Biological Confinement of Zebrafish using RNAi

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Abstract

The increasing demand for fish in the food industry has resulted in the extensive overfishing of wild fisheries. In efforts to alleviate the demand from the food industry, genetically modified (GM) fish were developed possessing traits such as larger mass, faster growth, and increased resistance to disease. However, the greater fitness advantage of GM fish presents potential risks for wild type populations in the event of release or escape from a confined fish facility. In addition to physical barriers, it is critical to develop a genetic mechanism in order to ensure that the spread of GM transgene to the natural populations does not occur.

RNA interference (RNAi) is an endogenous mechanism used to regulate gene expression by destroying targeted mRNA molecules. Manipulation of this biological process has been successfully utilized to knockdown specific genes through the introduction of synthetic transgenes in organisms such as *C. elegans, M. musculus,* and *D. melanogaster*. Although the use of RNAi as a biological tool is still relatively new in zebrafish, recent work has explored elements of this mechanism allowing for greater knockdown efficiencies.

The *deadend* (*dnd*) gene is required for primordial germ cell (PGC) development and survival. Previous studies have shown that zebrafish *dnd* knockouts develop into sterile adults without disrupting somatic development. In efforts to induce sterility in zebrafish, short hairpin RNA (shRNA) constructs targeting *dnd* were designed to exploit the endogenous RNAi pathway. Upon qualitative analysis in transient and transgenic zebrafish subjected to the synthetic RNAi construct, a reduction in the germ cell population at early stages of development was observed. However, quantification of *dnd* mRNA in fish from the

same time points did not show significant changes in expression levels compared to their wildtype counterparts. Adult fish subjected to the transgene construct produced viable gametes.

The use of RNAi as a tool for bioconfinement relies on sterility among all individuals subjected to the shRNA bearing transgene. Based on the results obtained, the verdict is still unclear as to whether shRNA is a viable mechanism for large scale bioconfinement.

Résumé

La demande croissante pour les poissons dans l'industrie alimentaire a mené à la surpêche de poissons sauvages. Afin de réponde à la demande de l'industrie alimentaire, des poissons génétiquement modifiés (GM) furent mis au point avec des caractéristiques tel qu'une masse accrue, une croissance accélérée et une résistance accrue aux maladies. Toutefois, les avantages des poissons GM présentent aussi des risques pour les populations sauvages dans le cas d'une évasion ou d'une libération accidentelles des poissons d'une ferme d'élevage. En plus de barrières physiques, il est essentiel de mettre au point des mécanismes génétiques visant à empêcher toute dissémination des transgènes des poissons GM aux populations naturelles.

Les ARN interférents (ARNi) font partie d'un mécanisme endogène utilisé dans le contrôle de l'expression génique par lequel les ARNi causent la destruction des ARN messagers cibles. La manipulation de ce processus biologique a été accomplie avec succès pour causer la perte de fonction de gènes spécifiques par l'introduction de transgènes synthétiques chez des organismes tel *que C. elegans*, *M. musculus*, et *D. melanogaster*. Quoique l'usage des ARNi est relativement nouveau chez le poisson-zèbre, des études récentes ont exploré diverses composantes de ce mécanisme pour mener à une meilleure efficacité.

Le gène *deadend* (*dnd*) est nécessaire au bon développement et à la survie des cellules de la lignée germinale. Des études antérieures ont montré que des poissons-zèbres ayant perdu la fonction de *dnd* devenaient des adultes stériles sans pour autant affecter le développement des cellules somatiques. Afin de provoquer la stérilité des poissons-zèbres,

des constructions ARN à courte épingle à cheveux (shARN) ciblant *dnd* furent élaborées afin de mettre à contribution la voie endogène des ARNi. L'analyse qualitative des poissons exprimant une construction shARN de manière transitoire ou stable, a montré une diminution apparente du nombre de cellules germinales primordiales aux stades précoces du développement. Cependant, la quantification de l'ARNm de *dnd* dans les poissons au même stade n'a pas montré de changements significatifs dans les niveaux de transcrits comparé aux poissons non-transgéniques. Les poissons adultes arborant les diverses constructions transgéniques ont produit des gamètes viables.

L'utilisation des ARNi en tant qu'outil pour le bio-confinement requiert la stérilité des tous les individus portant le transgène shARN. En se basant sur les résultats obtenus, il reste à déterminer si les shARN constituent une approche prometteuse pour le bio-confinement à grande échelle.

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Abbreviations and Acronyms

3'UTR: 3 prime untranslated region

BCIP: 5-bromo-4-chloro-3'-indolyphosphate

bp: base pairs

CMV: Cytomegalovirus

CRISPR: clustered regularly interspaced short palindromic repeats

***dnd*:** *deadend*

dpf: days post fertilization

EDTA: ethylenediaminetetraacetic acid

Exp5: Exportin-5

GM: genetically modified

hpf: hours post fertilization

miRNA: microRNA

miR-30e: microRNA 30e

MO: morpholino oligonucleotides

NBT: nitroblue tetrazolium

ORF: open reading frame

PBST: phosphate buffered saline solution

PCR: polymerase chain reaction

PFA: paraformaldehyde

PGC: primordial germ cell

PK: proteinase K

Poly-A Tail: polyadenylated tail

Pri-miRNA: primary microRNA

Pre-miRNA: precursor microRNA

PTU: phenylthiourea

q-RT PCR: quantitative real time polymerase chain reaction

RISC: RNA-inducing silencing complex

RNAi: RNA inhibition

rps1: *ribosomal protein subunit 18*

shRNA: short-hairpin RNA

siRNA: short interfering RNA

SSC: saline-sodium citrate buffer

TALENs: transcription activator-like effector nucleases

UAS: upstream activation sequence

WISH: whole-mount *in situ* hybridization

WT: wildtype

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Statement of Contributions

Susan Boratynska executed all experiments presented in this thesis. However, co-op students Manon Colas and Alexandra Antoine assisted in genotyping various zebrafish lines used in this thesis.

The *vasa* probe used for *in situ* hybridization were designed and synthesized by Susan Boratynska and a former laboratory technician, Evan Byers. Evan Byers also assisted in designing primers used in this experiment as well as the injections of both the first generation *dnd*RNAi constructs.

The first generation constructs were designed and funded with our collaborator, Robert H. Devlin from Fisheries and Oceans Canada prior to Susan Boratynska joining Dr. Ekker's laboratory. The second generation constructs also were funded by Robert Devlin.

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I am thankful to my family for their unconditional support and encouragement.

1. Introduction

1.1. General Overview

The increasing demand for fish in the food industry has resulted in the extensive overfishing of wild fisheries. This demand has caused widespread habitat destruction, overfishing, and an overall loss of biodiversity within both freshwater and marine ecosystems. To compensate for the issues surrounding industrial overfishing of wild populations, farmed fisheries in sheltered coastal waters, rivers, ponds and tanks were introduced. The innovation of genetically modified (GM) farm-raised fish that possess traits such as larger mass, faster growth, and increased resistance to diseases may alleviate the industrial pressure from the food industry for consumer-relevant species.

However, developing a transgenic GM fish does present a risk to wild populations in the event that GM fish escape a confined facility. GM fish would have a greater fitness advantage compared to wild type (WT) populations by their genetic modifications which could establish a selection pressure that may deplete natural fish populations. It is therefore critical to isolate these fish from open waters. Nonetheless, fish escapes owing to equipment failure are inevitable; this may occur as a result of events such as accidents, predators, storms, wave intensities, or vandalism. The escape of GM fish could have grave repercussions for the natural environment with the disruption of new ecological niche. The introduction of a new niche will likely alter predator-prey relationships and in turn result in loss of habitat and/or species extinction. It is for these reasons that it is important to develop a reliable method of containing GM fish.

Research on aquaculture species is both costly and time-consuming. Atlantic Salmon (*Salmon salar*) reach sexual maturity at 1 year of age whereas zebrafish are sexually mature at ~3 months post fertilization (Gjerde, B., 1984). The use of zebrafish (*Danio rerio*) as a model organism is quickly gaining a reliable reputation in aquaculture research as it resolves these disadvantages. The sequenced genome of zebrafish also offers a huge advantage by allowing the use of technologies for molecular analysis such as genotyping. Zebrafish has proven itself to be a representative system in understanding biological characteristics of a larger group of organisms. For these reasons, we used zebrafish in this study to explore a transgenic approach for biological confinement.

1.2. Bioconfinement

The introduction of GM fish in the food industry has provided some relief to the population demand for food. However, this relief comes with the concern of possible release or escape of transgenic species into the wild and the effects that it would have on natural environment.

The two primary methods of biological confinement are physical and genetic confinement. Physical confinements includes the use of barriers to prevent escape within an environment. In aquaculture, this can involve the use of conventional net pen systems or land-based facilities. However, these confinement methods has inherent risks which cannot ensure restraint on the proliferation of GM fish in the event of escape into the wild. Genetic control can include methods such as dietary dependence factors, such as vitamins only available through supplementation within an environment, or sterility. Inducing sterility,

defined by the absence of functional gametes, appears to be the most effective mechanism for large scale biological confinement as it inhibits the propagation of the transgene.

1.3. Gonad Development in Zebrafish

In early embryonic stages, a population of primordial germ cells (PGCs) is reserved for the differentiation of gametes in sexually reproductive animals (Houston, D., & King, M., 2000). These cells eventually lead to the production of eggs in females and sperm in males. Early PGCs are specified extragonadally as four random clusters during early embryogenesis (3 hours post fertilization) that eventually migrate towards the genital ridge (Raz, E., 2003; Yoon, C., Kawakami, K., & Hopkins, N., 1997).

Using intermediate targets found within the embryo, zebrafish PGCs migrate to the gonad through six steps during the first 24 hours of embryonic development (Weidinger, G., Wolke, U., & Koprunner, M., 1999). The first step (i) begins with migration of the four clusters towards the dorsal side of the embryo. At ~60% epiboly, the PGCs no longer form four distinct clusters as they make their way to the dorsal end. Ventrally located PGCs migrate more slowly towards the dorsal, with some cells remaining at the ventral end. Cells that are close to the dorsal end begin to travel away from the dorsal midline (step ii). By ~80% epiboly, dorsally located PGCs align along the anterior mesoderm while ventrally located PGCs align along the lateral mesoderm (step iii). At the 2-somite stage, a majority of the PGCs have migrated to the anterior end as two lines parallel to the first somite. These cells form two lateral PGC clusters that migrate along the side of the embryo (step iv). By the 8-somite stage, the trailing ventral PGCs migrate anteriorly until they are found laterally

in clusters developing along the first to third somite (step v). The final step (vi) occurs when the PGCs begin to position themselves posteriorly along the eighth and tenth somite by 24 hpf. The specification of PGCs in zebrafish relies on maternally supplied cytoplasmic determinants (Weidinger, G., Wolke, U., & Koprunner, M., 1999). PGC migration, colonization, and gamete formation rely on three identified genes; *vasa, nanos,* and *dead end* (Nagasawa, K., Fernandes, J. M. O., Yoshizaki, G., Miwa, M., & Babiak, I., 2013).

The *vasa* gene was first identified in *Drosophila melanogaster* (fruit fly) and is a DEAD-box RNA helicase specifically expressed in germ cells (Hay, B., Jan, L. Y., & Jan, Y. N., 1988; Lasko, P. F., & Ashburner, M., 1988). The DEAD-box family are highly conserved RNA helicases present in all eukaryotic cells which are required for the metabolic processes of RNA from transcription to degradation (Linder, P., & Jankowsky, E., 2011). Although *vasa* is maternally supplied, it maintains strong expression throughout zebrafish gonad development (Köprunner, M., Thisse, C., Thisse, B., & Raz, E., 2001; Yoon, C., Kawakami, K., & Hopkins, N., 1997). As early as the 2-cell stage, *vasa* transcripts have localized to specific regions within the embryo that direct the formation of the developing germ line (Yoon, C., Kawakami, K., & Hopkins, N., 1997).

The *nanos* gene encodes an RNA-binding zinc finger protein that are also required for PGC formation and migration (Saito, T., Fujimoto, T., Maegawa, S., Inoue, K., Tanaka, M., Arai, K., & Yamaha, E., 2006). Knockout experiments in zebrafish have shown that the absence of *nanos* prevents PGC migration which subsequently causes this population of cells to undergo apoptosis (Köprunner, M., Thisse, C., Thisse, B., & Raz, E., 2001). Knockout experiments in mice revealed that PGCs fail to survive in the absence of the *nanos* ortholog, suggesting that its role in PGC survival is evolutionarily conserved (Tsuda, M., Sasaoka,

Y., Kiso, M., & Abe, K., 2003). In the germ line, *nanos* is resistant to the silencing effects of microRNAs (miRNA) through the protection of another PGC specific maternal gene, *Dead end* (*dnd*) (Mishima, Y., Giraldez, A. J., Takeda, Y., Fujiwara, T., Sakamoto, H., Schier, A. F., & Inoue, K., 2007). *Dnd* interacts with the 3'UTR of *nanos,* preventing miRNA from binding to *nanos*.

Similar to *vasa* and *nanos, dnd* also plays a critical role in proper PGC development through migration and survival. *Dnd* encodes an RNA binding protein that is required to confine PGCs to the deep blastoderm layers during early stages of embryogenesis (Weidinger, G., Stebler, J., & Slanchev, K., 2003). *Dnd* knockdown experiments inhibited polarization and proper PGC migration, triggering PGC death without disrupting somatic development (Weidinger, G., Stebler, J., & Slanchev, K., 2003). Embryos whose germ cells were ablated with *dnd* antisense morpholino oligonucleotides (MO) developed into sterile adult males despite presenting typical male sexual behaviour, defined by their ability to trigger spawning in females (Slanchev, K., Stebler, J., de la Cueva-Méndez, G., & Raz, E., 2005). Typical courtship behaviour is defined by the male closely circling the female with his fins raised which stimulates the female to release her eggs which triggers the male to simultaneously discharge sperm (Spence, R., Gerlach, G., Lawrence, C., & Smith, C., 2008). The lack of females in *dnd* MO injected fish suggests that *dnd* function may be required for female development (Slanchev, K., Stebler, J., de la Cueva-Méndez, G., & Raz, E., 2005). It was later discovered that the presence of a germ line is vital for ovary development (female sex determination) but not necessary for testis determination (Siegfried, K. R., & Nüsslein-Volhard, C., 2008). The distinctive expression patterns and minimal pleiotropic effects of

vasa, *nanos*, and *dnd* allows these genes to be good potential candidates in exploring transgenic sterilization in fish.

While PGCs are specified early in zebrafish development, sexual differentiation does not begin until later stages (Uchida, D., & Yamashita, M., 2002). PGCs only migrate to the gonadal position at approximately the 6-somite stage (Yoon, C., Kawakami, K., & Hopkins, N., 1997). Although it is still unknown how sex is determined in zebrafish, studies have implied that it can be influenced by both multiple (>1) genetic factors (polygenic sex determination) as well as environmental factors (Siegfried, K. R., & Nüsslein-Volhard, C., 2008). By 21-23 dpf, the sex of the individual can be identified; however, sexual maturity does not occur until approximately three months post fertilization (Uchida, D., & Yamashita, M., 2002). Prior to sexual differentiation, all individuals begin the default process of oogenesis by developing a non-functional ovary (Maack, G., & Segner, H., 2003; Wang, X. G., Bartfai, R., Sleptsova-Freidrich, I., & Orban, L., 2007). The immature ovary begins to develop at 10 dpf up until 20 dpf. Between 21-30 dpf, testis begin to form in developing males coinciding with the apoptosis of oocytes, eventually leading to spermatogenesis (Maack, G., & Segner, H., 2003; von Hofsten, J., & Olsson, P.-E., 2005). Oogenesis continues in developing females to form a fully differentiated ovary.

1.4. Manipulating Gene Expression

1.4.1. RNA Interference

There are various biological mechanisms that are used to manipulate gene expression. RNA interference (RNAi) is an endogenous gene regulatory mechanism whereby double stranded small non-coding RNA molecules block post-transcriptional gene expression by the destruction of target mRNA molecules (Figure 1.1**)**. RNAi was first discovered in *Caenorhabditis elegans* but has since been identified to be an evolutionarily conserved gene silencing mechanism in most eukaryotic cells (Fire, A., Xu, S., Montgomery, M., & Kostas, S., 1998).

Two key players in RNAi are microRNAs (miRNAs) and small interfering RNAs (siRNAs). The miRNAs are produced from endogenous non-coding RNA regions that fold back onto themselves forming a stem loop structure (Bartel, D., 2004). The siRNAs are generated from long exogenous dsRNA which enters the cell through vectors such as viruses (Chen, M., Du, Q., Zhang, H., Wahlestedt, C., & Liang, Z., 2005).

RNAi regulates post-transcriptional gene expression using several mechanisms. RNAi can suppress translation or it can direct destruction of target mRNA via the ribonucleoprotein, RNA-induced silencing complex (RISC) through mRNA cleavage or deadenylation (Tamura, Y., Yoshida, M., Ohnishi, Y., & Hohjoh, H., 2009). RNA-polymerase II transcribes miRNA genes to form primary mRNA (pri-miRNA) with a 5' cap and a polyadenylated tail (Poly-A tail) in the nucleus (Lee, Y., Kim, M., Han, J., Yeom, K.-H., Lee, S., Baek, S. H., & Kim, V. N., 2004). These transcripts are further processed by an RNase III enzyme, Drosha and a nuclear dsRNA binding protein, Pasha into ~70-nucleotide

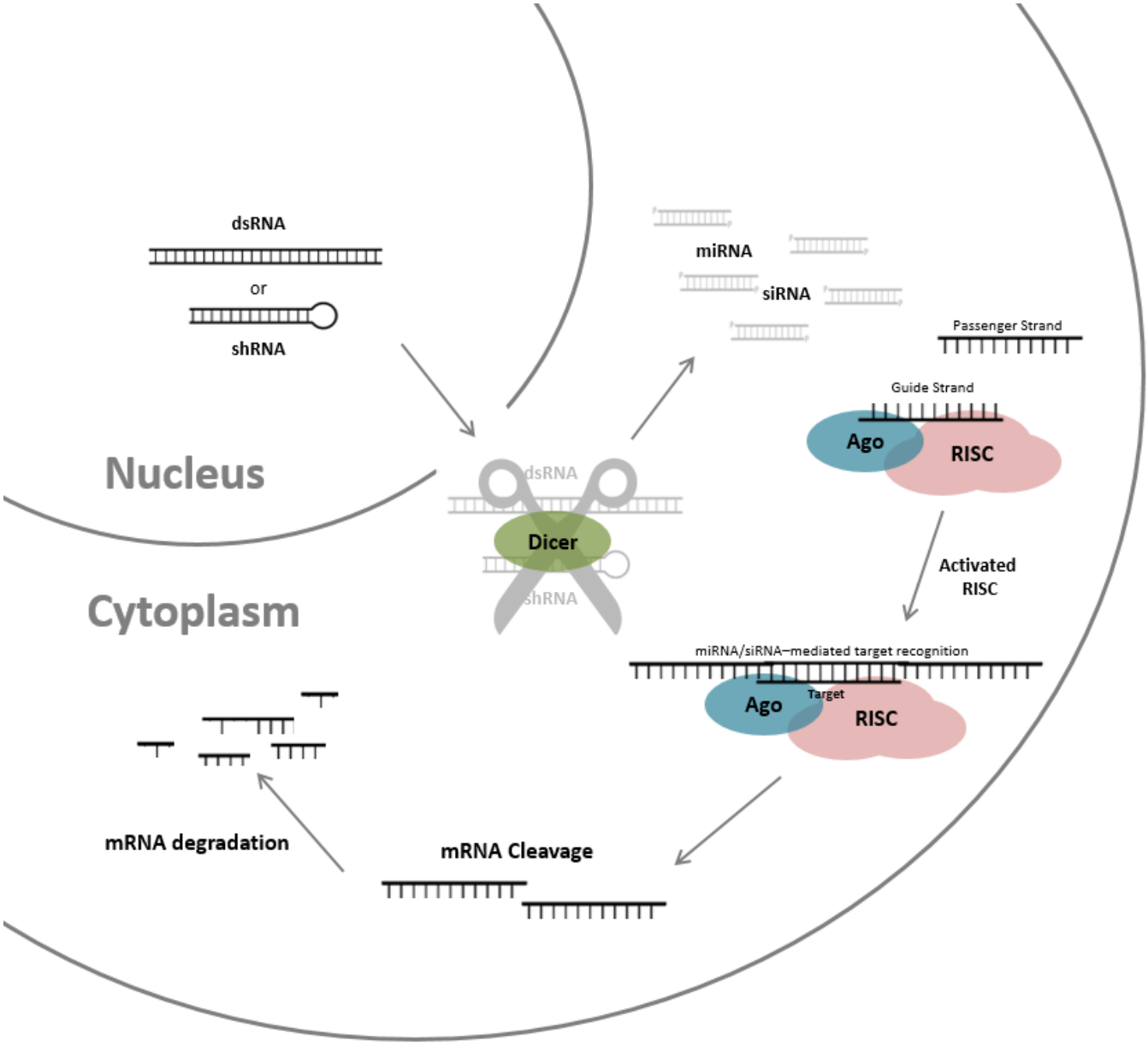
stem loop structures known as precursor-miRNA (pre-miRNA) (Yeom, K.-H., Lee, Y., Han, J., Suh, M. R., & Kim, V. N., 2006). The pre-miRNA then gets exported into the cytoplasm by binding toa karyopherin protein, exportin-5 (Exp5) (Yi, R., Qin, Y., Macara, I., & Cullen, B., 2003). In the cytoplasm, an RNA polymerase III endoribonuclease, Dicer, cleaves dsRNA into 19-23 nucleotide long short interfering RNA (siRNA) segments with a 3' overhang (Bernstein, E., Caudy, A. A., Hammond, S. M., & Hannon, G. J., 2001). These overhangs allows the siRNAs to bind to the RNA induced silencing complex (RISC) which initiates the RNAi pathway (Hammond, S. M., 2005). The RISC complex is what produces the gene silencing effects of RNAi (Hammond, S. M., Bernstein, E., Beach, D., & Hannon, G. J., 2000).

Each siRNA segment is comprised of complementary short RNA molecules referred to as the guide strand, and a passenger strand (Castel, S. E., & Martienssen, R. a, 2013). The RNAse component of RISC complex, Argonaute, is activated upon binding of the dsRNA to RISC which cleaves and destroys the passenger strand. Only the remaining guide strand is incorporated into the RISC complex and serves as a template to find target messenger RNA molecules with complementary sequence (Castel, S. E., & Martienssen, R. a, 2013). Once the target mRNA is bound to the guide strand, the endonuclease activity of Argonaute catalyzes cleavage of the mRNA which is then degraded by exonucleases.

The miRNAs guide RISC to knockdown gene expression through two known mechanisms; translational repression or mRNA cleavage. The mechanism employed is dependent on the level of complementarity of the miRNA sequence to its target gene. The translational repression of miRNA occurs when there is partial complementarity with the target gene which is typically found with endogenously expressed miRNA's (Filipowicz, W.,

Jaskiewicz, L., Kolb, F. a, & Pillai, R. S., 2005). RNAi mediated mRNA cleavage occurs when there is complete complementarity between the miRNA and the target gene.

Figure 1.1. Post transcriptional gene silencing RNAi pathway. Double stranded RNA (dsRNA) found in the nucleus is exported into the cytoplasm of the cell where Dicer (a Ribonuclease-III family member) cuts it into 21-23 nucleotide segments. The short double stranded segments, known as siRNA (small interfering RNA) or miRNA (microRNA) bind to the Argonaut protein attached to the RNA-inducing silencing complex (RISC) and begin to unwind. The antisense strand (guide strand) remains bound to Argonaut while the passenger strand is cleaved off and degraded. The guide strand base pairs with a complementary mRNA sequence where it cleaves and destroys the corresponding RNA by the catalytic module of the RISC complex.


dsRNA
or
shRNA
Nucleus
Cytoplasm
dsRNA
Dicer
shRNA
miRNA
siRNA
Passenger Strand
Guide Strand
Ago
RISC
Activated
RISC
miRNA/siRNA–mediated target recognition
Target
Ago
RISC
mRNA Cleavage
mRNA degradation

RNAi has since been used in a variety of species as a tool for target-specific gene silencing to better understand the role of genes in cellular pathways (Singh, S., Narang, A. S., & Mahato, R. I., 2011). The initial attempts to use RNAi in zebrafish proved to be unreliable owing to non-specific off-target effects. This led to the use of morpholino oligonucleotides as a transient gene silencing tool in zebrafish. However, recent reports have suggested that loss-of-function phenotypes obtained using MO also need to be reassessed due to irreproducible results obtained using newer knockdown technologies (Schulte-Merker, S., & Stainier, D. Y. R., 2014).

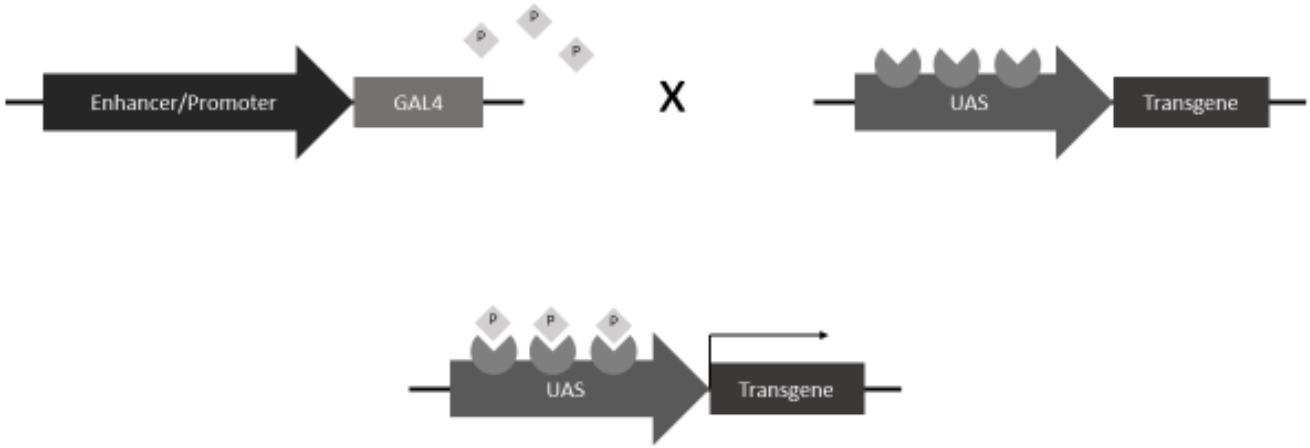
Although RNAi has been used successfully to knockdown target genes in various organisms, the verdict is still unclear as to whether or not RNAi can be used to reliably silence genes in zebrafish (Singh, S., Narang, A. S., & Mahato, R. I., 2011). Early accounts of successful RNAi in zebrafish were contended with reports of nonspecific off-target effects (Oates, a C., Bruce, a E., & Ho, R. K., 2000; Zhao, Z., Cao, Y., Li, M., & Meng, a, 2001). However as more information surrounding RNAi is being uncovered, elements that allow RNAi to be tailored to the zebrafish have also surfaced. This has led to the recent reports of efficient knockdown effects seen in zebrafish (De Rienzo, G., Gutzman, J. H., & Sive, H., 2012). Studies have reported higher knockdown effects designing RNAi expressing constructs with elements such as multiple target hairpin structures and as well as inserting sequences that mimic the natural backbone of endogenous primary microRNAs (Dong, M., Fu, Y.-F., Du, T.-T., Jing, C.-B., Fu, C.-T., Chen, Y., Jin, Y., Deng, M., & Liu, T. X., 2009).The use of RNAi as a tool to manipulate gene expression in zebrafish is still relatively recent and new discoveries on how to optimize its effectiveness are still being explored.

While some studies have shown the potential loss of function effects of RNAi on endogenous RNA expression in F_0 generation experiments, the nature of transient expression leads to variable knockdown effects. Transient expression is only expressed for a fixed period of time before the transgene is lost through factors such as cell divison. Using an inducible system, such as GAL4/UAS, transgenic lines expressing shRNA can possibly lead to higher and more uniform knockdown effects as a result of integration of the transgene into the host genome where it can be replicated and passed on to future generations.

1.4.2. GAL4-UAS System

The GAL4-UAS system requires two different transgenic lines referred to as the activator and effector lines (Brand, A., & Perrimon, N., 1993). The activator line contains an enhancer or promoter sequence driving the expression of GAL4, a yeast transcriptional activator (Figure 1.2.). The effector line contains the DNA-binding motif of GAL4 known as the Upstream Activating Sequence (UAS) which is fused to a desired sequence or gene of interest. In the absence of GAL4 protein, the effector line containing the target sequence remains silent. However when the effector line is crossed with a transgenic GAL4 activator line, the UAS sequence is then turned on allowing transcription of the gene of interest to occur. GAL4 protein has high binding affinity for the UAS sequence 5'-CGG-N_{11}-CCG-3' (Giniger, E., Varnum, S., & Ptashne, M., 1985). Changes in ±1 nucleotide(s) significantly decreases the affinity of GAL4 binding to this sequence. Therefore, expression of the effector gene is only seen in the progeny in the presence of GAL4 protein. The expression pattern and specificity can be manipulated by the promoter sequence used drive the activating sequence.

Figure 1.2. Binary GAL4/UAS Expression System. Transgenic construct bearing the yeast transcription factor GAL4 under the control of a specified promoter is crossed with a transgenic fish bearing a transgene downstream of the Upstream Activation Sequence (UAS). GAL4 protein (P) binds to the UAS which leads to the transcriptional activation of the transgene.


Enhancer/Promoter
GAL4
P
P
P
X
UAS
Transgene
P
P
P
UAS
Transgene

1.5. Zebrafish as a Model for Transgenic Sterilization

A good model species contains characteristics allowing it to be representative of a larger group of organisms. The zebrafish has become established as a reputable model organism in the study of vertebrate development. It offers structural simplicity while possessing cellular processes found in a range of species (Ribas, L., & Piferrer, F., 2013). The rapid external embryonic development in zebrafish, with optically clear embryos makes it easier to administer genetic manipulation early on in development. Their short generation time (approximately 3 months to reach sexual maturity) along with the fact that a single clutch may contain 100-200 embryos per week, allows for a high throughput of experimental data (Stern, H., & Zon, L., 2003).

Zebrafish have a relatively small and stable genome receptive to genetic manipulation using forward and reverse strategies (Stuart, G., McMurray, J., & Westerfield, M., 1988). The complete genome sequenced by the Sanger Institute has been pivotal in facilitating reverse genetic strategies in zebrafish by offering advantages such as various strains and transgenic lines (Link, V., Shevchenko, A., & Heisenberg, C.-P., 2006; Ribas, L., & Piferrer, F., 2013). Antisense morpholino oligonucleotides are shown to be effective at knocking down endogenous genes (Nasevicius, A., & Ekker, S. C., 2002). However, knockout studies using antisense morpholino oligonucleotides have recently been challenged by newer scientific technology such as CRISPR (clustered regularly interspaced short palindromic repeats), TALENs (transcription activator-like effector nucleases), and RNAi (Kok, F. O., Shin, M., Ni, C.-W., Gupta, A., Grosse, A. S., van Impel, A., Kirchmaier, B. C., Peterson-Maduro, J., Kourkoulis, G., Male, I., DeSantis, D. F., Sheppard-Tindell, S., Ebarasi, L., Betsholtz, C., Schulte-Merker, S., Wolfe, S. A., & Lawson, N. D., 2014). The increasing

availability of zebrafish genomic resources makes this species a favourable model for studying fields such as developmental biology and genetics research. These characteristics of zebrafish along with the accessibility of genetic tools to which they are amenable, make them an excellent model organism for the study of developmental processes.

Zebrafish are teleost fish species, similar in this respect to many aquaculture species such as tilapia (*Oreochromis niloticus*) and Atlantic salmon (*Salmon salar*). As genomic resources of commercially relevant fish have been uncovered, aquaculture research has increasingly benefitted from the use of zebrafish as a model species.

1.6. Summary of Background Information and Objectives of Current Study

With the increasing demand on the commercial fish industry, GM fish have been engineered to have traits such as rapid growth rate, increased weight, and disease resistance. However, the fitness advantage possessed by GM fish presents itself as a risk to the natural environment in the event of escape. These risks may include population shifts resulting in detrimental changes beyond the capacity of the given environment. For these reasons, a reliable bioconfinement strategy is critical with the introduction of GM fish. Among the current strategies, induced sterility holds promise as an effective method for the bioconfinement of aquaculture relevant species.

PGCs are specified early in zebrafish development and rely on signaling molecules to migrate to the developing gonad. The specification of these cells depend on maternally provided determinants; *vasa, nanos,* and *dnd* (Lasko, P. F., & Ashburner, M., 1988; Saito,

T., Fujimoto, T., Maegawa, S., Inoue, K., Tanaka, M., Arai, K., & Yamaha, E., 2006; Weidinger, G., Stebler, J., & Slanchev, K., 2003). Slanchev et al. (2005) reported that *dnd* knockdown in zebrafish using MO yielded individuals that developed into sterile adult fish without affecting somatic development. Such uses of MO had offered great insight into the effects of genes through reverse genetics. However owing to the artificial nature of MO, it is only amenable via microinjection or electroporation. Due to the fact that MO's do not possess gene regulatory elements, they are only effective for a short duration of time (2-4 days) as they become diluted by cell division.

To achieve a sustainable approach to knockdown/knockout *dnd*, we look to utilize genetically transmissible techniques to induce sterility. The use of endogenous RNAi pathway has shown promise in gene silencing effects through a variety of organisms (Fire, A., Xu, S., Montgomery, M., & Kostas, S., 1998). However, effectiveness of RNAi in zebrafish is still unclear with conflicting reports of successful gene ablation as well as nonspecific off-target effects (Huang, W.-T., Hsieh, J.-C., Chiou, M.-J., Chen, J.-Y., Wu, J.-L., & Kuo, C.-M., 2008; Oates, a C., Bruce, a E., & Ho, R. K., 2000). A methods improve, the effectiveness of applying RNAi as a biological tool in zebrafish continues increase (Dong, M., Fu, Y.-F., Du, T.-T., Jing, C.-B., Fu, C.-T., Chen, Y., Jin, Y., Deng, M., & Liu, T. X., 2009; Leong, I. U. S., Lan, C.-C., Skinner, J. R., Shelling, A. N., & Love, D. R., 2012). Studies have reported higher knockdown effects designing RNAi expressing constructs with elements such as multiple target hairpin structures and as well as inserting sequences that mimic the natural backbone of endogenous primary microRNAs (Dong, M., Fu, Y.-F., Du, T.-T., Jing, C.-B., Fu, C.-T., Chen, Y., Jin, Y., Deng, M., & Liu, T. X., 2009).

With the absence of pleiotropic effects in sterile zebrafish generated by *dnd* MO knockdowns, we hope to attain heritable knockdown effects using RNAi targeting *dnd*. In order to verify that *dnd* knockdown effects can be achieved by transgenesis, our first objective is to insert a constitutively expressed construct to test and characterize the efficiency of synthetic RNAi in knocking down *dnd* expression in zebrafish. The same *dnd* target sequences in the constitutive approach were also tested using the GAL4/UAS inducible system in attempt to produce a transmissible reproductive containment strategy.

Using zebrafish as a proof of concept, the focus of this project is to create a sustainable transgenic approach for large scale bioconfinement by inducing sterility through manipulation of germ cell specific gene expression.

2. Materials and Methods

2.1 Animal Care and Maintenance

Experiments were completed according to the guidelines of the Canadian Council on Animal Care. Zebrafish were reared at 28.5^{o}C using conditions corresponding to guidelines described by Westerfield (Westerfield, M., 2000). Adult zebrafish were raised and bred in tanks supplied with aerated, flowing, dechloraminated City of Ottawa tap water and subjected to a 14 hour light/dark cycle. Embryos collected at the one-cell stage were raised in 28.5^{o}C incubators in embryo medium solution (5 mmol/L NaCl, 0.17 mmol/L KCl ,0.33 mmol/L $CaCl_2$, 0.33mmol/L $MgSO_4$) for 5-6 days before being transferred into static tanks.

Microinjections were performed on a minimum of 100 wild-type embryos at the one-cell stage per a given construct.

2.2 Construct Design

2.2.1. First Generation RNAi Constructs

Two custom-designed shRNA constructs were previously created and synthesized by GenScript USA, Inc. Each construct contained an mCherry reporter gene under the control of the ubiquitous ef1α promoter (Figure 2.1). The RNAi portion of the construct contained a dual hairpin that consisted of two different target sequences from the open reading frame (ORF) of *dnd* driven by the constitutive CMV (cytomegalovirus) promoter. The *dnd* target sequences were obtained using the NCBI zebrafish database (GenBank accession number AY225448.1). The target sequences used to make up the dual hairpin structure were located in the open reading frame of the *dnd* mRNA sequences at 645-665bp and 1,116-1136bp (Table 2.1). The same construct was also created with the UAS promoter to be used in an inducible system. Flanking the construct were Tol2 arms which facilitate integration of the target vector (200 ng/µl) into the zebrafish genome when co-injected with synthetic Tol2 transposase mRNA (100 ng/µl) at the one-cell stage (Kawakami, K., Koga, A., Hori, H., & Shima, A., 1998).

2.2.2. Second Generation RNAi Constructs

Using the NCBI zebrafish database, the mRNA sequence of *dnd* (GenBank accession number AY225448.1) and the microRNA 30e (miR-30e) (found on Chromosome 13 NCBI Reference Sequence: NC_007124.6) were obtained. The *dnd* target sequences used to design the *CMVmiR30-endndRNAi-A* and *CMVmiR30-endndRNAi-B* are located at 1350-1373bp and 1692-1717 bp of *dnd* mRNA respectively (Table 2.1). The same regulatory elements and framework as for the first generation custom-designed shRNA constructs were used except that the dual hairpin structure was replaced with a single 24 nucleotide hairpin flanked with the endogenous miR30-e flanking sequence (Figure 2.2). Separate constructs using both the constitutive (CMV) and inducible (UAS) promoters were generated to drive *dnd* RNAi expression. Using the Life Technology BLOCK-iT^{TM} RNAi Designer software, two *dnd* candidate sequences were obtained in the 3' untranslated region (UTR) and were selected as target sequences for the RNAi hairpin (Figure 2.3). Based on the software parameters, these two target sequences were predicted to deliver greater knockdown of mRNA compared to other potential sequences found in the 3'UTR of *dnd.* The selected *dnd* target sequences were used to replace the endogenous hairpin found within the flanking regions of the native miR-30e precursor. These constructs, *CMVmiR30-endndRNAi-A* and *CMVmiR30-endndRNAi-B* were synthesized and purchased through GenScript USA, Inc. Microinjections of constructs were followed by fluorescence screening for ubiquitous mCherry expression. Primary injected fish were fixed at 2, 4, and 6 dpf for experimentation. A subset of fish was raised to adulthood and screened for germline transmission by pair-wise breeding with WT fish.

The second generation RNAi constructs were also created alongside a ubiquitous EGFP sensor mRNA assay system (Sensor A and Sensor B) with perfectly complementary

dnd target sites found in the *CMVmiR30-edndRNAi-A* and *CMVmiR30-edndRNAi-B* constructs (Figure 2.4). The rationale behind the *in vivo* EGFP sensor assay was to visualize potential sequence specific knockdown effects through transient expression of the custom-designed shRNA. The sensors would also be a valuable tool in assessing the knockdown efficiency for established transgenic lines as well as the activity of the shRNA transgene over several generations.

A decrease in EGFP expression in fish co-injected with the RNAi expressing construct and the corresponding sensor mRNA would suggest that the synthetic RNAi was able to knockdown gene expression by inhibiting translation of EGFP. The sensor sequences were designed with the EGFP-N1 Clontech sequence synthesized between the *Bam*H1 and *Eco*R1 cloning sites of the pCS2^{+}-EGFP vector with the 24 nucleotide that form the hairpin target sequence inserted downstream of the reporter gene. The sensor mRNA vectors were also purchased through GenScript USA, Inc.

Table 2.1. *dnd* target sequences used to design the short hairpin region in both the first and second generation shRNA constructs.

Construct Name	Location of Target Sequence in *dnd* mRNA Sequence	*dnd* Target Sequence
First Generation Dual Hairpin shRNA *dnd* Targets	645-665	5’-ATCCATGGCTAAGAAAGTGCT-3’
	1116-1136	5’-TCCTGACGGATTCCTCTACTT-3’
Second Generation *dnd* Target	1350-1373	5’-CAGTATTATGTACTGTTCCGGTTA-3’
Second Generation *dnd* Target	1692-1717	5’-TGTGCTCCTTGTGTTTCCTTGTTTG-3’

2.3 Microinjections

Microinjections of the synthesized constructs were performed using glass needles controlled by a micromanipulator. The day prior to injection, adult WT zebrafish were set up in breeding tanks with dividers in a 1♂:2♀ (male:female) ratio. At the start of the light cycle the following morning, the dividers were removed to allow for mating. Embryos were collected at the one-cell stage and transferred to a microinjection chamber with a few drops of embryo medium solution. The injection solution containing the DNA target vector diluted to 100 ng/µl, Tol2 transposase mRNA (100 ng/µl), and 0.1% Phenol Red (Sigma-Aldrich) was kept on ice and prepared immediately prior to injection. Phenol red is added to visualize injection into the embryo. Co-injection of the second generation constructs contained the same DNA construct concentration with the addition of 1µl of sensor mRNA.

Microinjections were performed using a Narishige IM-300 Pneumatic Microinjector. The solution was injected into the yolk close to the cell interface to ensure the solution diffuses into the cytoplasm of the cell via cytoplasmic streaming. Injected embryos were transferred into a Petri dish with embryo medium solution and raised in 28.5^{o}C incubators.

2.4 Transgene Expression Screening

Embryos injected with DNA constructs containing ubiquitous expressing ef1α promoter fused to mCherry were screened at 24-48 hpf for red fluorescence. A Nikon SMZ 1500 stereoscope with an integrated fluorescent filter system were used to visualize embryos. Only embryos expressing clearly detectable mCherry fluorescence was used for analysis.

Primary injected fish were fixed and/or collected at 2, 4, and 6 dpf time points for analysis by in situ hybridization and qRT-PCR (Figure 2.5). A subset of positive embryos was raised to adulthood to screen for stable germline transmission by examining embryos obtained from outcrossing with wild-type fish.

2.5 Gamete Viability

In order to determine the viability of gametes, individuals were outcrossed with wild-type to screen for the presence of fertile embryos. Typical zebrafish courtship behaviour involves a female being chased by a male in tight circles with raised fins in an attempt to align their genital pores (Spence, R., Gerlach, G., Lawrence, C., & Smith, C., 2008). This behaviour stimulates the female to release her eggs which simultaneously triggers the male to discharge sperm. Normal male sexual behaviour was evaluated by female oviposition while gamete viability was evaluated based on the presence of embryos.

2.6 Genomic DNA Extraction

Fin clippings were obtained from adult zebrafish *Tg:SAGFF73A* (a gift from the Kawakami Lab, National Institute of Genetics). Tissue samples were digested in a solution containing 10 µl of Proteinase K (10 mg/mL; Life Technologies) solution per 100 µl of digestion buffer (100 mM NaCl, 1% SDS, 100 mM EDTA, and 50 mM Tris-HCl pH 8.0) at 55^{o}C. Upon complete tissue lysis, 0.05X the volume of 5M NaCl was added to the digestion buffer/Proteinase K solution and centrifuged at maximum speed (13,500 rpm) for 5 minutes. The supernatant was transferred to a new tube with the addition of 0.8X the volume of

isopropanol to precipitate the genomic DNA by inverting the tube. The DNA pellet was acquired after a 10 minute centrifugation at maximum speed. The supernatant was removed and the pellet was washed with 70% ethanol. Once all the ethanol was removed, the pellet was resuspended in TE buffer (100 mM Tris-HCl pH 8.0 and 100 mM EDTA) and stored at -20^{o}C.

2.7 Genotyping

Fish were screened for presence of the *GAL4* gene using polymerase chain reaction (PCR) with primers designed to amplify *GAL4* (forward primer: ATGAAGCTACTGTCTTCT -3'; reverse primer: TCTAGATTAGTTACCCGG -3'). Individual PCR reactions consisted of 2 µl of genomic DNA from fin clippings, 10x PCR buffer (100mM Tris-HCl pH 8.3, 500mM KCl, 12mM $MgCl_2$), 2mM dNTPs, forward and reverse primers (10pmol) and 1 unit of Taq Polymerase. The PCR were performed under the following conditions: 95^{o}C for 3 minutes (polymerase activation), 95^{o}C for 1 min, 58^{o}C for 1 min, 72^{o}C for 2 min (PCR amplification), followed by 72^{o}C for 5 min (extension) and 4^{o}C (cool reaction); 30 amplification cycles were performed. PCR products were run on a 1% agarose gel and observed under an ultraviolet light for products. Presence of a band size of 441 bp indicated fish screened positive for the GAL4 cassette. *β-actin* primers (forward primer: ACCACGGCCGAAAGAGAA-3'; reverse primer: ATACCCAGGAAGGAAGGCTG-3') were used as a positive control for each genomic sample.

2.8 Whole-Mount *in situ* Hybridization (WISH) Using *vasa* Probe

Presumptive PGCs were identified by expression of *vasa* mRNA detected by WISH (Thisse, C., & Thisse, B., 2008). Using the NCBI zebrafish database, we obtained the mRNA sequence of zebrafish *vasa* (GenBank accession number BC095001.1; Figure 2.6) in order to design primers (forward primer: CTGTGTGCTACTCCTGGAAG-3'; reverse primer: CTCTCGCTGTTCCCGATCAC-3') to PCR-amplify a 480 bp sequence to synthesize an antisense RNA probe using T7 polymerase mMESSAGE mMACHINE® kit (Ambion, Inc.). The amplified template was then used to synthesize a digoxigenin-labelled antisense riboprobe.

Embryos were treated with 1-phenyl 2-thiourea (PTU) ~6 hours post fertilization (hpf) in order to maintain transparency in larvae and to better visualize the final staining pattern. Embryos were fixed with RNase-free 4% paraformaldehyde (PFA) at 4^{o}C overnight to secure the target mRNA within the embryo from degradation from RNase activity. Embryos were permeabilized with Proteinase K (PK) solution to ensure that the probe is able to access the target gene mRNA. To decrease potential background staining, acetylation treatment was performed using 0.25% acetic anhydride in triethanolamine. Digoxigenin-labelled *vasa* probe was diluted in hybridization mix [50% deionised formamide, 5x saline-sodium citrate (SSC) buffer, 0.1% Tween-20, citric acid, 500ng/ml yeast tRNA, 50 µg/ml heparin, ddH_2O] and incubated overnight at 70^{o}C. Embryos were stained with nitrobluetetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) for 1-2 hours. The staining reaction was stopped by washing embryos with 1X phosphate-buffered saline with Tween-20 (PBST) and ethylenediaminetetraacetic acid (EDTA). Embryos were scored in a single-blind fashion based on *vasa* patterning according to the criteria outlined in Table 2.2.

2.9 RNA Extraction and cDNA Synthesis

Total RNA was extracted from 2, 4, and 6 days post fertilization (dpf) embryos using RNeasy Plus Mini Kit (Qiagen, Inc.). Total RNA quality was assessed using gel electrophoresis and determined by the presence of two clean bands (without smears) representative of the 18s and 28s subunits. The quality and quantity were also analyzed using a NanoDrop™ ND 2000c UV-Vis spectrophotometer (Thermo Scientific™) by obtaining a 260/280 ratio of ≥2 and a concentration measured in nanograms per microliter (ng/µl). Complementary DNA (cDNA) synthesis was prepared using 500ng of total RNA and SuperScript® II reverse transcriptase (Invitrogen™) in a 40µl reaction volume.

2.10 Semi-Quantitative Real-Time Polymerase Chain Reaction (q-RT-PCR)

Quantitative real-time PCR was performed on cDNA synthesized from total RNA to compare relative *dnd* levels between fish injected with the second generation RNAi constructs and their wildtype counterparts. Total RNA of three biological replicates, each containing a pool of 25 zebrafish larvae, were collected at 2, 4, and 6 dpf and tested in biological triplicates. The *dnd* primers (forward: AAGTCAATGGGCAGAGGAAA-3'; reverse: TCATGAGGCGAAACTCGTAA-3') were used to detect changes in *dnd* expression between control and experimental samples. The ribosomal protein subunit 18 (*rps18*) gene primers (forward: TGAGGTTGAGAGGGTGGTGA-3'; reverse: TGACCACGCACACGCAG-3') were used as the reference gene to normalize all results. The amplicon lengths of *dnd* and *rps18* were 168 bp and 216 bp respectively. The PCR amplification specificity was confirmed using melting curve analysis. Primer efficiency was

determined by performing a standard curve analysis using a serial dilution of template cDNA for each primer pairs. Each qRT-PCR reaction contained 7.5 µl of 2x iQ™ SYBR® Green Supermix (Bio-Rad Laboratories, Inc.), 0.5 µl of 10 µM forward and reverse primers, 3 µl of cDNA template, and 4.5 µl of ddH_2O to make 15 µl reaction volumes. The qRT-PCR were performed in technical triplicates using an Eco™ Real-Time PCR System (Illumina, Inc.) under the following conditions: 95°C for 3 minutes (polymerase activation), 95°C for 30s, 58°C for 30s, 72°C for 30s (PCR amplification), followed by 95°C for 15s, 55°C for 15s, and 95°C for 15s (melt curve); 40 amplification cycles were performed.

2.11 Statistical Analysis

Statistical analysis of qRT-PCR data were calculated using Microsoft Excel 2010 based on the Pfaffl method (Pfaffl, M. W., 2001). Statistical significance was determined by performing a one-sample t-test.

Statistical analysis of the frequency (%) of larvae showing low *vasa* staining between wildtype and *CMVmiR30-endndRNAi-A* and *CMVmiR30-endndRNAi-B* fish were performed using chi-square tests ($\chi 2$) using Microsoft Excel.

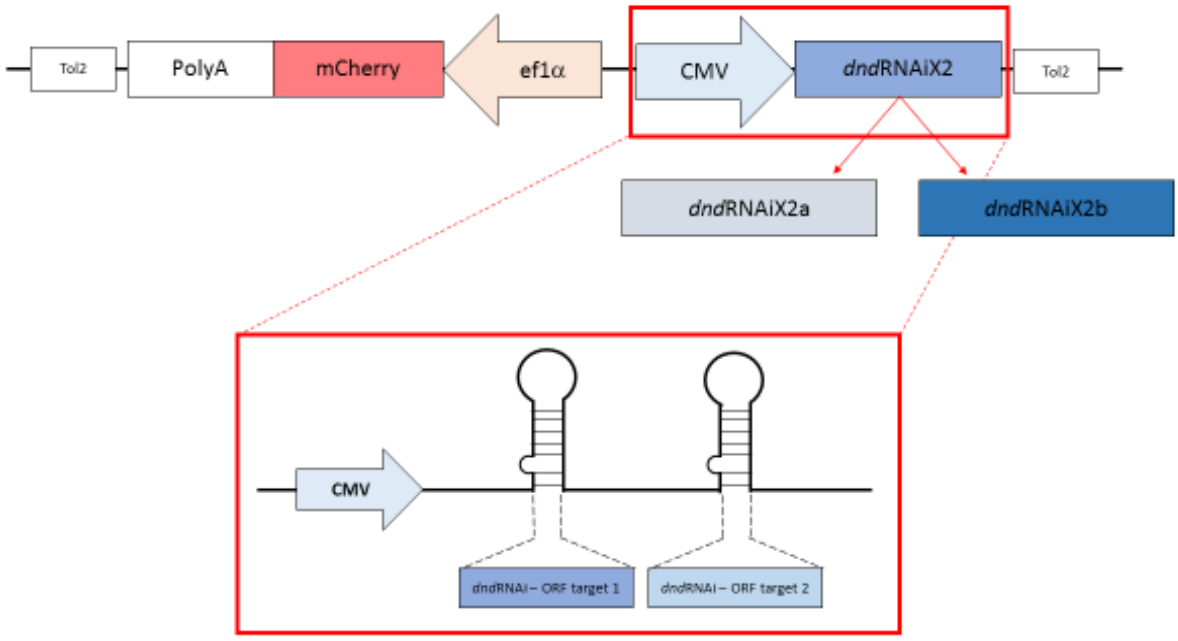
Table 2.2. Classification of *vasa* staining pattern.

Score	*vasa* Staining Pattern
Normal	Normal *vasa* staining density; by late somitogenesis (~24 hpf) PGCs form a V-shaped pattern as a string of cells on each side of the midline.
Low	Only a few cells with *vasa* staining relative to wildtype controls.
Ectopic	Cells expressing *vasa* outside of the characterized midline location of PGCs found in wildtype counterparts.

Zebrafish larvae were fixed at 2, 4, and 6 dpf followed by whole-mount in situ hybridization using a *vasa* digoxigenin-labelled antisense riboprobe. The expression patterns were scored according to the above criteria in a single-blind fashion.

Figure 2.1. First Generation RNAi Constructs. Each construct contained a mCherry reporter gene driven by the ubiquitous ef1α promoter to screen for transgene expression. (A) The RNAi portion of the construct (outlined in red) contained a dual hairpin consisting of two different target sequences from the open reading frame (ORF) of *dnd* driven by the constitutive CMV (cytomegalovirus) promoter. (B) The same construct was also created with the inducible UAS promoter. Flanking the construct were Tol2 arms which facilitated integration of the target vector.

A


Tol2
PolyA
mCherry
ef1α
CMV
dndRNAiX2
Tol2
dndRNAiX2a
dndRNAiX2b
CMV
dndRNAi – ORF target 1
dndRNAi – ORF target 2

B


Tol2
PolyA
mCherry
ef1α
UAS
dndRNAiX2
Tol2

Figure 2.2. Second Generation RNAi Constructs. Each construct contains an mCherry reporter gene driven by the ubiquitous ef1α promoter to screen for transgene expression. (A) The RNAi portion of the construct (outlined in red) contains a sing hairpin with a target sequence from the 3'UTR of *dnd* driven by the constitutive CMV (cytomegalovirus) promoter. (B) The same construct was also created with the inducible UAS (upstream activation sequence) promoter. Flanking the construct are Tol2 arms which facilitate integration of the target vector.

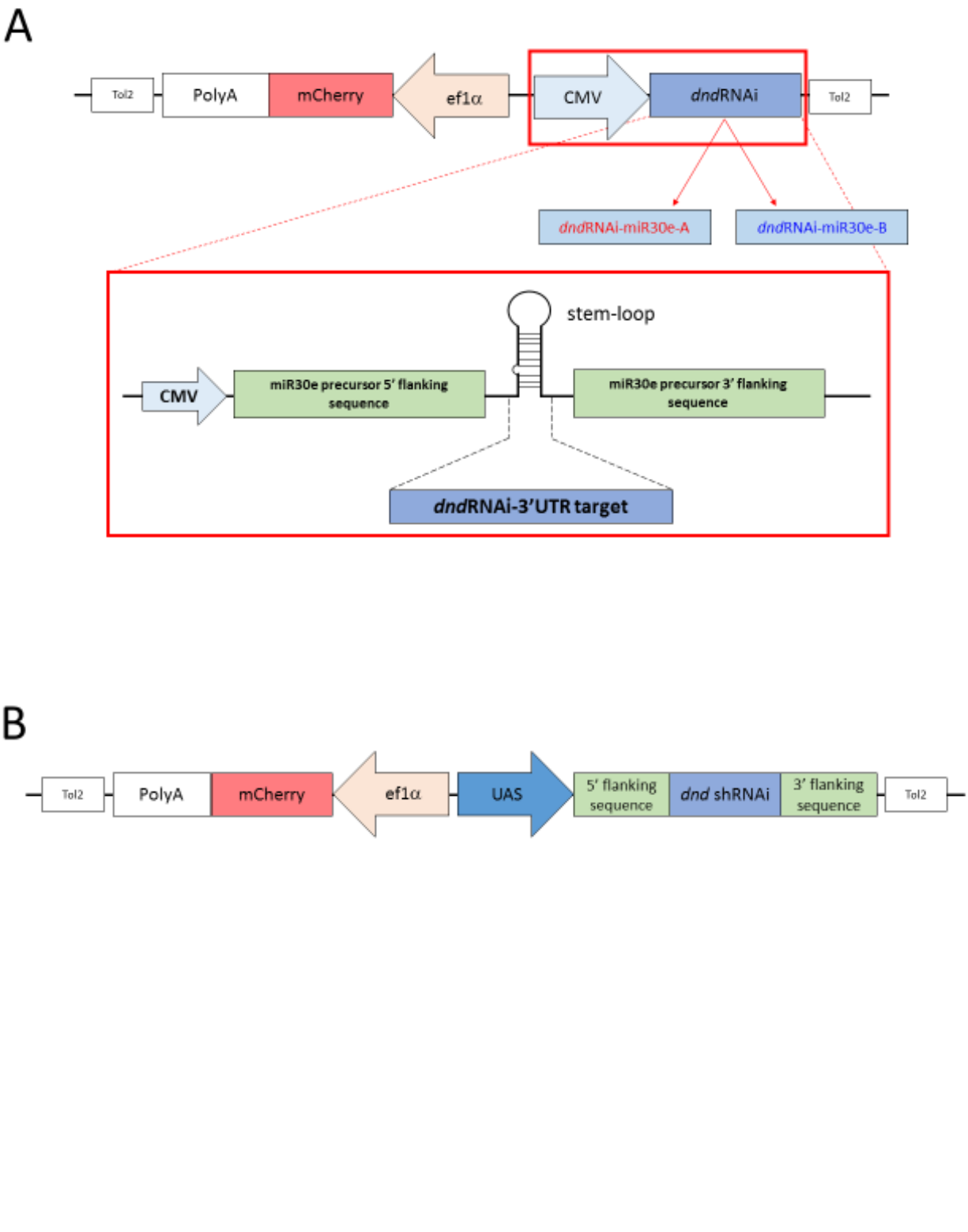

A
Tol2
PolyA
mCherry
ef1α
CMV
dndRNAi
Tol2
dndRNAi-miR30e-A
dndRNAi-miR30e-B
stem-loop
CMV
miR30e precursor 5' flanking sequence
miR30e precursor 3' flanking sequence
dndRNAi-3'UTR target
B
Tol2
PolyA
mCherry
ef1α
UAS
5' flanking sequence
dnd shRNAi
3' flanking sequence
Tol2

Figure 2.3. Zebrafish *dnd* mRNA sequence (1765 bp) GenBank accession number AY225448.1. Lowercase denote untranslated regions (UTR) and uppercase denote open reading frame (ORF). Green underlined sequences represent the target regions selected for the first generation construct (CMVdndRNAiX2) hairpins. Red and blue underlined sequences represent the target regions selected for the second generation CMVmiR30-edndRNAi-A and CMVmiR30-edndRNAi-B constructs respectively.

ctttaatgacctttcttgacttttccaccaatttacaggtgtgtctatcatcatcacagATGGTCGGAGACATGGATGCCC
AGCAGCAGGAGCTTCAGCAGATTCTGAACCCGCAGAAACTCAAGTCTCTGCAGGAATG
GATGCAGAGGAACTCCATCACTTTAACCCAAGTCAATGGGCAGAGGAAATATGGTGGT
CCTCCTCCAGGTTGGCAGGGTCCTGCTCCTGGTTCGGGCTGTGAGGTTTTCATCAGTCA
GATCCCGAACGACGTGTACGAGGACCGCCTGATCCCTCTCTTCCAGAGCATCGGCACCA
TTTACGAGTTTCGCCTCATGATGAACTTCAGCGGGCAGACCCGGGGCTTCGCCTATGCT
AAGTACGGTGACCCTCTTACAGCCTCGGCTGCCGTCACCACGCTGCATCAGTACCGGCT
GCCGGAGGGGGGCTGCCTGACCGTGCGCAGGAGCACCGAGAAGCGCCAGCTGCGTTTG
GGGGATCTGCCCGTCAGCATGAATGAATCGAAGCTGCTGATGGTGCTCCAGATGCTCTC
TGACGGTGTAGAGGACGTCCTGCTCAAGCCACCGGGGCCCAAAGGGAAAGAGGTTGTG
GCTCTAGTCAACTACACGTCCCATTACGCCGC**ATCCATGGCTAAGAAAGTGCT**CGTGG
AAGCTTTTCGGAACCGGTACGGCATTTCCATCACCGTCAGATGGACTTCCTTCTCCAAG
TCCAAGCGCGTCGAGGACACTCCCCAGGAAGACAGCTGCGTAACCCCACTTGTTCTGA
AGCCGCTTTCTAAACCATCACTCCTGCATTATGACGTCCCAGCTCACCAGTCTCTGCTTC
CTCTCTTCCGGGCTGTTGGGGGTCCGACCACCAGTGAGCAGAGAGATGAGATGATTCCT
CAACCCACCATAATGTCAAGAAATGAGCTGATTCCTCAATCGTCCATAAGGCAGAGAG
ATGAGATGGTTCCTCAGCTCCCCATAAGGCCGAGAGATGGGATGGCTCCCCAATCCCCC
ATTAGTCTCGACGCCGTGTCTCATCTGCAGTGGATGTGCGAGGTCAACAGACTCGGCTC
TCCGCAATATGAAGTCCACTTCCATCACGCGGC**TCCTGACGGATTCCTCTACTT**CGCC
TTCAAAGTGCTGATCCCAGGCCTGCCGCTGCCCCTGTATGGGTTCGTCCAGATCCTGCC
AGGCACCAGCGCACGAGCCATGAAGAGTGAAGTTTACCGGGCCGCCGCTGAGCAGGTG
ATCCAAACCTTGTGCCGAGTCTCAAATTTACGGCCTTTCTAAgaatgtcagattatggcttgatcgaatgt
gattgtgatcagttttacgtt**cagtattatgtactgttccggtta**tagatgatgaatatgtggaaatgtaatgaaaaataagcatttagtttactgtt
gatgaagagaaaaaaaaaaggtgaccaaggcagtattacttttatttgattttatttttttcaagctcttgaatttagtggtttgaagttttatgttctcg
tcgttttataatattttaactatgtaatattaataattgagttgttttagtcagcctcatcatattaggatgactgcatgttttcacgcttttcttttgagtgt
ttttcactgtatttcgacttcactttggtttgcgtttgtcacgattgttctttttgcatgg**tgtgctccttgtgtttccttgtttg**atgggttgtactgact
ataaatgacttttgtacaataaataagttgtt

Figure 2.4. EGFP Sensors. The second generation RNAi constructs were used alongside an *in vivo* EGFP sensor assay. Ubiquitously expressed EGFP sensor mRNA was designed with complementary sequences to the 24 nucleotide *dnd* target sites found in the custom-designed shRNA constructs. The *dnd* target sequence was inserted downstream of the EGFP reporter gene and co-injected with the corresponding shRNA bearing construct in order to evaluate the knockdown efficiency. A decrease in EGFP fluorescence in co-injected individuals would suggest that knockdown of the target mRNA was occurring *in vivo*. Sensor A contained the following *dnd* target sequence: 5'-CAGTATTATGTACTGTTCCGGTTA-3'; Sensor B contained the following *dnd* target sequence: 5'-TGTGCTCCTTGTGTTTCCTTGTTTG-3'.

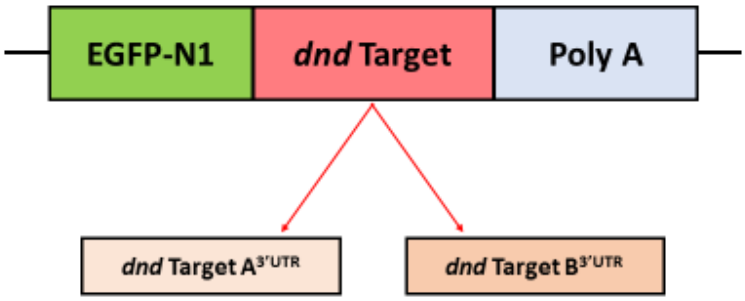

EGFP-N1
dnd Target
Poly A
dnd Target A3'UTR
dnd Target B3'UTR

Figure 2.5. Transgenesis in Zebrafish screened with a fluorescent reporter construct. Primary injected embryos (F_0) screened positive for the mCherry fluorescent reporter can be used for transient assays. A subset is raised to adulthood and can be screened for stable transgenesis by crossing with widtype fish. F_1 embryos screened positive for the expression of the fluorescent reporter gene would make the F_0 parent a founder fish.

Injection in Wildtype Embryos

One Cell Stage

Fluorescence Screening on Primary Injected Embryos

Transient Assays

Transient Fish (F_0)

Stable Transgenic Screening

x

Founder Fish (F_0)

Wildtype

Stable Transgenic Assays

Transgenic (F_1)

Figure 2.6. Zebrafish *vasa* mRNA sequence (2811 bp) GenBank accession number BC095001.1. Lowercase denote untranslated regions (UTR) and uppercase denote open reading frame (ORF).

ttttctttctctcgcgaggctcttcacgcgtgtccacctgctaccggctcttctgaaaaaaagtctcacctaaaattacccaatATGGATGACTGGGA
GGAAGATCAGAGTCCCGTTGTGTCTTGCAGCTCAGGGTTTGGAGGTGCTGGAAATGACAAGTCAA
ACAGTGAAGGTACTGAAGGTAGCTCATGGAAAATGACTGGTGATTCGTTCAGGGGGAGAGGAGG
CAGGGGAGGATCACGAGGAGGCAGAGGAGGCTTTAGCGGTTTTAAATCAGAAATTGATGAGAAT
GGCAGCGATGGAGGTTGGAACGGAGGTGAAAGTCGAGGAAGAGAACGCGGTGGTTTCCGTGGTG
GTTTTCGCAGTGGCTCCCGTGATGAAAATGATGAAAATGGAAATGATGATGGTTGGAAAGGAGGT
GAAAGCCGAGGTAGAGGTCGAGGTGGTTTCGGGGGTAGTTTTCGTGGAGGTTTCCGTGATGGTGG
TAATGAAGACACTGGGAGAAGAGGCTTTGGAAGAGAAAATAATGAAAATGGAAACGATGAGGG
AGGAGAAGGCAGAGGAAGAGGTCGTGGTGGTTTCCGGGGTGGTTTTCGTGATGGTGGTGGTGATG
AGAGTGGAAAAAGAGGCTTCGGAAGAGGAGTCACAACTGCAGACAAGTTGGATCAAGAAGGAA
GTGAAAATGCAGGACCCAAGGTTGTTTATGTGCCGCCGCCTCCTCCAGAGGAGGAGAGCTCGATA
TTCTCCCATTATGCAACAGGCATTAATTTTGACAAATATGATGATATTCTTGTGGACGTGAGTGGC
AGCAATCCTCCAAAAGCAATTATGACTTTTGAGGAAGCAGGACTTTGTGACTCACTGAGCAAAAA
TGTATCAAAGTCTGGATATGTAAAGCCTACACCTGTTCAGAAACATGGCATTCCCATCATTTCAGC
TGGACGGGATCTAATGGCTTGTGCTCAGACTGGATCAGGAAAAACGGCGGCCTTCCTACTGCCTA
TCCTACAGCGCTTTATGACTGATGGTGTGGCAGCCAGCAAGTTCAGTGAGATACAGGAGCCTGAG
GCCATAATCGTGGCTCCCACCAGAGAACTTATCAATCAGATCTATCTGGAGGCCAGGAAGTTTGC
ATACGGGACCTGTGTACGACCTGTTGTGGTTTATGGAGGTATAAATACTGGATATACTATCCGAG
AGGTGTTAAAGGGCTGCAATGTTCTGTGTGCTACTCCTGGAAGATTGCATGACCTCATTGGTCGTG
GAAAGATTGGCCTGAGTAAAGTGCGCTATCTAGTTCTGGATGAAGCAGACAGAATGCTGGACATG
GGCTTTGAGCCAGAAATGCGCAAACTGGTGGCGTCTCCTGGTATGCCTTCAAAAGAGGAACGACA
AACCCTCATGTTCAGTGCCACCTACCCTGAAGATATTCAAAGAATGGCTGCTGACTTTCTTAAAGT
GGACTATATTTTCCTTGCTGTTGGCGTGGTGGGTGGAGCATGCAGTGATGTGGAGCAAACCATTGT
TCAGGTGGACCAGTACTCAAAGAGGGACCAGCTGCTTGAATTGCTCAGAGCAACAGGTAATGAGC
GCACAATGGTTTTTGTGGAAACCAAAAGAAGTGCTGATTTCATAGCAACATTTCTCTGTCAAGAG
AAGATCTCAACCACAAGCATCCATGGTGATCGGGAACAGCGAGAGCGAGAGAAAGCTCTCAGTG
ATTTTCGCCTTGGCCAGTGTCCAGTTCTGGTCGCCACATCTGTAGCTGCTAGAGGCCTGGATATTG
AGCAAGTCCAGCATGTTGTGAATTTTGACATGCCCAGCAGCATCGATGAGTATGTCCATCGCATA
GGAAGAACTGGACGCTGTGGGAACACCGGTCGGGCCGTGTCCTTTTTTAACCCAGAGTCTGACAC
TCCACTAGCTCGCTCCCTGGTCAAAGTCCTTTCAGGGGCCCAACAAGTAGTTCCAAAATGGCTGG
AGGAAGTTGCCTTCAGTGCTCATGGGACAACAGGCTTCAACCCACGTGGGAAGGTGTTTGCATCT
ACAGACTCGCGGAAGGGAGGATCCTTCAAGAGCGATGAGCCGCCACCATCACAAATATCTGCCCC
GAGCGCAGCAGCTGCTGCAGACGATGAGGAATGGGAATAActggcctcacacctgttatatttatttttatttttatttgt
gcgagcttcttcaataccatttcaggatatttgttaatgttgaatgactaaagtagcattaggataaagctgttgtatttagagtcacattgaatgctgtaggtttgac
ctcaaggacgataatcaagactaataccttttggcagtttgatttgatcatctttttggtatcactcgtttggactggagcacaactgaattagtggatttg ttttacta
gagcacaagtgcagaagtgggtgggtttgtttaatcacattttaacacttttgctaggagtgtattgagttttgtaagctttgacctaaaggtgtttcctacagttaa
atctattaatcagttctattgttgattgtgactgtttgtaggttatttttcttgacatttccgtcttgaagccagagtatagaacaagaaacttgttttgcagatgtacctt
gccagataaaggtagctaaagtcattgtttaacagacatctctttaattttaaagtcaaaatgttgataaaattcagtcaagaggcagtgactgactgtaacagag
cggtgcttcctcactagctgaagtggctcaaaataaagacggatactggtgaaaaaaaaaaaaaaaa

3. Results

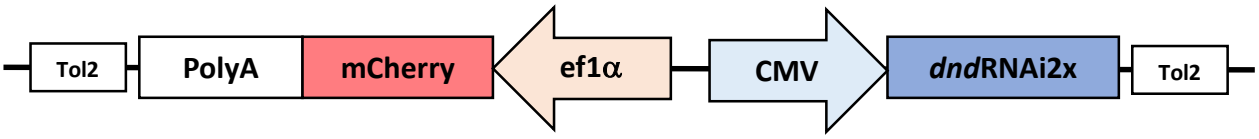
3.1 Targeting Zebrafish Primordial Germ Cells with *dnd* RNAi: First Generation Constructs

3.1.1 First generation CMVdndRNAi2X construct did not show consistent down regulation of PGCs in primary injected fish.

In zebrafish, PGCs rely on the *dnd* gene for migration and survival (Weidinger, G., Stebler, J., & Slanchev, K., 2003). Wildtype embryos were injected with the *CMVdndRNAiX2* DNA construct (50 ng/µl) (Figure 3.1A) and *Tol2* transposase mRNA (100 ng/µl) at the one cell stage. Embryos harbouring the transgene were identified by fluorescence microscopy screening for mCherry expression (Figure 3.1B). Whole-mount *in situ* hybridization was performed using an antisense digoxigenin-labelled *vasa* probe to identify and compare PGCs in *CMVdndRNAiX2* injected fish and in wildtype counterparts. All *CMVdndRNAiX2* injected fish (*n*=22) observed at 6 dpf displayed *vasa* staining with some individuals showing slightly fewer *vasa* stained cells compared to the wildtype uninjected controls (*n*=45; Figure 3.2).

Figure 3.1. CMVdndRNAiX2 construct injections. (**A**) A schematic representation of the *CMVdndRNAiX2* construct. The *ef1α* is a ubiquitously expressed promoter directing mCherry expression for fluorescence screening. CMV is also a ubiquitous promoter used to drive *dnd*RNAi2x expression containing dual stem-loop sequences with two ORF *dnd* target sequences. (**B**) Ubiquitous mCherry expression in zebrafish injected with the *CMVdndRNAiX2* construct at 6 dpf. Scale bar: 500μm.

A



B

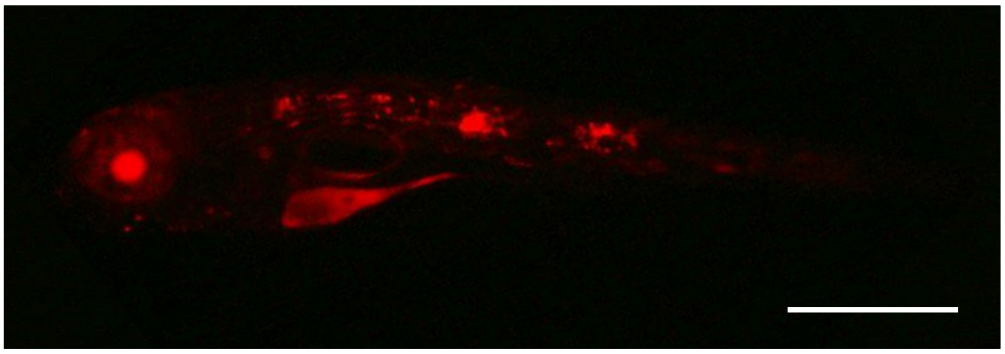
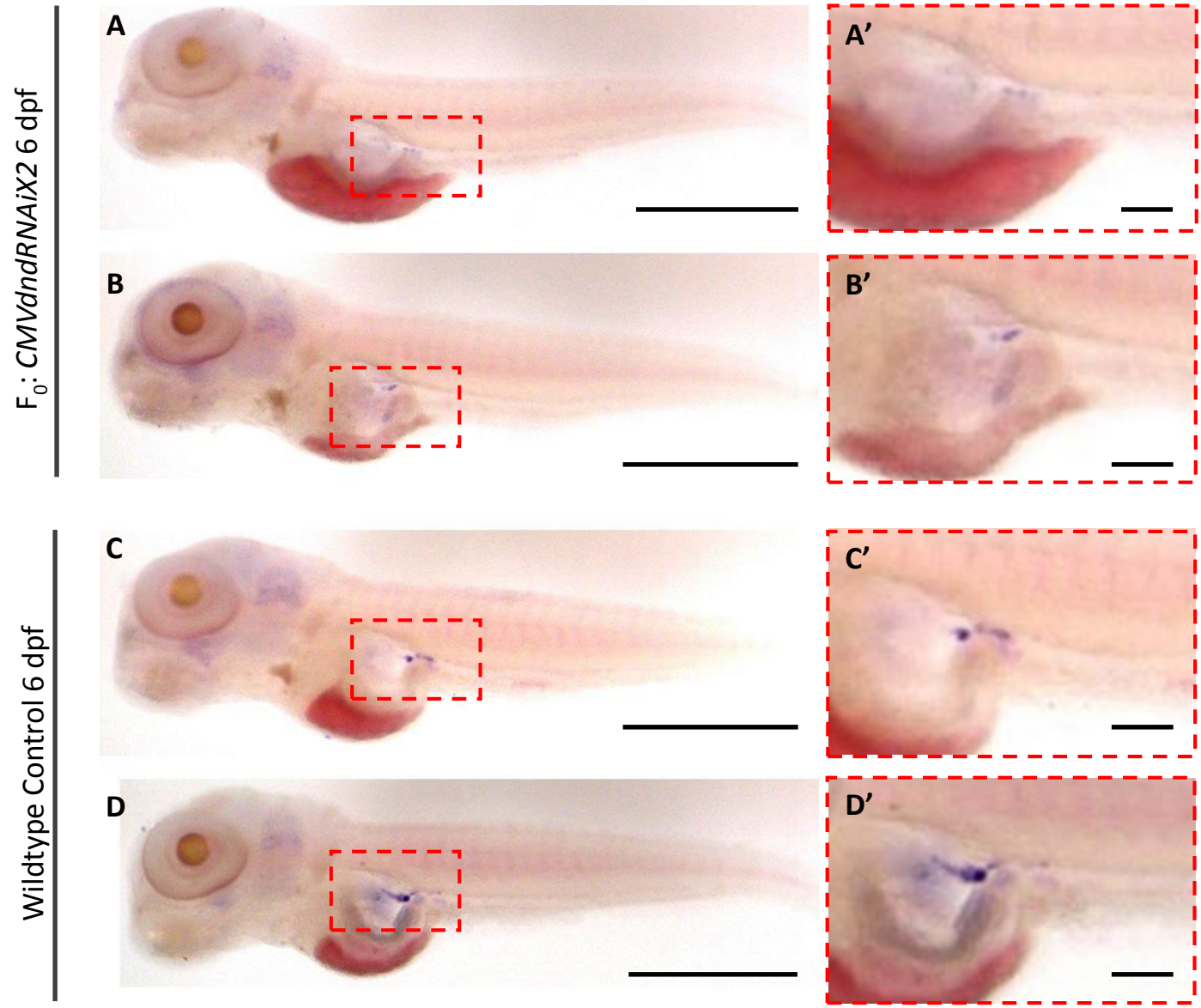


Figure 3.2. Whole-mount in situ hybridization on 6 dpf zebrafish showing decreased *vasa* staining in *F_0:CMVdndRNAiX2* fish. All *CMVdndRNAiX2* injected embryos displayed *vasa* staining in the presumptive gonadal regions. However, some *CMVdndRNAiX2* injected individuals (**A-B**) displayed low *vasa* expression (dashed regions) compared to wildtype embryos (**C-D**) when observed at 6 dpf. Scale bar: 500µm images **A**-**D**; 100µm in enlarged images **A'**-**D'**.


F0: CMVdndRNAiX2 6 dpf
Wildtype Control 6 dpf
A
A'
B
B'
C
C'
D
D'

3.1.2 Founder CMVdndRNAiX2 produced viable gametes.

To determine whether *CMVdndRNAiX2* founder fish could produce viable gametes, adults were outcrossed with wildtype fish. The presence of viable gametes was determined by the presence of developing embryos. All 10 founder fish tested were able to produce embryos.

To evaluate PGCs in F_1 fish, whole-mount *in situ* hybridization was performed on mCherry positive embryos obtained from *CMVdndRNAiX2* and wildtype crosses. There were no apparent differences in *vasa* expression between *Tg:(CMVdndRNAiX2* mCherry positive and negative (non-transgenic) embryos. All fish exhibited expected expression patterns in the presumptive gonadal regions with similar staining densities (data not shown).

3.1.3 UASdndRNAiX2/GAL4 crosses produced viable adults.

The production of *Tg(UASzfdndRNAiX2);Tg(SAGFF73A)* double transgenic fish, *Tg:UASzfdndRNAiX2* individuals were crossed with *Tg(SAGFF73A)* fish that ubiquitously express GAL4 in order to activate the UAS driven promoter. Embryos positive for mCherry fluorescence were raised to adulthood and genotyped by PCR for the presence of the *GAL4* gene using fin clips. The *Tg(SAGFF73A)* fish do not bear a visual marker and therefore required genotyping to confirm the presence of the GAL4 transgene. Fish determined to be positive for both mCherry fluorescence and GAL4 expression were outcrossed with wildtype fish to observe mating behaviour and viability of gametes. All five (two females and 3 males) double transgenic individuals displayed typical mating behaviour and were able to produce viable gametes.

3.1.4 Summary of first generation construct results

Consistent changes in *in situ* hybridization data using a *vasa* probe was not observed in first generation fish. Neither the constitutive CMV promoter nor the inducible GAL4/UAS approach had an effect on the fertility in adult fish.

3.2 Targeting Zebrafish Primordial Germ Cells with *dnd* RNAi: Second Generation Constructs

Due to the inconclusive ability of the *CMVdndRNAiX2* construct to impair germ cell formation in transient and stable transgenic zebrafish, it was decided to modify the construct used to knockdown *dnd* using RNAi. A study by Dong et al. (2009) reported increased knockdown efficiency when the shRNA was bordered by a sequence mimicking the endogenous microRNA-30e precursor flanking region. Based on their knockdown success of endogenous genes, it was decided to implement this strategy in a second attempt to eliminate *dnd* expression using RNAi. Applying the same construct design used by Dong et al., two target sequences selected from the 3'UTR of *dnd* mRNA (located at positions 1350-1373 and 1692-1717) were inserted as the hairpin sequence. These *dnd* sequences were used to generate two constructs which were designated as *CMVmiR30-edndRNAi-A* and *CMVmiR30-edndRNAi-B*. The constructs were designed in order to compare the efficacy of each hairpin sequence as an RNAi effector.

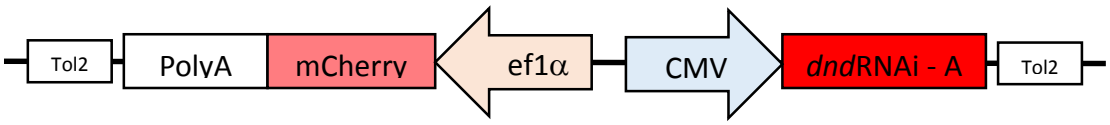
3.2.1 Disruption of PGC migration was observed in primary injected larvae

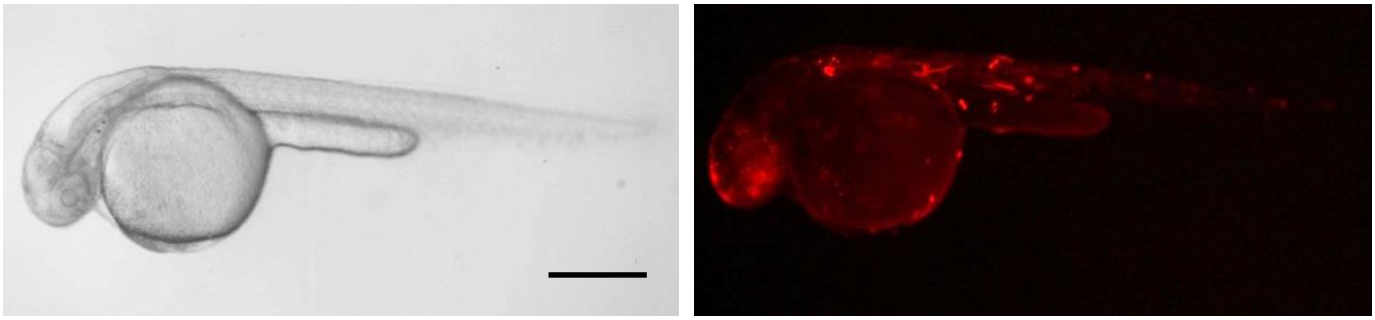
Wildtype embryos were injected at the one cell stage with either the *CMVmiR30-edndRNAi-A* or the *CMVmiR30-edndRNAi-B* DNA construct (50 ng/µl) along with *Tol2* transposase mRNA (100 ng/µl). Embryos harbouring the transgene were identified by fluorescence microscopy for mCherry (Figure 3.3). The PGCs in *F_0:CMVmiR30-edndRNAi-A* and *F_0:CMVmiR30-edndRNAi-B* positive embryos were identified using an antisense digoxigenin-labelled *vasa* probe. The staining patterns were examined.

When compared to the normal distribution of PGCs seen in wildtype embryos (*n*=144) at 2 dpf, approximately 68.7% (*n*=67) of *CMVmiR30-edndRNAi-A* fish and 40.9% (*n*=84) of *CMVmiR30-edndRNAi-B* fish showed decreased and/or irregular staining patterns (Figure 3.4). Both *CMVmiR30-edndRNAi-A* and *CMVmiR30-edndRNAi-B* founder fish comprised individuals displaying ectopic *vasa* expression, something that was never observed in wildtype fish (Figure 3.5).

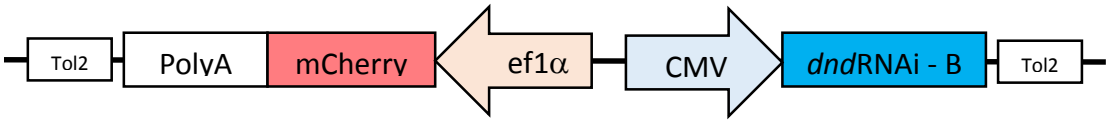
Figure 3.3. *CMVmiR30-edndRNAi-A* and *CMVmiR30-edndRNAi-B* construct injections. A schematic representation of the *CMVmiR30-edndRNAi-A* (**A**) and *CMVmiR30-edndRNAi-B* (**B**) constructs. The *ef1α* is an ubiquitously expressed promoter directing mCherry expression for fluorescence screening. CMV is also an ubiquitous promoter used to drive *dnd*RNAi-A and *dnd*RNAi-B expression. Each construct contains a single stem-loop with a distinct 3'UTR *dnd* targeting sequence (differentiated by A and B), flanked by 3' and 5' flanking sequences of the endogenous miR30-e precursor. Below each construct representation are characteristic examples of the ubiquitous transient mCherry expression observed in 2 dpf zebrafish injected with either *CMVmiR30-edndRNAi-A* or *CMVmiR30-edndRNAi-B* constructs. Scale Bar: 500µm

A


Tol2
PolyA
mCherry
ef1α
CMV
dndRNAi - A
Tol2



B


Tol2
PolyA
mCherry
ef1α
CMV
dndRNAi - B
Tol2

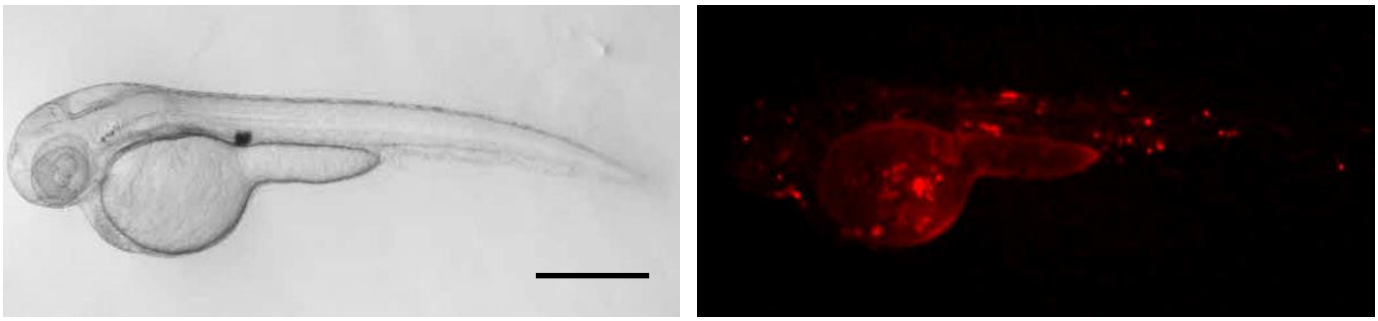


Figure 3.4. Zebrafish larvae injected with the *CMVmiR30-endndRNAi-A* and *CMVmiR30-endndRNAi-B* constructs displayed low *vasa* expression when observed at 2, 4, and 6 dpf. Zebrafish injected with *CMVmiR30-endndRNAi-A and CMVmiR30-endndRNAi-B*observed at 2, 4, and 6 dpf showed low *vasa* staining compared to their wildtype counterparts. 2 dpf (*n*: WT=144, A=67, B=84); 4 dpf (*n*: WT=70 CMVA=26 CMVB=12); 6 dpf (*n*: WT=53 CMVA=14 CMVB=83). (Chi-square significance; p=<0.05).

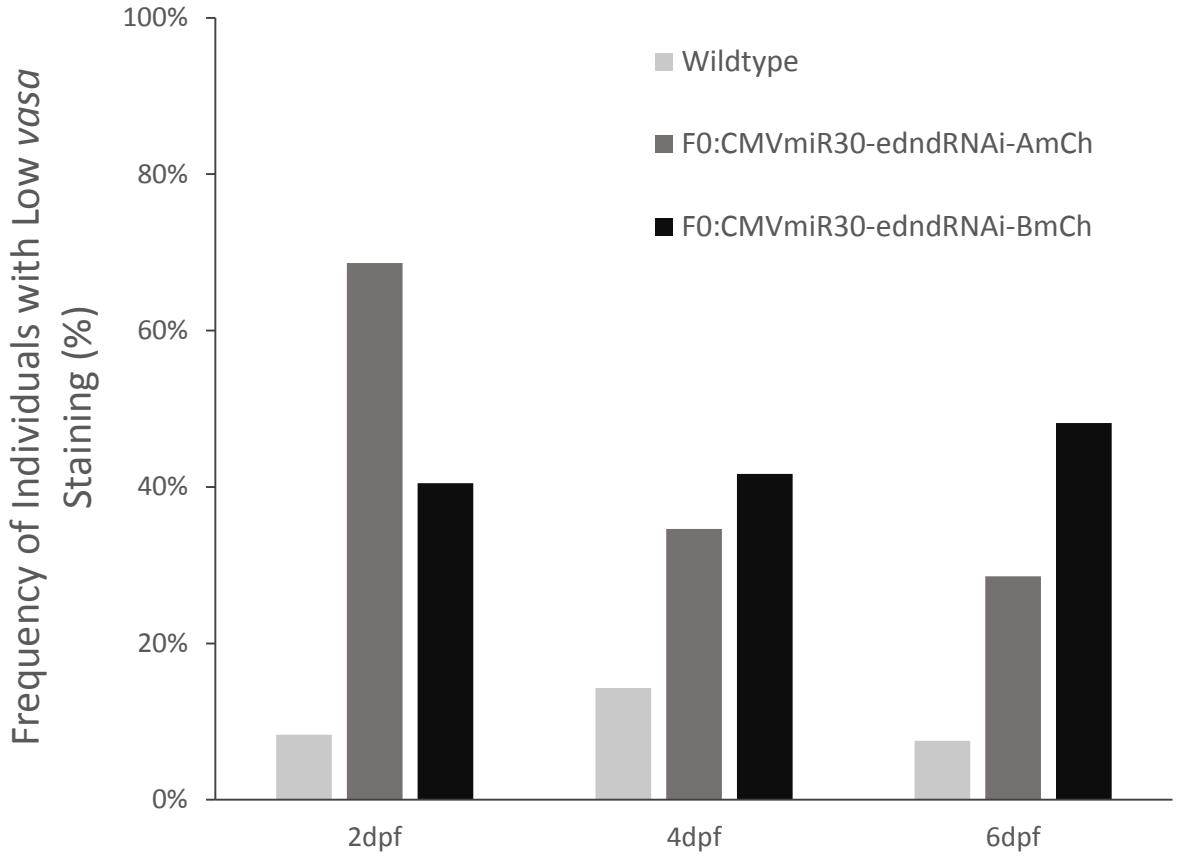
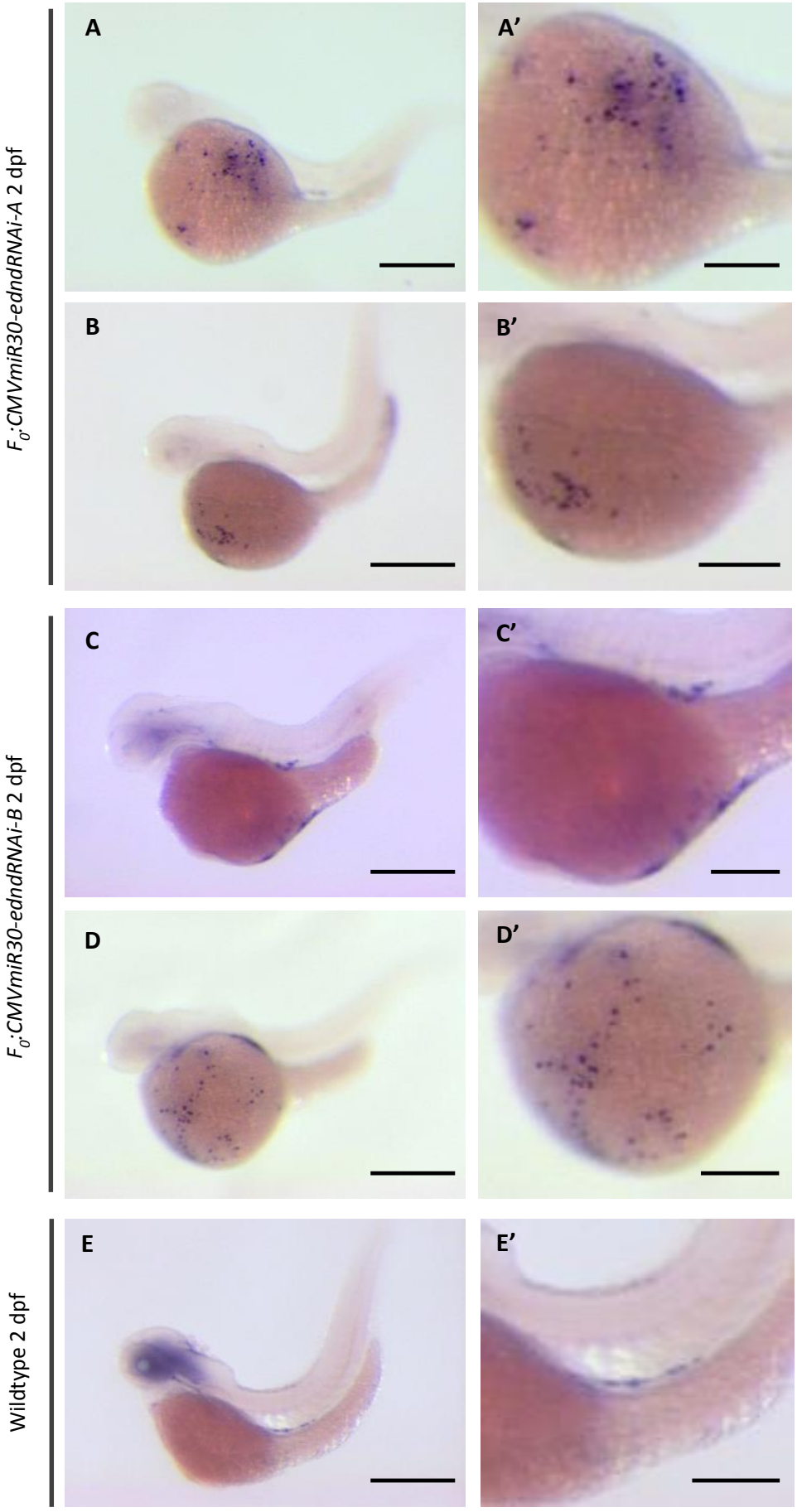

Frequency of Individuals with Low vasa Staining (%)
100%
80%
60%
40%
20%
0%
Wildtype
F0:CMVmiR30-endnRNAi-AmCh
F0:CMVmiR30-endnRNAi-BmCh
2dpf
4dpf
6dpf

Figure 3.5. Whole-mount in situ hybridization showing ectopic *vasa* staining in both *F_0:CMVmiR30-endndRNAi-A* and *F_0:CMVmiR30-endndRNAi-B* founder fish at 2 dpf. *F_0*:*CMVmiR30-endndRNAi-A* (**A-B**) and *F_0*:*CMVmiR30-endndRNAi-B* (**C-D**) larvae both showed instances of ectopic *vasa* expression when compared to wildtype fish, particularly evident at the surface of the yolk sac (**E**). Staining of PGCs in wildtype larvae was only observed in the presumptive gonadal region. Scale bar: 500µm images **A**-**E**; 250µm in enlarged images **A'**-**E'**.

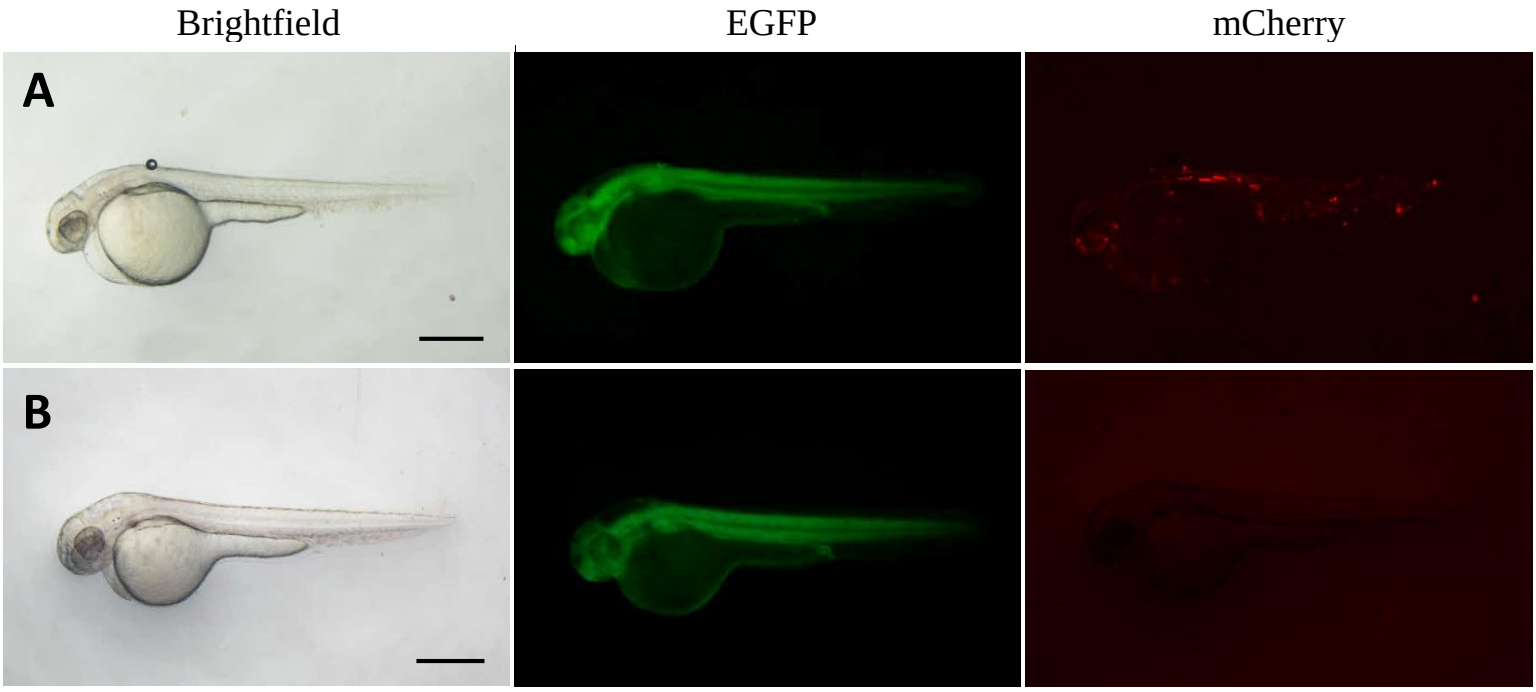

A
A'
B
B'
C
C'
D
D'
E
E'
F0:CMVmiR30-ednd RNAi-A 2 dpf
F0:CMVmiR30-ednd RNAi-B 2 dpf
Wildtype 2 dpf

3.2.2 dnd shRNA constructs do not alter expression of a sensor mRNA in transient assays

The second generation constructs were co-injected with an EGFP sensor mRNA designed with a sequence corresponding to the *dnd* sequence targeted by the hairpins found in the *CMVmiR30-edndRNAi-A* and *CMVmiR30-edndRNAi-B* constructs. These sensors were named Sensor A and Sensor B respectively. The purpose of creating an EGFP sensor mRNA was to serve as a visual indicator of the down regulatory effects of the shRNAs. When compared to individuals injected with the sensor mRNA alone, a decrease in EGFP fluorescence in co-injected individuals would suggest that the shRNA construct was able to effectively locate and knockdown a specific target mRNA *in vivo*. No changes in EGFP fluorescence would suggest that the shRNA was inefficient at inhibiting translation of the *dnd* target sequences.

Fish co-injected with the shRNA bearing construct (*CMVmiR30-edndRNAi-A* or *CMVmiR30-edndRNAi-B*) along with their corresponding sensor mRNA (Sensor A or Sensor B) were examined at 24 hpf (data not shown) and 48 hpf (Figure 3.6). No visible reduction in EGFP expression was observed when comparing co-injected embryos with those only injected with the EGFP sensor mRNA.

Figure 3.6. Co-injection of *CMVmiR30-edndRNAi* with their corresponding sensor mRNA did not reveal changes in EGFP expression. (**A**) Representative images of 2 dpf zebrafish co-injected with *CMVmiR30-edndRNAi-B* and Sensor B mRNA showing no changes in EGFP expression compared to (**B**) fish injected with the sensor mRNA alone. Injections with *CMVmiR30-edndRNAi-A* and Sensor A mRNA yielded similar results. Scale bar: 500µm.


Brightfield
EGFP
mCherry
A
B

3.2.3 CMVmiR30-edndRNAi-A and CMVmiR30-edndRNAi-B transgenic zebrafish were able to produce viable gametes

Both *F_0:CMVmiR30-edndRNAi-A* and *F_0:CMVmiR30-edndRNAi-B* fish were raised to adulthood (~3-4 months) and outcrossed with wildtype fish to look for viable gametes and germline transmission of the transgene. The production of embryos from pairwise breeding suggested that each contributing parent produced viable gametes. These embryos were then examined for mCherry fluorescence to indicate germline transmission of the transgene from the founder parent (F_0).

Ten fish (5 males and 5 females) from each construct were outcrossed with wildtype fish. All ten *F_0:CMVmiR30-edndRNAi-A* fish produced viable gametes. One female from the ten *F_0:CMVmiR30-edndRNAi-B* fish did not produce eggs after five breeding attempts (Table 3.1).

Fish injected with either construct generated a heterogeneous population comprising both male and female adults. We obtained 18 males and 9 females with the *CMVmiR30-edndRNAi-A* construct and 10 males and 12 females from *CMVmiR30-edndRNAi-B.*

Table 3.1. Individuals injected with either the *CMVmiR30-endndRNAi-A* or *CMVmiR30-endndRNAi-B* constructs produced viable gametes.

Construct (F_0)	Sex	Individuals with Viable Gametes
CMVmiR30-endndRNAi-A	Male	5 of 5
	Female	5 of 5
CMVmiR30-endndRNAi-B	Male	5 of 5
	Female	4 of 5

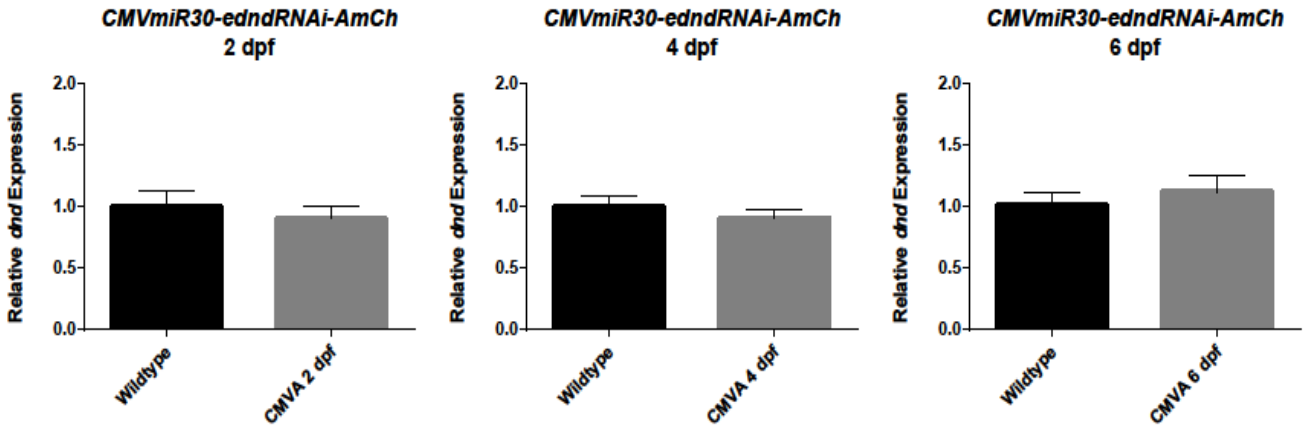
3.2.4 No significant changes in dnd mRNA expression observed in dnd-shRNA transgenic zebrafish (second generation constructs)

To assess the relative changes in *dnd* mRNA expression levels in *dnd*-shRNA transgenic zebrafish, q-RT PCR assays were performed on fish injected with the *CMVmiR30-edndRNAi-A* or *CMVmiR30-edndRNAi-B* constructs. Using cDNA synthesized from total RNA extracted from a pool of 25 embryos, *dnd* mRNA levels from either injected or wildtype control fish were assayed at three time points: 2, 4, and 6 days post fertilization (Figure 3.7). Expression levels were analyzed using six biological replicates, each comprised of a pool of 25 embryos, and each biological replicate was assayed in experimental triplicates.

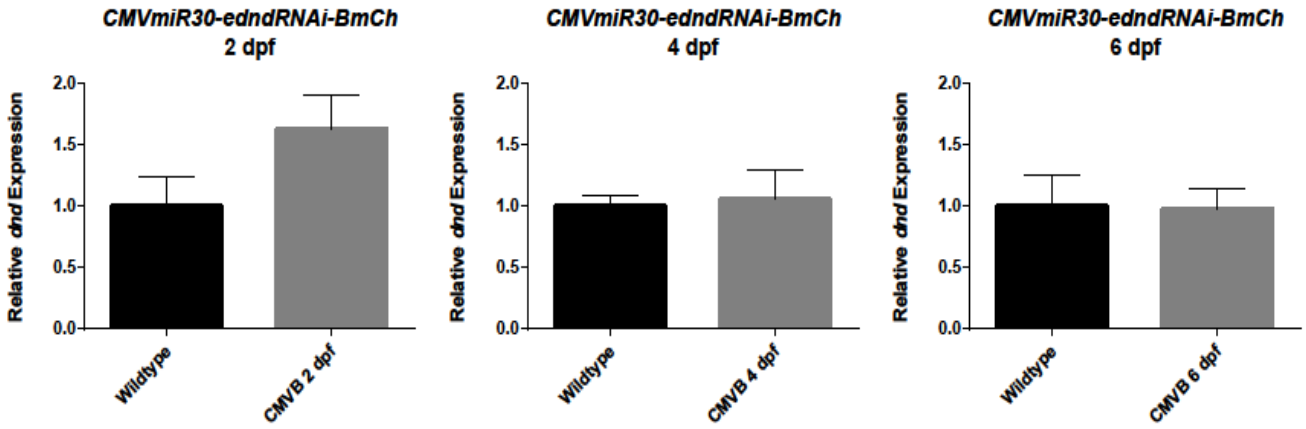
The results from the q-RT PCR assays showed no significant changes in relative *dnd* mRNA expression levels between the uninjected controls and fish injected with either the *CMVmiR30-edndRNAi-A* or the *CMVmiR30-edndRNAi-B* constructs as determined using a one-sample t-test.

Figure 3.7. The *dnd*-shRNA constructs do not significantly affect *dnd* mRNA expression levels. Quantitative RT-PCR analysis of relative *dnd* mRNA transcript levels at 2, 4, and 6 days post fertilization in embryos injected with the *CMVmiR30-endndRNAi-A* (**A**) and *CMVmiR30-endndRNAi-B* (**B**) constructs. Relative *dnd* gene expression is presented as a fold change (± standard error of the mean) compared to uninjected controls and is based on six biological replicates, each containing a pool of 25 embryos per given treatment. Each wildtype replicate was specific to the construct and timepoint that it was compared to. Data were normalized to ribosomal protein subunit 18 (*rps18*) transcript levels. Statistical analysis was determined using a one-sample t-test.

A


CMVmiR30-endndRNAi-AmCh
2 dpf
CMVmiR30-endndRNAi-AmCh
4 dpf
CMVmiR30-endndRNAi-AmCh
6 dpf
Relative dnd Expression
2.0
1.5
1.0
0.5
0.0
Wildtype
CMVA 2 dpf
CMVA 4 dpf
CMVA 6 dpf

B


CMVmiR30-endndRNAi-BmCh
2 dpf
CMVmiR30-endndRNAi-BmCh
4 dpf
CMVmiR30-endndRNAi-BmCh
6 dpf
Relative dnd Expression
2.0
1.5
1.0
0.5
0.0
Wildtype
CMVB 2 dpf
CMVB 4 dpf
CMVB 6 dpf

3.2.5 Summary of second generation dnd targeting constructs

Although changes in *vasa* expression density and location were observed in both second generation construct fish, there were no significant changes in relative *dnd* mRNA levels between wildtype and injected fish. Pairwise crosses of adult fish confirmed that fertility was not affected by either of the *dmd* targeting shRNA constructs.

4. Discussion

4.1 First Generation RNAi Targeting *dnd* Constructs.

The objective of the first generation construct was to characterize the knockdown ability of dual shRNAs targeting *dnd*. The two target sequences were located at positions 702-722 and 1278-1298 in the ORF of *dnd* mRNA.

4.1.1. Dual shRNA controlled by a constitutive CMV promoter was not able to induce sterility.

Whole-mount *in situ* hybridization revealed that fish injected with the *CMVdndRNAiX2* construct did not differ from wildtype in the density or location of *vasa* stained cells. Although there were some instances of mispatterned and/or fewer stained cells, these findings were variable and infrequent.

One explanation for the results is the fact that primary injected fish are transiently transfected. The DNA construct injected into embryos is unevenly distributed and not necessarily integrated into the genome. Without genome integration, the introduced construct will not be replicated, causing it to be diluted out as cells divide. This may explain why knockdown effects were not consistently observed in *F_0:CMVdndRNAiX2* fish.

Stable transgenic fish on the other hand are individuals that have the DNA construct integrated into their genome, allowing for replication of the transgene to occur. This allows the transgene to be inherited, resulting in a stable transgenic line. However, expression patterns may vary between each stable transgenic line due to characteristics of the integration site (Parinov, S., Kondrichin, I., Korzh, V., & Emelyanov, A., 2004; Williams, D. W., Müller, F., Lavender, F. L., Orbán, L., & Maclean, N., 1996).

Transient assays are advantageous as they allow rapid analysis of transgene expression levels within a large sample size. It has been shown that performing assays on transient transgene expression provides reliable results despite suffering from variability among individuals (Chatterjee, S., Min, L., Karuturi, R. K. M., & Lufkin, T., 2010; Roberts, J. A., Miguel-Escalada, I., Slovik, K. J., Walsh, K. T., Hadzhiev, Y., Sanges, R., Stupka, E., Marsh, E. K., Balciuniene, J., Balciunas, D., & Müller, F., 2014). This is particularly true when analyzing gene expression in large pools of sample.

The analysis of the whole-mount *in situ* hybridization on *F_0:CMVdndRNAiX2* larvae did not allow us to confidently say that a specific knockdown effect was achieved. To confirm that these fish were able to develop functional gonads, pairwise breeding between *F_0:CMVdndRNAiX2* and wildtype fish were performed. All breeding pairs produced healthy

embryos indicating fertility from each contributing parent. The presence of functionally mature gonads in *F_0:CMVdndRNAiX2* suggests that if there was *dnd* knockdown attributed to the dual shRNA construct, it was not effective enough to compromise fertility among these fish.

A study by Slanchev, Stebler, de la Cueva-Méndez, & Raz (2005) ablated PGCs using *dnd* antisense morpholinos. All injected fish developed into phenotypic males as judged by their body shape, colour, and courtship behaviour displayed during pairwise breeding. In the same study, all (*n*=85) phenotypic males lacked gonadal structures. Our results do not corroborate these findings as we obtained a fertile population consisting of both male (*n*=19) and female (*n*=12) in our *F_0:CMVdndRNAiX2* injected fish.

4.1.2. Dual shRNA controlled by an inducible promoter was not able to induce sterility.

To verify our findings from the Tol-2 based transient assays, we looked at transgenic fish to see whether *dnd* knockdown effects could be induced. Similar to the constitutive approach, F_1 crosses of *UASzfdndRNAiX2* with *Tg:SAIGFF* (producing GAL4 expression) produced embryos that developed into both male and female fertile fish. This confirmed that the dual shRNA was not able to effectively knockdown *dnd* with either a constitutive or an inducible approach.

4.2 Second Generation RNAi Targeting *dnd* Constructs

Based on the results obtained from the first generation construct, it was decided to refine our approach to knock down *dnd* using shRNA. It has been shown that the 5' and 3' flanking regions of a miRNA precursor are required for the optimal processing and maturation of mammalian miRNA (Bartel, D., 2004). These sequences can be used to create a synthetic stem-loop precursor containing the flanking regions of human miR-30 to express a hairpin shRNA to induce RNAi (Zeng, Y., Wagner, E. J., Cullen, B. R., & Carolina, N., 2002). A study by Dong et al. (2009) identified zebrafish homologues of mammalian miR-30 and used the zebrafish pri-miR-30e flanking sequences to successfully produce mature miR-30e *in vivo*. Based on the knockdown success of endogenous genes achieved using the miR-30e flanking regions, it was decided to implement this strategy in another attempt to eliminate *dnd* expression using RNAi. Applying the same design used by Dong et al., we selected two target sequences from 3'UTR of *dnd* mRNA (located at positions 1350-1373 and 1692-1717) and inserted a single hairpin creating our second generation RNAi constructs; *CMVmiR30-edndRNAi-A* and *CMVmiR30-edndRNAi-B.*

4.2.1 Second generation shRNA controlled by a constitutive CMV promoter showed changes in vasa expression patterns compared to wildtype fish.

Qualitative analysis from whole-mount *in situ* hybridization results from both *F_0:CMVmiR30-edndRNAi-A* and *F_0:CMVmiR30-edndRNAi-B* suggested that there was a knockdown effect on PGCs occurring in fish at the 2, 4, and 6 dpf time points. Within these three time points, ~28% to 68% of the population of injected fish showed decreased and/or

disrupted *vasa* staining. Both constructs also presented instances of severe ectopic *vasa* expression.

Dnd is an RNA binding protein explicitly expressed in the PGCs. It is required to confine PGCs to the deep blastoderm layers during early stages of embryogenesis (Weidinger, G., Stebler, J., & Slanchev, K., 2003). Knockdown experiments have shown an absence of *dnd* results in improper PGC migration (Weidinger, G., Stebler, J., & Slanchev, K., 2003). The ectopic expression observed in *F_0:CMVmiR30-edndRNAi-A* and *F_0:CMVmiR30-edndRNAi-B* fish could be attributed to decreased *dnd* mRNA levels as a result of the shRNA bearing construct. PGCs are specified extragonadally during early embryogenesis (3hpf) and eventually migrate towards the genital ridge (Raz, E., 2003; Yoon, C., Kawakami, K., & Hopkins, N., 1997). Although the PGCs may not have been completely eliminated by the shRNA, reduced *dnd* expression levels could have been what caused the PGCs to migrate to areas other than the gonadal region.

4.2.2 Second generation shRNA expressed by a constitutive CMV promoter showed no changes in vasa mRNA expression levels compared to wildtype fish.

The q-RT PCR data revealed no significant changes in *dnd* mRNA expression levels between wildtype and *F_0:CMVmiR30-edndRNAi-A* and *F_0:CMVmiR30-edndRNAi-B* fish analyzed at the 2, 4, and 6 dpf time points as determined using the one-sample t-test.

Semi-quantitative real-time PCR (qRT-PCR) analysis utilizes fluorescent reporter molecules to measure amplification products generated after each PCR cycle. Although qRT-PCR has become a standard assay to detect and compare relative mRNA levels, it is susceptible to inconsistencies due to its high sensitivity. When target templates are rare (only present in a small population of cells within the sample) results tend to be more vulnerable to variability (Karrer, E., & Lincoln, J., 1995). Zebrafish *dnd* mRNA is explicitly expressed in the PGCs, which represent a small fraction of the cells within the organism (Liu, W., & Collodi, P., 2010). The efficiency of a qRT-PCR reaction can also be affected by background RNA, which refers to the presence of non-target RNA present in the sample. Background RNA is more likely to hinder precise priming of the RNA template when the target transcript is rare (Curry, J., McHale, C., & Smith, M., 2002). The qRT-PCR data obtained from *F_0:CMVmiR30-edndRNAi-A* and *F_0:CMVmiR30-edndRNAi-B* fish did not show significant changes in *dnd* expression compared to levels observed in wildtype. This could be explained by the sample containing low initial *dnd* transcript levels which might cause the mRNA detection to be masked by contamination and/or background RNA.

4.2.3 Second generation shRNA expressed by a constitutive CMV promoter developed into fertile male and female population.

A subset of *F_0:CMVmiR30-edndRNAi-A* and *F_0:CMVmiR30-edndRNAi-B* positive embryos was raised to adulthood. Unlike the results obtained from Slanchev et al. (2005) where *dnd* morpholino injections resulted in sterile males, our second generation injected fish yielded a population of both male and female fish that were fertile fish. Following this report, it has been confirmed that the germ line is required for ovary development, whereas the absence of a germ line results in testis formation in zebrafish (Siegfried, K. R., & Nüsslein-Volhard, C., 2008). In the present study, it is apparent that any *dnd* knockdown effects observed with either *CMVmiR30-edndRNAi-A* or *CMVmiR30-edndRNAi-B* constructs were unable to completely inhibit germ line development in order to drive a male only population. This being said, the mechanisms that regulate sex determination and differentiation in zebrafish are still unknown. Neither sex-linked genes nor sex chromosomes have been identified in zebrafish. However, along with environmental factors, there have been genes that affect the sex determination and differentiation process (Siegfried, K. R., & Nüsslein-Volhard, C., 2008; von Hofsten, J., & Olsson, P.-E., 2005).

Another explanation for the presence of functional gametes found in *F_0:CMVmiR30-edndRNAi-A* and *F_0:CMVmiR30-edndRNAi-B* fish could be due to a partial inhibition of *dnd*. Transient transfection may result in the loss of the foreign DNA construct expression as cells replicate. Early experimental time points could explain why we see disruptive changes in *vasa* expression based on the whole-mount *in situ* hybridization results. Without germline transmission, it is possible that fish injected with *CMVmiR30-edndRNAi-A* or *CMVmiR30-edndRNAi-B* constructs only experience knockdown effects early on in development. This

early transient expression may only trigger partial inhibition of *dnd*, allowing the zebrafish to recover PGC development as the shRNA becomes diluted out. This could also explain the presence of a males and females in both *F_0:CMVmiR30-edndRNAi-A* and *F_0:CMVmiR30-edndRNAi-B* fish. Zebrafish have been known, and continue to be studied, as a model system for vertebrate regeneration (Kikuchi, K., Holdway, J. E., Werdich, A. a, Anderson, R. M., Fang, Y., Egnaczyk, G. F., Evans, T., Macrae, C. a, Stainier, D. Y. R., & Poss, K. D., 2010; Poss, K. D., Keating, M. T., & Nechiporuk, A., 2003). If the knockdown effects of either shRNA constructs were not able to completely eliminate *dnd* expression, the existing PGCs may be sufficient to give rise to functional gonadal structures.

It is apparent that based on the obtained results, sterility was not induced using the selected *dnd* target sequences in either the first or second generation shRNA constructs. A potential pitfall with RNAi is the possibility of off-target effects which can make it difficult to unravel the phenotypic effects observed in knockout experiments. By introducing synthetic shRNAs, we may have saturated the endogenous RNAi machinery resulting in unsuccessful *dnd* knockdown effects.

At the late stages of this project, a paper by Zhang et al. (2015) was published with the equivalent objective of developing a heritable approach to reproductive containment in fish. Using a GAL4/UAS system similar to the one described in this document, they reported reduced fertility among *dnd* knockdowns. A decrease in endogenous *dnd* expression was observed in GAL4 driver (TG1) fish crossed with transgenic fish bearing a 5x UAS promoter to drive antisense *dnd* transcription (TG2). This triggered apoptosis of the PGCs due to mismigration, resulting in sterility or relegated fertility in hybrid adult fish (TG3). Although having the same objective, Zhang et al. (2015) used an antisense *dnd* mRNA as opposed to

RNAi. Antisense RNA stops translation by base pairing to complementary mRNA molecules within a cell. This causes changes in the stoichiometry of the translation machinery and in turn inhibits translation.

At the early stages of development (between 0-3.66 hpf), q-RT PCR analysis revealed that *dnd* expression levels between wildtype and TG3 embryos were similar, likely due to maternal mRNA contributions (Zhang, Y., Chen, J., Cui, X., Luo, D., Xia, H., Dai, J., Zhu, Z., & Hu, W., 2015). However, a significant decrease in *dnd* mRNA was observed in TG3 embryos analyzed at 50% epiboly (5.25 hpf) and 3-somite (10 hpf) stages. *In situ* hybridization using an antisense *vasa* probe, also revealed that PGC migration was disrupted in TG3 embryos. An overall decrease in PGC number was also reported based on the WISH analysis.

TG3 fish raised to adulthood were assessed for fertility at 4.5 months post fertilization. TG3 male fish displayed typical mating behaviour which stimulated spawning when paired with WT females. However, 30.8% (53/172) of these males were unable to fertilize eggs (Zhang, Y., Chen, J., Cui, X., Luo, D., Xia, H., Dai, J., Zhu, Z., & Hu, W., 2015). Approximately 31% (15/48) of TG3 females did not spawn when paired with WT males. The TG3 males and females who were able to reproduce however, had lower relative fecundity compared to their WT counterparts. Zhang et al. (2015) explain that the variable reproductive capacity seen in TG3 fish could be due to the two CpG methylation target sites found in each of the five UAS repeats. The authors of the study decided to use the five tandem UAS repeats in their effector line after seeing higher transgene activation levels when testing a range of UAS repeats. However, repetitive sequences are susceptible to the epigenetic effects caused by methylation (Goll, M. G., Anderson, R., Stainier, D. Y. R., Spradling, A.

C., & Halpern, M. E., 2009). Single copies of UAS promoters are less vulnerable to these silencing effects compared to those with multiple UAS insertions (Akitake, C. M., Macurak, M., Halpern, M. E., & Goll, M. G., 2011). Despite the benefit of higher transcription levels, the use of five tandem UAS repeats could affect its regulatory abilities causing mosaic gene expression.

Similarly to the findings of Zhang et al., (2015), PGC migration in *CMVmiR30-edndRNAi-A* and *CMVmiR30-edndRNAi-B* primary transgenic fish appeared to be disrupted at larval stages (2-6 dpf). However, complete sterility was not observed in adult fish suggesting that the mismigration seen in *CMVmiR30-edndRNAi-A* and *CMVmiR30-edndRNAi-B* fish in this project was not sufficient to affect gonadal function. Previous reports of *dnd* knockdowns using morpholino oligonucleotides resulted in a strictly male population. However similar to Zhang et al., (2015), a population consisting of both males and females was obtained in *CMVmiR30-edndRNAi-A* and *CMVmiR30-edndRNAi-B* transgenic fish.

Although we were unable to reduce fertility in transgenic fish using both first and second generation constructs, Zhang et al. (2015) reported successful sterilization of approximately 30% of their tested population using the GAL4/UAS system. Their findings offer optimism in developing a heritable biocontainment strategy. However, the use of this approach is contingent on complete sterility in all affected subjects. We can see how various factors may influence the efficiency of gene silencing mechanisms when comparing our findings with those of Zhang et al. (2015). Once an effective shRNA target is identified, it would be in our best interest to further investigate the long term efficacies of the UAS promoter in order to develop a system that is 100% effective in subsequent generations.

Therefore, methods to avoid methylation of UAS repeats while maintaining high transcription activation must be explored.

In recent years, the use of CRISPR (clustered regularly interspaced short palindromic repeats) technology has shown promise as a cost effective gene editing tool in zebrafish. The CRISPR/Cas9 system can add and/or delete base pairs in specific DNA regions. Using this strategy could evade possible methylation effects endured by the UAS promoter. Future research should explore the use of the CRISPR/Cas9 approach as a method to knockout the *dnd* gene and apply a heritable system as a bioconfinement strategy.

The variable *dnd* knockdown effects attained by Zhang et al. (2015) demonstrate the complexity in developing a heritable approach for the reproductive confinement of fish. Different regulatory elements driving transcription of the target sequence, or the efficacy of the target sequence itself may confer different results. Although we were unable to induce sterility with the shRNA bearing constructs used in this report, there is evidence suggesting that RNAi could be used as a possible mechanism for bioconfinement.

As more is being learned about the RNAi, we are discovering new ways to modify and utilize this endogenous pathway to render higher knockdown efficiencies. Despite this, RNAi has been progressing slowly since its first attempt in zebrafish. Our results remain inconclusive as to whether it is an applicable gene silencing tool as the limitations of RNAi in zebrafish are still being uncovered.

References

Akitake, C. M., Macurak, M., Halpern, M. E., & Goll, M. G. (2011). Transgenerational analysis of transcriptional silencing in zebrafish. *Developmental Biology*, *352*(2), 191–201. http://doi.org/10.1016/j.ydbio.2011.01.002

Bartel, D. (2004). MicroRNAsGenomics, Biogenesis, Mechanism, and Function. *Cell*, *116*(2), 281–297. http://doi.org/10.1016/S0092-8674(04)00045-5

Bernstein, E., Caudy, A. A., Hammond, S. M., & Hannon, G. J. (2001). Role for a bidentate ribonuclease in the initiation step of RNA interference . *Nature*, *409*(6818), 363–366. Retrieved from http://dx.doi.org/10.1038/35053110

Brand, A., & Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development*, *415*, 401–415. Retrieved from http://dev.biologists.org/content/118/2/401.short

Castel, S. E., & Martienssen, R. a. (2013). RNA interference in the nucleus: roles for small RNAs in transcription, epigenetics and beyond. *Nature Reviews. Genetics*, *14*(2), 100–12. http://doi.org/10.1038/nrg3355

Chatterjee, S., Min, L., Karuturi, R. K. M., & Lufkin, T. (2010). The role of post-transcriptional RNA processing and plasmid vector sequences on transient transgene expression in zebrafish. *Transgenic Research*, *19*(2), 299–304. http://doi.org/10.1007/s11248-009-9312-x

Chen, M., Du, Q., Zhang, H., Wahlestedt, C., & Liang, Z. (2005). Vector-based siRNA delivery strategies for high-throughput screening of novel target genes. *Journal of RNAi and Gene Silencing*, *1*(1), 5–11.

Curry, J., McHale, C., & Smith, M. (2002). Low Efficiency of the Moloney Murine Leukemia Virus Reverse Transcriptase during Reverse Transcription of Rare t(8;21) Fusion Gene Transcripts. *Biotechniques*, *32*(4), 768–775.

De Rienzo, G., Gutzman, J. H., & Sive, H. (2012). Efficient shRNA-mediated inhibition of gene expression in zebrafish. *Zebrafish*, *9*(3), 97–107. http://doi.org/10.1089/zeb.2012.0770

Dong, M., Fu, Y.-F., Du, T.-T., Jing, C.-B., Fu, C.-T., Chen, Y., Jin, Y., Deng, M., & Liu, T. X. (2009). Heritable and lineage-specific gene knockdown in zebrafish embryo. *PloS One*, *4*(7), e6125. http://doi.org/10.1371/journal.pone.0006125

Filipowicz, W., Jaskiewicz, L., Kolb, F. a, & Pillai, R. S. (2005). Post-transcriptional gene silencing by siRNAs and miRNAs. *Current Opinion in Structural Biology*, *15*(3), 331–41. http://doi.org/10.1016/j.sbi.2005.05.006

Fire, A., Xu, S., Montgomery, M., & Kostas, S. (1998). Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature*, *391*(6669), 806–11. http://doi.org/10.1038/35888

Giniger, E., Varnum, S., & Ptashne, M. (1985). Specific DNA binding of GAL4, a positive regulatory protein of yeast. *Cell*, *44*(April). Retrieved from http://www.sciencedirect.com/science/article/pii/0092867485903368

Gjerde, B. (1984). Response to individual selection for age at sexual maturity in Atlantic salmon. *Aquaculture*.

Goll, M. G., Anderson, R., Stainier, D. Y. R., Spradling, A. C., & Halpern, M. E. (2009). Transcriptional silencing and reactivation in transgenic zebrafish. *Genetics*, *182*(3), 747–55. http://doi.org/10.1534/genetics.109.102079

Hammond, S. M. (2005). Dicing and slicing: the core machinery of the RNA interference pathway. *FEBS Letters*, *579*(26), 5822–9. http://doi.org/10.1016/j.febslet.2005.08.079

Hammond, S. M., Bernstein, E., Beach, D., & Hannon, G. J. (2000). An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells. *Nature*, *404*(6775), 293–296. Retrieved from http://dx.doi.org/10.1038/35005107

Hay, B., Jan, L. Y., & Jan, Y. N. (1988). A protein component of Drosophila polar granules is encoded by vasa and has extensive sequence similarity to ATP-dependent helicases. *Cell*, *55*(4), 577–587. http://doi.org/http://dx.doi.org/10.1016/0092-8674(88)90216-4

Houston, D., & King, M. (2000). Germ plasm and molecular determinants of germ cell fate. *Current Topics in Developmental Biology*, *50*. Retrieved from http://www.sciencedirect.com/science/article/pii/S0070215300500088

Huang, W.-T., Hsieh, J.-C., Chiou, M.-J., Chen, J.-Y., Wu, J.-L., & Kuo, C.-M. (2008). Application of RNAi technology to the inhibition of zebrafish GtHalpha, FSHbeta, and LHbeta expression and to functional analyses. *Zoological Science*, *25*(6), 614–21. http://doi.org/10.2108/zsj.25.614

Karrer, E., & Lincoln, J. (1995). In situ isolation of mRNA from individual plant cells: creation of cell-specific cDNA libraries. *Proceedings of the National Academy of Sciences of the United States of America*, *92*(April), 3814–3818. Retrieved from http://www.pnas.org/content/92/9/3814.short

Kawakami, K., Koga, A., Hori, H., & Shima, A. (1998). Excision of the Tol2 transposable element of the medaka fish, Oryzias latipes, in zebrafish, Danio rerio. *Gene*, *225*(1-2), 17–22. http://doi.org/10.1016/S0378-1119(98)00537-X

Kikuchi, K., Holdway, J. E., Werdich, A. a, Anderson, R. M., Fang, Y., Egnaczyk, G. F., Evans, T., Macrae, C. a, Stainier, D. Y. R., & Poss, K. D. (2010). Primary contribution to zebrafish heart regeneration by gata4(+) cardiomyocytes. *Nature*, *464*(7288), 601–5. http://doi.org/10.1038/nature08804

Kok, F. O., Shin, M., Ni, C.-W., Gupta, A., Grosse, A. S., van Impel, A., Kirchmaier, B. C., Peterson-Maduro, J., Kourkoulis, G., Male, I., DeSantis, D. F., Sheppard-Tindell, S., Ebarasi, L., Betsholtz, C., Schulte-Merker, S., Wolfe, S. A., & Lawson, N. D. (2014). Reverse Genetic Screening Reveals Poor Correlation between Morpholino-Induced and Mutant Phenotypes in Zebrafish. *Developmental Cell*, 1–12. http://doi.org/10.1016/j.devcel.2014.11.018

Köprunner, M., Thisse, C., Thisse, B., & Raz, E. (2001). A zebrafish nanos-related gene is essential for the development of primordial germ cells. *Genes & Development*, *15*(21), 2877–85. http://doi.org/10.1101/gad.212401

Lasko, P. F., & Ashburner, M. (1988). The product of the Drosophila gene vasa is very similar to eukaryotic initiation factor-4A. *Nature*, *335*(6191), 611–617. Retrieved from http://dx.doi.org/10.1038/335611a0

Lee, Y., Kim, M., Han, J., Yeom, K.-H., Lee, S., Baek, S. H., & Kim, V. N. (2004). MicroRNA genes are transcribed by RNA polymerase II. *The EMBO Journal*, *23*(20), 4051–60. http://doi.org/10.1038/sj.emboj.7600385

Leong, I. U. S., Lan, C.-C., Skinner, J. R., Shelling, A. N., & Love, D. R. (2012). In vivo testing of microRNA-mediated gene knockdown in zebrafish. *Journal of Biomedicine & Biotechnology*, *2012*, 350352. http://doi.org/10.1155/2012/350352

Linder, P., & Jankowsky, E. (2011). From unwinding to clamping - the DEAD box RNA helicase family. *Nature Reviews. Molecular Cell Biology*, *12*(8), 505–16. http://doi.org/10.1038/nrm3154

Link, V., Shevchenko, A., & Heisenberg, C.-P. (2006). Proteomics of early zebrafish embryos. *BMC Developmental Biology*, *6*, 1. http://doi.org/10.1186/1471-213X-6-1

Liu, W., & Collodi, P. (2010). Zebrafish dead end possesses ATPase activity that is required for primordial germ cell development. *FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology*, *24*(8), 2641–50. http://doi.org/10.1096/fj.09-148403

Maack, G., & Segner, H. (2003). Morphological development of the gonads in zebrafish. *Journal of Fish Biology*, *62*(4), 895–906. http://doi.org/10.1046/j.1095-8649.2003.00074.x

Mishima, Y., Giraldez, A. J., Takeda, Y., Fujiwara, T., Sakamoto, H., Schier, A. F., & Inoue, K. (2007). NIH Public Access, *16*(21), 2135–2142.

Nagasawa, K., Fernandes, J. M. O., Yoshizaki, G., Miwa, M., & Babiak, I. (2013). Identification and migration of primordial germ cells in Atlantic salmon, Salmo salar: characterization of vasa, dead end, and lymphocyte antigen 75 genes. *Molecular Reproduction and Development*, *80*(2), 118–31. http://doi.org/10.1002/mrd.22142

Nasevicius, A., & Ekker, S. C. (2002). Effective targeted gene " knockdown " in zebrafish, *26*(october 2000), 2–6.

Oates, a C., Bruce, a E., & Ho, R. K. (2000). Too much interference: injection of double-stranded RNA has nonspecific effects in the zebrafish embryo. *Developmental Biology*, *224*(1), 20–8. http://doi.org/10.1006/dbio.2000.9761

Parinov, S., Kondrichin, I., Korzh, V., & Emelyanov, A. (2004). Tol2 transposon-mediated enhancer trap to identify developmentally regulated zebrafish genes in vivo. *Developmental Dynamics : An Official Publication of the American Association of Anatomists*, *231*(2), 449–59. http://doi.org/10.1002/dvdy.20157

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research*, *29*(9), 45e–45. http://doi.org/10.1093/nar/29.9.e45

Poss, K. D., Keating, M. T., & Nechiporuk, A. (2003). Tales of regeneration in zebrafish. *Developmental Dynamics*, *226*(2), 202–210. http://doi.org/10.1002/dvdy.10220

Raz, E. (2003). Primordial germ-cell development: the zebrafish perspective. *Nature Reviews. Genetics*, *4*(9), 690–700. http://doi.org/10.1038/nrg1154

Ribas, L., & Piferrer, F. (2013). The zebrafish (Danio rerio) as a model organism, with emphasis on applications for finfish aquaculture research. *Reviews in Aquaculture*, n/a–n/a. http://doi.org/10.1111/raq.12041

Roberts, J. A., Miguel-Escalada, I., Slovik, K. J., Walsh, K. T., Hadzhiev, Y., Sanges, R., Stupka, E., Marsh, E. K., Balciuniene, J., Balciunas, D., & Müller, F. (2014). Targeted transgene integration overcomes variability of position effects in zebrafish. *Development (Cambridge, England)*, *141*(3), 715–24. http://doi.org/10.1242/dev.100347

Saito, T., Fujimoto, T., Maegawa, S., Inoue, K., Tanaka, M., Arai, K., & Yamaha, E. (2006). Visualization of primordial germ cells in vivo using GFP-nos1 3'UTR mRNA. *The International Journal of Developmental Biology*, *50*(8), 691–9. http://doi.org/10.1387/ijdb.062143ts

Schulte-Merker, S., & Stainier, D. Y. R. (2014). Out with the old, in with the new: reassessing morpholino knockdowns in light of genome editing technology. *Development (Cambridge, England)*, *141*(16), 3103–4. http://doi.org/10.1242/dev.112003

Siegfried, K. R., & Nüsslein-Volhard, C. (2008). Germ line control of female sex determination in zebrafish. *Developmental Biology*, *324*(2), 277–87. http://doi.org/10.1016/j.ydbio.2008.09.025

Singh, S., Narang, A. S., & Mahato, R. I. (2011). Subcellular fate and off-target effects of siRNA, shRNA, and miRNA. *Pharmaceutical Research*, *28*(12), 2996–3015. http://doi.org/10.1007/s11095-011-0608-1

Slanchev, K., Stebler, J., de la Cueva-Méndez, G., & Raz, E. (2005). Development without germ cells: the role of the germ line in zebrafish sex differentiation. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(11), 4074–9. http://doi.org/10.1073/pnas.0407475102

Spence, R., Gerlach, G., Lawrence, C., & Smith, C. (2008). The behaviour and ecology of the zebrafish, Danio rerio. *Biological Reviews of the Cambridge Philosophical Society*, *83*(1), 13–34. http://doi.org/10.1111/j.1469-185X.2007.00030.x

Stern, H., & Zon, L. (2003). Cancer genetics and drug discovery in the zebrafish. *Nature Reviews Cancer*, *3*(July), 1–7. Retrieved from http://www.nature.com/nrc/journal/v3/n7/abs/nrc1126.html

Stuart, G., McMurray, J., & Westerfield, M. (1988). Replication, integration and stable germ-line transmission of foreign sequences injected into early zebrafish embryos. *Development*, *412*. Retrieved from http://dev.biologists.org/content/103/2/403.short

Tamura, Y., Yoshida, M., Ohnishi, Y., & Hohjoh, H. (2009). Variation of gene silencing involving endogenous microRNA in mammalian cells. *Molecular Biology Reports*, *36*(6), 1413–20. http://doi.org/10.1007/s11033-008-9330-4

Thisse, C., & Thisse, B. (2008). High-resolution in situ hybridization to whole-mount zebrafish embryos. *Nature Protocols*, *3*(1), 59–69. Retrieved from http://dx.doi.org/10.1038/nprot.2007.514

Tsuda, M., Sasaoka, Y., Kiso, M., & Abe, K. (2003). Conserved role of nanos proteins in germ cell development. *Science*, *5*. Retrieved from http://www.sciencemag.org/content/301/5637/1239.short

Uchida, D., & Yamashita, M. (2002). Oocyte apoptosis during the transition from ovary-like tissue to testes during sex differentiation of juvenile zebrafish. *Journal of Experimental Biology*, *718*(6), 711–718. Retrieved from http://jeb.biologists.org/content/205/6/711.short

Von Hofsten, J., & Olsson, P.-E. (2005). Zebrafish sex determination and differentiation: involvement of FTZ-F1 genes. *Reproductive Biology and Endocrinology : RB&E*, *3*, 63. http://doi.org/10.1186/1477-7827-3-63

Wang, X. G., Bartfai, R., Sleptsova-Freidrich, I., & Orban, L. (2007). The timing and extent of "juvenile ovary" phase are highly variable during zebrafish testis differentiation. *Journal of Fish Biology*, *70*(sa), 33–44. http://doi.org/10.1111/j.1095-8649.2007.01363.x

Weidinger, G., Stebler, J., & Slanchev, K. (2003). dead end, a Novel Vertebrate Germ Plasm Component, Is Required for Zebrafish Primordial Germ Cell Migration and Survival. *Current Biology*, *13*, 1429–1434. http://doi.org/10.1016/S

Weidinger, G., Wolke, U., & Koprunner, M. (1999). Identification of tissues and patterning events required for distinct steps in early migration of zebrafish primordial germ cells. *Development*, *5307*, 5295–5307. Retrieved from http://dev.biologists.org/content/126/23/5295.short

Westerfield, M. (2000). *The zebrafish book. A guide for the laboratory use of zebrafish (Danio rerio)* (4th editio). Eugene: University of Oregon Press.

Williams, D. W., Müller, F., Lavender, F. L., Orbán, L., & Maclean, N. (1996). High transgene activity in the yolk syncytial layer affects quantitative transient expression assays in zebrafish (Danio rerio) embryos. *Transgenic Research*, *5*(6), 433–442. http://doi.org/10.1007/BF01980208

Yeom, K.-H., Lee, Y., Han, J., Suh, M. R., & Kim, V. N. (2006). Characterization of DGCR8/Pasha, the essential cofactor for Drosha in primary miRNA processing. *Nucleic Acids Research*, *34*(16), 4622–9. http://doi.org/10.1093/nar/gkl458

Yi, R., Qin, Y., Macara, I., & Cullen, B. (2003). Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. *Genes & Development*, 3011–3016. http://doi.org/10.1101/gad.1158803.miRNA

Yoon, C., Kawakami, K., & Hopkins, N. (1997). Zebrafish vasa homologue RNA is localized to the cleavage planes of 2-and 4-cell-stage embryos and is expressed in the primordial germ cells. *Development*, *3166*, 3157–3165. Retrieved from http://dev.biologists.org/content/124/16/3157.short

Zeng, Y., Wagner, E. J., Cullen, B. R., & Carolina, N. (2002). Both Natural and Designed Micro RNAs Can Inhibit the Expression of Cognate mRNAs When Expressed in Human Cells Technique, *9*, 1327–1333.

Zhang, Y., Chen, J., Cui, X., Luo, D., Xia, H., Dai, J., Zhu, Z., & Hu, W. (2015). A controllable on-off strategy for the reproductive containment of fish. *Scientific Reports*, *5*, 7614. http://doi.org/10.1038/srep07614

Zhao, Z., Cao, Y., Li, M., & Meng, a. (2001). Double-stranded RNA injection produces nonspecific defects in zebrafish. *Developmental Biology*, *229*(1), 215–23. http://doi.org/10.1006/dbio.2000.9982