MIRNA FUNCTION DURING EARLY VERTEBRATE DEVELOPMENT

By

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To my parents, Xiaolu and Yulan,

To my sister and bother in law, Li and Bangmin

and

To Jingbo

your love and support made this possible

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AGO	Argonaute
A/P	anterior-posterior
D/V	dorsal-ventral
DFC	dorsal forerunner cell
dpf	day post-fertilization
dsRBD	double-stranded RNA binding domain
DSE	dorsal surface epithelial
dsRNA	double-stranded RNA
EVL	enveloping layer
hpf	hour post-fertilization
IRES	internal ribosome entry site
ISH	in situ hybridization
KV	Kupffer's vesicle
L/R	left-right
LPM	lateral plate mesoderm
MBT	mid-blastula transition
MET	mesenchymal to epithelial transition
miRNA	microRNA
MO	morpholino
MRE	miRNA recognition element
NIC	non-injected control

RISC	RNA induced silencing complex
SEM	standard error of the mean
UTR	untranslated region
YSL	yolk syncytial layer

CHAPTER I

INTRODUCTION

microRNAs (miRNAs) are a family of 22-25 nucleotide endogenous non-coding RNAs that regulate gene expression in a sequence-specific manner. Typically, miRNAs down regulate target genes by recognizing and recruiting protein complexes to 3' untranslated regions (3'UTR) followed by either translation repression or degradation. Studies in vertebrates have revealed indispensable functions for miRNAs in various developmental and physiological events. Understanding the biology of miRNAs will not only increase our basic knowledge of development and disease, but also help to develop novel therapeutic approaches.

Identification of miRNAs

To date, the miRNA registry (www.mirbase.org) contains 174 *C. elegans* miRNAs, 157 *D. melanogaster* miRNAs, 360 *D. rerio* miRNAs, 721 *H. sapiens* miRNAs and 190 *A. thaliana* miRNAs. It has been estimated that miRNAs comprise 2-3% of the total number of genes in humans (Kim and Nam 2006). Three approaches have been used to the identify miRNA genes: forward genetic screens, direct cloning, and bioinformatic predictions.

The founding members of miRNAs were identified by forward genetic screens. *lin-4* and *let-7* were discovered in screens for developmental timing defects in C. elegans (Lee, Feinbaum et al. 1993; Wightman, Ha et al. 1993; Reinhart, Slack et al. 2000). *lin-4* turns

out to be worm specific but *let-7* homologs exist from worms to flies and humans. The other two miRNAs identified using genetic screens are *bantam* in Drosophila and *Lsy-6* in worms (Brennecke, Hipfner et al. 2003; Johnston and Hobert 2003). Because miRNAs are small (22nt), identification through mutagenic screens is rare.

Large-scale miRNA identification was achieved by directional cloning (Ambros and Lee 2004; Chen, Manninga et al. 2005). All cloned small RNA sequences are subject to the following criteria before classification as a miRNA (Ambros, Bartel et al. 2003). First, the candidate sequence should be present in the arm of a hairpin structure, usually ~80nt in animals. Second, the candidate sequence should be phylogenetically conserved in closely related species. Lastly, stable accumulation of the candidate sequence should be confirmed by northern blotting. Such strategies have worked well to identify highly expressed miRNAs but not those expressed at low levels or in specific cell types. Next generation sequencing has now provided the sensitivity to detect very low abundance miRNAs (Creighton, Benham et al.; Ansorge 2009).

The third approach is based on bioinformatic prediction and has served as a good alternative to direct cloning. Many miRNA identification algorithms have been developed to look for novel miRNA candidates and are primarily based on phylogenetic conservation and the structural characteristics of miRNA precursors (Lai, Tomancak et al. 2003; Lim, Lau et al. 2003; Bentwich, Avniel et al. 2005; Berezikov, Guryev et al. 2005; Nam, Shin et al. 2005). The combination of high throughput sequencing techniques and the refinement of bioinformatic prediction algorithms has dramatically increased the numbers of identified miRNAs although not all have been experimentally

verified (Jagadeeswaran, Zheng et al.; Bar, Wyman et al. 2008; Burnside, Ouyang et al. 2008; Friedlander, Chen et al. 2008; Rathjen, Pais et al. 2009).

Genomic organization of miRNAs

Roughly 50% of genes encoding mammalian miRNAs are found in close proximity in the genome to other miRNA genes. These clustered miRNA genes are often transcribed as polycistronic transcription units. The remaining 50% of miRNAs are monocistronic (Olena and Patton; Griffiths-Jones, Saini et al. 2008; Thatcher, Bond et al. 2008; Kim, Han et al. 2009). miRNAs within one cluster can belong to different families (meaning they have distinct 5'ends) and have distinct mRNA targets. Interestingly, proteins in the same interaction networks are often regulated by miRNAs from the same cluster (Yuan, Liu et al. 2009).

In humans, around 50% of all miRNA genes are in intergenic regions of the genome, while the other half are located within defined transcription units, including the intronic regions of protein coding genes (20%), the exonic regions of protein coding genes (5%), the intronic regions of non-coding genes (20%), and the exonic regions of non-coding genes (5%) (Griffiths-Jones, Saini et al. 2008; Kim, Han et al. 2009). Around 2% can be both intronic and exonic, depending on alternative splicing of the host gene. The exact percentage for each category may vary between species (Olena and Patton; Thatcher, Bond et al. 2008).

Biogenesis and regulation of miRNAs

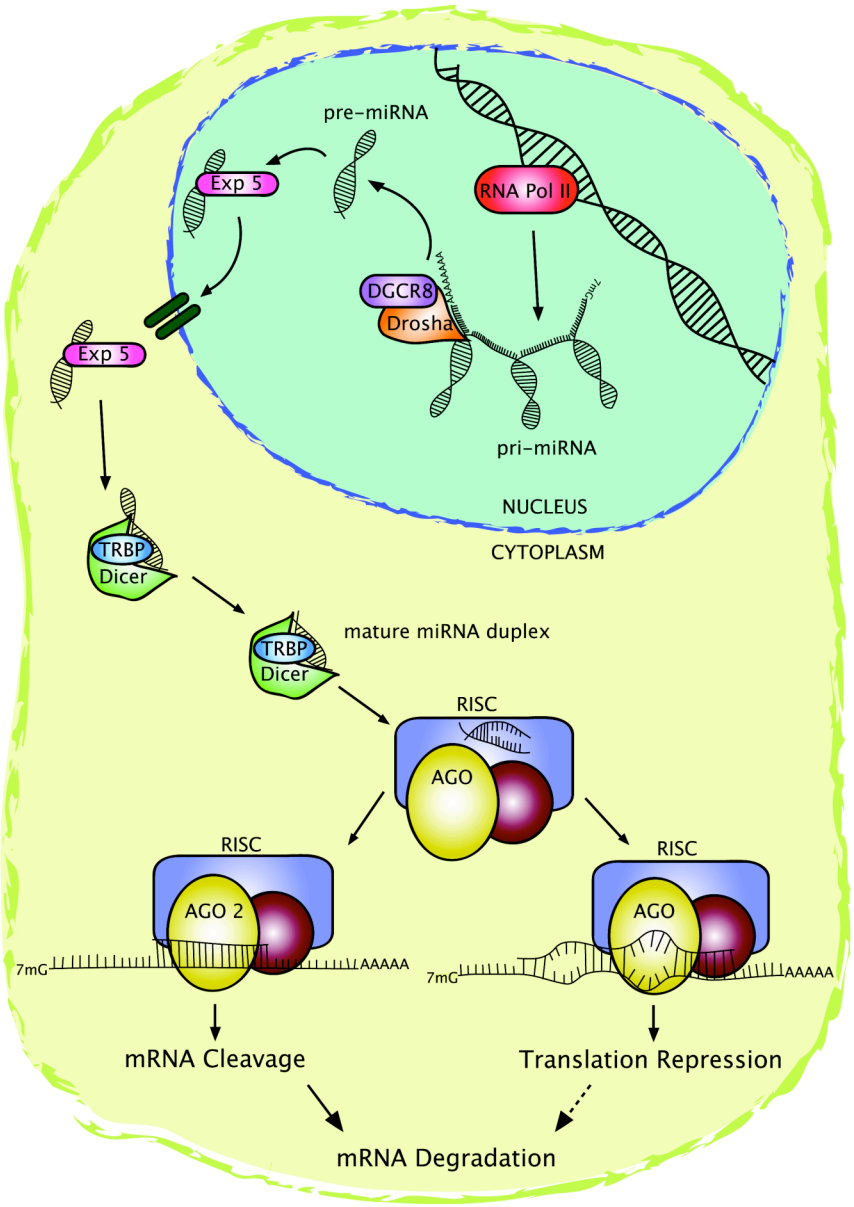

Figure 1. miRNA biogenesis.
miRNAs are typically transcribed by RNA polymerase II, processed by Drosha and Dicer and incorporated into RNA Induced Silencing Complex (RISC) for consequent cellular function. By Olena et al. 2009.

Transcription of pri-miRNA

The majority of miRNAs are transcribed by RNA polymerase II as either polycistronic or monocistronic transcription units (Lee, Jeon et al. 2002; Cai, Hagedorn et al. 2004; Lee, Kim et al. 2004). Primary miRNA transcripts (pri-miRNAs) originate as

long transcripts in which one or more hairpin structures with ~33bp stem regions and terminal loops are embedded (Bartel 2004). These pri-miRNAs are capped and polyadenylated and can be as long as several kilobases (Cai, Hagedorn et al. 2004). Only a few miRNA genes are transcribed by RNA polymerase III, such as those that associate with Alu repeats (Borchert, Lanier et al. 2006; Ozsolak, Poling et al. 2008).

A genome wide study using a strategy combining nucleosome-positioning patterns with ChIP-chip screens for promoter signatures identified transcription initiation regions for 175 miRNAs in human cell lines (Ozsolak, Poling et al. 2008). One-third of intronic miRNA genes have transcription initiation regions independent from their host genes. The promoters of RNA Polymerase II transcribed miRNA genes are generally similar to that of protein coding genes. Consistent with this, many miRNAs are subject to regulation by known transcription factors. For example, the *miR-17-92* cluster is directly regulated by the transcription factors cMyc and E2Fs (O'Donnell, Wentzel et al. 2005; Sylvestre, De Guire et al. 2007; Woods, Thomson et al. 2007), while Myogenin and MyoD1 (myoblast determination1) induce the transcription of *miR-1* and *miR-133* during myogenesis (Chen, Mandel et al. 2006; Rao, Kumar et al. 2006). Epigenetic control, such as DNA methylation, also contributes to the regulation of miRNA expression (Saito, Liang et al. 2006; Brueckner, Stresemann et al. 2007; Bueno, Perez de Castro et al. 2008; Lujambio, Calin et al. 2008).

Editing of pri-miRNA

Many pri-miRNAs (~16% in humans) are subject to A-to-I (adenosine to inosine) RNA editing mediated by ADARs (adenosine deaminases acting on RNA) (Luciano,

Mirsky et al. 2004; Blow, Grocock et al. 2006; Yang, Chendrimada et al. 2006; Kawahara, Megraw et al. 2008). This process alters the base pairing and structural properties of the transcripts, thereby post-trancriptionally regulating the processing and target recognition of a given miRNA (Scadden 2005; Yang, Chendrimada et al. 2006; Kawahara, Zinshteyn et al. 2007; Kawahara, Zinshteyn et al. 2007; Kawahara, Megraw et al. 2008).

Release of pre-miRNA

Hairpin structures embedded within pri-miRNA transcripts are recognized and excised by the microprocessor complex in the nucleus (Lee, Ahn et al. 2003). The RNase III-like enzyme Drosha and double-stranded RNA (dsRNA) binding protein DGCR8 (DiGeorge syndrome critical region gene 8, also known as Pasha (partner of Drosha) in *D. melanogaster* and *C. elegans*) are components of the microprocessor complex (Denli, Tops et al. 2004; Gregory, Yan et al. 2004; Han, Lee et al. 2004; Landthaler, Yalcin et al. 2004) (Fig. 2A). The double-stranded stem and two single-stranded flanking regions upstream and downstream of the hairpin structure are crucial for DGCR8 binding and Drosha cleavage (Zeng and Cullen 2003; Zeng and Cullen 2005; Han, Lee et al. 2006). DGCR8 binds to the base of pri-miRNAs and acts as a molecular ruler to determine the precise cleavage site for Drosha (Fig. 2D). The two RNase domains of Drosha endonucleolytically cleave the 5' and 3' arms of the hairpin 11 bases away from the single-stranded RNA/double-stranded RNA junction (Zeng and Cullen 2005; Han, Lee et al. 2006). The released hairpin structure is typically ~65-70nt with a monophosphate group at the 5' end and a dinucleotide overhang at the 3' end, referred to as a precursor

miRNA (pre-miRNA) (Lee, Jeon et al. 2002; Basyuk, Suavet et al. 2003; Lee, Ahn et al. 2003; Zeng and Cullen 2003). For intronic generated miRNAs, this process occurs concomitant with trancription and splicing (Kim and Kim 2007; Morlando, Ballarino et al. 2008; Pawlicki and Steitz 2008). pri-miRNA processing and RNA splicing do not seem to interfere with each other. For exonic generated miRNAs, processing of the pri-miRNA can destabilize the host transcript (Han, Pedersen et al. 2009).

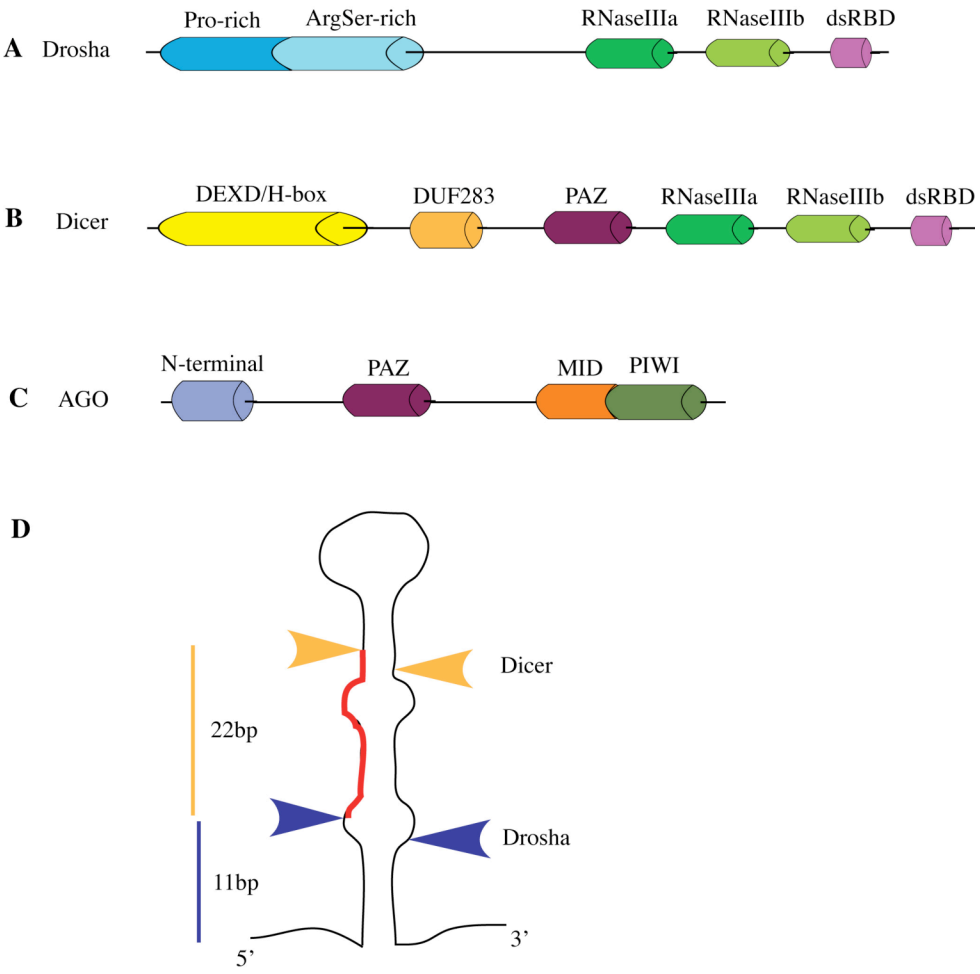

Figure 2. miRNA machinery.
A-C. Schematic representation of the domain structure of human Drosha, Dicer and AGO proteins. D. Schematic representation of the cleavage sites in pre-miRNAs by Drosha and Dicer.

In mammals, flies and worms, a group of miRNAs that bypass Drosha cleavage have been discovered. Hairpin-shaped pre-miRNAs are formed from debranching of the lariat-shaped introns, which are then directly released from host transcripts by the splicing machinery (Berezikov, Chung et al. 2007; Okamura, Hagen et al. 2007; Ruby, Jan et al. 2007). These miRNAs, referred to as mirtrons, have a similar biogenesis once they reach the cytoplasm.

Post-transcriptional processing of pri-miRNAs by the microprocessor complex is subject to regulation in a miRNA-specific manner. During stem cell differentiation, the levels of *pri-let-7* remain constant, while the levels of mature *let-7* increase. One of the mechanisms responsible for post-transcriptional suppression of *let-7* in undifferentiated cells is through direct binding of Lin-28 to the loop region of the *pri-let-7* hairpin, which blocks microprocessor cleavage (Newman, Thomson et al. 2008; Piskounova, Viswanathan et al. 2008; Viswanathan, Daley et al. 2008). The levels of mature *miR-21* are induced in response to Bone Morphogenetic Protein (BMP) signaling without an increase in transcription. The SMAD proteins, downstream effectors of BMP signaling, interact with Drosha and the RNA helicase DDX5 (p68) and enhance Drosha-mediated processing (Davis, Hilyard et al. 2008). Moreover, Drosha cleaves hairpin structures in the 5' UTR and the coding region of *dgcr8* mRNA, leading to down-regulation of DGCR8. DGCR8 in turn stabilizes Drosha through direct protein-protein interaction, resulting a negative feedback loop between the two components of the microprocessor (Yeom, Lee et al. 2006; Han, Pedersen et al. 2009).

Export of pre-miRNA

Pre-miRNAs are first generated in the nucleus and then exported into the cytoplasm by Exportin-5 (EXP5) in a Ran-GTP dependant manner (Yi, Qin et al. 2003). EXP5 belongs to the nuclear transport receptor family and export pre-miRNA as well as some tRNAs (Calado, Treichel et al. 2002). Cargos recognized by EXP5 contain >14bp dsRNA stems and 1-8nt overhangs at the 3' termini (Lund, Guttinger et al. 2004; Zeng and Cullen 2004). This step is usually the rate-limiting step of miRNA biosynthesis, as saturation of EXP5 is lethal (Grimm, Streetz et al. 2006). The nuclear export step can also be regulated in a miRNA-specific manner. The pre-miRNAs of human *miR-31*, *miR-128*, *miR-105* are all retained in the nucleus instead of being processed into mature miRNAs in certain cell types (Lee, Baek et al. 2008).

Creation of mature miRNA

In the cytoplasm, the other end (opposite to Drosha cleavage) of the mature miRNA is defined by endonucleolytic cleavage by another RNase III enzyme, Dicer, yielding a 22-25nt duplex with a 5' phosphate group and 3' 2nt overhang on each strand (Bernstein 2001; Grishok, Pasquinelli et al. 2001; Hutvagner, McLachlan et al. 2001; Ketting, Fischer et al. 2001; Knight 2001). This process seems to be ATP independent in mammals but is ATP dependent in flies (Nykanen, Haley et al. 2001; Provost, Dishart et al. 2002; Zhang, Kolb et al. 2002; Ma, MacRae et al. 2008). Dicer is highly conserved throughout species and contains an amino-terminal DEXH/H-box domain, a DUF283 domain (with unknown function), a PAZ domain, two RNase III domains in tandem arrangement, and a single dsRNA-binding domain (Fig. 2B). Crystal structures reveal

that the PAZ domain holds the 3' overhang of the pre-miRNA while the RNase III domains are responsible for the hydrolysis of both strands of the stem (Lingel, Simon et al. 2003; Song, Liu et al. 2003; Yan 2003). The connector sequence between the PAZ domain and the RNase III acts as a molecular ruler to position the hydrolytic site (MacRae, Zhou et al. 2006; MacRae, Zhou et al. 2007) (Fig. 2D). The amino-terminal DEXH/H-box domain is thought to exhibit an auto-inhibitory role on the catalytic activity of Dicer. Removal of the domain increases the cleavage rate. The dsRNA-binding protein TRBP (Tar RNA binding protein) binds to Dicer in this region and can release the inhibition through conformational changes (Haase, Jaskiewicz et al. 2005; Ma, MacRae et al. 2008).

The second cleavage step in the miRNA biosynthesis is also subject to regulation. As mentioned above, post-transcriptional processing of *let-7* in stem cells is regulated by Lin-28. Besides inhibiting the activity of the microprocessor, Lin-28 also prevents *pre-let-7* from being diced in the cytoplasm by inducing polyuridylation at its 3' end via a terminal uridylyl transferase (TUTase) (Heo, Joo et al. 2008; Rybak, Fuchs et al. 2008; Heo, Joo et al. 2009). The polyuridylated *pre-let-7* is then subject to degradation by an unknown pathway. Interestingly, Dicer is down regulated by *let-7*, creating a negative feedback loop (Forman, Legesse-Miller et al. 2008). Lin28 and *let-7* form a double negative feedback loop during neural stem cell commitment (Rybak, Fuchs et al. 2008).

Loading of guide strands into RISC

Once the mature miRNA duplex is created, one strand of the duplex is loaded into a multi-protein complex (RNA induced silencing complex, RISC) for subsequent target

selection and silencing (Schwarz 2003; Chendrimada, Gregory et al. 2005; Gregory, Chendrimada et al. 2005; Preall and Sontheimer 2005; MacRae, Ma et al. 2008). This strand is called the guide strand (miRNA), while the other strand is called the passenger strand (miRNA*), which usually undergoes degradation. There are cases where both strands can mediate subsequent gene silencing. The determination of guide/passenger strand is believed to depend on the thermodynamic stability of the base pairing at the ends of the duplex. The strand whose 5' end displays less stability will become the guide strand (Khvorova, Reynolds et al. 2003). Two strands of mature miRNA get separated through a yet unknown mechanism. It is still unknown whether a helicase-mediated unwinding step occurs during this process. On one hand, multiple helicases have been found to complex with RISC (Tomari, Matranga et al. 2004; Meister, Landthaler et al. 2005; Chu and Rana 2006; Robb and Rana 2007; Salzman, Shubert-Coleman et al. 2007). On the other hand, RISC loading and reconstitution experiments in vitro in the absence of ATP suggest that helicases are not generally required (Gregory, Chendrimada et al. 2005; Maniataki and Mourelatos 2005; MacRae, Ma et al. 2008).

RISC is the final miRNA effector machinery that directs miRNA:mRNA interaction and targets repression. The key component of the RISC is one or more Argonaute (AGO) proteins (Hutvagner and Zamore 2002; Pillai, Artus et al. 2004; Eulalio, Huntzinger et al. 2008; Filipowicz, Bhattacharyya et al. 2008). AGO proteins contain three characteristic domains: PAZ, MID, and PIWI (Cerutti, Mian et al. 2000) (Fig. 2C). The PAZ domain, which is also present in Dicer, holds the 3' end of the mature miRNA (Lingel, Simon et al. 2003; Song, Liu et al. 2003; Yan 2003), while the MID domain and PIWI domain anchors the 5' end of the mature miRNA (Ma, Yuan et al. 2005; Parker, Roe et al. 2005).

The PIWI domain is similar in structure to RNase H, an endonuclease that cuts the RNA strand of an RNA-DNA hybrid (Ma, Yuan et al. 2005). The PIWI domains of some AGO proteins maintain cleavage activity, so called slicer activity (Parker, Roe et al. 2004; Song, Smith et al. 2004; Nowotny, Gaidamakov et al. 2005). There are 2 AGO proteins in Drosophila, 4 in humans and potentially 4 in zebrafish (Hutvagner and Simard 2008). Both of the fly AGO proteins possess slicer activity, while only AGO2 in human does so (Liu, Carmell et al. 2004; Meister, Landthaler et al. 2004; Forstemann, Horwich et al. 2007). Three key residues are critical for slicer activity of human AGO2 protein: an Asp-Asp-His motif (Rivas, Tolia et al. 2005). Mutations in the motif abolish cleavage activity (Liu, Carmell et al. 2004). AGO proteins that do not possess slicer activity induce translational repression and/or mRNA decay with the help of many other proteins within the RISC.

An interesting question is how many kinds of RISCs are there? In Drosophila, at least two distinct RISCs exist: miRNP and siRNP. In flies, miRNAs are preferentially loaded onto AGO1 (miRNP) with the help of DCR-1 (Dicer-1) and LOQS (Loquacious, a double-stranded RNA binding protein), while siRNAs are preferentially loaded onto AGO2 (siRNP) with the help of DCR-2 and R2D2 (a double-stranded RNA binding protein) (Liu 2003; Tomari, Du et al. 2004; Jiang, Ye et al. 2005; Matranga, Tomari et al. 2005; Saito, Ishizuka et al. 2005; Liu, Jiang et al. 2006; Forstemann, Horwich et al. 2007; Tomari, Du et al. 2007). It is not clear whether similar sorting mechanisms exist in higher organisms (Azuma-Mukai, Oguri et al. 2008). It is still possible that different AGO proteins preferentially interact with different protein partners, comprising distinct RISCs. Also, it is possible that RISC represents a pool of dynamic protein complexes with

different components in specific cell types or physiological conditions. It is also unknown how many RISCs (either different or identical to each other) can associate with a single target mRNA simultaneously.

AGO proteins also seem to play a positive role in maintaining miRNA abundance (Diederichs and Haber 2007; O'Carroll, Mecklenbrauker et al. 2007; Diederichs, Jung et al. 2008). The activity and stability of AGO proteins are post-translationally regulated by phosphorylation or hydroxylation (Qi, Ongusaha et al. 2008; Zeng, Sankala et al. 2008).

Degradation of miRNA

Very few studies have addressed the degradation of miRNA. It is generally believed that once incorporated into RISC, mature miRNAs are quite stable (>14hr), as indicated by the long persistence of most miRNAs after depletion of Drosha or Dicer (Lee, Ahn et al. 2003; Gregory, Yan et al. 2004; Hwang, Wentzel et al. 2007). However some miRNAs seem to have a much shorter lifetime, for example *miR-29b* (Hwang, Wentzel et al. 2007). Moreover, the enrichment of guide strands but not passenger strands in the cytoplasm clearly indicates an unknown mechanism that quickly and selectively turns over these small RNAs. Several exonucleases have been suggested to be responsible for miRNA/siRNA degradation. The 3'→ 5' exonuclease ERI1 degrades siRNAs in C. elegans (Kennedy 2004). A family of exoribonucleases that degrade miRNAs (named SDN, small RNA degrading nuclease) have been identified in *A. thaliana* (Ramachandran and Chen 2008). Consistent with this, loss of bases at either end of the miRNA has been frequently observed (Katoh, Sakaguchi et al. 2009)(Wei and Patton manuscript in preparation).

In plants, miRNAs are sometime methylated, resulting in increased half-life (Li, Yang et al. 2005). Addition or deletion of 1-2nt at either end of mature miRNAs is often observed, and 3' ends tend to be more variable than 5' ends (Azuma-Mukai, Oguri et al. 2008; Seitz, Ghildiyal et al. 2008). Heterogeneity in the 5' end may be due to imprecise or alternative processing by Drosha and Dicer (Wu, Ye et al. 2009), whereas 3' termini often contain untemplated U or A residues, as a result of terminal uridyl/adenyl transferases activity (Katoh, Sakaguchi et al. 2009) (Wei and Patton manuscript in preparation). These modifications may play a role in miRNA stability (Chen, Sinha et al. 2000; Li, Yang et al. 2005; Heo, Joo et al. 2008; Katoh, Sakaguchi et al. 2009).

Mechanism of miRNA function

It is generally believed that miRNAs down-regulate translation by binding to the 3'UTR of their targets, although there are a few studies suggesting that miRNAs can target other regions including coding regions, 5' UTRs, and promoter regions (Rhoades, Reinhart et al. 2002; Jopling, Yi et al. 2005; Duursma, Kedde et al. 2008; Forman, Legesse-Miller et al. 2008; Henke, Goergen et al. 2008; Jopling, Schutz et al. 2008; Lal, Kim et al. 2008; Orom, Nielsen et al. 2008; Tay, Zhang et al. 2008; Voinnet 2009). Mostly, binding inhibits translation but there are also a limited number of reports that suggest translational activation or even transcriptional activation (Bao, Lye et al. 2004; Vasudevan and Steitz 2007; Vasudevan, Tong et al. 2007; Henke, Goergen et al. 2008; Jopling, Schutz et al. 2008; Kim, Saetrom et al. 2008; Orom, Nielsen et al. 2008; Place, Li et al. 2008). For the majority of miRNAs, near perfect complementarity between the miRNA and its targets directs endonucleolytic cleavage, whereas partial complementarity

inhibits translation which may or may not be accompanied by mRNA destabilization (Hutvagner and Zamore 2002; Zeng, Wagner et al. 2002; Aukerman and Sakai 2003; Zeng, Yi et al. 2003; Chen 2004). mRNA cleavage is the most common consequence of miRNA-induced silencing in plants (Llave, Xie et al. 2002; Kasschau, Xie et al. 2003; Tang, Reinhart et al. 2003; Xie, Kasschau et al. 2003), whereas translation inhibition is the dominant outcome in animals (Yekta, Shih et al. 2004; Hornstein, Mansfield et al. 2005; Filipowicz, Bhattacharyya et al. 2008).

The mechanism underlying miRNA-mediated post-transcriptional silencing in animals has been intensively studied and hotly debated. Several models have been proposed: 1) translational repression at the initiation step, 2) translational repression post-initiation, and 3) mRNA destabilization (Filipowicz, Bhattacharyya et al. 2008; Chekulaeva and Filipowicz 2009).

Translational repression at the initiation step

This model is supported by the fact that miRNA-mediated silencing is m^7G-cap dependent. The translation of mRNAs containing an IRES (internal ribosome entry site) or a non-functional ApppN-cap was not subject to repression by miRNAs (Humphreys, Westman et al. 2005; Pillai, Bhattacharyya et al. 2005; Mathonnet, Fabian et al. 2007; Thermann and Hentze 2007; Wakiyama, Takimoto et al. 2007). Tethering AGO2 to the 3'UTR of luciferase reporters repressed their translation in the presence of a m^7G-cap but not an IRES (Kiriakidou, Tan et al. 2007). Polysome gradient analysis in mammalian cells also provides strong evidence for this model. Reporter mRNAs that contain multiple artificial *let-7* binding sites were found to shift toward the top of the gradient in the

presence of endogenous *let-7*, indicating reduced ribosome loading (Pillai, Bhattacharyya et al. 2005). Similarly, in human hepatoma cells, starvation-induced release of *cat1* mRNA from *miR-122*-mediated repression was accompanied by more efficient association of *cat1* mRNA with polysomes (Bhattacharyya, Habermacher et al. 2006).

How do miRNAs inhibit translation initiation? One model suggests that miRNPs repress translation initiation by competing with eIF4E for cap-recognition (Kiriakidou, Tan et al. 2007; Mathonnet, Fabian et al. 2007). A motif within the MID domain of AGO proteins exhibits high sequence similarity to the cap-binding domain of eIF4E, an essential translation initiation factor. Human AGO2 binds to m^7GTP Sepharose in vitro and the interaction can be competed by m^7GpppG but not by unmethylated GpppG. Substitution of two crucial phenylalanine residues by valines abrogated the m^7GTP binding capacity and silencing activity without affecting its interaction with miRNA or catalytic activity.

A different model proposes that miRNPs repress translation initiation by preventing the assembly of 80S ribosomes via recruitment of eIF6 (Chendrimada, Finn et al. 2007). Human RISC can be co-immunoprecipitated with proteins of the 60S ribosome subunit and eIF6, a ribosome inhibitory protein known to prevent joining of the 60S subunit with the 40S subunit. Depletion of eIF6 abrogated silencing of reporter mRNAs by *let-7* in human cell lines and the repression of LIN-14 and LIN-28 by *lin-4* in *C. elegans*.

The third model suggests that miRNPs repress translation initiation by preventing mRNA circularization (Wakiyama, Takimoto et al. 2007). This is based on the observation that both the m^7G-cap and the polyA tail of mRNAs are necessary but not sufficient for silencing (Humphreys, Westman et al. 2005; Wang, Love et al. 2006;

Wakiyama, Takimoto et al. 2007). It is therefore possible that miRNPs prevent the formation of closed mRNA loops by some unknown mechanism, such as promoting deadenylation (see below).

Post-initiation repression

Inhibition of translation initiation is unlikely to be the only mechanism by which miRNAs regulate their targets. The observation that both repressed mRNAs and miRNAs sediment with polyribosomes strongly argues that repression occurs post-initiation (Olsen and Ambros 1999; Seggerson, Tang et al. 2002; Kim, Krichevsky et al. 2004; Nelson, Hatzigeorgiou et al. 2004; Maroney, Yu et al. 2006; Nottrott, Simard et al. 2006; Petersen, Bordeleau et al. 2006; Vasudevan and Steitz 2007). The seemingly conflicting conclusions from the ribosome sedimentation experiments may reflect differences in experimental design or different interpretation of results. Also, since the repression of mRNA targets by miRNAs is partial, co-sedimentation of miRNPs or target mRNAs with polysomes does not preclude the possibility of repression at the initiation step (Filipowicz, Bhattacharyya et al. 2008). Post-initiation repression is also supported by the finding that IRES-dependent translation of reporter mRNAs was sensitive to miRNA-mediated silencing (Petersen, Bordeleau et al. 2006; Lytle, Yario et al. 2007). Translation repression was observed by tethering human AGO2 to the 3'UTR of IRES-driven reporters (opposite to the observation from Kiriakidou et al. 2007) (Lytle, Yario et al. 2007). Notably, the experimental conclusions regarding whether IRES-dependent translation is subject to miRNA regulation are again controversial. Lytle et al (2007)

proposed that the conflicting results from different labs may be due to different transfection methods (Lytle, Yario et al. 2007).

What step do miRNPs target after initiation? Petersen et al (2006) hypothesize that miRNAs repress translation by causing ribosome drop-off during translation elongation. They found that when translation initiation was blocked with hippuristanol (an eIF4A inhibitor), reporter mRNAs shifted away from polyribosome regions more rapidly in the presence of miRNA mimics, indicating ribosomes on repressed mRNAs disassociated more rapidly than those on active mRNAs (Petersen, Bordeleau et al. 2006).

Nottrott et al (2006) suggested another hypothesis, proposing that miRNAs repress translation by promoting nascent polypeptide degradation. In their study, a Myc antibody was used to immunoprecipitate nascent polypeptides of Myc-tagged luciferase reporters from Hela cell extracts. Reporter mRNAs were not detected in the presence of target 3'UTRs. Unfortunately, this hypothesis is based on negative results and neither the degradation pathway nor the specific proteases responsible have been identified (Nottrott, Simard et al. 2006).

mRNA destabilization

At the beginning, miRNA-mediated gene silencing was characterized by a reduction in protein production without any decrease in RNA levels (Humphreys, Westman et al. 2005; Behm-Ansmant, Rehwinkel et al. 2006; Eulalio, Rehwinkel et al. 2007; Mathonnet, Fabian et al. 2007; Thermann and Hentze 2007). However, it is now known that miRNAs can also lead to a reduction in mRNA levels (Bagga, Bracht et al. 2005; Jing, Huang et al. 2005; Krutzfeldt, Rajewsky et al. 2005; Lim, Lau et al. 2005; Wu and Belasco 2005;

Behm-Ansmant, Rehwinkel et al. 2006; Esau, Davis et al. 2006; Schmitter, Filkowski et al. 2006). The decrease in transcript abundance is, importantly, not due to the slicer activity of AGO proteins, but due to accelerated mRNA decay (Behm-Ansmant, Rehwinkel et al. 2006; Giraldez, Mishima et al. 2006; Wu, Fan et al. 2006). Eukaryotic mRNA decay most often starts with shortening or removal of polyA tails (deadenylation), followed by removal of m^7G-caps (decapping) and then degradation from both ends by exonucleases (Coller and Parker 2004). mRNA decay, at least the later steps, are believed to occur in compartmentalized cytoplasmic foci called P-bodies (processing body) or GW-bodies (Eystathioy, Chan et al. 2002). Consistent with this, decapping enzymes DCP1, DCP2 and the 5'->3' exonuclease XRN1 have been localized to P-bodies (Sheth and Parker 2003). In P-bodies, mRNAs are sequestered from the active translation machinery and can be effectively targeted by these enzymes. However, under certain circumstances, mRNAs in P-bodies can re-enter the translation cycle instead of undergoing subsequent turnover (Brengues, Teixeira et al. 2005; Bhattacharyya, Habermacher et al. 2006).

Consistent with the idea that miRNAs induce mRNA decay, AGO proteins, miRNAs and their targets have been found to be enriched in P-bodies (Liu, Valencia-Sanchez et al. 2005; Pillai, Bhattacharyya et al. 2005; Bhattacharyya, Habermacher et al. 2006). In addition, depletion of P-body components DCP1, DCP2, GW182, and RCK/P54 (decapping activator, Dhh1 in yeast) impairs miRNA function (Jakymiw, Lian et al. 2005; Liu, Valencia-Sanchez et al. 2005; Meister, Landthaler et al. 2005; Rehwinkel, Behm-Ansmant et al. 2005; Barbee, Estes et al. 2006; Behm-Ansmant, Rehwinkel et al. 2006; Chu and Rana 2006; Eulalio, Rehwinkel et al. 2007; Eulalio, Helms et al. 2009;

Eulalio, Tritschler et al. 2009). Current evidence in both Drosophila and human cells agrees with the model that GW182 is recruited to mRNAs by AGO proteins though direct interaction between the N-terminal domain of GW182 and the PIWI domain of AGO proteins (Jakymiw, Lian et al. 2005; Behm-Ansmant, Rehwinkel et al. 2006; Till, Lejeune et al. 2007; Eulalio, Huntzinger et al. 2008). Target mRNAs are then subject to degradation by deadenylase, decapping enzymes, and exonucleases in the P-body (Liu, Rivas et al. 2005). Interestingly, accelerated deadenylation impairs mRNA circularization, which can affect translation efficiency. Thus, this model, together with the translation initiation model, supports the idea that miRNPs target two ends of mRNAs: the cap and polyA tail.

One hotly debated question is whether miRNA-dependent mRNA decay is a cause or consequence of translational repression. Some evidence suggests that mRNA decay does not require translational repression. miRNA-dependent mRNA deadenylation is intact when active translation is blocked by cycloheximide or by a start site morpholino (Giraldez, Mishima et al. 2006; Eulalio, Rehwinkel et al. 2007; Wakiyama, Takimoto et al. 2007). Also, reporters with either strong stem-loop structures in their 5'UTRs or with Appp-caps exhibit impaired translation but undergo miRNA-dependent deadenylation (Mishima, Giraldez et al. 2006; Wu, Fan et al. 2006; Wakiyama, Takimoto et al. 2007; Eulalio, Helms et al. 2009). There is also evidence that translational repression does not require mRNA decay. Reporters without polyA tails can be translationally repressed by miRNAs (Pillai, Bhattacharyya et al. 2005; Wu, Fan et al. 2006; Eulalio, Huntzinger et al. 2008; Eulalio, Helms et al. 2009). Also genome-wide profiling studies suggest that many miRNA targets are silenced at the protein level yet their transcript levels remain mostly

unchanged (Baek, Villen et al. 2008; Selbach, Schwanhausser et al. 2008). Therefore, it seems that miRNA-dependent mRNA decay and translational repression may be two independent effects of miRNPs.

Regulation of miRNA-mediated post-transcriptional silencing

Under certain conditions, miRNA-mediated silencing can be reversed or blocked (Tomari, Du et al. 2004; Ashraf, McLoon et al. 2006; Schratt, Tuebing et al. 2006; Huang, Liang et al. 2007). Bhattacharyya et al found that *miR-122*-mediated repression of CAT1 (cationic amino acid transporter1) can be alleviated in human cell lines as a result of starvation or other types of cell stress. Derepression is accompanied by the release of *cat1* mRNA from P-bodies and recruitment to polyribosomes. This process is mediated by the HuR protein, which translocates from the nucleus to the cytoplasm upon stress and binds to the AU-rich-element in the 3'UTR of *cat1* (Bhattacharyya, Habermacher et al. 2006). In zebrafish, the expression of *nanos* is post-transcriptionally restricted to PGCs (primordial germ cells) through multiple mechanisms including miRNA-induced silencing in somatic cells. Kedde et al (2007) provided evidence that Dnd1 (Dead end 1), an evolutionarily conserved RNA-binding protein essential for germ cell survival and migration both in zebrafish and in mouse (Weidinger, Stebler et al. 2003; Youngren, Coveney et al. 2005), protects *nanos* from miRNA repression by binding to uridine-rich regions in the 3'UTR (Mishima, Giraldez et al. 2006; Kedde, Strasser et al. 2007). In contrast to factors alleviating miRNA function, TRIM32 and NHL-2 have been suggested to physically interact with RISC components and enhance miRNA silencing in mouse and worm respectively (Hammell, Lubin et al. 2009; Schwamborn, Berezikov et al.

2009). In Drosophila, the ARE (AU-rich element) binding protein TTP (tristetraprolin) associates with the AGO protein and assists in the ARE-mediated mRNA degradation by *miR-16* (Jing, Huang et al. 2005). Taken together, the crosstalk of miRNPs with RNA binding proteins on the 3'UTR as well as other factors in the cytoplasm is important for modulating miRNA function in a cell-specific manner.

Target identification of miRNAs

In order to fully understand the biological and physiological function of miRNAs, the downstream targets of miRNAs need to be identified. It has been estimated that mammalian miRNAs can regulate up to 30% of protein coding genes (John, Enright et al. 2004; Lewis, Burge et al. 2005; Xie, Lu et al. 2005). What are the targets for each miRNA? How is interaction specificity achieved? Efforts towards answering these questions are ongoing. To date, there are two strategies for target identification: 1) bioinformatic approaches followed by experimental validation and 2) direct experimental approaches.

Computational target prediction

miRNA targets can be easily identified in plants where complementarity is perfect but this process is difficult in animals because of imperfect complementarity (Rhoades, Reinhart et al. 2002; Chen, Li et al. 2004; Mallory, Reinhart et al. 2004; Guo, Xie et al. 2005). Many prediction algorithms have been created to try to identify miRNA targets. Popular algorithms include TargetScan (Lewis, Shih et al. 2003; Grimson, Farh et al. 2007; Ruby, Stark et al. 2007; Friedman, Farh et al. 2009), Pictar (Krek, Grun et al. 2005;

Lall, Grun et al. 2006) and Miranda (John, Enright et al. 2004). Although these algorithms are helpful for target prediction, serious flaws exist. Typically, hundreds to thousands of targets are predicted, not all of which are true. Bona fide targets are not always identified by these algorithms. Worse, predicted targets from different algorithms often do not overlap. These problems are due to a lack of knowledge regarding rules for functional miRNA:mRNA interaction. For current algorithms, combinations of the following criteria are used to predict targets. 1) The "seed rule", perfect Watson-Crick base paring between the "seed" region (2-7nt from the 5' end) of the miRNA and its target. 2) Thermodynamic stability of miRNA:mRNA duplex. 3) Evolutionary conservation of the predicted miRNA recognition element (MRE). 4) Number of MREs within the 3'UTR.

The seed rule is currently the fundamental criterium for most algorithms. It was first proposed by Lewis et al (2003) in their attempt to systematically predict mammalian miRNA targets using bioinformatic approaches (Lewis, Shih et al. 2003). The seed region is the only continuous region of the miRNA that captures more evolutionarily conserved target sites than expected by chance. Also, perfect seed pairing seems to remarkably reduce the number of false positive predictions. Further, mutagenesis studies support the notion that base pairing at the 5' end of miRNA is more important for target recognition, as compared to 3' end (Doench and Sharp 2004; Mallory, Reinhart et al. 2004; Brennecke, Stark et al. 2005). Lastly, large-scale transcriptomics and proteomics studies following miRNA overexpression or depletion also detected enrichment of seed pairings in target mRNAs (Lim, Lau et al. 2005; Baek, Villen et al. 2008; Selbach, Schwanhausser et al. 2008).

Nevertheless, considerable evidence exists to also argue that the seed pairing is either not required or not sufficient for miRNA:mRNA interaction (Doench and Sharp 2004; Brennecke, Stark et al. 2005; Didiano and Hobert 2006; Hon and Zhang 2007; Kertesz, Iovino et al. 2007; Nielsen, Shomron et al. 2007). Other features within 3'UTRs in addition to the seed pairing have been demonstrated as important determinants including accessibility of the MRE, position of the MRE related to the stop codon, and local AU rich elements (Didiano and Hobert 2006; Hon and Zhang 2007; Kertesz, Iovino et al. 2007). Even evolutionary conservation in target prediction is not always foolproof, as many non-conserved sites have been identified (Lee, Feinbaum et al. 1993; Reinhart, Slack et al. 2000). Thus, specific rules that would enable rapid identification all known functional targets have not been devised, making it a big challenge for target prediction in animals.

Experimental target identification

While reporter constructs can be used to validate individual targets, several approaches have been taken to try to experimentally identify miRNA targets on a genome-wide scale. Lim et al (2005) combined mRNA arrays with miRNA gain-of-function experiments and identified hundreds of genes whose expression was altered by miR-1 or miR-124 overexpression in cell culture (Lim, Lau et al. 2005). Similarly, Mishima et al (2009) identified a large number of potential targets for miR-1 and miR-133 during zebrafish muscle development by comparing the muscle cell transcriptome before and after miRNA depletion (Mishima, Abreu-Goodger et al. 2009). One disadvantage of this approach is that the levels of mRNA transcripts are determined

rather than protein levels. Although miRNAs can induce mRNA destabilization, transcript levels of many bona fide targets may not be significantly altered. Baek et al (2008) and Selbach et al (2008) overcame this problem by directly measuring protein expression levels using quantitative mass spectrometry before and after alteration of miRNAs, leading to identification of more accurate mRNA target pools (Baek, Villen et al. 2008; Selbach, Schwanhausser et al. 2008).

In addition to mRNA profiling, biochemical purification of RISC has recently been developed as a miRNA target identification approach (Landthaler, Gaidatzis et al. 2008; Chi, Zang et al. 2009). Since both miRNAs and their targets associate with AGO proteins simultaneously, miRNA:mRNA duplexes were retrieved by isolating native AGO protein via crosslinking and immunoprecipitation (CLIP). The sequences of both the miRNAs and their targets were then determined via high throughput sequencing or microarray analysis.

Biological function of miRNAs

miRNA function during zebrafish development

Depletion of both maternal and zygotic Dicer results in arrest of zebrafish embryogenesis at 5 days post-fertilization. Although dorsal-ventral, anterior-posterior axes and major patterning of the embryos are not affected, severe morphogenesis defects in gastrulation, somitogenesis, heart, and brain development were observed. These observations suggest essential regulatory function of miRNAs during early zebrafish morphogenesis (Giraldez, Cinalli et al. 2005).

One of the better characterized miRNAs that function during development is the *miR-430* family which is expressed at the onset of zygotic transcription and facilitates destabilization and clearance of maternal transcripts (Giraldez, Mishima et al. 2006). Also, *miR-430* can regulate signaling pathways during early zebrafish development. Choi et al (2007) identified both the Nodal-related ligand Squint (sqt) and its antagonists Lefty1 and 2 as targets of *miR-430*. By suppressing both Nodal agonists and antagonists simultaneously, *miR-430* reduces the absolute expression levels of both and balances their relative levels in order to precisely regulate Nodal signaling (Choi, Giraldez et al. 2007).

miRNAs have been frequently identified as regulators of tissue or organ differentiation during later stages of development. Two muscle-specific miRNAs *miR-1* and *miR-133* were found to control actin organization during zebrafish sarcomere assembly (Mishima, Abreu-Goodger et al. 2009). *miR-214* specifies muscle cell types by modulating Hedgehog signaling (Flynt, Li et al. 2007), *miR-9* regulates formation and function of midbrain-hindbrain boundary by targeting Fgf signaling (Leucht, Stigloher et al. 2008), *miR-145* directs gut smooth muscle differentiation by targeting Gata6 (Zeng, Carter et al. 2009), and *miR-375* is thought to regulate the morphology of the pancreatic islet (Kloosterman, Lagendijk et al. 2007).

Finally, due to the ability to induce rapid changes at the protein level, miRNAs are good candidates for regulators of stress responses (Bhattacharyya, Habermacher et al. 2006). *miR-200a* and *b* are expressed in ionocytes and involved in osmoregulation by modulating Nherf1, which is required for apical trafficking of ion transporters (Flynt, Thatcher et al. 2009).

miRNAs as tumor suppressors

miR-15a and *miR-16-1* were identified as tumor suppressors during attempts to clone defective genes at region 13q14.3, a chromosomal region frequently depleted in B cell chronic lymphocytic leukemia (CLL) (Calin, Dumitru et al. 2002). Loss of *miR-15a* and *miR-16* was observed in ~70% of CLLs, consistent with up regulation of BCL-2, a well-known antiapoptotic gene which is a target of *miR-15a/16* (Cimmino, Calin et al. 2005). Ectopic expression of *miR-16* suppresses cell growth and induces apoptosis in human cancer cell lines (Cimmino, Calin et al. 2005; Linsley, Schelter et al. 2007). *let-7* family is another miRNA tumor suppressor. Many members of the *let-7* family are located in chromosomal regions that are lost in various types of tumors (Calin, Liu et al. 2004; Iorio, Ferracin et al. 2005; Johnson, Grosshans et al. 2005; Yanaihara, Caplen et al. 2006). Many oncogenes are targets of the *let-7* family, such as Ras, HMGA2 (high mobility group AT-hook 2) and c-Myc. Ectopic expression of *let-7* in human cancer cell lines inhibits cell cycle progression and induces apoptosis (Akao, Nakagawa et al. 2006; Lee and Dutta 2007; Sampson, Rong et al. 2007).

miRNAs as oncogenes

The *miR-17-92* cluster is located within a chromosomal region that is frequently amplified in various lymphomas and solid tumors (Ota, Tagawa et al. 2004; Volinia, Calin et al. 2006; Venturini, Battmer et al. 2007). This cluster of miRNAs is directly activated by c-Myc and E2Fs and inhibits apoptosis and cell cycle arrest by silencing tumor suppressors including Pten, p21 and Bim (O'Donnell, Wentzel et al. 2005;

Petrocca, Visone et al. 2008; Ventura, Young et al. 2008; Xiao, Srinivasan et al. 2008). The non-coding RNA BIC (B cell integration cluster) had been known as an oncogene long before it was identified as the primary transcript of *miR-155* (*pri-miR-155*) (Tam, Hughes et al. 2002; Metzler, Wilda et al. 2004; Kluiver, Poppema et al. 2005). *miR-155* is highly expressed in many types of leukemias and solid tumors (Metzler, Wilda et al. 2004; Calin, Ferracin et al. 2005; Kluiver, Poppema et al. 2005; Volinia, Calin et al. 2006; Garzon, Volinia et al. 2008). In mouse, B-cell-specific overexpression of *miR-155* causes B cell malignancy (Costinean, Zanesi et al. 2006). Interestingly, studies in knockout mice revealed that the proto-oncogene *miR-155* is required for normal function of mammalian immune system (Rodriguez, Vigorito et al. 2007; Thai, Calado et al. 2007).

miRNAs and tumor invasion and metastasis

Most cancer related miRNAs regulate the cell cycle, proliferation, and apoptosis. Very few miRNAs have been shown to function in tumor invasion and metastasis. *miR-10* was shown to promote migration and invasion of breast cancer cells. *miR-10* is up-regulated in metastatic breast cancer cells by the transcription factor Twist and down-regulates HOXD10 (homeobox D10), a suppressor of the pro-metastatic gene RHOC. Enforced expression of *miR-10* in non-metastatic breast cancer cells induces cell invasion and metastasis. In contrast, inhibition of *miR-10* abolishes Twist-induced cell migration and metastasis (Ma, Teruya-Feldstein et al. 2007). *miR-21* was found to function in both tumor growth and metastasis by targeting TPM1 (tropomyosin 1), PDCD4 (programmed cell death 4) and maspin (Zhu, Wu et al. 2008). Two other miRNAs *miR-373* and *miR-*

520 were identified as regulators of tumor invasion during a miRNA screen for stimulators of breast cancer cell migration (Huang, Gumireddy et al. 2008).

miRNAs as tumor diagnostic and prognostic tools

miRNA profiling has been found to be highly informative for distinguishing the tissue origin of tumor samples. Systematic miRNA expression analyses suggested that signature profiles from only a modest number of miRNAs could accurately cluster cancer samples based on their tissue origin (Lu, Getz et al. 2005; Volinia, Calin et al. 2006). This may allow improvement in cancer diagnosis by resolving one of the most demanding issues – determining the origin of metastatic cancers of unknown primary origin (Rosenfeld, Aharonov et al. 2008). miRNA profiling can also be used to predict the outcome or survival ratio of diverse cancers (Takamizawa, Konishi et al. 2004; Calin, Ferracin et al. 2005; Murakami, Yasuda et al. 2006; Roldo, Missiaglia et al. 2006; Bloomston, Frankel et al. 2007; Garzon, Volinia et al. 2008; Marcucci, Radmacher et al. 2008; Schetter, Leung et al. 2008).

CHAPTER II

DISPATCHED HOMOLOG 2 IS TARGETED BY *miR-214* THROUGH A COMBINATION OF THREE WEAK MICRORNA RECOGNITION SITES

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Abstract

microRNAs (miRNAs) regulate gene expression by inhibiting translation of target mRNAs through pairing with microRNA recognition elements (MREs), usually in 3' UTRs. Because pairing is imperfect, identification of *bona fide* mRNA targets presents a challenge. Most target recognition algorithms strongly emphasize pairing between nucleotides 2-8 of the miRNA (the "seed" sequence) and the mRNA but adjacent sequences and the local context of the 3' UTR also affect targeting. Here, we show that *dispatched 2* is a target of *miR-214*. In zebrafish, *dispatched 2* is expressed in the telencephalon and ventral hindbrain and is essential for normal zebrafish development. Regulation of *dispatched 2* by *miR-214* is via pairing with three, non-canonical, weak MREs. By comparing the repression capacity of GFP reporters containing different *dispatched 2* sequences, we found that a combination of weak sites, which lack canonical seed pairing, can effectively target an mRNA for silencing. This finding underscores the challenge that prediction algorithms face and emphasizes the need to experimentally validate predicted MREs.

Introduction

microRNAs (miRNAs) are highly conserved non-coding RNAs that post-transcriptionally regulate gene expression, usually by inhibiting translation (Ambros 2003; Bartel 2004; He and Hannon 2004). Mature miRNAs are generated from long endogenous primary transcripts by the RNAse III enzymes, Drosha and Dicer resulting in ~22nt double stranded RNAs (Hutvagner, McLachlan et al. 2001; Lee, Jeon et al. 2002; Lee, Ahn et al. 2003; Cai, Hagedorn et al. 2004). One strand of the duplex gets assembled into the RNA-induced silencing complex (RISC) coincident with target identification and pairing (Khvorova, Reynolds et al. 2003; Schwarz 2003). RISC identifies target mRNAs based on complementarity between the miRNA and mostly 3'UTR mRNA sequences resulting in translational repression or, in cases where the pairing is perfect, degradation of the mRNA (Valencia-Sanchez, Liu et al. 2006). It has been suggested that 30%-50% of human genes are regulated by miRNAs since a single miRNA can target multiple mRNAs and a given mRNA may be regulated by multiple miRNAs (Lewis, Burge et al. 2005; Lim, Lau et al. 2005; Xie, Lu et al. 2005).

miRNAs play essential roles in development, physiology, and disease processes (Alvarez-Garcia and Miska 2005; Wienholds and Plasterk 2005). Consistent with this, most miRNAs are expressed in a development-, tissue-, or cell type-specific manner (Wienholds, Kloosterman et al. 2005; Kloosterman, Wienholds et al. 2006). Direct cloning and genomic analyses suggest the presence of hundreds of miRNAs in higher eukaryotic genomes but only a small number have been fully characterized (Lee and Ambros 2001; Lagos-Quintana, Rauhut et al. 2002; Lagos-Quintana, Rauhut et al. 2003).

Besides identifying the full complement of miRNAs, a major problem in functional studies is the identification of the complete range of target mRNAs. Bioinformatic approaches to identify miRNA targets have been very effective in plants where complementarity between miRNAs and their target mRNAs is usually perfect (Rhoades, Reinhart et al. 2002). In contrast, pairing in higher eukaryotes is typically imperfect with numerous gaps, mismatches, and G:U base pairs (Lewis, Shih et al. 2003). Computational and experimental evidence led to the "seed rule" where base pairing between nucleotides 2-8 of the miRNA (the seed sequence) and its target mRNA is crucial (Lewis, Shih et al. 2003; Kloosterman, Wienholds et al. 2004; Brennecke, Stark et al. 2005; Wang, Love et al. 2006). While the "seed rule" has been useful, there are many instances where gene silencing is observed despite multiple gaps and mismatches in the seed region (Doench and Sharp 2004; Brennecke, Stark et al. 2005; Flynt, Li et al. 2007). Additional work has shown that other features in the 3'UTR beyond seed pairing can affect silencing (Brennecke, Stark et al. 2005; Didiano and Hobert 2006; Grimson, Farh et al. 2007; Hon and Zhang 2007; Nielsen, Shomron et al. 2007). Hence, a better understanding of the exact requirements for miRNA recognition is needed to facilitate predictive algorithms, functional characterization studies, and to better design siRNAs in order to reduce potential off-target effects.

Previously, we showed that *miR-214* functions to modulate the Hedgehog (Hh) pathway during zebrafish somitogenesis (Flynt, Li et al. 2007). Regulation of Hh signaling by *miR-214* is primarily through targeting of *Suppressor of Fused* (*sufu*). Here, we show that *miR-214* also targets *dispatched homolog 2 (disp2)*. Interestingly, for both *sufu* and *disp2*, we identified three possible miRNA recognition elements (MREs) but

none of these sites contain a perfect seed match for *miR-214*. Our results suggest that weak sites which by themselves are capable of only minimal silencing, can combine to effectively reduce gene expression to levels comparable to that observed in the presence of perfectly complementary sites.

Materials and methods

Microinjection

Fertilized one-cell zebrafish embryos were injected with 1nl volumes at the following concentrations: 2μg/μl of *miR-214*, 4μg/μl of *disp2*^{MO} (5'-TGGACCCGCTTTCCATGCTGGAGTA-3'), 100ng/μl of *in vitro* transcribed, capped *disp2* mRNA, 50ng/μl of *in vitro* transcribed, capped GFP reporter mRNAs.

Target protectors

Target protectors were named and designed as described (Choi, Giraldez et al. 2007). Disp2TPmir214.1 (5'-CTTGGTTGTGTAAAAGAACAGGCAC-3'), disp2TPmir214.2 (5'-ATGTATTCATGTGTAGAACAGTTAT-3'), disp2TPmir214.3 (5'-AGGTATTATTTACCACAACATGCGA-3') were injected into zebrafish embryos separately or in combination with 1nl at 1μg/μl concentrations.

Molecular cloning

The *disp2* (NM_212434 .1) 3'UTR was amplified by RT-PCR using a forward primer (5'-AGAATTCAATGGAAAGCGGGTCCATTTCC-3') and a reverse primer (5'-

GGTCTAGACCACAACATGCGATAGAATGTAT-3'). The resulting DNA was cloned downstream of the GFP ORF in the pCS2+ vector (Rupp, Snider et al. 1994). Deletion mutants were created by reverse PCR (Coolidge and Patton 1995) using the following primers. All clones were verified by DNA sequencing.

Reverse primer for Δ3: 5'-GGTCTAGAGGGTTCAAATGTCATATTGCAGT-3'

D1 forward primer: 5'-TTACACAACCAAGCCATGAGT-3'

D1 reverse primer: 5-TTGTACATTTGCAGTTCAAGG-3'

D2 forward primer: 5'-ATGAATACATTCTATCGCATG-3'

D2 reverse primer: 5'-ACGTTTAGAGTAAAATAACTG-3'

D3 forward primer: 5'-TACCTTTTCAAACTTGATTTG-3'

D3 reverse primer: 5'- TCATGTGTAGAACAGTTATAG-3'.

Immunoblotting

Proteins were extracted from deyolked 1 day post fertilization (dpf) embryos in lysis buffer (25mM HEPES, pH 7.5, 5mM $MgCl_2$, 300mM NaCl, 1mM EDTA, 0.2mM EGTA, 1Mm DTT, 10% glycerol, 1.0% Triton X-100 and 1mM PMSF). 20µg of total protein were then separated on 10% SDS-polyacrylamide gels and transferred to PVDF-plus membranes. Rabbit polyclonal antibodies against GFP (Torrey Pines Biolabs) and α-tubulin (Abcam) were used at concentrations of 1:1000 and 1:500, respectively. HRP conjugated secondary antibodies against rabbit (GE Healthcare) were then used for visualization with ECL. For quantification, GFP levels were normalized to a-tubulin control levels after which the ratio of GFP in the presence of *miR-214* was determined compared to that in the absence of *miR-214*.

Immunohistochemistry

Immunostaining was as described (Flynt, Li et al. 2007). Rabbit polyclonal antibodies against Prox1 (Abcam) and 4D9 mouse monoclonal antibodies against Engrailed were used at concentrations of 1:1000 and 1:100, respectively. Secondary antibodies against rabbit or mouse IgG were Cy3 or Cy2 conjugated (Jackson ImmunoResearch) and were used at 1:1000 and 1:500, respectively. Embryos were mounted in 50% glycerol and imaged as described (Flynt, Li et al. 2007).

Results

Disp2 is a target of *miR-214*

Previous studies have shown that the expression of *miR-214* in zebrafish starts from the 6-somite stage, suggesting an important role for this miRNA during early zebrafish development (Wienholds, Kloosterman et al. 2005; Flynt, Li et al. 2007; Thatcher, Flynt et al. 2007). Over-expression of *miR-214* in zebrafish results in embryos consistently exhibiting a ventrally curved body axis at 48 hours post fertilization (hpf)(Fig. 3B). A similar curling down phenotype was previously observed in embryos injected with three different antisense morpholino oligonucleotides directed against *disp2* (Nakano, Kim et al. 2004)(data not shown) (Fig. 3C). Interestingly, we found three possible MREs in the 3' UTR of *disp2* (Fig. 3D). None of the three sites contain perfect matches to the seed regions (nucleotides 2-7) but since we previously showed that *miR-214* targets *sufu*

without perfect seed pairing (Flynt, Li et al. 2007), we sought to determine whether *miR-214* could also target *disp2*.

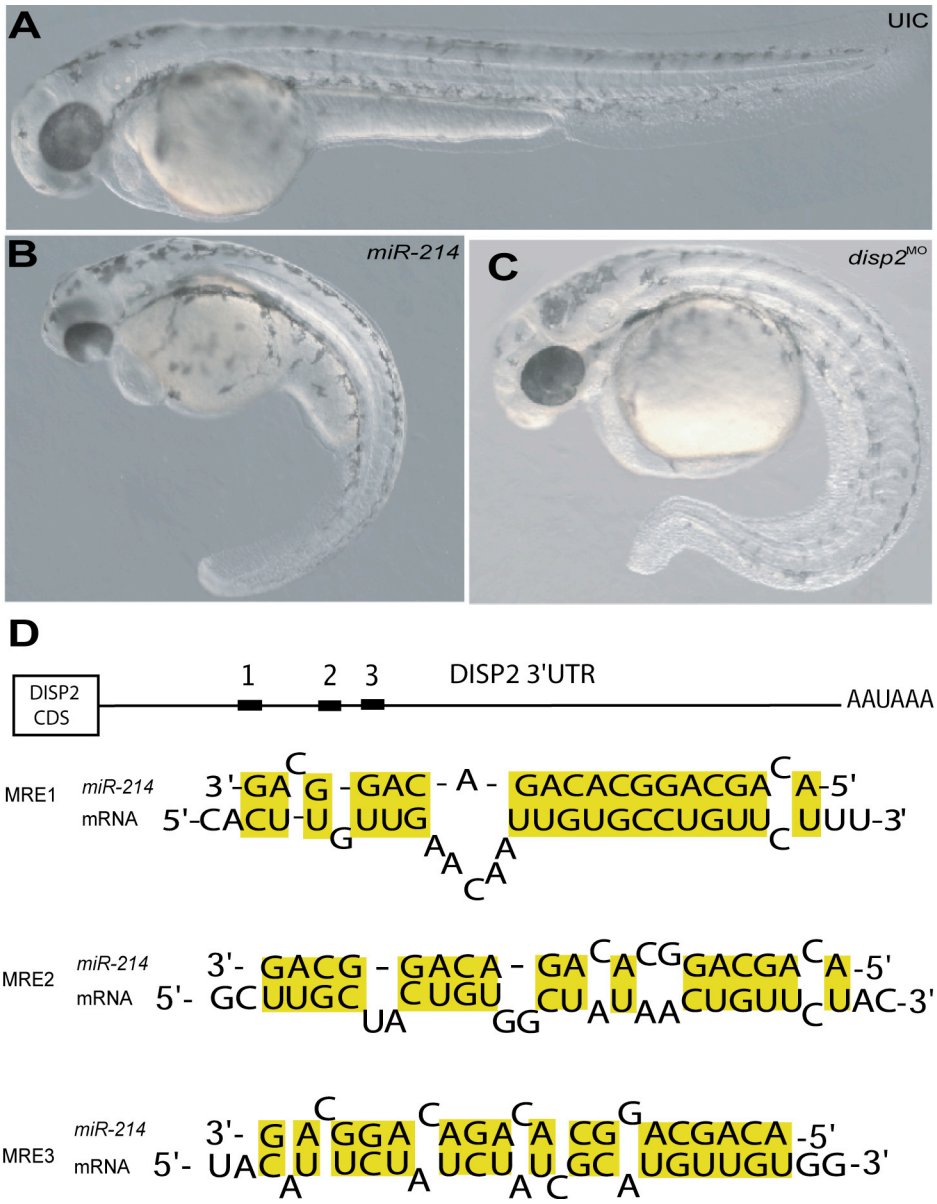

Figure 3. Curling Down Phenotypes in Zebrafish Embryos.
(A-C) Over-expression of *miR-214* results in ventrally curved embryos at 2 days post fertilization (dpf), a phenotype that mimics the effect of injection of antisense morpholino oligonucleotides against *dispatched homolog 2* (*disp2*^{MO}). A wild type, uninjected embryo at 2 dpf is shown in A (UIC). (D) The 3' UTR of *disp2* contains three predicted microRNA recognition elements (MREs) for *miR-214*.

To test whether *disp2* is targeted by *miR-214*, we created reporter constructs in which the entire *disp2* 3'UTR, or portions thereof, was cloned downstream of the coding region of GFP (Fig. 4A). As a control, we also created a construct in which two perfect MREs for *miR-214* were placed downstream of the GFP coding region (Fig. 4A). To assay silencing, synthetic mRNAs derived from these reporters were injected into single cell zebrafish embryos in the presence or absence of exogenous *miR-214* and fluorescence levels in live embryos were determined at 24hpf (Fig. 4B-K). As expected, the presence of two perfect MREs for *miR-214* led to efficient silencing of GFP in the presence of *miR-214* (Fig. 4F,G). Decreased fluorescence was also observed when the entire 3' UTR from *disp2* was inserted downstream of GFP (Fig. 4D,E). Deletion of the downstream half of the 3'UTR (Δ3) did not affect silencing, consistent with the fact the none of the three predicted MREs are located in this region (Fig. 4H,I). In contrast, deletion of the upstream portion, which contains all three predicted MREs (D5), abolished silencing (Fig. 4J,K).

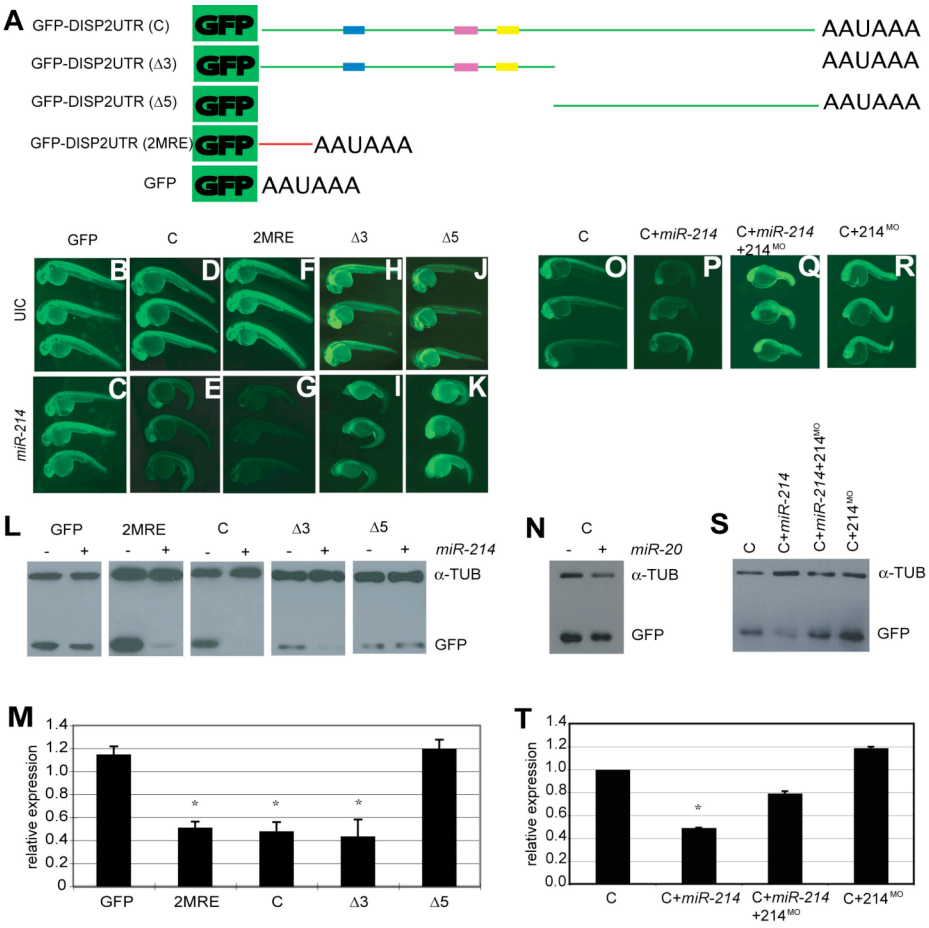

Figure 4. *disp2* is Targeted by *miR-214*.
(A) GFP reporters were constructed that contain the indicated 3' UTR sequences from *disp2* (C, D3, D5), a synthetic 3' UTR that contains two perfect pairing sites for *miR-214* (2MRE), or the normal GFP 3'UTR sequence (GFP). The predicted MREs for *miR-214* are indicated by the colored rectangles. (B-K) mRNAs derived from the reporters in A were injected into single cell embryos in the presence or absence of co-injection of *miR-214*. Fluorescence was examined at 1 dpf in living embryos. (L, M) Western blots of lysates from embryos injected as in B-K were performed with antibodies against GFP and the levels of GFP were quantitated as described in the methods. Relative GFP levels (+/- sem) were plotted with asterisks representing significant decreases between the control GFP construct and the indicated constructs. Significance was analyzed using Student's T test ($p<0.001$ for constructs 2MRE and C, $p<0.01$ for construct D3; $n>3$). (N) mRNAs encoding the complete *disp2* 3' UTR fused to GFP were injected in the presence and absence of *miR-20*. Embryo lysates were prepared, and GFP levels were examined by western blot as above. (O-R) Single cell embryos were injected as indicated in the presence or absence of antisense morpholino oligonucleotides against *miR-214* (214^{MO}). Fluorescence was examined at 1 dpf. (S,T) Western blots of embryo lysates were performed and quantitated as above. Relative GFP levels (+/- sem) were plotted with asterisks representing significant decreases between the GFP reporter alone and the indicated co-injections. Significance was analyzed using Student's T test ($p<0.001$, $n>3$).

To analyze silencing from the entire population of injected embryos, lysates were prepared from embryos injected as above and western blots were performed with antibodies against GFP (Fig. 4L,M). The presence of either the entire 3' UTR (construct C) or the downstream half (Δ3) led to a reduction of GFP levels by approximately 60% in the presence of *miR-214*, similar to that observed when the 3' UTR contained 2 perfect MREs (2MRE). As above, no silencing was observed upon deletion of the region containing the predicted MREs (D5) nor was silencing observed when the 3' UTR was derived from the GFP vector (GFP). As a specificity control, we also co-injected an unrelated miRNA (*miR-20*) with the C construct. No silencing of GFP was observed (Fig. 4N). Lastly, we injected antisense morpholino oligonucleotides against *miR-214* (214^{MO}) to determine whether inhibition of endogenous levels of *miR-214* during early zebrafish development would inhibit silencing of the GFP reporter. As shown (Fig. 4N-R), inhibition of endogenous levels of *miR-214* led to increased GFP levels. Together, the fluorescence assays and western blots demonstrate that *disp2* is targeted by *miR-214* and are consistent with silencing mediated by the three predicted MREs.

Genetic Interaction Between *miR-214* and *Disp2*

Injection of zebrafish embryos with any of three different antisense morpholino oligonucleotides against *disp2* (*disp2*^{MO}) results in embryos displaying a downward curvature of the tail at 48 hpf (Nakano, Kim et al. 2004)(Fig. 5E)(data not shown). If *miR-214* targets *disp2,* overexpression of *miR-214* in zebrafish embryos should recapitulate the curling down phenotype. As shown in Fig. 5, over 80% of *miR-214*

injected embryos displayed the curling down phenotype. Interestingly, the percent of embryos displaying the curling down phenotype was nearly identical between injection of *miR-214* and a morpholino against the translation start site for *disp2* (*disp2*^{MO}). If the effect of excess *miR-214* is specific, co-injection of *disp2* mRNA should be able to suppress the overexpression phenotype. As shown in Fig. 5C,F, there was a significant decrease in the fraction of ventrally curved embryos when both *miR-214* and *disp2* mRNA were co-injected (from 84% to 55%). Since most miRNAs target multiple mRNAs, it is likely that *miR-214* can still silence other mRNAs such that partial phenotypic rescue is the expected result. These results strongly suggest genetic interaction between *miR-214* and *disp2* and further demonstrate that *disp2* is indeed a target of *miR-214*.

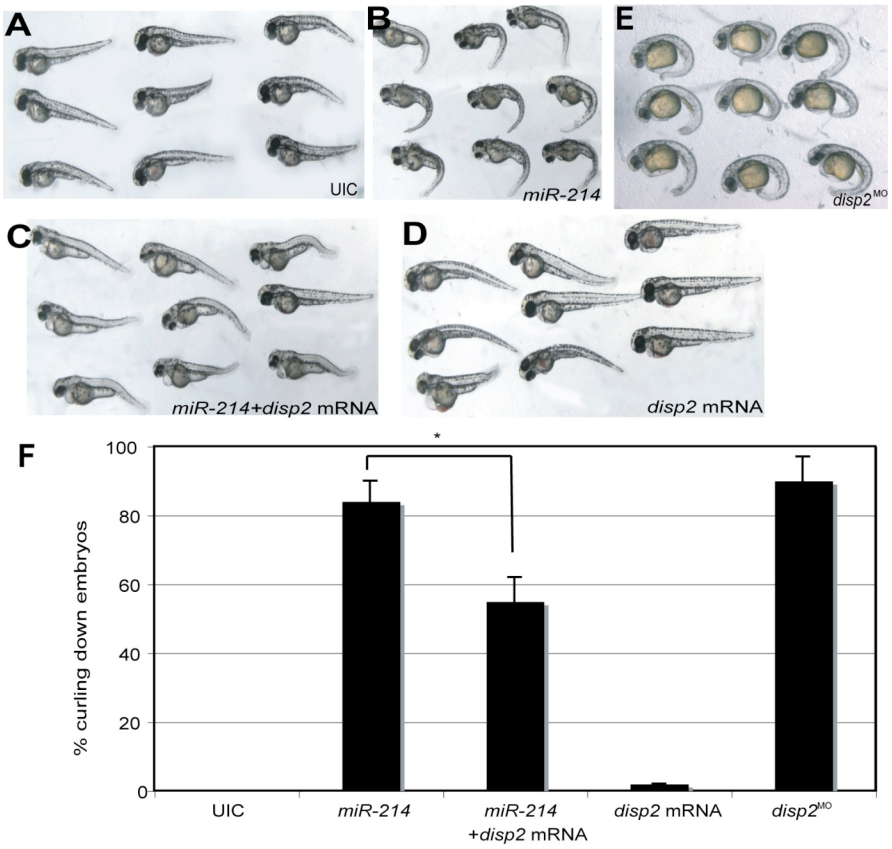

Figure 5. Rescue of the Curling Down Phenotype by *miR-214*.
Single cell zebrafish embryos, either uninjected (UIC; A) or injected with *miR-214* RNA (B), antisense morpholino oligonucleotides against *disp2* (*disp2*^{MO}; E), *disp2* mRNA (D), or a combination of *miR-214* and *disp2* (C) were allowed to develop for 48 hrs before examination and quantitation (F) of the fraction exhibiting a curling down phenotype. The number of embryos exhibiting the curling down phenotype for *miR-214* and *disp2* co-injection compared to *miR-214* injection alone was analyzed using Student's T test ($p<0.01$, $n>3$).

In early zebrafish embryos, *disp2* is expressed primarily in the central nervous system with highest expression in the telencephalon and ventral hindbrain (Nakano, Kim et al. 2004). While *disp1* and *disp2* are closely related, no Hh signaling defects have been observed with loss of *disp2* as compared to loss of *disp1* (Nakano, Kim et al. 2004). However, loss of *disp2* leads to loss of the neural marker transcription factor Prox1 in the hindbrain at 24 hpf (H.R. Kim, Y. Nakano, and P.W. Ingham, personal communication)(Fig. 6E). If *miR-214* targets *disp2*, overexpression of *miR-214* should also block *prox1* expression in the hindbrain at 24 hpf. To test this, we marked the hindbrain midbrain boundary by immunostaining with CY2 tagged antibodies against Engrailed (green) and co-stained to detect Prox1 expression in the hindbrain. As shown in Fig. 6, a significant decrease (more than 50%) in the number of Prox1 positive hindbrain neurons (red) was observed in embryos injected with *miR-214* at 24 hpf (Fig. 6B,F), similar to the decrease observed in the *disp2* morphants (Fig. 6E,F). Significantly, the decreased numbers of Prox 1 nuclei caused by injection of *miR-214* could be rescued by co-injection of *disp2* mRNAs (Fig. 6C,F). These data are consistent with regulation of *disp2* by *miR-214* during early zebrafish development.

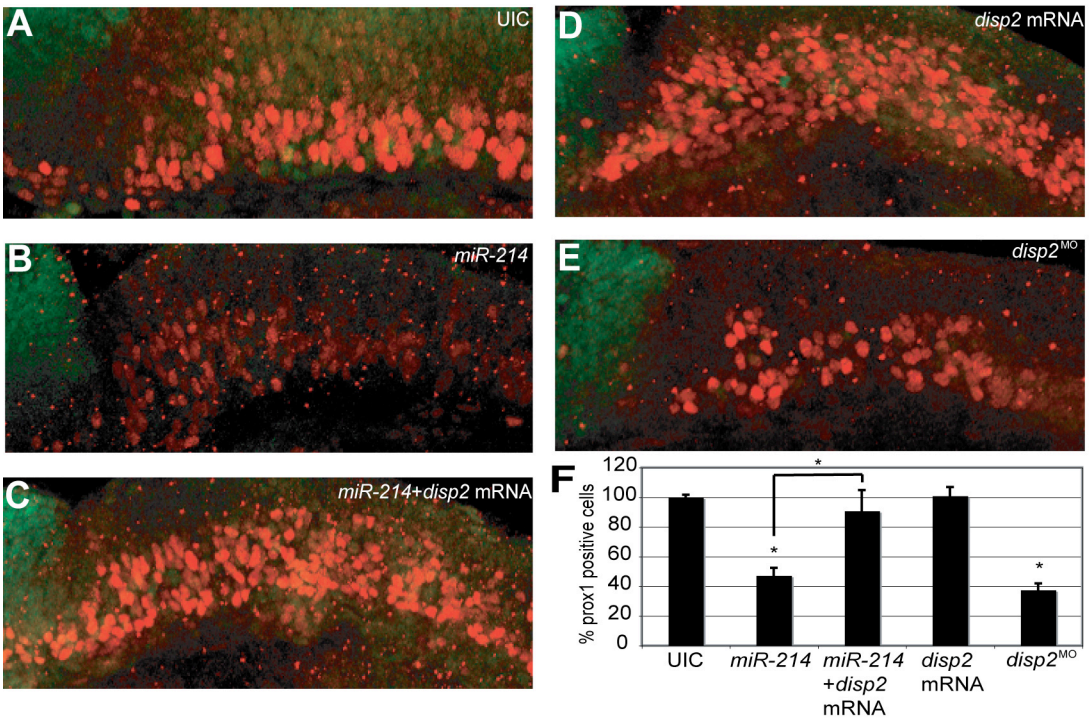

Figure 6. Genetic Interaction Between *disp2* and *miR-214*.
Whole mount immunostaining of zebrafish embryos was performed using antibodies against the neural marker Prox1 (red) and the midbrain hindbrain boundary marker Engrailed (green). Embryos were positioned dorsal to the top, anterior to the left. Single cell embryos were either uninjected (UIC; A) or injected with *miR-214* (B), the combination of *miR-214* and *disp2* mRNA (C), *disp2* mRNA (D), or *disp2*^{MO} (E). The relative number of Prox1 positive cells in the hindbrain compared to that in UIC was graphed in F. Significant differences were observed between UIC and *miR-214* injected embryos ($p<0.001$), between UIC and *disp2*^{MO} injected embryos ($p<0.001$) and between embryos injected with *miR-214* alone and co-injected with *miR-214* and *disp2* mRNA ($p<0.05$) by Student's T test. In all cases, $n>3$.

Regulation of *disp2* by *miR-214* requires multiple weak MREs

Based on the above results as well as previously published work (Flynt, Li et al. 2007), we have shown that *miR-214* targets both *disp2* and *sufu*. Both genes contain 3 predicted MREs but none of these elements obey the seed rule for miRNA:mRNA pairing (Lewis, Shih et al. 2003; Kloosterman, Wienholds et al. 2004; Brennecke, Stark et al. 2005; Wang, Love et al. 2006). One possibility is that multiple weak MREs can act combinatorially to enable efficient silencing similar to the effect of one or more perfect MREs. We therefore sought to determine whether multiple weak MREs are required for silencing *disp2*. For this, six GFP reporter constructs (Fig. 7A) were created by deletion of one or more of the three *disp2* MREs. RNA was prepared from each of the resulting constructs, injected into zebrafish embryos, and analyzed for fluorescence in living embryos (data not shown). Western blots were also performed on embryo lysates in the presence and absence of *miR-214* (Fig. 7). As in Fig. 4, co-injection of *miR-214* led to an almost 60% decrease in GFP levels when the 3' UTR contained all 3 weak *disp2* MREs (Fig. 7B,C). When only 2 MREs were present, silencing of GFP was roughly equivalent to that observed with all 3 sites, regardless of the combination (Fig. 7 D1, D2, D3). In contrast, single sites were mostly incapable of effective gene silencing although relatively small decreases were consistently observed, especially for MRE3 (Fig. 7 D12). The results from Figures 4 and 7 demonstrate that the combination of three weak MREs are as effective in mediating silencing as 2 perfect MREs followed closely by the presence of two weak sites which are far more effective than a single weak MRE. Thus, weak MREs can act combinatorially to silence gene expression.

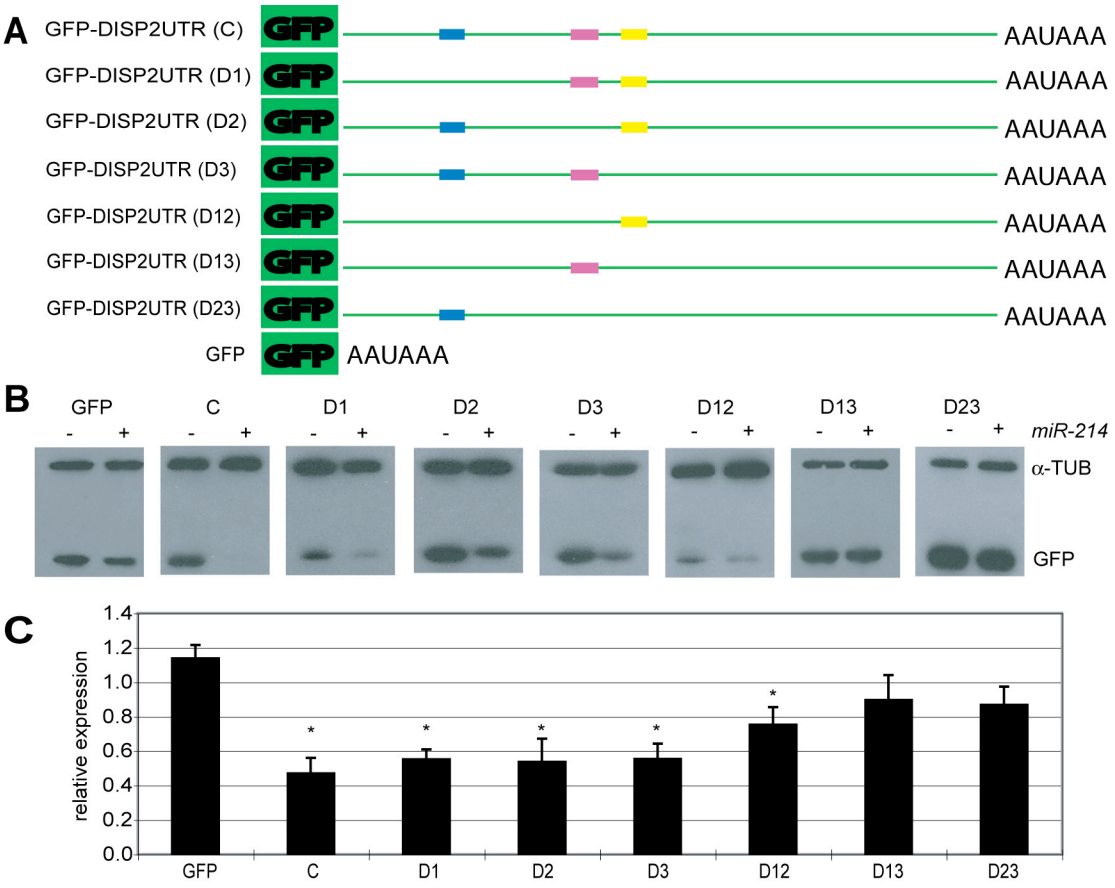

Figure 7. Deletion Analysis of *disp2* MRE Function.
(A) As in Figure 4, GFP reporters were constructed that contain the indicated 3' UTR sequences. (B, C) mRNAs derived from the reporters in A were injected into single cell embryos in the presence or absence of co-injection of *miR-214*. Western blots of embryo lysates were performed with antibodies against GFP and the level of GFP was quantitated as above. Relative GFP levels are shown (+/- sem) with asterisks representing significant differences between the control GFP levels and the indicated constructs as follows: $p<0.001$ for constructs C and D1, $p<0.01$ for constructs D2 and D3, $p<0.05$ for construct D12 by Student's T test, $n>3$.

To further validate the role of each of the three weak MREs, we would ideally like to create point mutations that abolish MRE function. However, the results thus far illustrate that the precise requirements for any particular base are apparently quite flexible. Thus, to selectively silence one or more of the three MREs, we chose to utilize antisense morpholino target protectors designed to hybridize to MREs and block the ability of miRNAs to effect silencing (Choi, Giraldez et al. 2007). Three target protectors were designed complementary to portions of each of the three MREs in the 3'UTR of *disp2* (TP1, TP2, TP3). First, we co-injected all three target protectors with the C construct and *miR-214*. The presence of the three target protectors impaired silencing in the presence of *miR-214* (Fig. 8A-F). Co-injection of all three target protectors was not quite as efficient at blocking silencing as was co-injection of antisense morpholino oligonucleotides against *miR-214* (see Fig. 4) but there was still a significant increase in GFP levels. Next, we co-injected single and pairwise combinations of target protectors (Fig. 8G,H). As shown, each individual target protector was able to inhibit silencing from 20-40% whereas pairwise combinations varied from a 20% increase in GFP levels to complete rescue in the presence of target protectors 1-2. Taken together, efficient silencing of *disp2* 3'UTR by *miR-214* requires contribution from multiple weak MREs. Although none of the three MREs contain perfect seed sequences, the three weak MREs can act combinatorially to silence gene expression.

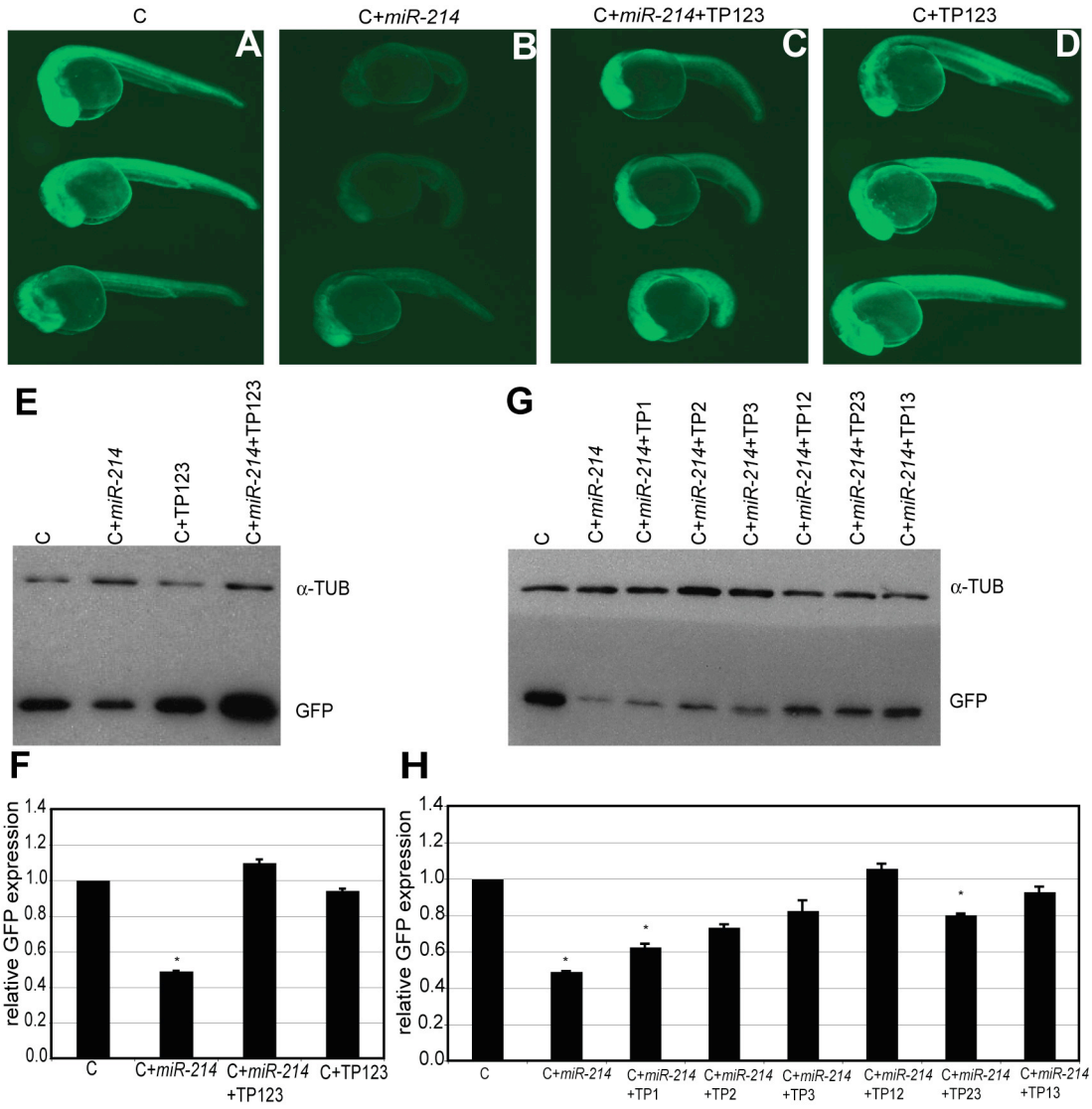

Figure 8. Combinatorial Action of Weak MREs.
mRNAs encoding GFP reporters containing the complete *disp2* 3'UTR sequence were injected into zebrafish embryos along with target protectors against the three *disp2* MREs (TP1,2,3) in the presence or absence of exogenous *miR-214*. Single cell embryos injected with all three target protectors were injected and examined for fluorescence at 1 dpf (A-D). Western blots of embryo lysates isolated from embryos injected either with all three target protectors or combinations thereof were performed with antibodies against GFP (E,G). Relative GFP levels were quantitated as above and values plotted (+/- sem) with asterisks representing significant decreases between the GFP reporter alone and the indicated co-injections (F,H). Significance was analyzed using Student's T test ($p<0.001$ for construct C and *miR-214* co-injection, $p<0.05$ for construct C, *miR-214*, TP1 co-injection and construct C, *miR-214*, TP2,3 co-injection, $n>3$).

Discussion

Dispatched Homolog 2 is a target of *miR-214*

Here, we provide several lines of evidence that support the hypothesis that *disp2* is a target of *miR-214*. First, using GFP reporters in zebrafish embryos, we were able to show that silencing by *miR-214* requires the presence of the *disp2* 3' UTR. Second, overexpression of *miR-214* produced a curling down phenotype similar to that observed in *disp2* morphants. Third, interference with *disp2* function led to the loss of the Prox1 positive nuclei in the hindbrain at 24 hpf and overexpression of *miR-214* phenocopied. Importantly, the loss of Prox1 nuclei by injection of *miR-214* could be rescued by co-injection of *disp2* mRNA. Similarly, the curling down phenotype could be partially suppressed by co-injection of *disp2* mRNA. Finally, consistent with regulation by *miR-214*, *disp2* is expressed in the neural tube at 1 dpf whereas *miR-214* is not (Nakano, Kim et al. 2004; Flynt, Li et al. 2007). These data are entirely consistent with regulation of *disp2* by *miR-214*.

One limitation of the above results is that the exact function of Dispatched 2 remains to be determined. Despite the fact that it is very similar to Dispatched 1, loss of Dispatched 2 does not lead to detectable Hh signaling defects (Nakano, Kim et al. 2004). Thus, while curling down of zebrafish embryos is generally indicative of Hh defects, this is not thought to be the case for Dispatched 2. Complete understanding of the significance of *miR-214* regulation of *disp2* will await further functional analyses of Dispatched 2.

Combinatorial Silencing

Many computational and experimental approaches have been used to formulate general rules that allow accurate identification of miRNA targets. Previous studies, as well as the results reported here, suggest that base pairing between the "seed" region (residues 2-8 from the 5'end) of the miRNA and the mRNA target is the most readily identifiable determinant for predicting and establishing specificity. However, perfect seed pairing is not necessarily sufficient for repression. The degree of repression can also be influenced by adjacent AU rich sequences, the distance between MREs and stop codons, and accessibility of the 3' UTR (Didiano and Hobert 2006; Grimson, Farh et al. 2007; Hon and Zhang 2007; Kertesz, Iovino et al. 2007; Nielsen, Shomron et al. 2007). Our results demonstrate that even sites that violate the pairing rules above can still serve to mediate silencing provided the presence of multiple weak sites. This finding further challenges prediction algorithms by increasing the number of sites that serve as *bona fide* targets.

We previously showed that targeting of *sufu* by *miR-214* is via three weak MREs and we extend that observation here to show that *disp2* is similarly regulated through the cooperative action of three weak MREs. For all three *disp2* sites, there are gaps and G:U base pairs within the seed region and the pairing with the 3' end of *miR-214* is even weaker. Individually, these sites are not effective targets but, surprisingly, in combination, can lead to silencing as effective as perfect sites. A different observation was made previously in an invertebrate model system (Brennecke, Stark et al. 2005) where multiple weak sites were not found to act combinatorially, concluding that weak sites, which by themselves cannot mediate silencing, do not do so in combination. This

suggests that the rules for miRNA-mRNA recognition are not absolute and that the mechanisms of silencing may be slightly different between species. Based on our study, an additive model does not accurately reflect silencing and instead, a synergistic model most closely approximates the combined effects of multiple weak MREs.

Acknowledgements

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Chapter III

REGULATION OF ENDODERM FORMATION AND LEFT-RIGHT ASYMMETRY BY *miR-92* DURING EARLY ZEBRAFISH DEVELOPMENT

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Abstract

microRNAs (miRNAs) are a family of 21-23 nucleotide endogenous non-coding RNAs that post-transcriptionally regulate gene expression in a sequence-specific manner. Typically, miRNAs down regulate target genes by recognizing and recruiting protein complexes to 3'UTRs followed by either translation repression or mRNA degradation. *miR-92* is a well studied oncogene in mammalian systems. Here, using zebrafish as a model system, we uncovered a novel tissue-inductive role for *miR-92* during early vertebrate development. Overexpression resulted in reduced endoderm formation during gastrulation with consequent cardia and viscera bifida. In contrast, depletion of *miR-92* increased endoderm formation which led to abnormal Kupffer's vesicle development and left-right patterning defects. Using target prediction algorithms and reporter constructs, we show that *gata5* is a target of *miR-92*. Alteration of *gata5* levels reciprocally mirrored the effects of gain- and loss-of-function of *miR-92*. Moreover, genetic epistasis experiments showed that *miR-92*-mediated defects could be substantially suppressed by modulating *gata5* levels. We propose that *miR-92* is a critical regulator of endoderm formation and left-right asymmetry during early zebrafish development and provide the first evidence for a regulatory function for *gata5* in the formation of Kupffer's vesicle and left-right patterning.

Introduction

The *miR-92* family

The *miR-92* family includes *miR-92a*, *miR-92b* and *miR-25*. These miRNAs share identical "seed" sequences (nucleotides 2-8 from the 5' end of the mature miRNA) and similar sequences in the middle and 3' portion of each mature miRNA. Members of the same family have overlapping functions by targeting similar mRNAs (Xu and Wong 2008; Yuan, Liu et al. 2009). From zebrafish to human, the genomic organization of the *miR-92* family is fairly conserved. Members of the *miR-92* family are encoded in clusters with other miRNA families, transcribed first as polycistronic RNAs and then processed into individual miRNAs. In human, two *miR-92a* loci, one *miR-92b* locus, and one *miR-25* locus exist. *miR-92a-1* is encoded in a cluster with *miR-17*, *miR-18a*, *miR-19a*, *miR-20a* and *miR-19b-1* on chromosome 13, region 13q32 (known as the *miR-17-92* cluster). *miR-92a-2* is encoded in a cluster with *miR-106a*, *miR-18b*, *miR-20b*, *miR-19b-2* and *miR-363* on the X chromosome, region q26.2 (*miR-106a-363* cluster). *miR-92b* is encoded alone. *miR-25* is encoded in a cluster with *miR-106b* and *miR-93* in the 13th intron of the protein coding gene MCM7 on chromosome 7 (*miR-106b-25* cluster). (Fig. 9)

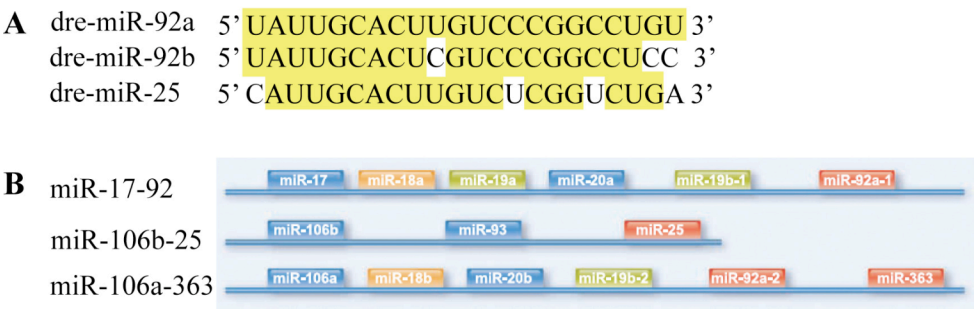


Figure 9. *miR-92* family.
A) Sequence alignment of the *miR-92* family. B) Genomic organization of the *miR-92* family in humans. miRNAs in identical colors belong to the same family. Adapted from Petrocca et al. 2008.

Several lines of evidence suggest that the *miR-17-92* cluster functions as an oncogene. First, amplification of the human *miR-17-92* cluster is frequently observed in hematopoietic malignancies and solid tumors (Ota, Tagawa et al. 2004; Volinia, Calin et al. 2006; Petrocca, Visone et al. 2008). Second, both loci encoding the *miR-17-92* and *miR-106a-363* clusters are common insertion sites in multiple types of retrovirally induced murine leukemias (Joosten, Vankan-Berkhoudt et al. 2002; Lund, Turner et al. 2002; Mikkers, Allen et al. 2002; Suzuki, Shen et al. 2002; Wang, Wang et al. 2006; Cui, Li et al. 2007; Landais, Landry et al. 2007). Third, in a mouse B-cell lymphoma model, enforced overexpression of the *miR-17-92* cluster significantly accelerated disease onset and progression (He, Thomson et al. 2005). Transcriptional activation of *miR-17-92* cluster is directly regulated by cMyc and E2F3, both of which are oncogenic transcription factors (O'Donnell, Wentzel et al. 2005; Sylvestre, De Guire et al. 2007; Woods, Thomson et al. 2007). Similarly, the *miR-106b-25* cluster and its host gene MCM7 are up-regulated by E2F1 in gastric cancer (Petrocca, Visone et al. 2008).

Multiple important downstream targets of the *miR-17-92*/*miR-106b-25* clusters have been identified that contribute to their tumorigenic function. *miR-17* and *miR-20a* target the transcription factor E2F1 and attenuate E2F1-induced apoptosis (O'Donnell, Wentzel et al. 2005). E2F1 is a critical cell cycle regulator that promotes the G1-S transition in mammalian cells (Bracken, Ciro et al. 2004). When its expression exceeds a threshold, E2F1 also generates an apoptotic signal (Lazzerini Denchi and Helin 2005). By contributing to a feedback loop including cMyc, E2F1, and E2F3, the *miR-17-92* cluster shifts the balance away from the pro-apoptotic E2F1 and toward the proliferative E2F3 network (Woods, Thomson et al. 2007). Other studies suggest that *miR-17*, *miR-20a*, *miR-106b* and *miR-93* promote cell cycle progression and suppress TGFβ-dependent cell cycle arrest and apoptosis by targeting p21/CDKN1A (Ivanovska, Ball et al. 2008; Petrocca, Visone et al. 2008). The other downstream effector of TGFβ signaling, the proapoptoic gene Bim/BCL2L11 is also targeted by *miR-92* and *miR-25* (Koralov, Muljo et al. 2008; Petrocca, Visone et al. 2008; Ventura, Young et al. 2008; Xiao, Srinivasan et al. 2008). Likewise, the tumor suppressor Pten is targeted by *miR-17* and *miR-19* in lymphocytes. Lastly, down-regulation of anti-angiogenic TSP1 (thrombospondin-1) and CTGF (connective tissue growth factor) by *miR-19* and *miR-18,* respectively, appears to contribute to cMyc-induced tumor angiogenesis (Dews, Homayouni et al. 2006).

As compared to the tumorigenic role revealed by intensive studies in fully differentiated cells, especially cancer cell lines, the physiological function of these miRNAs during development is poorly understood. Ventura et al (2008) generated targeted deletions in mice and found that mice deficient for the *miR-17-92* cluster are postnatal lethal with severe cardiac and lung defects. Also, the development of both fetal

and adult B-cells is impaired upon loss of *miR-17-92*, which may be due to increased Bim-mediated apoptosis in early B cell progenitors. No obvious abnormalities were observed in *miR-106b-25* or *miR-106a-363* deficient mice, which may indicate functional redundancy of these loci. However, *miR-106b-25* and *miR-17-92* double knockout and triple knockout mice die much earlier and exhibit much more severe defects than their single knockout counterparts (Ventura, Young et al. 2008). *miR-17*, *miR-20a* and *miR-106a* participate in monocytic differentiation and maturation by targeting the transcription factor Acute Myeloid Leukaemia-1 (AML1) (Fontana, Pelosi et al. 2007). Depletion of Dicer results in a block of pro- to pre- B cell transition, accompanied by up-regulation of target genes of the *miR-17-92* cluster, including Bim (Koralov, Muljo et al. 2008). Overexpression of *miR-17-92* cluster in mouse lymphocytes leads to lymphoproliferative disease, autoimmunity and death (Xiao, Srinivasan et al. 2008).

Overview of zebrafish development

Zebrafish provide a powerful model system for developmental studies in vertebrates. Advantages include high fecundity, external and rapid development, transparent embryos, and the ability to carry our forward genetic screens, chemical screens, reverse genetics, and simple experimental manipulation.

The development of zebrafish within the first 3 days post-fertilization (dpf) can be conceptually divided into zygote (0-0.75h), cleavage (0.75-2.25h), blastula (2.25-5.25h), gastrula (5.25-10h), segmentation (10-24h), pharyngula (24-48h), and hatching periods (48-72h) (Kimmel, Ballard et al. 1995). [Fig. 10]

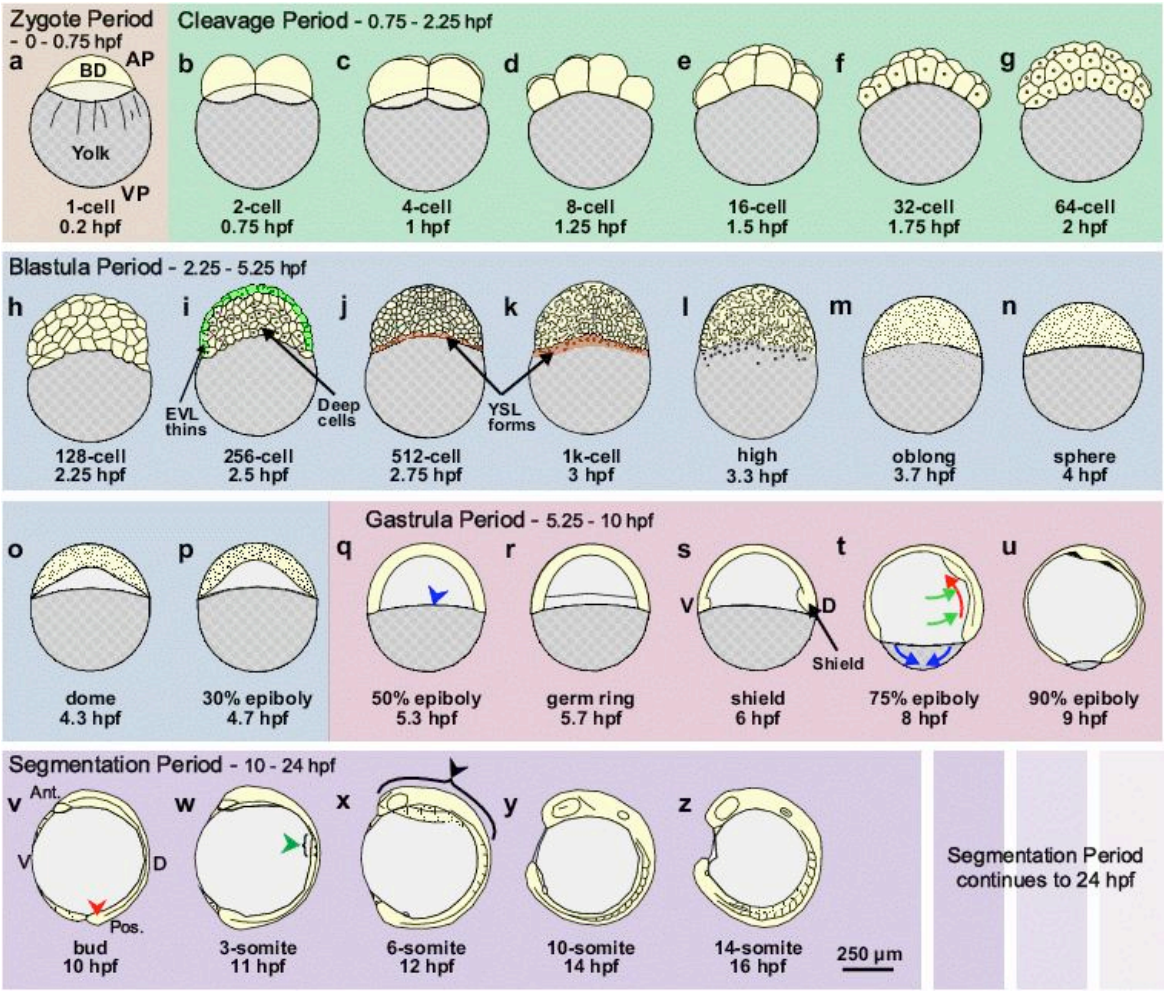


Figure 10. Diagram of early zebrafish development.
BD, blastodisc. AP, animal pole. VP, vegetal pole. EVL, enveloping layer. YSL, yolk syncytial layer. Blue, green and red arrows in panel (t) show epiboly, convergence and extension cell movement. Red arrowhead in panel (v) indicates tail bud. Green arrowhead in panel (w), somites. Black arrowhead in panel (x), brain. Adapted from Webb et al. 2007 (Webb and Miller 2007).

The zebrafish zygote is about 1mm in diameter, composed of a clear blastodisc which gives rise to the entire future animal and a yolk which provides nutrition for the embryo for the first 5 days. A membranous chorion surrounds and protects the entire embryo during the first 2 days.

The blastodisc first undergoes a series of vertical cell divisions. The first horizontal cleavage occurs at the sixth cycle and results in a top layer of cells referred to as the

enveloping layer (EVL) and a bottom layer of cells referred to as the deep cell layer. During the development that follows, the EVL remains as a flattened monolayer and eventually becomes the protective outer surface of the animal called the periderm, an extra-embryonic cell layer that is sloughed off during later development. The deep cells that are buried underneath the EVL will form the entire embryo proper.

The embryo is referred to as a blastula when multiple cell cleavages result in a group of ball-like cells sitting atop the yolk. Three important events occur during the blastula stage: the mid-blastula transition (MBT), formation of the yolk syncytial layer (YSL), and the start of epiboly (Kimmel, Ballard et al. 1995). At the tenth cell cycle, cell cycle lengthening marks the onset of the mid-blastula transition, which is accompanied by the loss of cell division synchrony and the beginning of zygotic transcription. Also, during the tenth cell cycle, the most marginal tier of blastomeres collapse and release their cellular components (cytoplasm and nucleus) into the adjoining cytoplasm of the yolk. This release gives rise to the YSL, a transient structure throughout embryogenesis which is originally a marginal ring around the blastomere and then quickly spreads underneath the blastodisc. The YSL mediates interactions between the deep cell layer and the yolk by lying between and separating the two. Because of this, the YSL, although not directly contributing to any part of the embryo, plays a critical role during early development in nutrient transport, epiboly, and the induction and patterning of the endoderm and mesoderm (see below). During the late blastula stage, the blastodisc starts thinning and spreading over the yolk. This vegetal cell movement is called epiboly and persists throughout the gastrula stage until the entire yolk is enveloped by the embryo.

As soon as blastoderm covers 50% of the yolk surface (50% epiboly), a second important cell movement starts – internalization, which marks the onset of gastrulation. Internalization is the movement of deep cells at the blastoderm margin folding inward and back upon themselves. As a result of internalization, two primary germ layers are formed in the deep cells, the outer epiblast and the inner hypoblast. Hypoblast cells give rise to future mesoderm and endoderm, while cells that remain in the epiblast by the end of gastrulation will become ectoderm. In addition to epiboly and internalization movements, deep cells in the gastrula stage undergo a third important movement – convergence and extension. Convergence is the movement of deep cells towards the future dorsal side of the embryo. Extension is the elongation and rearrangement of deep cells along the anterior-posterior axis. As a result of these rapid, highly coordinated movements (epiboly, internalization and convergence and extension), three germ layers, the dorsal-ventral and the anterior-posterior axes are established by the end of gastrulation.

Gastrulation is followed by segmentation where embryos form somites, which give rise to the myotome and sclerotome. Also, left-right axis specification occurs and organogenesis begins. By 24 hours post-fertilization (hpf), zebrafish embryos develop a heart and circulation begins. By 48hpf, the embryos hatch and start voluntary activities.

Endoderm formation in zebrafish

In zebrafish, endoderm gives rise to the gastrointestinal tract, liver, pancreas, pharyngeal pouches, gill clefts, thymus, thyroid, parathyroid and parts of the inner ear. Fate mapping experiments have shown that endoderm and mesoderm share common

progenitors (mesoendoderm), which are located within the marginal region next to the yolk during the early blastula stage (Warga and Nusslein-Volhard 1999). At the late blastula stage, these bipotential progenitors acquire a more restricted fate. Most endodermal progenitors are found no more than two cell layers away from the blastoderm margin, whereas mesodermal progenitors can be located up to 8 cell layers away. Coincident with internalization, internalized endodermal cells start to express the endoderm marker *sox17* and can be morphologically distinguished from mesodermal cells. Endoderm initially forms a discontinuous monolayer between the mesoderm and the YSL, and eventually coalesces at the midline to form a rod-like structure during segmentation.

Nodal signaling is both required and sufficient for mesoendoderm specification (Zorn and Wells 2007). Nodal-related genes belong to the activin-type subfamily of the Transforming Growth Factor β (TGFβ) family. In zebrafish, there are three nodal-related genes: Cyclops (Cyc), Squint (Sqt) and Southpaw (Spaw). Cyc and Sqt are involved in endoderm formation and Spaw is expressed later and has a role in left-right asymmetry (Schier 2003). Nodal proteins are first secreted as dimerized pre-pro-proteins that are activated by the kexin family of proprotein convertases [Fig. 11]. Active Nodal ligands bind to heteromeric transmembrane receptors with the help of an EGF-CFC co-receptor (One eyed pinhead in zebrafish), leading to phosphorylation of the cytosolic protein Smad2 (Gritsman, Zhang et al. 1999; Tremblay, Hoodless et al. 2000; Yeo and Whitman 2001). Phosphorylated Smad2 then translocates into the nucleus and directly regulates target gene expression by forming transcriptional protein complexes with other transcription factors. The spatial, temporal, and strength of Nodal signaling can be

modulated at multiple levels (Schier 2003). Importantly, Nodal signaling is activated by Nodal ligands themselves and is antagonized by one of its targets, Lefty. Formation of a proper Nodal signaling gradient is critical for endoderm specification. In zebrafish, expression of the Nodal-related ligands Cyclops (Cyc) and Squint (Sqt) are induced by maternal signals from the YSL. Cells located closer to the YSL receive higher levels of Nodal and differentiate as endoderm, whereas cells further away from the blastula margin receive lower levels of Nodal and differentiate as mesoderm (Schier, Neuhauss et al. 1997; Rodaway, Takeda et al. 1999).

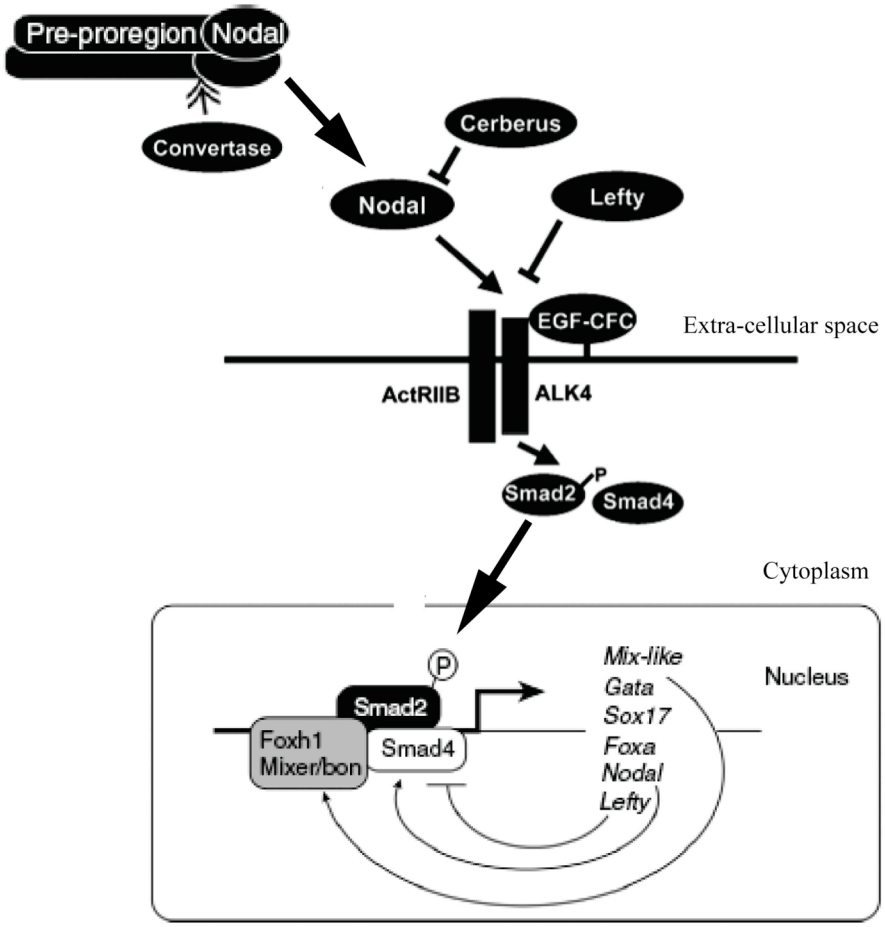


Figure 11. Nodal signaling pathway.
Active Nodal ligand binds to transmembrane receptors and consequent phosphorylation of cytosolic protein Smad2 directly activate transcription of downstream target genes. Adapted from Schier et al. 2003 and Zorn et al. 2007.

Expression of the endodermal marker *sox17* is activated by Casanova (Cas, Sox32) in a cell autonomous manner (Alexander, Rothenberg et al. 1999) [Fig. 12]. The induction of *sox17* by Cas also requires Oct4, a maternally and ubiquitously distributed POU domain transcription factor also known as Pou2 or Pou5f1 (Lunde, Belting et al. 2004; Reim, Mizoguchi et al. 2004). *cas* is activated transcriptionally by a protein complex that consists of Bon (Bonnie and clyde), Gata5 (Gata related protein 5) and a T-box gene Eomes (Eomesodermin). Both *bon* and *gata5* are direct downstream targets of Nodal signaling, whereas *eomes* is expressed maternally. Consistent with their hierarchy in the cascade, *bon*, *gata5*, *cas* and *sox17* transcripts are present in overlapping regions of the embryo. *bon* is found in a broad band (about 8 cell diameters) next to YSL; *gata5* is present within a 5 cell-diameter band; *cas* and *sox17* are restricted to the two cell layer adjacent to the margin.

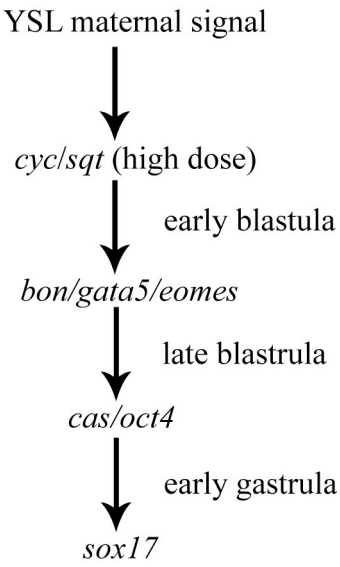


Figure 12. Simplified signaling cascade during endoderm formation.
The expression of endodermal maker *sox17* is induced by Nodal signaling.

Heart development in zebrafish

The primitive vertebrate heart consists of an outer epithelial layer (epicardium or pericardium), a middle muscular layer (myocardium) and an inner endothelial layer (endocardium), all of which originate from progenitors located bilaterally within the lateral plate mesoderm (LPM) (Stainier 2001; Serluca 2008). Cell lineage studies indicate that heart progenitors are located throughout the ventral and lateral regions of the embryo just before gastrulation. After involution, they migrate from the lateral edges of the embryo towards the midline where they coalesce to form a linear heart tube. This simple linear heart tube then undergoes complex changes leading to the formation of a fully developed heart with multiple chambers and valves.

Differentiation and migration of cardiac progenitors are independent events. Differentiation of myocardial precursors is marked by expression of homeobox transcription factors that belong to the NK2 family, such as *tinman* in Drosophila and

nkx2.5 in vertebrates (Bodmer, Jan et al. 1990; Komuro and Izumo 1993; Lints, Parsons et al. 1993; Tonissen, Drysdale et al. 1994; Evans, Yan et al. 1995; Schultheiss, Xydas et al. 1995; Chen and Fishman 1996; Lee, Xu et al. 1996). In zebrafish, *nkx2.5* expression in myocardial precursors is regulated by Gata5, Fgf8, Bmp2b (Bone morphogenetic protein 2b (TGFβ superfamily)) and Oep (one-eyed pinhead, a member of the EGF-CFC family that functions as a co-receptor for Nodal signaling) (Dale, Howes et al. 1992; Jones, Lyons et al. 1992; Kishimoto, Lee et al. 1997; Erter, Solnica-Krezel et al. 1998; Feldman, Gates et al. 1998; Rebagliati, Toyama et al. 1998; Osada and Wright 1999; Reiter, Alexander et al. 1999; Reiter, Kikuchi et al. 2001).

Migration of heart precursors is critical for heart morphogenesis. Disruption of cardiac migration progress leads to the formation of two separate hearts, a condition known as cardia bifida. Several mutants have been identified that cause cardia bifida in zebrafish and can be grouped into 3 categories (Chen, Haffter et al. 1996; Stainier, Fouquet et al. 1996). In the first category, cardia bifida is accompanied by the absence or severe disruption of endoderm formation, underscoring the developmental link between endoderm formation and heart morphogenesis (Jacobson and Sater 1988; Gannon and Bader 1995; Nascone and Mercola 1995). This group includes the mutants *faust* (*fau*; Gata5) (Reiter, Alexander et al. 1999), *casanova* (*cas*; Cas/Sox32) (Alexander, Rothenberg et al. 1999), *bonnie and clyde* (*bon*; Bon) (Kikuchi, Trinh et al. 2000) and *one-eyed pinhead* (*oep*; Oep) (Schier, Neuhauss et al. 1997). In the second category, cardiac bifida is accompanied by defects in myocardial differentiation. This group includes the mutants *faust* (*fau*; Gata5), *hands off* (*han*; Hand2) (Yelon, Ticho et al. 2000) and *one-eyed pinhead* (*oep*; Oep). In the third category, cardiac bifida seems to be

secondary to impaired cell-extracelluar interaction with effects on myocardial migration (Matsui, Raya et al. 2007). This group includes the mutants *miles apart* (*mil*; S1P2/sphingosine-1-phosphate receptor-2) (Kupperman, An et al. 2000), *ko157* (Spns2) (Kawahara, Nishi et al. 2009), *two-of-heart* (*toh*; Spinster-like) (Alexander, Stainier et al. 1998) and natter (nat; Fibronectin 1) (Trinh and Stainier 2004).

Gut and liver development in zebrafish

The zebrafish alimentary canal and associated digestive organs are endodermally derived (Zorn and Wells 2007). Endodermal progenitors are located along the blastoderm margin before gastrulation and then undergo internalization, convergence and extension movements to form a sparse and uniform monolayer by the end of gastrulation (Warga and Nusslein-Volhard 1999). During somitogenesis, endodermal cells converge dorsally and medially and transform from a sheet-like layer to a solid multicellular rod at the midline by 24hpf. These cells become polarized and subsequently form a lumen, transforming the endodermal rod to the endodermal tube (Horne-Badovinac, Lin et al. 2001). This tube gives rise to the gut through a series of steps (Roberts 2000) and also produces signals for the proper development of accessory organs, including the liver (Roberts 2000).

Viscera bifida, a condition where digestive organs (liver, pancreas) are duplicated along the midline, can be caused by defects in endoderm fusion during segmentation stages. Viscera bifida has been reported upon loss of a variety of genes, including Gata5 (Reiter, Alexander et al. 1999), Vegfc (vascular endothelial growth factor C) (Ober, Olofsson et al. 2004), Cxcl12b (a chemokine), Cxcr4a (a chemokine receptor) (Nair and

Schilling 2008), Sdc2 (syndecan2) (Arrington and Yost 2009), Mtx1 (Sakaguchi, Kikuchi et al. 2006), aPKCλ (Horne-Badovinac, Lin et al. 2001), Spt (Spade tail, a transcription factor) (Biemar, Argenton et al. 2001) and Kny (knypek, a glypican) (Biemar, Argenton et al. 2001). These studies together suggest that viscera bifida seems to be secondary to defects in endoderm formation or endoderm/mesoderm migration. Interestingly, genes implicated in cardia or viscera bifida do not completely overlap.

Left-right determination in zebrafish

The position of organs and the direction of looping appear to be evolutionally conserved in vertebrates. Defects in left-right asymmetry often couple with disease. The normal disposition of internal organs is called situs solitus. Situs inversus totalis (or Kartagener's syndrome) refers to a situation where the positions of all internal organs are completely reversed. This is the only type of L/R asymmetry defect that does not cause major effects on health and is often under-detected. In contrast, other L/R asymmetry defects that fail to either properly specify L/R axes or coordinate asymmetric development of multiple organs can have grave consequences, such as isomerism (symmetrical organ morphology, for example, asplenia or polysplenia) or heterotaxia (partial organ reversal, for example dextrocardia) (Capdevila, Vogan et al. 2000; Mercola and Levin 2001; Fliegauf, Benzing et al. 2007).

The left-right axis of the embryo is determined after the establishment of dorsal-ventral (D-V) and anterior-posterior (A-P) axes and is a result of cascades of asymmetrically expressed genes (Raya and Izpisua Belmonte 2006). The process of left-right determination can be conceptually divided into three continuous steps: 1) breaking

of initial symmetry, 2) stabilization and propagation of L/R asymmetry signals in the lateral plate mesoderm (LPM) and 3) induction of asymmetric morphology of internal organs. Although there is not a unique and universal model regarding how the asymmetric signals are initiated, the later steps of L/R patterning are quite conserved from zebrafish to chick and mouse.

Breaking of initial symmetry

At the very beginning of embryonic development, the two sides of the embryo are indistinguishable. Initial symmetry is broken in a process that is not yet completely understood. Evidence suggests that leftward "nodal flow" is indispensable for and probably initiates the symmetry breaking in both mouse and zebrafish. Nodal flow is generated by ciliary movement in the "node", a transient embryonic structure that is conserved throughout vertebrates (Hensen's Node in bird, Spemann's organizer in Xenopus and Kupffer's Vesicle in teleost fish) (Essner, Vogan et al. 2002; Nonaka, Yoshiba et al. 2005). Studies in mouse indicate that the leftward nodal flow is sensed by the left side of the node, inducing left-sided intracellular Ca2+ release and asymmetric expression of downstream genes only on the left side of the embryos (Nonaka, Tanaka et al. 1998; McGrath, Somlo et al. 2003; Tabin and Vogan 2003; Tanaka, Okada et al. 2005; Fliegauf, Benzing et al. 2007). In zebrafish, the structure equivalent to the mouse node is called "Kupffer's vesicle" (KV). Both the formation and function of the KV are required for proper left-right patterning. The KV is derived from dorsal forerunner cells (DFCs), which derive from ingression of dorsal surface epithelial (DSE) cells at the dorsal blastoderm margin between the sphere and 50% epiboly stages (prior to gastrulation)

(Oteiza, Koppen et al. 2008) (Fig. 13). This process is under the control of Nodal signaling. Reduced Nodal signaling decreases DFC number, while enhanced Nodal signaling increases DFC number, leading to ectopic KV formation (Choi, Giraldez et al. 2007; Oteiza, Koppen et al. 2008). During early gastrulation, DFCs migrate at the leading edge of the blastoderm margin towards the vegetal pole by attaching to dorsal marginal enveloping layer (EVL) cells. At the end of gastrulation, DFCs dissociate from dorsal marginal EVL cells and rearrange into rosette-like cell clusters (Oteiza, Koppen et al. 2008). During early somitogenesis, DFC clusters fuse into a single rosette structure and subsequently undergo a series of maturation steps, including mesenchymal to epithelial transition (MET), apical membrane clustering, formation and expansion of the KV lumen, and ciliogeneis (Amack, Wang et al. 2007). The KV reaches maturaty at the 6-somite stage, represented by a spherical structure with a fluid-filled interior lumen and monocilia on the lumen apical surface. During late somitogenesis, the KV collapses and DFCs incorporate into notochord, somites, and tail mesenchyme. Defects in KV morphogenesis impair L/R determination (Amack and Yost 2004; Amack, Wang et al. 2007; Kreiling, Balantac et al. 2008; Schneider, Houston et al. 2008).

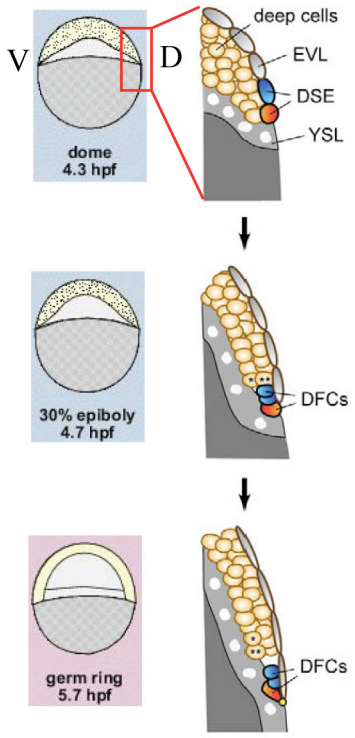


Figure 13. Ingression of dorsal forerunner cells.
Dorsal forerunner cells (DFC), the progenitors of Kupffer's vesicle, are derived from ingression of dorsal surface epithelial (DSE) cells. Adapted from Oteiza et al. 2008.

Cilia distribution and positioning and ciliary motility are essential for KV function and generation of leftward nodal flow. In zebrafish, more cilia are found on the anterior-dorsal surface of the KV as compared to the posterior-ventral surface, protruding towards the KV lumen (Kreiling, Williams et al. 2007; Okabe, Xu et al. 2008). As in mice, zebrafish cilia tilt posteriorly (Cartwright, Piro et al. 2004; Kramer-Zucker, Olale et al. 2005; Okabe, Xu et al. 2008; Supatto, Fraser et al. 2008). Such distribution and positioning of cilia ensures the generation of a net leftward flow instead of a vortex in the KV lumen as a result of the clockwise rotation of cilia. Mice or zebrafish lacking primary cilia or ciliary motility have randomized L/R patterning (Nonaka, Tanaka et al. 1998;

Marszalek, Ruiz-Lozano et al. 1999; Okada, Nonaka et al. 1999; Supp, Brueckner et al. 1999; Takeda, Yonekawa et al. 1999; Murcia, Richards et al. 2000). In zebrafish, Fgf signaling and downstream effectors Foxj1, Rfx2, Ier2 and Fibp1 regulate general ciliogenesis (Stubbs, Oishi et al. 2008; Yu, Ng et al. 2008; Hong and Dawid 2009; Neugebauer, Amack et al. 2009; Yamauchi, Miyakawa et al. 2009). Intracellular calcium levels and soluble inositol phosphates also have important functions in ciliary motility (Sarmah, Latimer et al. 2005; Sarmah, Winfrey et al. 2007; Shu, Huang et al. 2007).

Propagation and reinforcement of asymmetric signals

In all vertebrates, the transient and subtle asymmetric signals generated by the initial symmetry breaking events need to be converted into robust asymmetric expression of Nodal signaling in the left lateral plate mesoderm (LPM). Nodal is a determinant for leftness (possibly by inducing expression of *pitx2*, as discussed below). Bilateral LPM expression of nodal leads to left isomerism (Meno, Shimono et al. 1998) whereas, lack of nodal expression leads to right isomerism (Oh and Li 1997; Gaio, Schweickert et al. 1999; Yan, Gritsman et al. 1999).

Transferring of asymmetric signals from the KV to the LPM in zebrafish is not well understood but evidence suggests a mechanism similar to the "self-enhancement and lateral-inhibition" model in mice (Nakamura, Mine et al. 2006). In mice, Nodal positively regulates itself, as well as Lefty1 and 2, whereas both Lefty1 and 2 antagonize Nodal, thus creating a negative feedback loop (Raya and Izpisua Belmonte 2006) (Fig. 14). Nodal flow initially generates a small difference in Nodal expression levels between the left and right side of node. Leftward diffusion of Nodal signals to the LPM is enhanced

by Nodal itself while rightward diffusion is blocked by Lefty1 expression in the midline (Brennan, Norris et al. 2002; Saijoh, Oki et al. 2003; Saijoh, Oki et al. 2005).

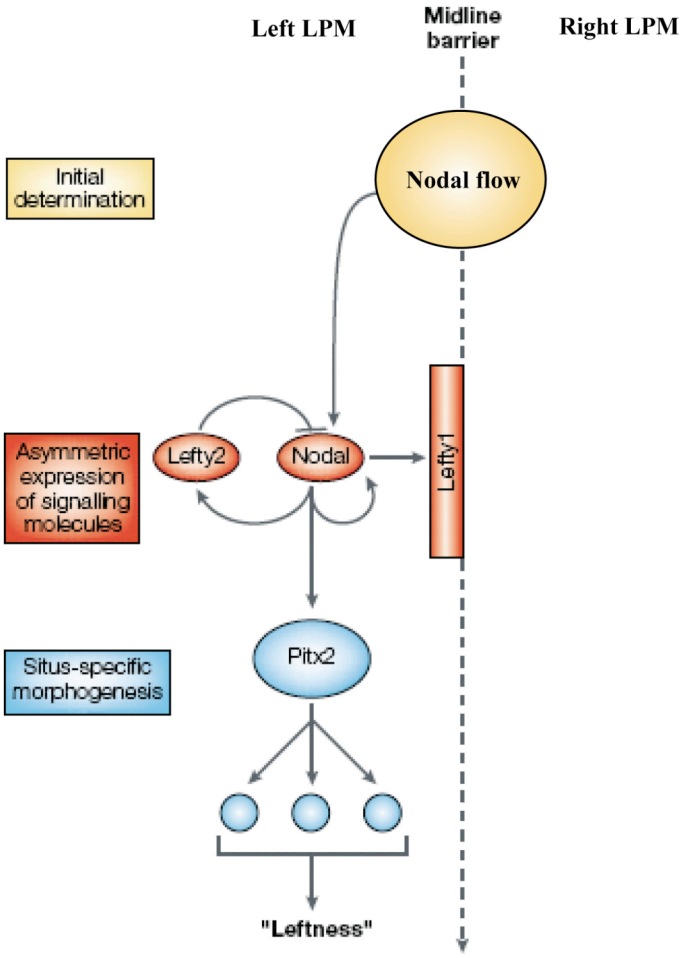


Figure 14. Propagation and reinforcement of Nodal signaling in the left LPM in mouse.
Nodal signaling is enhanced by itself and antagonized by Lefty. Adapted from (Hamada, Meno et al. 2002).

In zebrafish, the nodal-related ligand *southpaw* (*spaw*) is expressed bilaterally in the region peripheral to KV at the 4-6-somite stage (Long, Ahmad et al. 2003), in good agreement with the self-enhancement and lateral-inhibition model, Starting from the 10-

12-somite stage, *spaw* is present in the LPM, first bilaterally in the posterior region of the embryos, and then asymmetrically on the left as it expands anteriorly.

Asymmetric morphogenesis of internal organs

The final step of L/R patterning is the interpretation of asymmetric signaling by individual organ primordia, resulting in morphological or positional asymmetry of individual organs. Pitx2, a bicoid-type homeobox transcription factor is the major downstream effector of Nodal signaling and responsible for translating Nodal signaling to organ-specific morphogenesis (Logan, Pagan-Westphal et al. 1998; Piedra, Icardo et al. 1998; Ryan, Blumberg et al. 1998; Yoshioka, Meno et al. 1998; Campione, Steinbeisser et al. 1999; Shiratori, Sakuma et al. 2001). The involvement of Pitx2 in lateral development is conserved in all vertebrates (Campione, Steinbeisser et al. 1999; Patel, Isaac et al. 1999; Bisgrove and Yost 2001). Consistent with its function, *pitx2* is present in the left LPM shortly after nodal expression and persists much longer in many organ promordia in an asymmetric pattern (Logan, Pagan-Westphal et al. 1998; Piedra, Icardo et al. 1998; Ryan, Blumberg et al. 1998; St Amand, Ra et al. 1998; Yoshioka, Meno et al. 1998). The exact pathways downstream of Pitx2 that are required for asymmetric morphogenesis are organ-specific (Schilling, Concordet et al. 1999; Bisgrove, Essner et al. 2000).

Midline barrier

A midline barrier, which may be physical as well as molecular in nature, is required to separate left from right once asymmetric signals are established. Vertebrates with

impaired midline structures, such as the floor plate and notochord, display randomized L/R patterning or discordance in positioning among different organs (heterotaxia) (Danos and Yost 1996; Lohr, Danos et al. 1997; Bisgrove, Essner et al. 2000; Chin, Tsang et al. 2000; Liang, Etheridge et al. 2000). At the molecular level, Lefty1 (Antivin in Xenopus) has been suggested to function as a midline barrier to prevent left-sided Nodal signaling from crossing the midline (Long, Ahmad et al. 2003; Wang and Yost 2008). Embryos lacking Lefty1 develop normal floor plate and notochord but bilaterally expressed nodal signaling and isomerism (Meno, Shimono et al. 1998).

Gata5

Members of the GATA family are transcription factors that bind to the consensus DNA sequence (A/T) GATA (A/G) (Evans, Reitman et al. 1988). They contain one or two zinc-finger DNA-binding domains (Patient and McGhee 2002). Six Gata members have been identified in vertebrates and can be categorized into two groups based on sequence similarity and expression patterns (Reiter, Alexander et al. 1999; Heicklen-Klein, McReynolds et al. 2005). Gata1/2/3 function in hematopoiesis, whereas Gata4/5/6 regulate development of endoderm and numerous endoderm and mesoderm derived organs. Members within each group possess redundant but distinctive expression patterns and functions (Heicklen-Klein, McReynolds et al. 2005).

Interestingly, in addition to regulating differentiation of endoderm and internal organs, Gata4-6 have also been implicated in cancer progression. Gata4 and Gata5 have been suggested to act as tumor suppressors, whereas Gata6 seems to have oncogenic properties (Capo-chichi, Roland et al. 2003; Shureiqi, Zuo et al. 2007; Kwei, Bashyam et

al. 2008). Hypermethylation of Gata4 and Gata5 promoter regions has been reported in human lung, colorectal, gastric and esophageal squamous cancer cell lines, accompanied with reduced Gata4/5 expression. (Akiyama, Watkins et al. 2003; Guo, Akiyama et al. 2004; Hellebrekers, Lentjes et al. 2009).

In this study, we showed that *miR-92* regulates endoderm specification and formation of Kupffer's vesicle, with effects on left-right patterning in zebrafish. Gata5 is a target of *miR-92*. Alteration of Gata5 can partially rescue *miR-92* gain- and loss-of-function defects. Our results reveal a novel and essential role of *miR-92* during early vertebrate development.

Materials and methods

Zebrafish lines and maintenance

Wild-type AB and *sox17:gfp* (Sakaguchi, Kikuchi et al. 2006) lines of zebrafish were used. Embryos were grown at 28^{o}C in egg water and staged according to morphology (Kimmel, Ballard et al. 1995) and age (hours post-fertilization).

Microinjection

Zebrafish embryos were injected at the one cell stage with miRNAs, morpholinos, or mRNA reporters. *miR-92a*, *miR-92b*, and control miRNAs were prepared by annealing single-stranded RNAs synthesized by IDT:

miR-92a sense: 5'-UAUUGCACUUGUCCCGGCCUGUUU-3'

miR-92a antisense: 5'-ACAGGCCGGGACAAGUGCAAUAUU-3'

miR-92b sense: 5'-UAUUGCACUCGUCCCGGCCUCCUU-3'

miR-92b antisense: 5'-GGAGGCCGGGACGAGUGCAAUAUU-3'

ctrl mRNA sense: 5'-CUCUAGGUUAAACUCCUGGUU-3'

ctrl miRNA antisense: 5'-UUGAGAUCCAAUUUGAGGACC-3.

Annealing was performed by mixing equal amounts of sense and antisense strands, heating to 65^{o}C for 5 min., and gradually cooling to room temperature. Annealed duplexes were aliquoted and stored at -80C. Unless otherwise indicated, 0.5ng of *miR-92a* and *b* were injected. Control miRNAs were injected at 1ng per embryo.

All morpholinos were obtained from Gene Tools LLC with the following sequences:

MO92a1: 5'-ACAGGCCGGGACAAGTGCAATA-3'

MO92a2: 5'-CACACAGCATTGCTACCAATCCCAA-3'

MO92a2': 5'-CACAGAGCATTGCGGCCGATCCCAA-3'

MO92b1: 5'-GGAGGCCGGGACGAGTGCAATA-3'

MO92b2: 5'-TGAACAACACTGCACAACATCCCAC-3'

Unless otherwise indicated, 1ng of MO92a1 and MO92b1 were injected per embryo and referred to as MO1; 3ng of MO92a2, MO92a2' and MO92b2 were injected per embryo and referred to as MO2. Standard control morpholinos (GeneTools) were injected at 10ng per embryo. 1pmol of a *gata5* translation blocker morpholino (5'-AAGATAAAGCCAGGCTCGAATACAT-3') were injected per embryo (Holtzinger and Evans 2007). *In vitro* transcribed, capped GFP reporter mRNAs and *gata5* mRNAs were injected at 25pg and 20pg per embryo, respectively.

Dorsal forerunner cell injections were performed as described (Amack and Yost 2004). For delivery of *miR-92* into DFCs, embryos were injected with 1ng of *miR-92 a* and *b* mixture at 3hpf.

Molecular cloning

The *gata5* (NM_131235.2) 3'UTR was amplified by RT-PCR using a forward primer (5'-CCACCGAATTCTGATCCGAGACC-3') and a reverse primer (5'-GGAGGCTCGAGAAACGATATAATTCC-3'). The resulting cDNA was cloned downstream of the GFP open reading frame in the pCS2+ vector (Rupp, Snider et al. 1994). Deletion of both MREs was created by reverse PCR (Coolidge and Patton 1995) using the following primers: gata5 D1F, 5'-TCCACCAAAAATATGGTGGATG-3'; gata5 D1R, 5'-ACATCATAGATATGCCACCATAAATCA-3'; gata5 D2F, 5'-GACCCGCGCCGCTT-3'; gata5 D2R, 5'-GGAATACAATACAACATTGACAGAGTC-3'. All clones were verified by DNA sequencing.

In situ hybridization

Embryos were fixed in 4% paraformaldehyde (PFA)/1xPBS. Digoxygenin-labeled RNA probes were synthesized using a Roche DIG RNA labeling kit. cDNA templates included *foxa3* (Field, Ober et al. 2003), *cmlc2* (Yelon, Horne et al. 1999), *sox17* (Amack and Yost 2004), *ntl* (Schulte-Merker, Hammerschmidt et al. 1994), *lrdr* (Essner, Amack et al. 2005), *foxj1* (Neugebauer, Amack et al. 2009), *fgf8* (Yamauchi, Miyakawa et al. 2009). Whole-mount in situ hybridization was performed as described (Thisse and Thisse 2008).

Embryos were mounted in 100% glycerol and images were obtained using a Zeiss Axiophot camera.

Northern blotting

Total RNA from zebrafish embryos was separated on 12% acrylamide gels and electroblotted to positively charged nylon membranes. DNA oligonucleotides complementary to *miR-92* were labeled with α-^{32}P-dATP using Starfire labeling kits (IDTDNA). Hybridizations were carried out in 7% SDS and 0.2M $NaPO_4$, pH7.2 for 16hr followed by washes in 2XSSPE-0.1%SDS.

qRT-PCR

Total RNA from embryos at 90% epiboly was isolated using TRI reagent (Molecular Research Center, Inc). cDNAs were prepared from 20 ng of total RNAs and quantitative PCR was performed using the Power SYBR Green PCR Master Mix (Applied Biosystems) on an iCycler iQ Multicolor machine (Bio-Rad). Primer sequences were 5'-GGTGTGGGCGAAAGATGAGC-3' (forward) and 5'-CTCGTAGACGTTCGGCCTCC-3' (reverse). The annealing temperature was 60^{o}C.

Immunoblotting

Proteins were extracted from deyolked 1dpf embryos in lysis buffer (25mM HEPES, pH 7.5, 5mM $MgCl_2$, 300mM NaCl, 1mM EDTA, 0.2mM EGTA, 1Mm DTT, 10% glycerol, 1.0% Triton X-100 and 1mM PMSF). 20µg of total protein were then separated on 10% SDS-polyacrylamide gels and transferred to PVDF-plus membranes. Rabbit polyclonal

antibodies against GFP (Torrey Pines Biolabs) and α-tubulin (Abcam) were used at concentrations of 1:1000 and 1:500, respectively. Anti-rabbit HRP conjugated secondary antibodies (GE Healthcare) were then used for visualization with ECL. For quantification, GFP levels were normalized to a-tubulin control levels after which the ratio of GFP in the presence of *miR-92* was determined compared to that in the absence of *miR-92*.

Immunohistochemistry

Embryos were fixed in 4% paraformaldehyde (PFA)/1xPBS, permeabilized in 0.5% Triton X-100/1xPBS for 1hr., followed by incubation in blocking buffer (5% donkey serum, 5mg/ml BSA, 1%DMSO, 0.1% Tween-20 in 1xPBS) at room temperature for 2hrs. Mouse polyclonal antibodies against acetylated tubulin (Sigma) were diluted to 1:800. Cy3 conjugated secondary antibodies against mouse IgG (Jackson ImmunoResearch) were used at 1:100 dilution. Embryos were mounted in GVA mount (Invitrogen). Samples were imaged on a Zeiss LSM 510META confocal microscope.

Results

Overexpression of *miR-92* results in partial viscera and cardia bifida

To characterize the function of *miR-92* during early vertebrate development, we performed both gain- and loss-of-function experiments. *miR-92* is among the earliest expressed miRNAs detected during zebrafish development following analysis by deep sequencing (Wei and Patton, manuscript in preparation). It localizes to the developing

gut, liver and heart by 2-3 days post-fertilization (dpf) (Wienholds, Kloosterman et al. 2005). Gain-of-function experiments were performed by injecting *miR-92* into zebrafish embryos at the one-cell stage followed by assessment of the effects on gut and liver tissues by *in situ* hybridization with the pan-endodermal marker *foxa3* (*forkhead box a3*) (Reiter, Alexander et al. 1999). In non-injected controls (NIC), *foxa3* localized to the developing gut tube, liver and pancreas primordia at 50 hours post-fertilization (hpf) with liver on the left and pancreas on the right side of midline (Fig. 15A,B). Strikingly, *miR-92* injection caused over 70% of the embryos to display aberrant *foxa3* localization, showing a bifurcated gut tube with duplication of liver primordia (Fig. 15A,B). A similar phenotype was previously observed and referred to as viscera bifida (Nair and Schilling 2008). For the remaining embryos, 20% showed localization of *foxa3* along the midline, indicating a lack of gut looping, and another 3% had undetectable levels of *foxa3*, suggesting possible defects in endodermal specification.

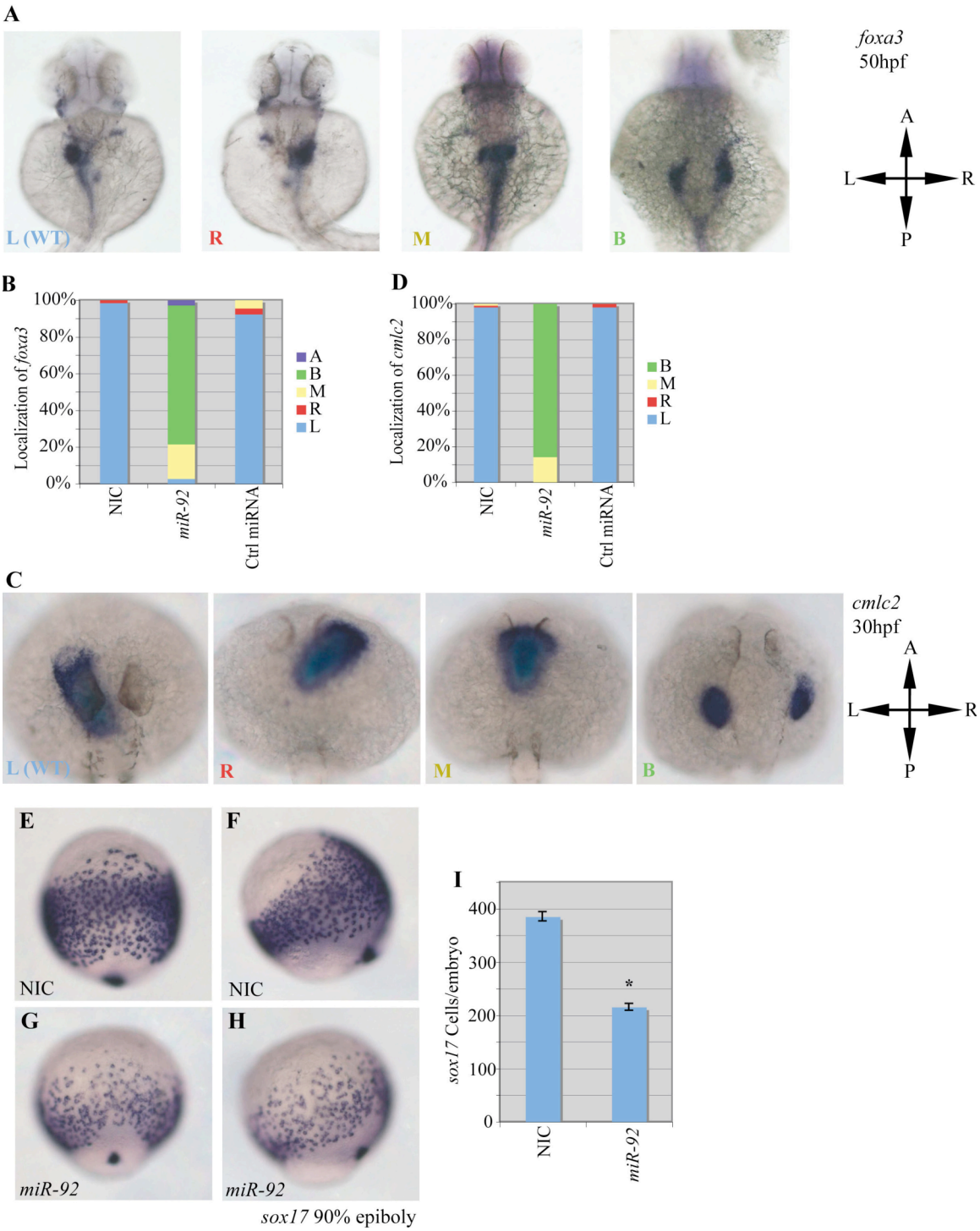

A
L (WT)
R
M
B
foxa3
50hpf
A
L
R
P
B
Localization of foxa3
100%
80%
60%
40%
20%
0%
NIC
miR-92
Ctrl miRNA
A
B
M
R
L
D
Localization of cmlc2
100%
80%
60%
40%
20%
0%
NIC
miR-92
Ctrl miRNA
B
M
R
L
C
L (WT)
R
M
B
cmlc2
30hpf
A
L
R
P
E
NIC
F
NIC
G
miR-92
H
miR-92
sox17 90% epiboly
I
sox17 Cells/embryo
400
300
200
100
0
*
NIC
miR-92

Figure 15. *miR-92* Gain-of-Function.
Gain-of-function experiments were performed by injection of single cell embryos with *miR-92* followed by localization of specific markers, as indicated.
(A) Localization of *foxa3* in wild type embryos and those injected with *miR-92* at 50hpf. All views are dorsal with anterior to the top. Pictures show representative embryos with liver primordia localized to either the left (L), right (R), midline (M), or bilateral (B) positions.
(B) Percentages of left (L), right (R), midline (M), or bilateral (B) localization of *foxa3* in non-injected control (NIC) (n=188), *miR-92* injected (n=37) and control miRNA injected embryos (n=90). In rare cases, no expression of *foxa3* was detected (absent; A).
(C) Localizion of *cmlc2* in wild type and embryos injected with *miR-92* at 30hpf. All views are dorsal with anterior to the top. Pictures show representative embryos with cardiac primordia localized to the left (L), right (R), midline (M), or bilateral (B).
(D) Percentages of left (L), right (R), midline (M), and bilateral (B) localization of *cmcl2* in non-injected controls (NIC) (n=103), *miR-92* injected (n=98), and control miRNA injected embryos (n=53).
(E-H) Localization of *sox17*-positive cells in wild type embryos and *miR-92* overexpressing embryos at 90% epiboly. (E,G) Dorsal views with anterior to the top. (F,H) Lateral views with dorsal to the right of pictures.
(I) Numbers of *sox17*-positive cells in NIC (n=16) and *miR-92* injected embryos (n=11). Error bars represent SEM. Asterisk represents statistical significance determined by Student's t-test, $p<0.01$.

The developing heart is also enriched for *miR-92* and its development depends indirectly on the proper establishment of endodermal fates. Thus we also tested the effects of *miR-92* overexpression on heart development by examining the localization of the cardiac marker *cmlc2* (*cardiac myosin light chain 2*) (Reiter, Alexander et al. 1999). In NICs, *cmlc2* localized to cardiac primordia on the left side of the embryo at 30hpf, as expected (Fig. 15C,D). In contrast, injection of *miR-92* caused over 85% of the embryos to display bilateral *cmlc2* expression indicating failure of heart fusion, referred to as cardia bifida {Reiter, 2001 #16240;Reiter, 1999 #16226;Holtzinger, 2007 #16559;Stainier, 1996 #22913}. The remaining 15% of embryos showed *cmlc2* along the midline, indicating an inability of the heart tube to undergo normal looping. The specificity of these the gain-of-function experiments was supported by the absence of

defects caused by injection of unrelated and control miRNAs on *foxa3* or *cmlc2* expression (Fig. 15B,D). Also, the effects of *miR-92* were dose dependent (Table 1).

Table 1. Dose dependent effects of *miR-92* gain-of-function.
(A) Percentages of left (L), right (R), midline (M), bilateral (B) localization of *foxa3* in non-injected controls (NIC), *miR-92* injected, and control miRNA injected embryos. Occasionally, no *foxa3* was detected (absent, A).
(B) Percentages of left (L), right (R), midline (M) and bilateral (B) localization of *cmlc2* in non-injected controls (NIC), *miR-92* injected, and control miRNA injected embryos. N represents number of total embryos analyzed in each condition.

A

foxa3	N	L	R	M	B	A
NIC	188	98.4%	1.6%	0.0%	0.0%	0.0%
miR-92 200pg	72	23.6%	22.2%	22.2%	26.4%	5.6%
miR-92 1ng	37	2.7%	0.0%	18.9%	75.7%	2.7%
miR-92 2ng	74	0.0%	0.0%	14.9%	70.3%	14.9%
Ctrl miRNA	90	92.2%	3.3%	4.4%	0.0%	0.0%

B

cmlc2	N	L	R	M	B
NIC	103	98.1%	1.0%	1.0%	0.0%
miR-92 200pg	67	11.9%	4.5%	38.8%	44.8%
miR-92 1ng	98	0.0%	0.0%	14.3%	85.7%
Ctrl miRNA	53	98.1%	1.9%	0.0%	0.0%

Overexpression of *miR-92* results in a reduction of endoderm

Viscera and cardia bifida result from the failure to coalesce the relevant mesendodermal organ progenitors at the midline during the segmentation stages and may be secondary to a variety of earlier defects in endoderm or mesoderm formation (Schier, Neuhauss et al. 1997; Alexander, Rothenberg et al. 1999; Reiter, Alexander et al. 1999; Kikuchi, Trinh et al. 2000; Reiter, Kikuchi et al. 2001; Ober, Olofsson et al. 2004; Nair and Schilling 2008). To address this issue, we examined the effects of *miR-92* gain-of-function on the expression of genes that act early in the specification and determination of endoderm and mesoderm. No significant defects were detected in mesoderm

formation with or without *miR-92* injection, as indicated by the normal expression pattern of *ntl* (Fig. 16I-P). In contrast, *miR-92* injection caused a clear and dramatic decrease in the number of early endoderm-specified cells, as indicated by decreased numbers of *sox17*- and *sox32*-(*casanova*) positive cells at the end of gastrulation (Fig. 15E-I and Supplemental Fig. 16A-H). This suggests a selective impairment in the formation of endodermal cells with no effect on the formation of the mesodermal germ layer.

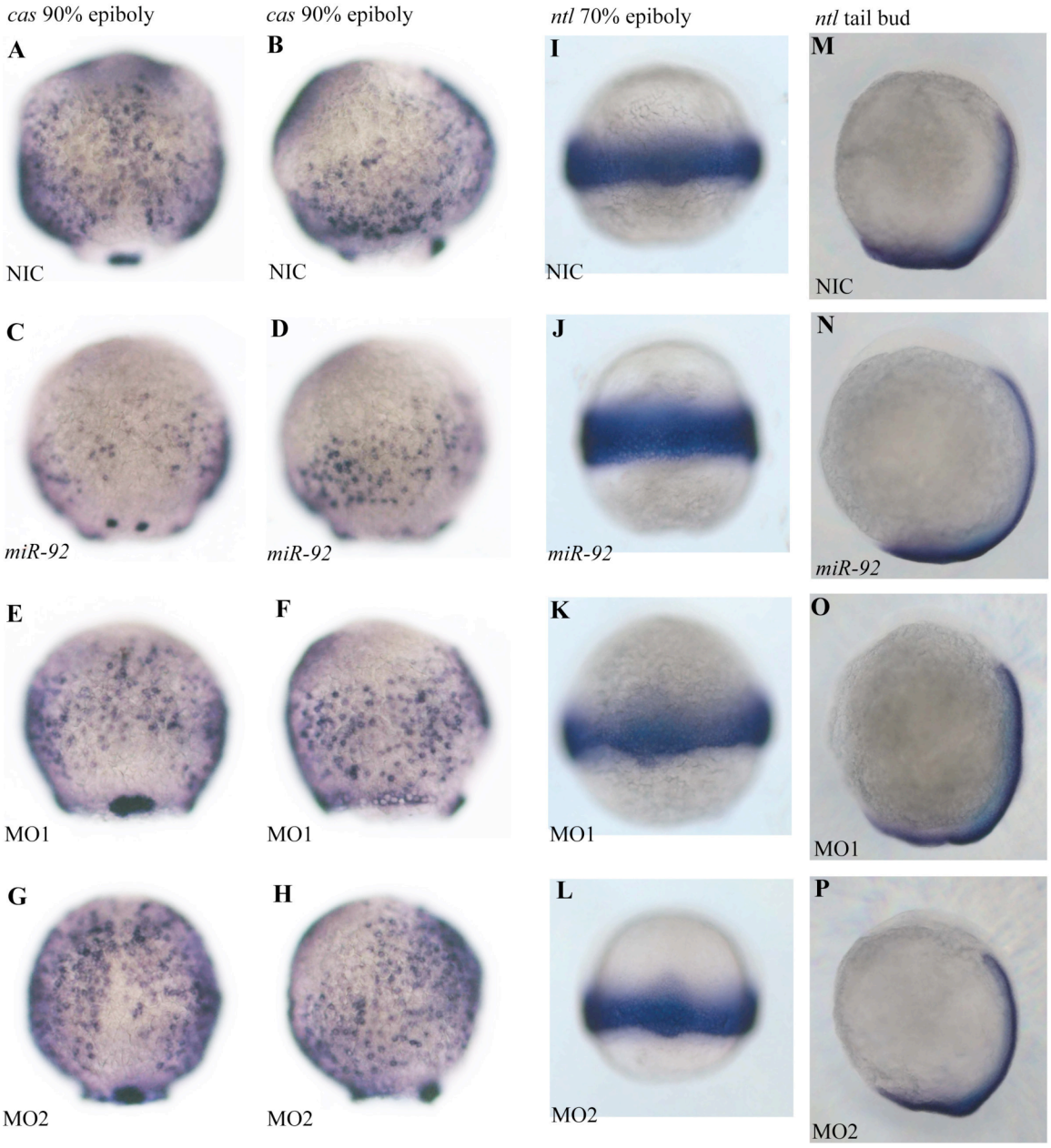


Figure 16. Expression of *casanova* and *no tail* transcripts in wild type, *miR-92* gain-of-function, and *miR-92* loss-of-function embryos.
(A,C,E,G,I-L) Dorsal views with anterior to the top. (B,D,F,H,M-P) Lateral views with embryonic dorsal to the right of pictures. *cas* expression is down-regulated by *miR-92* gain-of-function and up-regulated by *miR-92* loss-of-function. *ntl* expression is not significantly altered by mis-regulation of *miR-92*.

Depletion of *miR-92* results in aberrant left-right patterning of internal organs

We next performed loss-of-function experiments using antisense morpholinos to block *miR-92* activity during early development (Fig. 17,18). In NICs, the localization of *foxa3* to the developing liver was primarily on the left side at 50hpf (Fig. 19A). However, injection of morpholinos against *miR-92* resulted in 20-40% of the embryos displaying abnormal left-right localization of *foxa3* in the developing liver. Similarly, 40% of the morphants showed localization of *cmcl2* to either the right or the middle, compared to normal left sided heart patterning in NICs (Fig. 19B). Thus, loss of *miR-92* resulted in a significant incidence of aberrant left-right patterning.

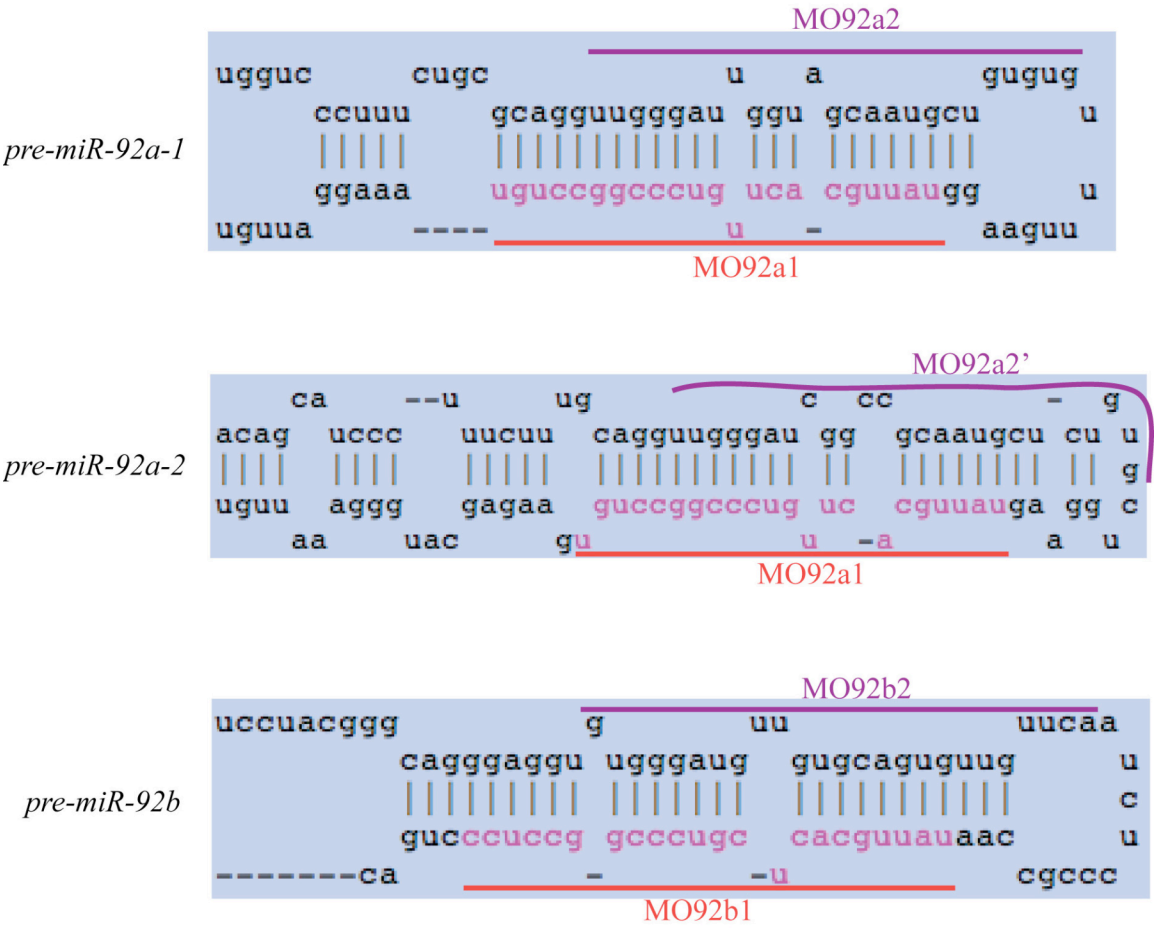


Figure 17. Morpholino design.
MO1 is a mixture of two morpholinos that target mature *miR-92a* and *b*. MO2 is a mixture of three morpholinos that target the loop regions of *pre-miR-92a* and *b*.

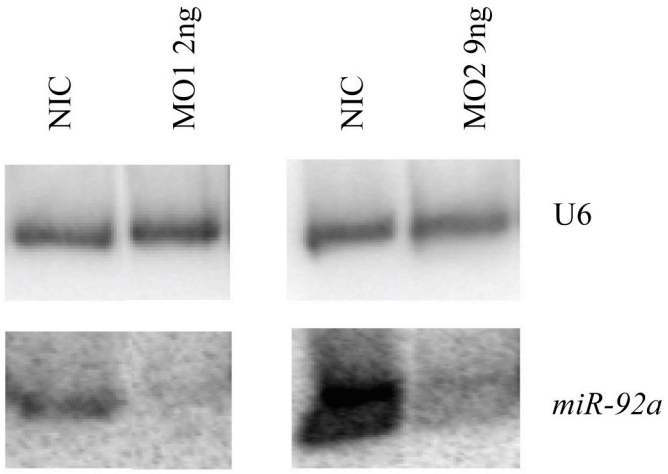
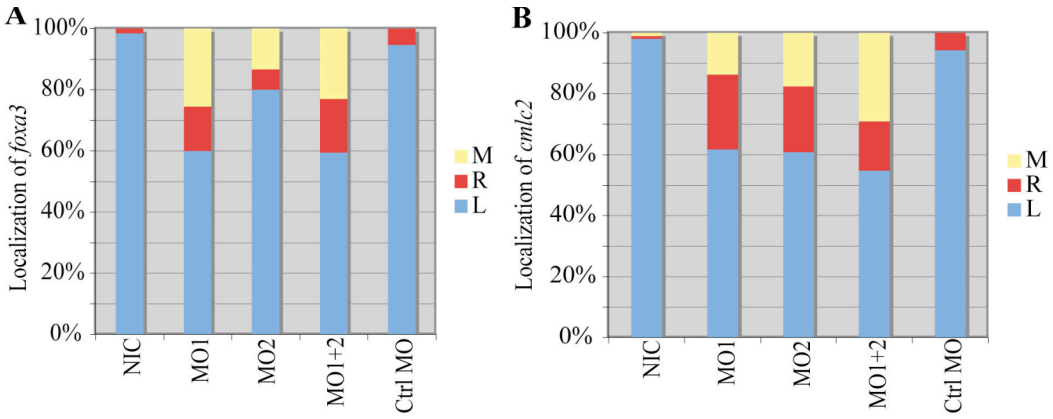
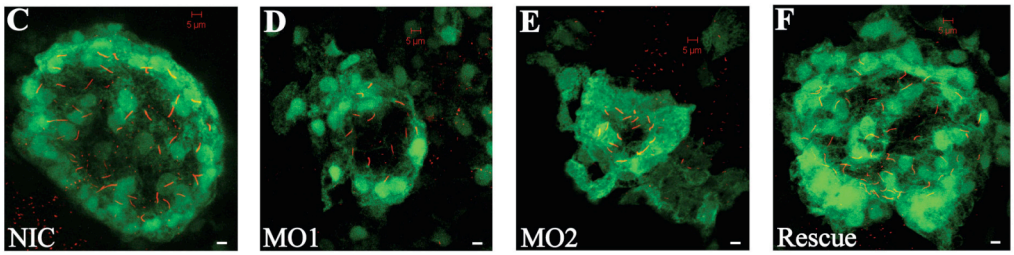
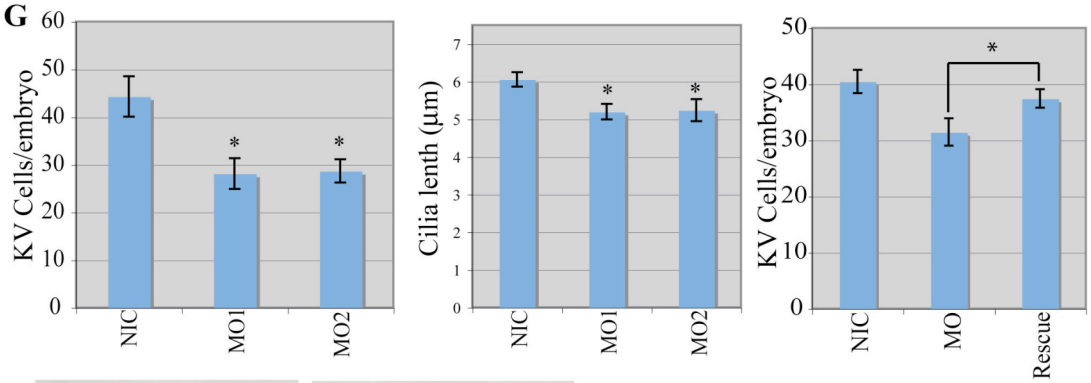


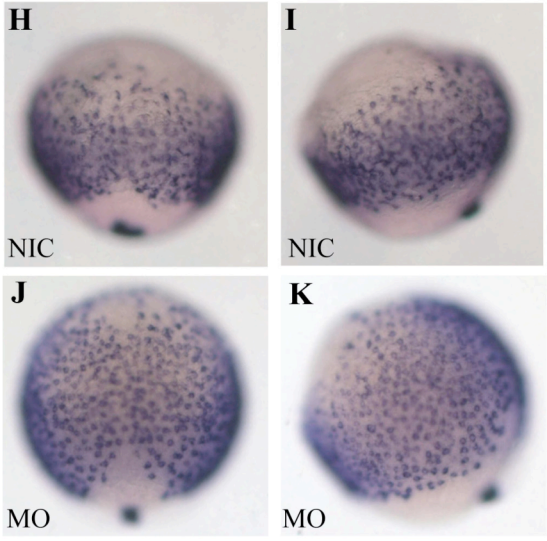
Figure 18. Knockdown of *miR-92*.
Northern blot of *miR-92* levels in the absence or presence of combinations of morpholinos targeting *miR-92*.

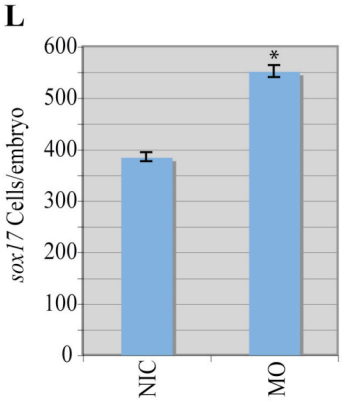




sox17:GFP Acetylated tubulin 10s







sox17 90% epiboly

Figure 19. *miR-92* loss-of-function.
Loss-of-function experiments were performed by injection of antisense morpholinos against *miR-92* into single cell embryos followed by localization of markers, as indicated.
(A) Percentages of left (L), right (R) and midline (M) localized *foxa3* in non-injected controls (NIC) (n=188), MO1 injected (n=55), MO2 injected (n=15), MO1+2 injected (n=126), and control MO injected embryos (n=56).
(B) Percentages of left (L), right (R) and midline (M) localized *cmcl2* in non-injected controls (NIC) (n=103), MO1 injected (n=102), MO2 injected (n=97), MO1+2 injected (n=62), and control MO injected embryos (n=53).
(C-F) Confocal z-stack images of Kupffer's vesicle in NICs, MO1 injected (MO1), MO2 injected embryos (MO2) and *miR-92* morphants co-injected with *miR-92* RNA into the dorsal forerunner cells (Rescue) at the10-somite stage using a *sox17:gfp* (green) transgenic line which labels KV cells green. Motile cilia were identified by immunohistochemistry with antibodies against acetylated tubulin (red).
(G) GFP-positive KV cells were counted and cilia length measured in the indicated embryos. Error bars represent SEM. Asterisks represent statistical significance as determined by Student's t-test, $p<0.01$ for KV cell number between *miR-92* morphants and NICs, $p<0.05$ for cilia length and $p<0.05$ for KV cell number between *miR-92* morphants and rescue embryos.
(H-K) Localization of *sox17*-positive cells in wild type embryos and *miR-92* morphants at 90% epiboly. (H,J) Dorsal views with anterior to the top. (I,K) Lateral views with dorsal to the right of pictures.
(L) Numbers of *sox17*-positive cells in NICs (n=16) and *miR-92* morphants (n=13). Error bars represent SEM. Asterisk represents statistical significance determined by Student's t-test, $p<0.01$.

Several lines of evidence suggest that the observed left-right asymmetry defects are specific to depletion of *miR-92*. First, two independent morpholinos (MO1 and MO2) yielded identical dose dependent results (Fig. 19A,B). Second, synergistic and similar effects were observed upon co-injection of both morpholinos at much lower doses compared to individual injections. Third, the defects were dose dependent (Table 2). Fourth, injection of control MOs did not affect left-right localization of either marker (Fig. 19A,B).

Table 2. Dose dependent effects of *miR-92* loss-of-function.
Percentages of left (L), right (R) and midline (M) localized *foxa3* in non-injected controls (NIC), MO injected, and control MO injected embryos. N represents number of total embryos analyzed in each condition.

foxa3	N	L	R	M
NIC	188	98.4%	1.6%	0.0%
MO1 400pg	10	100.0%	0.0%	0.0%
MO1 1ng	59	94.9%	3.4%	1.7%
MO1 2ng	55	60.0%	14.5%	25.5%
MO1 4ng	35	57.1%	22.9%	20.0%
MO2 1.5ng	59	88.1%	11.9%	0.0%
MO2 3ng	49	98.0%	2.0%	0.0%
MO2 6ng	48	87.5%	8.3%	4.2%
MO2 9ng	15	80.0%	6.7%	13.3%
MO1+2	126	59.5%	17.5%	23.0%
Ctrl MO	56	94.6%	5.4%	0.0%

Depletion of *miR-92* alters Kupffer's vesicle function

Specification of left-right patterning is highly regulated and propagated through several stages of embryogenesis in a complex genetically controlled program (Hamada, Meno et al. 2002; Raya and Izpisua Belmonte 2006; Bakkers, Verhoeven et al. 2009). In zebrafish, motile cilia in Kupffer's vesicle (KV) appear to be critical for left-right asymmetry, analogous to their counterparts in the node in amniotes (Amack and Yost 2004; Amack, Wang et al. 2007). Evidence gathered from both zebrafish and mouse embryos suggests a strong connection between node/vesicle structures and proper left-right patterning, especially the flow generating function of primary cilia (Nonaka, Tanaka et al. 1998; Marszalek, Ruiz-Lozano et al. 1999; Okada, Nonaka et al. 1999; Supp, Brueckner et al. 1999; Takeda, Yonekawa et al. 1999; Murcia, Richards et al. 2000; Amack and Yost 2004; Amack, Wang et al. 2007; Kreiling, Balantac et al. 2008; Schneider, Houston et al. 2008). We therefore tested for KV defects in the *miR-92*

morphants. To visualize the KV, we used a *sox17:gfp* transgenic line to label KV cells with GFP. Immediately obvious was a substantial reduction of cell numbers within KV in the *miR-92* morphants (Fig. 19C-E,G). To evaluate further the structural deficits in KV function in the *miR-92* morphants, we examined the number and length of monocilia within KV by performing immunohistochemistry using antibodies against acetylated tubulin (Essner, Amack et al. 2005). A significant reduction in cilia number was observed and a modest but significant decrease in length was also observed in *miR-92* morphants (Fig. 20, Fig. 19G). These results suggest that *miR-92* is required for the proper development of KV and cilia within KV.

To ensure that the KV defects were specific to loss of *miR-92*, we performed rescue experiments in which exogenous *miR-92* was expressed in dorsal forerunner cells (Amack and Yost 2004). Dorsal forerunner cells are the progenitor cells that form KV and if loss of *miR-92* from these cells is responsible for the observed defects, we should be able to rescue the defects by targeted *miR-92* gain-of-function experiments in the dorsal forerunner cells of morphant embryos. As shown in Fig. 19F-G, specific restoration of *miR-92* expression in dorsal forerunner cells was able to partially but significantly rescue KV size in morphant embryos. This indicates that the observed KV defects are specific to *miR-92* depletion in dorsal forerunner cells.

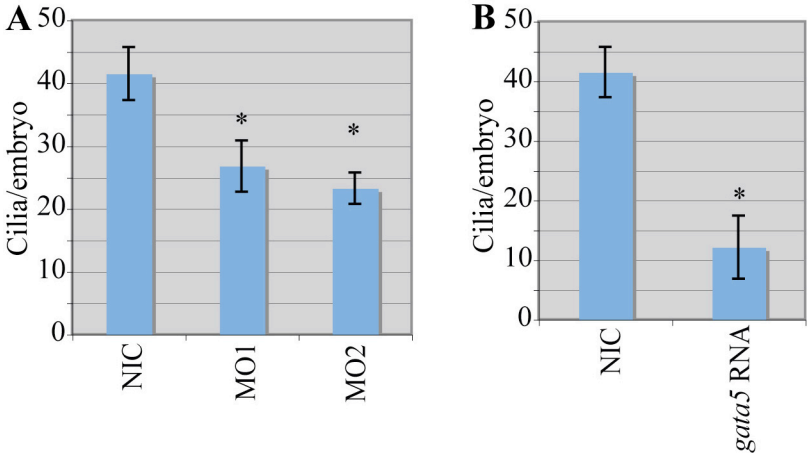


Figure 20. Kupffer's vesicle cilia number is reduced in *miR-92* morphants and *gata5* gain-of-function embryos.
(A) KV cilia were counted in NICs, MO1 injected and MO2 injected embryos.
(B) KV cilia were counted in NICs and *gata5* RNA injected embryos. Error bars represent SEM. Asterisks represent statistical significance as determined by Student's t-test, $p<0.01$.

Depletion of *miR-92* results in increased endoderm

Normal KV development from dorsal forerunner cells requires both endodermal and mesodermal signals, in addition to genes involved in general ciliogenesis (Alexander, Rothenberg et al. 1999; Amack and Yost 2004; Amack, Wang et al. 2007; Oteiza, Koppen et al. 2008; Neugebauer, Amack et al. 2009). We therefore examined the expression and localization of markers involved in both general ciliogenesis and the formation of endoderm and mesoderm. No significant defects in general ciliogenesis or mesoderm formation were detected, as indicated by normal expression of *lrdr*, *foxj1*, *fgf8* and *ntl*, as well as the normal development of motile cilia in the pronephros and inner ear (Fig. 16, 21). In contrast, loss of *miR-92* caused a dramatic increase in the number of *sox17*-positive endodermal cells and these cells were much more spread out at the end of gastrulation, indicating defects in endoderm formation (Fig. 19H-L). Consistent with

increased cell number, there was an overall 1.6-fold increase in *sox17* mRNAs in the *miR-92* morphants, as detected by quantitative RT-PCR. This increase could be suppressed by co-injection of *miR-92* (Fig. 22). Taken together, these results suggest that *miR-92* is required for proper endoderm specification and for the development of structures critical for controlling later fundamental aspects of organogenesis and overall body patterning.

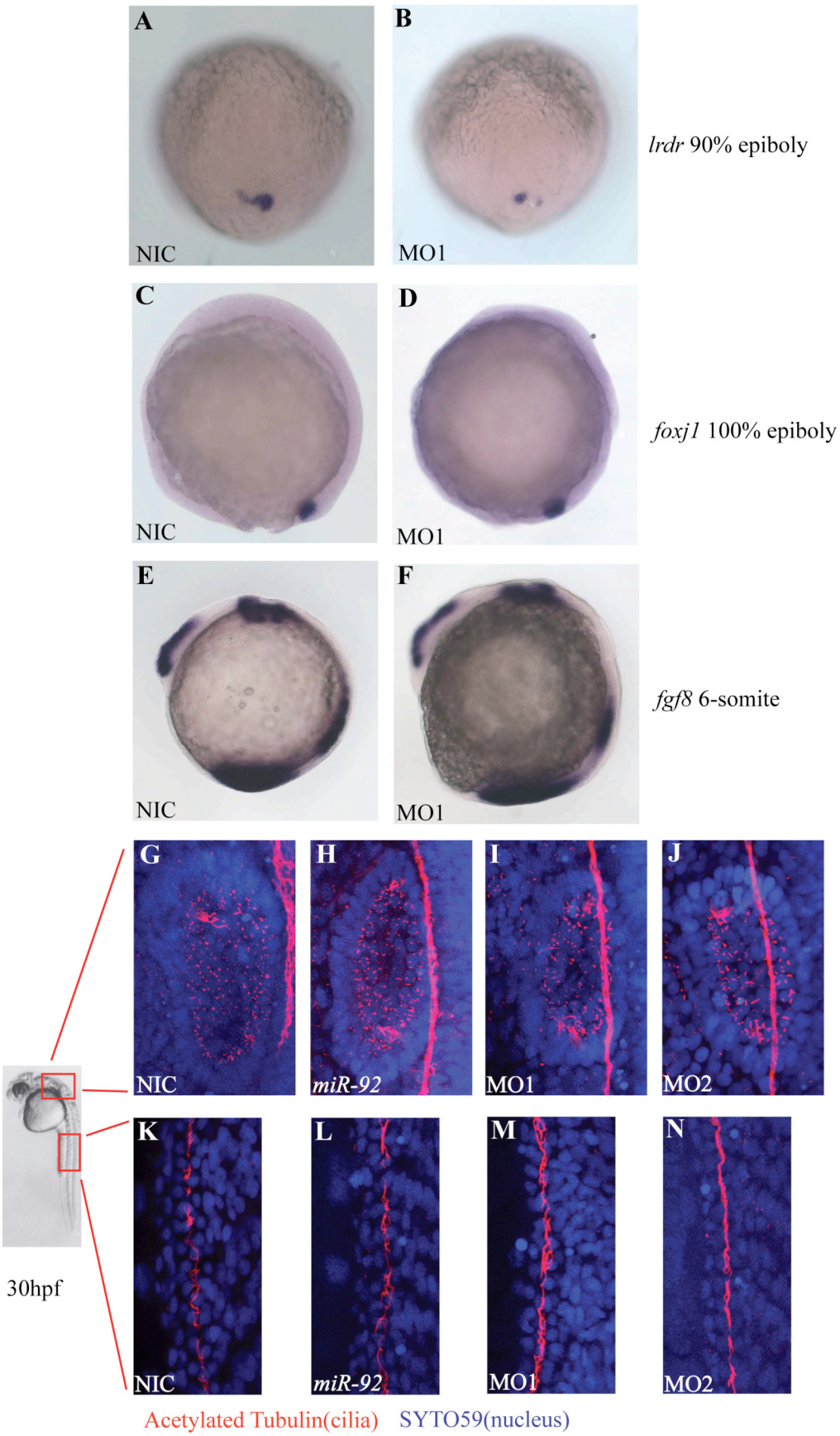

A
NIC
B
MO1
lrdr 90% epiboly
C
NIC
D
MO1
foxj1 100% epiboly
E
NIC
F
MO1
fgf8 6-somite
G
NIC
H
miR-92
I
MO1
J
MO2
30hpf
K
NIC
L
miR-92
M
MO1
N
MO2
Acetylated Tubulin(cilia)
SYTO59(nucleus)

Figure 21. Ciliogenesis is not disrupted in *miR-92* morphants.
(A-B) Expression of *lrdr* in wild type and MO injected embryos at 90% epiboly.
(C-D) Expression of *foxj1* in wild type and MO injected embryos at 100% epiboly.
(E-F) Expression of *fgf8* in wild type and MO injected embryos at the 6-somite stage.
(G-J) Motile cilia in the inner ear are intact in wild type embryos, *miR-92* gain-of-function, and *miR-92* loss-of-function embryos at 1dpf.
(K-N) Motile cilia in the pronephros are intact in wild type embryos, *miR-92* gain-of-function, and *miR-92* loss-of-function embryos at 1dpf. Red, cilia staining using antibodies against acetylated tubulin. Blue, SYTO59 staining of nuclei.

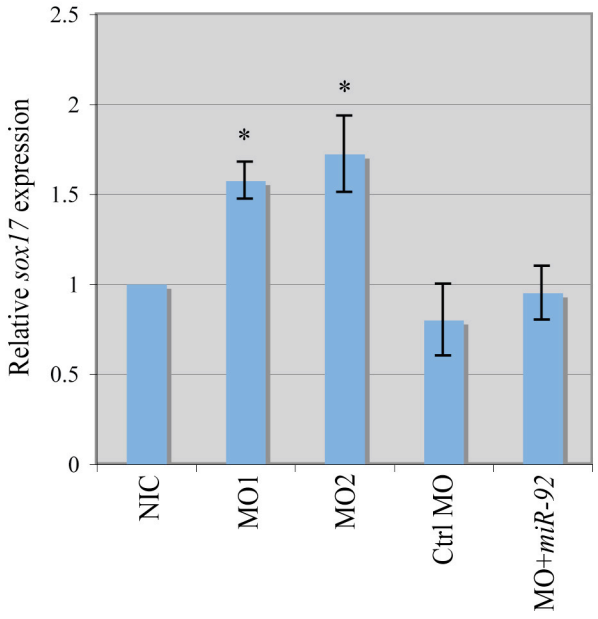


Figure 22. Quantitation of *sox17* expression.
Real time RT/PCR was performed on RNA isolated from whole embryos at 90% epiboly using primers complementary to *sox17*. A 1.6-fold increase in *sox17* levels was observed in *miR-92* morphants, but not in embryos injected with control morpholino. Co-injection of *miR-92* rescued this increase. Error bars represent SEM. Asterisks represent statistical significance between injected embryos and NICs as determined by Student's t-test, $p<0.01$, $n>3$.

gata5 is a target of *miR-92*

Online target prediction algorithms were used to search for potential targets of *miR-92*. Zinc finger transcription factor Gata-binding protein 5 (Gata5) was identified as a potential target containing two potential miRNA recognition elements (MREs) in its

3'UTR (Fig. 23A). Gata5 is a critical regulator of vertebrate endoderm development as overexpression of Gata5 results in an increase in endodermal cell numbers while reduced Gata5 expression causes in a reduction in endodermal cell numbers (Reiter, Alexander et al. 1999; Reiter, Kikuchi et al. 2001). Hence, *gata5* is a compelling *miR-92* target in the process of endoderm development.

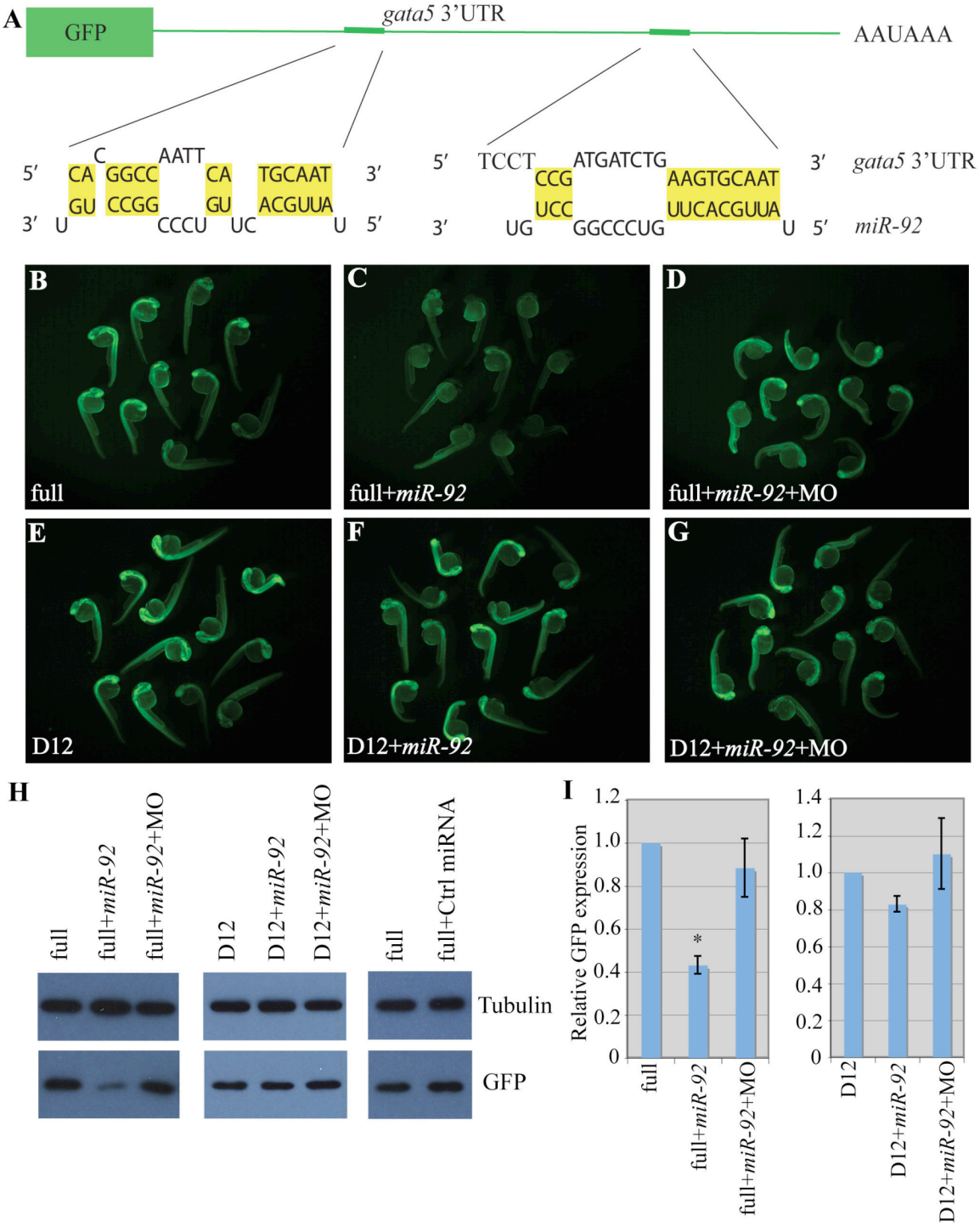

A
GFP
gata5 3'UTR
AAUAAA
5′ CA GGCC CA TGCAAT 3′
C AATT
3′ GU CCGG GU ACGUUA 5′
U CCCU UC U
5′ TCCT ATGATCTG 3′ gata5 3'UTR
CCG AAGTGCAAT
UCC UUCACGUUA
3′ UG GGCCCUG U 5′ miR-92
B full
C full+miR-92
D full+miR-92+MO
E D12
F D12+miR-92
G D12+miR-92+MO
H
full
full+miR-92
full+miR-92+MO
D12
D12+miR-92
D12+miR-92+MO
full
full+Ctrl miRNA
Tubulin
GFP
I
Relative GFP expression
1.2
1.0
0.8
0.6
0.4
0.2
0
*
full
full+miR-92
full+miR-92+MO
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
D12
D12+miR-92
D12+miR-92+MO

Figure 23. *gata5* is a target of *miR-92*.
(A) Schematic of GFP reporter fused to the 3' UTR of *gata5*. Base pairing between *miR-92* and two MREs is shown.
(B-G) Single cell embryos were injected with mRNAs encoding GFP reporters and fluorescence was monitored at 1dpf. Embyros were injected with reporters containing either the full-length *gata5* 3'UTR (full) or a construct in which both MREs were deleted (D12).
(H) Western blot of lysates from embryos injected as above were performed with antibodies against GFP or α-tubulin as a loading control.
(I) Quantification of relative GFP expression. Error bars represent SEM. Asterisk represents statistical significance between indicated injection and control embryos as determined by Student's t-test, $p<0.01$, $n>3$.

To determine if *gata5* is a *bona fide* target of *miR-92*, we analyzed the interaction between the *gata5* 3'UTR and *miR-92* using GFP reporter assays. The full-length *gata5* 3'UTR and a deletion construct lacking both MREs were cloned downstream of the GFP open reading frame. RNA transcripts from these constructs were then injected into single cell zebrafish embryos in the presence or absence of *miR-92*. The following day, GFP expression levels were monitored by fluorescence microscopy and by western blotting with antibodies against GFP. In both assays, GFP levels were reduced by *miR-92* co-injection, dependent on the presence of intact *miR-92* MREs (Fig. 23). Importantly, co-injection of *miR-92* antisense morpholinos rescued expression of GFP in the presence of *miR-92* (Fig. 23D,G-I). Also, injection of a control siRNA had no effect on GFP levels (Fig. 23H). These results suggest that *gata5* is a *bona fide* target of *miR-92*.

Regulation of *gata5* by *miR-92* during early zebrafish development

The hypothesis that *miR-92* targets *gata5* is not only supported by the reporter assays but also by previous work that showed that altered levels of *gata5* control endoderm formation, as monitored by changes in *sox17* positive cell numbers (Reiter, Alexander et

al. 1999; Reiter, Kikuchi et al. 2001). Also, loss of *gata5* has been shown to cause cardia and viscera bifida (Reiter, Alexander et al. 1999; Reiter, Kikuchi et al. 2001). However, no prior reports have implicated *gata5* in KV defects or consequent left right asymmetry defects. Thus, we sought to test directly whether *miR-92* control of *gata5* would induce KV defects and changes in body plan. If *gata5* is regulated by *miR-92* during early zebrafish development, altered expression of Gata5 should reciprocally mirror the effects caused by gain and loss of *miR-92* which then enables powerful genetic epistasis experiments to verify interaction between *miR-92* and *gata5*.

First, we confirmed that knockdown of *gata5* caused a reduction of *sox17*-positive cells at the end of gastrulation and that injection of *gata5* mRNA caused an increase in *sox17*-positive cells (Fig. 24A-G)(Reiter, Alexander et al. 1999; Reiter, Kikuchi et al. 2001). Second, we confirmed that *gata5* knockdown caused cardia and viscera bifida defects, as observed in Gata5 mutants (*faust*) and *gata5* morphants (Reiter, Alexander et al. 1999; Reiter, Kikuchi et al. 2001; Holtzinger and Evans 2007)(Fig. 24L,M). After confirming that our experimental system recapitulated earlier work, we then sought to test the effects of excess Gata5 expression on organogenesis and left-right patterning. Thus, we injected *gata5* mRNA and determined the localization of *foxa3* and *cmcl2*. We found that 45% and 7% of embryos displayed altered left-right patterning of cardia and viscera primordia, respectively (Fig.24L,M). These results show that the increase or reduction of endodermal cell numbers caused by raising or lowering Gata5 levels, together with the finding that increased and decreased levels of *miR-92* have converse effects which can be offset by altered *gata5* expression, support the idea that *miR-*

92/*gata5* regulatory interaction is involved in allocating correct endodermal cell numbers and maintaining proper left-right patterning.

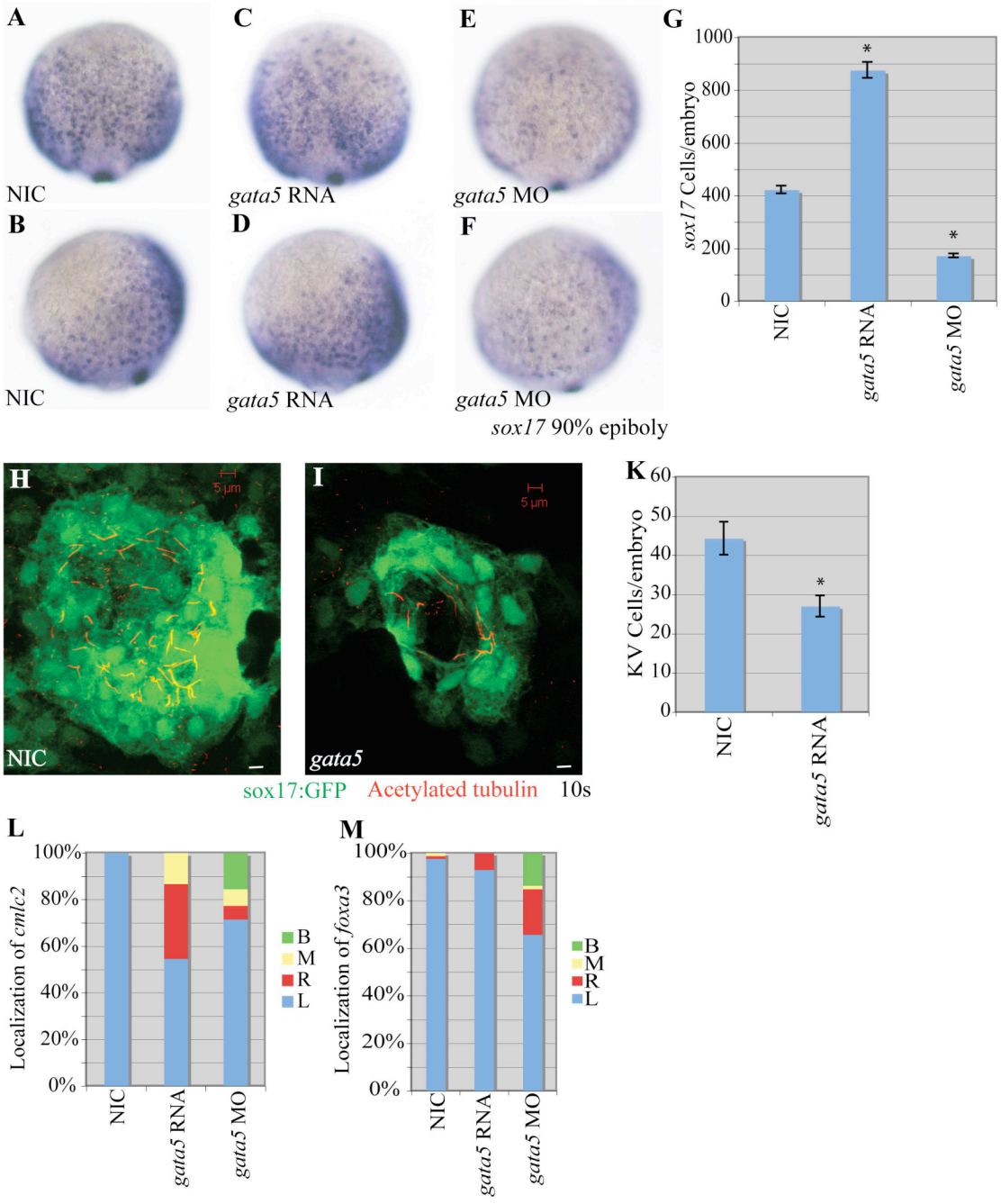

A
NIC
C
gata5 RNA
E
gata5 MO
G
sox17 Cells/embryo
1000
800
600
400
200
0
NIC
gata5 RNA
gata5 MO
B
NIC
D
gata5 RNA
F
gata5 MO
sox17 90% epiboly
H
5 µm
NIC
I
5 µm
gata5
sox17:GFP Acetylated tubulin 10s
K
KV Cells/embryo
60
50
40
30
20
10
0
NIC
gata5 RNA
L
Localization of cmlc2
100%
80%
60%
40%
20%
0%
B
M
R
L
NIC
gata5 RNA
gata5 MO
M
Localization of foxa3
100%
80%
60%
40%
20%
0%
B
M
R
L
NIC
gata5 RNA
gata5 MO

Figure 24. *gata5* effects on early zebrafish development.
(A-F) Localization of *sox17*-positive cells in wild type embryos, *gata5* gain-of-function, and *gata5* loss-of-function embryos at 90% epiboly. (A,C,E) Dorsal views with anterior to the top. (B,D,F) Lateral views with dorsal to the right of pictures.
(G) Numbers of *sox17*-positive cells in NICs (n=4), *gata5* gain-of-function (n=7) and *gata5* loss-of-function embryos (n=8). Error bars represent SEM. Asterisks represent statistical significance between injected embryos and NIC as determined by Student's t-test, $p<0.01$.
(H,I) Confocal z-stack images of Kupffer's vesicle in NIC and *gata5* injected embryos at the 10-somite stage. KVs were labeled using a *sox17:gfp* (green) transgenic line, as above. Motile cilia were labeled with antibodies against acetylated tubulin (red).
(K) KV cell number was counted, as above. Error bars represent SEM. Asterisks represent statistical significance between NIC and *gata5* gain-of-function as determined by Student's t-test, $p<0.01$.
(L) Percentages of left (L), right (R), midline (M), and bilateral (B) localized *cmcl2* in non-injected controls (NIC) (n=49), *gata5* gain-of-function (n=75), and *gata5* loss-of-function (n=84) embryos.
(M) Percentages of left (L), right (R), midline (M) and bilateral (B) localized *foxa3* in non-injected controls (NIC) (n=83), *gata5* gain-of-function (n=142), and *gata5* loss-of-function (n=73) embryos.

Because we discovered a link between miR-92 expression in dorsal forerunner cells and KV formation, it was important to determine if the Gata5-induced left-right asymmetry defects could be explained by defective KV formation. Using the *sox17:gfp* transgenic line that allows visualization of KV formation, we found a dramatic reduction in KV cell number after Gata5 overexpression (Fig. 24H-K, Fig. 20).

miR-92-mediated defects can be partially suppressed by modulation of Gata5

Genetic epistasis experiments were next performed to test the hypothesis that *miR-92* regulates *gata5*. If *miR-92* acts as a negative regulator of *gata5*, co-injection of *gata5* mRNA with *miR-92* should suppress the *miR-92* gain-of-function defects. Likewise, blocking *gata5* function should suppress the *miR-92* loss-of-function defects. As shown in Fig. 25, the reduction in the number of *sox-17* positive cells caused by overexpression

of *miR-92* could be suppressed by co-injection of *gata5* RNA. Similarly, co-injection of *gata5* morpholinos with *miR-92* morpholinos suppressed the increase in the *sox17*-positive cells (Fig. 25A-F).

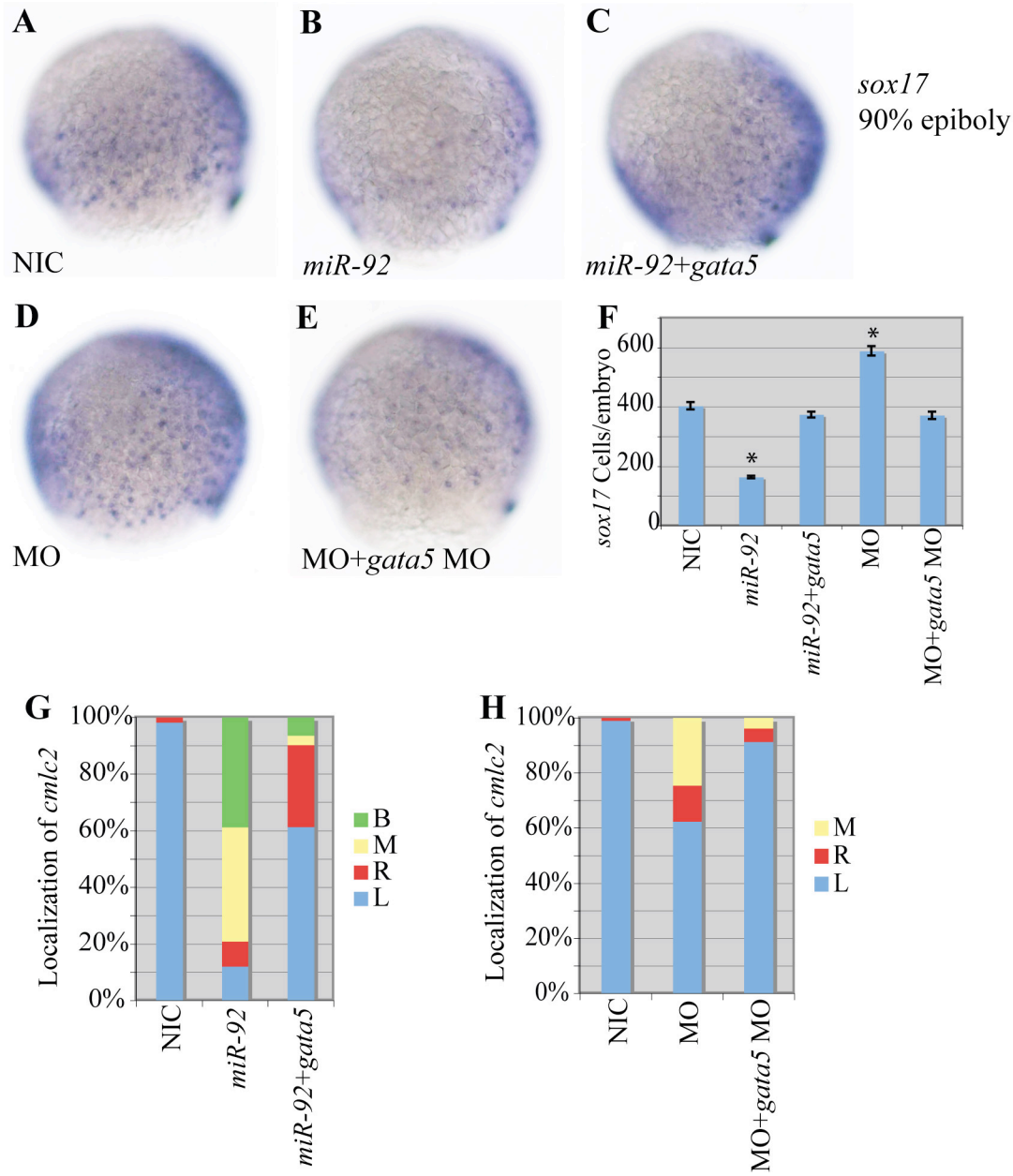

A
NIC
B
miR-92
C
miR-92+gata5
sox17
90% epiboly
D
MO
E
MO+gata5 MO
F
sox17 Cells/embryo
600
400
200
0
*
*
NIC
miR-92
miR-92+gata5
MO
MO+gata5 MO
G
Localization of cmlc2
100%
80%
60%
40%
20%
0%
B
M
R
L
NIC
miR-92
miR-92+gata5
H
Localization of cmlc2
100%
80%
60%
40%
20%
0%
M
R
L
NIC
MO
MO+gata5 MO

Figure 25. Epistatic interaction between *miR-92* and *gata5.*
(A-E) Localization of *sox17*-positive cells in wild type and injected embryos at 90% epiboly. All views are lateral with dorsal to the right.
(F) Numbers of *sox17*-positive cells in NICs (n=4), *miR-92* gain-of-function (n=4), *miR-92* and *gata5* co-injected (n=7), *miR-92* loss-of-function (n=4) and *miR-92* and *gata5* co-injected (n=7) embryos. Error bars represent SEM. Asterisks represent statistical significance between injected embryos and NIC as determined by Student's t-test, $p<0.01$.
(G) Percentages of left (L), right (R), midline (M), and bilateral (B) localized *cmlc2* in non-injected controls (NIC) (n=112), *miR-92* gain-of-function (n=67), and *miR-92* and *gata5* co-injected (n=31) embryos.
(H) Percentages of left (L), right (R), midline (M), and bilateral (B) localized *cmlc2* in non-injected controls (NIC) (n=97), *miR-92* loss-of-function (n=61), and *miR-92* and *gata5* morpholino injected (n=81) embryos.

For cardiac morphogenesis, we observed a partial but significant rescue of *cmlc2* localization upon co-injection of both *miR-92* and *gata5* RNA resulting in a reduction of cardia bifida from 40% to 6% (Fig. 25G). Similarly, blockage of *gata5* function in *miR-92* morphants resulted in a significant suppression (from 40% to 10%) of the left-right patterning defects (Fig. 25H). Thus, the effects of altered *miR-92* levels can be partially suppressed by modulating *gata5* levels. These results strongly support the hypothesis that *miR-92* regulates endoderm formation and left-right asymmetry by controlling *gata5* expression.

Discussion

Our data suggest a model in which *miR-92* is a critical regulator of early zebrafish development by precisely controlling *gata5* expression. During the blastula and gastrula stages, excess *miR-92* causes a reduction in endoderm and, at later stages, cardia/viscera bifida by repressing *gata5* expression. In contrast, reduced levels of *miR-92* cause increased endoderm formation, defects in Kupffer's vesicle development, and, at later

stages, abnormal left-right patterning of internal organs. Together, we have uncovered a novel function for *miR-92* in controlling endoderm formation and left-right asymmetry by modulating *gata5*.

Function of *miR-92* during early development

The *miR-92* family is among the earliest expressed miRNAs during zebrafish development beginning at the mid-blastula stage. Only a limited number of miRNAs have been analyzed for their roles during these early stages when the germ layers develop and the body axes are patterned (Choi, Giraldez et al. 2007; Martello, Zacchigna et al. 2007; Rosa, Spagnoli et al. 2009). The zebrafish *miR-430* family is extraordinarily abundant at the mid-blastula transition to target maternal messages for decay (Giraldez, Mishima et al. 2006). In addition, zebrafish *miR-430* and its Xenopus homolog, miR-427 are known to dampen and balance both agonists and antagonists of nodal signaling (Choi, Giraldez et al. 2007; Rosa, Spagnoli et al. 2009). Loss of these miRNAs leads to an overall reduction of nodal signaling output with reduced endoderm formation. Also, *miR-15* and *miR-16* restrict the size of the organizer in Xenopus by targeting the nodal receptor Acvr2a. These miRNAs are ventrally enriched by canonical wnt signaling and act as translators of wnt gradients to control nodal responsiveness along the dorsal-ventral axis (Martello, Zacchigna et al. 2007). Here, we have shown a novel role for *miR-92* in early zebrafish development in the process of endoderm formation, Kupffer's vesicle function, and left-right patterning via the control of *gata5*. To our knowledge, this is the first time that a miRNA has been identified as regulator of endoderm formation, vertebrate left-right asymmetry, and KV development.

Regulation of *gata5* by *miR-92*

The precise regulation of Gata5 expression is not well understood. During zebrafish endoderm formation, *gata5* is transcriptionally up regulated by phosphorylated Smad2 (Shivdasani 2002). Hypermethylation of the Gata5 promoter region has also been reported in various human cancer cell lines, concomitant with down-regulation of *gata5* transcription (Akiyama, Watkins et al. 2003; Guo, Akiyama et al. 2004; Guo, House et al. 2006; Hellebrekers, Lentjes et al. 2009). Our results suggest that *gata5* is subject to post-transcriptional regulation by *miR-92*. Three lines of evidence support this hypothesis. 1) *miR-92* silenced GFP reporter expression dependent on an intact *gata5* 3'UTR (Fig. 23). 2) The effects of gain- and loss-of-function experiments with *gata5* are opposite to those observed with *miR-92* (Fig. 24). 3) *miR-92*-induced defects could be substantially suppressed by changing concentrations of *gata5* in epistasis experiments (Fig. 25).

As a downstream effector of Nodal signaling and a regulator of endoderm specification, Gata5 needs to be precisely controlled, both temporally and spatially. In zebrafish, *gata5* mRNA is found within 5 cell diameters from the blastoderm margin at the onset of gastrulation and overlaps with definitive endodermal cells during gastrulation (Warga and Nusslein-Volhard 1999; Reiter, Kikuchi et al. 2001). Either excess expression or depletion of Gata5 alters endodermal cell numbers at the end of gastrulation (Reiter, Kikuchi et al. 2001). We hypothesize that *miR-92* contributes to the establishment of proper *gata5* expression patterns during the blastula and gastrula stages which guide differentiation and allocation of proper endodermal cell numbers during gastrulation.

Interestingly, the *miR-17-92* cluster is up regulated in lung cancer cell lines whereas *gata5* is down regulated (Guo, Akiyama et al. 2004; Volinia, Calin et al. 2006). It is tempting to hypothesize that both transcriptional regulation via DNA methylation and post-transcriptional regulation via *miR-92* contribute to the silencing of *gata5* in lung cancer. It will be interesting to determine if *gata5* is a general target of *miR-92* in other types of tumors.

Misregulation of Gata5

We observed a decrease in *sox17*-positive cell numbers upon *miR-92* overexpression or *gata5* knockdown (Fig. 15,24). We also observed both cardia and viscera bifida in these embryos. The mechanisms underlying both cardia and viscera bifida are not fully understood. It will be interesting to elucidate how endoderm defects cause cardia/viscera bifida. An increase in endoderm formation was observed upon *miR-92* knockdown or *gata5* overexpression (Fig. 19,24), but it is yet not clear whether endoderm cell numbers increase at the expense of reduced mesoderm cell numbers. Endoderm and mesoderm share common progenitors; endodermal cells become specified from mesodermal cells as a result of stronger Nodal signaling (Schier, Neuhauss et al. 1997; Warga and Nusslein-Volhard 1999). Perhaps *miR-9*2 regulation of *gata5* affects endoderm versus mesoderm fate decisions or perhaps reflects control of overall cell numbers within the endodermal lineage.

Examining the localization of *cmlc2* and *foxa3* surprisingly revealed left-right patterning defects in internal organs upon either loss of *miR-92* or overexpression of *gata5*. This is the first evidence, to our knowledge, for a regulatory function for *gata5* on

left-right asymmetry and also the first to demonstrate defects in viscera morphogenesis upon *gata5* overexpression. Previously, overexpression of *gata5* was found to cause expansion and ectopic development of cardiac tissue (Reiter, Alexander et al. 1999; Holtzinger and Evans 2007). Here, we did not observe significant expansion of *cmlc2* expression and we did not observe ectopic localization except for on the right side or midline in both the *gata5* gain-of-function and *miR-92* loss-of-function experiments. One possible explanation for the observed differences could be due to different dosages used for *gata5* RNA injection in the two studies.

The exact causes of the observed KV defects upon altered *miR-92* and *gata5* levels remain to be determined. Development of Kupffer's vesicle from dorsal forerunner cells (DFCs) is a complex procedure and is not fully understood (Oteiza, Koppen et al. 2008). Both cell autonomous and non-autonomous signals contribute to the induction, migration, proliferation and polarization of DFCs (Amack and Yost 2004; Amack, Wang et al. 2007; Choi, Giraldez et al. 2007; Oteiza, Koppen et al. 2008). We observed impaired KV development upon *miR-92* loss-of-function which could be suppressed by restoration of *miR-92* expression specifically within DFCs of *miR-92* morphants. This suggests that the requirement for *miR-92* during KV development is cell autonomous although other signaling or mechanisms cannot be excluded. Previously, diminished KVs were found in embryos with decreased endodermal cell numbers (Alexander, Rothenberg et al. 1999). Here, both *miR-92* loss-of-function and *gata5* gain-of-function experiment resulted in an increase in endodermal cell numbers and smaller KVs, but it remains unclear at what stage of development the KV defects arise.

miR-92 targets

One explanation for the partial suppression of *miR-92* defects in epistasis experiments with *gata5* is that *gata5* is not likely the only target of *miR-92*. Consistent with an oncogenic function for *miR-92*, many genes involved in cell cycle control, proliferation or apoptosis are predicted to be targets of *miR-92*. During normal gastrulation movements, endodermal cell migrate dorsally in a characteristic pattern. Besides an increase in endodermal cell number, depletion of *miR-92* caused endodermal cells to spread out more during migration which may indicate additional defects in cell adhesion or migration. This phenotype was not observed in *gata5* overexpressing embryos suggesting that *miR-92* may regulate genes involved in cell adhesion or migration. If true, this might be another way in which *miR-92* contributes to cancer by controlling cell migration and metastasis.

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CHAPTER IV

SUMMARY AND CONCLUSIONS

The research presented in this dissertation demonstrates that weak MREs (miRNA recognition elements) can work synergistically for gene silencing. This advances our understanding towards the sequence requirement for miRNA:mRNA interaction and facilitates the construction of better rules for computational target prediction. We have also identified a novel regulatory function for *miR-92* during endoderm formation, KV function, and left-right patterning. My data suggest a model in which *miR-92* is a critical regulator of early zebrafish development by precisely controlling *gata5* expression. During the blastula and gastrula stages, excess *miR-92* leads to a reduction in endoderm and, at later stages, cardiac/viscera bifida by repressing *gata5* expression. In contrast, reduced levels of *miR-92* lead to increased endoderm, defects in Kupffer's vesicle development and, at later stages, abnormal left-right patterning of internal organs by up-regulation of *gata5*. Taken together, we have uncovered a novel function for *miR-92* in controlling endoderm formation and left-right asymmetry by modulating *gata5*.

This work demonstrates that miRNAs play important roles during early development contributing to the establishment of signals that are required for proper cell specification and differentiation.

Significance and Future Directions

miRNA:mRNA interaction

To date, it remains a challenge to computationally identify miRNA target in animals, largely due to the lack of reliable rules for target prediction. Current bioinformatic prediction algorithms rely heavily on the extensive seed pairing between miRNAs and their targets (Bartel 2009). Perfect seed pairing has been considered to be both required and sufficient for miRNA function (Kloosterman, Wienholds et al. 2004; Wang, Love et al. 2006; Easow, Teleman et al. 2007). However, our data provide evidence that perfect seed pairing is not necessary for miRNA:mRNA interaction. A combination of multiple weak sites can work synergistically and induce silencing as effectively as a single perfect site. This finding is important because it indicates that current algorithms have probably underestimated a large number of *bona fide* targets by sticking to the seed rule. Indeed, in agreement with this possibility, experimental studies have often identified targets that violate the seed rule (Reinhart, Slack et al. 2000; Johnston and Hobert 2003; Flynt, Li et al. 2007; Stern-Ginossar, Elefant et al. 2007). Therefore, an update of current algorithms is clearly needed. The weight placed on seed pairing should be reduced and instead, other features in the 3'UTR, including multiple MREs, should be granted a much higher weight.

Here, we showed that a single miRNA can efficiently down regulate gene expression by simultaneously targeting multiple MREs in the 3'UTR. Since a given 3'UTR usually contains potential MREs for different miRNAs, I hypothesize that different miRNAs can

also act synergistically on a single 3'UTR in a similar manner. How can multiple weak sites work synergistically? Perhaps transient and unstable recruitment of RISCs to the 3'UTR may be guided by interactions between the miRNA and each weak MRE. When several weak MREs are present in the 3'UTR in tandem, it is possible that persistent and stable recruitment of RISCs to the 3'UTR can be achieved as a result of association-dissociation dynamics, leading to comparable effects on gene silencing. In other words, small silencing signals from individual miRNA:MRE pairs can facilitate dynamic RISC recruitment, resulting in larger effects on gene silencing. This is promising because it suggests that instead of an "all or none" model, a synergistic model may be more precise for miRNA-mediated gene silencing. This mode of gene regulation allows more subtle control of gene expression instead of an "on or off" switch. It also allows the cell to precisely respond to different combinations of miRNAs.

Regulation of protein coding genes by miRNAs

It is known that protein production and activity can be regulated at multiple levels, such as transcription, mRNA localization, mRNA stability, protein modification and degradation. How does the cell choose one mechanism over another? Obviously, the answer is that all modes of regulation combine to properly regulate gene expression. Here, I have focused on another layer of gene regulation mediated by miRNAs. One question is why regulate gene expression by miRNAs? Why utilize miRNA regulation over other possible regulatory cascades?

I have shown that Gata5 is post-transcriptionally down regulated by *miR-92* during early zebrafish development. At the same time, Gata5 is transcriptionally induced by

nodal signaling (Schier 2003). Why is Gata5 regulated through distinct mechanisms? I speculate that Nodal signaling is required to turn on Gata5 expression, while *miR-92* is required to fine tune its expression level and pattern. For proper endoderm formation, both Nodal signaling and downstream transcription factors need to be precisely controlled (Schier 2003). *gata5*, as a downstream effector of Nodal signaling, is expressed within 5 cell layers from the blastoderm margin. It is very likely that *miR-92* contributes to the establishment of the restricted expression pattern of *gata5*, by locating in adjacent domains. It is also possible that *miR-92* regulates the absolute expression level of Gata5 by locating in the same expression domains.

More generally speaking, my work, along with others, suggests that the advantages of miRNA-mediated regulation include partial, reversible and rapid silencing of targets. As seen in the GFP reporter assays, miRNAs seldom completely abolish protein production, but result in partial silencing. Also, it has been shown that in some circumstances the repression of targets can be released (Bhattacharyya, Habermacher et al. 2006; Mishima, Giraldez et al. 2006; Schratt, Tuebing et al. 2006). Finally, compared to transcriptional regulation, miRNA-mediated regulation can exhibit much more rapid effects on protein production through inhibiting translation. As a result, miRNAs can first, "tune" the levels of actively transcribed genes (Mishima, Abreu-Goodger et al. 2009; Shkumatava, Stark et al. 2009), second, sharpen gene expression domains (Leucht, Stigloher et al. 2008; Morton, Scherz et al. 2008; Woltering and Durston 2008; Zeng, Carter et al. 2009), and third, quickly modulate the cells response to extracellular signals (Choi, Giraldez et al. 2007; Flynt, Li et al. 2007; Clouthier 2008; Eberhart, He et al. 2008; Flynt, Thatcher et al. 2009). These features make miRNA-mediated silencing a very powerful mechanism for

regulation of gene expression, in addition to just cleaning up junk messages in the cell (Giraldez, Mishima et al. 2006).

Formation of Kupffer's vesicle

Genetic pathways underlying KV morphogenesis have not been clearly elucidated, although the roles of Nodal signaling, Ntl, Tbx16, and intracellular calcium have been implicated in some studies (Amack and Yost 2004; Essner, Amack et al. 2005; Amack, Wang et al. 2007; Oteiza, Koppen et al. 2008; Schneider, Houston et al. 2008). Here, I uncovered two potential regulators of KV morphogenesis, *miR-92* and Gata5. Whether they function through direct or indirect means remains a question.

I have shown that reductions in KV function are accompanied by an increase in endoderm formation upon altered *miR-92* or Gata5 expression. It is, however, not clear whether reduced KV function is due to the increase in endoderm formation or due to altered Gata5 expression. Here, I propose two hypotheses. First, reduced KV function is secondary to increased endoderm through a mechanical mechanism. It is possible that an increase in endoderm results in a more crowed neighborhood around the DFCs/KV and more intensive cell-cell physical interactions, which may negatively affect ingression or migration of DFCs or enlargement of the KV lumen. Reduced KV function may therefore result from altered physical and/or mechanical pressure from surrounding cells. Second, reduced KV function may be an indirect effect of excess Gata5 which negatively affects Nodal signaling through a feedback loop. Nodal signaling positively regulates the number of DFCs in a non-cell-autonomous manner (Oteiza, Koppen et al. 2008). Some of its downstream effectors are known to enhance or antagonize signaling (Schier 2003). I

speculate that such a regulatory role may also be true for Gata5. Excess Gata5 expression may somehow down regulate Nodal signaling, resulting in reduced DFCs and at later stage, reduced KV. In this case, the KV morphogenesis defect is secondary to altered Gata5 expression in a non-cell-autonomous manner.

Regulation of miRNAs

I have shown that altered *miR-92* expression causes defects in endoderm formation. This strongly argues that both temporal and spatial regulation of *miR-92* expression is critical for proper specification of zebrafish endoderm. *miR-92* is expressed beginning at the mid-blastula stage and persists through gastrulation, consistent with its regulatory function in endoderm formation. However the exact localization of *miR-92* during these stages is unclear. One possibility is that *miR-92* expression is either in overlapping domains with *gata5* to dampen its absolute level or in neighboring domains to shape its expression boundaries. One interesting question is how the temporal and spatial expression of *miR-92* is achieved. Transcriptional regulation may account for one of the mechanisms. Since *miR-92* is transcriptionally regulated by c-Myc and E2Fs in mammalian cell lines (O'Donnell, Wentzel et al. 2005; Sylvestre, De Guire et al. 2007; Woods, Thomson et al. 2007), it will be interesting to know whether this is also true during early zebrafish development.

miRNAs regulate a variety of biological and physiological events. Many miRNAs exhibit temporal- or spatial-specific expression patterns. Mis-regulation of miRNAs expression can cause severe consequences such as morphological defects and cancers (see chapter I). What regulates the regulators remains one of the most intriguing

questions in the miRNA field. It has been found that miRNAs can be regulated at multiple levels (see section I). Regulation of pri-miRNA transcription is relatively easy to study since miRNAs share similar promoters as protein coding genes (Ozsolak, Poling et al. 2008). In contrast, post-transcriptional regulation of miRNA is barely understood. Answers to these questions will be helpful to obtain better knowledge on miRNA function as well as specific biological or physiological events.

Summary

In closing, my studies have improved our knowledge about miRNAs focusing on two areas. First, I have shown that multiple weak pairing elements can silence as efficiently as a single perfect site illustrating deficiencies in current prediction algorithms. Second, my work also demonstrates that miRNA regulation is crucial for early vertebrate development.

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