SMALL RNAS AND GENE SILENCING IN ZEBRAFISH

By

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Dissertation

Submitted to the Faculty of the

Graduate School of Vanderbilt University

In partial fulfillment of the requirements

For the degree of

DOCTOR OF PHILOSPHY

In

Biological Sciences

August 2014

Nashville, Tennessee

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ACKNOWLEDGEMENTS

Matriculation through graduate school has been a challenging yet fulfilling journey achieved with the aid of key individuals. First of all, I want to thank my mentor, Dr. James "Jim" Patton for accepting me as a transfer student into his laboratory and allowing me the independence to conduct ground breaking research in the world of small noncoding RNAs. He provided me with sound advice, intellectual input, and demanded the best in terms of quality of research, oral presentations, and scientific communication. He is a fantastic mentor that is able to recognize strengths and weaknesses of each of his students and helps to improve these areas. Members of the Patton laboratory work as a team and each student brings incredible insight and intellect to the work environment. I learned so much from every one especially Dr. Chunyao Wei, Kamya Rajaram, Diana Cha, and Nergis Kara. The entire atmosphere was unique and being with these highly motivated individuals helped shape me as a scientist and I am grateful to have been in the lab with them. I also want to thank Qiang Guan who took excellent care of the fish facility, helped me tirelessly by providing embryos for my experiments, and was very supportive. I would also like to thank the entire fish community (with special thanks to Daniel Levic and Chuck Williams) for their helpful input, Brian McKenna for his kindness and teaching me ChIP as well as the program of developmental biology that provided me with a breadth of knowledge in the field of development biology.

I give many thanks to my thesis committee members, Dr. David Bader, Dr. Joshua Gamse, Dr. Charles Hong, Dr. Charles Singleton, and Dr. Christopher Williams for their unwavering support, open door policy, with critical criticisms and useful suggestions in my ongoing research. They are all incredible scientists and I truly valued the lessons learned and

opportunity to scientifically engage with them throughout my tenure at Vanderbilt University. An extraordinary thank you goes to Dr. Roger Chalkley who was an unofficial member of my committee and the main support system that allowed me to transition from my former lab to the Patton laboratory. Dr. Chalkley listened to me and supported me academically as well as financially through the IMSD program during the turning point of my graduate career and I am forever grateful for his belief and investment in me. I also want to thank Dr. Linda Sealy for her support throughout my graduate career and especially allowing me to attend ABRCMS conference in San Jose, California to recruit for IMSD.

I want to thank my mom, Francisca Enaholo, who has been my rock and motivation for persevering through this journey. Her faith in my abilities and encouragement throughout has been invaluable. I also want to thank my grandmother for always being there. A heartfelt thank you goes to my friends and extended family in Maryland, U.S.A, Africa, & Europe for their love and support, my college girlfriends for keeping me grounded, my adopted families in Nashville for their love and keeping me focused, and for the friends I've made throughout Vanderbilt who have helped me grow as a person. I would especially like to thank Dr. Ernest Yufenyuy, Dr. Ashok Sharma, Dr. Dawit Jowhar and soon to be PhD holders Neil Dani and Gloria Laryea for walking this journey with me till the end.

The work reported below was supported by IMSD (Initiative for Maximizing Student Diversity) grant 5GM062459-10 awarded to Dr. Roger Chalkley and Dr. Linda Sealy from NIH.

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LIST OF ABBREVIATIONS

cDNA ..... complementary deoxyribonucleic acid

CG ..... Convergent Genes

ChIP ..... Chromatin Immunoprecipitation

CMV ..... Cytomegalovirus

CRISPR ..... Clustered regularly interspaced short palindromic repeats

CT ..... Convergent Transcription

DGCR8 ..... DiGeorge Syndrome Critical Region 8

DIC ..... Dye injected control

DNA ..... deoxyribonucleic acid

DOC ..... sodium deoxycholate

dpf ..... days post fertilization

dsRNA ..... double stranded RNA

EDTA ..... ethylenediaminetetraacetic acid

ENU ..... N-ethyl-N-nitrosourea

F1 ..... first filial

F2 ..... second filial

GAPDH ..... Glyceraldehyde 3-phosphate dehydrogenase

GFP ..... green fluorescent protein

gRNA ..... guide ribonucleic acid

HCL ..... hydrochloric acid

Hela cells ..... Henrietta Lacks cancer cells

hpf ..... hours post fertilization

HP1 ..... heterochromatin binding protein 1

H3K9me3 ..... trimethylation of lysine 9 on histone 3

IgG ..... immunoglobulin G

LiCl ..... lithium chloride

lncRNAs ..... long noncoding RNAs

miRNA ..... microRNA

MO ..... morpholino

mRNA ..... messenger RNA

NaCl ..... sodium chloride

$NaHCO_3$ ..... sodium bicarbonate

NLS ..... nuclear localization signal

NON-CT ..... Non Convergent Transcription

NOSpro ..... nopaline synthase promoter

nt ..... nucleotide

OEP ..... One Eyed Pinhead

ORF ..... Open Reading Frame

PK ..... Proteinase K

PTEN ..... Phosphatase and tensin homolog

PTGS ..... Post-transcriptional gene silencing

qPCR ..... quantitative polymerase chain reaction

RDRC ..... RNA-directed RNA polymerase complex

RISC ..... RNA-induced silencing complex

RITS ..... RNA-induced transcriptional silencing complex

RNA ..... Ribonucleic acid

RNAi ..... Ribonucleic acid interference

SDS ..... sodium dodecyl sulfate

shRNA.....short hairpin ribonucleic acid

siRNA ..... small interfering ribonucleic acid

ssDNA..... single stranded deoxyribonucleic acid

TALENs.....transcription activator like effector nucleases

TE..... Tris-EDTA buffer solution

TGS ..... Transcriptional gene silencing

TRBP.....the human immunodeficiency virus transactivating response RNA-binding protein

UIC.....uninjected control

UTR..... Untranslated region

WT .....wildtype

ZFN ..... zinc finger nucleases

CHAPTER 1

INTRODUCTION

RNAi discovery and downstream mediators

RNA interference (RNAi) was demonstrated in a landmark article that revealed potent sequence specific gene silencing via double-stranded RNA injection in *C. elegans* (Fire et al., 1998). These ground-breaking experiments revealed that double-stranded sequences corresponding to exonic sequences produced silencing of complementary mRNA transcripts, whereas intronic and promoter sequences had no effect. Specifically, sense or antisense RNA individually had modest to little effect on the target gene compared to mutant phenotypes observed with double-stranded RNA (dsRNA). A similar twitching phenotype in progeny of injected animals was produced following injection of *unc-22* dsRNA when compared to animals with loss-of-function mutations, consistent with disruption of myofilament protein motile function. This novel silencing mechanism was capable of crossing cellular boundaries and traversing into the germline. Additionally, the robust silencing and targeting of genes distant from the site of injection suggested the involvement of a catalytic component and/or RNA-transport mechanism. This essential discovery ushered in an era of reverse genetic studies aimed at deciphering the RNAi pathway and elucidating key mediators of this process. The rest of this introduction will highlight major milestones in elucidating the RNAi pathway and focus on two important mediators of RNAi---microRNAs (miRNAs) and small interfering RNAs (siRNAS). The history of these small ~23-25 nucleotide (nt) noncoding RNA molecules and their roles in gene regulation including post-transcriptional gene silencing

(PTGS – referring to RNA suppression) and transcriptional gene silencing (TGS – referring to DNA suppression) will be expanded upon.

miRNA and siRNA biogenesis and Post-Transcriptional Gene Silencing

Vertebrate model systems paved the way in the discovery of miRNAs, with the initial discovery in *C. elegans*. Consistent with the interference of mRNA expression through binding with an RNA via sequence complementarity, the detection of miRNAs as small RNA molecules with antisense sequences to mRNAs suggested an RNAi-like mechanism. However, the novel discovery of the first miRNA *lin-4* in 1993 wasn't fully appreciated until after the Fire and Mello breakthrough of RNAi five years later (Lee et al., 1993). RNAi provided a logical explanation for the behavior observed with inhibition of LIN-14 protein production by *lin-4* during the first larval steps of *C. elegans* development. Chromosomal walking and transformation rescue analysis showed that *lin-4* encoded a small RNA and genomic comparison indicated that *lin-4* had sequence complementarity to the 3'untranslated region (UTR) of the mRNA encoding LIN-14. These observations led to the hypothesis that *lin-4* inhibited LIN-14 protein levels via an antisense RNA-RNA interaction. Interestingly, *lin-4* was found to reside in an intronic region of an unknown host gene which suggested temporal regulation of its expression. Although premature, the authors proposed the potential involvement of a stable complex mediating the hypothesized RNA-RNA interaction with possible crosstalk with the translation machinery.

Despite progress on the mechanism of RNAi, the miRNA field remained limited until the discovery of a second miRNA in *C. elegans* (Reinhart et al., 2000). RNA

expression analysis and mutant allele sequencing demonstrated that *let-7* encoded a small untranslated RNA of 21nt in length and its expression was shown to be temporally regulated with peak expression at early L4 and adult stages but not in the L1 or L2 stages. Similar to *lin-4, let-7* sequence was found to be complementary to the 3'UTR of the LIN-41 gene, in addition to other genes. Further experiments indicated that *let-7* functioned as a temporal switch between larval and adult fates. Based on sequence conservation, numerous miRNAs were then discovered in multiple species prompting studies aimed at elucidating miRNA regulation and mechanism (Bartel D., 2004).

An effective method for studying mechanism is generating an *in vitro* system with limited components to recapitulate *in vivo* events. Using a cell free system derived from syncytial blastoderm *Drosophila* embryos, incubation of reporter mRNAs with corresponding dsRNAs produced sequence specific silencing (Tuschl et al., 1999). The effects of the dsRNA suggested direct destabilization of the target mRNA and serial dilution of dsRNA up to 64-fold produced silencing similar to undiluted dsRNA (Tuschl et al., 1999). Another key finding using cell free assays demonstrated sequence specific RNAi using *Drosophila* S2 cells, in which a Ca^{2+} dependent nuclease with an RNA component mediated silencing. Biochemical fractionation suggested that the nuclease might be a ribonucleoprotein. The term RNA-Induced Silencing Complex or RISC was coined to describe this complex. Due to an association of RNA species of 25 nt homologous to the target, a model developed where small RNAs served as guides for targeting of specific messages based upon sequence recognition (Hammond et al. 2000). A strikingly similar observation was shown in plants, whereby 25 nt antisense transcripts were detected after transforming plants with cDNA sequences, further supporting small

RNAs as determinants of PTGS (Hamilton and Baulcombe, 1999). Around the same time, another report observed degradation of homologous RNAs and showed that the dsRNA was processed to small ~21-23 nt RNA fragments (Zamore et al., 2000). These studies proposed the hypothesis that the 21-23 nt RNA fragments mediated RNAi via detection of radiolabeled 21-23 nt RNAs following incubation of radiolabeled dsRNA with *Drosophila* embryo lysates. These synthetic RNA species were termed small interfering RNAs or siRNAs with the characteristic of perfect complementarity to their target. Purification of 21-23 nt RNAs revealed the presence of 2' and 3' hydroxyl groups, and responsiveness to alkaline phosphatase implied the presence of a 5' phosphate group, which suggested processing of dsRNA in an RNase III- like manner. Furthermore, these authors showed that chemically synthesized siRNA duplexes harboring overhanging 3' ends mediated gene silencing (Elbashir et al., 2000). Taken together, these studies illustrated that exogenous delivery of dsRNA elicited sequence specific silencing, similar to the PTGS mechanism of silencing in plants (Bartel, D., 2004).

PTGS in plants produced a reduction of RNA molecules with sequence similarity to foreign or endogenous DNA that was initially introduced. Triggers of PTGS include internal inverted repeat transgenes or co-expression of sense and antisense transgenes (Waterhouse et al, 1998, Hamilton et al., 1999, Chuang et al., 2000). A set of related proteins (SGS2/QDE-1/EGO-1) shared by PTGS in plants, quelling in *Neurospora*, and RNAi in animals provided proof that these pathways are functionally linked. A related protein encoded the *AGO1* gene was shown to be required for PTGS in *Arabidopsis thaliana* plants as *AGO1* mutants were defective in gene silencing (Fagard et al., 2000). This finding was further corroborated with purification and identification of AGO2 as an

essential component of RISC, a required protein for RNAi *in vitro* (Hammond et al., 2001), and later described as the catalytic enzyme responsible for mRNA cleavage or 'slicer' activity in mammals (Liu et al., 2004)

Subsequent experiments identified the RNase III enzyme, Dicer as responsible for cleavage of long dsRNA into smaller dsRNA duplexes in *Drosophila* (Bernstein et al., 2000). Dicer was found to belong to the class of RNase III-like enzymes (dependent on ATP) harboring two RNAse III signature motifs for cleavage, a dsRNA-binding domain, a PAZ (Piwi, Argo, and Zwille/Pinhead) domain, and an amino-terminal helicase domain. Cleavage products of RNAse III enzymes contain 2 to 3 nt 3' overhangs, 5'phosphate, and 3'hydroxyl termini. In addition, fractionation methods demonstrated that Dicer activity was distinct from RISC, providing a model of sequential processing of dsRNA with Dicer acting upstream of RISC (Bernstein et al., 2001). Through analysis of mature *let-7* miRNA transcripts after maturation *in vitro* from longer *let-7* precursor transcripts, human Dicer was shown to regulate *let-7*, thus, linking siRNA and miRNA pathways. Following Dicer inhibition, precursor *let-7* RNA accumulated in Hela cells with reduction of mature *let-7* transcripts, implicating the RNAi enzyme Dicer for maturation of human *let-7* RNA (Hutvágner et al., 2001).

Further association between the two families of small noncoding RNAs was demonstrated with the finding that siRNAs silenced target mRNAs via base pairing, similar to miRNAs (Doench et al., 2003). Initially, pairing of a miRNA with its target mRNA was thought to result in translational inhibition whereas siRNA pairing results in degradation (Doench et al, 2003). To further identify other enzymes responsible for miRNA processing with particular focus on primary miRNA transcripts, the RNase III

enzyme Drosha was identified as the enzyme responsible for cleavage of primary transcripts in the nucleus. Drosha was shown to release precursor miRNA transcripts *in vitro* and Drosha depletion produced accumulation of primary transcripts, further supporting a stepwise route of miRNA processing (Lee et al., 2003). A comparison between siRNAs and miRNAs is shown in Table 1 (Bartel, D., 2004) and a model of the miRNA biogenesis pathway is illustrated in Fig. 1. Of note, exogenously delivered siRNAs intersect with the miRNA biogenesis pathway from the Dicer processing step all the way to the target degradation step.

Table 1.Comparison of siRNAs and miRNAS in PTGS

siRNAs	**miRNAS**
~22 nt in size	~21-25 nt in size
Usually generated from exogenous introduction	Usually endogenously transcribed from intronic or intergenic regions
Derived from long dsRNA	Derived from stem loop regions of long primary / precursor transcripts
Cleavage by Dicer	Stepwise cleavage by Drosha and Dicer
Interact with RISC	Interact with RISC
Perfect complementarity to mRNA target	Mostly imperfect complimentarity to mRNA target except ‘seed region’
3’UTR, also CDS	Mostly 3’UTR
Mostly facilitate mRNA cleavage	Mostly facilitate translational repression
Multiple duplexes generated	Single miRNA:miRNA* duplex from each hairpin precursor molecule
Rarely conserved	Mostly conserved in related organisms

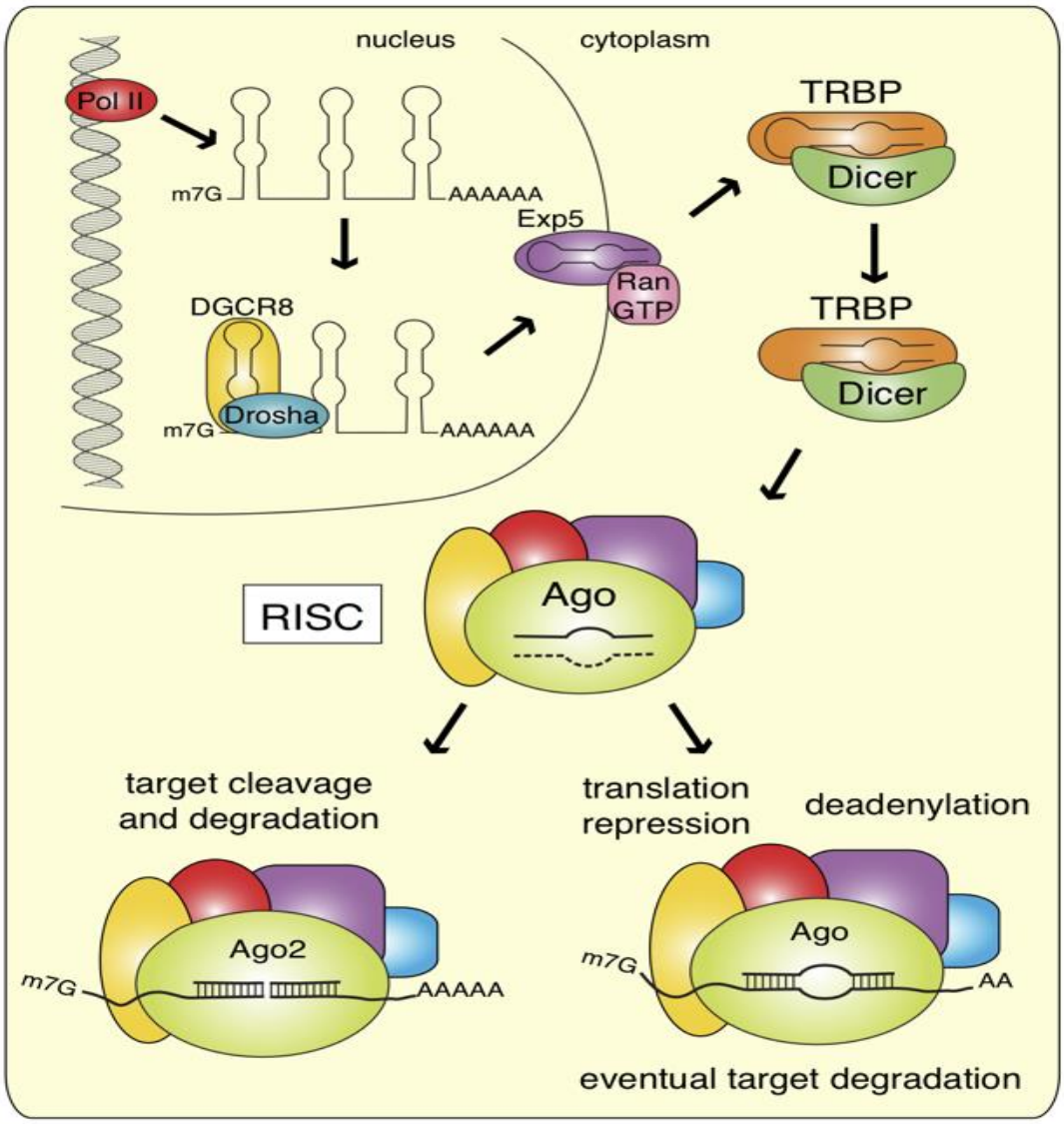

Figure 1. The miRNA biogenesis pathway

miRNAs are transcribed by RNA polymerase II as long primary transcripts called pri-miRNAs with independent promoters or from intronic/exonic regions of protein-coding genes either in a polycistronic or monocistronic fashion. Nuclear Drosha and cofactor DGCR8 cleave the base of the stem-loop to release precursor miRNAs (pre-miRNAs), which are transported to the cytoplasm by Exportin-5. Pre-miRNAs are recognized and cleaved by Dicer and cofactor TRBP releasing small ~22nt RNA duplexes. A guide strand is selected for incorporation into cytoplasmic RISC containing Ago proteins with the degradation of the passenger miRNA strand. The single stranded mature miRNA guides RISC to the 3'UTR

region of specific mRNA targets where AGO and GW182 families facilitate cleavage. Most miRNAs bind their target with imperfect complimentarity followed by translation repression with subsequent degradation, while perfect complementarity (usually observed with siRNAs) results in mRNA cleavage. Figure by Abby Olena.

miRNAs in disease

Initially discovered as mediators of gene silencing during animal development and plant viral defense, miRNAs have been implicated in a variety of human diseases including neurodegenerative, cardiovascular, liver, and cancer (Fernández-Hernando, C., & Baldán, A., 2013, Goodall et al., 2013, Szabo, G. & Bala S., 2013, Esteller, M., 2011, Andrews, O., & Patton, J.P. , 2014). Cancer in particular is characterized by misregulated and/or altered cell division. Most commonly, changes in cell cycle control arise from accumulated mutations leading to chromosomal instability, proliferation, and aggressive metastatic behavior. Changes in cancer cell behavior lead to a progression from benign to malignant stages (Fig. 2). Cancer is also fascinating because developmental pathways are often re-activated and commonly drive tumor progression. During animal development, miRNA expression has been shown to be temporally and spatially regulated (Thatcher et al., 2008 and Wei at al., 2012) and miRNAs have been shown to regulate multiple genes during development (Flynt et al., 2007, Li et al., 2011, Giraldez et al., 2006, Weinholds et al., 2005, Bernstein et al., 2003). Due to the observation of some miRNA target genes acting in common cancer pathways, the discovery of miRNAs as cancer promoters came as no surprise. More importantly, miRNAs have been shown to play roles in specific hallmarks of cancer as identified by Hanahan and Weinberg. These hallmarks are defined as acquired functional capabilities that allow cancer cells to survive, proliferate, and disseminate. These shared characteristics include self-sufficiency from growth signals,

insensitivity to anti-growth signals, the ability to evade apoptosis, limitless replicative potential, sustained angiogenesis, tissue invasion, and metastasis (Hanahan, D., & Weinberg, R.A., 2000). Broadly, there are two defining feature of cancers: upregulation of oncogenes (genes having gain-of-function or abundant expression which promote cancer) and downregulation of tumor suppressor genes (genes having inhibitory cancer function whereby their loss-of-function promote cancer). In the following sections, key oncogenic and tumor suppressor miRNAs will be described and a summary of miRNAs in steps of tumorigenesis will be shown in Table 2.

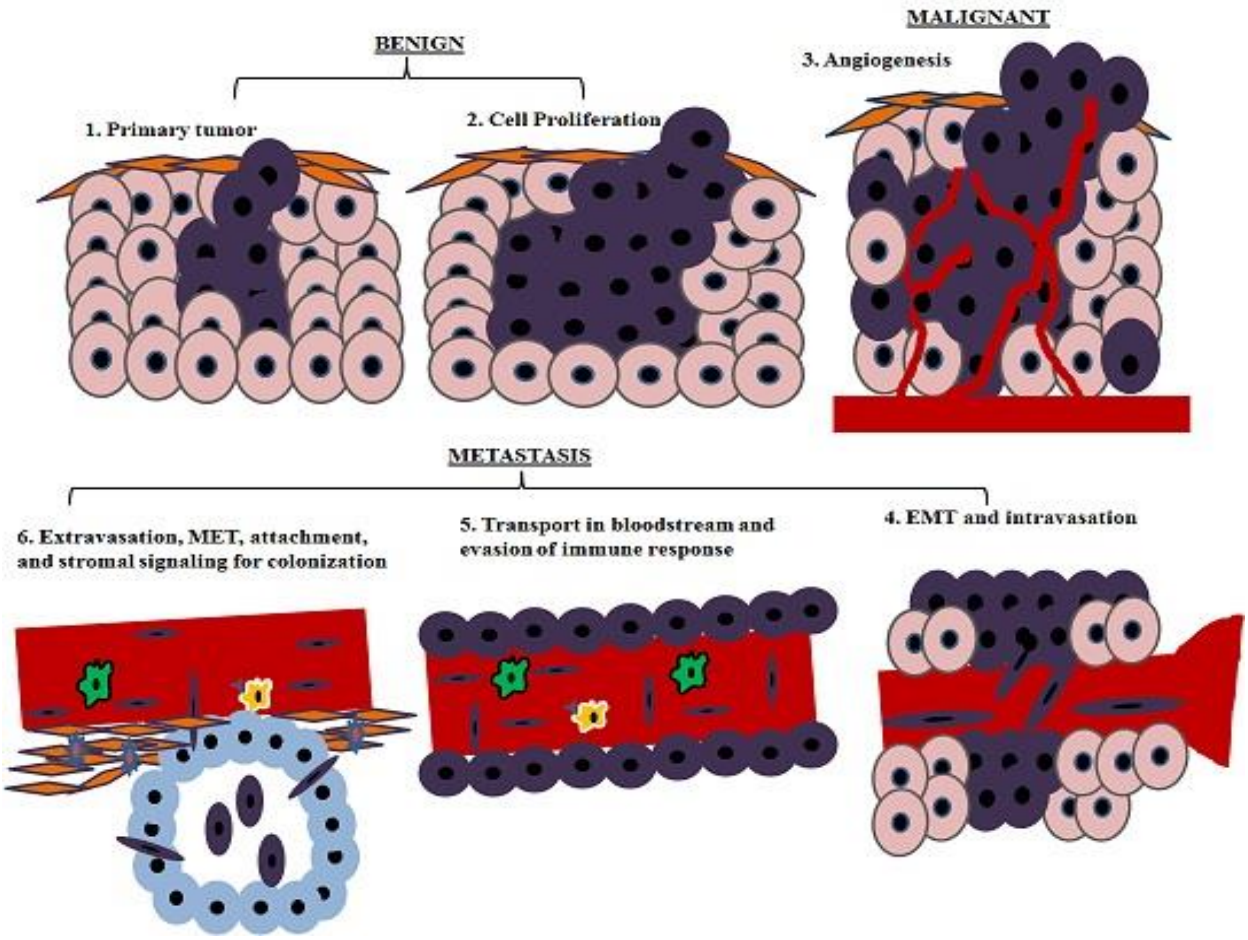

Figure 2. Model of cancer progression

During the benign stage, cells acquire multiple mutations and form the primary tumor. With increasing tumor growth, angiogenesis is initiated and cells locally invade during the malignant phase. Aggressive metastasis results when tumor cells undergo epithelial-to-mesenchymal transition (EMT), enter blood vessels, and colonize secondary sites through crosstalk in a tumor permissive microenvironment (Andrews, O., & Patton, J.G., 2014).

miRNAs as oncogenes

The first report of an oncogenic miRNA showed that the *miR-17-92* polycistron cluster is highly expressed in human B-cell lymphomas (He et al., 2005). The *miR-17-92* cluster is located within a genomic locus on the chromosome 13q31 that was previously established as a frequently amplified region in cases of diffuse large B-cell lymphoma, follicular lymphoma, mantle cell lymphoma, primary cutaneous B-cell lymphoma, and other cancer types (Ota et al., 2004). The cluster encodes *miR-17-5p, 17-3p, 18, 19a, 20, 19b-1,* and *92-1*. One of the defining characteristics of an oncogene is chromosomal amplification and as a proof-of-principle, serial transplant assays are typically used to examine the tumor forming potential of a gene of interest. Using a mouse model of human B-cell lymphoma, where the c-Myc oncogene is over-expressed by the immunoglobulin heavy chain enhancer (Eμ), He and colleagues (2005) demonstrated that the *miR-17-19b* cluster accelerated tumorigenesis (He et al., 2005). Transplantation of reconstituted hematopoietic stem cells (derived from Eμ-Myc mice) with *miR-17-19b* expression into irradiated recipient mice led to lymphoma development, invasion of tumor cells into other organs, and decreased survival. Tumors continued to form after two rounds of serial transplantation and analysis of the tumor cell population for markers of pre-B and mature B cells (CD19 and IgM respectively) suggested that over-expression of the cluster favored transformation of B-cell progenitors (He et al., 2005). Another

study implicating the *miR-17-92* cluster in lung tumorigenesis used northern blotting and detected increased *miR-17-92* expression, increased copy number, and functionally enhanced lung cancer cell growth through cell proliferation assays (Hayashita et al., 2005). Thus, the *miR-17-92* cluster can accurately be classified as oncogenic.

Further investigation of oncogenic miRNAs, or "oncomiRs" as they are designated, led to the detection of elevated *miR-155* expression in lymphomas derived from B cells of different developmental stages (Eis et al., 2005). Expression of *miR-155* (found on chromosome 21q21) was derived from sequences present in the *bic* RNA, which was previously discovered as a target for insertional mutagenesis in avian B-cell lymphomas (Tam et al., 1997). *bic* cooperates with c-Myc (an established oncogene) in enhancing the growth and transformation potential of cultured chicken embryo fibroblasts. In pancreatic ductal adenocarcinoma, *miR-155* expression is upregulated and functional assays identified tumor protein 53 induced nuclear protein 1 (TP53INP1) as its target (Gironella et al., 2007). Decreased TP53INP1 occurred through translational inhibition, providing a link between *miR-155* and a regulator of cell cycle progression and apoptosis. Further evidence for the oncogenic nature of *miR-155* was observed in breast cancer (Jiang et al., 2010). Over-expression of *miR-155* triggered constitutive activation of growth pathways including the STAT3 and JAK pathways and its effects were mediated through targeting the tumor suppressor gene, Socs1 (Jiang et al., 2010). These experiments provided support for the contribution of miRNAs in tumorigenesis by downregulation of genes controlling cell growth and division, a mandatory prerequisite for primary tumor formation.

While initial findings of pro-tumorigenic miRNAs were mostly derived from

DNA copy number analyses, few have been identified through targeted approaches i.e. genetic screens. Voorhoeve and colleagues (2006) designed a library of vectors expressing the majority of cloned human miRNAs and used microarrays to examine expression (Voorhoeve et al., 2006). Through the use of an oncogenic stress model in which human fibroblasts express a constitutively active Ras, they discovered that vectors encoding *miR-372* and *miR-373* conferred a selective growth advantage to cells that would otherwise undergo a stress response, known as oncogene-induced senescence. Their results indicated that these miRNAs act in cooperation with Ras to promote tumorigenesis and also provided evidence implicating both miRNAs in tumors that retained a wild type (WT) copy of p53 but were nevertheless sensitive to DNA-damaging agents. Analysis of chemosensitive testicular germ cell tumors harboring WT p53 indicated high expression of *miR-372* and *miR-373* in tumors classified as embryonal carcinoma (Voorhoeve et al., 2006). Also, germ cell lines failed to undergo growth arrest in the presence of a cell cycle inhibitor and *miR-372* and *miR-373*. Using prediction algorithms to identify targets whose 3'UTRs contained putative binding sites for both miRNAs, the serine threonine kinase Large Tumor Suppressor homolog 2 (LATS2) was identified and validated using a luciferase reporter assay. Negative regulation of LATS2 by *miR-372* and *miR-373* occurs through a combination of RNA ablation and inhibition of protein synthesis. Since LATS2 is a tumor suppressor, its loss in mouse embryonic fibroblasts provides a growth advantage (Voorhoeve et al., 2006). Together, *miR-372* and *miR-373* fit the oncogenic criteria because downregulation of their mRNA targets prevent exit from the cell cycle resulting in uncontrolled cell growth.

The classification of miRNAs as oncogenes was further substantiated when

specific miRNAs were found to be overexpressed within diverse tumor types (Lujambio et al., 2008, Esquela-Kerscher et al., 2004, Dalmay et al., 2006). One such miRNA that appears to be ubiquitously required for aggressive metastatic potential is *miR-21*. Encoded on chromosome 17q, *miR-21* was originally identified as an oncogene in human glioblastoma cells (Chan et al., 2005). Through analysis of RNA isolated from neoplastic and non-neoplastic glioma samples, *miR-21* was found to be upregulated in gliomas. Inhibition of *miR-21* expression in cultured glioblastoma cells caused a marked increase in apoptosis through activation of the caspase machinery (Chan et al., 2005). Additional evidence for pro-tumorigenic *miR-21* was illustrated through microarray analyses that showed significant upregulation of *miR-21* in all tumors (breast, colon, lung, pancreas, prostate, and stomach) irrespective of disease status (Volinia et al., 2006). Consistent with this, the anti-apoptotic protein Bcl-2 was found to be a direct target of *miR-21* in breast cancer cells and in a xenograft model of breast cancer (Si et al., 2007). Other tumor suppressor targets of *miR-21* have been identified including PTEN, which inhibits the oncogene PI3K in AKT-mediated cell proliferation (Liu et al., 2013). *miR-21* modulates PTEN levels in lung cancer cells resulting in increased cell growth, migration, and invasion (Liu et al., 2013).

Several reports have referred to the phenomenon of dependence on a single oncogene as "oncogenic addiction" (Weinstein et al., 2002, Jain et al., 2002, and Chin et al., 1999). A landmark in miRNA cancer research was the *in vivo* demonstration of oncogenic addiction in mice conditionally expressing *miR-21* (Medina et al., 2010). Using Cre recombinase and the Tet-off system, over-expression of *miR-21* in hematopoietic tissues accelerated pre-B malignant lymphoid-like tumor formation,

whereas loss of *miR-21* resulted in regression of tumors. This clearly demonstrates that *miR-21* is a *bona fide* oncomiR. Overexpression of *miR-21* in different cancer types illustrates the dysregulation and dependence on common miRNA pathways and common mRNA targets in the acceleration of tumorigenesis (Krichevsky et al., 2009, Wang et al., 2009, Zhong et al., 2012, and Reis et al., 2012).

Similar to *miR-21*, the *miR-221/222* family encoded on the X chromosome has been implicated in several cancer types after initial detection in glioblastoma multiforme (GBM) (Ciafre et al., 2005). Global miRNA expression profiles (miRNome) showed increased expression of *miR-221/222* in patient tissue samples and GBM cell lines. Similar microarray analyses uncovered an increase in *miR-221/222* expression in papillary thyroid carcinoma, consistent with decreased Kit receptor expression, a gene with *miR-221/222* binding sites (He et al., 2005). Also, prostate cancer cells showed increased *miR-221/222* expression, particularly in highly aggressive PC3 cells (cells derived from a distal metastasis) compared to LNCaP cells (slow growing cells derived from local lymph-node metastasis) (Galardi et al., 2007). Overexpression of both miRNAs in the slowly growing cell line increased the number of cells entering S-phase. For these experiments, targeted inhibition of both miRNAs caused increased expression of $p27^{Kip1}$. Similarly, treatment of tumor-bearing mice with intravenous injection of antisense oligonucleotides against *miR-221* resulted in increased survival in mice injected with human hepatocellular carcinoma (HCC) (Park et al., 2011).

miRNAs as tumor suppressors

Located in a cluster on chromosome 13q14.3, the first described tumor suppressor

miRNA was the *miR-15/16* family (Calin et al., 2002). Deletions and translocations in this region were found in ~65% of B-cell chronic lymphocytic leukemia (CLL). An initial inverse correlation was recognized between *miR-15/16* and the anti-apoptotic regulator protein Bcl2, which is over-expressed and a hallmark of CLL (Cimmino et al., 2005). Other *miR-15/16* family members harbor the same 9 base-pair Bcl2-complementary sequence, providing evidence for a putative interaction consistent with experiments that showed that *miR-15/16* post-transcriptionally regulates Bcl2. Re-introduction of *miR-15/16* in a leukemia-derived cell line lacking both miRNAs resulted in strong reduction in Bcl2 levels. Also, cells transfected with plasmids expressing *miR-15/16* demonstrated increased apoptosis as measured by DNA fragmentation, activation of caspases, and TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) staining of individual cells (Cimmino et al., 2005).

Tumor growth relies on genetic alterations coupled with external communication to and from the environment. A critical process for tumor expansion is the establishment of stromal progression and the metastatic niche (Whiteside et al., 2008). Tumor cells interact with the extracellular matrix (stroma) and upon metastasis, distant stromal-tumor interactions form a niche that facilitates tumor growth. Distinct cell types configure the stromal architecture including fibroblasts that often receive signals (cytokine and chemokine) from tumor cells that prime the stroma for tumor growth. Investigation of prostate cancer-stromal interaction showed down-regulation of *miR-15/16* in comparison to stroma surrounding non-cancerous tissues (Musumeci et al., 2011). Candidate mRNA targets were screened using bioinformatic prediction algorithms and luciferase reporter assays and found that the 3'UTR of FGF-2 and FGFR1 can be silenced by *miR-15/16,*

consistent with increased FGF-2 and FGFR1 in prostate cancer (Musumeci et al., 2011). Reconstitution of carcinoma-associated fibroblasts with *miR-15/16* also showed a reduction of FGF-2 and FGFR1. Lastly, subcutaneous co-injection of prostate cancer cells with fibroblasts transduced with *miR-15/16* expression vectors dramatically reduced tumor growth in immunocompromised mice (Musumeci et al., 2011). Histological analyses showed decreased parenchymal invasion, impaired angiogenic behavior, and reduction of FGF-2 expression in the stroma. These experiments show how miRNAs can regulate multiple aspects of cancer development including primary tumor growth and metastatic spreading.

Let-7, the first conserved miRNA discovered in *C. elegans*, plays a pivotal role in regulating animal development and has been implicated in tumorigenesis (Boyerinas et al., 2010, and Johnson et al., 2005). In humans, there are 13 *let-7* family members encoded on 9 different chromosomes (Roush et al., 2008). Target identification experiments support the idea that *let-7* negatively regulates Ras, consistent with increased levels of oncogenic Ras in lung cancer (Johnson et al., 2005). Indeed, *let-7g* reduced luciferase activity when the K-Ras 3'UTR was fused to luciferase in mouse lung adenocarcinoma cells. *let-7* also repressed the levels of c-Myc using the same reporter system.Typically, *let-7* loss is due to chromosomal deletions but mutations in the 3' UTR of a *let-7* target can also block *let-7* function (Mayr et al., 2007). Chromosomal translocations associated with human tumors disrupted repression of the oncogenic chromatin-associated *let-7* target Hmga2 (High Mobility Group protein A2; Hmga2) by deletion of 3' UTR regions targeted by *let-7* (Mayr et al., 2007). *Let-7* regulates Hmga2 by interacting with seven conserved 3'UTR elements. Expression of truncated Hmga2

lacking the *let-7* sites induced higher colony formation on soft agar and anchorage-independent behavior. Consistent with this, subcutaneous injection of mouse embryonic fibroblast cells with full length, wild type Hmga2 produced no tumors, whereas fibroblasts harboring mutated *let-7* sites or 3' UTR truncations in Hmga2 generated tumors at the sites of injections (Mayr et al., 2007). These data provide *in vivo* support for a tumor suppressor function for *let-7* through Hmga2 regulation.

One of the most widely studied anti-tumorigenic proteins is p53, the most commonly mutated gene in numerous cancers (Finlay et al., 1989). Originally identified as a transcription factor, p53 also acts as a DNA damage response protein and apoptotic regulator. Global changes in miRNA levels were examined in p53-deficient mouse embryonic fibroblasts and found that *miR-34* expression correlates with p53 status (He et al., 2007). Conditional activation of p53 or induction of p53 increased *miR-34* levels. Analysis of p53 binding showed that p53 occupies specific *miR-34* promoter regions. Ectopic expression of *miR-34* inhibited growth of human primary fibroblast cells, in addition to inducing cell cycle arrest in G1 after addition of *miR-34* in immortalized mouse cells and human tumor cells. Upregulation of *miR-34* also produced changes in mRNA expression patterns among a large number of genes implicated in cell proliferation including cyclin-E and CDK-4 (He et al., 2007). These experiments suggest a novel mechanism of p53-mediated regulation of cell proliferation through activation of *miR-34*. *miR-34* can therefore be considered a tumor suppressor and indeed, the chromosomal region 1p36 encoding *miR-34* is frequently deleted in various cancers including colon cancer (Di Vinci et al., 1996). For targets of *miR-34*, increased SIRT1 (Silent mating type Information Regulator 1) expression was observed after *miR-34a*

inhibition (Yamakuchi et al., 2008). SIRT1 is known to regulate apoptosis in response to oxidative and genotoxic stress through NAD-dependent deacetylation (reviewed in [Bosch-Presegue et al., 2011]). Cell survival decreased when *miR-34a* was introduced into colon cancer cells but SIRT1 re-expression partially blocked *miR-34a*-mediated cell death. Interestingly, SIRT1 can deacetylate histones associated with the p53 gene, suggesting a positive feedback loop between p53, *miR-34a*, and SIRT1 (He et al., 2007).

Besides regulating histone acetylation, miRNAs can also regulate DNA methylation. DNA methylation patterns are frequently altered in cancer (El-Osta et al., 2003). The human DNA methyltransferases DNMT3A and -3B are targets of *miR-29* (Fabbri et al., 2007). The *miR-29* family includes *miR-29a, miR-19b-1, miR-29b-II*, and *miR-29*, located on chromosome 7q32, a region frequently deleted in various leukemias. Lung cancer cells transfected with *miR-29* showed a global reduction of DNA methylation and reduced promoter methylation and therefore expression of the tumor suppressor genes FHIT and WWOX (normally hypermethylated and silenced in lung cancer) (Fabbri et al., 2007). In a mantle cell lymphoma model, *miR-29* levels were found to be controlled by c-Myc (Zhang et al., 2012). Promoter analysis showed that Myc, HDAC3, and EZH2 form a repressive complex to epigenetically repress *miR-29* transcription in Myc-expressing lymphoma cells. Loss of *miR-29* results in upregulation of CDK6 and IGF-1R, which are pro-growth genes that promote Myc-associated lymphomagenesis (Zhang et al., 2012). *miR-29* loss therefore confers a growth advantage, supporting its designation as a tumor suppressor miRNA.

Small RNAs have also been shown to regulate downstream steps of tumorigenesis including angiogenesis and metastasis (Table 2). The versatile role of miRNAs in cancer

coupled to their essential roles during animal development make miRNAs attractive targets for therapeutic strategies (Fig.3).

Table 2: miRNAs in **tumor associated processes**

Features of tumorigenesis	**Prominent miRNAs**
Oncogenes	miR-17-92 cluster, miR-155, miR-372, miR-373, miR-21, miR-221/222
Tumor Suppressor genes	miR-15/16, *let-7*, miR-34, miR-29
Angiogenesis	miR-296, miR-9
Epithelial-to-Mesenchymal transition	miR-200a/b, miR-205
Metastasis	miR-335, miR-126, miR-206

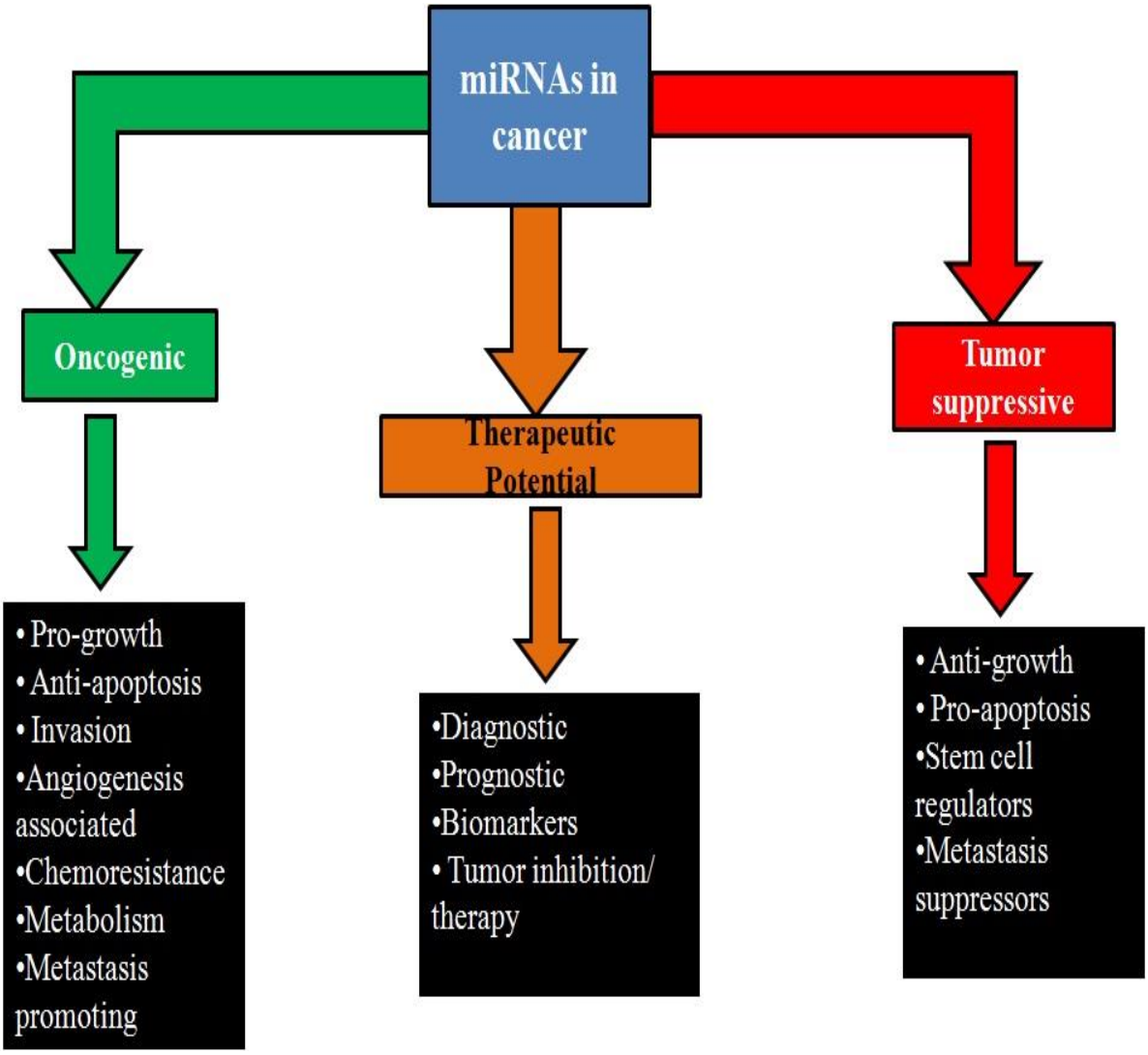

Figure 3: Overview of miRNAs in cancer

miRNAs can be oncogenic or tumor suppressive, highlighting the therapeutic potential of these small noncoding RNAs. New therapeutic approaches to cancer treatment now include targeting of small RNAs particularly miRNAs. In the future, miRNAs are likely to serve as diagnostic, prognostic, and reliable biomarker information during cancer therapy (Andrews, O., & Patton, J.G., 2014).

Transcriptional Gene Silencing and RNAi

One of the pioneering studies in TGS demonstrated *de novo* methylation on corresponding viroid cDNA sequences upon viroid RNA-RNA replication in a transgenic *Tobacco* plant system. Briefly, viroids (plant pathogens) harbor non-coding, highly base-paired RNAs that are replicated in the nucleus by the host RNA polymerase II. Infection with incompetent viroid cDNAs lacking replicative capacity resulted in an unmethylated state, leading the authors to propose a model whereby methylases are guided to DNA target sequence for methylation via sequence specific RNAs (Wassenegger et al., 1994). The upregulation of methyl groups on DNA substrates represents an epigenetic modification frequently associated with gene inactivation. More direct evidence for TGS and RNAi was shown when dsRNAs targeting the nopaline synthase promoter (NOSpro) triggered methylation and transcriptional inactivation of homologous copies of the NOSpro *in trans*. NOSpro dsRNA degradation produced small RNAs ~23 nt in length, supporting a connection between TGS and PGTS (Mette et al., 2000). Cooperation of TGS and PTGS was further demonstrated when *Brassica napus* plants suppressed viral gene expression after systemic infection with a DNA virus. Due to shared sequence homology between the transgene and virus, co-suppression was mediated via degradation of homologous RNA molecules in a post-transcriptional manner. Gene silencing was also observed in plants infected with transgenes sharing homology with viral promoter sequences, suggesting potential interaction with chromatin to inhibit transcription (Al-Kaff et al., 1998). Another investigation observed homology-based, RNA-directed methylation associated with silencing following infection of transgenic plants encoding GFP with viral RNA vectors with promoter sequences or ORF sequences of GFP (Jones

et al., 1999). The above studies supported sequence specific RNA regulation of chromatin. In addition to the association of DNA methylation with gene silencing, methylation of specific histones also correlates with gene silencing.

Extensive studies made in *Schizosaccharomyces pombe* (*S. pombe* or fission yeast) revealed regulation of repressive histone modification by RNAi. Although *S. pombe* lack DNA methylation, particular regions of the genome is silenced via heterochromatin formation. Distinct from euchromatin (active chromatin domains), heterochromatic regions are generally characterized by a high density of repeat sequences and transposons, with a prominent feature of modified histones that stimulate inactive chromatin conformation (Rea et al., 2000, Fischer et al., 2006). In particular, trimethylation of lysine 9 on histone H3 is a distinguishing signature. DNA regions harboring H3K9me3 are almost always heterochromatic or repressed (Fischer et al., 2006). In the fission yeast genome, centromeric regions undergo heterochromatic silencing (other regions include telomeres and silent mating type loci). Upon deletion of RNAi related genes, centromeric silencing is suppressed and H3K9me3 modification is reduced. These RNAi mutants also display delocalization of *Swi6*, which is the fission yeast homolog of HP1 (heterochromatin binding protein in higher eukaryotes), that specifically recognizes and binds to H3K9me3 (Volpe et al., 2002). Heterochromatin assembly was eloquently shown to be regulated by a newly discovered nuclear complex containing the RNAi specific protein Ago1 with other binding partners Tas3 and Chp1 (a heterochromatin-associated chromodomain protein) in *S. pombe* (Verdel et al., 2004). This complex is referred to as the RNA-induced initiation of transcriptional gene silencing or RITS complex with similarity to PTGS associated RISC except for

differences in localization and distinct protein components (Creamer K.M., & Partridge, J.F., 2011, Denli, A.M., & Hannon, G..J., 2003). Deletion of Dicer leads to loss of small RNA species that co-purify with members of the RITS complex from centromeric regions, suggesting that these RNAS are Dicer-dependent siRNAs that interact with the RITS complex (Verdel et al., 2004). This directly links RNAi, heterochromatin formation, and TGS.

Convergent transcription, RNAi, and heterochromatin

Analysis of the fission yeast genome showed that genes arranged in a convergent orientation produce overlapping transcripts via convergent transcription (CT) by Pol II. Synchronization experiments demonstrated that overlapping dsRNA transcripts are specific to the G1 phase of the cell cycle but not G2. During G2, intergenic cohesin acts to promote gene-proximal transcription termination and prevents further dsRNA production during this phase. Further investigation of G1-specific dsRNA derived from convergent genes (CG) showed an induction of localized and transient heterochromatin signatures (H3K9me3) and recruitment of *Swi6* in an RNAi dependent manner (requiring RITS and Dicer activity). siRNAs apparently direct RITS to the homologous convergent chromatin region suggesting an autoregulated and cell-cycle dependent mechanism of cohesin recruitment to CGs (Gullerova et al., 2008). Interestingly, 80% of RNAi genes were discovered to be arranged in a convergent orientation in *S.pombe* and are down-regulated in the G1-S phase of the cell cycle by forming transient heterochromatin. Repositioning RNAi genes in a tandem orientation at their chromosomal location abolished gene silencing during G1-S and generated defects in cell division and

morphology (Gullerova et al., 2011). Transfection of *S. pombe* with CT plasmids harboring *ura4* promoter or ORF sequences flanked by convergent *nmt1* promoters induced silencing of the endogenous ura4 gene *in trans.* Chromatin immunoprecipitation (ChIP) analysis with histone H3K9me3 specific antibodies showed enrichment of H3K9me3 at the *ura4* locus. Similarly, transfection of Hela cells with CT constructs targeting ɣ–actin or TDP-43 genes produced dsRNA and increased H3K9me3 levels, supporting a mechanism of RNAi-mediated heterochromatin formation (Gullerova et al., 2012). Based on these studies, a model is shown that describes how convergent transcription mediates gene silencing via RNAi (Fig. 4).

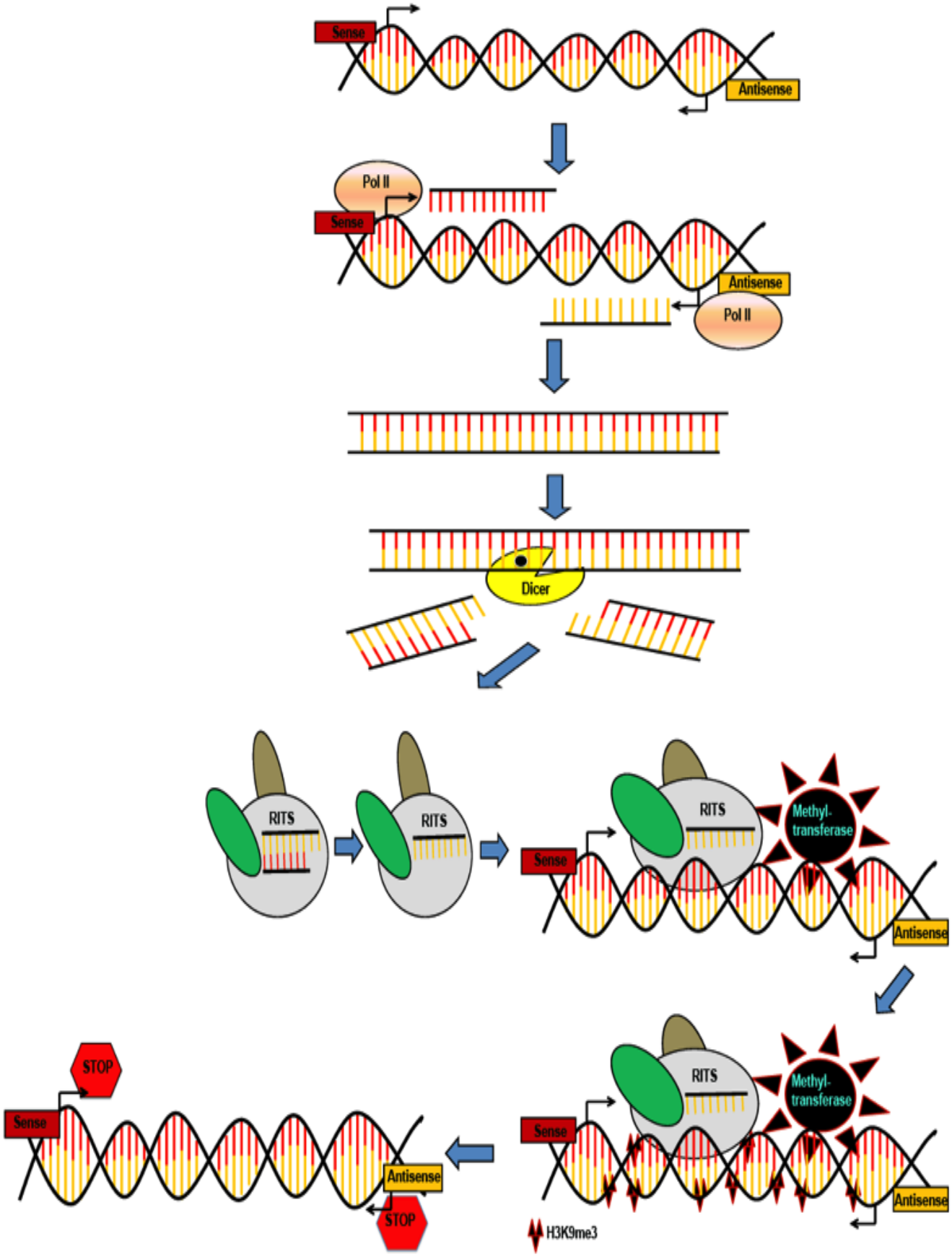
Sense
Antisense
Pol II
Sense
Antisense
Pol II
Dicer
RITS
RITS
RITS
Methyl-
transferase
Sense
Antisense
RITS
Methyl-
transferase
Sense
Antisense
H3K9me3
STOP
Sense
Antisense
STOP

Figure 4: Gene silencing via convergent transcription
Convergent transcription triggers gene silencing. Overlapping transcripts with complementary sequence are generated that form dsRNAs that are recognized by nuclear Dicer and cleaved to form short siRNA duplexes. These RNAs become incorporated into nuclear effector RITS (RNA-Induced Transcriptional Silencing) complexes. The guide siRNAs direct RITS to the convergent chromatin location, leading to recruitment of methlytransferases and subsequent increases in H3K9 trimethylation. As a result, genes are silenced at the level of transcription (model adapted from Nick Proudfoot studies) (Gullerova et al., 2012).

Zebrafish model system

Zebrafish (*Danio rerio*) has emerged as a powerful model organism for the study of vertebrate development and disease. Zebrafish studies began when George Streisinger initiated the application of mutational analysis to study vertebrate embryonic development during the 1960s. As the organism began to be studied by others (Charles Kimmel, Christiane Nüsslein-Volhard, Marc Fishman, Wolfgang Driever, Monte Westerfield, and Judith Eisen) during the 1980s, cell lineage and mapping information became established. By the 1990s, forward genetic screens were used in zebrafish to identify embryonic lethal mutations and extensive investigation focused on stages of zebrafish development began (Grunwald, D.J., & Eisen, J.S., 2002). As an organism that fertilizes externally and produces optically transparent embryos with high fecundity, zebrafish were ideal for visually studying development. A landmark publication fully described zebrafish development defining seven broad periods of embryogenesis – the zygote, cleavage, blastula, gastrula, segmentation, pharyngula, and hatching periods. These stages were based on morphological features of zebrafish embryos under observation with dissecting stereomicroscope (Kimmel et al., 1995). Genetic studies and cloning of mutants identified genes involved in early patterning of the zebrafish embryo.

Within the last decade, zebrafish has also been used to model human disease including hematopoietic disorders, cardiovascular disorders, and kidney disorders (Dooley, K., & Zon, L.I., 2000). Large scale sequencing of the zebrafish genome identified over 26,000 protein-coding genes and comparison to the human reference genome showed that ~70% of human genes have at least one zebrafish orthologue (Howe et al., 2013). Zebrafish also express small noncoding RNAs – miRNAs - (Weinholds et al., 2005, Thatcher et al., 2008, Wei et al., 2012).

Gene silencing tools in zebrafish

Zebrafish has been used to study reverse genetics to determine gene function. The most widely used strategy to knockdown genes post-transcriptionally in this organism are antisense morpholinos (MO). MOs are chemically modified oligonucleotides that inhibit RNA by steric hindrance and by blocking translation of mRNA. The unique structure of MOs derives from the use of morpholine rings to replace the ribose backone of nucleic acids (Summerton, J., & Weller, D., 1997) which confer resistance to nuclease digestion and enhanced affinity for binding to complementary RNA sequences (Summerton, J., 1999). The first study that paved the way for using MOs in zebrafish showed specfic silencing of GFP and the *uroporphyrinogen decarboxylase* gene, which generated embyos mimicking a human disease (hepatoerythropoietic porphria) (Nasevicius, A., & Ekker, S.C., 2000). While the MOs have proven quite useful, several disadvantages have been uncovered including non-specific effects, limited duration, and toxicity. Large mutagnesis screens either by retrovirus mediated insertions or chemical exposure to ENU (N-ethyl-N-nitrosourea) have also been used to mutate zebrafish genes. Disadvantages of

these mutagenesis screens include the labor-intensive step of mapping insertion sites and the generation of large mutant libraries to identify desired mutations. Silencing of genes in zebrafish has also been accomplished using a gene targeting approach by site-specific or programmable genome modifications. These include zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and the CRISPR-Cas sytem (clustered regulatory interspaced short palindromic repeat). The common features of site-specific ZFNs and TALENs are the chimeric nature of the nucleases which harbor sequence-specific DNA- binding modules linked to a nonspecific DNA cleavage domain. Genome editing by ZFNs and TALENs is achieved by induction of double stranded breaks in the DNA and activation of DNA damage response pathways that utilize either error-prone nonhomologous end joining or homology-directed repair at specific genomic locations to repair the damage. Successful targeting of zebrafish genes using ZFN (*gol and ntl)* induced somatic loss-of-function phenotypes with embryos displaying decreased pigmentation or truncated body axis with no tail, respectively (Doyon et al., 2008). Using a modified TALEN systen (GoldyTALEN) with single-stranded DNA oligonucleotides as donor templates, successful homology-directed repair was achieved in zebrafish (Bedell et al., 2012). Despite the successes of ZFNs and TALENs, limitations and drawbacks include off-target activity, cellular toxicity, and high cost (Gaj et al., 2013). Unlike ZFNs and TALENs, the CRISPR/Cas system uses small RNAs to guide DNA endonucleases to specific sites which can differ by changing the RNA guide sequence. The CRISPR nuclease system has been used to silence genes in zebrafish embryos with high efficiency (Hwang et al., 2013) and to knock-in via homology-independent DNA repair (Auer et al., 2013). Improved designs to the system include a chimeric guide RNA (gRNA) consisting

of a fused CRISPR RNA and transacting RNA sequences and purified Cas9 (endonuclease) that induce site-specific DNA double stranded breaks. One report has demonstrated robust silencing with the improved chimeric guide when targeting a reporter gene and four endogenous loci. Within their system, high mutagenesis rates (75-99%) were observed in zebrafish (Jao et al., 2013). Although these genome editing tools have demonstrated high efficiency in silencing, however, the subsequent mutation is irreversible. Current studies are working to make CRISPR technology temporally and spatially regulated but so far, there's none at hand. Thus, an effective gene silencing tool that is easily regulated both temporally and spatially is missing in zebrafish.

The RNAi pathway has been explored in zebrafish in a PTGS context and yielded inconsistent results. While few studies showed specific gene silencing, other reports showed nonspecific phenotypes following dsRNA injection into zebrafish embryos. In order to understand the feasibility and functionality of RNAi, a collaborative effort was made to investigate RNAi using a TGS approach. Using the *Tol2* vector transfer system, Diana Cha generated convergent plasmid constructs targeting mCherry protein, and we observed silencing in embryos. I optimized and used chromatin immunoprecipitation methods to determine that suppression of mCherry was mediated by a histone modification associated with silencing–H3K9me3. I detected enrichment of H3K9me3 on convergent chromatin from transient transgenics and a significant increase of H3K9me3 in F1 and F2 embryos. Convergent transcription was also used to suppress transcription of One Eyed Pinhead, producing embryos with similar mutant phenotypes. To analyze the ability to silence noncoding RNAs, I generated convergent plasmid constructs targeting *miR-27a/b* family members, which were previously shown to be required for proper

craniofacial development. I showed that injection of convergent plasmids phenocopied *miR-27a/b* morphants using cartilage specific staining in a specific population of embryos.

Summary

Small RNAs including miRNAs and siRNAs play important roles in gene regulation in diverse species. Through interaction with mRNA targets, miRNAs control developmental events and normal cellular behavior. The discovery of miRNAs in diseases including cancer highlight the importance of investigating miRNAs during aberrant cellular behavior and exploring them for future therapeutic strategies. While great progress has been made in understanding siRNA and miRNA function at the post-transcription level, there are still questions regarding roles for small RNAs at the level of transcription. Since most of the studies of RNAi-based TGS occur in plants and fission yeast, it is not entirely clear how applicable these mechanisms will apply in higher vertebrates. The work in this thesis explores TGS in a higher vertebrate model- the zebrafish.

CHAPTER 2

RNAi-MEDIATED GENE SILENCING IN ZEBRAFISH TRIGGERED BY CONVERGENT TRANSCRIPTION[1]

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[1]This work has been accepted for publication. Andrews, O.E., Cha, D.J., Wei, C., and Patton, J.G. (2014). "RNAi-mediated gene silencing in zebrafish triggered by convergent transcription." *Sci. Rep.*

[2]All authors contributed to the design of the experiments. OEA and DJC performed experiments, developed methods, analyzed data. CW contributed Figure 11. OEA and JGP wrote the manuscript.

[*]These authors contributed equally to this work.

Abstract

RNAi based strategies to induce gene silencing are commonly employed in numerous model organisms but have not been extensively used in zebrafish. We found that introduction of transgenes containing convergent transcription units in zebrafish embryos induced stable transcriptional gene silencing (TGS) in *cis* and *trans* for reporter (mCherry) and endogenous (One-Eyed Pinhead (OEP) and *miR-27a*/b) genes. Convergent transcription enabled detection of both sense and antisense transcripts and silencing was suppressed upon Dicer knockdown, indicating processing of double stranded RNA. By ChIP analyses, increased silencing was accompanied by enrichment of the heterochromatin mark H3K9me3 in the two convergently arranged promoters and in the intervening reading frame. Our work demonstrates that convergent transcription can induce gene silencing in zebrafish providing another tool to create specific temporal and spatial control of gene expression.

Introduction

Zebrafish is a powerful model vertebrate organism to elucidate mechanisms regulating development and disease (Lieschke et al., 2007). The availability of large numbers of external, optically transparent developing embryos, combined with genetics and imaging, have provided exceptionally useful tools to study development and as powerful animal models of numerous human diseases. However, a critical missing tool is a simple, straightforward way to post-transcriptionally knock down genes in a sequence specific manner, especially at later stages of development. Recent reports have described the possibility that shRNA approaches might be useful for temporal and spatial knockdown of genes in zebrafish (Dong et al., 2013, De Rienzo et al., 2012). More commonly, antisense morpholinos have been used to knockdown gene expression during early development and have proven quite useful but with important caveats related to toxicity, limited duration, and potential off-target effects (Bill et al., 2009). Here, we describe a novel RNA interference (RNAi) mediated mechanism to silence gene expression in zebrafish. First discovered in *C. elegans*, the discovery of RNAi ushered in a new era of reverse genetics allowing sequence specific knockdown of genes in multiple model organisms (Fire et al., 1998). As an umbrella term, RNAi encompasses a number of gene regulatory mechanisms that ultimately depend on the production of small dsRNAs. Post-transcriptional gene silencing (PTGS) methods utilize small RNAs derived either from endogenous genes (miRNAs) or from exogenously delivered small interfering RNAs (siRNAs) (Hammond et al., 2001). Primary transcripts encoding miRNAs are initially processed to precursor miRNAs (pre-miRNAs) in the nucleus by Drosha (Lee et al., 2003) followed by a second processing step in the cytoplasm by the

enzyme Dicer that produces mature 21-23 nucleotide dsRNAs (Bernstein et al., 2001). Following Dicer processing, one of the strands is incorporated into a multi-component complex called the RNA Induced Silencing Complex (RISC) containing Argonaute proteins (Cenik et al., 2011). Complementary base pairing then determines the fate of targeted mRNAs. Perfect pairing (siRNAs) results in mRNA cleavage whereas imperfect pairing (miRNAs) results in translational repression, deadenylation, and subsequent degradation of target mRNAs (Djuranovic et al., 2012).

Despite the fact that the zebrafish genome encodes Dicer, Drosha, and RISC components (Flynt et al., 2009), siRNA-mediated gene knockdown remains controversial with the limited number of reports claiming successful knockdown countered by results suggesting that the effects are entirely nonspecific (Wargelius et al., 1999, Acosta et al., 2005, Zhao et al., 2008, Li et al., 2000, Oates et al., 2000, Mangos et al., 2001, Gruber et al., 2005, Wang et al., 2010). These conflicting reports account for the fact that morpholino-mediated knockdown is prevalent in zebrafish, especially during early development. Given the limitations of morpholinos, we sought to determine whether RNAi-mediated chromatin silencing could silence genes in zebrafish. Transcriptional gene silencing (TGS) directed by small RNAs has been widely reported and recent work has raised the possibility that similar mechanisms may apply in higher eukaryotes (Martienssen et al., 2005, Sabin et al., 2013, and Moazed, D., 2009). While examining transcription termination in *Schizosaccharomyces pombe*, the Proudfoot lab discovered that overlapping transcripts derived from genes organized in a convergent manner can generate dsRNAs that activate nuclear RNAi leading to histone methylation, recruitment of cohesin, and silencing of the convergent genes (Gullerova, M. & Proudfoot, N.J.,

2008). The methylation marks that regulate chromatin are removed during G2 enabling transient production of overlapping RNAs that then silence the genes during G1/S. A number of genes encoding RNAi components in the *S. pombe* genome are arranged in a convergent manner enabling autoregulation (Gullerova et al., 2011). Interestingly, the same mechanism can silence genes in mammalian cells (Gullerova, M. & Proudfoot, N.J., 2012, Calero-Nieto et al., 2010). Based on the presence of functional RNAi genes in the zebrafish genome, we hypothesized that convergent transcription could be used to specifically silence genes via a comparable mechanism. Our data demonstrate that transgenic zebrafish harboring convergently arranged genes generate dsRNAs that direct chromatin silencing of both reporter constructs and endogenous genes. Silencing leads to H3K9me3 deposition and can be suppressed by Dicer knockdown, consistent with the *S. pombe* data. This paper is the first paper to demonstrate that RNAi-mediated chromatin silencing is possible in zebrafish with the ability to precisely control spatial and temporal gene expression in embryos and adult zebrafish.

Results

Convergent transcription induces gene silencing in zebrafish.

To determine if we could trigger RNAi-mediated gene silencing in zebrafish via convergent transcription, we created plasmids with transcriptional promoters arranged in either a convergent (CT) or nonconvergent (non-CT) manner. The experimental setup included insertion of open reading frame (ORF) sequences between the two promoters with no 5' or 3' UTR sequences. For proof of principle, we first targeted mCherry for

rapid visualization of silencing. For the non-CT mCherry construct, the full length zebrafish β-actin promoter was inserted upstream of the mCherry open reading frame (ORF). The CT mCherry construct harbored a β-actin promoter at the 5' end and an inverted CMV promoter at the 3' end (Fig. 5A). Transient transgenic animals were created by co-injecting embryos at the 1-2 cell stage with the non-CT mCherry construct and *Tol2* transposase mRNA (Kawakami, K., 2009). As a marker of transgenesis, the cardiac myosin light chain promoter (cmlc2) was fused to GFP driving fluorescent expression in the heart (Kwan et al., 2007). As expected, transgenic animals containing the non-CT mCherry construct displayed robust expression of mCherry throughout the entire developing embryo (Fig. 5B). In contrast, there was a striking absence of mCherry in transgenic animals created by injection of the CT mCherry construct (Fig. 5B). Except for a few small puncta and some yolk autofluorescence, the levels of mCherry were dramatically reduced to near zero. The lack of mCherry was not due to the absence of the transgene as readily detectable levels of heart GFP were observed (Fig. 5B). Silencing was robust across multiple injections with undetectable levels of mCherry in greater than 92% of the transgenic CT-mCherry embryos. Nearly ~100% of non-CT mCherry transgenic embryos broadly expressed mCherry. Importantly, crossing of founders to generate F1 and F2 generations showed that silencing of mCherry is stable and continues to be maintained.

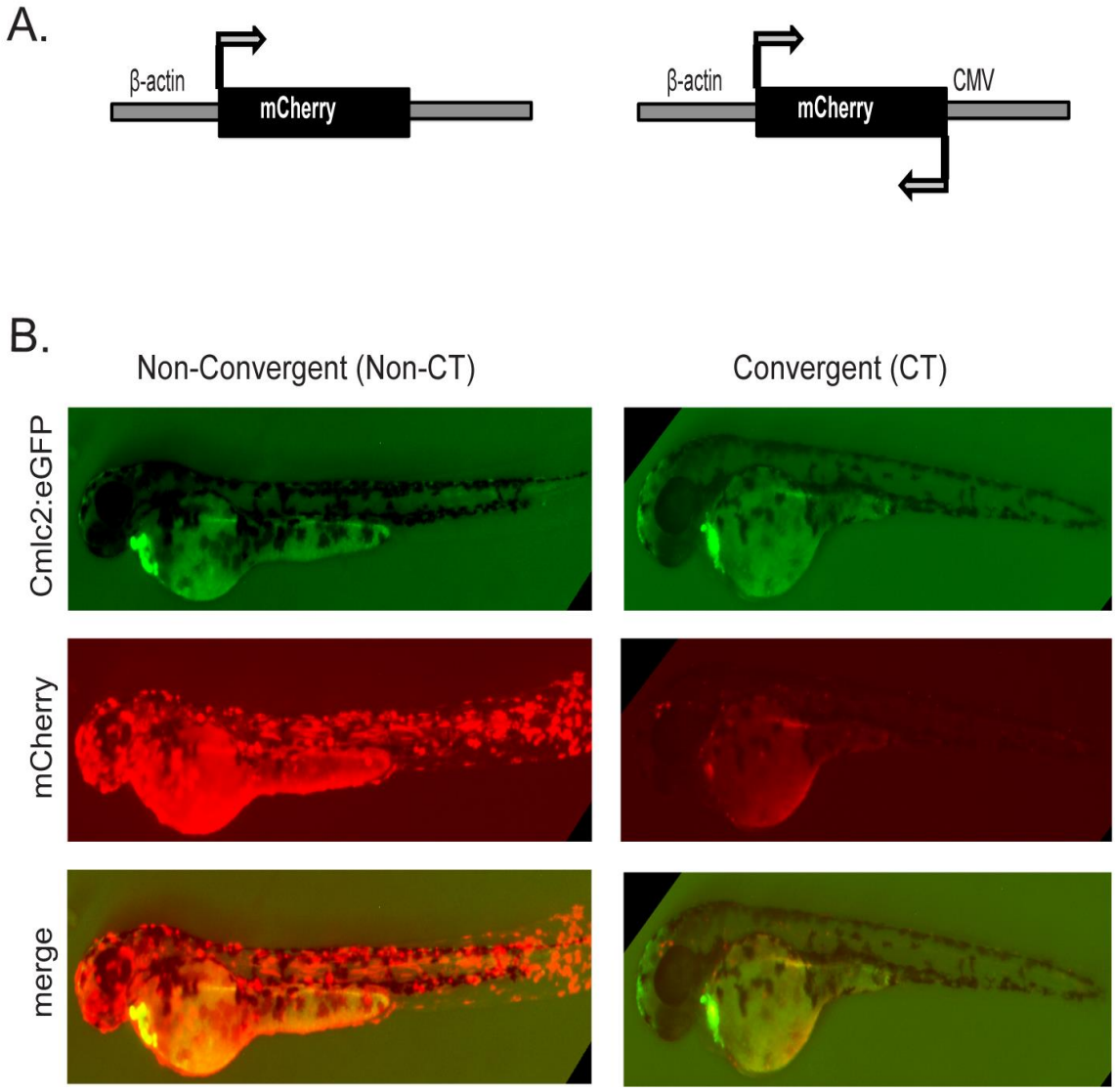

Figure 5. Convergent silencing of mCherry.

(A) Non-CT and CT constructs were designed using the *Tol2* transgenesis system with the β-actin promoter driving sense transcription and an inverted CMV promoter driving antisense transcription of the mCherry open reading frame (see Supplement for maps and sequence information). All constructs harbored heart-specific GFP (cmlc2-GFP) which enabled identification of transgenic embryos. (B) Widespread expression of mCherry was observed in non-CT embryos (386 out of 386 transgenic embryos), whereas near complete loss of mCherry was observed in CT-mCherry embryos at 2dpf (370 out of 402 transgenic embryos).

Convergent transcription produces dsRNA and silencing is suppressed upon knockdown of Dicer

In order to determine whether the mechanism of mCherry silencing was as predicted based on convergent silencing in *S. pombe* (Gullerova, M. & Proudfoot, N.J., 2012), we designed primers to amplify sense and antisense mCherry transcripts. We reasoned that convergent transcription should produce both sense and antisense transcripts. As expected, the levels of sense mCherry transcripts in non-CT F2 embryos were abundant, 30-fold more than the levels detected in CT-mCherry animals, whereas the levels of antisense transcripts were at or just barely above background levels. In contrast, RNA isolation and qPCR analyses from F2 embryos derived from multiple F1s confirmed elevated levels of both sense and antisense mCherry transcripts with slight variation between lines (Fig. 6A). These data argue against the idea that the loss of mCherry is due to simple steric interference from colliding convergent polymerases.

If the mechanism of silencing that we observe is an RNAi-mediated event, as suggested by the presence of sense and antisense transcripts, it should require Dicer activity to cleave dsRNA for packaging into RNA Induced Silencing Complexes (Castel et al., 2013). To test whether decreased levels of Dicer would suppress CT-mCherry silencing, we used antisense morpholinos to knockdown Dicer. Compared to scrambled control morpholinos, Dicer morphants displayed an increase in mCherry levels (Fig. 6B). Not only could we detect increased mCherry transcript levels after Dicer knockdown, we also observed increased mCherry protein levels using Western blots (Fig. 6C; full gel in Fig. 7). Taken together, these results indicate that silencing of mCherry is suppressed upon Dicer knockdown and consistent with the formation of dsRNA from sense and

antisense transcripts. The fact that the levels of suppression are not complete is expected and is likely due to the efficiency of Dicer knockdown but could also indicate a requirement for other components involved in nuclear RNAi mediated silencing.

RNAi-mediated chromatin modification

To further elucidate the mechanism of sustained mCherry gene suppression, we tested whether increased levels of H3K9me3 could be detected in the convergent promoters and within the mCherry open reading frame. Increased levels of H3K9me3 would be indicative of heterochromatin formation *in vivo* and would be further confirmation that convergent silencing in zebrafish utilizes a similar mechanism as proposed in *S. pombe*. To test for histone modification, we utilized formaldehyde-based *in vivo* cross-linking/immunoprecipitation (Chromatin immunoprecipitation; ChIP) with antibodies against H3K9me3. Chromatin was isolated from non-CT and CT mCherry injected embryos and specific primers were then used to amplify the border region between the β-actin promoter and the mCherry ORF, within the mCherry ORF, and in the inverted CMV promoter. We performed ChIP on F1 and F2 embryos (Figs. 8 9) and observed enrichment of H3K9me3 on convergent mCherry promoters and the mCherry ORF. Some embryos demonstrated significant enrichment of H3K9me3 after normalizing the histone silencing signal to the IgG negative control signal (Figs. 8 and 9A). As shown in Fig. 6D in F2 embryos, compared to the IgG control, we detected a 16-fold enrichment for H3K9me3 modified DNA on the β-actin promoter-mCherry border, a 15 fold enrichment within the mCherry ORF, and a 13 fold enrichment within the inverted CMV promoter. As an additional control, we examined the levels of H3K9me3

on an unrelated, active zebrafish gene, the flk-1 promoter (Jin et al., 2005). We were not able to detect any enrichment for H3K9me3 in the flk-1 promoter (data not shown). Combining experiments to detect sense and antisense transcripts, suppression upon Dicer knockdown, and H3K9me3 marks, our data are consistent with a model of RNAi-mediated heterochromatin silencing in zebrafish.

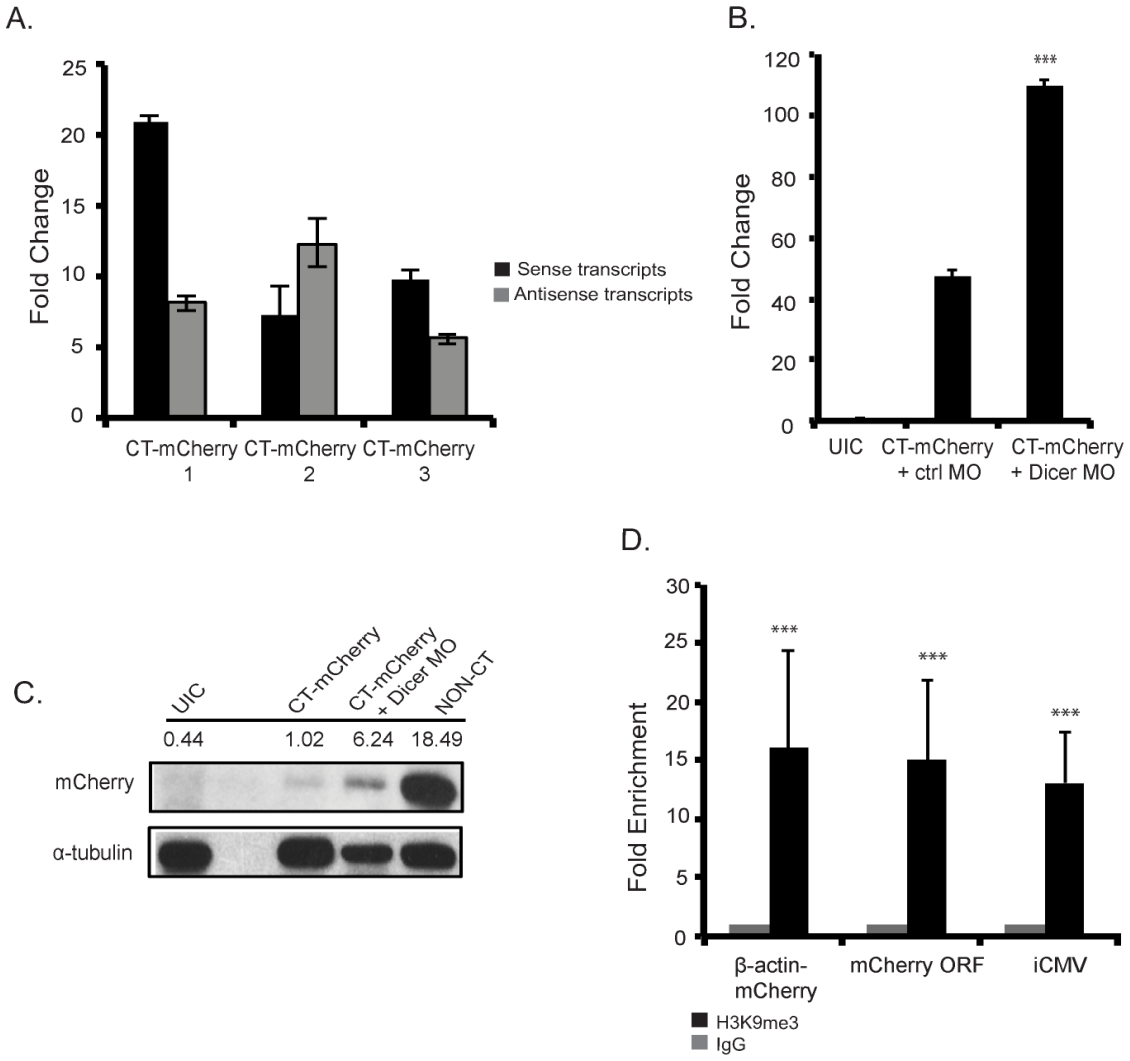

Figure 6. mCherry silencing is Dicer dependent and results in increased levels of

H3K9me3 chromatin modification.

(A) Detection of sense and antisense mCherry transcripts via qPCR analysis in F2 embryos from 3 different CT-mCherry F1 lines. (B) Increased mCherry sense mRNA levels upon co-injection of a Dicer MO compared to control (ctrl) MO injected transient transgenics at 54hpf. (C) Rescue of mCherry protein levels upon co-injection of Dicer MO. Protein lysates were prepared from embryos as in (B) and Western blots were prepared with antibodies against mCherry and α-tubulin. The full gel is shown in Fig. 7. For comparison, protein levels in a non-CT embryo are as shown. (D) ChIP-qPCR showing significant enrichment of H3K9me3 levels on convergent chromatin from CT-mCherry F2 embryos (F1 line 9119). Enrichment was determined compared to negative IgG control at 5dpf. ChIP values and standard deviations are shown from three independent biological experiments. ***p<.001 based on unpaired, two-tailed distribution Student's t-test.

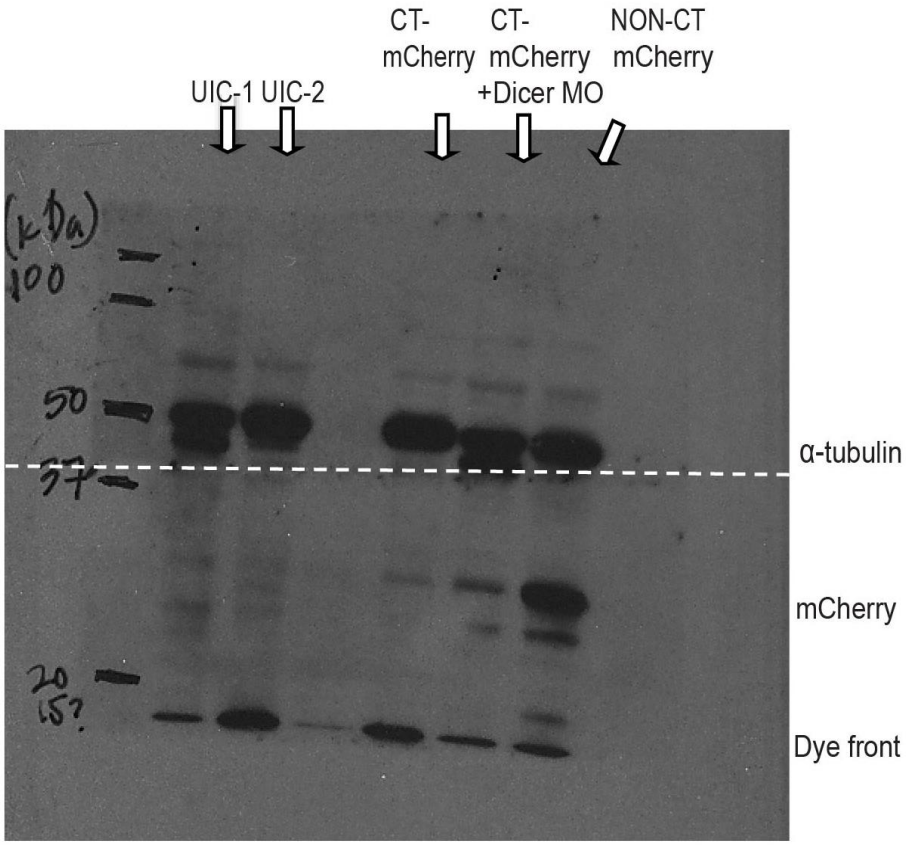

Figure 7. Rescue of mCherry by Dicer knockdown.
The full Western blot from Figure 6 is shown. The blot was cut in half to probe for either α-tubulin or mCherry. Lanes 1 and 2 correspond to UIC embryos from 2 separate clutches. Lane 3 (empty). Lane 4: CT-mCherry injected embryos. Lane 5: CT-mCherry + 2.5ng Dicer MO. Lane 6: non-CT mCherry injected embryos. Both bands correspond to α-tubulin. Band at ~27kDa corresponds to mCherry. The dye front is as indicated.

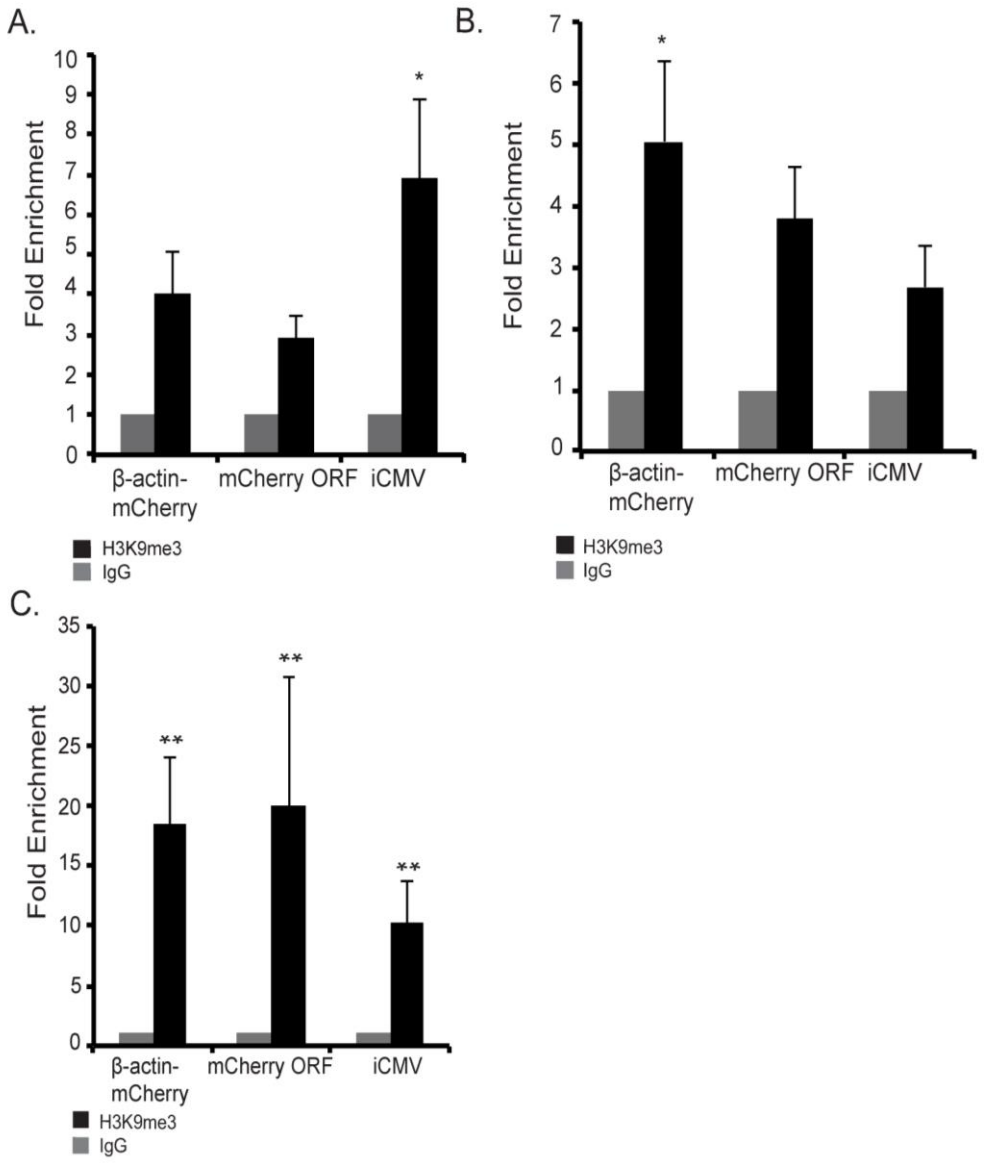

Figure 8. Enrichment of H3K9me3 in F1 CT-mCherry embryos.
(A-C) ChIP-qPCR indicating increased H3K9me3 occupancy on convergent chromatin in F1 embryos from 3 different male founders mated with wild type AB females at 5dpf. ChIP values and standard deviations are from three independent experiments. *($p<.05$) and **($p<.01$) based on unpaired, two-tailed distribution Student's t-test.

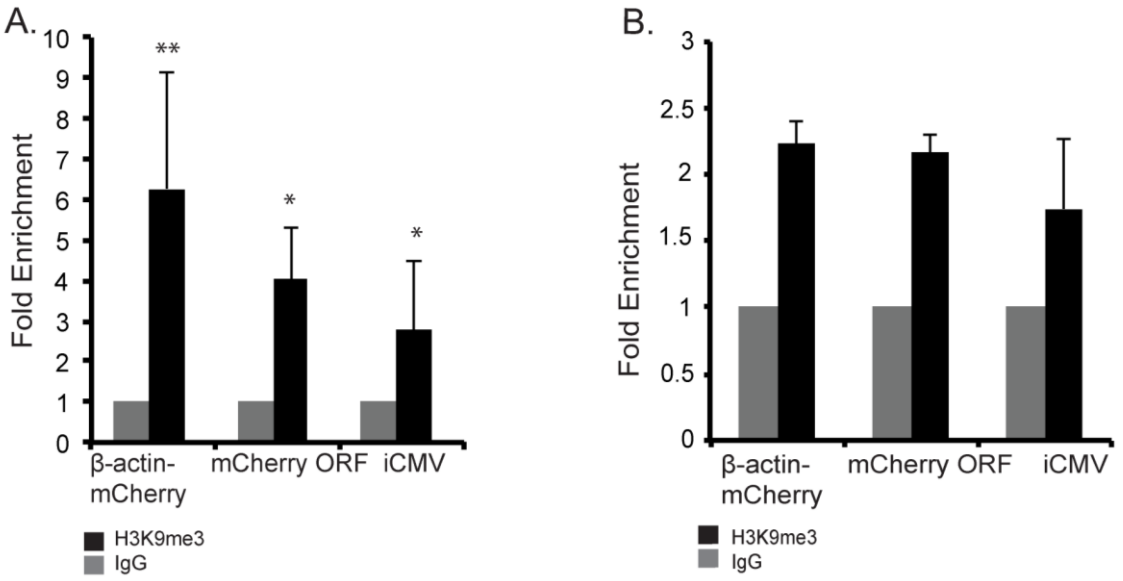

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Figure 9. Enrichment of H3K9me3 in F2 CT-mCherry embryos.
(A-B) ChIP-qPCR indicating increased H3K9me3 occupancy on convergent chromatin in F2 CT-mCherry embryos from 2 different F1 lines mated with either wild type AB fish or siblings at 5dpf. ChIP values and standard deviations are from three independent experiments. *($p < .05$) and **($p < .01$) based on unpaired, two-tailed distribution Student's t-test. Rescue experiments were performed to determine whether knockdown of Dicer would decrease the levels of H3K9me3 but because our ChIP experiments were optimized for 5 dpf, the effects were not robust, likely due to the half life of the Dicer morpholino.

Convergent silencing in *trans*: One Eyed Pinhead

To address the ability of convergent transcription to silence genes *in trans*, we generated a convergent construct targeting the endogenous One-Eyed Pinhead (OEP) gene (Schier et al., 1997) (Fig. 10A). We chose this gene because the OEP phenotype is well characterized and easy to score. As shown in Fig. 10B-G and quantified in Fig. 10H, 43% of transient transgenic animals injected with CT-OEP displayed cyclopia, strong ventral curvature of the body axis, and a reduced/misshapen notochord, consistent with OEP mutants and morpholino knockdown of OEP (Schier et al., 1997, Nasevicius, A. & Ekker, S.C., 2000). More severe defects were observed in 12% of the embryos with eyeless, headless, or severely reduced body axis. Interestingly, the more severe phenotypes resemble those observed with high concentrations of OEP MOs which were proposed to be due to the loss of both maternal and zygotic OEP expression (Nasevicius, A. & Ekker, S.C., 2000). In our experiments, the more severe effects would not be due to destruction of maternal transcripts but are consistent with increased silencing of the endogenous OEP gene.

To test whether silencing of OEP *in trans* could be suppressed by knockdown of Dicer, embryos were co-injected with the Dicer MO. As shown in Fig. 10I, knockdown of Dicer resulted in a substantial decrease in the number of embryos displaying the OEP phenotype from 43% to 15%. The OEP experiments suggest that convergent silencing can be used to silence endogenous zebrafish genes in *trans*.

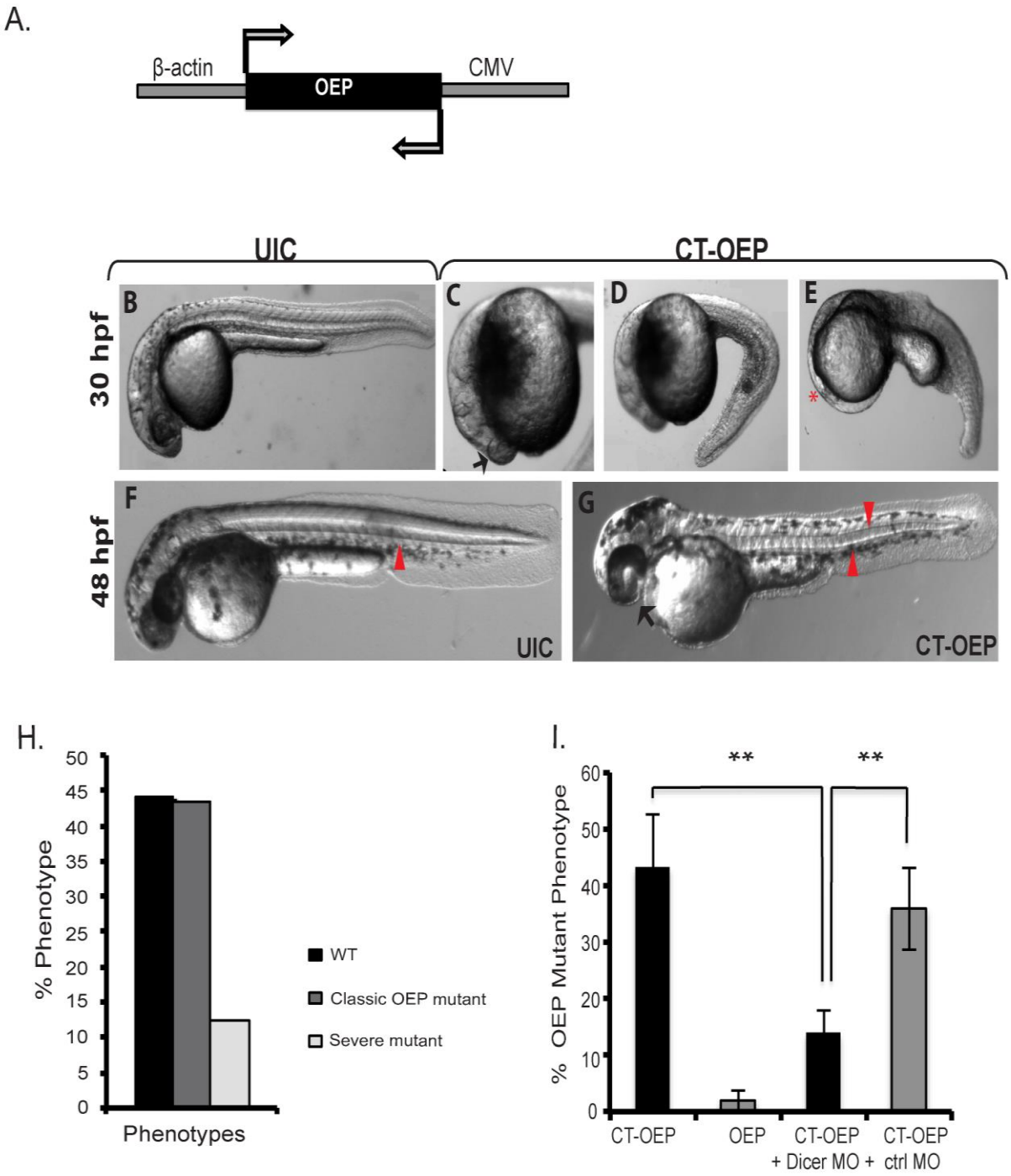

Figure 10. Silencing of One Eyed Pinhead

Convergent silencing of the zebrafish One Eyed Pinhead (OEP) gene with the β-actin promoter driving sense transcription and an inverted CMV promoter driving antisense transcription. The OEP open reading frame was directly cloned between the two promoters. (B-G) Compared to uninjected control embryos (UICs), CT-OEP injected embryos

phenocopied OEP mutants with curved body axis, cyclopia (black arrow in C), and reduced notochord (red arrows in F compared to G). In 12% of the CT-OEP embryos, we observed a more severe phenotype with complete loss of eye development (red asterisk in E). (H) Graph showing % transient transgenics showing WT, classic OEP, or more severe phenotypes. n=167. (I). Silencing of OEP is Dicer dependent. Embryos were injected as indicated in the absence or presence of Dicer MO or a control mismatch morpholino. **p<0.005.

Convergent silencing of *miR-27a/b*

To further demonstrate the utility of targeting endogenous genes with convergent transcription, we decided to target a miRNA gene. We chose *miR-27a/b* because morpholino and CRISPR knockdown of *miR-27a/b* results in dramatic craniofacial defects and impairment of pectoral fin outgrowth that can be readily detected both visually, and by staining extracellular matrix (ECM) and cartilage with alcian blue (Kara et al, manuscript in preparation). *Tol2* based CT and non-CT constructs were generated with identical convergently arranged zebrafish Ubiquitin promoters flanking a fusion of DNA sequences encoding the precursors of both *miR-27a* and *miR-27b* (Fig. 12A). We chose to use the Ubiquitin promoter as a means to test the utility of multiple promoters and to begin to address questions related to differential promoter strength. Although the frequency of the defect was lower than what we observed with mCherry and OEP convergent silencing, we found that transgenic animals containing the CT construct showed a nearly identical phenotype to that produced upon morpholino knockdown (Fig. 11) of *miR-27a/b* (Fig. 12B). This included loss of craniofacial structures, loss of upper and lower jaws, and impaired pectoral fin outgrowth. Only ~8-10% of transgenic animals displayed the phenotype but this was significantly higher than that compared to DICs where much less than1% of the embryos showed some form of jaw defect or slight changes in alcian blue staining patterns (Fig. 12C). While the reasons for less efficient

silencing of the *miR-27a/b* genes are not clear, the results indicate that convergent transcription can be used to silence noncoding RNAs *in trans*.

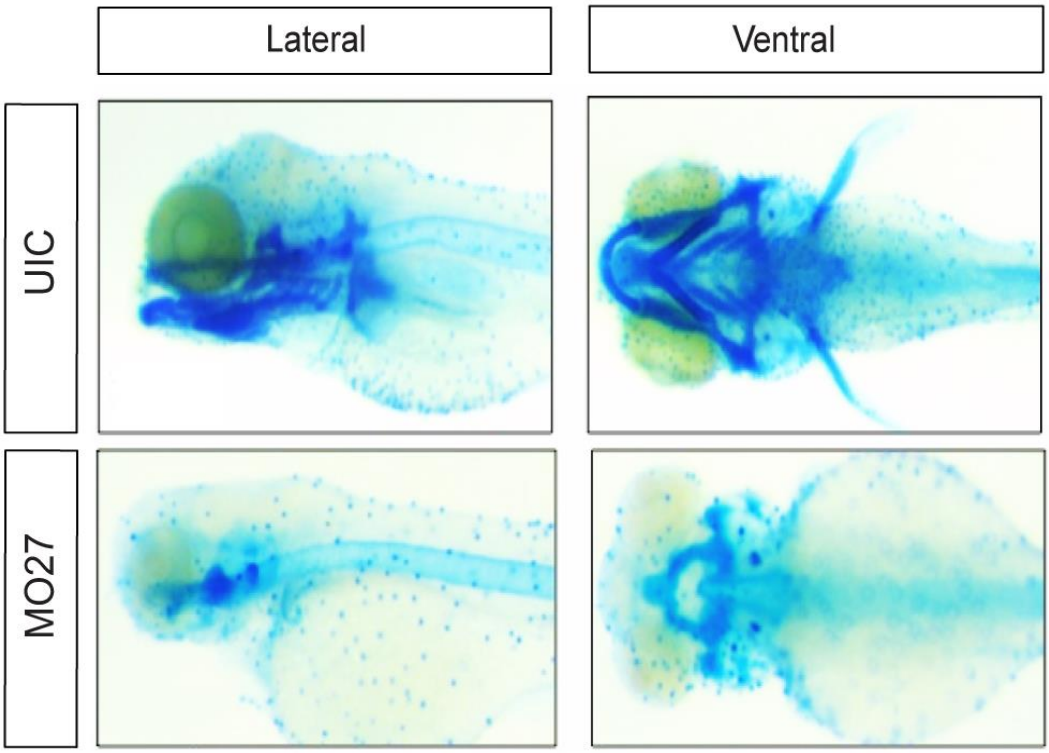

Figure **11. *miR-27a/b* morphants.**
Uninjected (UIC) embryos or embryos injected with morpholinos against *miR-27a/b* were stained with alcian blue at 4dpf to detect extracellular matrix and developing cartilage.

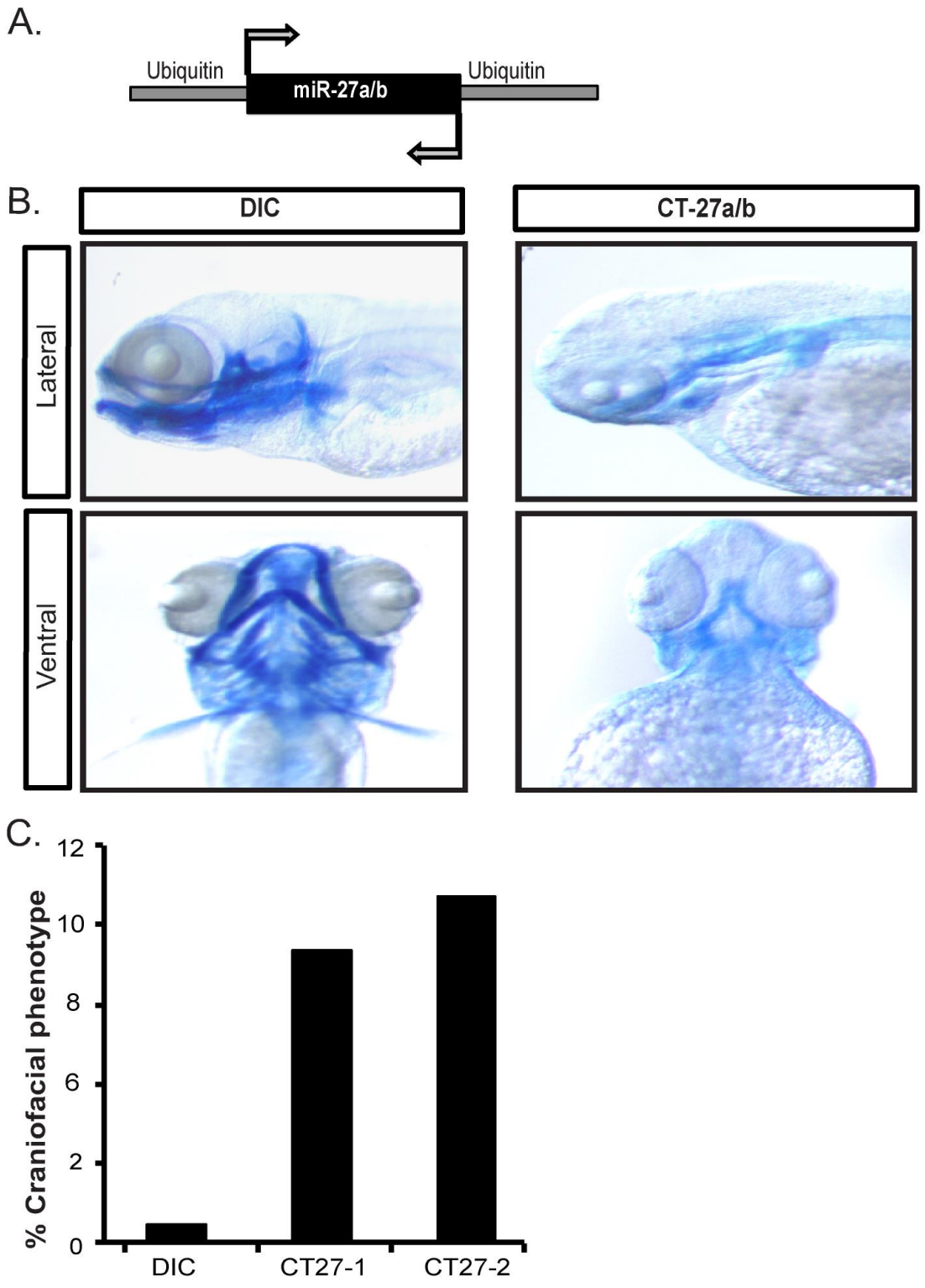

Figure 12. Silencing of *miR-27a/b*.
(A) Convergent silencing of the *miR-27a/b* genes with ubiquitin promoters driving both sense and antisense transcription. A synthetic DNA sequence encompassing precursor sequences for both miR-27a and miR-27b were directly cloned between the two promoters. (B) Compared to control embryos injected with dye only (DIC), lateral and ventral views show that CT-*miR-27a/b* injected embryos phenocopy *miR-27a/b* morphants (see Supplemental

Figure 4) with defects in pharyngeal arch morphogenesis, craniofacial defects, and inhibition of pectoral fin outgrowth as shown by decreased staining of ECM (cartilage) with alcian blue. (C) Compared to DICs, craniofacial defects observed with alcian blue staining were observed in 8-9.5% of CT-*miR-27a/b* embryos using two different plasmid clones of the construct in (A). n=191 for DIC, n=458 for CT-*miR-27a/b* clone 1, and n=272 for CT-*miR-27a/b* clone 2. All images are at 4dpf.

Discussion

Using convergent transcription to induce nuclear RNAi-mediated gene silencing, we achieved targeted silencing of exogenous mCherry and endogenous mRNA (OEP) and miRNA (*miR-27a/b*) encoding genes. This paper is the first to show that nuclear RNAi mediated gene silencing can be used in zebrafish to trigger heterochromatin formation. The mechanism of silencing in zebrafish is hypothesized to follow that described in fission yeast, which is supported by two sets of experiments. First, Dicer knockdown suppressed transcriptional gene suppression of both mCherry and OEP, suggesting that long dsRNAs derived from the convergent promoters fail to be efficiently processed and thereby unable to elicit TGS. Second, our ChIP analyses showed increased H3K9me3 levels coincident with silencing. Enrichment of H3K9me3 was maintained and became even more pronounced from generation to generation, with greatest H3K9me3 occupancy observed in F2 generations. These novel findings demonstrate that convergent transcription can trigger nuclear RNAi pathways to allow reverse genetics in zebrafish. With multiple tools available to regulate transcription in zebrafish, precise spatial and temporal control of gene expression is possible using convergent transcription (Halpern et al., 2008, Bai et al., 2009).

Currently, the most common method to knockdown genes in zebrafish utilizes antisense morpholinos to block mRNA translation, inhibit pre-mRNA splicing, or

interfere with miRNA function (Nasevicius, A. & Ekker, S.C., 2000, Flynt et al., 2007). Despite ongoing work, the use of RNAi in zebrafish remains elusive (Kelly, A. & Hurlstone, A.F., 2011). Reports of successful knockdown via RNAi have been countered by reports of broad nonspecificity. Recently, shRNA expression vectors have been generated to produce siRNAs mimicking miRNA pathways (Dong et al., 2013, De Rienzo et al, 2012, Dong et al., 2009) but it remains to be determined how useful or efficient these methods will be in generating stable knockdown lines. For mCherry knockdown, we observed stable, ongoing silencing. However, we also observed gene dependent differences in efficiency. Silencing of mCherry was greater than 92%, silencing of OEP was approximately 50%, and silencing of *miR-27a/b* was the least efficient at ~9%. Differences in efficiency can be due to numerous causes with obvious challenges for silencing high copy genes with stable mRNAs. Additionally, as more is learned about how small RNAs direct sequence specific silencing, we will likely learn more about how chromatin is altered and how chromosome location and positioning might affect silencing. Nevertheless, the ability to generate stable convergent lines creates an advantage over standard morpholinos that can lack precise spatial and temporal control, not to mention possible off target and nonspecific effects. As we develop this strategy further, we will examine the expansion of heterochromatin marks on different promoters and ORFs as well as discern the exact requirements for the size and abundance of the convergent transcripts to gain a better understanding of the mechanism and functional relevance of convergent silencing in zebrafish.

In addition to variable efficiencies of silencing in mCherry, OEP and *miR-27a/b*, we also found that some genes were not able to be targeted for silencing. Convergent

constructs targeting different pigment genes (*tyrosinase, golden, sandy*) showed a delay or only very limited silencing of pigment expression, provided the embryos survived, especially for tyrosinase. Despite changes in the use of different strength promoters or different length ORF sequences in the convergent constructs, we were never able to generate non-pigmented embryos. A potential explanation for the lack of silencing with the CT-pigment constructs could be due to limiting levels of Dicer since Dicer over-expression was shown to promote primary siRNA generation in a genome wide dependent manner in *S. pombe* (Yu et al., 2014). Further, sequence context and chromosomal position can also inhibit siRNA-mediated heterochromatin formation (Yu et al., 2014). Lastly, even though we have focused extensively on the role of Dicer, other components of the RNAi pathway, such as Argonaute proteins (Lund et al., 2011) are likely required and might be limiting for nuclear RNAi. Future work will be necessary to answer such questions as well as positional and sequence-dependent effects on convergent gene silencing. Nevertheless, our results demonstrate that RNAi-mediated heterochromatin formation can be used to silence genes in zebrafish.

Tools to efficiently knockdown gene expression or generate gene knockouts in zebrafish are rapidly evolving with CRISPR mediated gene editing rapidly becoming a common technique in zebrafish (Jao et al., 2013). In our hands, knockdown of *miR-27a/b* is most efficient with antisense morpholinos, followed by CRISPR knockout, followed by convergent silencing. As discussed above, morpholino based knockdowns suffer from temporal and spatial limitations. Although spatial restrictions can be overcome using CRISPR technology, temporal and reversible strategies are still lacking and it may not always be desirable to create irreversible genetic mutations. For some applications,

convergent silencing could prove useful, especially with tissue specific and inducible promoters that can be further used to control temporal and spatial production of dsRNA.

Material and Methods

Ethics Statement

All zebrafish experiments and methods were carried out in accordance with the approved guidelines from the Vanderbilt Institutional Animal Care and Use Committee (IACUC) under protocol M-09-398.

Plasmid Constructs

Convergent vectors were created by inserting ORF sequences directly between convergent promoters with no 5' or 3' UTR sequences. For *miR-27a/b*, a region encompassing the precursor sequence for both miRNAs was inserted directly between convergent Ubiquitin promoters (see below). All plasmid constructs were created using Gateway technology (Kwan et al., 2007) as shown in Figure 13. Destination vectors were created in the pDEST2G2 backbone by Gateway LR Clonase II reactions (Invitrogen) using the following vectors:

For non-CT-mCherry: p5E-β-Actin, pME-mCherry, p3E-pA. CT-mCherry: p5E-β-Actin, pME-mCherry, p3E-iCMV. non-CT OEP: p5E-β-Actin, pME-OEP, p3E-pA. CT-OEP: p5E-β-actin or p5E-CMV, pME-OEP, p3E-iCMV or p3E-iβ-Actin.

Inverted promoters were inserted into p3E (3'-entry vectors) to transcribe the antisense strand of target genes. P3E-iCMV, p3E-iβActin and p3E-Ubi promoter sequences were

PCR amplified with Phusion polymerase (NEB) from p5E-CMV/SP6, p5E-β-Actin (Tol2 Kit) and p5E-Ubi (a kind gift from Dr. Josh Gamse), respectively. Forward and reverse primers were flanked by attB2 and attB3 sites (lowercase, respectively:

Table 3. List of inverted promoter primers

iCMV	Forward- 5'ggggacagctttcttgtacaaagtggaTCACCTAAATCAAGCTTGCTC 3'
iCMV	Reverse - 5' ggggacaactttgtataataaagttgccAAGGCCTCTTCGC 3'
iβ-Actin	Forward- 5' ggggacagctttcttgtacaaagtggaGGATCCGGCTGAACTGTAAAAGAAAG 3'
iβ-Actin	Reverse- 5' ggggacaactttgtataataaagttgccggtaccAATTCCAGTTTGAAG 3'
iUbi	Forward – 5' ggggacagctttcttgtacaaagtggaGGATCCCTGTAAACAAATTCAAAG 3'
iUbi	Reverse – ggggacaactttgtataataaagttgCCCTCGAGACCAGCAAAGTTCTAG

pME-OEP: OEP cDNAs were generated using 500ng of total RNA from sphere stage wild type embryos using Superscript II reverse transcriptase (Invitrogen). Forward and reverse primers flanked by attB1 and attB2 sites (lowercase) were then used for PCR amplification:

F- 5' ggggacaagtttgtacaaaaaagcaggctTGCCACCATGACGAGTCAACTGTTCG 3'
R- 5' ggggaccactttgtacaagaaagctgggtGCATACGGAGCGTTACAGTACA 3'

pME-*miR27a/b*: The following sequence was ordered from GeneArt (Life Technologies) containing a fusion of precursor sequences for *miR-27a* and *miR-27b*:

GGGGACAAGTTTGTACAAAAAAGCAGGCTTAGGGCCAATAGCTAATATGGCC
AATGATTTggagagcaTCTGGATATGATGTCTGCTGAAGTTTCGTGAGGTGCAGG
ACTTAGCTCACTCTGTGAACAGATCTCGGATATCCTATGTTCACAGTGGCTAA

GTTCCGCTCCTCTGAGGCCCACACTCGAAATCAGCCAGGaggtgagaacacaaacatgac
GCGGCCGCTCTTTTCTAGCAGGTGCAGAGCTTAGCTGATTGGTGAACAGTGAT
TGAACTCTTTGTTCACAGTGGCTAAGTTCTGCATCTGAGGAGAGGACAGTGTA
CCCAGCTTTCTTGTACAAAGTGGTCCCC

BP reactions (Invitrogen) were performed to recombine the PCR amplicons into entry vectors following the manufacturers protocol. All entry vectors were sequence verified with M13 F and M13 (-21) R primers. The maps of the various vectors are shown in Figure 13-15.

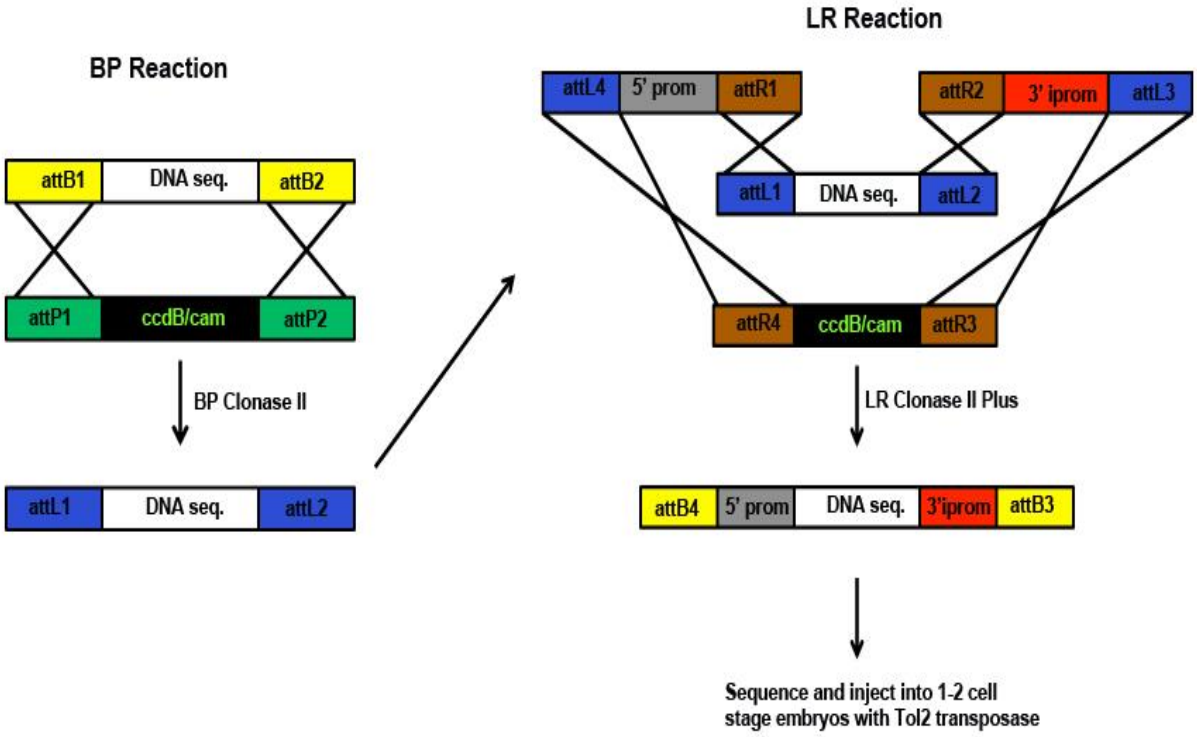

Figure 13. Model of Gateway Vector Transfer
Convergent plasmid constructs were created using Gateway technology (Kwan et al., 2007). The BP Clonase II enzyme catalyzes the transfer of specific DNA sequence of interest (ORF etc) into a donor vector using a recombination reaction releasing the ccdB/cam cassette and producing new recombination sites (attL1/L2). The BP clone is further recombined with two other plasmids containing different recombination sequences. One plasmid contains the sense promoter (5'prom) and another containing the inverted promoter on the 3' end (3' iprom) along with the BP clone recombine via LR clonase II Plus into a pDEST2G2 backbone encoding heart-specific GFP.

Convergent Transcription Vector Maps

Shown below are maps for the three Convergent Transcription (CT) vectors. Fully annotated sequences are available from Addgene.

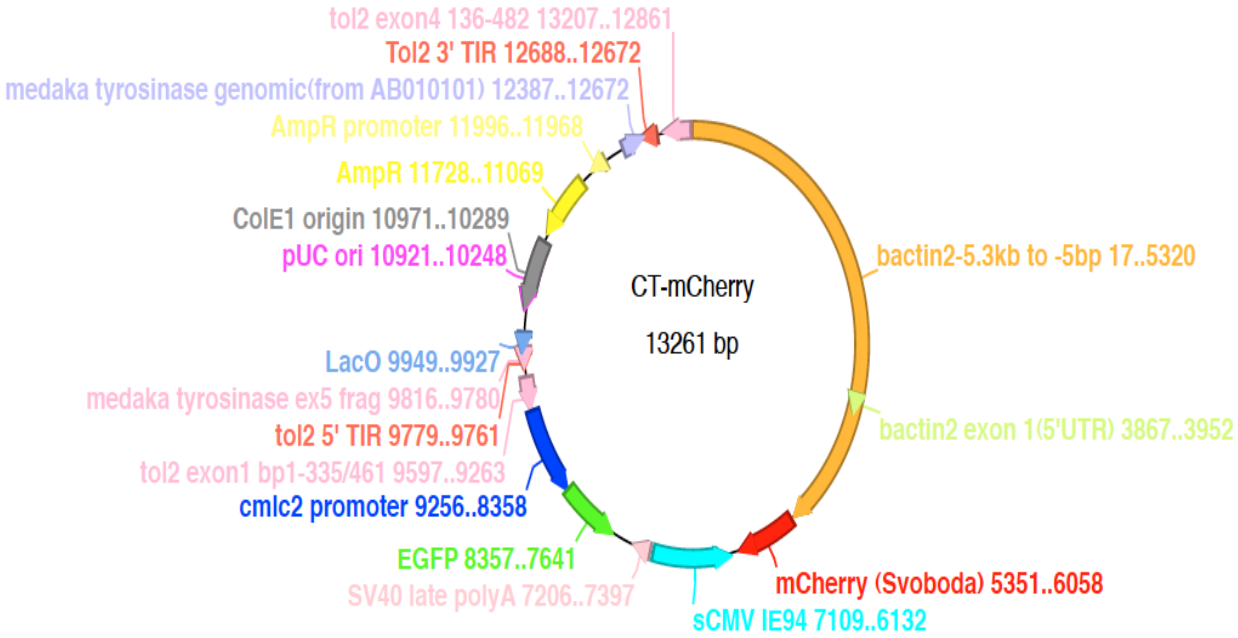

Figure 14. CT-mCherry Map.
The mCherry ORF was cloned directly between the zebrafish β-actin promoter and an inverted CMV promoter. The β-actin and CMV promoters were obtained as 5' entry clones from the Tol2 kit (http://tol2kit.genetics.utah.edu/index.php/Main_Page). The mCherry ORF was recombined into the above vector from a middle entry mCherry clone from the Tol2 kit. The non-CT-mCherry vector is identical except it lacks the inverted CMV promoter.

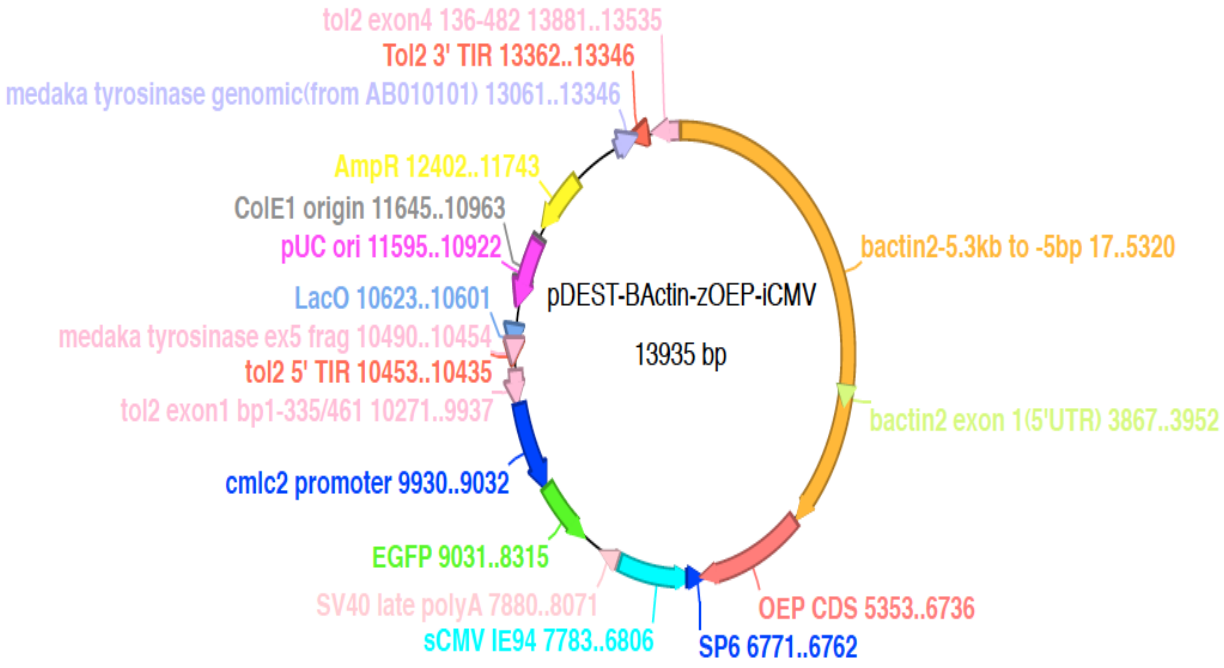

Figure 15. CT-OEP Map
The zebrafish One Eyed Pinhead ORF was cloned directly between the zebrafish β-actin promoter and an inverted CMV promoter. The OEP ORF was amplified by PCR and recombined into an empty middle entry vector from the Tol2 kit. The non-CT-OEP vector is identical except it lacks the inverted CMV promoter.

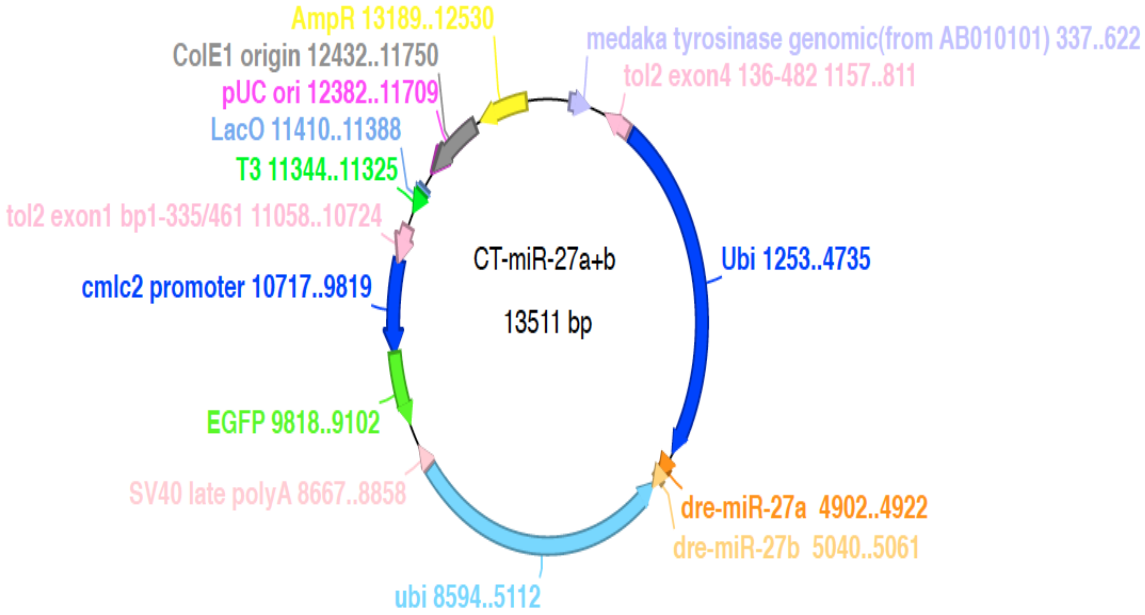

Figure 16. CT-miR-27a/b Map
Sequences encompassing the precursor transcripts from miR-27a and miR-27b were cloned directly between two zebrafish ubiquitin promoters. Two ubi promoters from 5' and 3' entry vectors (see Methods) were recombined with a middle entry vector containing the precursor sequences for miR-27a/b. The non-CT-miR-27a/b vector is identical except it lacks the reverse Ubi promoter.

Transgenic Animals

Transgenic animals were created using Gateway vectors and the *Tol2* transgenesis system (http://chien.neuro.utah.edu/tol2kitwiki/index.php/Main_Page) (Kwan et al., 2007, Villefranc et al., 2007). For injections, embryos from wild type AB fish were collected from a 15-minute mating period and microinjections of pDEST-based plasmids were performed (Suster et al., 2009). Briefly, plasmid DNA and *Tol2* transposase mRNAs were injected into 1-2 cell stage embryos. For non-CT-mCherry and CT-mCherry, 22.5pg plasmid DNA and 7.5pg transposase mRNAs were injected. For CT-OEP, 11.25pg DNA and 7.5pg transposase mRNAs was injected. For CT-*miR-27a/b*, 30pg DNA and 7.5 pg transposase mRNAs were injected.

RNA isolation and sense/antisense transcript detection

Total RNA from GFP+ 4 dpf embryos was isolated using Tri-Reagent (MRC). 20 embryos were used per 1ml Tri-reagent. 800ng of RNA was reversed transcribed with M-MLV RT (Promega) using Oligo$(dT)_{16}$ (Applied Bioscience) or strand-specific primers. Sense and antisense transcripts were generated and quantified by SYBR green (BioRad) in real-time PCR (BioRad). Transcripts were normalized to GAPDH and relative fold changes were normalized to values of WT embryos, set at 1, and determined by the $\Delta\Delta C_t$ method (Livak et al., 2001).

Strand-specific primers:
mCherry anti-sense detection: 5’ CGACATCCCCGACTACTTGAAGC
mCherry sense detection: 5’ TCTTGGCCTTGTAGGTGGTCTT

qRT-PCR primers:
GAPDH: F- 5’ GGCAGAAGGCGGCAAACT; R- 5’ CTGGGTCCCTCTCGCTATAGA
mCherry: F- 5’ CCCCGTAATGCAGAAGAAGA; R- 5’TCTTGGCCTTGTAGGTGGTC

Dicer knock-down

Fluorescein-tagged antisense morpholinos (Gene Tools) against the 5’UTR of Dicer (*dicer*^{MO1}) (Weinholds et al., 2003) or the translational start (*dicer*^{start}) (Thatcher et al., 2008) were co-injected with the CT construct and transposase mRNAs into 1-2 cell stage wild-type embryos of the AB strain. As a control, a mismatched Dicer MO (*dicer*^{mm2}) (Wienholds et al., 2003) was used. Injection of either *dicer*^{MO1} and *dicer*^{start} produced the same phenotype and comparable levels of rescue. Embryos were examined at 54hpf.

Western blots

At 72 hpf, GFP+ embryos were de-yolked and protein lysates were prepared from 30 embryos in lysis buffer as described (Flynt et al., 2007). 25ug of total protein was loaded per lane as determined by Bradford assays (Biorad). Blots were probed with anti-mCherry1C51 (Novus Biologicals) and anti-α-Tubulin (Abcam). For detection, ECL Mouse- and ECL Rabbit-IgG-HRP-linked secondary antibodies (GE Sciences-NA931) were used followed by visualization with ECL (Perkin Elmer).

ChIP and qPCR

Approximately 25-60 embryos were washed in 1X PBS. Embryos were incubated

with 2.22% formaldehyde for 10 minutes at 25°C with gentle rotation. 150mM of glycine was used to quench the reaction for 10 minutes at 25°C with gentle rotation. Embryos were then washed three times with 1X PBS for 5 minutes each and then dissociated in ChIP Whole Cell Lysis Buffer (10mM Tris-HCl, pH 8.1, 10mM NaCl, 3mM MgCl2, 1% NP40, 1% SDS, 0.5% DOC, and 1X protease inhibitor cocktail). The protease cocktail was from Sigma (P8340). DNA was fragmented using a probe sonicator (Heat Systems Ultrasonics) followed by water bath sonication (Diagenode) at the high setting (3 rounds of 5 minutes each). After centrifugation for 20 minutes, supernatants were collected. Reverse crosslinking of chromatin aliquots, DNA isolation, and fragmentation checks were performed by incubation at 65°C overnight, phenol/chloroform extraction, and agarose gel electrophoresis, respectively. Immunoprecipitation was conducted using 10ug of chromatin with 1ug of either negative control rabbit IgG (Cell Signaling) or H3K9me3 antibody (Diagenode pAb-056-050) incubated overnight at 4°C. Magnetic Protein A beads (Millipore) were pre-washed with chip dilution buffer (16mM Tris-HCl, pH 8.1,162mM NaCl, .0096mg SDS, 2% Triton-X 100) and then blocked by incubation overnight with 1.5% BSA, 0.03% protease inhibitor cocktail, and 0.006% of Herring sperm ssDNA at 4°C. An equal volume of blocked bead solution was then added to each chromatin-antibody bound sample for 1 hour at 4°C. Samples were then washed for 5 minutes each with three different buffers. Wash Buffer 1 (20mM Tris-HCl, pH 8.1, 150mM NaCl, 2mM EDTA, 0.1% SDS, and 1% Triton X-100), Wash Buffer 2 (20.5mM Tris-HCl, pH 8.1, 493mM NaCl, 2mM EDTA, 0.103% SDS, and 1% Triton X-100) and Wash Buffer 3 (10 mM Tris-HCl, pH 8.1, 1 mM EDTA, 250mM LiCl, 1% NP-40, and 1% DOC). Following two washes in TE buffer, antibody

bound chromatin was eluted in 1% SDS and 10mM $NaHCO_3$. Tubes were then incubated at 65°C overnight after addition of 200mM NaCl and then placed at 45°C after addition of 40mM Tris-HCl (pH 7.5), 10mM of EDTA (500mM) and 20ug of PK (20mg/mL) for 1-2 hours. Following reverse crosslinking, DNA was isolated (Qiagen) and dissolved in H_20. ChIP-qPCR was performed and quantified using Sybr green (Bio-Rad). Fold enrichment was calculated by normalization of signal (H3K9me3 CT values) to background (IgG CT values) using the ΔΔCt method.

Acknowledgements

We thank Qiang Guan for excellent zebrafish care at the Vanderbilt University Stevenson Center Fish Facility. This study was supported by a grant from the National Eye Institute–National Institutes of Health to J.G.P (R21 EY 019759). OEA was supported in part by grants from the NIH (IMSD GM062459) and the Microenvironmental Influences in Cancer training grant (T32 CA009592). OEA, DJC, and CW were supported in part by the Department of Biological Sciences and the Gisela Mosig Fund. The authors wish to thank Dr. Josh Gamse and Sataree Khuansuwan for *Tol2* based reagents and constructs. We also thank Brian McKenna and Dr. Roland Stein for help and advice with ChIP experiments and Daniel Levic for alcian blue staining.

CHAPTER 3

DISCUSSION

Significance

The field of small RNA biology and the RNAi pathway in particular has advanced tremendously since the initial discovery of *lin-4* and *let-7*. Extensive progress has been made towards elucidating miRNA and siRNA biogenesis including the discovery of key processing enzymes ---Dicer and Argonaute--- and genome wide analysis techniques have aided in the identification of conserved miRNAs across diverse animal species (Bernstein et al., 2001, Carmell et al., 2002, Bartel, D., 2004). Molecular and computational strategies have illuminated functionally relevant targets of many miRNAs and uncovered roles in development and disease (Andrews, O., & Patton, J.G., 2014, Singh, S.R. & Rameshwar, P., 2014). Remarkably, the gene silencing function of small RNAs has expanded to include regulation of chromatin at the transcriptional level, in addition to targeting mRNAs at the post-transcriptional level. Pioneering studies in plants have shown that transfection with promoter specific dsRNA produces small RNAs of ~23 nt and induces methylation of homologous DNA sequences (Mette et al., 2000). In depth studies in *S. pombe* have also shown RNAi-mediated regulation of heterochromatin via H3K9me3 deposition at centromeres and regions containing CG repeats. However, the majority of RNAi-based gene silencing strategies in higher vertebrates rely almost exclusively on the PTGS mechanism.

In zebrafish, the use of RNAi has remained controversial in achieving sequence

specific gene knockdown. Silencing inefficiency is not due to the lack of functionality of the RNAi machinery as Dicer deficiency produces organ abnormalities and growth arrest at 10dpf, suggesting that Dicer and miRNA processing are crucial for zebrafish development (Wienholds et al., 2003). Given that the RNAi machinery is intact, the inconsistency in RNAi-based silencing in zebrafish is puzzling. Injection of dsRNA or siRNA has produced inconsistent results with some reports claiming specific silencing and others demonstrating high 'off target' effects (Wargelius et al., 1999, Li et al., 2000, Oates et al., 2000, Zhao et al., 2001, Mangos et al., 2001, Dodd et al., 2004, and Gruber et al., 2005). As a result, the majority of loss-of-function studies in zebrafish utilize morpholinos, which are highly specific but are short-lived with potential off-target effects (Bill et al., 2009). The significance of the work in this thesis is that this is the first to investigate and demonstrate TGS via an RNAi pathway in zebrafish. The utility of the convergent silencing strategy will enable spatial and temporal control by using inducible or tissue specific promoters. This novel strategy provides a blueprint for a simple, cheap, and straightforward way to silence genes in a sequence specific manner. Additionally, the studies outlined in this thesis may serve as a foundation to discover how widespread and functionally relevant endogenous convergent transcription is in zebrafish. One potential link to CT-like mechanism may occur with long noncoding RNAs (lncRNAs), which have been shown in models including mice and mammalian cells to regulate gene expression in *cis* and *trans* as well as interacting with chromatin modifying machinery (Pauli et al., 2012). The identification of over 1100 multi-exonic noncoding transcripts in zebrafish embryos including intronic overlapping lncRNAs and exonic antisense overlapping lncRNAs that can regulate specific genes in a spatial and temporal manner is

promising evidence for CT-mediate silencing. In the future, this hypothesis can be tested by knocking down target lncRNAs and examining RNAi-mediated heterochromatin formation.

Transcriptional Gene Silencing in zebrafish

The observation that CT triggered sequence specific RNAi-mediated gene silencing in *S. pombe* and human cells prompted us to explore convergent silencing in zebrafish. Our data has successfully demonstrated that convergent silencing of mCherry expression occurs via Dicer activity and H3K9me3 deposition. Targeting of endogenous OEP and *miR-27a/b* genes successfully produced embryos that phenocopied OEP mutants and *miR-27a/b* morphants. Our work has demonstrated the utility of TGS in zebrafish and showed that stable silencing is achievable as observed with silencing of mCherry in F2 embryos via RNAi. This work also provides evidence to support the conservation of RNAi-mediated heterochromatin formation mechanism exemplified in *S. pombe* (Gullerova et al., 2012).

Future Directions

Convergent silencing provides an alternative tool to knockdown genes with the option for reversal of gene silencing. However, there are particular limitations with our strategy as we observed reduced efficiency when silencing in *trans* compared to *cis*. One likely hindrance stems from the differing promoter strengths. We observed greater silencing when using distinct β–actin and CMV promoters compared to dual zebrafish

Ubiquitin promoters. This may suggest that using distinct promoters is more favorable for generating overlapping transcripts that form dsRNA and also more favorable for growth in competent bacteria since we observed inhibition of growth of plasmids containing dual β–actin promoters. The size of the promoters and the subsequent final size of the destination vector may also be rate limiting giving that CT-plasmids containing dual Ubiquitin promoters (3.5kb for each) grew compared to dual β-actin promoters (5.3kb for each). Another reason for reduced inefficiency could be targeting genes of high copy number and since the majority of our experiments were performed in AB strain, the finding that laboratory strains including AB contain higher number of copy number variants than natural "outdoor" strains supports this supposition (Brown et al., 2011).

One intrinsic limitation is the lack of directionality with the *Tol2* transposase. A more improved system would precisely regulate where the convergent plasmids become inserted in the zebrafish genome instead of random insertion. Another limitation is the possible saturation of nuclear Dicer with overlapping transcripts, which may lead to inefficient silencing. It has been recently shown that Dicer over-expression produces genome wide increase of small RNAs in *S. pombe* (Yu et al., 2014). One approach to overcoming this issue is to co-inject *in vitro* transcribed Dicer containing a NLS (nuclear localization signal) with CT-constructs to increase silencing efficiency of endogenous genes. While this seems fairly straightforward, proper primer design and selection of high-fidelity polymerases is necessary to amplify Dicer due to its large size (6kb). However, we have to be mindful that injection of high amounts of RNA – *Tol2* transposase and NLS-tagged Dicer--- might generate some non-specific effects and reduce viability of embryos. Another strategy would be to either generate a stable

transgenic line using the Gateway vector transfer system to constitutively express tagged Dicer harboring an NLS signal or generate an inducible system by using a Tet-on system that expresses nuclear Dicer upon doxycycline addition (Campbell et al., 2012). Since we observed that pigment genes are refractory to CT-mediated silencing, we cannot exclude the possibility that other regulatory mechanisms exist to prevent TGS of specific mRNAs in zebrafish. Since polyadenylation signals in the 3'UTR of specific mRNAs have been shown to inhibit TGS in *S. pombe* (Yu et al., 2014), one approach would be to generate CT-plasmids containing 3'UTR sequences flanked by convergent promoters. If silencing efficiency increases, this would indicate that sequestration of the 3'UTR from interacting partners allows for TGS to proceed. While this experiment would not prove that signals within 3'UTRs prevent TGS, it may provide useful information, especially since it has been previously shown that shRNA vector-mediated silencing targeting the 3'UTR of GFP provided appreciable knockdown (Kelly, A., & Hurlstone, A.F., 2011).

One of the critical future aims will be to demonstrate the specificity of convergent silencing by examining the spread of H3K9me3 in CT-mCherry F2 embryos. ChIP-seq (chromatin immunoprecipitation sequencing) is the most sensitive way to select for H3K9me3 bound DNA, identify associated DNA sequences, and to map them back to the zebrafish genome to determine the localization of this heterochromatin signature. The expected outcome would be localized and specific H3K9me3 signature without extension or overlap with neighboring genes. A high throughput experiment could also be performed in presence and absence of Dicer using control and Dicer MO in CT-mCherry F2 embryos to quantitatively determine if Dicer deficiency globally reduces H3K9me3 levels or whether specificity is restricted at convergent regions. Another avenue to

explore will be to investigate the regulation of siRNA production in zebrafish independent of Dicer activity. In *S. pombe*, it has been shown that Rdp1 (RNA-dependent RNA polymerase) as part of a RDRC (RNA-directed RNA polymerase complex) helps to generate siRNAs that feedback and physically interact with the RITS complex to mediate RNAi-induced heterochromatin formation (Motamedi et al., 2004). While there is no evidence for Rdp1 expression in vertebrates including zebrafish (Zong et al., 2009), it is unclear if zebrafish have devised an alternative way to amplify siRNA or if an enzyme (not yet discovered) is responsible for mediating this process.

Another key aim that will provide valuable insight about TGS is to determine the mechanism of recognition of convergent DNA regions by siRNA-RITS complexes. Given the distribution and large genomes of zebrafish and the dynamic chromosomal landscape, it will be very interesting to discover if siRNAs bind to DNA directly or if other surveillance proteins remodel the chromatin to direct these siRNAs to their target. A potential experiment to address this would be to verify expression of RITS components in zebrafish and perform sequential ChIP using Ago-specific antibody for example to determine if RITS-associated sequences also harbor the H3K9me3 modification. Alternatively, convergent transcription itself might relay an epigenetic signal that then recruits ribonucleoprotein complexes to the convergent region. Future work is necessary to answer some of these questions and to gain a better understanding of CT-mediated gene silencing in zebrafish.

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