA to phosphorylate NHE3 (Weinman et al., 2000). Phosphorylation results in internalization of NHE3 thereby downregulating ion exchange across the membrane. Interestingly, cAMP production is coupled to a variety of stress responses. Among these is hypertonicity, hypotonicity, and acidosis, all of which increase cAMP levels several fold (Disthabanchong et al., 2002; Orlic et al., 2002; Sheikh-Hamad and Gustin, 2004). Increased cAMP levels are thought to play an important role in the response to osmotic stress by abrogating the negative effects of stress responsive genes whose activation can induce apoptosis (d'Anglemont de Tassigny et al., 2004; Pascual-Ahuir et al., 2001; Saran et al., 2002). If cAMP levels are elevated in ionocytes experiencing osmotic stress this should, through a Nherf1 dependent mechanism, result in the inhibition of Na^+/H^+ exchange activity. This would be a deleterious outcome since NHE activity is required to balance Na^+ accumulation and H^+ efflux as well as for the retention of Na^+ in hypotonic

solution. The *miR-8* family may function to ameliorate cAMP mediated inhibition of NHEs during stress. This would allow Na^+/H^+ exchange to occur independently of protective cAMP elevation.

We have also shown that regulation of *nherf1* by the *miR-8* family is responsible for maintaining the apical character of ionocytes. The apical domains of ionocytes were revealed using FITC-conA staining. While the exact identify of the specific zebrafish glycoproteins that are recognized by conA remains to be determined, the overall resemblance of the ionocytes studied here to mammalian renal brush border cells is striking (Tyska et al., 2005). In brush border cells, Nherf1 has been shown to be recruited to apical membranes by overexpression of podocalyxin, an obligate apical glycoprotein (Nielsen et al., 2007). Due to the large number of apical glycoproteins on the membranes of HRC ionocytes, Nherf1 may be constitutively recruited to the membranes of these cells. This would necessitate attenuation of *nherf* 1 expression to permit NHE activity in these cells. Additionally, other types of MRCs in zebrafish, specifically neuromasts and nasal epithelium, are also strongly labeled by conA (data not shown). Down-regulation of *nherf1* may be essential for the appropriate presentation of specific glycoproteins on the apical membranes of these cell types.

Nherf1 is predicted to be a target of *miR-200b* in mammals

The miRanda algorithm predicts that *miR-200b* should target both zebrafish, and mammalian *nherf1* (John et al., 2004). In mammals, *miR-200b* is expressed in colon, kidney, prostate, pancreas, and thymus all of which contain polarized secretory cells (Beauchamp et al., 2007). In colon and kidney, Nherf1 is known to be an active

participant in the regulation of many ion transporters, in addition to Na^+/H^+ exchange (Stemmer-Rachamimov et al., 2001). Both of these organs contain brush border membranes that are reactive to conA staining (Nielsen et al., 2007; Tyska et al., 2005). If *miR-200b* regulation of *nherf1* in colon and kidney has effects similar to our observations in zebrafish ionocytes, it will be critical to determine whether expression of *miR-200b* is restricted to specific cell types within these organs. Nherf1 expression in colon is restricted suggesting precise regulation of expression between cell types, potentially through the activity of *miR-200b* in these tissues (Stemmer-Rachamimov et al., 2001). Additionally, the cells of both the prostate and pancreas, which express *miR-200b*, are highly secretory and similarly reactive to conA, requiring apical localization of multiple membrane proteins (Arenas et al., 1999; Gheri et al., 1997). It is also noteworthy that Nherf1 is up-regulated in proliferative endometrium compared to secretory endometrium (Stemmer-Rachamimov et al., 2001). Down-regulation of *nherf* 1 by *miR-200b* may be essential for secretory epithelial cells to adjust their physiology towards a permanently differentiated state. Indeed, increased expression of *nherf1* has been observed in breast and liver cancer cells (Stemmer-Rachamimov et al., 2001).

miRNAs and stress

The function of the *miR-8* family may be required for mounting appropriate stress responses in mammalian cells, as we have shown in zebrafish. During our efforts to describe the role of the *miR-8* family in zebrafish, we attempted to determine if the expression of *miR-200b* changes in response to salt concentration or pH. The results of these experiments demonstrated little alteration in the level of *miR-8* family expression in

whole embryo RNA extracts, at least at the time points tested. However, this may be due to a lack of sensitivity when comparing whole embryos versus ionocytes, especially given the high expression levels observed in nasal epithelium.

Originally, miRNAs were found to regulate developmental timing in worms, and a role for miRNAs in development is a continuous theme, translating into other phyla (Bartel and Chen, 2004). However, miRNAs have been found to have diverse functions beyond regulating development. Studies in Drosophila uncovered a role for *miR-14* in fat metabolism and stress (Xu et al., 2003) and miRNAs have been shown to play a role in triggering cardiac hypertrophy in response to stress (van Rooij et al., 2006). Additionally, the activity of the cationic amino acid transporter 1 (CAT-1) is controlled by *miR-122* in response to nutrient starvation (Bhattacharyya et al., 2006). The expression of a subset of miRNAs also appears to be up regulated by p53 in response to oncogenic stress (He et al., 2007; Raver-Shapira et al., 2007). When coupled to our findings related to osmotic stress, a clear theme emerges in which miRNAs serve to regulate the response to a variety of cellular stresses (Leung and Sharp, 2007).

miRNA targeting of transcripts is through the activity of the RISC (Hutvagner and Zamore, 2002; Zamore et al., 2000). Core factors of this complex are the Argonaute (Ago) proteins, which bind small RNAs and mediate pairing between a small RNA and its target (Carmell et al., 2002). The sub-cellular localization of Ago proteins shows that while the majority of Argonaute proteins are distributed in the cytoplasm, distinct foci of accumulated Ago proteins can be detected in structures called Processing Bodies (P-bodies) (Liu et al., 2005). Ago proteins also accumulate in a stress dependent manner in separate cytoplasmic foci called stress granules (SG) (Leung et al., 2006). Interestingly,

miRNAs localize to SGs and have been shown to dynamically accumulate and dissociate from SGs in a stress dependent manner. The unique mechanism of miRNA mediated gene regulation may be used as a method of effecting rapid changes in gene expression, particularly during stress. Regulation of *nherf1* by the *miR-8* family serves as a particularly crucial stress response in that it links extracellular events to membrane trafficking, enabling sensitive and precise control of gene expression due to changes in environmental cues and stresses.

Methods

Live imaging of zebrafish embryos

Embryos were raised in egg water (0.03% Instant Ocean marine salt mix) for the initial 24 hrs. of development (Esaki et al., 2007). After 24 hrs., embryos were transferred to 1x Danieau buffer: 58mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6mM $Ca(NO_3)_2$ and 10mM HEPES (Solnica-Krezel et al., 1994). The pH of the solution was controlled by buffering HEPES prior to addition of component salts. For fluorescent staining, embryos were incubated in 1x Daneiau buffer containing 0.25 nM of DASPEI (Molecular Imaging) (Harris et al., 2003), 0.5µM of MitoTracker deep red 633, or 10µM of Sodium Green (Molecular Probes, Invitrogen) (Esaki et al., 2007). Embryos were rinsed briefly three times and mounted in 1x Danieau buffer. Fluorescent staining was visualized by using a 40x objective on a Zeiss LSM510 Meta Laser Scanning microscope. Images were processed and Z-projections made with Zeiss LSM510 software before import into Adobe Photoshop for orientation and cropping. Quantification of Na^+ accumulation in ionocytes was accomplished using NIH ImageJ

software. Z-projections of images of Sodium Green fluorescence were imported into NIH ImageJ. The average intensity of fluorescent ionocytes was calculated and divided by local background to yield fold fluorescence of ionocyte/background. (Figures 18,22,24) or with a Leica MZFIII dissecting scope (Figure 18A and Figure 20).

In situ hybridization and Northern Blots

Detection of mature *miRNA-200b* was accomplished by *in situ* hybridization using digoxigenin labeled LNA oligonucleotides (Kloosterman et al., 2006b; Wienholds et al., 2005). Visualization of *miR-200b* expression was by NBT/BCIP color development after mounting in 50% glycerol and photographed in color with a 20x objective in Figure 18B',18C', and 18D' and by DIC in Figure18E. Images of embryos stained by NBT/BCIP were acquired by using a Zeiss Axiophot compound microscope and an Axiocam digital camera. Images were acquired with use of Axiovision software and imported into Adobe Photoshop for orientation. Northern blotting was performed as described (Flynt et al., 2007; Sempere et al., 2003).

Microinjection

Fertilized 1-cell zebrafish embryos were injected with 1nl volumes. Morpholino oligonucleotides were injected as follows per embryo: 2ng A^{MO1}, 2ng B^{MO1}, 2ng B^{MO2}, and 1ng *nherf1*^{MO1}. 40pg of *in vitro* synthesized, capped mRNA encoding either GFP without UTR (-UTR) or GFP fused to the *nherf1* UTR (GFPnherfUTR) were injected alone or with 200pg of synthetic *miR-200b* into embryos.

Induction of Osmotic stress

Embryos were raised in 1x egg water for the initial 24 hrs. of development before transfer into 1x or 10x Danieau buffer. After 24hrs., embryos were transferred to distilled water by multiple brief washes. The percent of embryos exhibiting edema was calculated 24 hrs. and 48 hrs. after transfer to distilled water. Paired Student t-tests were performed to determine significant differences between embryos exhibiting edema. Images acquired by Lecia MZFIII dissecting scope using an Axiocam digital camera were captured with Axiovision software.

GFP Reporter Analyses

Reporter analyses and western blotting was as described (Flynt et al., 2007). To generate the *nherf1* GFP reporter, the GFP ORF was fused to the 3' UTR sequence of zebrafish *nherf1*. The *nherf1* UTR was cloned from zebrafish embryo RNA extracts using oligo d(T) primed reverse transcription followed by PCR amplification with gene specific primers (5'-GCCTCCTGCGTGC-3' and 5'-GACTTTTCATAATATTTAATAACAAAAATCAT-3"). Images were acquired by Lecia MZFIII dissecting scope equipped with a fluorescent laser using a Qimaging camera with Qimaging software, and imported into Adobe photoshop for orientation and cropping.

ConA Labeling

Embryos were first incubated for 30 min. in 1x Danieau buffer containing MitoTracker red followed by the addition for 10 mins. of 50µg/ml FITC conjugated conA (Esaki et al.,

2007). Excess conA was removed by several brief washes in 1x Danieau buffer. After 1 hr., embryos were mounted in 1x Danieau buffer and FITC-ConA labeled cells were visualized by fluorescent confocal microscopy using a 100x objective on Zeiss LSM510 laser scanning confocal microscope. The average number of unclustered and internalized conA foci was determined by examining Z-stacks. In both assays, statistical differences between UIC and embryos injected with AB^{MO}, *nherf1*^{MO}, and *nherf1*^{MO}+AB^{MO}, were determined by ANOVA at $\alpha \leq 0.05$.

CHAPTER V

SIGNIFICANCE AND DISCUSSION

Here we have shown that miRNAs have diverse roles in zebrafish embryos. These molecules participate in both patterning by modulating fundamental signaling pathways and in the regulation of animal physiology. We demonstrated through our analysis of *miR-214* that this miRNA regulates the Hh signaling pathway through targeting *su(fu)*. The activity of *miR-214* is required to appropriately pattern cell types in developing somites. This finding demonstrates that like miRNAs in other organisms zebrafish miRNAs are critical mediators of developmental processes (Bartel, 2004). We also show that the zebrafish *miR-8* family has a critical role in regulating ion balance through targeting of *nherf1*. As discussed earlier, miRNAs seem to work as especially effective mediators of the stress responses. Our findings indicate that, like other organisms, zebrafish employ miRNAs during stress response (Leung and Sharp, 2007). Our underlying discoveries that *miR-214* and the *miR-8* family function can be attributed to regulation of a single mRNA is similar to discoveries in other organisms where the function of an miRNA in a particular context can be reduced to the regulation of a single target. This suggests that while miRNAs may have a large number of potential targets, only a few may be critical in a given situation. Most significantly, the diversity of miRNA function along with the composition of effector molecules of the RNAi pathway seems to be well conserved between fish and mammals. Zebrafish will prove to be a powerful tool to examine miRNA function in animal development and physiology. Discoveries made in this system will likely inform, in significant ways, outstanding questions concerning miRNAs and RNAi in mammals.

Impact on Medicine

The discovery of RNAi will be likely seen as a watershed event that ushered in the age of personalized medicine. The discovery that 21-23 nucleotide RNA duplexes (siRNAs) mediate RNAi in mammalian cells opened the door to the therapeutic use of siRNAs. Critical to reaching the goal of clinical application of RNAi is the use of model organisms, such as zebrafish, to understand the nature of RNAi phenomena. Indeed, many of the current outstanding questions that prohibit clinical use can be solved through basic research. Describing the interactions necessary for specificity of miRNAs could likely be used in the design of clinically employed siRNAs. Efforts that seek to determine which genes are targeted by a miRNA will also contribute towards this end. Algorithms could be designed to take into account miRNA/mRNA base-pairing dynamics. Describing how organisms utilize RNAi phenomena to guide development or respond to stress will likewise give insight into how external or circumstantial factors such as cell type or physiology may contribute to targeting of transcripts by RNAi. Much work remains to optimize delivery and maintain specificity, however, the therapeutic advantages of siRNAs for treatment of viral infection, dominant disorders, cancer, and neurological disorders show great promise. While many obstacles remain, with the current pace of discovery in RNAi research, it is likely we will see breakthroughs at an astonishing rate.

Synthetic small molecules are currently relied on for a large number of therapies. For many diseases, small molecules can be effective treatment, such as the treatment of infection with antibiotics. There are many cases, however, where relying on small

molecules results in the inability to cure disease. Mutations that result in cancer or dominantly inherited genetic disease remain, for the large part, incurable by current technologies. Development of therapeutic small molecules is a long and costly process. While many of these molecules are specific enough to target a particular gene product, the ability to generate a new molecule to specifically target any particular cellular factor desired may be untenable. Also, the design of a particular drug will be unlikely to translate in a meaningful way to target a significantly different type of factor. Using RNAi could circumvent these problems. Employing a well-designed siRNA could yield a therapy that very precisely targets a particular transcript. Once an ideal targeting sequence is determined, the siRNA molecule could be produced immediately with current technology. Ultimately, a situation could be envisioned where a mutation in a patient's genome, occurring in an mRNA that results in a dominant phenotype, could be immediately addressed. The single most attractive aspect of RNAi to the clinic will prove to be the ability to instantly design new oligos, tailored to a patient's needs. The targeting of viral transcripts is also an exciting application of RNAi. Mutations occurring in viral populations could be easily compensated for by the production of a different siRNA. While much work remains to optimize delivery and maintain specificity, the therapeutic advantages of siRNAs for treatment of viral infection, dominant disorders, and cancer show great promise.

The simplest siRNA design takes advantage of sequence differences between wild type and mutant RNAs (Figure 23a). This strategy has been used in a cellular model of frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17) (Miller et al., 2003). Small deletions can also be targeted in this fashion. DYT1 dystonia is an

incompletely penetrant, dominant disorder in which the overwhelming majority of patients inherit a 3 nt deletion that eliminates a glutamic acid residue in the torsinA protein (Gonzalez-Alegre et al., 2003). Using siRNAs that placed the 3 nt sequence difference near the central portion of the siRNA resulted in allele-specific degradation. Diseases such as DYT1 dystonia, where a common mutation is present in greater than 50% of the disease population, represent the ideal situation because the design and optimization of a single siRNA would apply to many patients. In contrast, patient-specific or family-specific therapy and specialized optimization would be required for diseases caused by variable mutations.

Disease-associated splicing isoforms (Faustino and Cooper, 2003; Lewis et al., 2002) can be classified into two categories: intron retention (inclusion of specific introns or activation of cryptic splice sites within introns) or exon skipping (complete skipping or activation of cryptic splice sites within exons). Both groups can be degraded using sequence specific siRNAs. Exon-specific siRNA has also been successful with more traditional approaches using siRNAs complementary to alternatively spliced isoforms (Figure 23B) (Cho et al., 2000; Ge et al., 2003). Thus, properly designed siRNAs can be used to specifically degrade aberrant or alternatively spliced mRNAs (Figure 23B and 23C).

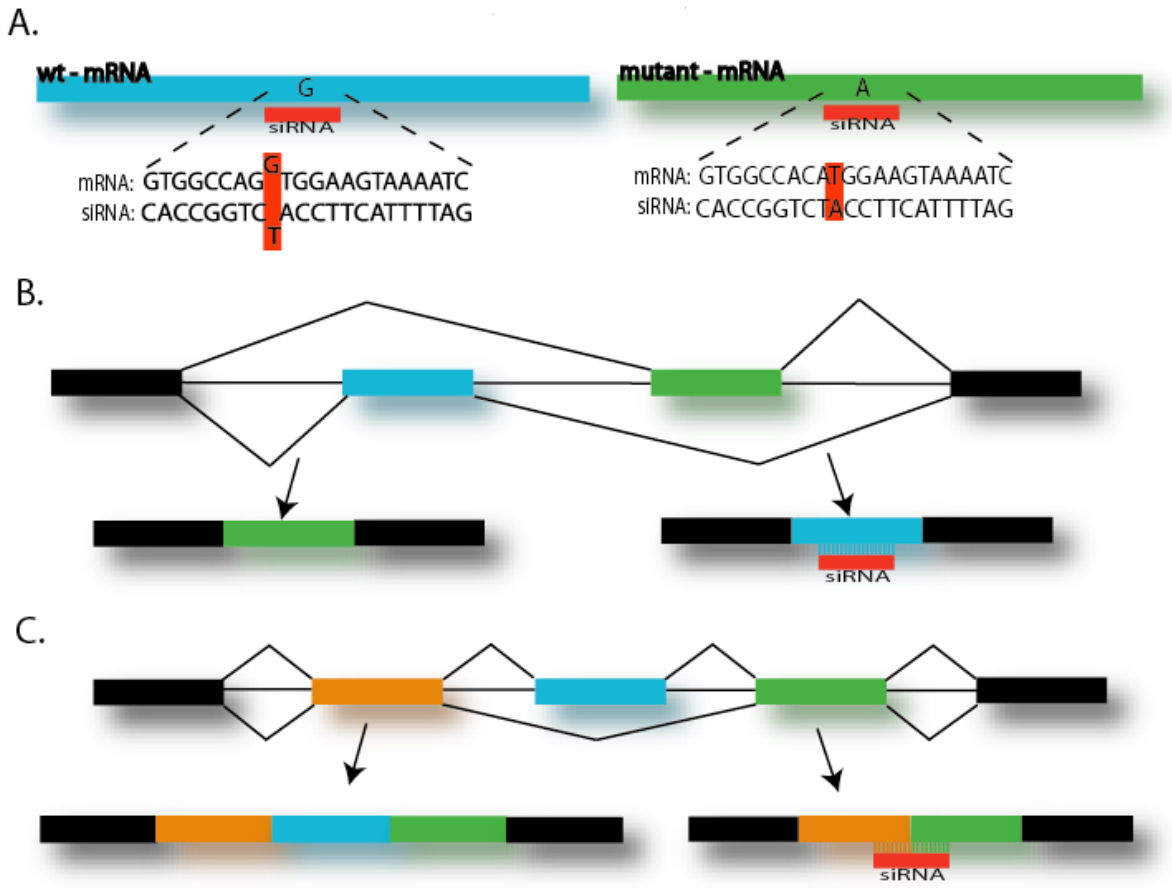


Figure 23. siRNA Mutation Targeting

(a) Direct Targeting. siRNAs are designed to perfectly match mutant alleles but contain one or more mismatches with wild type alleles, leading to specific degradation of the matching, mutant transcripts. (b) Exon-specific targeting. siRNAs targeted against specific exons (boxes). (c) Targeting exon skipped transcripts. Aberrant and/or alternative splicing creates unique exon-exon boundaries that can be targeted with specific siRNAs.

Obstacles to Effective siRNA Therapeutics

For all siRNAs, target specificity needs to be validated. In general, it appears that specificity can be attained depending on the position and sequence of a given siRNA. To examine whether global gene expression patterns change in the presence and absence of

siRNAs, microarrays have been used (Jackson et al., 2003; Semizarov et al., 2003). These experiments suggest that small numbers of non-target transcripts with sequence homology as short as 9 nt may be affected. This demonstrates the critical importance of designing appropriate siRNAs and testing them *in vivo*. Fortunately, it appears that most nonspecific responses can be minimized or eliminated with small changes in siRNA sequences (Ryther et al., 2004).

As one might expect, the exquisite specificity of siRNA-mediated degradation is both its greatest strength and its potential downfall as an effective antiviral or chemotherapeutic agent. In fact, it is thought that the normal, endogenous RNAi pathway developed for protection against viruses in plants and fungi (Bernstein, 2001a). Fittingly, siRNAs have been tested extensively against HIV-1, hepatitis, herpes, and other viruses (Gitlin and Andino, 2003; Kitabwalla and Ruprecht, 2002; Palliser et al., 2006; Silva et al., 2002; Stevenson, 2003). Two reports have indicated that siRNA-resistant HIV strains can emerge in as little as 25 days (Boden et al., 2003; Das et al., 2004). Resistance has also been observed with other viral targets (Gitlin et al., 2002). To combat resistance, multiple sequences per target and multiple targets per viral genome will likely be necessary.

Another form of resistance is the fact that not all sequences can be targeted by siRNAs. This is likely due to a lack of accessibility of the RNA sequence, either hidden by RNA-binding proteins or by complex secondary structures. Lastly, cells may also develop resistance to RNAi through loss of genes essential for RISC complex formation or selection of suppressors that inhibit degradation. Cymbidium ringspot virus is resistant to RNAi via production of p19, a protein that inhibits RNAi by sequestering

dsRNAs (Lakatos et al., 2004). Although these forms of resistance are largely hypothetical in humans, appropriate selective pressure could lead to similar problems.

To be widely applicable in clinical settings, siRNAs must exert their effects over time. Multiple studies have indicated that degradation generally peaks 36-48 hours after introduction and is complete after 96 hours. This can be extended with repeated siRNA delivery and obviously depends on the rate of target turnover. Several modifications can be used to extend the life of the dsRNAs themselves, notably, 2'-O-methylation (Amarzguioui et al., 2003; Chiu and Rana, 2003; Czauderna et al., 2003). In contrast, phosphorothioate backbones appear to be cytotoxic and 2'-O-allylation inhibits activity (Amarzguioui et al., 2003). Additionally, many companies have produced their own proprietary chemical modifications.

A variety of strategies have been used to deliver dsRNAs to cells, either directly or by introduction of expression vectors. The advent of lentiviral, adeno-associated, and other retroviral, short hairpin vectors that produce siRNAs allows the use of traditional gene therapy delivery systems. As such, the clinical utility of siRNAs will depend at least in part, on the development of safe and effective delivery systems. The majority of studies performed in mice have used high-pressure, large-volume tail vein injections that allows delivery of dsRNAs into multiple organs, notably liver, spleen, lung, kidney, and pancreas (Lewis et al., 2002; McCaffrey et al., 2002). However, except for limb delivery, this method is unlikely to be useful in humans. For *in vivo* delivery, cationic liposomes bearing siRNAs have been intravenously injected into mice (Sorensen, 2003) and electroporation has been used to deliver siRNAs and short hairpin RNAs (shRNAs) to postimplantation embryos and post-natal retinas (Calegari, 2002; Chou et al., 2004;

Matsuda and Cepko, 2004; Trezise et al., 2003). Direct injection into the portal vein with lipiodol (Zender et al., 2003) may be feasible in humans although injection volumes were still quite large as a percentage of total blood volume. In addition, siRNAs have been successfully delivered via intranasal delivery to the lungs (Zhang et al., 2004). One report examined direct application of siRNAs in the rat brain (Iascson et al., 2003). Although siRNAs against the dopamine D1 receptor effectively degraded D1 transcipts *in vitro*, delivery with a mini-osmotic pump via a cannula into the caudate-putamen failed to induce RNAi *in vivo*, suggesting that the development of effective delivery systems may be the key barrier to siRNA therapeutics. Contempory efforts to induce RNAi in cells have taken a wide range of techniques to specifically deliver therapeutic siRNAs. Among these is nanoparticles that have moieties that specifically associtate with transmemebrane proteins on target cells, or the complexing of siRNAs with cell penetrating peptides (Schiffelers et al., 2004; Veldhoen et al., 2006). Overall, unless direct delivery of siRNA to target tissues proves feasible, many of the same delivery problems that plague traditional forms of gene therapy will still need to be overcome.

RNA at the edge of evolution

The great scandal of the genome age is that protein-coding gene diversity does not scale with the apparent complexity of organisms. For example, yeast share 40% of their protein coding genes with humans. The magnitude of complexity between humans and yeast is clearly greater than comparison of their respective repetoire of protein coding genes would indicate. This problem begs the question: From where does the complexity of higher organisms arise? Analyses of non-coding regions of the genome show that

vertebrates have undergone a dramatic expansion of “junk” DNA compared to less complex forms of life. This suggests that it is additional layers of regulation on the function of protein coding genes that higher animals use to increase their complexity. More and more it is appreciated that trans-acting DNA elements, epigenetic modifications, and non-coding transcriptional units, are major players in the regulation of the genome and that nuances in these phenomena are responsible for differences between species.

RNA has played a unique role in the evolution of species. This is mostly a consequence of the dual role RNA plays as a vehicle for genetic information, being able to encode information in the sequence of bases, and as a functional molecule that can participate in catalytic reactions. It is this dual role that has formed the prevailing theory of the origin of life. This theory predicts that “ribo-organisms” were the first forms of life on earth. These primordial organisms are hypothesized to use RNA as genetic material and for folding into essential catalytic molecules. Efforts to recreate the conditions necessary for RNA polymerization by an RNA molecule showed some success (Johnston et al., 2001). This demonstrates to some degree that RNA could be the original bio-molecule, and suggests it pre-dates both DNA and proteins in biological processes. In modern organisms the vestiges of the RNA world are still apparent. Functional RNAs, such as rRNA and tRNA, are central to the process of protein-coding gene expression. Indeed, the catalytic activity of the ribosome that synthesizes polypeptides is carried out by the RNA component of the ribosome (Ban et al., 2000; Nissen et al., 2000).

While RNA is likely the most ancient bio-molecule, and participates in the most

fundamental biological processes there are very dramatic differences in the RNA biology in different organisms. This can be particularly highlighted by the differences in the RNAi pathway in different organisms. The *C. elegans* genome encodes upwards of 27 Argonaute proteins, and a single dicer (Carmell et al., 2002). *D. melanogaster*, meanwhile, has five argonautes and a two dicer homologs (Carmell et al., 2002). Even within the *Ceanorhabiditis* genus, dramatic differences are seen. In *C. elegans* a transmembrane protein called SID-1 acts as a pore that transports dsRNA into cells. This activity is responsible for the systematic nature of RNAi in this organism (Feinberg and Hunter, 2003). Another species in the *Ceanorhabiditis* genus is *C. Briggasae*. The genome of *C. Briggasae* encodes a SID-1 homolog, but RNAi does not act in a systematic manner in this organism. Interstingly, flies do not possess a homolog of SID-1, while vertebrates do. These dramatic differences in some of the fundamental components of the RNAi pathway indicate that the role of RNAi varies greatly between species. This also suggests that the role of RNAi maybe to modulate the activity of fundamental signaling pathways that are less flexible, which we have demonstrated (Flynt et al., 2007).

Further evidence of RNAi playing species specific roles in regulating more critical processes comes from phylogenic analysis of miRNAs. Unlike the lack of correlation between organismal complexity and diversity of protein coding genes the number of distinct miRNAs scales more accurately with complexity (Sempere et al., 2006). Major changes in miRNA diversity have accompanied revolutionary changes in body plan. Fourteen miRNA families appeared in organisms that developed a bilateral body plan (Sempere et al., 2006). Likewise, vertebrates show 76 distinct families

(Sempere et al., 2006). Evidence that miRNAs have driven morphological complexity is that unlike transcription factors and ligands, which only diversify and gain new functions through mutation, entirely new families of miRNAs have arisen. In plants large scale sequencing has uncovered many unique Arabidopsis miRNAs (Fahlgren and al, 2007). Further analysis of this population revealed that miRNAs arise at a frequent rate, and while many persist and acquire function, many also decay. Approximately 500 new miRNAs were recently cloned from human brain RNA extract with many being unique to primates (Berezikov et al., 2006). This suggests that a similar mechanism of rapid, spontaneous birth and death of miRNAs could be involved in vertebrate evolution.

miRNA Research in Zebrafish

During our analysis of miRNA function in zebrafish we showed that *miR-214* plays a role in specifying cell types during somitogenesis, and how the *miR-8* family of miRNAs cooperate in the osmotic stress response. Interestingly, the sequence elements in *su(fu)* that we described above, are not conserved in mammals, while the elements of the *miR-8* family in *nherf1*, are conserved. This may be a result of the fact that *miR-214* is a more recently evolved miRNA. Further evidence for this is *miR-214* is the only member of its family. While we have shown that the function of *miR-214* is essential for normal development in zebrafish embryos, due to its recent appearance it may have not acquired an essential function before the split of the bony fish from rest of the vertebrate lineages. The *miR-8* family, as indicated above, has evolved from a founder that also is encoded in insect genomes. With these miRNAs we observe that one of the functionally significant targets of this family is conserved in fish and mammals. Unlike *miR-214*, the

miR-8 family is a diversified group of five miRNAs that are conserved in invertebrates. It would seem that during evolution this family of miRNAs acquired a critical role in regulating the function of *nherf1*. It will be interesting to determine if the *miR-8* family has a role in the stress response in vertebrates. This could demonstrate which aspects of a miRNA's function are most well conserved; whether particpation in a specific process, or regulation of a particular gene is the most significant selective pressure on miRNA evolution.

While it appears that efforts to describe the total number of miRNAs in the zebrafish genome are reaching saturation (Kloosterman et al., 2006a), many may still be uncovered as the genome assembly reaches completion. Description of miRNA diversity is indisputably a first step in characterization of miRNA function in a specific animal. These analyses can be carried out on a large scale simultaneously describing the expression of numerous miRNAs (Kloosterman et al., 2006a). Understanding the role of individual miRNAs is a more complex undertaking, and will require careful analysis of each miRNA. While this may be a protracted effort, techniques currently available in zebrafish may expedite this process. The ability to introduce molecules that produce gain-of-function and loss-of-function phenotypes directly into embryos makes for a powerful, rapid system for analysis of miRNA function. It may be useful to begin this analysis on miRNAs that are well conserved in other phyla. This may increase the likely hood of identifying miRNAs that have similar functions and targets in both mammals and fish.

Zebrafish may also prove to be useful in solving some of the issues associated with the use of therapeutic siRNAs. We have shown that zebrafish and humans share a

similar repetoire of RNAi pathway components. This suggests that the basic role of miRNAs may be very similar in fish and man. This may also indicate that efforts to describe miRNA/mRNA pairing rules in zebrafish may translate well (Kloosterman et al., 2004). Beyond base-pairing in MREs, analysis of auxillary RISC components in zebrafish, or proximal regulatory sequence elements in mRNAs could yield evolutionary conserved regulatory mechanisms of RNAi. Zebrafish may also be useful to test the stability of siRNAs with modified backbone chemistry in both inter- and extracellular environments. Experiments that use adult fish could be designed to test the stability of siRNAs in a variety of organs.

Zebrafish represents an excellent model system for the study of miRNAs. Through a combination of ease of manipulation and similarity to mammals, major breakthroughs in the RNAi field will be made through using this model organism. In the immediate future careful analysis of individual miRNA function is a necessary step. Additionally, mechanistic analysis of the differences in zebrafish and mammalian RNAi pathways will yield critical information concerning miRNA function. Ultimately, zebrafish could serve as a system to test the pharmacokinetics of siRNAs. The field of miRNAs/RNAi is still in its infancy. The rapid advancement of this field has resulted in, and will continue to produce major discoveries. These findings will, in the near future, have profound effects on how man benefits from modern medicine, and reshape how researchers view gene regulation.

REFERENCES

Aaronson, D. S., and Horvath, C. M. (2002). A road map for those who don't know JAK-STAT. Science *296*, 1653-1655.

Amarzguioui, M., Holen, T., Babaie, E., and Prydz, H. (2003). Tolerance for mutations and chemical modifications in a siRNA. Nuc Acid Res *31*, 589-595.

Ambros, V. (2001). microRNAs: tiny regulators with great potential. Cell *107*, 823-826.

Ambros, V., Bartel, B., Bartel, D. P., Burge, C. B., Carrington, J. C., Chen, X., Dreyfuss, G., Eddy, S. R., Griffiths-Jones, S., Marshall, M., *et al.* (2003). A uniform system for microRNA annotation. Rna *9*, 277-279.

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

Aravin, A. A., Lagos-Quintana, M., Yalcin, A., Zavolan, M., Marks, D., Snyder, B., Gaasterland, T., Meyer, J., and Tuschl, T. (2003b). The small RNA profile during Drosophila melanogaster development. Dev Cell *5*, 337-350.

Arenas, M. I., Romo, E., De Gaspar, I., De Bethencourt, F. R., Sánchez-Chapado, M., Fraile, B., and Paniagua, R. (1999). A lectin histochemistry comparative study in human normal prostate, benign prostatic hyperplasia, and prostatic carcinoma. Glycoconjugate Journal *16*, 375.

Babak, T., Zhang, W., Morris, Q., Blencowe, B. J., and Hughes, T. R. (2004). Probing microRNAs with microarrays: Tissue specificity and functional inference. Rna *10*, 1813-1819.

Ban, N., Nissen, P., Hansen, J., Moore, P. B., and Steitz, T. A. (2000). The Complete Atomic Structure of the Large Ribosomal Subunit at 2.4 A Resolution. Science *289*, 905.

Barnfield, P. C., Zhang, X., Thanabalasingham, V., Yoshida, M., and Hui, C.-c. (2005). Negative regulation of Gli1 and Gli2 activator function by Suppressor of fused through multiple mechanisms. Differentiation *73*, 397-405.

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. Nat Rev Genet *5*, 396-400.

Beauchamp, L., Conrad, R., Labourier, E., and Powers, P. (2007). New Tools for miRNA and siRNA Analysis.

Berezikov, E., Thuemmler, F., van Laake, L. W., Kondova, I., Bontrop, R., Cuppen, E., and Plasterk, R. H. A. (2006). Diversity of microRNAs in human and chimpanzee brain. Nat Genet *38*, 1375.

Bernstein, E., Ahmet M. Denli, and Gregory J Hannon (2001a). The rest is silence. RNA *7*, 1509-1521.

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

Bernstein, E., Kim, S.Y., Carmell, M.A., Murchison, E.P., Alcorn, H., Li, M.Z., Mills, A.M., Elledge, S.J., Anderson, K.V., Hannon G.J. (2003). Dicer is essential for mouse development. Nature Genetcs *35*, 215-217.

Bhattacharyya, S. N., Habermacher, R., Martine, U., Closs, E. I., and Filipowicz, W. (2006). Relief of microRNA-Mediated Translational Repression in Human Cells Subjected to Stress. Cell *125*, 1111.

Boden, D., Pusch, O., Lee, F., Tucker, L., and Ramratnam, B. (2003). Human immunodeficiency virus type 1 escape from RNA interference. J Virology *77*, 11531-11535.

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.

Brennecke, J., Stark, A., Russell, R. B., and Cohen, S. M. (2005). Principles of MicroRNATarget Recognition. PLoS Biology *3*, e85.

Calegari, F., Haubensak, W., Yang, D., Huttner, W.B., and Buchholz, F. (2002). Tissue-specific RNA interference in postimplantation mouse embryos with endoribonuclease-prepared short interfering RNA. PNAS *99*, 14236-14240.

Carmell, M. A., Xuan, Z., Zhang, M. Q., and Hannon, G. J. (2002). The Argonaute family: tentacles that reach into RNAi, developmental control, stem cell maintenance, and tumorigenesis. Genes Dev *16*, 2733-2742.

Castelli, J., Hassel, B., Maran, Z., Paranjape, J., Hewitt, J., Li, X., Hsu, Y., Silverman, R., and Youle, R. (1998). The role of 2'-5' oligoadenylate-activated ribonuclease L in apoptosis. Cell Death Differ *5*, 313-320.

Caudy, A. A., Myers, M., Hannon, G. J., and Hammond, S. M. (2002). Fragile X-related protein and VIG associate with the RNA interference machinery. Genes Dev *16*, 2491-2496.

Chen, P. Y., Manninga, H., Slanchev, K., Chien, M., Russo, J. J., Ju, J., Sheridan, R., John, B., Marks, D. S., Gaidatzis, D., *et al.* (2005). The developmental miRNA profiles of zebrafish as determined by small RNA cloning. Genes Dev *19*, 1288-1293.

Chiu, Y.-L., and Rana, T. (2003). siRNA function in RNAi: a chemical modification analysis. RNA *9*, 1034-1048.

Cho, J., Bandyopadhyay, J., Lee, J., Park, C.-S., and Ahnn, J. (2000). Two isoforms of sarco/endoplasmic reticulum calcium ATPase (SERCA) are essential in *Caenorhabditis elegans*. Gene *261*, 211-219.

Chou, T., Biswas, S., and Lu, S. (2004). Gene delivery using physical methods: an overview. Methods Mol Biol *245*, 147-166.

Ciruna, B., Weidinger, G., Knaut, H., Thisse, B., Thisse, C., Raz, E., and Schier, A. F. (2002). Production of maternal-zygotic mutant zebrafish by germ-line replacement. PNAS *99*, 14919-14924.

Claiborne, J. B., Edwards, S. L., and Morrison-Shetlar, A. I. (2002). Acid-base regulation in fishes: cellular and molecular mechanisms. Journal of Experimental Zoology *293*, 302-319.

Culp, P., Nusslein-Volhard, C., and Hopkins, N. (1991). High-Frequency Germ-Line Transmission of Plasmid DNA Sequences Injected Into Fertilized Zebrafish Eggs. PNAS *88*, 7953-7957.

Czauderna, F., Fechtner, M., Dames, S., Aygun, H., Klippel, A., Pronk, G., Giese, K., and Kaufmann, J. (2003). Structural variations and stabilising modifications of synthetic siRNAs in mammalian cells. Nuc Acid Res *31*, 2705-2716.

d'Anglemont de Tassigny, A., Ghaleh, B., Souktani, R., Henry, P., and Berdeaux, A. (2004). Hypo-Osmotic Stress Inhibits Doxorubicin-Induced Apoptosis via a Protein Kinase A-Dependent Mechanism in Cardiomyocytes. Clinical and Experimental Pharmacology and Physiology *31*, 438-443.

Das, A., Brummelkamp, T., Westerhout, E., Vink, M., Madiredjo, M., Bernards, R., and Berkhout, B. (2004). Human immunodeficiency virus type 1 escapes from RNA interference-mediated inhibition. J Virology *78*, 2601-2605.

Disthabanchong, S., Martin, K. J., McConkey, C. L., and Gonzalez, E. A. (2002). Metabolic acidosis up-regulates PTH/PTHrP recpetors in UMR 106-01 oestoblast-like cells. Ingenta *62*, 1171-1177.

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

Driever, W., Solnica-Krezel, L., Schier, A. F., Neuhauss, S. C., Malicki, J., Stemple, D. L., Stainier, D. Y., Zwartkruis, F., Abdelilah, S., Rangini, Z., *et al.* (1996). A genetic screen for mutations affecting embryogenesis in zebrafish. Development *123*, 37-46.

Dunaeva, M., Michelson, P., Kogerman, P., and Toftgard, R. (2003). Characterization of the Physical Interaction of Gli Proteins with SUFU Proteins. J Biol Chem *278*, 5116-5122.

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

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

Erter, C., Solnica-Krezel, L., and Wright, C. V. E. (1998). Zebrafish nodal-related2 Encodes an Early Mesendodermal Inducer Signaling from the Extraembryonic Yolk Syncytial Layer. Developmental Biology *204*, 36100-36372.

Esaki, M., Hoshijima, K., Kobayashi, S., Fukuda, H., Kawakami, K., and Hirose, S. (2007). Visualization in zebrafish larvae of Na+ uptake in mitochondria-rich cells whose differentiation is dependent on foxi3a. AJP - Regulatory, Integrative and Comparative Physiology *292*, R470.

Fahlgren, N., and al, e. (2007). High-Throughput Sequencing of Arabidopsis microRNAs: Evidence for Frequent Birth and Death of MIRNA Genes. PLoS One *e219*.

Faustino, N., and Cooper, T. (2003). Pre-mRNA splicing and human disease. Genes & Dev *17*, 419-437.

Feinberg, E. H., and Hunter, C. P. (2003). Transport of dsRNA into Cells by the Transmembrane Protein SID-1. Science *301*, 1545.

Feng, X., Adiarte, E. G., and Devoto, S. H. (2006). Hedgehog acts directly on the zebrafish dermomyotome to promote myogenic differentiation. Developmental Biology 300(2): 736-46.

Fire, A., SiQun Xu, Mary K. Montgomery, Steven A Kostas, Samuel E. Driver, Craig C. Mello (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorahbditis elegans*. Nature *391*, 806-811.

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

Francisco, P. (2003). Maternal factors in zebrafish development. Developmental Dynamics *228*, 535-554.

Fukuda, K., Tsujita, T., Matsumoto, M., Seya, T., Sakiyama, H., Nishikawa, F., Nishikawa, S., and Hasegawa, T. (2006). Analysis of the interaction between human TLR3 ectodomain and nucleic acids. Nucleic Acids Symp Ser *50*, 249-250.

Ge, B., Ziong, X., Jing, Q., Mosley, J., Filose, A., Bian, D., Huang, S., and Han, J. (2003). TAB1B (transforming growth factor-B-activated protein kinase 1-binding protein 1B), a novel splicing variant of TAB1 that interacts with p38α but not TAK1*. J Biol Chem *278*, 2286-2293.

Gheri, G., Gheri Bryk, S., and Sgambati, E. (1997). Glycoconjugate saccharidic moieties of the exocrine and endocrine pancreas in the chick embryo, newborn and adult. Biotch Histochem *72*, 158-167.

Giraldez, A. J., Cinalli, R. M., Glasner, M. E., Enright, A. J., Thomson, J. M., Baskerville, S., Hammond, S. M., Bartel, D. P., 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.

Gitlin, L., and Andino, R. (2003). Nucleic acid-based immune system: the antiviral potential of mammalian RNA silencing. J Virology *77*, 7159-7165.

Gitlin, L., Karelsky, S., and Andino, R. (2002). Short interfering RNA confers intracellular antiviral immunity in human cells. Nature *418*, 430-434.

Gonzalez-Alegre, P., Miller, V., Davidson, B., and Paulson, H. (2003). Toward therapy for DYT1 dystonia: allele-specific silencing of mutant TorsinA. Ann Neurol *53*, 781-787.

Goodrich, L. V., Johnson, R. L., Milenkovic, L., McMahon, J. A., and Scott, M. P. (1996). Conservation of the hedgehog/patched signaling pathway from flies to

mice: induction of a mouse patched gene by Hedgehog. Genes and Development *10*, 301.

Grad, Y., Aach, J., Hayes, G. D., Reinhart, B. J., Church, G. M., Ruvkun, G., and Kim, J. (2003). Computational and experimental identification of C. elegans microRNAs. Mol Cell *11*, 1253-1263.

Griffiths-Jones, S. (2004). The microRNA Registry. Nucleic Acids Res *32 Database issue*, D109-111.

Griffiths-Jones, S., Grocock, R. J., van Dongen, S., Bateman, A., and Enright, A. J. (2006). miRBase: microRNA sequences, targets and gene nomenclature. Nucleic Acids Research *34*, D140.

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.

Gritsman, K., Zhang, J., Cheng, S., Heckscher, E., Talbot, W. S., and Schier, A. F. (1999). The EGF-CFC Protein One-Eyed Pinhead Is Essential for Nodal Signaling. Cell *97*, 121.

Haffter, P., and Nusslein-Volhard, C. (1996). Large scale genetics in a small vertebrate, the zebrafish. Int J Dev Biol *40*, 221-227.

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., Caudy, A., and Hannon, G. J. (2001). Post-transcriptional gene silencing by double-stranded RNA. Nat Rev Gen *2*, 110-119.

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.

Harris, J. A., Cheng, A. G., Cunningham, L. L., MacDonald, G., Raible, D. W., and Rubel, E. W. (2003). Neomycin-Induced Hair Cell Death and Rapid Regeneration in the Lateral Line of Zebrafish (Danio rerio). JARO - Journal of the Association for Research in Otolaryngology *4*, 219.

Hatfield, S. D., Shcherbata, H. R., Fischer, K. A., Nakahara, K., Carthew, R. W., and Ruohola-Baker, H. (2005). Stem cell division is regulated by the microRNA pathway. Nature *435*, 974.

He, L., He, X., Lim, L. P., de Stanchina, E., Xuan, Z., Liang, Y., Xue, W., Zender, L., Magnus, J., Ridzon, D., *et al.* (2007). A microRNA component of the p53 tumour suppressor network. Nature *447*, 1130.

Hemann, M. T., Fridman, J. S., Zilfou, J. T., Hernando, E., Paddison, P. J., Cordon-Cardo, C., Hannon, G. J., and Lowe, S. W. (2003). An epi-allelic series of p53 hypomorphs created by stable RNAi produces distinct tumor phenotypes in vivo. Nat Genet *33*, 396-400.

Henry, C. A., and Amacher, S. L. (2004). Zebrafish Slow Muscle Cell Migration Induces a Wave of Fast Muscle Morphogenesis. Developmental Cell *7*, 917.

Hsiao, C.-D., May-Su, Y., Ying-Jey, G., Ming, M., Yun-Jin, J., and Pung-Pung, H. (2007). A Positive Regularoty Loop between foxi3a and foxi3b Is Essential for Specification and Differentiation of Zebrafish Epidermal Ionocytes. PLoS One.

Hutvagner, G., and Zamore, P. D. (2002). A microRNA in a Multiple-Turnover RNAi Enzyme Complex. Science *297*, 2056-2060.

Iascson, R., Kull, B., Salmi, P., and Wahlestedt, C. (2003). Lack of efficacy of 'naked' small interfering RNA applied directly to rat brain. Acta Physiol Scand *179*, 173-177.

Inbal, A., Topczewski, J., and Solnica-Krezel, L. (2006). Targeted gene expression in the zebrafish prechordal plate. genesis *44*, 584-588.

Ingham, P. W., and Kim, H. R. (2005). Hedgehog signalling and the specification of muscle cell identity in the Zebrafish embryo. Experimental Cell Research *306*, 336.

Ishizuka, A., Siomi, M. C., and Siomi, H. (2002). A Drosophila fragile X protein interacts with components of RNAi and ribosomal proteins. Genes Dev *16*, 2497-2508.

Jackson, A., Bartz, S., Schelter, J., Kobayashi, S., Burchard, J., Mao, M., Li, B., Cavet, G., and Linsley, P. (2003). Expression profiling reveals off-target gene regulation by RNAi. Nat Biotech *21*, 635-637.

Janicke, M., Carney, T. J., and Hammerschmidt, M. (2007). Foxi3 transcription factors and Notch signaling control the formation of skin ionocytes from epidermal precursors of the zebrafish embryo. Developmental Biology *307*, 258.

Jepsen, J. S., Sorensen, M. D., and Wengel, J. (2004). Locked Nucleic Acid: A Potent Nucleic Acid Analog in Therapeutics and Biotechnology. Oligonucleotides *14*, 130-146.

John, B., Enright, A. J., Aravin, A., Tuschl, T., Sander, C., and Marks, D. S. (2004). Human MicroRNA Targets. PLoS Biology *2*, e363.

Johnston, R. J., and Hobert, O. (2003). A microRNA controlling left/right neuronal asymmetry in Caenorhabditis elegans. Nature *426*, 845-849.

Johnston, W. K., Unrau, P. J., Lawrence, M. S., Glasner, M. E., and Bartel, D. P. (2001). RNA-Catalyzed RNA Polymerization: Accurate and General RNA-Templated Primer Extension. Science *292*, 1319.

Jonz, M. G., and Colin A., N. (2006). Epithelial mitochondria-rich cells and the associtated innervation in adult and developing zebrafish. The Journal of Comparative Neurology *497*, 817-832.

Kawakami, K., Shima, A., and Kawakami, N. (2000). Identification of a functional transposase of the Tol2 element, an Ac-like element from the Japanese medaka fish, and its transposition in the zebrafish germ lineage. Proceedings of the National Academy of Sciences *97*, 11403.

Kennerdell, J. R., Yamaguchi, S., and Carthew, R. W. (2002). RNAi is activated during Drosophila oocyte maturation in a manner dependent on aubergine and spindle-E. Genes Dev *16*, 1884-1889.

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

Khvorova, A., Reynolds, A., and Jayasena, S. D. (2003). Functional siRNAs and miRNAs Exhibit Strand Bias. Cell *115*, 209.

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.

Kimmel, C. B., Kane, D. A., Walker, C., Warga, R. M., and Rothman, M. B. (1989). A mutation that changes cell movement and cell fate in the zebrafish embryo. Nature *337*, 358.

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 Dev *18*, 1165-1178.

Kishimoto, Y., Lee, K. H., Zon, L., Hammerschmidt, M., and Schulte-Merker, S. (1997). The molecular nature of zebrafish swirl: BMP2 function is essential during early dorsoventral patterning. Development *124*, 4457-4466.

Kitabwalla, M., and Ruprecht, R. (2002). RNA interference - a new weapon against HIV and beyond. N Engl J Med *347*, 1364-1367.

Kloosterman, W. P., Lagendijk, A. K., Ketting, R., F, Moulton, J. D., and Plasterk, R. H. A. (2007). Targeted Inhibition of miRNA Maturation with Morpholinos Reveals a Role for miR-375 in Pancreatic Islet Development. PLoS Biology *5*, e203.

Kloosterman, W. P., and Plasterk, R. H. A. (2006). The Diverse Functions of MicroRNAs in Animal Development and Disease. Developmental Cell *11*, 441.

Kloosterman, W. P., Steiner, F. A., Berezikov, E., de Bruijn, E., van de Belt, J., Verheul, M., Cuppen, E., and Plasterk, R. H. A. (2006a). Cloning and expression of new microRNAs from zebrafish. Nucl Acids Res *34*, 2558-2569.

Kloosterman, W. P., Wienholds, E., de Bruijn, E., Kauppinen, S., and Plasterk, R. H. A. (2006b). In situ detection of miRNAs in animal embryos using LNA-modified oligonucleotide probes. Nat Meth *3*, 27.

Kloosterman, W. P., Wienholds, E., Ketting, R. F., and Plasterk, R. H. A. (2004). Substrate requirements for let-7 function in the developing zebrafish embryo. Nucl Acids Res *32*, 6284-6291.

Koudijs, M. J., den Broeder, M. J., Keijser, A., Wienholds, E., Houwing, S., van Rooijen, E. M. H. C., Geisler, R., and van Eeden, F. J. M. (2005). The Zebrafish Mutants dre, uki, and lep Encode Negative Regulators of the Hedgehog Signaling Pathway. PLoS Genetics *1*, e19.

Krek, A., Grun, D., Poy, M. N., Wolf, R., Rosenberg, L., Epstein, E. J., MacMenamin, P., da Piedade, I., Gunsalus, K. C., Stoffel, M., and Rajewsky, N. (2005). Combinatorial microRNA target predictions. Nat Genet *37*, 495.

Krutzfeldt, J., MN, P., and M, S. (2006). Strategies to determine the biological function of microRNAs. Nat Genet *38*.

Krutzfeldt, J., Rajewsky, N., Braich, R., Rajeev, K. G., Tuschl, T., Manoharan, M., and Stoffel, M. (2005). Silencing of microRNAs in vivo with 'antagomirs'. Nature *438*, 685.

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. Curr Biol *12*, 735-739.

Lai, E. C. (2002). Micro RNAs are complementary to 3' UTR sequence motifs that mediate negative post-transcriptional regulation. Nat Genet *30*, 363-364.

Lai, E. C. (2004). Predicting and validating microRNA targets. Genome Biol *5*, 115.

Lai, E. C., Tomancak, P., Williams, R. W., and Rubin, G. M. (2003). Computational identification of Drosophila microRNA genes. Genome Biol *4*, R42.

Lakatos, L., Szittya, G., Silhavy, D., and Burgyan, J. (2004). Molecular mechanism of RNA silencing suppression mediated by p19 protein of tombusviruses. EMBO J *23*, 876-884.

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.

Lau, N. C., Seto, A. G., Kim, J., Kuramochi-Miyagawa, S., Nakano, T., Bartel, D. P., and Kingston, R. E. (2006). Characterization of the piRNA Complex from Rat Testes. Science *313*, 363-367.

Lederer, E. D., Khundmiri, S. J., and Weinman, E. J. (2003). Role of NHERF-1 in Regulation of the Activity of Na-K ATPase and Sodium-Phosphate Co-transport in Epithelial Cells. Journal of the American Society of Nephrology *14*, 1711.

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., Radmark, O., Kim, S., and Kim, V. N. (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. (2004a). MicroRNA genes are transcribed by RNA polymerase II. Embo J *23*, 4051-4060.

Lee, Y. S., Nakahara, K., Pham, J. W., Kim, K., He, Z., Sontheimer, E. J., and Carthew, R. W. (2004b). Distinct roles for Drosophila Dicer-1 and Dicer-2 in the siRNA/miRNA silencing pathways. Cell *117*, 69-81.

Leung, A. K. L., Calabrese, J. M., and Sharp, P. A. (2006). Quantitative analysis of Argonaute protein reveals microRNA-dependent localization to stress granules. PNAS *103*, 18125-18130.

Leung, A. K. L., and Sharp, P. A. (2007). microRNAs: A Safeguard against Turmoil? Cell *130*, 581-585.

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.

Lewis, D. L., Hagstrom, J. E., Loomis, A. G., Wolff, J. A., and Herweijer, H. (2002). Efficient delivery of siRNA for inhibition of gene expression in postnatal mice. Nat Gen *32*, 107-108.

Lewis, K. E., Currie, P. D., Roy, S., Schauerte, H., Haffter, P., and Ingham, P. W. (1999). Control of Muscle Cell-Type Specification in the Zebrafish Embryo by Hedgehog Signalling. Developmental Biology *216*, 469.

Li, Y.-X., Farrell, M. J., Liu, R., Mohanty, N., and Kirby, M. L. (2000). Double-Stranded RNA Injection Produces Null Phenotypes in Zebrafish. Developmental Biology *217*, 394.

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

Lin, L.-Y., Horng, J.-L., Kunkel, J. G., and Hwang, P.-P. (2006). Proton pump-rich cell secretes acid in skin of zebrafish larvae. AJP - Cell Physiology *290*, C371.

Liu, J., Carmell, M. A., Rivas, F. V., Marsden, C. G., Thomson, J. M., Song, J. J., Hammond, S. M., Joshua-Tor, L., and Hannon, G. J. (2004). Argonaute2 is the catalytic engine of mammalian RNAi. Science *305*, 1437-1441.

Liu, J., Valencia-Sanchez, M. A., Hannon, G. J., and Parker, R. (2005). MicroRNA-dependent localization of targeted mRNAs to mammalian P-bodies. Nat Cell Biol *7*, 719.

Liu, Q., Rand, T.A., Kalidas, S., Du, F., Kim, H., Smith, D.P., Wang, X. (2003). R2D2, a Bridge Between the Initiation and Effector Steps of the *Drosophila* RNAi Pathway. Science *301*, 1921-1925.

Matsuda, T., and Cepko, C. (2004). Electroporation and RNA interference in the rodent retina in vivo and in vitro. Proc Natl Acad Sci *101*, 16-22.

McCaffrey, A. P., Meuse, L., Pham, T.-T. T., Conklin, D. S., Hannon, G. J., and Kay, M. A. (2002). RNA interference in adult mice. Nature *418*, 38-39.

Meister, G., Landthaler, M., Dorsett, Y., and Tuschl, T. (2004). Sequence-specific inhibition of microRNA- and siRNA-induced RNA silencing. RNA *10*, 544-550.

Miller, V., Xia, H., Marrs, G., Gouvion, C., Lee, G., Davidson, B., and Paulson, H. (2003). Allele-specific silencing of dominant disease genes. Proc Natl Acad Sci *100*, 7195-7200.

Mishima, Y., Giraldez, A. J., Takeda, Y., Fujiwara, T., Sakamoto, H., Schier, A. F., and Inoue, K. (2006). Differential Regulation of Germline mRNAs in Soma and Germ Cells by Zebrafish miR-430. Current Biology *16*, 2135.

Miska, E. A., Alvarez-Saavedra, E., Townsend, M., Yoshii, A., Sestan, N., Rakic, P., Constantine-Paton, M., and Horvitz, H. R. (2004). Microarray analysis of microRNA expression in the developing mammalian brain. Genome Biol *5*, R68.

Morales, F. C., Takahashi, Y., Momin, S., Adams, H., Chen, X., and Georgescu, M.-M. (2007). NHERF1/EBP50 Head-to-Tail Intramolecular Interaction Masks Association with PDZ Domain Ligands. Mol Cell Biol *27*, 2527-2537.

Murthy, A., Gonzalez-Agosti, C., Cordero, E., Pinney, D., Candia, C., Solomon, F., Gusella, J., and Ramesh, V. (1998). NHE-RF, a Regulatory Cofactor for Na+-H+ Exchange, Is a Common Interactor for Merlin and ERM (MERM) Proteins. Journal of Biological Chemistry *273*, 1273.

Myers, D., Sepich, D., and Solnica-Krezel, L. (2002). Convergence and extension in vertebrate gastrulae: cell movements according to or in search of identity? Trends Genet *18*, 447-455.

Naguibneva, I., Ameyar-Zazoua, M., Polesskaya, A., Ait-Si-Ali, S., Groisman, R., Souidi, M., Cuvellier, S., and Harel-Bellan, A. (2006). The microRNA miR-181 targets the homeobox protein Hox-A11 during mammalian myoblast differentiation. Nat Cell Biol *8*, 278-284.

Napoli, C., Lemieux, C., and Jorgensen, R. (1990). Introduction of a Chimeric Chalcone Synthase Gene into Petunia Results in Reversible Co-Suppression of Homologous Genes in trans. THE PLANT CELL *2*, 279.

Nasevicius, A., and Ekker, S. C. (2000). Effective targeted gene 'knockdown' in zebrafish. Nature Genetics *26*, 216-220.

Neely, L. A., Patel, S., Garver, J., Gallo, M., Hackett, M., McLaughlin, S., Nadel, M., Harris, J., Gullans, S., and Rooke, J. (2006). A single-molecule method for the quantitation of microRNA gene expression. Nat Meth *3*, 41.

Nielsen, J. S., Graves, M. L., Chelliah, S., Vogl, A. W., Roskelley, C. D., and McNagny, K. M. (2007). The CD34-Related Molecule Podocalyxin Is a Potent Inducer of Microvillus Formation. PLoS One.

Nissen, P., Hansen, J., Ban, N., Moore, P. B., and Steitz, T. A. (2000). The Structural Basis of Ribosome Activity in Peptide Bond Synthesis. Science *289*, 920.

Oates, A. C., Bruce, A. E. E., and Ho, R. K. (2000). Too Much Interference: Injection of Double-Stranded RNA Has Nonspecific Effects in the Zebrafish Embryo. Developmental Biology *224*, 20.

Okamura, K., Ishizuka, A., Siomi, H., and Siomi, M. C. (2004). Distinct roles for Argonaute proteins in small RNA-directed RNA cleavage pathways. Genes Dev *18*, 1655-1666.

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. Dev Biol *216*, 671-680.

Orlic, T., Loomis, W. H., Shreve, A., Namiki, S., and Junger, W. G. (2002). Hypertonicity increases cAMP in PMN and blocks oxidative burst by PKA-dependent and -independent mechanisms. Am J Physiol Cell Physiol *282*, C1261-1269.

Palatnik, J. F., Allen, E., Wu, X., Schommer, C., Schwab, R., Carrington, J. C., and Weigel, D. (2003). Control of leaf morphogenesis by microRNAs. Nature *425*, 257-263.

Palliser, D., Chowdhury, D., Wang, Q.-Y., Lee, S. J., Bronson, R. T., Knipe, D. M., and Lieberman, J. (2006). An siRNA-based microbicide protects mice from lethal herpes simplex virus 2 infection. Nature *439*, 89.

Park, H.-C., Shin, J., and Appel, B. (2004). Spatial and temporal regulation of ventral spinal cord precursor specification by Hedgehog signaling. Development *131*, 5959-5969.

Pascual-Ahuir, A., Posas, F., Serrano, R., and Proft, M. (2001). Multiple Levels of Control Regulate the Yeast cAMP-response Element-binding Protein Repressor Sko1p in Response to Stress. Journal of Biological Chemistry *276*, 37373.

Perrimon, N. (1998). New advances in Drosophila provide opportunities to study gene functions. PNAS *95*, 9716-9717.

Piccin, A., Salameh, A., Benna, C., Sandrelli, F., Mazzotta, G., Zordan, M., Rosato, E., Kyriacou, C. P., and Costa, R. (2001). Efficient and heritable functional knock-out of an adult phenotype in Drosophila using a GAL4-driven hairpin RNA incorporating a heterologous spacer. Nucl Acids Res *29*, e55-.

Prioleau, M.-N., Buckle, R.S., and Mechali, M. (1995). Programming of a repressed but committed chromatin struture during early development. EBO J *14*, 5073-5084.

Raver-Shapira, N., Marciano, E., Meiri, E., Spector, Y., Rosenfeld, N., Moskovits, N., Bentwich, Z., and Oren, M. (2007). Transcriptional Activation of miR-34a Contributes to p53-Mediated Apoptosis. Molecular Cell *26*, 731.

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.

Rodriguez, A., Griffith-Jones, S., Ashurst, J. L., and Bradley, L. (2004). Identification of mammalian mircroRNA host genes and transcription units. Genome Res *14*, 1902-1910.

Roy, S., Wolff, C., and Ingham, P. W. (2001). The u-boot mutation identifies a Hedgehog-regulated myogenic switch for fiber-type diversification in the zebrafish embryo. Genes Dev *15*, 1563-1576.

Ryther, R. C. C., Flynt, A. S., Harris, B. D., Phillips III, J. A., and Patton, J. G. (2004). siRNA therapeutics: Big potential from small RNAs. Gene Therapy. (2005) 12(1): 5-11

Saran, S., Meima, M. E., Alvarez-Curto, E., Weening, K. E., Rozen, D. E., and Schaap, P. (2002). cAMP signaling in Dictyostelium. Journal of Muscle Research and Cell Motility *23*, 793.

Schafer, M., Kinzel, D., Neuner, C., Schartl, M., Volff, J.-N., and Winkler, C. (2005). Hedgehog and retinoid signalling confines nkx2.2b expression to the lateral floor plate of the zebrafish trunk. Mechanisms of Development *122*, 43.

Schauerte, H. E., van Eeden, F. J., Fricke, C., Odenthal, J., Strahle, U., and Haffter, P. (1998). Sonic hedgehog is not required for the induction of medial floor plate cells in the zebrafish. Development *125*, 2983-2993.

Schier, A. F., and Talbot, W. S. (2005). Molecular Genetics of Axis Formation in Zebrafish. Annual Review of Genetics *39*, 561-613.

Schiffelers, R. M., Ansari, A., Xu, J., Zhou, Q., Tang, Q., Storm, G., Molema, G., Lu, P. Y., Scaria, P. V., and Woodle, M. C. (2004). Cancer siRNA therapy by tumor selective delivery with ligand-targeted sterically stabilized nanoparticle. Nucleic Acids Research *32*, e149.

Schmajuk, G., Sierakowska, H., and Kole, R. (1999). Antisense Oligonucleotides with Different Backbones. J Biol Chem *274*, 21783-21789.

Schmittgen, T. D., Jiang, J., Liu, Q., and Yang, L. (2004). A high-throughput method to monitor the expression of microRNA precursors. Nucl Acids Res *32*, e43-.

Schratt, G. M., Tuebing, F., Nigh, E. A., Kane, C. G., Sabatini, M. E., Kiebler, M., and Greenberg, M. E. (2006). A brain-specific microRNA regulates dendritic spine development. Nature *439*, 283-289.

Schwarz, D. S., Hutvagner, G., Du, T., Xu, Z., Aronin, N., and Zamore, P. D. (2003). Asymmetry in the Assembly of the RNAi Enzyme Complex. Cell *115*, 199.

Semizarov, D., Frost, L., Sarthy, A., Kroeger, P., Halbert, D., and Fesik, S. (2003). Specificity of short interfering RNA determined through gene expression signatures. PNAS *100*, 6347-6352.

Sempere, L., Cole, C., McPeek, M., and Peterson, K. (2006). The Phylogenetic Distribution of Meazoan microRNAs: Insights into Evolutionary Complexity and Constraint. Journal of Experimental Zoology *306*, 575-588.

Sempere, L. F., Freemantle, S., Pitha-Rowe, I., Moss, E., Dmitrovsky, E., and Ambros, V. (2004). Expression profiling of mammalian microRNAs uncovers a subset of brain-expressed microRNAs with possible roles in murine and human neuronal differentiation. Genome Biology *5*, R13.11-R13.11.

Sempere, L. F., Sokol, N. S., Dubrovsky, E. B., Berger, E. M., and Ambros, V. (2003). Temporal regulation of microRNA expression in Drosophila melanogaster mediated by hormonal signals and broad-Complex gene activity. Dev Biol *259*, 9-18.

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

Sheikh-Hamad, D., and Gustin, M. C. (2004). MAP kinases and the adaptive response to hypertonicity: functional preservation from yeast to mammals. Am J Physiol Renal Physiol *287*, F1102-1110.

Silva, J., Hammond, S., and Hannon, G. (2002). RNA interference: a promising approach to antiviral therapy? TRENDS Mol Med *8*, 505-508.

Solnica-Krezel, L., Schier, A. F., and Driever, W. (1994). Efficient Recovery of ENU-Induced Mutations From the Zebrafish Germline. Genetics *136*, 1401-1420.

Sorensen, D. R., Leirdal, M., and Sioud, M. (2003). Gene Silencing by Systemic Delivery of Synthetic siRNAs in Adult Mice. J Mol Biol *327*, 761-766.

Stark, A., Brennecke, J., Russell, R. B., and Cohen, S. M. (2003). Identification of Drosophila MicroRNA Targets. PLoS Biol *1*, E60.

Stein, P., Zeng, F., Pan, H., and Schultz, R. M. (2005). Absence of non-specific effects of RNA interference triggered by long double-stranded RNA in mouse oocytes. Developmental Biology *286*, 464.

Stemmer-Rachamimov, A. O., Wiederhold, T., Nielsen, G. P., James, M., Pinney-Michalowski, D., Roy, J. E., Cohen, W. A., Ramesh, V., and Louis, D. N. (2001). NHE-RF, a Merlin-Interacting Protein, Is Primarily Expressed in Luminal Epithelia, Proliferative Endometrium, and Estrogen Receptor-Positive Breast Carcinomas. Am J Pathol *158*, 57-62.

Stevenson, M. (2003). Dissecting HIV-1 through RNA interference. Nat Rev Immun *3*, 851-857.

Tabara, H., Sarkissian, M., Kelly, W. G., Fleenor, J., Grishok, A., Timmons, L., Fire, A., and Mello, C. C. (1999). The rde-1 Gene, RNA Interference, and Transposon Silencing in C. elegans. Cell *99*, 123.

Thatcher, E. J., Alex S. Flynt, Nan Li, Jonathan R. Patton, 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.

Theisen, C. S., Wahl, J. K., III, Johnson, K. R., and Wheelock, M. J. (2007). NHERF Links the N-Cadherin/Catenin Complex to the Platelet-derived Growth Factor Receptor to Modulate the Actin Cytoskeleton and Regulate Cell Motility. Molecular Biology of the Cell *18*, 1220.

Thisse, C., Thisse, B., Schilling, T. F., and Postlethwait, J. H. (1993). Structure of the zebrafish snail1 gene and its expression in wild-type, spadetail and no tail mutant embryos. Development *119*, 1203-1215.

Topczewska, J. M., Topczewski, J., Shostak, A., Kume, T., Solnica-Krezel, L., and Hogan, B. L. M. (2001). The winged helix transcription factor Foxc1a is essential for somitogenesis in zebrafish. Genes and Development *15*, 2483.

Trezise, A., Palazon, L., Davies, W., and Colledge, W. (2003). In vivo gene expression: DNA electrotransfer. Curr Opin Mol Ther *5*, 397-404.

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 Dev *13*, 3191-3197.

Tyska, M. J., Mackey, A. T., Huang, J.-D., Copeland, N. G., Jenkins, N. A., and Mooseker, M. S. (2005). Myosin-1a Is Critical for Normal Brush Border Structure and Composition. Mol Biol Cell *16*, 2443-2457.

van Rooij, E., Sutherland, L. B., Liu, N., Williams, A. H., McAnally, J., Gerard, R. D., Richardson, J. A., and Olson, E. N. (2006). A signature pattern of stress-responsive microRNAs that can evoke cardiac hypertrophy and heart failure. PNAS *103*, 18255-18260.

Veldhoen, S., Laufer, S., Trampe, A., and Restle, T. (2006). Cellular delivery os small interfering RNA by a non-covalently attached cell-penetrating peptide: quatitative analysis of uptake and biological effect. Nucleic Acids Research *34*, 6561-6573.

Voltz, J. W., Weinman, E. J., and Shenolikar, S. (2001). Expanding the role of NHERF, a PDZ-domaing containing protein adapter, to growth regulation. Oncogene *20*, 6309-6314.

Voorhoeve, P. M., le Sage, C., Schrier, M., Gillis, A. J., Stoop, H., Nagel, R., Liu, Y. P., van Duijse, J., Drost, J., Griekspoor, A., *et al.* (2006). A genetic screen implicates miRNA-372 and miRNA-373 as oncogenes in testicular germ cell tumors. Cell *124*, 1169-1181.

Wargelius, A., Ellingsen, S., and Fjose, A. (1999). Double-Stranded RNA Induces Specific Developmental Defects in Zebrafish Embryos. Biochemical and Biophysical Research Communications *263*, 156.

Weinholds, E., Koudijs, M.J., van Eeden, F., Cuppen, E., and Plasterk, R., (2003). The microRNA-producing enzyme Dicer1 is essential for zebrafish development. Nature Genetcs *35*, 217-218.

Weinman, E. J., Steplock, D., Donowitz, M., and Shenolikar, S. (2000). NHERF Associations with Sodium-Hydrogen Exchanger Isoform 3 (NHE3) and Ezrin Are Essential for cAMP-Mediated Phosphorylation and Inhibition of NHE3. Biochemistry *39*, 6123-6129.

Wheeler, D., Sneddon, W. B., Wang, B., Friedman, P. A., and Romero, G. (2007). NHERF-1 and the Cytoskeleton Regulate the Traffic and Membrane Dynamics of G Protein-coupled Receptors. Journal of Biological Chemistry *282*, 25076.

Wienholds, E., Kloosterman, W. P., Miska, E., Alvarez-Saavedra, E., Berezikov, E., de Bruijn, E., Horvitz, H. R., Kauppinen, S., and Plasterk, R. H. A. (2005).

MicroRNA Expression in Zebrafish Embryonic Development. Science *309*, 310-311.

Wienholds, E., Koudijs, M. J., van Eeden, F. J., Cuppen, E., and Plasterk, R. H. (2003a). The microRNA-producing enzyme Dicer1 is essential for zebrafish development. Nat Genet *35*, 217-218.

Wienholds, E., van Eeden, F., Kosters, M., Mudde, J., Plasterk, R. H., and Cuppen, E. (2003b). Efficient target-selected mutagenesis in zebrafish. Genome Res *13*, 2700-2707.

Wolff, C., Roy, S., and Ingham, P. W. (2003). Multiple Muscle Cell Identities Induced by Distinct Levels and Timing of Hedgehog Activity in the Zebrafish Embryo. Current Biology *13*, 1169.

Wu, L., Fan, J., and Belasco, J. G. (2006). From the Cover: MicroRNAs direct rapid deadenylation of mRNA. PNAS *103*, 4034-4039.

Wu, S., and Kaufman, R. J. (1997). A Model for the Double-stranded RNA (dsRNA)-dependent Dimerization and Activation of the dsRNA-activated Protein Kinase PKR. J Biol Chem *272*, 1291-1296.

Xu, P., Vernooy, S. Y., Guo, M., and Hay, B. A. (2003). The Drosophila microRNA Mir-14 suppresses cell death and is required for normal fat metabolism. Curr Biol *13*, 790-795.

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

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 Dev *17*, 3011-3016.

Yun, C. H. C., Oh, S., Zizak, M., Steplock, D., Tsao, S., Tse, C.-M., Weinman, E. J., and Donowitz, M. (1997). cAMP-mediated inhibition of the epithelial brush border Na+/H+ exchanger, NHE3, requires an associated regulatory protein. PNAS *94*, 3010.

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

Zender, L., Hutker, S., Liedtke, C., Tillmann, H., Zender, S., Mundt, B., Waltemathe, M., Gosling, T., Flemming, P., Malek, N., *et al.* (2003). Caspase 8 small interfering RNA prevents acute liver failure in mice. PNAS *100*, 7797-7802.

Zeng, Y., Yi, R., and Cullen, B. R. (2003). MicroRNAs and small interfering RNAs can inhibit mRNA expression by similar mechanisms. PNAS *100*, 9779-9784.

Zhang, X., Shan, P., Jiang, D., Noble, P., Abraham, N., Kappas, A., and Lee, P. (2004). Small interfering RNA targeting heme oxygenase-1 enhances ischemia-reperfusion-induced lung apoptosis. J Biol Chem *279*, 10677-10684.

Zhao, Z., Cao, Y., Li, M., and Meng, A. (2001). Double-Stranded RNA Injection Produces Nonspecific Defects in Zebrafish. Developmental Biology *229*, 215.