

**Vector-Mediated RNA Interference in Zebrafish: A  
Feasibility Study**

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The University of Manchester, Doctor of Philosophy (PhD)  
Vector-Mediated RNA Interference in Zebrafish: A Feasibility Study  
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## Abstract

Zebrafish are becoming an increasingly popular model organism in which to model diseases with a genetic component. Their use is hindered however, by the lack of an efficient, reliable, stable and cost-effective method to carry out reverse genetics and model diseases which arise from a loss of function of a gene. RNAi is a method of post-transcriptional gene regulation and has been widely manipulated in other systems to knockdown genes at will. This thesis therefore looks at the feasibility of vector-mediated RNAi in zebrafish by attempting to knockdown green fluorescent protein (GFP) and the Parkinson's disease-associated gene PTEN Induced Kinase 1 (PINK1).

Initial results in HEK 293 cells and in G0 animals were encouraging, however low expression of the self-reporting vector made the identification of transgenic animals difficult. To improve expression levels the vector was modified to contain a Gal4-VP16/UAS amplification cassette. Inclusion of this cassette led to increased expression and knockdown capabilities of the vector in HEK 293 cells and led to the successful identification of transgenic zebrafish. Despite high level expression however, no knockdown of GFP or PINK1 was detected in transgenic zebrafish larvae expressing the RNAi vectors out to 5 dpf. This lack of knockdown was shown to be despite the expression of the main components of the RNAi pathway and the production of customised miRNAs throughout development and across tissues. Interestingly however, in adult transgenic zebrafish 50% knockdown of PINK1 was detected in brains expressing two independent PINK1 miRNAs compared to the control miRNA and wild type zebrafish brains. This knockdown coincided with increased transcript expression of the RNAi components and increased production of customised mature miRNA in the brain compared to embryos.

In an attempt to improve vector-mediated RNAi in zebrafish, the effect of over-expression of components of the RNAi machinery, including Argonaute 2, Dicer, Drosha and Exportin 5 was assessed in zebrafish cells. Of these, only over-expression of Argonaute 2 improved knockdown in HEK 293 cells and resulted in moderate knockdown in two independent zebrafish cell lines, PAC.2 and ZFL cells. This improvement in knockdown was shown to be a result of the RNase activity of Argonaute 2 as mutation of this domain abrogated the effect of Argonaute 2 over-expression. Despite the encouraging results in zebrafish cell lines, injection of Argonaute 2 mRNA into transgenic zebrafish failed to produce knockdown, suggesting perhaps, that in zebrafish embryos other factors apart from Argonaute 2 are also limiting

Given the difficulties of vector-mediated RNAi in zebrafish, this technology is at present not a feasible approach to knocking down genes in zebrafish, at least not to an extent as to model complete loss of gene function.

## **Declaration**

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

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## Abbreviations

aa	amino acid
Ago	argonaute
ATP	adenosine triphosphate
BCA	bicinchoninic acid
bp	base-pair
BSA	bovine serum albumin
BSU	biological services unit
CT	cycle threshold
DA	dopamine
ddH <sub>2</sub> O	double distilled water
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide
dpf	days post fertilisation
ds	double-stranded
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGF	epithelial growth factor
endoND	endonuclease domain
F1, F2 etc.	family one, family two etc.
FACS	fluorescence activated cell sorting
Fwd	forward
G0	generation zero
GDP	guanosine diphosphate
GFP	green fluorescent protein
GTP	guanosine triphosphate
HEPES	N-2-hydroxyethyl piperazine-N-(2-ethanesulphonic acid)
hpf	hours post fertilisation
HRP	horse radish peroxidase
hrs	hours
KAc	potassium acetate
KCl	potassium chloride
kDa	kilo-dalton
LB	luria broth
MgCl <sub>2</sub>	magnesium chloride
min	minutes
miRNA	microRNA
miRNP	microribonucleoprotein
mRNA	messenger RNA
MS222	tricaine methane sulphonate
MZ	maternal-zygotic
NaAc	sodium acetate
NaOH	sodium hydroxide
NPC	Nuclear pore complex
nt	nucleotide
NTS	non-transgenic siblings
ORF	open-reading frame
PAGE	polyacrylamide gel electrophoresis

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PAZ	Piwi and Zilli
PCR	polymerase chain reaction
PD	Parkinson's disease
PINK1	P-TEN induced kinase 1
PKR	protein kinase R
PMSF	phenylmethanesulfonylfluoride
pol	polymerase
pre-miRNA	precursor-RNA
pri-miRNA	primary-RNA
PVDF	polyvinylidene
RBD	RNA binding domain
Rev	reverse
RFP	red fluorescent protein
RIPA	radio-immunoprecipitation assay buffer
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
RNAi	RNA interference
rpm	revolutions per minute
rt	room temperature
RT-PCR	reverse transcription PCR
RT-qPCR	Real time- quantitative PCR
SDS	sodium dodecyl sulfate
sec	seconds
siRNA	small interfering RNA
SNpc	substantia nigra pars compacta
TAE	tris acetate EDTA
TBS	tris buffered saline
TBS-T	TBS-Tween
TE	tris EDTA
u	units
UAS	upstream activating sequence
UPS	ubiquitin-proteasome system
wks	weeks
Xpo5	Exportin5



## 1 Introduction

Animal models have been extremely important in understanding biological processes involved in both normal homeostasis and disease states. The zebrafish in particular, has proved extremely valuable due to its many advantages over other model organisms such as the mouse and flies. One such advantage is the amenability of zebrafish for phenotype-guided drug discovery in high-throughput screens to identify novel therapeutic agents. A major limitation in this drug discovery process is the lack of zebrafish disease models, caused not least by an inability to effectively model diseases which result from a loss of function of a particular gene. This research therefore explores the possibility for the use of vector-mediated RNA Interference (RNAi) in zebrafish in order to stably, reliably and cost-effectively knockdown genes. It is hoped that the development of this technology will enable the generation of new disease models, and in particular I am interested in developing a Parkinson's disease (PD) model. PD is a debilitating disease which affects the dopaminergic neurons of the *substantia nigra* and for which there are currently no effective treatments. Importantly, several forms of PD are caused by loss of function of gene products including *PARK6*, which results from the loss of function of the kinase, P-TEN induced kinase 1 (PINK1). The following therefore reviews gene knockdown in zebrafish and the potential use of RNAi and discusses the aetiology and genetic basis of PD with the view to developing an RNAi-induced model.

### 1.1 Use of the Zebrafish as a Model Organism

The zebrafish is a rapidly emerging model organism in which to study embryonic development and disease. The popularity of this organism is due to the ease of production and manipulation. The high fecundity of the zebrafish means that a mating pair can produce hundreds of embryos per breeding (Amatruda *et al.*, 2002). This is accompanied by the rapid and *ex utero* development of optically transparent embryos, which makes it possible to study embryogenesis and organogenesis and to detect functional and morphological changes in internal organs without having to kill or dissect the

organism (Eisen, 1996). Furthermore, easy and affordable husbandry, together with a relatively rapid generation time of just 3 months (Amatruda *et al.*, 2002), makes the zebrafish an economically viable option. Sequencing and annotation of the zebrafish and human genomes has revealed a high degree of genetic conservation. In addition, in contrast to other invertebrate models being used zebrafish and humans also have a lot of physiological similarities; both develop extensive and complex cardiovascular, nervous, digestive, immune, endocrine and excretory systems, thus allowing the use of zebrafish to study vertebrate specific processes (Eisen, 1996). Genetic and physiological conservation between humans and zebrafish, along with their small size and high fecundity also make them ideal for use in high-throughput screening to identify potential therapeutic and/or toxic compounds for a vast array of diseases (Zon and Peterson, 2005). Perhaps most importantly however, in relation to the aims of this research, is the ease with which the zebrafish can be genetically modified to generate transgenic animals.

### **1.1.1 Transgenesis in Zebrafish**

The production of transgenic zebrafish used to be carried out simply by the injection of plasmid or linear DNA into early embryos. This was a highly inefficient means of transgenesis and often resulted in the genome insertion of concatemers of DNA which were eventually silenced through DNA methylation. In 2002, the field of transgenesis was revolutionised when the Wittbrodt lab demonstrated that flanking transgenes with a meganuclease restriction site and co-injecting into embryos along with the appropriate meganuclease significantly improved rates of transgenesis and prevented the formation of concatemers (Thermes *et al.*, 2002). Further advancements came with the discovery of an autonomous transposon in medaka (*Oryzias latipes*) called *To12* (Koga *et al.*, 1996). Functional dissection of the transposable and transposase elements of *To12* meant that it was now possible to flank transgenes with the arms of the transposable element. Upon co-injection of the transposon-flanked transgene and transposase messenger RNA (mRNA) into the host, the transgene is transposed into the

host genome. This method has substantially improved rates of transgenesis in a host of organisms including, zebrafish (Kawakami and Shima, 1999; Kawakami *et al.*, 2000), *Drosophila* (Urasaki *et al.*, 2008), xenopus (Kawakami *et al.*, 2000), chicken (Sato *et al.*, 2007) and mouse (Kawakami and Noda, 2004).

As well as the technology to efficiently produce transgenics, numerous promoter elements have been identified in the zebrafish allowing tissue-specific expression of transgenes. Furthermore, numerous conditional systems have been shown to function in zebrafish, including the heat-inducible system using the heat-shock promoter, (HSP70) (Adam *et al.*, 2000), a doxycycline-inducible system using Tet repressors and *tet* operators (Huang *et al.*, 2005), a Cre/lox recombinase system (Langenau *et al.*, 2005), and the Gal4/UAS system (Scheer and Campos-Ortega, 1999). Indeed the Gal4/UAS system combined with *To2* has been used as an enhancer trap and has provided the zebrafish community with a wealth of driver lines (Asakawa and Kawakami, 2008; Davison *et al.*, 2007; Scott *et al.*, 2007).

## 1.2 Gene knockdown

Despite the wide-spread uptake of zebrafish as a versatile and tractable model organism, there is currently no reliable and affordable technology for stable gene knockout/ knockdown in zebrafish. The section evaluates possible techniques ranging from homologous recombination and mutagenesis to transient morpholino technologies and the possibility of RNAi.

### 1.2.1 Homologous Recombination

Homologous recombination is the knockout method of choice in mice. It relies on the endogenous recombination machinery of cells to replace genes with modified versions of themselves. These modified versions can be engineered to alter or eliminate gene function, for example through the introduction of a premature stop codon, or changes in the sequence encoding the active site of a protein. In mice, homologous recombination is carried out in embryonic stem cells (Doetschman *et al.*, 1987; Doetschman *et*

*al.*, 1988) which are then transplanted into embryos and result in the development of chimeras (Thompson *et al.*, 1989). Assuming the modified ES cells contribute to the germ line, a proportion of the offspring of these mice will carry the modified gene. Alternatively, in animals where ES cells have not been identified, fibroblast donor cells can be modified and then their nuclei transferred to enucleated oocytes (Lai *et al.*, 2002; McCreath *et al.*, 2000).

In zebrafish, ES-like cells have been isolated (Sun *et al.*, 1995), transplanted and shown to contribute to the germ line (Ma *et al.*, 2001). However, the rate of transmission, at 2-4%, is extremely low. Nuclear transfer of modified nuclei has also been achieved, however with similar transmission rates (Lee *et al.*, 2002). Despite these achievements gene inactivation in zebrafish via homologous recombination is still yet to be achieved and progress in this area has been very slow.

### **1.2.2 Random Mutagenesis**

An alternative method of knocking out gene function is to randomly induce mutations in genomes and then screen for these mutations. Mutagenesis can be brought about using chemical mutagens such as the alkylating agents ethyl methanesulfonate (EMS) or ethyl-N-nitrosourea (ENU) or insertional mutagens such as transposons (Sivasubbu *et al.*, 2006) or pseudotyped retroviruses (Amsterdam and Hopkins, 2004; Golling *et al.*, 2002). Where chemical mutagens are used, mutations can be identified by a method called Targeting Induced Local Lesions in Genomes (TILLING). In this method, mutations in genes of interest are identified by amplification and direct sequencing of that gene from individual fish DNA samples (Wienholds and Plasterk, 2004; Wienholds *et al.*, 2002). Where transposons or retroviruses are involved mutations can be somewhat more easily identified. A restriction enzyme which cuts near the 5' end of the vector is used to cut up the genome, linkers are then added to the ends of fragments and linker-mediated PCR performed, where one primer is specific for the vector and the other is specific for the linker. Amplified products can then be sequenced to identify where the insertion is (Ellingsen *et al.*, 2005).

A major disadvantage to the use of random mutagenesis is the huge amount of time, space and hence money required in the generation and breeding of mutagenised zebrafish. Indeed, Amsterdam and Hopkins estimated that given the size of the zebrafish genome ( $1.6 \times 10^9$  bp), 500,000 randomly placed retroviral insertions would have to be screened to reach saturation of the genome (Amsterdam and Hopkins, 2004). Furthermore, chemical and insertional mutations can often be silent or result in incomplete loss of function.

### **1.2.3 Zinc Finger Nucleases: Targeted Mutagenesis**

Zinc Fingers are protein domains which recognise and bind specifically to 3 base pairs (bp) of DNA or RNA. The specific binding capacity of zinc fingers can be exploited and numerous zinc fingers can be arrayed together to recognise longer lengths of DNA. In addition, nuclease domains can be fused to the zinc finger arrays which enable engineered zinc fingers to act like restriction enzymes and induce double strand breaks at sequence-specific locations in the genome. Double strand breaks are then repaired by the cell's endogenous non-homologous end joining machinery. This process is highly mutagenic, often resulting in random insertions or deletions at the break site, which can result in loss of function of the targeted gene. Using this method, targeted knockout mutations have been introduced in human cells (Zou *et al.*, 2009) and in *in vivo* animal models, including *Drosophila* (Beumer *et al.*, 2006) and zebrafish (Doyon *et al.*, 2008; Foley *et al.*, 2009; Meng *et al.*, 2008).

Despite the promise of zinc fingers, there are several limitations to this technology. Firstly, there is the potential for off-target effects which may cause detrimental developmental defects preventing the use of some zinc finger combinations. Secondly, there are as yet a limited number of validated zinc fingers, so identifying unique zinc finger target sites in the target regions of zebrafish genes may limit the use of this technology. Finally, the type of mutation introduced is random and may not have the desired effect.

### 1.2.4 Morpholinos

Morpholinos are antisense oligonucleotides with a modified backbone, which makes them resistant to nucleases and therefore extremely stable. Morpholinos can be designed to function in one of two ways. Firstly, they can be designed against the translation initiation site, in which case they prevent the translation of mRNA by preventing ribosome entry through steric hindrance. Alternatively, they can be designed to target splice acceptor or splice donor sites, this either results in incorrect splicing of the mRNA or in loss of the transcript via nonsense mediated decay. Morpholinos were initially used in zebrafish to phenocopy known mutants such as *no tail*, *nacre* and *sparse* (Nasevicius and Ekker, 2000) and since then have become a widely used tool to elucidate gene function and embryogenesis in zebrafish. The use of morpholinos is limited however, as there have been numerous reports of sequence-specific and non-specific 'off-target' effects, which often lead to phenotypes such as neuronal cell death, small eyes and heads and disrupted somites and notochord (Ekker and Larson, 2001). This makes deciphering what are on-target and off-target effects difficult. Another major limitation is the transient nature of morpholinos, which means that they cannot be used to study development and gene function past the first few days of embryogenesis.

### 1.2.5 RNA Interference (RNAi)

The recent discovery of RNAi as an innate and potent method of sequence-specific gene knockdown in organisms ranging from plants and fungi to worms, flies, mice and humans, combined with the ease in which it can be manipulated to target genes of interest, has led to the rapid uptake of RNAi as a general lab technique to knockdown genes. The presence of functional RNAi machinery in zebrafish combined with the possibilities of vector-mediated delivery of RNAi, means that this technique may hold the key to achieving efficient, stable and cost-effective gene knockdown in zebrafish. The following section discusses the RNAi pathway in detail and how it might be used in zebrafish to successfully target genes for knockdown.

### 1.3 Gene Knockdown by RNAi

RNAi is an evolutionary conserved pathway of post-transcriptional gene regulation, in which small RNA effector molecules sequence-specifically regulate gene expression. The small RNA effector molecules can either be endogenously expressed microRNAs (miRNAs) or exogenous small interfering RNAs (siRNAs).

#### 1.3.1 Micro RNAs (miRNAs) and Small Interfering RNAs (siRNAs)

miRNAs are small non-coding RNA molecules which post-transcriptionally regulate gene expression by base-pairing to mRNAs and either blocking the translation of the mRNA or causing its destabilisation and degradation. The first miRNA gene to be identified was *lin-4* in *Caenorhabditis elegans* (Lee *et al.*, 1993). *lin-4* encodes a miRNA which was identified as a translational suppressor of the *lin-14* gene, whose down regulation is required for progression of *C. elegans* from the first to second larval stage, and later *lin-28*, a protein which initiates the transition between the second and third larval stage (Moss *et al.*, 1997). *lin-4* regulation of *lin-14* and *lin-28* was shown to be dependent on the imperfect base pairing between the *lin-4* miRNA and the 3' Un-Translated Regions (UTRs) of *lin-14* and *lin-28* which blocked the translation of the mRNAs (Ha *et al.*, 1996; Lee *et al.*, 1993; Moss *et al.*, 1997; Olsen and Ambros, 1999; Wightman *et al.*, 1993). Initially, this type of post-transcriptional gene regulation was believed to be an oddity of *C. elegans* until a second miRNA was identified. This second miRNA was *let-7* which regulates in the same way the translation of *lin-41* mRNA (Reinhart *et al.*, 2000; Vella *et al.*, 2004). The importance of this discovery was the realisation of the evolutionary conservation of both *let-7* and *lin-41* among metazoans, raising the possibility that this method of gene regulation may be more far reaching than previously thought (Pasquinelli *et al.*, 2000). Today over 14,000 validated miRNA precursors have been identified across 133 distinct species ranging from fungi to mammals (Griffiths-Jones, 2004; Griffiths-Jones *et al.*, 2006; Griffiths-Jones *et al.*, 2008). The human genome itself has been shown to contain over 900 miRNA precursors and miRNAs are believed to regulate up to 30% of the genome (Lewis *et al.*, 2005).

Concurrently with the identification of miRNAs, it was realised by chance through knockdown experiments using sense and antisense RNAs, that the exposure of *C. elegans* to double-stranded (ds)RNA caused potent and sequence-specific gene silencing through binding to mRNA (Fire *et al.*, 1998) and inducing its cleavage (Montgomery *et al.*, 1998). In 2001, it was realised that dsRNA was being processed *in vivo* to form small dsRNA of between 21-25 nucleotides (nt) in length which closely resemble endogenous miRNAs and that these siRNAs were the mediators of silencing (Elbashir *et al.*, 2001b). Furthermore, the mechanism is also conserved, as this form of silencing is common to plants, fungi, worms and flies, and might be an endogenous defence mechanism against viral infection or parasitic nucleic acids (Hannon, 2002).

Although not appreciated at first, analysis of the mechanisms of these two methods of gene silencing proved that they both make use of the same pathway, which is described below.

### **1.3.2 The miRNA/RNAi Pathway: An Overview**

The effector molecules of both siRNA- and miRNA-induced silencing are ~21-25 nt dsRNA with 5' phosphate groups, 3' hydroxyl groups and asymmetrical 3' 2 nt overhangs (Elbashir *et al.*, 2001b), however they differ in their modes of biogenesis.

miRNAs are transcribed in the nucleus either as genes in their own right, or embedded within protein-coding and non-coding host genes, in which case their expression is regulated by the promoter elements of the host. Often, they are found clustered together in polycistronic transcripts transcribed from the same promoter region and may contain representatives of distinct miRNA families, indicating the coordinated regulation of expression of numerous miRNAs (Griffiths-Jones *et al.*, 2008; Thatcher *et al.*, 2008a). During transcription, the primary or pri-miRNA transcript, which consists of a double stranded stem, a terminal loop and flanking ssRNA is cleaved by a microprocessor complex. This microprocessor complex consists of the RNaseIII-like enzyme Drosha and cofactors, including DGCR8 (DiGeorge Syndrome Critical Region 8, also known as Pasha in flies

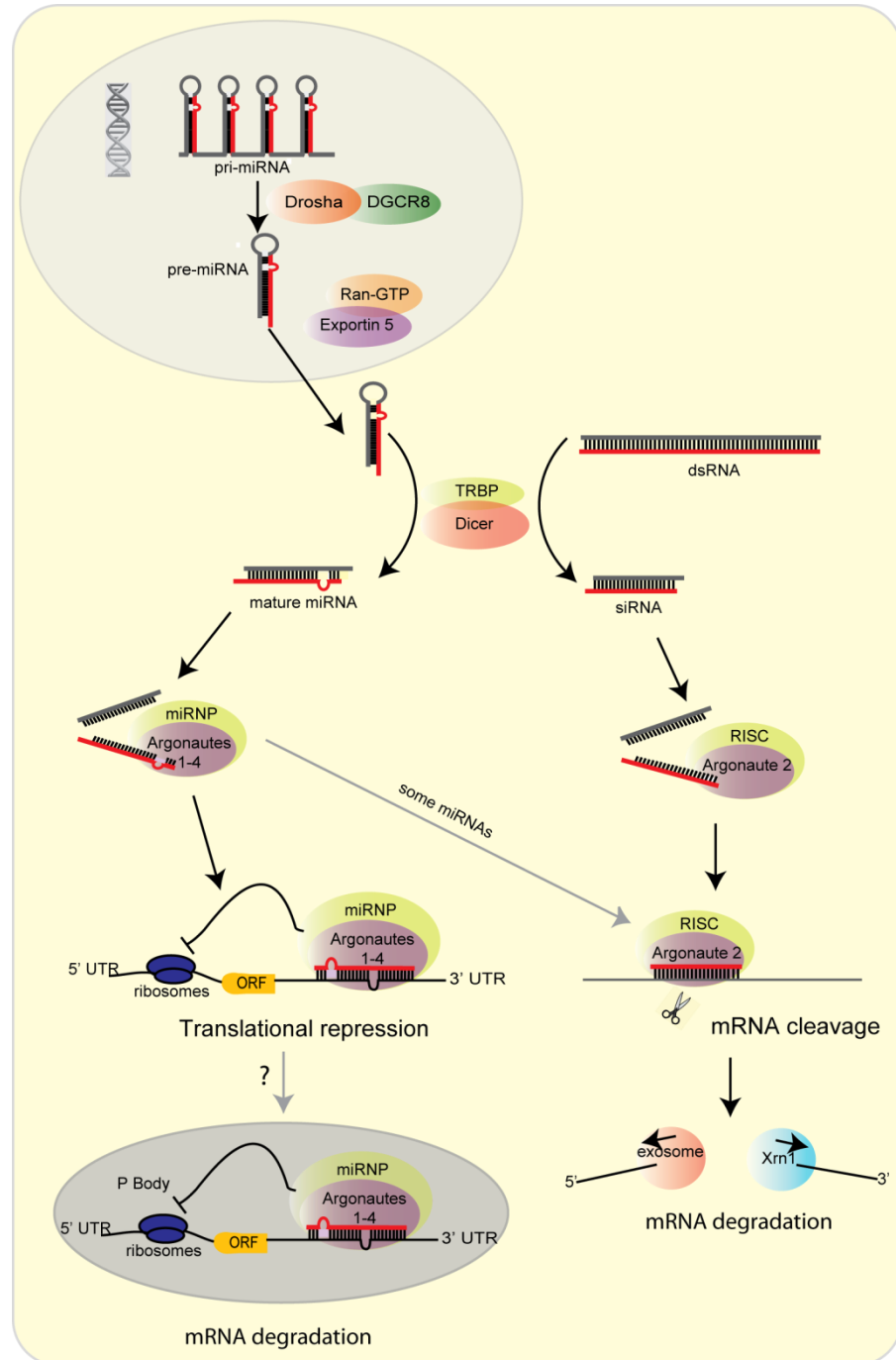


and worms). Cleavage of pri-miRNAs by the microprocessor creates ~70 nt stem-loop structures termed precursor, or pre-miRNAs which have 5' phosphate groups, 3' hydroxyl groups and 2 nt overhangs at their 3' ends (Figure 1.1) (Denli *et al.*, 2004; Gregory *et al.*, 2004; Han *et al.*, 2004; Landthaler *et al.*, 2004; Lee *et al.*, 2003). Pre-miRNA is then transported out of the nucleus into the cytoplasm by a nuclear transport receptor complex, Exportin 5 (Xpo5)-RanGTP (Bohnsack *et al.*, 2004; Lund *et al.*, 2004; Yi *et al.*, 2003). The loop structure is then removed via cleavage by the cytoplasmic RNaseIII-like endonuclease, Dicer to form a ~ 22 nt miRNA duplex with 5' phosphate groups and asymmetrical 2 nt 3' overhangs. This processing also occurs within a microprocessor complex which also contains the human immunodeficiency virus transactivating response RNA-binding protein (TRBP) which stabilises the interaction of Dicer with pre-miRNA through its three dsRNA binding domains. Processing by Dicer is coupled with the preferential assembly of one strand, the guide strand, into the effector complex, known as the RNA-induced silencing complex (RISC) or the micro ribonucleoprotein (miRNP), while the other strand, the passenger strand, is degraded (Bartel, 2004).

Once loaded into the silencing complex, the miRNA acts on its target either by binding through a seed region (usually nucleotides 2-8) to its 3' UTR and causing translational repression or mRNA destabilisation or by binding with complete complementarity to the mRNA and inducing cleavage of the mRNA (Figure 1.1). In the majority of cases, endogenous miRNAs function by binding through a seed region to the 3' UTRs of their targets. The exception to this is the miRNA *miR-196* which has been shown to bind with almost complete complementarity to the Hoxb8 mRNA and cause its cleavage in mouse embryos and in cell culture (Yekta *et al.*, 2004).

Small interfering RNAs (siRNAs) can either be chemically synthesised or are generated from an exogenous source of dsRNA (e.g. from viral infection). In the case of the latter, Dicer cuts the dsRNA up randomly into small double-stranded 21-25 nt siRNAs. These siRNA duplexes then enter into the same pathway as described above (Figure 1.1). However, in contrast to miRNAs the majority of siRNAs induce gene silencing by binding

to a target with complete complementarity, resulting in the cleavage of the mRNA. Despite this distinction, endogenous miRNAs have been shown to be able to induce cleavage of mRNA containing completely complementary target sequences (Zeng *et al.*, 2003) and conversely, when siRNAs imperfectly complement the 3' UTR of their target, translational repression or mRNA destabilisation may ensue (Doench *et al.*, 2003; Zeng *et al.*, 2003).

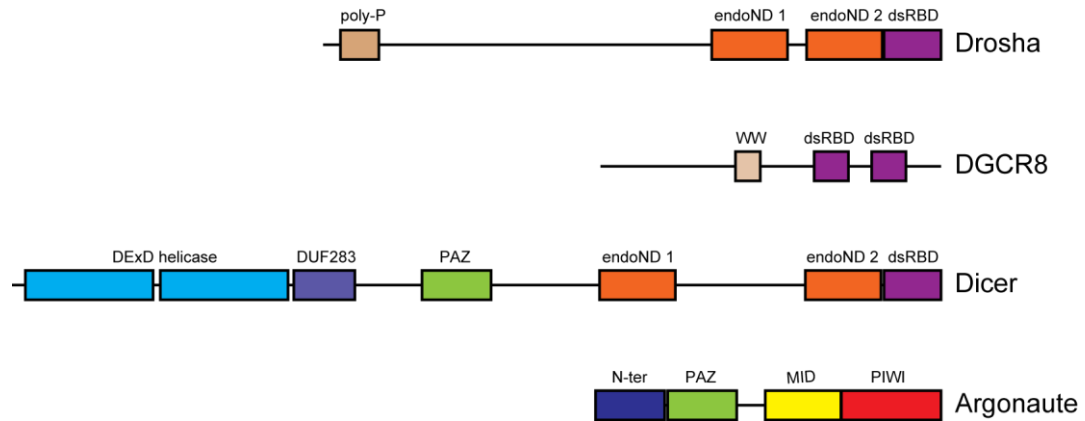


**Figure 1.1: Biogenesis of and Post-transcriptional Suppression by microRNAs and small interfering (si)RNAs.** The nascent primary miRNA (pri-miRNA) transcripts are first processed into ~70-nucleotide precursor miRNAs (pre-miRNAs) by Drosha inside the nucleus. Pre-miRNAs are transported to the cytoplasm by Xpo5-Ran-GTP and are processed into mature miRNA duplexes by Dicer. Dicer also processes long dsRNA molecules into siRNA duplexes. One strand of the miRNA or siRNA duplex is preferentially assembled into the RNA-induced silencing complex (RISC) or microribonucleoprotein (miRNP), which subsequently acts on its target by binding through a seed region in the 3'UTR and causing translational repression or mRNA destabilisation or, by binding with complete complementarity and cleaving the mRNA.

### 1.3.3 RNAi Processes in Depth

#### 1.3.3.1 Drosha Processing

Drosha is a class II RNase III-like enzyme which consists of two tandem endonuclease domains (endoND), and a double stranded RNA binding domain (dsRBD) (Figure 1.2). The two tandem endonuclease domains fold back on each other and create a pseudo-dimer catalytic core in which the first and second endonuclease domains independently cut the dsRNA in a staggered manner and produce 3' 2-nt overhangs (Han *et al.*, 2004). In order for this to happen in the correct place, Drosha needs to recognise and orientate itself properly along the stem loop structure. Originally, it was proposed that Drosha itself may recognise the terminal loop structure and unstructured ssRNA flanking the stem loop (Zeng *et al.*, 2005b). However, more recent evidence suggests that recognition of primary miRNA transcripts is dependent on binding of the co-factor DGCR8, a protein containing two dsRBDs, to the ssRNA/dsRNA junction at the base of the stem loop. Once DGCR8 is bound to the ssRNA/dsRNA junction Drosha then orientates itself along the stem loop so that the catalytic domain cleaves ~ 11bp from the flanking ssRNA (Han *et al.*, 2006). Indeed, both Drosha and DGCR8 are essential (Denli *et al.*, 2004; Gregory *et al.*, 2004; Han *et al.*, 2004) and sufficient for processing of primary miRNA transcripts (Han *et al.*, 2004).



**Figure 1.2: Domain Composition of Components of Animal RNAi.** Double stranded RNA binding domains (dsRBDs) bind to pri- and pre- miRNAs. Endonuclease domains (endoND) form intermolecular dimers which cleave pri- and pre- miRNAs. PAZ domains bind to 3' overhangs of mature miRNAs. DExD helicase modifies the catalytic activity of Dicer. PIWI domains contain an RNase H-like fold which in the case of Ago2 cleaves target mRNA in a sequence-specific manner. MID domain binds to the 5' phosphorylated ends of guide strand miRNAs. Modified from Nowotny and Yang (2009).

### 1.3.3.2 Export from the Nucleus

After processing by Drosha, pre-miRNA is exported from the nucleus. Export from the nucleus is carried out by the karyopherin  $\beta$  transporter, Exportin 5 (Xpo5) in a RanGTP dependent manner (Bohnsack et al., 2004; Lund et al., 2004; Yi et al., 2003). Binding of the RanGTP•Xpo5 complex to pre-miRNA is sequence independent, but requires a terminal double stranded 'minihelix' of  $\geq 14$  bp, a based paired 5' end and 3' overhang of  $\leq 3$  nt (Gwizdek et al., 2003). Once bound the RanGTP•Xpo5•pre-miRNA complex is translocated through the nuclear pore complex (NPC) into the cytoplasm. In the cytoplasm RanGTP is hydrolysed to RanGDP, which in turn causes the release of the pre-miRNA. Once the pre-miRNA is released exportin 5 is transported back into the nucleus and the process begins again.

### 1.3.3.3 Dicing and Loading

Once transported to the cytoplasm pre-miRNA is further processed by Dicer to form mature miRNA duplexes. Dicer is a class III RNaseIII-like endonuclease which contains two endoND domains, a dsRBD, a PAZ domain and two DExD helicase domains (Figure 1.2). Dicer binds pre-miRNAs through its dsRBD and the PAZ domain. The dsRBD recognises

dsRNA without sequence-specificity, whereas the PAZ domain specifically binds to the 3' ends of ssRNA or to the protruding 3' 2 nt overhang generated by Drosha processing. RNA cleavage takes place in a mechanism similar to Drosha cleavage, in which the two tandem endonucleolytic domains fold back on themselves to create a catalytic core. The site of Dicer cleavage is determined by the distance between the PAZ domain, bound to the 3' end of the stem loop, and the active endonucleolytic site (Zhang *et al.*, 2004). Like Drosha processing, Dicer processing also results in 3' 2 nt overhangs. The DExD helicase domain has a dual role. Firstly, it acts as an autonomous inhibitor of catalytic Dicer activity, as removal of the DExD helicase domain enhances catalytic activity. Secondly, it is required for the association between Dicer and co-factors such as TRBP. Notably, increasing availability of TRBP also enhances the catalytic activity of full-length Dicer, but not of Dicer with the DExD helicase domain removed. This suggests that the binding of the DExD helicase domain to Dicer co-factors acts as a molecular switch between low and high catalytic activity (Deddouche *et al.*, 2008).

After Dicer cleavage one strand of the miRNA/siRNA duplex is preferentially loaded into RISC. Guide strand selection is believed to be dependent on the thermodynamic stability at the ends of the dsRNA duplex, with the strand with the lower stability at the 5' end being preferentially selected for loading into RISC (Khvorova *et al.*, 2003; Schwarz *et al.*, 2003). In *Drosophila*, guide strand selection was shown to be dependent on the orientation of the Dicer-2 RNA binding partner R2D2 which binds to the 5' end of the most thermodynamically stable strand and prevents its assembly into RISC (Tomari *et al.*, 2004). Recently, TRBP, which had already been shown to recruit Argonaute 2 to siRNAs (Chendrimada *et al.*, 2005), has been shown to be the possible human equivalent of R2D2 (Gredell *et al.*, 2010). However, detection of 5'-thermodynamically stable miRNA strands in some tissues and tissue-specific differences in strand selection, suggests that strand selection may be more complex and be dependent on further signals outside of the miRNA duplex sequence and perhaps also on expression of other factors (Hu *et al.*, 2009; Ro *et al.*, 2007).

#### 1.3.3.4 RNA Induced Silencing Complexes: translational repression, mRNA destabilisation or mRNA degradation

The major component of RISC is a member of the argonaute (Ago) family of proteins. Ago proteins are defined by the presence of PAZ, Mid and PIWI domains (Figure 1.2). PAZ domains recognise and bind to the single stranded 3' ends of miRNAs/siRNAs (Song *et al.*, 2003) while the Mid domain recognises the phosphate at the 5' end of the miRNA/siRNA (Nowotny and Yang, 2009). The PIWI domain encodes the endonuclease activity of argonaute and is structurally similar to the RNase H family of proteins, which cleave RNA in RNA/DNA hybrids (Liu *et al.*, 2004; Song *et al.*, 2004). In mammals there are four argonaute proteins Ago1-Ago4 and four argonaute-related proteins, called PIWIs which are restricted in expression to the germ cells. Although, Ago proteins are the only component to be continuously found in RISC, numerous accessory proteins have been shown to associate, including RNA helicases such as RNA helicase A (Robb and Rana, 2007), MOV10 (Meister *et al.*, 2005) gemin 3 and gemin 4 (Mourelatos *et al.*, 2002), nucleases such as the staphylococcal nuclease Tudor-SN (Caudy *et al.*, 2003) and RNA-binding proteins such as fragile X-related protein (dFXR) (Caudy *et al.*, 2002), VIG (Caudy *et al.*, 2002), TNRC6B/KIAA1093 (Meister *et al.*, 2005), TRBP (Chendrimada *et al.*, 2005), PACT (Lee *et al.*, 2006) and GW182 (Liu *et al.*, 2005a). The vast array of accessory proteins means that there may be a huge number of combinatorial RISC complexes with distinct functions.

##### 1.3.3.4.1 Translation Repression and mRNA Destabilisation

The majority of endogenous silencing complexes loaded with miRNA carry out gene regulation through translational repression or destabilisation of the target mRNA. All four mammalian Ago proteins have been shown to induce translation repression/mRNA destabilisation by binding of single or multiple miRNAs through a seed region (usually bases 2-8) to the 3' UTR of the target. As yet the precise mechanisms by which this takes place are not fully understood, but may be down to one or a combination of the following mechanisms:

- Prevention of competent ribosome assembly and hence translation. This prevention is possibly mediated through association of RISC with EIF6, which has been shown to prevent the association between 40S and 60S ribosomal subunits and hence prevent the assembly of the 80S competent ribosome (Chendrimada *et al.*, 2007; Thermann and Hentze, 2007).
- Repression of the formation and binding of translation initiation complexes to the m7G cap of mRNAs (Humphreys *et al.*, 2005; Mathonnet *et al.*, 2007). Indeed, Ago2 was found to bind to m7G cap and competes with the translation initiation factor eIF4E for binding to the cap (Kiriakidou *et al.*, 2007).
- Interaction of RISC with active ribosomes, rendering them prone to premature termination (Petersen *et al.*, 2006).
- Induction of deadenylation of target mRNA, which subsequently leads to mRNA destabilisation (Bagga *et al.*, 2005; Behm-Ansmant *et al.*, 2006; Giraldez *et al.*, 2006; Wu *et al.*, 2006). Consistent with this is the finding that miRISCs have been shown to co-localise with Processing bodies (P-bodies), granules which contain the enzymes necessary for mRNA degradation, such as deadenylases, decapases and exonucleases (Liu *et al.*, 2005b; Sen and Blau, 2005).

Methods of miRNA mediated repression of target genes are reviewed in Filipowicz (2008).

#### 1.3.3.4.2 mRNA Cleavage

mRNA cleavage is dependent on complete (or almost complete) complementarity between the miRNA/siRNA and the target mRNA. Unlike in translational repression or mRNA destabilisation only one mammalian Ago protein, Ago2 is able to induce silencing via mRNA cleavage. Cleavage is mediated through the PIWI domain of Ago2. The PIWI domain in Ago2 contains a unique RNase catalytic domain consisting of two aspartates (D) and a Histidine (H) (DDH) at positions 597, 669 and 807 (relative to human Ago2) respectively (Rivas *et al.*, 2005). These three active sites are conserved in Ago2 proteins (or equivalents) in all species including *Drosophila*, zebrafish, mice and humans. The DDH motif however is also



found in the mammalian Ago3 protein, however other residues around the active site are not conserved and mutation of the non-conserved residues of Ago2 to those residues found in Ago3 have been shown to inactivate the RNase activity of Ago2 (Liu *et al.*, 2004). That Ago2 is the protein responsible for RNAi mediated knockdown by siRNAs targeted to the open-reading frame (ORF) has been confirmed by the finding that knockout of Ago2 renders mouse embryo fibroblasts (MEFs) incapable of gene knockdown in response to siRNAs (Liu *et al.*, 2004). As stated previously, cleavage of the mRNA is dependent on sequence complementarity, but especially important is complementarity at position 10 and 11 where mRNA cleavage takes place. A single mismatch at either of these two positions completely disrupts mRNA cleavage.

#### **1.3.4 Delivering Targeted Gene Knockdown using RNAi**

The discovery of RNAi in *C. elegans* meant that it was now possible to knockdown target genes in a sequence-specific manner. The next major hurdle was how to deliver the siRNA effector molecules of RNAi. While organisms such as *C. elegans* and *Drosophila melanogaster* (Kennerdell and Carthew, 1998) responded as expected to injection or ingestion of dsRNA, its use in mammalian systems was hampered by the fact that long dsRNAs elicit an immune response. During this immune response, interferon activates RNaseL and Protein Kinase R (PKR). Activation of RNaseL causes degradation of several RNA species including ribosomal RNA, while Protein Kinase R (PKR) is a dsRNA-dependent protein kinase which blocks protein synthesis through the phosphorylation of the translation initiation factor eIF2 $\alpha$  (Stark *et al.*, 1998; Williams, 1997). Fortunately, it was soon realised that delivery of short, chemically synthesised dsRNAs which resemble Dicer-processed siRNAs do not elicit such an immune response and are able to effectively knockdown targets via mRNA cleavage in both invertebrate and vertebrate systems, including human cells (Caplen *et al.*, 2001; Elbashir *et al.*, 2001a). Since then, the use of chemically synthesised siRNAs to knockdown genes in mammalian cells and animals has been a wide-spread reverse genetic tool in elucidating and manipulating gene function. Despite

the wide-spread use of chemically synthesised siRNAs there are major limitations to their use. Unlike in plants (Dalmay *et al.*, 2000; Mourrain *et al.*, 2000), fungi (Cogoni and Macino, 1999) and worms (Sijen *et al.*, 2001; Smardon *et al.*, 2000) where RNAi is long lived due to the amplification of siRNAs by RNA-dependent RNA polymerases, mammals, flies and fish have no such polymerase, and so siRNA mediated gene knockdown is only transient. This makes the use of siRNAs at best expensive and at worst impossible in situations where gene knockdown should be maintained. To overcome this, vector-mediated mechanisms for stably producing siRNAs have been developed. These vectors fall into two major categories. The first are polymerase III- (pol III-) responsive vectors, and the second are pol II-responsive vectors.

#### 1.3.4.1 Pol III-responsive Vectors

Pol III-responsive vectors utilise polymerase III promoters, usually U6 or H1 promoters to drive expression of short hairpin (sh)RNAs. Pol III-responsive promoters are short, well defined enhancers which drive expression of short RNAs which lack polyA tails and instead transcription is terminated by a run of four or more Thymidine (T) residues. Importantly, cleavage of the transcribed RNA occurs after the second in a run of uridines. By inserting the sense and antisense sequence of the target mRNA separated by a spacer downstream of a pol III promoter a transcript which folds back on itself to form a short hairpin with 3' 2 nt overhangs is produced. These shRNAs resemble the short stem-loop structures of pre-miRNA, and so enter into the miRNA processing pathway and are cut by Dicer to release a ~ 21nt siRNA. This system was first successfully used by Brummelkamp *et al.* (2002) to silence E-cadherin and p53 in the breast cancer cell line MCF-7. Since then, they have been widely and successfully used by many laboratories. Despite their ability to successfully induce silencing of targeted genes, there are limitations in the use of pol III-responsive promoters. Firstly, it is not possible to identify when and where the shRNA is being expressed by means of a tandem-expressed marker protein such as green fluorescent protein (GFP). Secondly, spatial and temporal specificity of

shRNA expression, which may be required when using vector-mediated RNAi in organisms, cannot be easily controlled using pol III-responsive vectors. Despite this, conditional pol III-based RNAi systems have been described. One such system is created through the insertion of a tet operator (*tetO*) just upstream of the transcription start site. TetO interferes with the TATA box through the recruitment of the exogenously expressed tetracycline repressor (*tetR*). Upon addition of doxycycline the *tetR* is sequestered and transcription of the shRNA is enabled. This system was used by Hoeflich *et al.*, (2006) to conditionally knockdown BRAF in a mouse tumour xenograft model. However, this system does not guarantee complete suppression of the shRNA. In an improvement upon this system fusion of the *tetR* to the Krüppel-associated box (KRAB) protein has been shown to result in stringent silencing of the gene downstream of the *tetO* through the formation of heterochromatin (Wiznerowicz and Trono, 2003). Finally, conditional knockdown systems have been created through the insertion of *loxP* sites within the shRNA vector. In this system, tissue-specific or temporal expression of Cre results in the excision of DNA between the *loxP* sites. In this way vectors have been designed so that shRNA can be expressed either in the presence or absence of Cre (Garcia-Otin and Guillou, 2006).

#### 1.3.4.2 Pol II-responsive Vectors

Pol II-responsive vectors utilise polymerase II promoters to deliver miRNAs. An effective method of pol II-responsive expression of customised miRNAs has been described Cullen and colleagues (Zeng *et al.*, 2005a; Zeng *et al.*, 2002). In this system, pol II promoters are used to transcribe a naturally occurring human primary miRNA transcript, in this case hsa-miR-30a, but with the target sequence modified to target a gene of interest. Use of an endogenous primary miRNA backbone ensures the correct Drosha and Dicer processing of the customised miRNA. It also means that by embedding the primary miRNA sequence within an intron, miRNA and a reporter gene can be co-expressed from the same promoter. The vast array of pol II promoters also means that the expression of the miRNA could be controlled in a tissue-

specific and/or temporal manner. Finally, multiple customised miRNAs, targeting the same or distinct genes can be embedded within one polycistronic transcript (Zeng *et al.*, 2005a)

### **1.3.5 RNAi in Zebrafish**

Zebrafish have been shown to have fully functional RNAi machinery. Homologs of both mammalian *Dicer* and *Drosha* have been identified in zebrafish and a putative *exportin 5* and 4 putative *argonaute* genes have been found with a high degree of conservation to their mammalian counterparts. The most recent count of cloned and validated miRNAs in zebrafish puts the total number of miRNAs expressed at 217 (Soares *et al.*, 2009), and inclusion of bioinformatically predicted miRNAs takes the total number of miRNAs to over 400 (Thatcher *et al.*, 2008a). They have been shown to be involved in numerous biological processes (Dore *et al.*, 2008; Pase *et al.*, 2009; Thatcher *et al.*, 2008b; Woltering and Durston, 2008; Yin *et al.*, 2008), expressed in a wide range of tissues (Flynt *et al.*, 2007; Giraldez *et al.*, 2005; Kapsimali *et al.*, 2007; Kloosterman *et al.*, 2007; Mishima *et al.*, 2009; Zeng *et al.*, 2009) and are present at a variety of developmental time points (Begemann, 2008; Kloosterman and Plasterk, 2006). Indeed the role of miRNAs has been shown to be extremely important in zebrafish development. Disruption of *Dicer* by TILLING leads to growth arrest at 8 dpf and lethality between 14 and 15 days. Morpholinos targeting *Dicer* induce a more severe phenotype and growth arrest occurs at about 4 dpf. The difference in severity here is down to the contribution of maternal mRNA in the mutant fish which is inhibited in the morpholino knockdown studies (Wienholds *et al.*, 2003). In a further study, maternal-zygotic (MZ) *Dicer* mutants were generated by a germ line replacement technique to ensure no maternal contribution of *Dicer* mRNA or protein. These fish all underwent axis formation and differentiation of multiple cell types, however they all displayed abnormal morphogenesis during gastrulation, brain formation, somitogenesis and heart development (Giraldez *et al.*, 2005). Thus miRNAs play an essential role in the normal development of zebrafish. In particular the miRNA miR-430 was shown to be essential in normal zebrafish development, as injection of *Dicer* processed

miR-430, a miRNA which promotes mRNA deadenylation and clearance of maternal mRNAs, into *MZDicer* mutants rescued many of the associated brain and gastrulation defects (Giraldez *et al.*, 2005; Giraldez *et al.*, 2006). Furthermore, endogenous miRNAs were shown to be able to silence exogenous GFP with specific miRNA target sites in its 3' UTR (Giraldez *et al.*, 2005; Kloosterman *et al.*, 2004) and in the ORF and 5' UTR (Kloosterman *et al.*, 2004). However, despite the importance of miRNAs during zebrafish development, the production of *MZDicer* mutant embryos demonstrates that miRNAs and functional RNAi machinery are not required for germ line formation.

The first targeted RNAi-mediated gene knockdown in zebrafish was demonstrated through injection of dsRNA into embryos. This technique was successfully used by Wargelius *et al.*, (1999) to knockdown the zebrafish gene *no tail (ntl)*, *flathead (flh)* and *pax2.1/no isthmus (noi)*, by Li *et al.*, (2000) to knockdown *ntl*, *pax.6.1* and exogenous GFP, and by Acosta *et al.*, (2005) to knockdown *myostatin (mstn)*. Li *et al.*, (2000) and Acosta *et al.*, (2005) both reported highly efficient levels of knockdown, with little or no off-target effects. In contrast, Wargelius *et al.*, (1999) despite achieving good knockdown also reported a high level of off-target defects, that were present in all injected fish regardless of the dsRNA sequence injected. Studies by Oates *et al.*,(2000), Zhao *et al.*, (2001) and Mangos *et al.*, (2001) went further, suggesting that they found no evidence of specific gene silencing, but a more global knockdown of mRNA most likely attributable to an interferon response. As a result the use of dsRNA as a technique to study gene knockdown in zebrafish has not been widely adopted.

Chemically synthesised siRNAs were first used in zebrafish by Dodd *et al.*, (2004) to knockdown the zebrafish *Duchenne muscular dystrophy (dmd)* gene. Efficient knockdown of dystrophin was detected via qPCR analysis and injected embryos exhibited delayed development of the myotubules resulting in a disruption to the somites, characteristic of muscular dystrophy. In agreement with Dodd *et al.*, (2004), Kloosterman *et al.*, (2004)Liu *et al.*, (2005c), Chang and Nie, (2008) and Blidner *et al.*, (2008) have also reported specific knockdown of target genes using siRNAs in

zebrafish. However, work by Gruber *et al.*, (2005) suggested that although siRNAs could induce specific gene silencing in zebrafish cell lines when injected into zebrafish embryos they resulted in morphological defects, abnormal development and early death, similar to the morphological defects seen with the injection of dsRNA. This may suggest that siRNAs like dsRNAs are able to induce an interferon response resulting in non-sequence-specific defects. Alternatively, it is possible that high levels of exogenous siRNA compete with endogenous miRNAs for components of the RNAi machinery, thus titering out the activity of essential endogenous miRNAs. This hypothesis is supported by Zhao *et al.*, (2008), who demonstrated that injection of siRNAs leads to a reduction in levels of processed miRNAs. In particular, they showed reduced levels of miR-430. Co-injection of pre-processed miR-430 with siRNAs was shown to significantly reduce the off-targets effects, suggesting that injection of siRNA results in non-specific defects in zebrafish embryos due to a titering out of the miR-430.

As well as concerns over off-target and toxic effects of siRNAs, a major limitation to the use of siRNA in zebrafish embryos is the brief temporal manner in which they can be used. Clearly, if RNAi is to be used as an effective tool to target genes in zebrafish, a more permanent means of targeted gene knockdown is required. To this end I propose the use of a pol II vector-mediated delivery strategy of RNAi, based on the hsa-miR-30a system established by Zeng *et al.* (2005a). It is hoped that such a delivery strategy which more closely mimics the natural production of miRNAs will effectively knockdown target genes without producing the unwanted off-target effects. Expression of the RNAi transgene will only occur with the onset of zygotic transcription and hence it is hoped that detrimental defects shown to result from reduced early processing of miR-430 due to competition with siRNAs will be eliminated. Indeed, since embarking on this project it has been demonstrated that single-cell injection of constructs expressing shRNAs under the control of both pol III (Wang *et al.*, 2007) and pol II (Su *et al.*, 2008) promoters are capable of silencing exogenous GFP and zebrafish

*ntl* in zebrafish embryos. Importantly however, neither paper demonstrated heritable transmission of targeted gene knockdown.

This research therefore aims to demonstrate heritable vector-mediated targeted gene knockdown in zebrafish. Proof of principle will be sought by knocking down GFP in zebrafish embryos. The kinase, P-TEN Induced Kinase 1 (PINK1), a gene whose loss of function is associated with Parkinson's disease, will then be targeted in the hope of generating a novel Parkinson's disease model.

## 1.4 Parkinson's Disease

Parkinson's disease (PD) is the second most prevalent neurodegenerative disease after Alzheimer's in the western world. The pathological hallmark of the disease is the progressive loss of dopaminergic neurons from a region of the midbrain called the *substantia nigra pars compacta* (SNpc), commonly accompanied by the development of proteinacious neuronal inclusions called Lewy bodies.

Normal motor function is dependent on the regulated synthesis and release of dopamine (DA) by the neurons projecting from the SNpc to the striatum (Chase *et al.*, 1998). Damage and loss of these neurons leads to a reduction in dopamine present in the striatum and ensuing motor impairment. Motor impairments associated with PD, including bradykinesia, tremor, rigidity and postural impairments, become apparent when about 70% of the striatal DA and 50% of the nigral dopaminergic neurons are lost (Dunnett and Bjorklund, 1999). In addition, patients also suffer from non-motor symptoms including, olfactory impairments, gastrointestinal dysfunction, depression, sleep disturbances, and cognitive impairment. These non-motor symptoms however, are likely related to alterations in brain regions other than the SNpc and usually occur at later stages of disease progression.

Currently the treatments for PD focus on relieving the motor symptoms, most commonly by administering Levodopa (L-dopa), a metabolic precursor of dopamine, to replace that lost through the death of the dopaminergic neurons. The beneficial effects of this therapy are limited, as

side-effects such as the onset of L-dopa-induced dyskinesias become apparent after 3-5 years treatment (Nutt, 2001). No treatments to date however, can stop or slow the disease progression by preventing the loss of dopaminergic neurons. In order to identify possible therapeutic compounds animal models which adequately mimic the complex nature of the disease are of great urgency.

### **1.4.1 The Aetiology of Parkinson's Disease**

Current understanding of the mechanistic basis for the loss of dopaminergic neurons in Parkinson's disease centres around three distinct but interrelated mechanisms. These are oxidative stress, mitochondrial dysfunction and dysfunction of the ubiquitin-proteasome system (UPS).

#### 1.4.1.1 Oxidative Stress

Oxidative stress is caused by the unregulated production of reactive oxygen species (ROS), such as hydrogen peroxide, nitric oxide and highly reactive hydroxyl radicals. Excessive generation of ROS leads to deregulation of intracellular calcium signalling and eventually cell death. The brain has a very high oxygen consumption and therefore propensity to generate these ROS, but a relatively low level of antioxidants and so is susceptible to oxidative damage. Dopamine production and transport by the dopaminergic neurons of the SNpc can result in the production of vast quantities of ROS, making these cells even more susceptible and eventually leading to selective dopaminergic cell death (Barnham *et al.*, 2004). The SNpc of sporadic PD patients' brain post-mortems shows evidence of substantial oxidative damage to lipids, proteins and DNA and a depletion of endogenous antioxidants (Jenner, 2003).

#### 1.4.1.2 Mitochondrial Dysfunction

The mitochondria are the primary site for the generation of cellular energy. Mitochondrial dysfunction can therefore lead to energy failure and eventually cell death. Indeed neurotoxins known to induce parkinsonism, such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and rotenone function by



inhibiting mitochondrial complex 1, resulting in energy failure and cell death (von Bohlen und Halbach *et al.*, 2004).

#### 1.4.1.3 The Ubiquitin-Proteasome System

The ubiquitin-proteasome system is the major way in which misfolded and unwanted proteins are removed. The presence of Lewy bodies in PD patients' brains which stain heavily for  $\alpha$ -synuclein, a protein usually degraded by the proteasome, suggests deregulation of the ubiquitin-proteasome system may be involved in Parkinson's disease. Indeed, proteasomal inhibition in PC12 cells leads to the formation of ubiquitin- $\alpha$ -synuclein immunoreactive inclusions (Biasini *et al.*, 2004) and biochemical studies have demonstrated reduced proteasome function in the substantia nigra of patients with sporadic PD (McNaught and Jenner, 2001).

### 1.4.2 The Genetics of Parkinson's disease

In the majority of cases PD is a late-onset sporadic disease usually caused by the interaction between multiple genetic susceptibilities and environmental factors, the most important of which being age. However, monogenic forms of the disease have also been identified and so far mutations in seven genes have been identified as causal factors in development of PD, often giving rise to early onset forms. The genes involved include, three autosomal dominant genes,  *$\alpha$ -synuclein (SNCA)* (Chartier-Harlin *et al.*, 2004; Kruger *et al.*, 1998) and *leucine-rich repeat kinase 2 (LRRK2)* (Paisan-Ruiz *et al.*, 2004) and *ubiquitin C-terminal hydrolase L1 (UCHL1)* (Leroy *et al.*, 1998) and four autosomal recessive genes including *parkin (PRKN)* (Kitada *et al.*, 1998), *DJ-1* (Bonifati *et al.*, 2003), *PTEN-induced putative kinase 1 (PINK1)* (Valente *et al.*, 2004b) and *ATPase type 13A2 (ATP13A2)* (Ramirez *et al.*, 2006). In line with current thinking about the mechanistic basis of sporadic PD, all of the genes so far identified as causative genes are in some way associated with UPS, responding to oxidative stress and mitochondrial function.

In the case of the autosomal recessive genetic factors, *Parkin*, *DJ-1*, *PINK1* and *ATP13A2*, PD is caused by loss of function mutations in the gene. *PINK1* mutations are the second most common cause of autosomal

recessive PD, the first being *Parkin*. Furthermore, it is believed that parkin may act downstream of PINK1 so understanding PINK1 function in the disease may shed light on *Parkin* forms of PD.

#### 1.4.2.1 PINK1

The *PINK1* gene encodes a serine threonine kinase, which localises to the mitochondria (Valente *et al.*, 2004a). Most of the mutations in the *PINK1* gene are missense mutations, particularly in the kinase domain and result in loss of function of the gene, however whole gene deletions have also been described. PINK1 has been shown to be involved in several mitochondrial functions including protecting cells from oxidative stress-induced apoptosis, facilitating normal respiration, aiding the ubiquitin-proteasome system (UPS) and enabling calcium signalling. It also plays a possible role in mitochondrial fusion and fission events and facilitates mitochondrial trafficking. For a review see Deas *et al.*, (2009)

The first evidence that PINK1 protected cells from stress-induced apoptosis came when cells deficient in PINK1 were shown to be more susceptible to mitochondrial toxins (Deng *et al.*, 2005; Valente *et al.*, 2004a; Wood-Kaczmar *et al.*, 2008) and conversely over-expression of PINK1 was shown to protect cells (Deng *et al.*, 2005; Valente *et al.*, 2004a). Subsequently, it was shown that cells deficient in PINK1 had increased cytoplasmic and mitochondrial reactive oxygen species (ROS) (Gandhi *et al.*, 2009; Wood-Kaczmar *et al.*, 2008). ROS are a natural by-product of oxygen metabolism, but their accumulation causes oxidative stress by damaging DNA, proteins and lipids and makes cells more vulnerable to stress-induced apoptosis. PINK1 is believed to protect cells from oxidative stress by phosphorylating the mitochondrial chaperone tumour necrosis factor receptor-associated protein 1 (TRAP1). Once phosphorylated TRAP1 prevents the release of cytochrome c from the mitochondria and H<sub>2</sub>O<sub>2</sub> induced apoptosis. Mutation of the kinase domain of PINK1 impairs the phosphorylation of TRAP1 and makes cells more prone to oxidative-stress induced cell death. Conversely the protective effect of PINK1 over-expression is abolished in cells lacking TRAP1 (Pridgeon *et al.*, 2007).

PINK1 has also been shown to be involved in mitochondrial respiration as PD mutations in PINK1 result in reduced oxygen consumption and ATP production (Liu *et al.*, 2009). One explanation may be that PINK1 has a direct effect on either the expression or activity of the electron transport chain. Alternatively, an accumulation of ROS in response to loss of PINK1 may possibly inhibit glucose transport and so result in a lack of metabolic substrate delivery to the mitochondria (Deas *et al.*, 2009).

PINK1 has also been linked to the UPS. In particular, PINK1 has been shown to be an upstream activator of the ubiquitin ligase parkin (Yang *et al.*, 2006). Ubiquitination is required for removal of proteins by the UPS and so reduced parkin activity may lead to an increase in unwanted proteins. The importance of this pathway in loss of PINK1 neurotoxicity is confirmed by the fact that loss of dopaminergic neurons in the PINK1 deficient *Drosophila* is alleviated by over-expression of parkin. Protein ubiquitination is also an ATP dependent process, so reduced ATP production due to mitochondrial dysfunction may be another way in which PINK1 mutations affect the UPS (Deas *et al.*, 2009). In agreement with UPS impairment in the absence of PINK1, increased, compensatory autophagic vacuoles and lysosomes have been detected in PINK1 deficient cells (Deas *et al.*, 2009). PINK1 has also been shown to interact with the protease HtrA2 (Plun-Favreau *et al.*, 2007). Little is known about the function of HtrA2; however it has been proposed to be involved in the mitochondrial protein quality control system (Spiess *et al.*, 1999; Walsh *et al.*, 2003).

Finally, PINK1 has been shown to regulate mitochondrial calcium flux through the  $\text{Na}^+/\text{Ca}^{2+}$  exchanger, and PINK1 deficiency results in the accumulation of calcium in mitochondria (Marongiu *et al.*, 2009). This in turn stimulates ROS production, and leads to ensuing toxic effects and reduced respiration. Interestingly, dopaminergic neurons rely on calcium channels instead of sodium channels to maintain their action potential, and so are commonly exposed to high calcium levels. The reduced capacity to buffer calcium in the absence of PINK1 may therefore be one explanation why dopaminergic neurons in particular are affected in Parkinson's disease (Deas *et al.*, 2009).

### 1.4.3 Animal models of Parkinson's disease

To date most of the *in vivo* data about PINK1 has been collected from loss of function mutations in *Drosophila*. *Drosophila* lacking PINK1 display a wide range of phenotypes including, male sterility, disorganised mitochondrial, reduced mitochondrial mass, lowered concentrations of ATP, muscle degeneration, minor loss of dopamine neurons and increased sensitivity to oxidative stressors such as paraquat and rotenone (Clark *et al.*, 2006; Park *et al.*, 2006; Yang *et al.*, 2006).

PINK1 knockout mice also show evidence of impaired mitochondrial respiration in the striatum and reduce striatal plasticity and hence reduced DA release. However, no impairments were found in overall DA levels or the number of dopaminergic neurons (Kitada *et al.*, 2007). Thus, it has been suggested that mouse models recapitulate the early stages of Parkinson's disease (Gautier *et al.*, 2008; Kitada *et al.*, 2007).

Although the *Drosophila* has proved invaluable in dissecting out PINK1 signalling pathways, their use as invertebrates for therapeutic research is limited. Meanwhile, mice as vertebrates are perhaps more similar to humans in terms of aetiology and how they respond to therapeutics, but are limited by the fact that they cannot be easily used in high-throughput screens. Therefore, zebrafish may be a good intermediate. As vertebrates they have a similar neuronal complexity to mice and humans, but their small size and high fecundity make them a viable option for use in high-throughput screens.

### 1.4.4 Zebrafish: Suitability to Modelling PD

The feasibility of using zebrafish to model complex neurological diseases relies on there being some degree of anatomical and functional similarity between the zebrafish and human brain, and in particular dopaminergic neurons equivalent to those of the SNpc must be present. Recently, a catecholaminergic system similar to that present in mammals has been described by immunohistochemical experiments. In light of these experiments, a zebrafish dopaminergic system has been identified. In

contrast to mammals, zebrafish dopaminergic neurons are in the diencephalon and telencephalon, but not in the midbrain, the location of the mammalian SNpc (Ma, 2003; Rink and Wullimann, 2001). However, a group of dopaminergic neurons in the ventral diencephalon were shown to project into the striatum in the zebrafish brain, and thus this region in the forebrain is believed to be the anatomical equivalent of the SNpc (Rink and Wullimann, 2001). Figure 1.3 shows the position of zebrafish dopaminergic neurons and the ventral diencephalon.



**Figure 1.3: Dopaminergic Neurons in the Zebrafish Larvae.** Whole mount in situ hybridisation with a riboprobe against the dopamine transporter (DAT) on 4 dpf zebrafish larvae. Neurons of the ventral diencephalon the anatomical equivalent of the human SNpc are circled in white. Arrows point to dopaminergic neurons in the bilateral pretectal clusters of the telencephalon (Adapted from (McKinley *et al.*, 2005)).

Furthermore, PD models created by the application of MPTP have already been produced in zebrafish (Bretaud *et al.*, 2004; McKinley *et al.*, 2005). McKinley *et al.* (2005) showed significant neurodegeneration of dopaminergic neurons particularly in the pretectal clusters and in the ventral diencephalon. They also showed that this neurodegeneration could be prevented by co-incubation with either the monoamine oxidase (MAO-B) inhibitor L-deprenyl or the dopamine transporter (DAT) inhibitor nomifensine, thus indicating that the mechanism of dopamine neuron toxicity in mammals is conserved in fish.

Taken together the evidence provided here suggests that zebrafish would make an appropriate model organism in which to model Parkinson's disease. The importance of PINK1 in both sporadic and familial forms of PD makes it

an interesting target for knockdown to further elucidate its involvement in PD and potentially to generate a PD model. Moreover, the PINK1 gene has recently been successfully targeted by RNAi in mice and so PINK1 has been shown to be an accessible target for RNAi induced knockdown (Zhou *et al.*, 2007).

## 1.5 Aim

The aim of this PhD is to develop a method and investigate the viability of stable and heritable vector-mediated RNAi in zebrafish. This will be done through targeted knockdown of stably expressed GFP and endogenous PINK1.

It is hoped that knockdown of PINK1 will lead to the generation of a Parkinson's disease model which could be used in high-throughput therapeutic screens and in further elucidation of how loss of PINK1 leads to the development of Parkinson's disease.

## 2 Materials and Methods

### 2.1 Buffers and Solutions

Solution	Procedure	Components
Chorion water + methylene blue (optional)	Embryo culture	60 mg Instant Ocean salts ((Tropic Marin®), 10-5% methylene blue, 1L dH <sub>2</sub> O
MS222	Anesthetising/ killing fish	7.7mM MS222, 1mM tris, pH 8.5 in dH <sub>2</sub> O
Alkaline Lysis Solution I	Plasmid miniprep	100 mM Tris pH 8.0, 50 µg/ml RNase A
Alkaline Lysis Solution II	Plasmid miniprep	0.2 N NaOH in 1% SDS
Alkaline Lysis Solution III	Plasmid miniprep	4 M KAc, 11.5% glacial acetic acid
TAE buffer	DNA electrophoresis	40 mM Tris base, 1 mM EDTA, 0.1% glacial acetic acid
DNA Lysis Buffer	DNA Extraction	50 mM Tris (pH 8), 100 mM EDTA, 0.5% SDS (w/v), pH 7.2
TE Buffer	Reconstituting DNA	10 mM Tris (pH 7.5), 1 mM EDTA
Annealing buffer	annealing shRNAs	10 mM tris pH 8.0, 50 mM NaCl, 1 mM EDTA
IP Lysis Buffer	Protein Extraction (cells)	1mM HEPES pH 7.4, 1 mM EDTA, 15 mM NaCl, 0.5% Triton X-100
½ Ginzburg Fish Ringers	Deyolking embryos	55 mM NaCl, 1.8 mM KCl, 1.25 mM NaHCO <sub>3</sub>

Solution	Procedure	Components
SDS Lysis Buffer	Protein extraction (embryos)	20mM HEPES pH 7.4, 0.1 M NaCl, 1 mM DTT, 1% SDS (w/v)
4% Triton-X 100 Lysis Buffer	Protein extraction (embryos)	20 mM HEPES pH 7.4, 0.1 M NaCl, 1 mM DTT, 1% SDS (w/v)
RIPA Lysis buffer	Protein Extraction (zebrafish tissues)	25 mM Tris (pH 7.6), 150 mM NaCl, 1% NP- 40 (v/v), 1 % sodium deoxycholate (w/v), 0.1% SDS (w/v)
Running Buffer	SDS-PAGE	25 mM tris, 192 mM glycine, 1% SDS (w/v)
Transfer Buffer	Western Blot Transfer	25 mM Tris, 192 mM glycine, 1% SDS (w/v), 20% methanol

**Table 2.1: Solutions used in this thesis.**



## 2.2 Zebrafish husbandry

Zebrafish were housed at the BSU (Biological Services Unit), the University of Manchester and maintained according to standard conditions described in the zebrafish handbook (Westerfield, 2000). Tap water, used for housing zebrafish, was first passed through activated carbon, mechanical and biological filters. Water temperature was maintained at 28-26°C, while light/dark cycles of 14hrs light and 10hrs dark were used. Embryos up to 5 dpf were cultured at 28.5°C in chorion water (Table 2.1). Larvae were transferred to the BSU at 5 dpf from which point they were fed daily on commercial powdered fish flakes. At one month old adult fish were fed daily on both dried fish food and live *Artemia* nauplia. Animals were sacrificed using schedule I methods. This entailed overdosing the fish with 4% MS222 (Table 2.1) for 30 minutes and the pithing the brain with a needle.

### 2.2.1 Breeding and Embryo Collection

Zebrafish were placed in thoron boxes (Aquatics, Inc.) with males and females separated with a plastic divide the night before breeding. After the switching on of lights in the morning, divides were removed, initiating spawning. Embryos fell through the mesh of the inside tank of the thoron boxes into the outer tank and could be easily collected.

## 2.3 Microinjecting Embryos

DNA and RNA were injected into the cytoplasm of single-cell embryos. Morpholinos were injected into embryos up until the 4 cell stage. An injection volume of 1 nl (around 1/100th of the egg volume) was injected using a PLI-90 Pico-Injector microinjection station. Injected embryos were incubated at 28.5°C. Embryos were either transferred to the BSU at 5 dpf or destroyed.

### 2.3.1 Injection Solutions

#### 2.3.1.1 *I-SceI*-mediated Transgenesis

Injection mixes comprised of 300 ng of plasmid DNA, 1 x *I-SceI* buffer, 5 u of meganuclease enzyme (NEB), 0.05% (w/v) phenol red in a final volume of 15 µl ddH<sub>2</sub>O.

#### 2.3.1.2 *To2*-mediated Transgenesis

Injection mixes comprised of 125 ng of plasmid DNA, 125 ng *To2* transposase RNA and 0.05% phenol red in a final volume of 10 µl ddH<sub>2</sub>O.

### 2.3.2 Screening and Imaging Transgenic Zebrafish

Embryos were monitored on a daily basis up to 4 dpf for fluorescent protein expression and phenotype development using Leica MZFLIII microscope. Multidimension acquisition images were taken using a Stereo Lumar.V12 microscope (Zeiss) attached to a monochrome AxioCam and using AxioVision 4 software. Z-stack images were taken using an AxioPlan 2 microscope (Zeiss) equipped with an apotome and compiled to generate extended focus images. In order to take images embryos were first anaesthetized with a 1 in 20 dilution of MS222 (Table 2.1).

## 2.4 General Cloning Methods

### 2.4.1 PCR

The following basic protocol was used for all DNA amplification. 1 µl of template genomic DNA (approximately 5ng), 100 ng plasmid DNA or 1 µg cDNA was added to a PCR mastermix containing 1 x GoTaq PCR buffer (Promega), 0.5 mM MgCl<sub>2</sub> (Promega), 0.1 µM dNTPs (Bioline), 0.15 µM of both forward and reverse primers, 1 u GoTaq DNA polymerase (Promega) made up to 20 µl using DEPC H<sub>2</sub>O. Cycling conditions consisted of: 95°C for 5 mins and then 28 cycles of 95°C for 30 secs, specific annealing temperature (usually 55°C) for 40 secs, and 72°C for 1 min. This was followed by a 10 mins extension at 72°C. Specific changes to cycle number, annealing temperature and elongation time can be viewed in the methods section of each results chapter.

### 2.4.2 p-GEM T Cloning

PCR products were cloned into the p-GEM T vector (Promega) following manufacturer's guidelines.

### 2.4.3 DNA Digestion

DNA digestion was carried out using approximately 10 µg of plasmid DNA, 1 x appropriate restriction enzyme buffer (Roche or NEB) and 5 u of the

appropriate restriction enzyme (Roche or NEB). The digestion mixtures were made up to 20  $\mu$ l with DEPC H<sub>2</sub>O, and 100  $\mu$ g/ml BSA (Bovine Serum Albumin) added where necessary. The mixtures were then incubated for between 2 and 16 hrs depending on the restriction enzyme used at 37°C. Digest mixtures were run on an appropriate percentage agarose gel and the desired fragment purified using a GFX purification kit (Amersham Biosciences) and following manufacturer's guidelines.

#### **2.4.4 DNA Blunting Reaction**

To blunt unwanted overhangs created by restriction enzyme digestion, reactions were set up as follows: 40  $\mu$ l purified DNA, 2 mM dNTPs, 100  $\mu$ g/ml BSA, 1 x T4 DNA polymerase buffer (Roche), 5 u T4 DNA polymerase (Roche) made up to a final volume of 60  $\mu$ l with DEPC water. The reaction was then incubated at 12°C for 15 mins. DNA was then purified using a GFX purification kit (Amersham Biosciences) and following manufacturer's guidelines.

#### **2.4.5 Ligation Reactions**

Ligations used 1 u T4 DNA ligase enzyme (Invitrogen) and 1 x ligase buffer (Invitrogen). Reactions were set up with a 3:1 molar ratio of insert to vector (approximately 200ng of vector was used in each reaction), made up to 20  $\mu$ l with DEPC H<sub>2</sub>O and incubated at 16°C overnight or room temperature (RT) for 2 hrs.

#### **2.4.6 Transformations**

Ligation mixtures were added to pre-thawed One Shot® competent *E. coli* cells (Invitrogen) and incubated on ice for 20 mins. Cells were then heat-shocked at 42°C for 45 secs, placed immediately back on ice for 5 mins and then incubated at RT for 5 mins. After incubation the cells were plated onto pre-warmed LB agar plates containing 50  $\mu$ g/ml of the appropriate antibiotic (ampicillin or kanamycin). The plate was incubated at 37°C overnight.

#### **2.4.7 Isolation of Plasmid DNA (mini prep)**

Bacterial colonies were inoculated into 2 ml LB broth containing 50  $\mu$ g/ml of the appropriate antibiotic (ampicillin or kanamycin). Following overnight

incubation in a shaking incubator (250 rpm, 37°C), plasmid isolation was performed via alkaline lysis. 1 ml of culture was decanted into a 1.5 ml microcentrifuge tube and centrifuged for 60 seconds at 13 000 rpm. After discarding the supernatant the bacterial pellet was resuspended in 100 µl of ice cold Alkaline Lysis Solution I (Table 2.1). 200 µl of Solution II (Table 2.1) was added and the contents mixed by inverting the tube 5-6 times. 160 µl of Solution III (Table 2.1) was then added and the mixture mixed again by inverting 5-6 times. After addition of 200 µl of chloroform the contents of the tube was mixed vigorously and was then centrifuged for 15 minutes at 13 000 rpm, producing three layers. 400 µl from the upper aqueous layer was then transferred to a fresh tube and an equal volume of propan-2-ol added. This mixture was centrifuged for a further 10 minutes at 13 000 rpm, and the supernatant removed and discarded, leaving a DNA pellet. The DNA pellet was washed with 1 ml 70% ethanol and centrifuged at 13 000 rpm for 10 mins. The supernatant was discarded and the pellet allowed to air dry. Once dry, the DNA was re-suspended in 50 µl ddH<sub>2</sub>O.

#### **2.4.8 PCR Colony Screening**

Single colonies from transformation plates were screened for the presence of the correct construct by inoculating a PCR reaction of 1 x GoTaq PCR buffer (Promega), 0.5 mM MgCl<sub>2</sub>, 0.1 µM dNTPs, 0.15 µM fwd primer, 0.15 µM rev primer, 1 u GoTaq DNA polymerase (Promega) made up to 20 µl using DEPC. Cycling conditions consisted of: 95°C for 5 mins and then 28 cycles 95°C for 30 secs, 55°C for 40 secs, and 72°C for 1 min. This was followed by a 10 minute extension at 72°C. The PCR products were then run on an appropriate agarose gel.

#### **2.4.9 DNA Sequencing**

Sequencing reactions contained 400 ng plasmid DNA or 100 ng PCR product, 3.3 pmol primer, 2 µl Terminator Ready reaction mix (Big dye version 1.1, ABI) and 3 µl sequencing buffer made up to a final volume of 20 µl. The cycling conditions consisted of a denaturation step at 94°C for 5 mins, 35 cycles of 96°C for 30 secs, 50°C for 10 secs, 60°C for 5 mins, and an extension at 72°C for 10 mins. Amplified DNA was transferred to a 1.5 ml microcentrifuge tube and precipitated by adding 2 µl NaAc (pH5.2) and 50 µl

96% ethanol, and incubating at rt for 20 mins. Precipitated DNA was pelleted by centrifugation at 13 000 rpm for 30 mins and washed in 250  $\mu$ l 70% ethanol, before air drying at room temperature. Samples were sent to the University of Manchester Sequencing Facility, where the reaction products were separated by polyacrylamide gel electrophoresis. Analysis of sequence data was performed using BioEdit software.

#### **2.4.10 Maxi Prep**

Large scale plasmid isolation of cloned constructs was carried out using QIAfilter<sup>TM</sup> Plasmid Maxi kit (Qiagen) according to the manufacturer's instructions.

#### **2.4.11 Site Directed Mutagenesis (SDM)**

Site-directed Mutagenesis was performed using a Quikchange<sup>®</sup> site directed mutagenesis kit (Stratagene) and following manufacturer's instructions. Primer sequences are given in the relevant chapters.

#### **2.4.12 *In vitro* Transcription**

mRNAs were transcribed using a T7 message machine (Ambion) and following manufacturer's instructions. Once synthesised mRNA was purified using an RNeasy Mini kit (Qiagen) and a test sample was run on a 2100 Bio-analyser (Agilent Technologies).

### **2.5 Generating miRNA Vectors**

#### **2.5.1 Cloning miRNAs into pGEM-T**

Appropriate miRNA target sequences were identified for each gene to be knocked down using BLOCK-IT<sup>TM</sup> RNAi Designer (Invitrogen) and blasting sequences against the zebrafish genome to check they do not share high sequence homology with other genes, with particular attention paid to the seed region of the miRNA. Forward and reverse primers which incorporate the hsa-miR-30a backbone, the sense and antisense target sequence, and *KpnI* and *EcoRI* restriction sites were designed (primer sequences for miRNAs are given in the appropriate results chapter). miRNA primers were annealed and extended in PCR reaction conditions consisting of 1  $\mu$ g forward and reverse primers, 1 x GoTaq Buffer (Promega), 2.5 mM MgCl<sub>2</sub>,

100  $\mu$ M dNTPs and 1 u GoTaq polymerase (Promega), made up to 20  $\mu$ l with deionised water. Cycling conditions consisted of 95°C for 5 mins, followed by 10 cycles of 95°C for 20 secs, 37°C for 20 secs, 68°C for 10 secs, and a final extension of 68°C for 5 mins. Annealed PCR products were separated from un-annealed single stranded DNA by electrophoresis on a 2.5% agarose gel made up with 1 x TAE buffer containing 0.5  $\mu$ g/ml ethidium bromide. The annealed PCR product was excised from the gel and the DNA extracted using a GFX purification kit (GE Healthcare) following the manufacturer's instructions. All miRNAs were first cloned into the pGEM-T vector (Promega) following manufacturer's guidelines. Positive clones were identified by *KpnI/EcoRI* restriction digest of mini preps.

### **2.5.2 Cloning miRNAs into Silencing Vectors**

miRNAs were liberated from pGEM-T vector (Promega) with *EcoRI/KpnI* restriction digest (see 2.4.3) and ligated (see 2.4.5) into the appropriate silencing vector which had also been linearised with *EcoRI/KpnI* restriction digest. Ligations were then transformed (see 2.4.6) and positive colonies were identified by PCR colony screening (see 2.4.8) using miRNA forward (5' - TGCTGTTGACAGTGAGCGA - 3') and mcherry 5' reverse (5' - TGGCCATGTTATCCTCCTCG - 3') primers.

## **2.6 Genotyping Zebrafish**

### **2.6.1 Genomic DNA Extraction from Fish Larvae/Tissues for Genotyping**

To check the identity of miRNAs present in transgenic zebrafish, transgenic offspring or tail clippings from transgenic fish were lysed in 380  $\mu$ l of DNA Lysis buffer (Table 2.1) supplemented with 0.5 mg/ml Proteinase K (Sigma-Aldrich). Samples were incubated at 55°C overnight with gentle agitation, after which 400  $\mu$ l isopropanol was added and samples were mixed by shaking. Samples were then centrifuged at 13 000 rpm for 15 mins to pellet the DNA. The supernatant was then discarded and the pellet was washed in 70% ethanol and centrifuged for a further 10 mins at 13000 rpm. The supernatant was discarded and samples left to air dry for 10 mins. DNA was then resuspended in 50  $\mu$ l TE buffer (Table 2.1).

### 2.6.1.1 Nested PCR and Sequencing

#### 2.6.1.1.1 1<sup>st</sup> PCR

1 µl genomic DNA was used in a general PCR mix (see 2.4.1) along with the first set of genotyping primers listed in Table 2.2. Cycling conditions consisted of: 95°C for 5 mins and then 24 cycles of 95°C for 30 secs, 55°C for 40 secs and 72°C for 1 min, followed by a final extension of 72°C for 10 mins.

#### 2.6.1.1.2 2<sup>nd</sup>/Nested PCR

PCR products from the first round PCR were diluted 2.5-fold and 1 µl transferred to a second PCR reaction (see 2.4.1) along with the second set of genotyping primers (Table 2.2) which contain binding sites for M13 fwd and rev primers. Cycling conditions were as above (see 2.6.1.1.1). The resulting PCR product was again diluted 2.5-fold and 1 µl was used in sequencing reactions (see 2.4.9) using M13 fwd and rev primers (Invitrogen). Sequences were analysed using BioEdit to check for the presence of the correct miRNA.

Primer Name	Sequence
Genotyping Fwd 1	CAGCAGCTGAGGAGTGATC
Genotyping Rev 1	TTACTTGTACAGCTCGTCCATG
Genotyping Fwd 2 (M13 fwd)	<u>TGTAAAACGACGGCCAGT</u> CCTTGGTGAGTACTATAGGCT
Genotyping Rev 2 (M13 rev)	CAGGAAACAGCTATGACCTCCTTGATGATGGCCATG

**Table 2.2: Primers Used to Genotype Transgenic Zebrafish.** Sequences are given in a 5' to 3' format. M13 fwd and rev binding sites are underlined.

## 2.7 Tissue Culture

### 2.7.1 Cell Maintenance and Passage

#### 2.7.1.1 HEK 293 Cells

HEK 293 (Human Embryonic Kidney) cells were cultured in DMEM (Dulbecco's Modified Eagle Medium): F12 (1:1) + GlutaMAX™ (Invitrogen) supplemented with 10% FCS (Fetal Calf Serum) and 1% penicillin/streptomycin and grown at 37°C in 5% CO<sub>2</sub>.

#### 2.7.1.2 AB.9 Cells

Zebrafish AB.9 cells originating from the adult caudal fin (ATCC® Number: CRL-2298TM) were cultured in DMEM (Dulbecco's Modified Eagle Medium): F12 (1:1) + GlutaMAX™ (Invitrogen) supplemented with 10% FCS and 1% penicillin/streptomycin and grown at 28°C in 5% CO<sub>2</sub>.

#### 2.7.1.3 PAC.2 Cells

PAC2 cells, a fibroblast cell line derived from 24 hpf embryos (Lin *et al.*, 1994) were cultured in Leibovitz's L-15 medium (Invitrogen) supplemented with 15% FCS and 1% penicillin/streptomycin and grown at 28°C without CO<sub>2</sub>.

#### 2.7.1.4 ZFL Cells

ZFL cells, an adult zebrafish liver epithelial cell line (ATCC® Number: CRL-2643TM) were cultured in DMEM (Dulbecco's Modified Eagle Medium): F12 (1:1) + GlutaMAX™ (Invitrogen) supplemented with 10% FCS, 1% pen/strep, 0.1 ug/ml epithelial growth factor (EGF, Sigma-Aldrich) , 0.01 mg/ml insulin (diluted in HCL, Sigma-Aldrich), 0.1% Lipid mixture (v/v, Sigma-Aldrich) and grown at 28°C in 5% CO<sub>2</sub>.

All cells were passaged upon confluency by washing twice in Dulbecco's Phosphate Buffered Saline (DPBS) without calcium and magnesium chloride (Sigma) and detaching in TrypLE (Invitrogen). HEK 293 cells were seeded into new flasks/wells at a 1:10 ratio. Zebrafish cells were seeded at a ratio of 1:3.

### **2.7.2 Transfections**

24 hours prior to transfection cells were detached using TrypLE (Invitrogen) and then passaged into 6 well culture plates (Costar). 30 mins prior to transfection cells were washed in PBS and the appropriate fresh medium was added. For zebrafish cell transfection fresh medium lacked antibiotics. Per well to be transfected 87 µl of antibiotic free media and 3 µl FuGENE HD (Roche) were mixed together and incubated at room temperature for 5 mins. 1 µg (10 µl) plasmid DNA was added to the DMEM/FuGENE HD mix and



incubated at room temperature for a further 45 mins. 100 µl transfection mix was then added to each well of cells. Transfections were carried out in triplicate. 24 hrs post transfection zebrafish cells had the medium containing FuGENE HD removed and fresh medium containing antibiotics replenished.

## 2.8 Fluorescent Flow Cytometric Analysis

### 2.8.1 Transfected Cells

Transfected cells were harvested 3 days post transfection by first washing in DPBS and then detaching from the culture plate by incubating for 5 mins in TrypLE. Once detached cells were transferred to a 1.5 ml centrifuge tube and spun for a few secs at <5500 rpm. The supernatant was then removed and cells were resuspended in DPBS, before analysing on a FACS Aria (BD Biosciences) equipped with a 488 nm and 594 nm laser for analysis of GFP and mcherry fluorescent levels respectively. Forward and side scatter measurements enabled the removal of aggregated and dead cells/debris from the analysis. In HEK 293 and AB.9 cells where GFP and the RNAi vector (mcherry) were co-transfected the ratio of GFP:mcherry in mcherry positive cells was calculated. In PAC.2 YFP and ZFL GFP cells the YFP/GFP content of mcherry positive cells was calculated.

### 2.8.2 Zebrafish

Between 10-15 transgenic zebrafish were transferred to a 1.5 ml microcentrifuge tube and incubated in 1x trypsin (Sigma-Aldrich) with agitation for approx. 15 mins. Once trypsinised, samples were spun down at < 5500 rpm for a few secs. Supernatant was then removed and cells were resuspended in PBS before passing through a 50 micron filter, to remove debris. Trypsinised zebrafish cells were then analysed on FACS Aria (BD Biosciences) equipped with a 488 nm and 594 nm laser for analysis of GFP and mcherry fluorescent levels respectively. Forward and side scatter measurements enabled the removal of aggregated and dead cells/debris from the analysis. The GFP content of mcherry positive cells was compared to GFP levels in non-transgenic sibs (NTS) with regard to RNAi vector.

## 2.9 Quantitative PCR (qPCR)

### 2.9.1 RNA Extraction

Total RNA was extracted from cells grown in a well of a six well plate, zebrafish embryos/larvae and adult zebrafish organs using the RNeasy® Lipid Tissue Mini Kit (Qiagen) and following manufacturer's instructions. An RNase-Free DNase Set (Qiagen) was also used following manufacturer's instructions to perform on the column DNase digestion. Extracted RNA was quantified using a Nanodrop spectrophotometer (Thermo Scientific) aliquoted into 1 µl aliquots and stored at -80°C.

#### 2.9.1.1 Time Course and Tissue Bank mRNA Extraction

To make each time course cDNA, RNA was extracted using the above method from 30 embryos and larvae at the 1 cell stage, 4 hpf, 12 hpf, 1 dpf, 2 dpf, 3 dpf, 4 dpf, 5 dpf, 6 dpf, 7 dpf and 3 weeks post fertilisation. For each tissue bank, organs were extracted from 6, 6 month old adult zebrafish (3 male and 3 female).

### 2.9.2 Reverse Transcription

First strand cDNA was reverse transcribed from RNA using Omniscript® RT Kit (Qiagen). Reaction mixtures consisted of 1 µg RNA, 1 x reverse transcription buffer, 0.5 mM of each dNTP, 1 µl oligo (dT) 15 (Promega), 10 u RNase inhibitor (Roche), 4 u Omniscript reverse transcriptase and made up to 20 µl with DEPC H<sub>2</sub>O. After assembly reaction mixtures were incubated at 37°C for 1 hr. Synthesised cDNA was quantified on a Nanodrop spectrophotometer (Thermo scientific) and diluted to 100 ng/µl

### 2.9.3 Real Time- qPCR (sybr-green)

Sybr-green-based qPCR reactions consisted of 1 x SYBR Green Jumpstart™ Taq Ready Mix™ (Sigma-Aldrich), 0.2 µM forward and reverse primers, 200 ng cDNA made up to 20 µl with DEPC H<sub>2</sub>O. Samples were analysed on a Peltier Thermal Cycler (PTC-200) connected to a Chromo4 Continuous Fluorescence Detector (MJ Research). Cycling conditions were as follows: 94°C for 2 mins followed by 40 cycles of 94°C for 15 secs and 60°C for 1 min. Melting curve analysis was performed from 45°C to 95°C.

### 2.9.4 Validation of qPCR Primers

Primers were validated by determining the CT values from a serial dilution of cDNA and plotting those values against the log quantity of cDNA used in each reaction to produce a straight line. A slope with a gradient of -3.32 indicates that the primers are 100% efficient. -3.1 is equivalent to 90% efficiency and -3.58 is equivalent to 110% efficiency. Only primers which fell between these ranges and gave rise to a correlation coefficient ( $R^2$ ) of at least 0.97 were accepted for use in cDNA quantification. Validated primer pairs are listed in the appropriate results chapters.

### 2.9.5 Analysis of qPCR Data: Comparative CT ( $\Delta\Delta CT$ ) Method

The cycle threshold (CT) level was set manually at the linear part of the curve at a point in which doubling the cDNA concentration leads to a doubling in signal intensity. The  $\Delta\Delta CT$  is then calculated using the following formula:

$$\Delta\Delta CT = \Delta CT \text{ Test Sample} - \Delta CT \text{ Calibrator Sample}$$

where,

$$\Delta CT = CT \text{ Target} - CT \text{ Housekeepers}$$

To convert the  $\Delta\Delta CT$  into a fold change on calibrator levels the  $\Delta\Delta CT$  is reversed using the formula:

$$2^{(-\Delta\Delta CT)}.$$

## 2.10 Immunoblotting

### 2.10.1 Protein Extraction from Cultured Cells

To extract protein from confluent cells, media was removed via aspiration and cells were washed twice in DPBS. 100  $\mu$ l of chilled IP buffer (Table 2.1), supplemented with 1% protease inhibitor cocktail (Amersham Biosciences) and 1 % phosphatase inhibitor cocktail (Set II; Calbiochem) inhibitors, was added to each well of a 6-well cell culture plate and cells were detached using a cell scraper. The lysate was transferred into a microcentrifuge tube and was incubated on ice for 5 mins. Samples were then centrifuged at 12

000 x g at 4°C for 15 mins, and the supernatant containing protein extract was transferred to a fresh tube. Protein was quantified in mg/ml using Advanced Protein Assay reagent (Cytoskeleton Inc.) and following manufacturer's instructions. Absorption was measured at 595 nm on a Jenway 6305 spectrophotometer (Jencons-PLS) and proteins were aliquoted before being snap frozen in liquid nitrogen and then transferred to a -80°C freezer.

### **2.10.2 Protein Extraction from Embryos and Larvae**

Embryos were dechorionated either manually or by soaking for 10 mins in a 2 mM Pronase solution (Sigma-Aldrich) and deyoked by transferring embryos (up to 20) into a tube containing 1 ml ½ Ginzburg buffer (Table 2.1) supplemented with 1% protease inhibitor cocktail, 0.75 mM EDTA (ethylenediaminetetraacetic acid), 0.3 mM PMSF (phenylmethanesulfonylfluoride) dissolved in isopropanol (stock concentration 100 mM). Embryos/larvae were dissociated from the yolk sac by pipeting up and down and then shaking at 11 000 rpm at 4°C for 5 mins. Embryos were then spun down at 2 000 rpm for 1 min and the supernatant removed and discarded. 150 µl chilled SDS Lysis Buffer (Table 2.1) supplemented with 1% protease inhibitor cocktail, 1% phosphatase inhibitor cocktail, 0.75 mM EDTA and 0.3 mM PMSF was added to the embryos, and embryos were disaggregated using a pestle for 1 min on ice. Samples were then heated to 95°C for 3 mins and a further 150 µl chilled Triton X-100 Lysis Buffer (Table 2.1) supplemented with 1% protease inhibitor cocktail and 1% phosphatase inhibitor cocktail was added to each tube. Samples were incubated for a further 2 mins on ice and then spun at 13 000 rpm for 1 min. Supernatants were transferred to a new tube and protein quantified using the Bicinchoninic Acid (BCA) kit for protein determination (Sigma-Aldrich) and following manufacturer's instructions. The remaining sample was aliquoted and snap frozen in liquid nitrogen before transferring to a -80°C freezer.

### **2.10.3 Protein Extraction from Adult Brain Samples**

Dissected brains were homogenised on ice using a pestle in RIPA Buffer (Table 2.1) supplemented with 1 % protease inhibitor cocktail and 1%

phosphatase inhibitor cocktail. Samples were incubated for 5 mins on ice before centrifuging at 13 000 rpm for 15 mins at 4°C. Supernatant was transferred to a fresh tube and protein quantification was performed using Advanced Protein Assay reagent (Cytoskeleton Inc.) and following manufacturer's instructions, before aliquoting protein, snap freezing in liquid nitrogen and storing at -80°C.

#### **2.10.4 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)**

Protein lysates were thawed on ice and equal amounts of protein (~30 µg) were mixed with 2 x NUPAGE® LDS sample buffer (Invitrogen). After thorough mixing, samples were boiled at 95°C for 10 mins. A 4-15% gradient SDS-polyacrylamide ready gel (Bio-Rad) was immersed in Running Buffer (Table 2.1) and protein samples and Precision Plus Protein™ Dual Colour Standards (Bio-Rad) were loaded. The gel was run at 100-130 V until the proteins were well separated.

#### **2.10.5 Electrophoretic Transfer**

Proteins were transferred from SDS-PAGE gel to an Immobilon-P transfer membrane (Millipore). First the membrane was activated by soaking in methanol for 15 secs, followed by a wash in ddH<sub>2</sub>O for 2 mins. The gel and membrane were assembled into a transfer cassette and immersed in chilled Transfer Buffer (Table 2.1). Protein transfer was performed at 100 V for 45 mins.

#### **2.10.6 Blocking, Primary Antibody and Secondary Antibody**

Following transfer, membranes were washed briefly in Tris-buffered saline (TBS) (Table 2.1), and were then blocked in 5% milk (marvel) made up with TBS/Tween (TBS, 0.05% (v/v) Tween (Sigma-Aldrich)) for 1 hr at rt with gentle agitation. After washing in TBS/T, membranes were incubated with the appropriate primary antibody, diluted as required in 1% milk TBS/T at 4°C overnight with gentle agitation. The following day, membranes were washed three times for 5 mins in TBS/T and then incubated with a 1:10 000 dilution of the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (GE Healthcare) in 5% milk/TBS/T for 1 hr at rt.

### **2.10.7 Detection: Enhanced Chemiluminescence (ECL)**

After incubation with the secondary antibody, membranes were washed 3 times for 5 mins in TBS/T and proteins were incubated for 1 min in ECL plus detection reagent (Amersham Biosciences) at rt and then exposed on Kodak Biomax MR film and developed using an Optimax film processor. To quantify western blots, blots were scanned and analysed by densitometry using ImageJ.

### **2.11 Statistics**

All quantitative data was analysed with the appropriate statistical test indicated in each figure and using the 5% significance threshold. Where a significant difference was found, this is indicated using the \* system (\* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ ). All graphical data is depicted with bars indicating standard error of the mean.

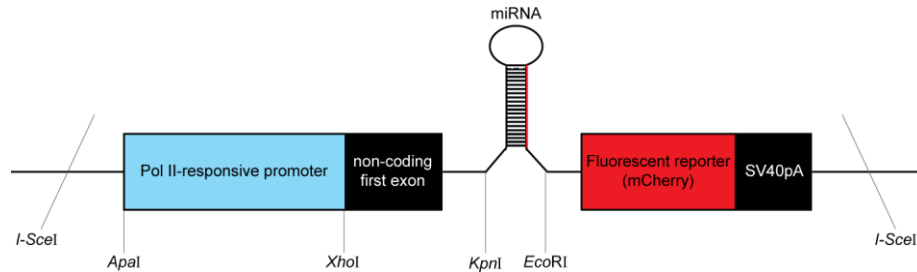
### **3 Development and Validation of Vector-Mediated RNAi in Zebrafish Embryos**

#### **3.1 Introduction**

The use of zebrafish to model disease and in reverse genetics has been limited by an inability to effectively and reliably knockdown genes. The discovery of RNAi as an evolutionary conserved mechanism of gene regulation offers a new avenue to explore in knocking down genes in zebrafish. Indeed siRNAs have already been used by various groups to knockdown zebrafish genes with varying degrees of success (Blidner *et al.*, 2008; Chang and Nie, 2008; Dodd *et al.*, 2004; Gruber *et al.*, 2005; Kloosterman *et al.*, 2004; Liu *et al.*, 2005c). However, a major limitation in the use of siRNAs was a high level of general toxicity (Gruber *et al.*, 2005; Zhao *et al.*, 2008) due to the delivery of large quantities of siRNA into early stage embryos, which seems to saturate the RNAi machinery to the point at which essential endogenous miRNAs, such as miR-430, are not successfully processed (Zhao *et al.*, 2008). Development of a vector-mediated system of RNAi in which primary miRNAs transcripts encoding customised miRNAs are only produced after the initiation of transcription and at more physiological levels may therefore avoid these problems as well as providing a more stable and heritable form of knockdown.

A vector suitable for RNA polymerase II (pol II)-driven expression of customised miRNAs has been developed by Dr. Paul Walker, the Hurlstone Laboratory, and is depicted in Figure 3.1. In this system a promoter of choice is cloned upstream of a non-coding first exon and first intron of Elongation Factor 1 alpha (*ef1 $\alpha$* ), downstream of which is the reporter gene, *mcherry*. Within the intron is a stuffer fragment surrounded by *KpnI* and *EcoRI* restriction sites which enables miRNAs to be easily cloned in. Activation of the promoter results in expression of both miRNA and the reporter gene. Pol II promoters can be easily cloned in and out using *ApaI* and *XhoI* restriction sites. In order to generate stable zebrafish transgenic lines expressing the miRNA the construct is flanked by *I-SceI* restriction

sites. Upon co-injection with the *I-SceI* meganuclease, these restriction sites aid integration of the construct into the zebrafish genome (Thermes *et al.*, 2002). For a full vector map see appendices section 8.1.1.



**Figure 3.1: Schematic Representation of the RNAi Vector.** A promoter of choice drives expression of both the miRNA and reporter gene. Different promoters can be cloned into the promoter position using *Apal* and *XhoI* restriction sites. miRNAs can be cloned in using *KpnI* and *EcoRI* restriction sites. The silencing cassette is surrounded by *I-SceI* restriction sites which aids transgenesis when co-injected with *I-SceI* meganuclease into embryos.

### 3.1.1 Aim

The aim of this chapter is to develop and validate a method of vector-mediated delivery of RNAi for use in zebrafish.

### 3.1.2 Objectives

- to clone and test control and GFP miRNAs in HEK 293 cells.
- to develop a vector suitable for delivery of these miRNAs *in vivo*.
- to demonstrate whether vector mediated delivery of miRNAs is sufficient to knockdown GFP *in vivo* in both a transient and stable transgenic manner.



## 3.2 Materials and Methods

For general material and methods see chapter 2.

### 3.2.1 Cloning miRNAs

General cloning methods as described in section 2.4 were used to clone RNAi vectors with appropriate promoter and miRNA combinations. *ef1 $\alpha$* , *H2A.Zf* and *krt18* promoters were inserted using *ApaI* and *XhoI* restriction sites. Control miRNA and miRNAs against the open-reading frame (ORF) of GFP were generated using the primers listed in Table 3.1, and cloned into the RNAi vectors using the incorporated *KpnI* and *EcoRI* restriction sites.

miRNA	Primer sequence 5'-3'	
GFP #1	Fwd	GC GG <b>GGTACC</b> TGCTGTTGACAGTGAGCGA <b>GCACAAGCTGGAGTACAAC</b> TAGTGAAGCCACAG ATG
	Rev	CCGC <b>GAATTC</b> CCTTGAAGTCCGAGGCAGTAGGCAG <b>GCACAAGCTGGAGTACAAC</b> TACATCTG TGGCTTCAC
GFP #2	Fwd	GC GG <b>GGTACC</b> TGCTGTTGACAGTGAGCGA <b>CGGCCACAAGTTCTCAGCGGGTCC</b> TAGTGAAGCCA CAGATG
	Rev	CCGC <b>GAATTC</b> CCTTGAAGTCCGAGGCAGTAGGCAG <b>CGGCCACAAGTTCTCAGCGGTCC</b> TACATCT GTGGCTTCAC
Control	Fwd	GC GG <b>GGTACC</b> TGCTGTTGACAGTGAGCGA <b>AGTCTGTGCGCAGAAATATAT</b> TAGTGAAGCCACAG ATG
	Rev	CCGC <b>GAATTC</b> CCTTGAAGTCCGAGGCAGTAGGCAG <b>AGTCTGTGCGCAGAAATATAT</b> TACATCTG TGGCTTCAC

**Table 3.1:** Primers used in the production of miRNAs. The sense sequence of the target is shown in grey on the forward primer and red on the reverse primer. *KpnI* and *EcoRI* sites are indicated in turquoise and yellow respectively. The rest of the sequence is based on the hsa-miR-30a backbone identified by Zeng *et al.* (2002).

### 3.2.2 Cloning a Gal4-VP16/UAS-responsive RNAi Vector

To generate a Gal4-VP16/UAS-responsive RNAi vector *KpnI* and *EcoRI* restriction sites were removed from pBluescript *I-SceI* (Thermes *et al.*, 2002) (for vector map see appendices section 8.1.2) by cutting the plasmid, blunting overhanging DNA bases and re-ligating the vector. A Gal4-VP16 SV40pA UAS E1b minimal promoter fragment was cut out of the SAGVG vector (Davison *et al.*, 2007) (for vector map see appendices section 8.1.3) with *MscI* and *ClaI* restriction digest, overhangs were blunted and the insert was ligated into the modified pBluescript *I-SceI* vector digested with *SmaI*

and *Bam*HI and also blunted. The *Eco*RI restriction site in the Gal4-VP16 sequence was then removed via site directed mutagenesis (see section 2.4.11) using the forward primer, 5'-GGTCGACCCCGGGTATACAAATCTCTCGAG-3' and reverse primer, 5'-CTCGAGAGATTTGTATACCCGGGGTTCGACC-3'. The non-coding first exon, miRNA-containing intron and mcherry fragment from the original RNAi vector was then removed by digestion with *Xho*I, then blunted and digested with *Sac*II and cloned downstream of the pBluescript Gal4-VP16/ UAS *I-Sce*I which had been cut with, *Bam*HI, then bunted, followed by *Sac*II. Promoters were adapted by PCR amplification followed by pGEM-T cloning to contain an *Eco*RV restriction site at the 3' end. The promoter of choice was then cloned into the Gal4-VP16-responsive RNAi vector using *Apa*I and *Eco*RV restriction sites. For a full vector map of the Gal4-VP16-responsive *I-Sce*I vector see appendices section 8.1.4

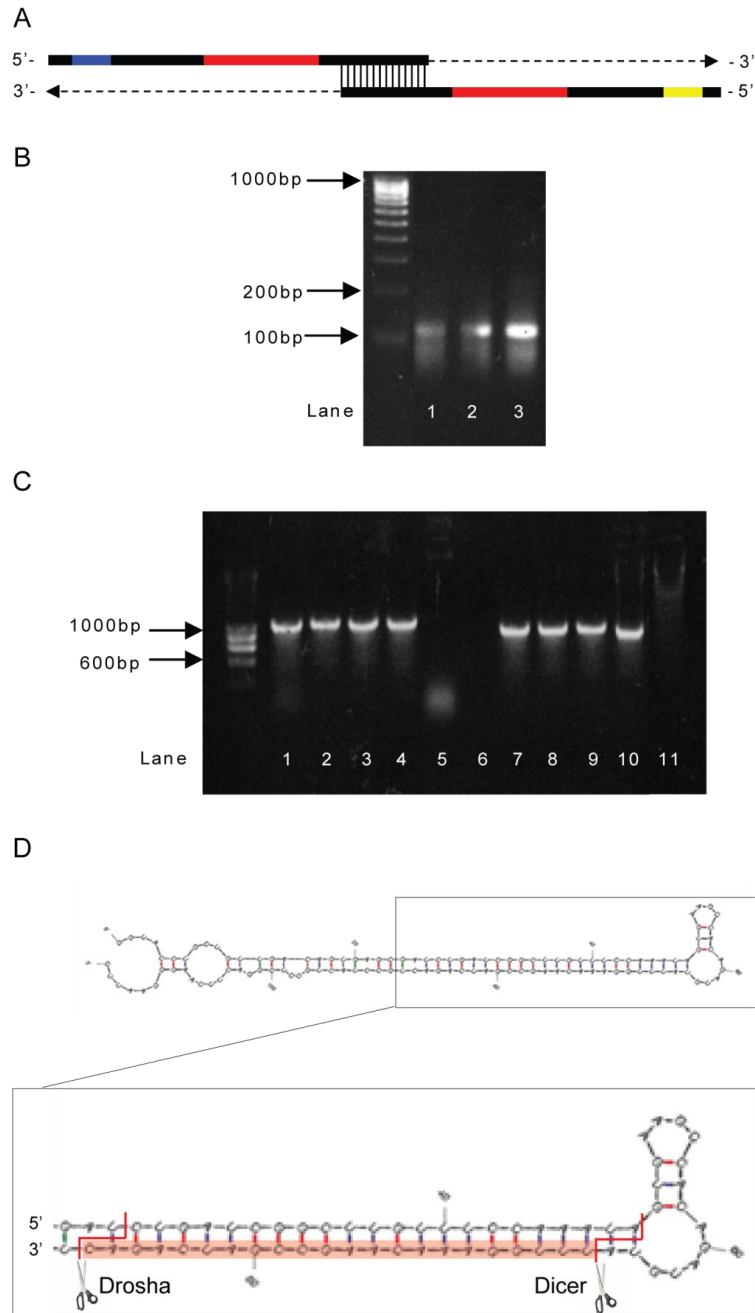
#### 3.2.2.1 Cloning a Gal4-VP16-responsive *To*2 Vector

*To*2 vectors are based on the pT2KXIGdeltaIN plasmid which incorporates transposable sequences derived from the medaka *To*2 transposable element (Urasaki *et al.*, 2006). The pT2KXIGdeltaIN vector was modified by Dr. Stephen Renshaw, University of Sheffield, UK, to contain a multi-cloning site between the transposable arms and to remove a *Kpn*I restriction site from the backbone. The Gal4-VP16-responsive RNAi cassette was then cloned in to this *To*2 vector as an *Apa*I, *Sac*II fragment (for full vector map see appendices section 8.1.5).

### 3.3 Results

#### 3.3.1 Production and Cloning of miRNAs

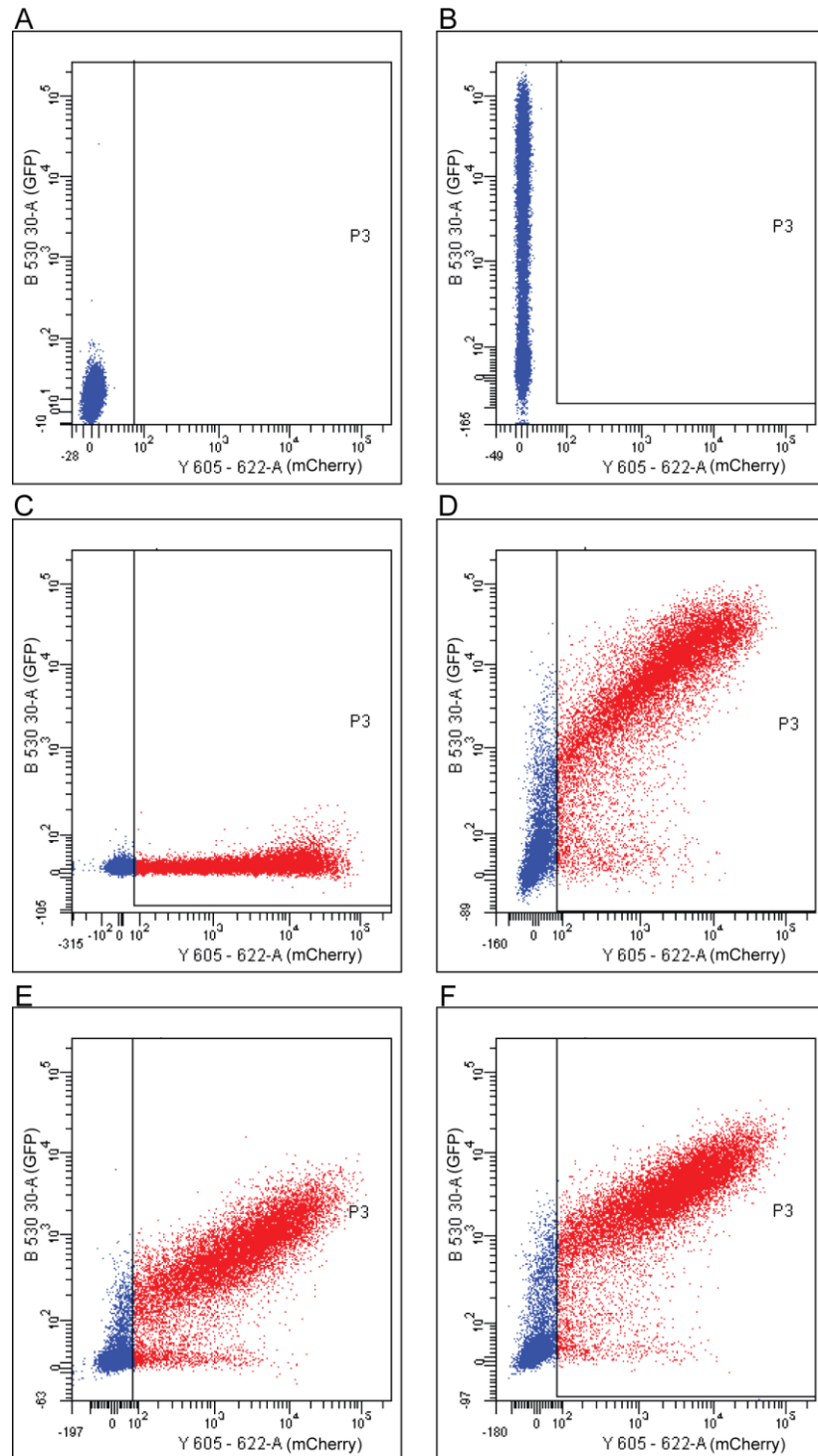
To generate RNAi vectors containing customised miRNAs, primer pairs listed in Table 3.1 were annealed and extended in a PCR reaction described in section 2.5.1; a schematic of this can be seen in Figure 3.2 A. The resulting PCR products were run on a 2.5% agarose gel (Figure 3.2 B). Such a high percentage gel was required to allow good separation of the double stranded DNA (120bp) from single stranded (non-annealed) DNA. Following gel extraction the double stranded PCR product was cloned into pGEM-T vector. Positive clones were identified by *KpnI/EcoRI* digestion of mini preps. For each miRNA approximately 5 plasmids positive for insert were sequenced due to the high error rates in sequences observed. The miRNA from one positive clone, as identified by sequence analysis, was sub-cloned as a *KpnI/EcoRI* fragment into the intron of the RNAi vector (Figure 3.1). Positive clones were identified by PCR colony screening with a forward primer specific for the hsa-miR-30a backbone (miRNA forward) and a mcherry 5' reverse primer (see section 2.5.2). Transcription of the intronic region of the RNAi vector results in a stem loop structure like that seen in Figure 3.2 D (as predicted by mfold, <http://mfold.bioinfo.rpi.edu/>).



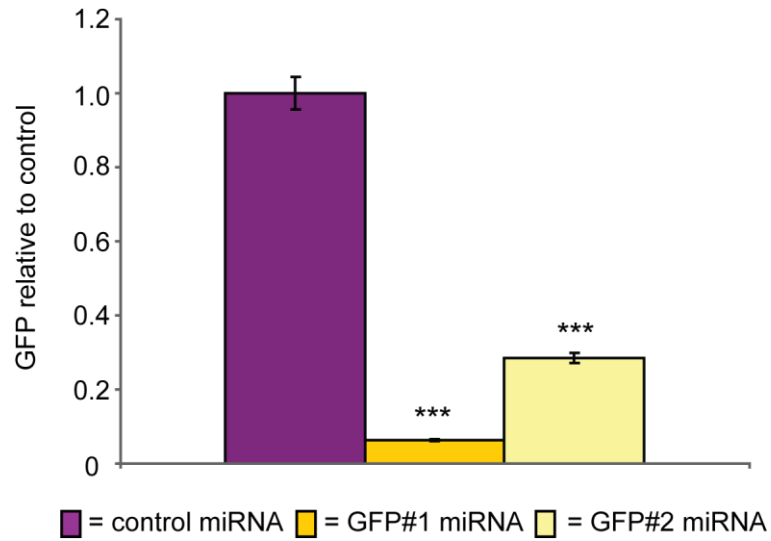
**Figure 3.2: Production and Cloning of miRNAs.** A: Schematic diagram showing the annealing of the two primers used to make the miRNAs. The miRNA sequence is highlighted in red on the forward and reverse strands. *KpnI* and *EcoRI* restriction sites are indicated in blue and yellow respectively. B: Annealed primers were run out on a 2.5% TAE agarose gel to allow good separation of annealed (120bp) and non-annealed product (smear) as seen in lanes 1-3. C: Example of a PCR colony screen used to identify constructs containing a miRNA. A forward primer specific to the hsa-miR30 backbone and a mcherry 5' reverse primer were used. PCR products were run on 1% agarose gels and positive clones were identified by a 1 Kb PCR product as can be seen in lanes 1-4 and 7-10. Previously identified constructs were used as positive controls (lane 10) and constructs lacking the miRNA were used as negative controls (lane 11). D: A schematic depiction of the transcribed pri-miRNA showing the hairpin loop, the position of the silencing sequence (highlighted in orange) and also the positions where Drosha and Dicer cut the primary and pre-miRNA respectively to form the mature miRNA. Folding predicted by mfold (<http://mfold.bioinfo.rpi.edu/>)

### **3.3.2 Efficient Vector-mediated RNAi in HEK 293 Cells**

In order to check that the RNAi vectors are correctly processed and capable of driving knockdown, their ability to knockdown GFP in HEK 293 cells was assessed. To do this, HEK 293 cells were co-transfected with ef1 $\alpha$ ::GFP and the RNAi vector with either a GFP or control miRNA under the control of the H2A.Zf promoter in a 3:1 ratio. Three days post transfection cells were prepared for flow cytometry analysis as per section 2.8.1. Figure 3.4 shows an example of the flow cytometry data collected. Cells singly transfected with empty pBluescript, ef1 $\alpha$ ::GFP or the RNAi vector were used as non-labelled, GFP labelled and RFP labelled cells respectively to calibrate the flow cytometer (Figure 3.3 A, B and C ). Measurements of the GFP intensity of cells are plotted on the y-axis and mcherry intensity on the x-axis. In cells transfected with either GFP miRNAs a downward shift can be seen in the GFP values compared to the control miRNA-transfected cells (Figure 3.3 D, E and F). This shift is greatest for cells transfected with the GFP #1 miRNA. To analyse the degree of knockdown in cells expressing control or GFP miRNAs the mean GFP:mcherry ratio of the mcherry positive cells (highlighted in red and labelled P3 in Figure 3.3) was used. Analysis of the mcherry positive population showed a 95% and 72% knockdown of GFP in cells transfected with the GFP #1 and GFP #2 miRNAs respectively compared to cells transfected with control miRNA (Figure 3.4).



**Figure 3.3: Fluorescent Flow Cytometry Analysis of GFP Knockdown in HEK 293 Cells.** The x-axis is a measure the RFP signal intensity and the y-axis is a measure of GFP signal intensity. A: non-labelled cells. B: GFP labelled cells. C: mcherry labelled cells. D-F: Cells co-transfected with GFP and RNAi silencing vector. D: Control miRNA. E: GFP #1 miRNA. F: GFP #2 miRNA. In cells transfected with the GFP miRNAs a downward shift can be seen in the intensity of GFP expression compared to cells expressing the control miRNA. This shift is largest with the GFP #1 miRNA.



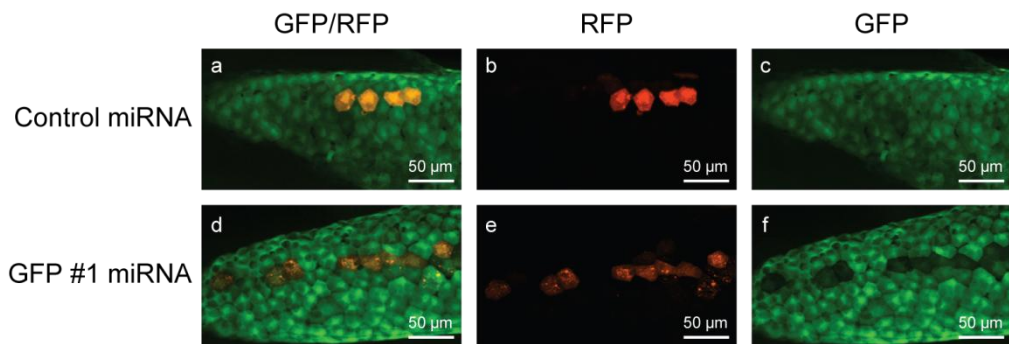
**Figure 3.4: Vector-mediated Knockdown of GFP in HEK 293 Cells.** Relative GFP levels after transfection with vectors containing a control miRNA (purple bars, n=9), GFP#1 miRNA (gold bars, n=9) or GFP#2 miRNA (yellow bars, n=9). GFP levels measured using fluorescent flow cytometry. Results were analysed by One-way ANOVA. \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ .

### **3.3.3 *in vivo* GFP Knockdown**

#### 3.3.3.1 Knockdown of Skin GFP in G0 Zebrafish

To test whether the RNAi vector could induce silencing in zebrafish embryos, transgenic *krt4::GFP* male zebrafish which express GFP in the keratinocytes of the skin were crossed with AB females, and the offspring were injected with the RNAi vector containing either a control or GFP #1 miRNA under the control of the *krt18* (keratinocyte-specific) promoter. Three days post injection, larvae were analysed for GFP and RFP expression using an Axioplan 2 microscope equipped with an apotome for optical sectioning. In larvae which had been injected with the control miRNA co-localisation of the GFP and RFP in keratinocytes was detected, and there was no difference in the GFP content of RFP positive and RFP negative cells. However, in larvae which had been injected with the GFP #1 miRNA reduced levels of GFP were detected in RFP positive cells compared to RFP negative cells (Figure 3.5). Therefore *krt18*-driven expression of GFP miRNA results in knockdown of GFP in zebrafish keratinocytes. The amount of knockdown achieved seems to be dependent on the amount of RFP expression in cells, as in cells in which only a trace amount of RFP can be detected GFP is still present, whereas strong expression of RFP coincides with complete knockdown of GFP (Figure 3.5).



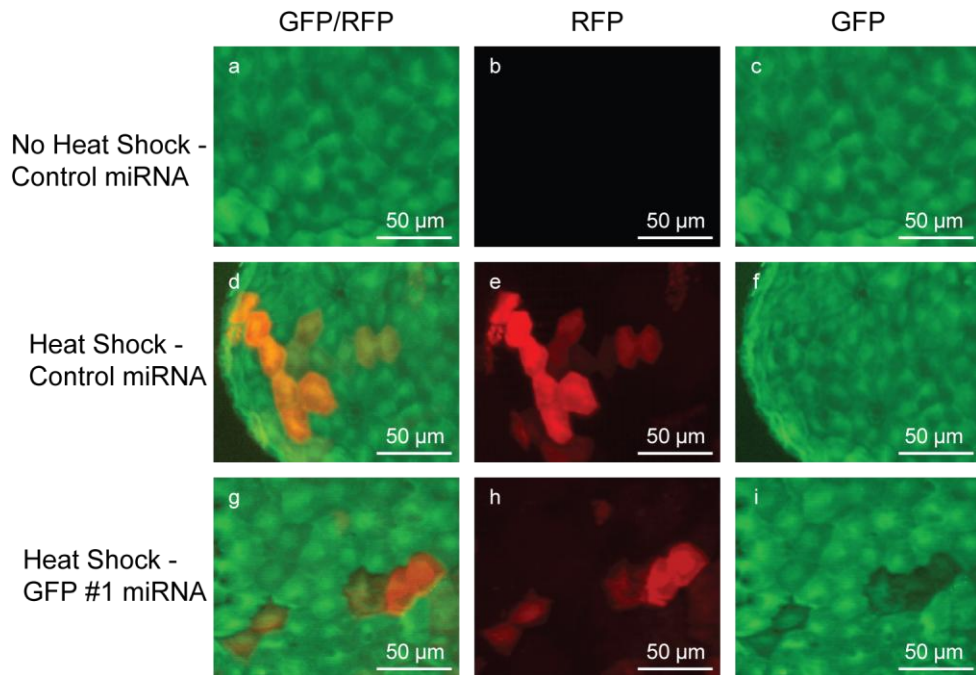


**Figure 3.5: Krt18-driven Knockdown of Skin GFP in G0 zebrafish.** Krt4::GFP transgenic zebrafish embryos expressing GFP in keratinocytes were injected with silencing vector expressing either a control or GFP #1 miRNA under the control of the krt18 promoter. Z stack images of the larvae were taken at 3 dpf and extended focus images analysed. a-c: GFP and RFP in zebrafish larvae which had been injected with control miRNA. Where the control miRNA is expressed, as indicated by RFP expression, co-expression of GFP and RFP can be seen (a). GFP levels in RFP positive cells are similar to levels in RFP negative cells as can be seen in the panel c. d-e: GFP and RFP of Zebrafish larvae which had been injected with GFP #1 miRNA. Where the GFP miRNA is expressed reduced levels of GFP can be detected (panels d and f). The amount of GFP knockdown seems to be proportional to the amount of miRNA (RFP) expressed as can be seen by comparing panels e and f.

RNAi vectors containing control or GFP #1 miRNAs under control of the ubiquitous promoters, H2A.Zf and ef1 $\alpha$ , were also injected into zebrafish expressing ubiquitous GFP. However, the complexity of expression due to mosaics in G0 zebrafish meant that analysis of these fish in a G0 setting was not possible.

### 3.3.3.2 Conditional Knockdown of GFP in G0 Zebrafish

To test whether knockdown could be induced in a conditional manner krt4::GFP male zebrafish were crossed with female AB zebrafish and the resulting embryos were injected with the RNAi vector expressing either a control or GFP #1 miRNA under the control of the heat shock protein 70 promoter (HSP70). One day post injection the embryos were heat-shocked at 37°C for 16 hrs before analysing for GFP and RFP expression in the skin at 3 dpf. In larvae which had been injected with the control miRNA but not heat-shocked, GFP but no RFP could be detected (Figure 3.6 a-c). In larvae which had been injected with the control miRNA and heat-shocked, co-localisation of the GFP and RFP in keratinocytes was detected and there was no difference in the GFP content of RFP positive and RFP negative cells (Figure 3.6 d-f). However, in larvae which had been injected with the GFP #1 miRNA and heat shocked, reduced levels of GFP were detected in RFP positive cells compared to RFP negative cells (Figure 3.6 g-i). Therefore, conditional, HSP70-driven expression of GFP miRNA results in knockdown of GFP in zebrafish keratinocytes. As with krt18-driven knockdown, the amount of knockdown achieved was dependent on the amount of RFP expression in cells. Cells with low level expression of RFP have an increased level of GFP compared to cells which express high levels of RFP.



**Figure 3.6: Conditional Knockdown of GFP in Skin of G0 Zebrafish.** Krt4::GFP transgenic zebrafish embryos expressing GFP in keratinocytes were injected with the RNAi vector expressing either a control or GFP #1 miRNA under the control of the HSP70 promoter. Zebrafish were heat shocked overnight at 37°C at 30 hpf, Z stack images of the larvae were taken at 3 dpf and extended focus images analysed. a-c: GFP and RFP in zebrafish larvae which had been injected with control miRNA with no heat-shock. Panels a and c show uniform GFP expression. Lack of expression of the RNAi vector as indicated by a lack of RFP expression can be seen in panel b. d-f: GFP and RFP in zebrafish larvae injected with control miRNA after heat-shock. Where the control miRNA is expressed, as indicated by RFP expression, co-expression of GFP and RFP can be seen (a). GFP levels in RFP positive cells are similar to levels in RFP negative cells as can be seen in panel f. g-i: GFP and RFP in zebrafish injected with GFP #1 miRNA after heat-shock. Where the GFP miRNA is expressed reduced levels of GFP can be detected (panels g and i). The amount of GFP knockdown seems to be proportional to the amount of miRNA (RFP) expressed as can be seen by comparing panels h and i.

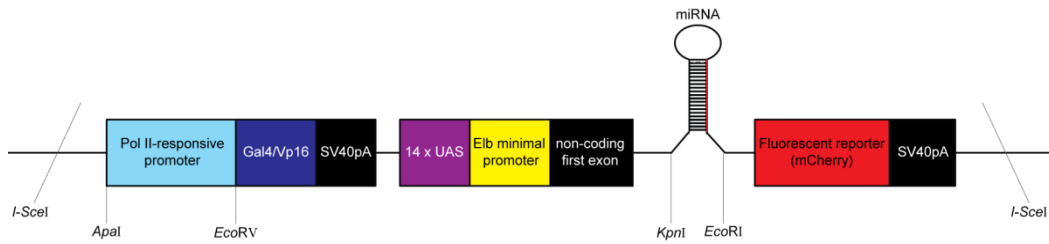
### 3.3.3.3 Generating Stable Transgenic (F1) Zebrafish

To generate stable transgenic F1 zebrafish G0 injected zebrafish were raised to sexually maturity and then inbred and the resulting zebrafish embryos screened under a fluorescent microscope for RFP expression. Unfortunately, after screening off-spring from approximately 50 mating pairs for each miRNA and promoter (krt18, H2A.Zf and ef1 $\alpha$ ) combination only one transgenic line (control miRNA under control of the ef1 $\alpha$  promoter) was detected. RFP levels in these animals were weak, making detection of transgenic zebrafish difficult. In order to be able to efficiently screen for transgenic zebrafish by virtue of RFP expression, increased levels of RFP would need to be expressed.

## **3.3.4 Boosting Expression of miRNA and RFP Expression with Gal4/UAS**

### 3.3.4.1 The Gal4-responsive RNAi Vector

In order to increase both the levels of RFP and miRNA expression achieved from RNAi vectors in stable transgenic zebrafish, the RNAi vector was modified to contain a tandem Gal4-VP16/UAS cassette (see section 3.2.2). A schematic representation of the final Gal4-VP16-responsive RNAi vector can be seen in Figure 3.7.



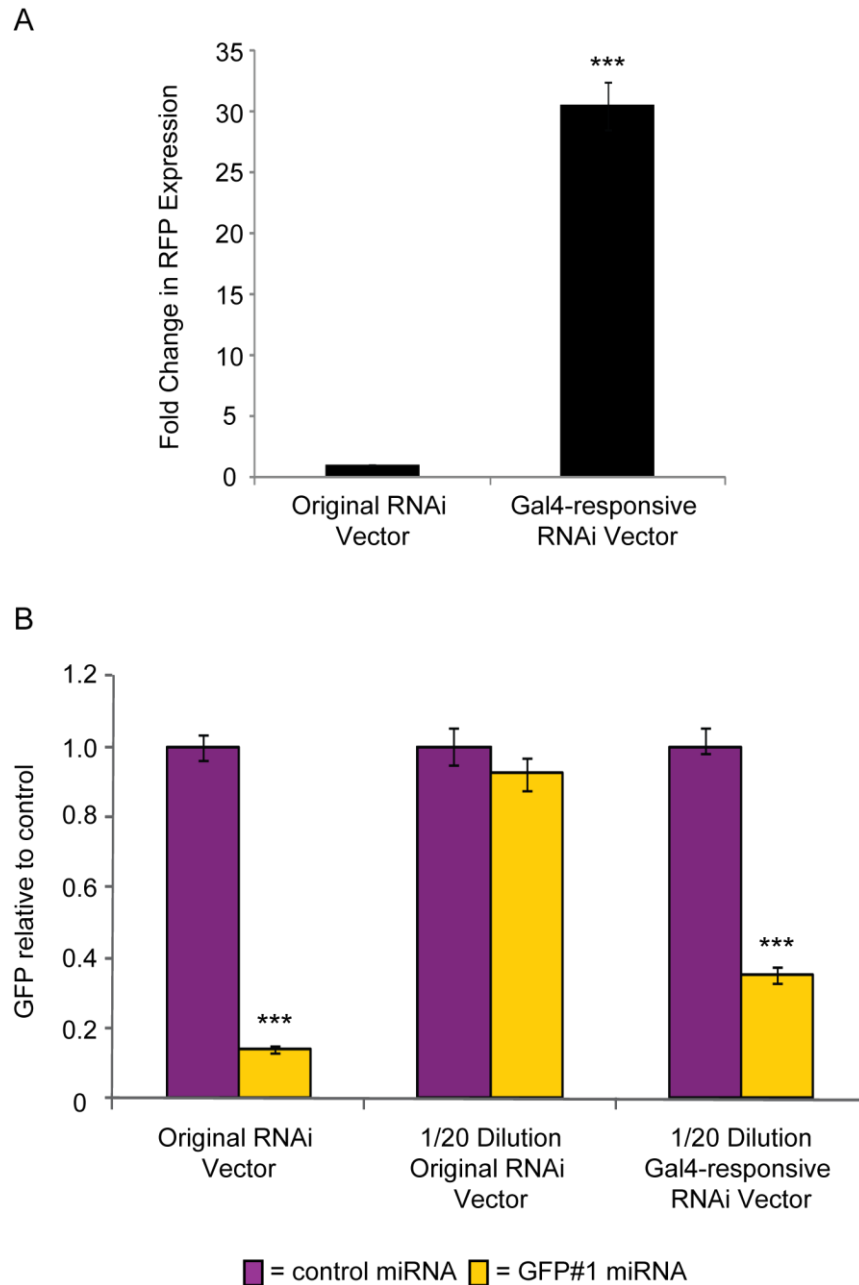
**Figure 3.7: Schematic representation of the Gal4-VP16-responsive RNAi vector.** This construct consists of a pol II-responsive promoter which drives expression the transcription factor Gal4/VP16. Gal4/VP16 then binds the 14 tandem Upstream Activation Sequence (UAS) repeats, which results in transcription of the non-coding first exon, miRNA-containing intron, and the fluorescent protein, mcherry. Pol II-responsive promoters can be easily cloned in using the *ApaI* and *EcoRV* restriction sites. miRNA sequences can be cloned in via *KpnI* and *EcoRI* restriction sites. The silencing cassette is flanked by *I-SceI* meganuclease restriction sites which aid integration of the vector into the zebrafish genome upon co-injection with *I-SceI* meganuclease.

In the Gal4-responsive RNAi vector a pol II-responsive promoter of choice drives transcription of the transcription factor, Gal4/VP16. Gal4/VP16 then binds to the 14 tandem Upstream Activation Sequence (UAS) repeats and results in transcription of the non-coding first exon, miRNA containing intron, and mcherry. The vector has been designed so that promoters can be easily exchanged via the *ApaI* and *EcoRV* restriction sites and miRNA sequences via the *KpnI* and *EcoRI* restriction sites. For a full vector map of Gal4-VP16-responsive RNAi vector see appendices 8.1.4.

#### 3.3.4.2 Gal4/UAS Increases Knockdown Efficiency in HEK 293 Cells

To test whether the insertion of a Gal4-VP16/UAS cassette into the RNAi vector improved transgene expression, whilst still able to efficiently knockdown genes, HEK 293 cells were co-transfected with *ef1α::GFP* and equal moles of either the original RNAi vector containing control or GFP #1 miRNA, or the Gal4-VP16-responsive RNAi vector containing control or GFP #1 miRNA. Due to the amount of RFP signal produced by Gal4-VP16-responsive RNAi vector being too high to accurately quantify, a 20-fold dilution of both the original and Gal4-VP16-responsive RNAi vector was used. Three days post transfection cells were analysed for GFP and RFP expression via fluorescent flow cytometry. Insertion of the Gal4-VP16/UAS

cassette into the RNAi vector led to a 30-fold increase in the expression of RFP (Figure 3.8 A). Whereas the original, undiluted RNAi vector resulted in an 85% knockdown of GFP the diluted vector induced only minimal silencing of 7%, however this was not shown to be statistically significant. The diluted Gal4-VP16-responsive vector on the other hand extremely significantly knocked down GFP by 65% (Figure 3.8 B). Therefore, insertion of the Gal4-VP16/UAS cassette led to an approximate 10-fold enhancement of knockdown efficiency.

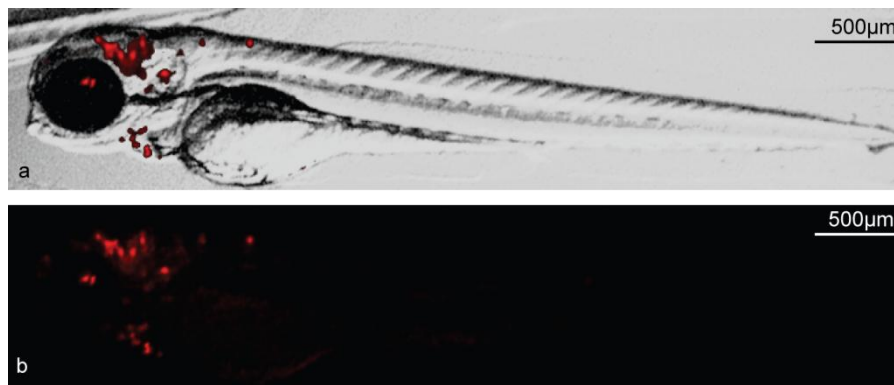


**Figure 3.8: Gal4-VP16/UAS Increases Transgene Expression and GFP Knockdown in HEK 293 cells.** A: Fold change in the level of RFP produced from the Gal4-VP16-responsive RNAi vector compared to the original RNAi vector. Equal moles of the original and Gal4-responsive RNAi vector were used. RFP levels were analysed by Independent samples T test. \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ . B: Relative GFP levels after transfection with the original RNAi vector, a 20-fold dilution of the original RNAi vector and a 20-fold dilution of the Gal4-VP16-responsive RNAi vector. Purple bars indicate GFP levels in control miRNA transfected cells. Gold bars indicate GFP levels in GFP #1 miRNA transfected cells. For each condition  $n=6$ . The original RNAi vector knocked down GFP by 86%. A 20-fold dilution resulted in no significant knockdown of GFP, whereas the same molar quantity of the Gal4-responsive vector resulted in knockdown of 65%. GFP and RFP levels measured using fluorescent flow cytometry. GFP levels in cells transfected with GFP #1 miRNA compared to the control miRNA were analysed by Independent samples T test. \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ .

#### 3.3.4.3 Generation of Gal4-responsive RNAi Transgenic Zebrafish

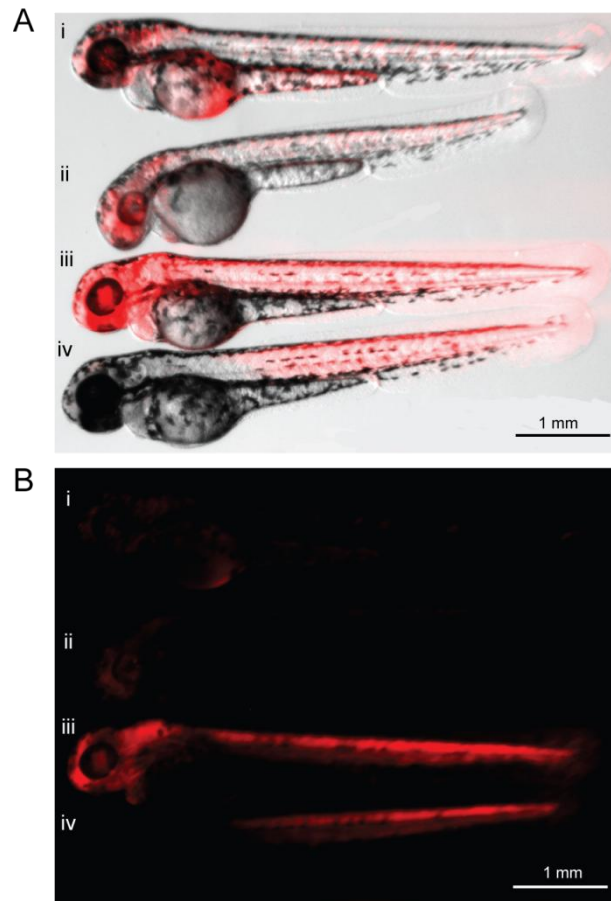
To generate stable transgenic zebrafish, the Gal4-VP16-responsive RNAi vectors containing the control and GFP #1 miRNA under the control of the ubiquitous promoter H2A.Zf were co-injected into zebrafish along with *I-SceI* meganuclease. Injected embryos were raised to adulthood and then inbred and the resulting off-spring screened under a fluorescent microscope for expression of the transgene. This time approximately 10% of breeding pairs gave rise to transgenic offspring. However, despite the use of a ubiquitous promoter, the expression pattern in all identified transgenic offspring was extremely mosaic with only a small minority of cells/tissues expressing the fluorescent protein. Figure 3.9 shows an example of the type of expression seen in *I-SceI*-mediated transgenic zebrafish. Expression is restricted to small clonal expansions of cells predominately in the brain. Varying degrees of expression in the brain were the most consistent expression pattern observed.





**Figure 3.9: *I-SceI*-mediated Transgenesis in F1 Zebrafish Expressing the Gal4-VP16-responsive RNAi Transgene under the Control of the Ubiquitous H2A.Zf Promoter.** A: merged brightfield and fluorescent image of F1 zebrafish at 4 dpf. B: fluorescent image of F1 zebrafish. Expression of the reporter gene mcherry is extremely mosaic in F1 zebrafish despite being under the control of the ubiquitous H2A.Zf promoter.

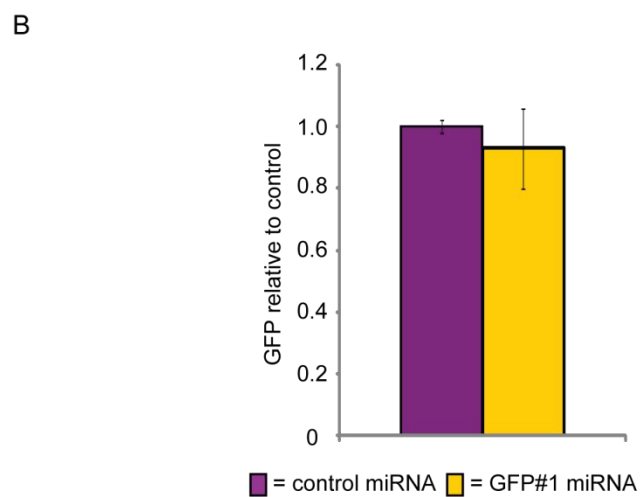
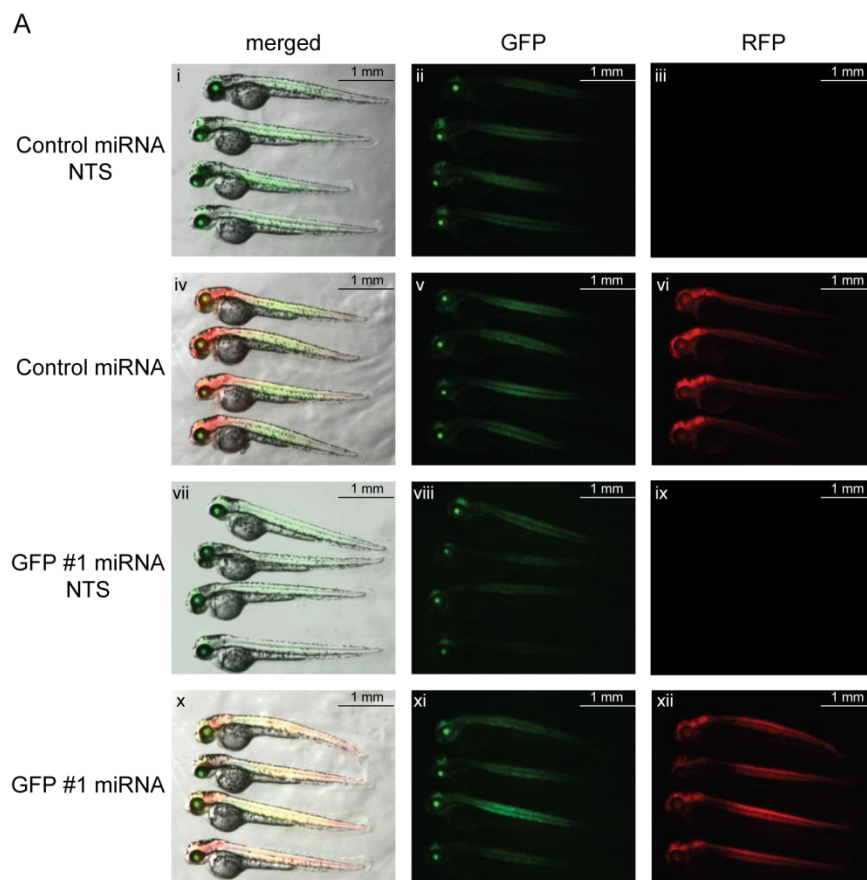
To see whether the rates of transgenesis could be improved and more ubiquitous expression of the transgene obtained, the Gal4-VP16-responsive RNAi cassette was transferred into a *To/2* background (see section 3.2.2.1). The *To/2* Gal4-VP16-responsive RNAi vector with control or GFP #1 miRNA was then co-injected into zebrafish embryos along with transposase mRNA. Analysis of the resulting offspring revealed a 37% rate of transgenesis. Of these the majority of fish still did not express the transgene ubiquitously, though the proportion of cells expressing the transgene was greatly improved. However, 32% of the founding zebrafish did give rise to embryos which appeared ubiquitous in fluorescent protein expression. Of the founders identified the germ line transmission of the transgene to their offspring ranged from less than 10% up to 100%. Figure 3.10 shows examples of the types of expression patterns detected in F1 zebrafish.

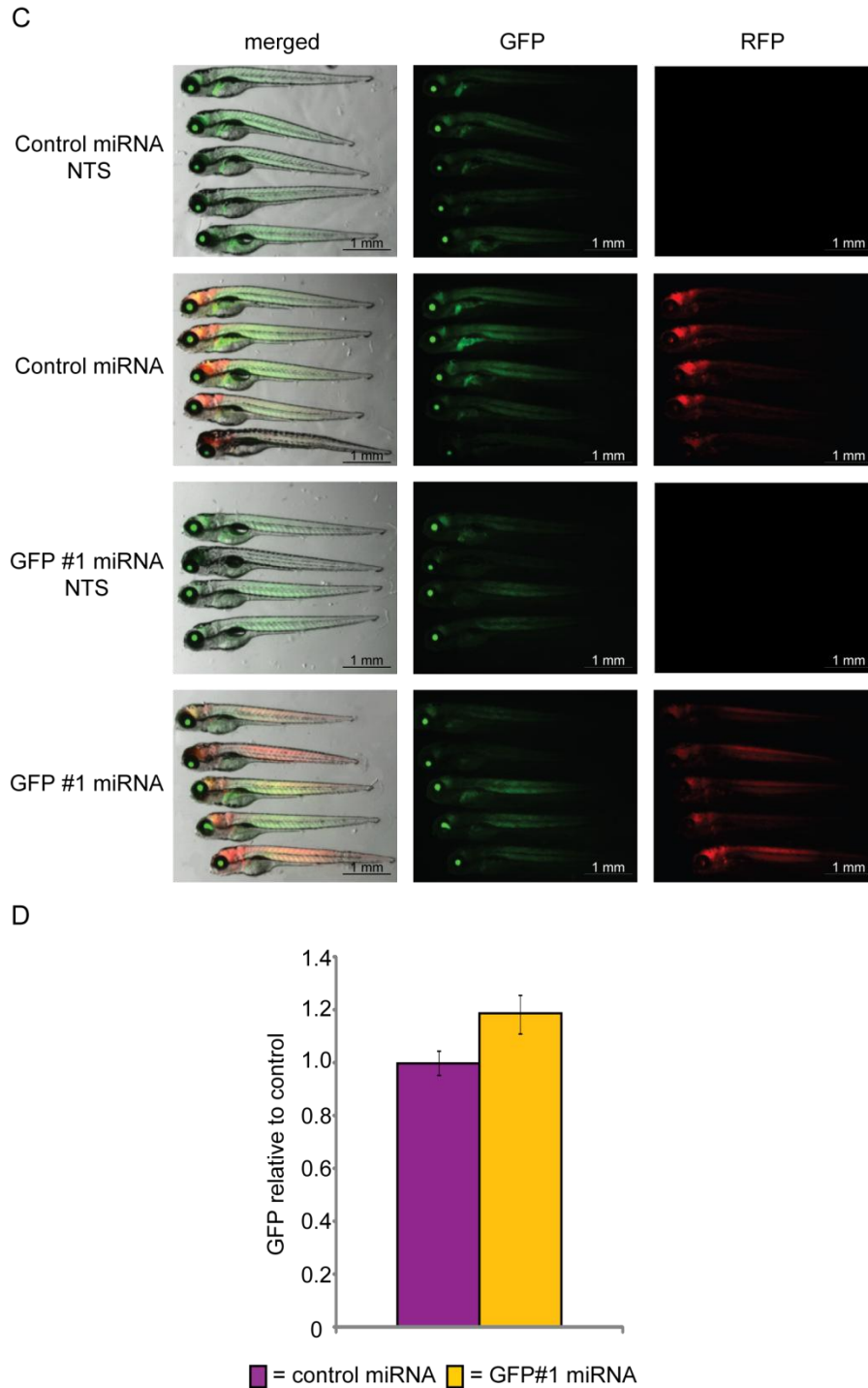


**Figure 3.10: *Tol2*-mediated Transgenesis in F1 Zebrafish Expressing the Gal4-reponsive RNAi Transgene under the Control of the Ubiquitous H2A.Zf Promoter.** A: merged brightfield and fluorescent image of F1 zebrafish at 2 dpf. B: fluorescent image of F1 zebrafish. The expression patterns achieved in the F1 generation varied from being extremely mosaic all over (i and ii) or being restricted to part of the zebrafish (iv) to being ubiquitous and uniform (iii).

#### 3.3.4.4 GFP Knockdown in Stable Transgenic Zebrafish

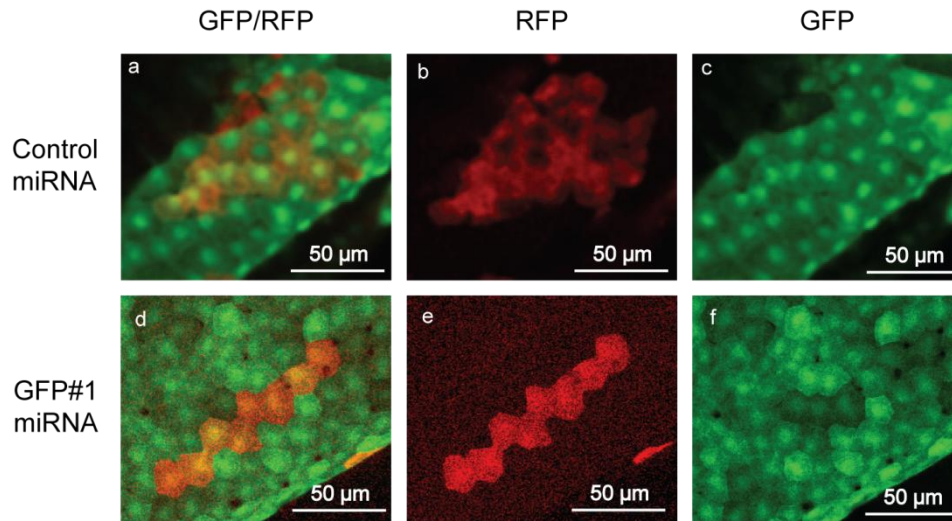
In order to test whether GFP can be knocked down in zebrafish stably expressing the Gal4-VP16-responsive RNAi vector under control of the H2A.Zf promoter, ubiquitous F1 zebrafish, expressing either the control or GFP #1 miRNA, as verified by genotyping (see section 2.6) were raised to adulthood and zebrafish with an approximate 50% transmission rate, indicative of single transgene integration were then crossed with the GFP transgenic line, *ef1α::GFP*. The double transgenic off-spring of these zebrafish (the F2 generation) were again raised to adulthood and the off-spring of adult male zebrafish (the F3 generation) were analysed for GFP and RFP expression. Figure 3.11 A and C show GFP levels present in transgenic zebrafish expressing either the control or GFP #1 miRNA at 2 and 5 dpf respectively. GFP levels in double transgenic animals were compared to siblings which only express the *ef1α::GFP* transgene and are referred to here as non-transgenic siblings (NTS). The presence of either the control miRNA or the GFP #1 miRNA, as indicated by the presence of red fluorescent protein, had no impact on the level of GFP compared to their relative NTS. In order to quantify the level of GFP in zebrafish expressing either the control or GFP #1 miRNA, *ef1α::GFP* zebrafish expressing miRNAs and their relative NTS were homogenised in trypsin and analysed by flow cytometry (see section 2.8.2). Figure 3.11 B and D shows that there is no significant difference in the relative GFP levels in zebrafish expressing either the control or GFP #1 miRNA compared to their NTS at 2 and 5 dpf respectively.





**Figure 3.11: Lack of GFP Knockdown in Stable Transgenic Zebrafish Expressing *ef1α::GFP* and a GFP miRNA.** A and C: merged and fluorescent images of *ef1α::GFP* zebrafish expressing either the control (iv-vi) or GFP #1 miRNA (x-xii) alongside their respective non-transgenic sibs (NTS) (i-iii and vii-ix respectively) A: 2 dpf. C: 5 dpf. B and D: flow cytometry quantification of GFP levels in *ef1α::GFP* zebrafish expressing either the control or GFP #1 miRNA. B: 2dpf zebrafish. D: 5dpf zebrafish. There is no significant reduction in GFP in zebrafish expressing the GFP #1 miRNA compared to zebrafish expressing the control miRNA (levels normalised to NTS levels). For each condition n=3. Results were analysed by Independent samples T test. \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ .

The discrepancy between GFP knockdown in keratinocytes in a G0 setting, but no global GFP knockdown in transgenic animals could be explained in one of two ways. Firstly, it could be that RNAi-mediated knockdown is effective in keratinocytes but not in other cell types. Alternatively, RNAi-mediated knockdown may be possible in a G0 setting, but knockdown is not transmissible. In order to try and address this, zebrafish transgenic lines expressing the Gal4-VP16-responsive RNAi transgene under the control of the *krt18* promoter and GFP under the control of the *krt4* promoter were created. It should be stated here however, that the identification of these lines was extremely difficult, as despite the use of Gal4-VP16-responsive vectors expression levels were extremely weak and expression was also, despite being a stable transgenic line, extremely mosaic. Furthermore, the expression levels deteriorated between 1 dpf when the embryos were first screened and 3 dpf when GFP levels were analysed. Figure 3.12, shows GFP levels in keratinocytes weakly expressing either the control or GFP #1 miRNA. Although some cells expressing the GFP #1 miRNA do appear to have slightly reduced GFP expression, this variation in GFP is no greater than background variation, as some cells which are not expressing the transgene also have reduced levels.



**Figure 3.12: Lack of GFP Knockdown in Stable Transgenic Zebrafish Expressing GFP and a GFP miRNA in Zebrafish Skin.** Double transgenic zebrafish expressing *krt4::GFP* and either the control (a-c) or GFP #1 (d-f) miRNA under the control of *krt18*-driven Gal4-Vp16/UAS. Where the control miRNA is expressed (a-c), as indicated by RFP expression, co-expression of GFP and RFP can be seen (a). GFP levels in RFP positive cells are similar to levels in RFP negative cells and can be seen in c. Where the GFP #1 miRNA is expressed (d-f) co-expression of GFP can also be seen. The level of GFP expression however, is reduced in some GFP #1 miRNA expressing cells, but this reduction is not beyond the background level of variation (f).



## 3.4 Discussion

The use of RNAi in a vector-mediated approach has the potential to provide the zebrafish research community with a much needed cheap and efficient technique for gene knockdown. The aim of this study was therefore to develop and validate a vector for vector-mediated RNAi and show that it can be used to knockdown genes in zebrafish in a stable and heritable fashion.

### 3.4.1 Validating a Vector for Vector-mediated RNAi

GFP miRNAs cloned into the pol II-responsive RNAi vector were shown to be able to effectively knockdown co-transfected GFP compared to the control miRNA in HEK 293 cells, thus demonstrating the vector is suitable for delivery of vector-mediated RNAi (Figure 3.4). The co-expression of red-fluorescent protein aids the analysis of knockdown as it is possible to evaluate the GFP content of the cherry red positive cells only.

In order to determine whether this vector was also capable of knocking down genes in zebrafish, vectors containing either the control or the most potent GFP miRNA, as determined in HEK 293 cells, were injected into single-stage zebrafish embryos stably expressing GFP. Detection of knockdown in a G0 setting is complicated by the fact that injection of plasmids into single stage zebrafish embryos results in a mosaic pattern of expression of the transgene due to progressive dilution of inherited episomal DNA and infrequent transgene insertion events. For this reason the keratinocytes of the zebrafish skin were chosen as the target for GFP knockdown, as their uniform and flat shape make them easily visualised and analysed for RNAi vector expression, as indicated by presence of the reporter, mcherry, and GFP content. In the first instance, efficient knockdown of skin-specific, Krt4-driven GFP was successfully demonstrated by Krt18-driven expression of the RNAi vectors containing the GFP miRNA compared to the control miRNA (Figure 3.5). Secondly, the heat-shock promoter HSP70 was used to drive expression of control and GFP miRNAs and was also shown to induce effective knockdown of GFP in the keratinocytes of 3 dpf zebrafish larvae (Figure 3.6). Thus, vector-mediated

RNAi seems to be possible in a G0 setting in zebrafish keratinocytes and this knockdown can be made conditional by the use of a heat-inducible promoter. The demonstration of GFP knockdown in G0 zebrafish is in line with work by Wang *et al.*, (2007) and Su *et al.*, (2008) who demonstrate efficient knockdown (~70%) of GFP and no tail (*ntl*) by means of *in vivo* T7 transcribed shRNAs and CMV transcribed shRNAs respectively.

To demonstrate that vector-mediated knockdown could be stably inherited from one generation to the next, G0 injected fish were raised to adulthood, inbred and the resulting offspring analysed for expression of the transgene. Unfortunately, after exhaustive breeding of zebrafish injected with either the control or GFP miRNA under the control of the Krt18, H2A.Zf and ef1 $\alpha$  promoters, only one stable transgenic line was identified. The lack of stable lines identified is most likely due to very low germ line *I-SceI*-mediated transmission rates combined with very low transgene expression levels which may have meant that some stable lines were missed because mcherry levels were not high enough to be detected on the Leica MZFIII fluorescent microscope. In keeping with the later theory HSP70 stable transgenic zebrafish were later identified as the HSP70 promoter leads to much stronger expression of mcherry than any of the other promoters tested. In order to generate a user-friendly system of vector-mediated RNAi in which transgenic zebrafish can be easily identified and high levels of miRNA produced, a method of increasing promoter activity was required.

#### 3.4.1.1 Increasing Promoter Activity and miRNA Expression with Gal4-

##### VP16/UAS

Gal4 is a transcription factor identified in the yeast *Saccharomyces cerevisiae* and induced by galactose. It binds to 17 bp upstream activation sequences (UASs) and leads to expression of GAL10 and GAL1 genes (Hashimoto *et al.*, 1983). Since the identification of this system it has been widely used in *Drosophila* where transgenic expression of Gal4 binds to multiple tandem UAS sites and leads to high level expression of transgenes (Duffy, 2002). It was first used in zebrafish by Scheer and Campos-Oriega in 1999 to generate stable transgenic zebrafish expressing *myc-notch:intra* (a

fusion gene encoding 6 myc epitopes fused to the intracellular domain of notch1)(Scheer and Campos-Ortega, 1999). Despite reporting robust and stable expression of the effector, expression levels were low. Use of Gal4 fused to the transcriptional activator domain VP16 of the herpes simplex virus (developed by Sadowski *et al.*, 1998) improved expression levels in transient zebrafish assays (Koster and Fraser, 2001), and recently the Gal4-VP16/UAS system has been used as an enhancer trap in both a binary (separate Gal4-VP16 activator and UAS effector transgenic lines) and a tandem system (single vector with both Gal4-VP16 activator and UAS effector cassettes) in zebrafish and has resulted in the high level, stable and robust reporter expression in several different cell types (Davison *et al.*, 2007; Scott *et al.*, 2007). Insertion of a Gal4-VP16/UAS cassette into the RNAi vector downstream of the required promoter should lead to high level expression of the transgene in this case miRNA and mcherry. This was tested in HEK293 cells and indeed the Gal4-VP16-responsive vector under control of the H2A.Zf promoter resulted in 30 times the amount of mcherry compared to the original RNAi vector and increased GFP knockdown 10-fold (Figure 3.8). The difference in the fold increase in mcherry expression and GFP knockdown may suggest that at some point the available RNAi machinery in the cell may become limiting and thus prevent any further knockdown.

Gal4-VP16-responsive vectors were then used to generate stable transgenic lines. Initially vectors incorporating *I-SceI* restriction sites either side of the transgene were used but these vectors although capable to driving high levels of transgenes resulted in extremely mosaic expression even when the ubiquitous promoter H2A.Zf was used (Figure 3.9). Using an alternative vector, in which the transgene is surrounded by arms of the medaka *To2* transposable element and injected along with transposase mRNA, lead to greatly improved levels transgenesis, and although transgene expression was in many cases still mosaic, many more cells were targeted, and it was possible to identify lines of zebrafish which expressed high levels of the transgene ubiquitously and uniformly (Figure 3.10).

Variegated expression of transgenes is a common problem in zebrafish transgenesis and there are three potential reasons for this. Firstly, transgene expression is extremely dependent on integration site, which at present cannot be easily controlled in zebrafish. If a transgene integrates close to transcriptional enhancers or repressors the expression of the transgene may be affected. Secondly, the heterochromatin state of DNA surrounding the insertion site may also play an important role, as heterochromatin renders DNA less accessible to transcription. Furthermore, the heterochromatin state of DNA may vary in a cell type dependent manner, thus generating variegated expression patterns of transgenes. Thirdly, injection of plasmid DNA can result in the integration of large concatemers of DNA into the genome and these have been shown to be silenced through chromatin modification (Garrick *et al.*, 1998). The use of a meganuclease or transposon based system of transgenesis however, should prevent concatemer formation and aid single transgene integration (Kawakami *et al.*, 2000; Thermes *et al.*, 2002) and so this is unlikely to be the cause for variegated expression in F1 zebrafish identified here. Recently however, the tandem UAS repeats used in Gal4/UAS systems have been shown to be especially liable to silencing induced by DNA methylation due to the large number of CpG dinucleotides in the UAS sequence (Goll *et al.*, 2009). Methylation of the UAS sequences may therefore explain the variegation in expression patterns seen here. As methylation events are inheritable UAS transgene expression may be subject to progressive silencing resulting in increased variegation with increasing generations. However, despite the variegation seen in some F1 zebrafish, with the aid of *in trans* reporter gene expression it was possible to identify zebrafish with fairly uniform and ubiquitous expression patterns which were maintained through several generations. Consistent with this, Davison and colleagues also report stable and robust expression patterns in most of their Gal4-VP16/UAS enhancer trap lines over 3 generations (Davison *et al.*, 2007). Where progressive loss of expression was seen, this was often associated with a greater than 50% germ line transmission rate, and so may simply reflect the segregation of multiple genome insertions. For analysis of gene knockdown in zebrafish as well as selecting fish which gave rise to robust and uniform expression, fish

with a 50% transmission rate, indicative of single transgene insertion, were also selected. Although it has been possible to identify appropriate zebrafish for analysis, the methylation induced silencing of tandem UAS sequences is a major limitation in the use of this transgenic technology. It may be possible that reducing the number of UAS repeats will reduce the silencing effect. Indeed, Asakawa and colleagues reported reproducible and robust expression patterns from their constructs containing 5 tandem UAS repeats (Asakawa and Kawakami, 2008). Moreover, co-expression of two independent UAS transgenes were consistently detected in the same cells, suggesting that UAS transgenes were not being silenced (Asakawa and Kawakami, 2008).

Differences in the amount of variegation observed between zebrafish generated by means of meganuclease and zebrafish generated by transposons may simply reflect the increased efficiency of transposon mediated transgenesis, which increases the likelihood of eventually identifying zebrafish with robust and uniform expression patterns. Alternatively, it may reflect differences in likely insertion sites. Indeed, mapping of *To12* insertion sites in human cells has revealed significant underrepresentation of chromosomal sites with H3K27me3 histone marks, which are typically associated with transcriptionally repressive heterochromatin (Grabundzija *et al.*, 2010). Another possibility is that the transposon arms of *To12* may act as insulators, protecting the transgene from the influences of heterochromatin.

As well as potential problems with the silencing of Gal4/UAS-responsive transgenes, there have also been concerns over the use of the transcriptional enhancer VP16 as it has been shown to have toxic effects in vertebrate cells (Gill and Ptashne, 1988; Sadowski *et al.*, 1988). It is thought that these toxic effects are due to the over-exploitation of important transcription factors. Whilst creating the transgenic lines required for this research a couple of the transgenic lines created did show signs of toxicity. In one case this coincided with multiple copy integration. In another line, zebrafish larvae developed normally, but as adults the fish had a shortened axis, curvature of the spine and reduced fertility. However, although widely

and strongly expressed, overall detectable toxicity was extremely low, <5%. Consistent with this Asakawa *et al.*, (2008) and Scott *et al.*, (2007) both report Gal4-VP16 toxicity at 4%. The toxic effects associated with Gal4-VP16 can according to Asakawa *et al.*, (2008) be eliminated altogether through the use of Gal4FF which consists of the DNA binding domain of Gal4 combined with two of the transcriptional enhancer modules from VP16.

The benefit of using a Gal4/UAS system to express miRNAs and reporter genes is not only that it results in high level expression of both the primary miRNA and the reporter gene, but also that this system could be used as a binary system. This means that separate transgenic lines could be identified, one expressing Gal4 (the activator line) and the other containing the UAS and transgene (the effector line). Combining activator and effector lines would then lead to the expression of miRNA and reporter. This may be particularly useful to maintain transgenic lines in which knockdown of the target gene results in lethality or infertility. In addition, it means that Gal4 driver lines could be employed to drive expression of miRNAs in particular lineages for which enhancer motifs have not yet been identified.

### **3.4.2 Lack of GFP Knockdown in Zebrafish Stably Expressing miRNAs**

To test whether GFP knockdown is heritable, stable transgenic male zebrafish expressing both GFP and the Gal4-VP16-responsive RNAi vector with either the control or GFP miRNA under the control of the ubiquitous H2A.Zf promoter were crossed to wild type female zebrafish. Visual and fluorescent flow cytometry analysis of the resulting offspring at 2 and 5 dpf showed no significant knockdown of GFP in fish expressing the GFP miRNA compared to fish expressing the control miRNA (Figure 3.11). This is contrary to GFP knockdown seen in the keratinocytes of the G0 fish, raising the possibility that GFP knockdown is not heritable or, that keratinocytes are amenable to RNAi whereas other cells in the zebrafish are not. To test this, I attempted to create double transgenic zebrafish expressing both the Gal4-VP16 vector expressing the GFP or control miRNA under the control of the Krt18 promoter and Krt4::GFP. Although, transgenic zebrafish were

identified the expression of the RNAi vector was extremely weak and mosaic throughout the zebrafish. In addition, the level of expression of the RNAi vector appeared to reduce over time. Limited and varied expression of the Gal4-VP16-responsive transgene in keratinocytes possibly indicates that these cells are silencing the vector, most likely through DNA methylation as discussed previously. Analysis of those zebrafish which did express both GFP and the RNAi vector, albeit in a mosaic manner, revealed however, that unlike in the G0 analysis where expression of the GFP #1 miRNA led to significant reductions in GFP beyond the background variation, expression of the GFP #1 miRNA in transgenic fish did not (Figure 3.12). Although, this seems to indicate that knockdown is not transmissible through generations, the extremely low expression levels detected in the cells means this analysis is not sufficient to draw such conclusions. However, taking together the results put forward here showing a lack of GFP knockdown in transgenic zebrafish expressing GFP miRNA ubiquitously, with previous reports by Su et al., (2008), and Wang et al., (2007) who detected knockdown of ubiquitous GFP in a G0 setting, seems to support the idea that while RNAi may be feasible in a G0 setting, knockdown is not stably transmitted to the next generation. There are several possible explanations for this. Firstly, expression in G0 zebrafish is often extremely high due to the presence of multiple copies of the transgene and high level expression of the transgene may be required in order for enough miRNA to be produced to impact GFP levels. If levels in stable transgenic zebrafish are lower, this may explain the lack of knockdown detected. However, the use of Gal4-VP16-responsive vectors in combination with the H2A.Zf promoter resulted in very high levels of expression in stable transgenic lines and so it is unlikely that expression levels were the limiting factor in these fish. Another, possible explanation is that the introduction of large amounts of foreign DNA into G0 zebrafish results in the induction of the RNAi machinery. This seems a highly plausible explanation, especially since the RNAi pathway is thought to have evolved as an innate response to foreign nucleic acids (Obbard *et al.*, 2009). In stable transgenic lines the integrated transgene however may not elicit such a response, resulting in the lack of knockdown observed. In order to test this hypothesis, stable transgenic embryos expressing both GFP and the RNAi

vector could have been injected with empty vector DNA or other components of the injection mix to see whether presence of exogenous DNA or any other injection mix component resulted in the induction of an RNAi response.

### **3.4.3 Conclusion**

A vector suitable for the delivery of miRNAs has been identified and shown to effectively knockdown GFP in HEK 293 cells and in transient assays in zebrafish. This vector however, led to extremely low level expression in transgenic zebrafish, making the identification of transgenic lines extremely difficult. The introduction of the Gal4-VP16/UAS cassette boosted the expression level of miRNA and reporter in HEK 293 cells and in stable transgenic zebrafish. However, despite being able to effectively knockdown GFP in HEK 293 cells, no knockdown was observed in stable transgenic lines. The discrepancy between knockdown in G0 zebrafish and the lack of knockdown in stable transgenic lines may suggest that the introduction of exogenous DNA into G0 zebrafish may provoke an RNAi response which leads to GFP knockdown.



## 4 Chapter Four: Knocking Down PINK1

### 4.1 Introduction

At the same time as targeting GFP for knockdown, and encouraged by the preliminary results of chapter one other similarly encouraging results by Wang *et al.*, (2007) and Su *et al.*, (2008), I also set about targeting the endogenous Parkinson's disease-associated gene, PINK1.

Parkinson's disease is the second most prevalent neurodegenerative disease and is characterised by the selective loss of the dopaminergic neurons of the *substantia nigra pars compacta* (SNpc) and accompanied by the development of proteinacious neuronal inclusions called Lewy bodies. P-TEN induced putative kinase 1 (PINK1) is a serine threonine kinase whose loss of function mutations have been shown to be the second most common cause of autosomal recessive juvenile Parkinson's disease (Valente *et al.*, 2004a) and mutations in PINK1 have also been shown to be involved in the sporadic forms of the disease (Valente *et al.*, 2004b).

PINK1 has been shown to localise to the mitochondria and to be important in protecting cells from oxidative stress induced cell death through the phosphorylation of TRAP1 (Pridgeon *et al.*, 2007), maintaining normal respiratory function (Liu *et al.*, 2009) and regulating mitochondrial calcium flux (Marongiu *et al.*, 2009). It has also been shown to be involved in protein turnover through the regulation of parkin, an ubiquitin ligase also associated with early onset parkinsonism (Yang *et al.*, 2006), and protein quality control through the association with HrtA2 (Plun-Favreau *et al.*, 2007).

Animal models PINK1 Parkinson's disease have so far predominantly relied on *Drosophila* and mice. While *Drosophila* have proved valuable in understanding pathways associated with loss of PINK1, PINK1 knockouts fail to recapitulate the complex aetiology of Parkinson's disease (Clark *et al.*, 2006; Park *et al.*, 2006; Yang *et al.*, 2006). Knockout mice on the other hand, although not showing signs of dopaminergic cell loss, do show some of the other hallmarks of Parkinson's disease, such as impaired mitochondrial respiration in the striatum, reduced striatal plasticity and

reduced dopamine release (Gautier *et al.*, 2008; Kitada *et al.*, 2007). An animal model which successfully recapitulates the aetiology of the disease while providing the advantages of small size, ease of manipulation and amenability for high-throughput screening, would therefore be extremely valuable and it is hoped that zebrafish as a vertebrate with its complex and analogous neuroanatomy will provide such a system.

In the hope of generating a PINK1 Parkinson's model, several groups have recently demonstrated and characterised knockdown of PINK1 by morpholinos in zebrafish embryos (Anichtchik *et al.*, 2008; Xi *et al.*, 2010). Reports by Anichtchik *et al.*, (2008) reported that PINK1 knockdown resulted in severe developmental defects with a >30% loss in tyrosine hydroxylase (TH, a marker of dopaminergic and noradrenergic neurons) positive cells. However, others have reported much more modest dopaminergic neuron cell loss (Anichtchik *et al.*, 2008; Sallinen *et al.*, 2010; Xi *et al.*, 2010). The discrepancy in results is probably due to common off-target and non specific effects associated with morpholinos, thus further emphasising the need for a different approach to knockdown genes and to determine the true effect of loss of PINK1.

Moreover, recent RNAi experiments in mice have demonstrated the accessibility of mouse PINK1 to vector-mediated RNAi gene knockdown (Zhou *et al.*, 2007), raising the possibility that zebrafish PINK1 may also be an accessible target for RNAi gene knockdown.

#### **4.1.1 Aims**

The aims of this chapter are therefore to:

- identify and characterise the zebrafish PINK1 homolog.
- identify potential polymorphic regions in the PINK1 gene.
- identify efficient PINK1 miRNAs in HEK293 cells.
- generate transgenic lines expressing PINK1 and control miRNAs and analyse transgenic lines for knockdown of PINK1.

## 4.2 Material and Methods

For general materials and methods see chapter two.

### 4.2.1 Cloning miRNAs

miRNAs against PINK1 were made using the primers listed in Table 4.1 as described in sections 2.5 and 3.3.1.

miRNA	Primer sequence	
PINK1 #1	Fwd	GCGG <b>GGTACC</b> TGCTGTTGACAGT <b>GAGCGA</b> GGGTTTGGGTCTGATTGAACA <b>TAGTGAAGCCACAG</b> ATG
	Rev	CCGC <b>GAATTC</b> CCTTGAAGTCCGAGGCAGTAGGCAG <b>GGTTTGGGTCTGATTGAACA</b> TACATCTG TGGCTTCAC
PINK1 #2	Fwd	GCGG <b>GGTACC</b> TGCTGTTGACAGT <b>GAGCGA</b> GCCTTCCGAAGCCCTTTACAT <b>TAGTGAAGCCACAG</b> ATG
	Rev	CCGC <b>GAATTC</b> CCTTGAAGTCCGAGGCAGTAGGCAG <b>GCCTTCCGAAGCCCTTTACAT</b> TACATCTG TGGCTTCAC
PINK1 #3	Fwd	GCGG <b>GGTACC</b> TGCTGTTGACAGT <b>GAGCGA</b> CCGACTCAATCCACATGGTTT <b>TAGTGAAGCCACAG</b> ATG
	Rev	CCGC <b>GAATTC</b> CCTTGAAGTCCGAGGCAGTAGGCAG <b>CCGACTCAATCCACATGGTTT</b> TACATCTG TGGCTTCAC
PINK1 #4	Fwd	GCGG <b>GGTACC</b> TGCTGTTGACAGT <b>GAGCGA</b> GCAAGGAGAGCTGACTTTGAA <b>TAGTGAAGCCACAG</b> ATG
	Rev	CCGC <b>GAATTC</b> CCTTGAAGTCCGAGGCAGTAGGCAG <b>GCAAGGAGAGCTGACTTTGAA</b> TACATCTG TGGCTTCAC
PINK1 #5	Fwd	GCGG <b>GGTACC</b> TGCTGTTGACAGT <b>GAGCGA</b> GGAAAGATTATGTGATTGGGAA <b>TAGTGAAGCCACAG</b> ATG
	Rev	CCGC <b>GAATTC</b> CCTTGAAGTCCGAGGCAGTAGGCAG <b>GGAAAGATTATGTGATTGGGAA</b> TACATCTG TGGCTTCAC
PINK1 #6	Fwd	GCGG <b>GGTACC</b> TGCTGTTGACAGT <b>GAGCGA</b> GCAGTGGAGATACCTGCTGAT <b>TAGTGAAGCCACAG</b> ATG
	Rev	CCGC <b>GAATTC</b> CCTTGAAGTCCGAGGCAGTAGGCAG <b>GCAGTGGAGATACCTGCTGAT</b> TACATCTG TGGCTTCAC
Control	Fwd	GCGG <b>GGTACC</b> TGCTGTTGACAGT <b>GAGCGA</b> AGTCTGTGCGCAGAAATATAT <b>TAGTGAAGCCACAG</b> ATG
	Rev	CCGC <b>GAATTC</b> CCTTGAAGTCCGAGGCAGTAGGCAG <b>AGTCTGTGCGCAGAAATATAT</b> TACATCTG TGGCTTCAC

**Table 4.1: Primers Used in the Production of PINK1 and Control miRNAs.** The sense sequence of the target is shown in grey on the forward primer and red on the reverse primer. *KpnI* and *EcoRI* sites are indicated in turquoise and yellow respectively. The rest of the sequence is based on the miR-30 backbone identified by Zeng *et al.* (2002).

### 4.2.2 Sequencing PINK1

PINK1 was amplified from wild type zebrafish cDNA (extracted from equal numbers of embryos from 8 breeding pairs) using *Pfu* DNA polymerase (Promega) and following manufacturer's guidelines in a nested PCR

approach similar to that described in section 2.6.1.1. Cycling conditions for both rounds of PCR were: 95°C for 5 mins and then 24 cycles of 95°C for 30 secs, 55°C for 40 secs and 72°C for 4 mins, followed by a final extension of 72°C for 10 mins. The primers for the first and second round PCR are listed in

Table 4.2. Amplified PINK1 was sequenced using M13 fwd and rev primers (Invitrogen).

Primer	Sequence
PINK1 Fwd 1	GTAAAGCATGTTCTCAGCCG
PINK1 Rev 1	GGCTGAGAGTTAGACATCAG
PINK1 Fwd 2 (M13 fwd)	<u>TGTA</u> AAACGACGGCCAGTGT <u>TTTTCCAGCTGGGACTTC</u>
PINK1 Rev2 (M13 Rev)	<u>CAGGAAACAGCTATGACGATGTTGGCGAGGAAAG</u>

**Table 4.2: Primers used in Nested PCR to Amplify and Sequence PINK1.** Sequences are given in a 5' to 3' format. M13 fwd and rev binding sites are underlined.

#### 4.2.3 Cloning GFP:PINK1 Fusion Expression Vector

To create a GFP:PINK1 fusion expression vector, full-length PINK1 was amplified using general PCR methods (see section 2.4.1) with the forward primer 5'-ATGTCAGTAAAGCATGTTCTCAGCC-3' and reverse primer 5'-CTATGGCTGAGAGTTAGACA-3' and cloned into NT-GFP Fusion TOPO® TA Expression Kit (Invitrogen) following manufacturers' instructions

#### 4.2.4 Detecting PINK1 mRNA: RT-qPCR

PINK1 transcript levels were measured using the primer listed below and following the RT- qPCR protocol detailed in section 2.9.

Gene	Accession Number	Primer Sequence	
<i>*β-actin1</i>	NM_131031.1	Forward	CGAGCTGTCTTCCCATCCA
		Reverse	TCACCAACGTAGCTGTCTTTCTG
<i>*ef1α</i>	NM_131263.1	Forward	CTGGAGGCCAGCTCAAACAT
		Reverse	ATCAAGAAGAGTAGTACCGCTAGCATTAC
† <i>ef1α</i> (human)	NM_001402.5	Forward	CTGAACCATCCAGGCCAAAT
		Reverse	GCCGTGTGGCAATCCAAT
<i>PINK1</i>	NM_001008628.1	Forward	GCGAGGAGTATCCTGATGTC
		Reverse	CAGGGGTAATTCTTCATGAC
<i>*rpl13 α</i>	NM_212784	Forward	TCTGGAGGACTGTAAGAGGTATGC
		Reverse	AGACGCACAATCTTGAGAGCAG

**Table 4.3: RT-qPCR Primer Sets used in PINK1 Gene Transcript Analysis.** Primers used for qPCR analysis are listed in a 5' to 3' manner. Reference genes used are highlighted in grey. Accession numbers of genes analysed are also given. \* indicates primers published by Tang *et al.*,(2007). † indicates primers published by Hamalainen *et al.*,(2001).

#### 4.2.5 Detection of PINK1 Protein

PINK1 protein levels were detected by western blot using methods described in section 2.10. The rabbit polyclonal anti-PINK1 antibody (Cayman Chemicals) was used at 1 µg/ml followed by an anti rabbit IgG HRP-conjugate used at 1:10 000.

## 4.3 Results

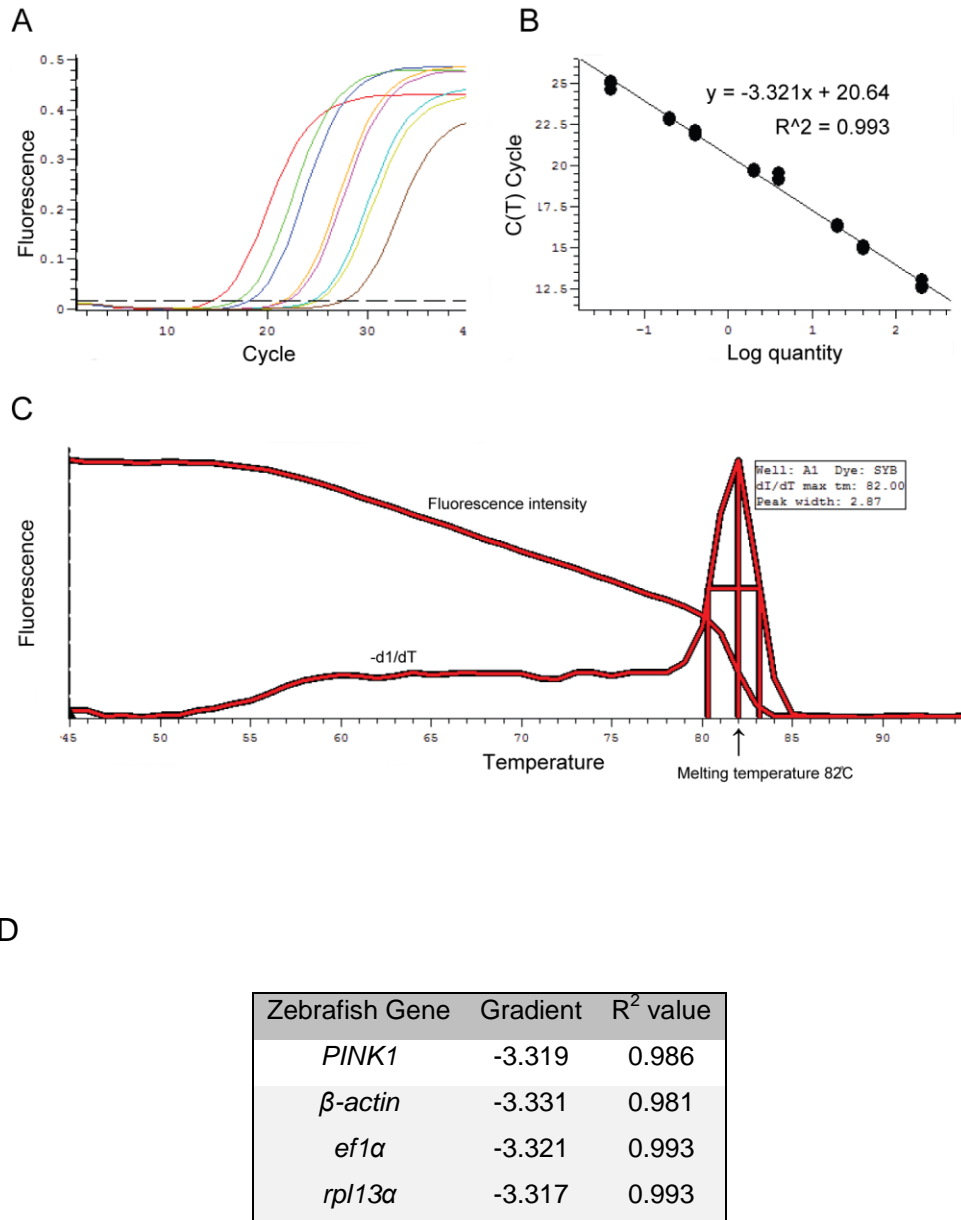
### 4.3.1 Identification and Expression of PINK1

Zebrafish *PINK1* was identified by performing tblastn analysis of the Ensembl zebrafish database for sequences with high homology to human *PINK1*. A putative *PINK1* transcript was identified and confirmed by PCR Topo cloning and sequencing. Zebrafish *PINK1*, resides on chromosome 23 and encodes a 574 aa protein which is 53.8% identical and 66.2% similar to the human *PINK1* protein. The PKc (protein kinase catalytic) domain (amino acids 271 to 501 in the human sequence) however is more highly conserved, being 71% identical and 80.1% similar and the kinase active site (amino acids 358 to 370 in the human sequence) is 92.3% identical and 100% similar. For a full alignment of human and zebrafish *PINK1* proteins see appendices section 8.2.

#### 4.3.1.1 Validation of qPCR Primers

In order to analyse the expression of *PINK1* throughout development and across tissues, Sybr green qPCR assays were designed and validated against zebrafish *PINK1* and the house-keepers *ef1 $\alpha$* ,  *$\beta$ -actin* and *rpl13 $\alpha$* . *ef1 $\alpha$* ,  *$\beta$ -actin* and *rpl13 $\alpha$*  were chosen as house-keepers because they were shown to have the greatest stability in a panel of zebrafish house-keepers throughout development and across tissues (Tang *et al.*, 2007). Sybr green qPCR works on the principle that Sybr green binds with high affinity to dsDNA and when it does it emits a green fluorescent signal. As the PCR reaction progresses, there is an increasing amount of dsDNA and therefore an increase in fluorescent signal. In order to compare the amount of PCR product, and hence the amount of starting DNA in different samples, a threshold fluorescent level is set and the number of cycles each sample takes to reach this threshold level is recorded. This level is known as the cycle threshold or CT value of a sample. The lower the CT value the higher the levels of starting template DNA. In order to validate primers for their PCR efficiency, the CT value for each sample of a serial dilution of cDNA (Figure 4.1 A) was determined and plotted against the log quantity of cDNA used in each reaction (Figure 4.1 B) to produce a straight line. A slope with

a gradient of -3.32 indicates that the primers are 100% efficient; i.e with each increase in cycle number there is a doubling in the amount of PCR product. Values of -3.1 and -3.58 are equivalent to 90% and 110% efficiency respectively. Only primers which fell between these ranges and gave rise to a correlation coefficient ( $R^2$ ) of at least 0.97 were accepted for use in cDNA quantification. A list of gradients and  $R^2$  values for each primer pair used can be seen in Figure 4.1 D. In order to further validate that qPCR primers were specific for a single cDNA species melting curve analysis between 45°C and 95°C was performed. Figure 4.1 C shows a drop in fluorescent intensity with increasing temperature, with a sharp drop appearing around 82°C. This drop in fluorescence is due to the strands of dsDNA dissociating and releasing the Sybr green (and thereby causing it to stop fluorescing) with increasing temperature. The sharp drop at 82°C indicates the melting temperature of the PCR product. The negative of the first derivative over time ( $-dI/dT$ ), a measure of rate of change, is also plotted and shows a single peak at 82°C. Presence of a single peak indicates the amplification of a single product, as other products would have a different melting temperature and so result in multiple peaks.

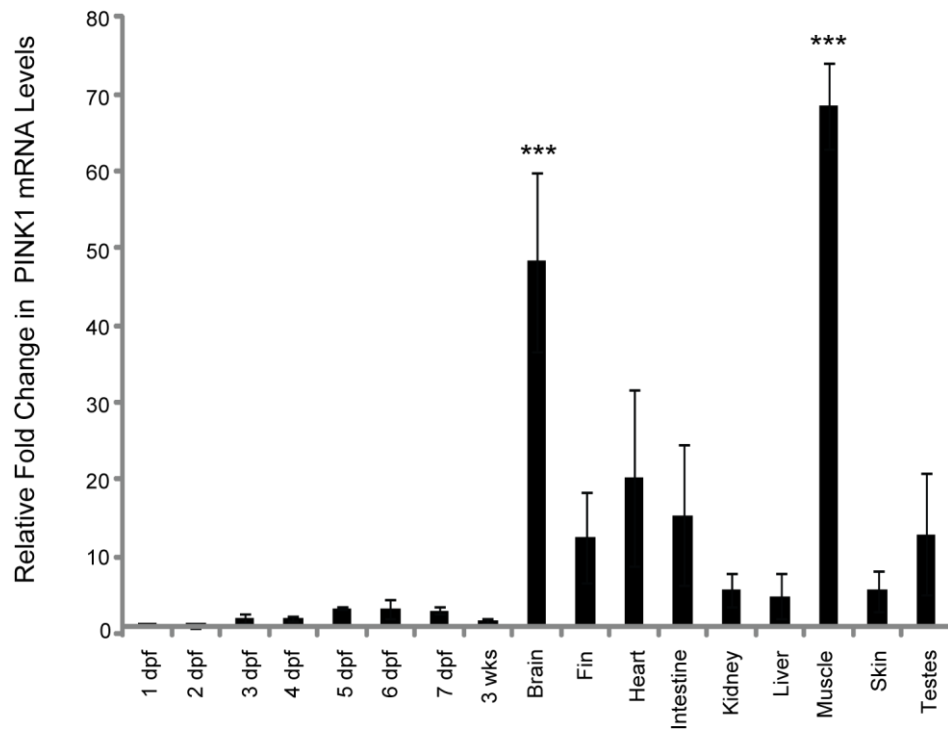


**Figure 4.1: Example of Validation of qPCR primers.** A: Plots of cycle number against fluorescent intensity across of serial dilution of cDNA. The dotted line indicates the threshold level. The number of cycles required for a sample to reach this threshold is known as the cycle threshold (CT) value of that sample. B: Plot of log concentrations against CT values. A gradient of -3.32 is indicative of 100% primer efficiency. C: Melting curve analysis. A drop in fluorescent intensity can be seen with increasing temperature. This drop in fluorescence is due to the strands of dsDNA dissociating and releasing the Sybr green. The sharp drop at 82°C indicates the melting temperature of the PCR product. The negative of the first derivative over time (-dI/dT), a measure of rate of change, is also plotted and shows a single peak at 82°C. Presence of a single peak indicates the amplification of a single product, as other products would have a different melting temperature and so result in multiple peaks. D: Gradient and R<sup>2</sup> values for primers used in the detection of PINK1, and the house-keepers *β-actin*, *ef1α* and *rpl13α* mRNAs.



#### 4.3.1.2 Quantification of PINK1 Expression throughout Development and Across Tissues

PINK1 mRNA levels were quantified and normalised to the average of three house-keepers, ef1 $\alpha$ ,  $\beta$ -actin and rpl13 $\alpha$  throughout development (from 1 dpf to 3 wks) and across 6 month old tissues (brain, fin, heart, intestines, kidney, liver, muscle, skin and testes) using the  $\Delta\Delta$ CT method described in section 2.9.5. Throughout development PINK1 levels remained fairly constant, increasing slightly, but non-significantly out to 6 dpf and then dropping off again by 3 wks. Across tissues, PINK1 levels are slightly elevated in many tissues including fin, heart, intestine and testes, but are only statistically significantly higher in brain and muscle which show an approximate 50-fold and 70-fold increase respectively above levels at 1 dpf (Figure 4.2).

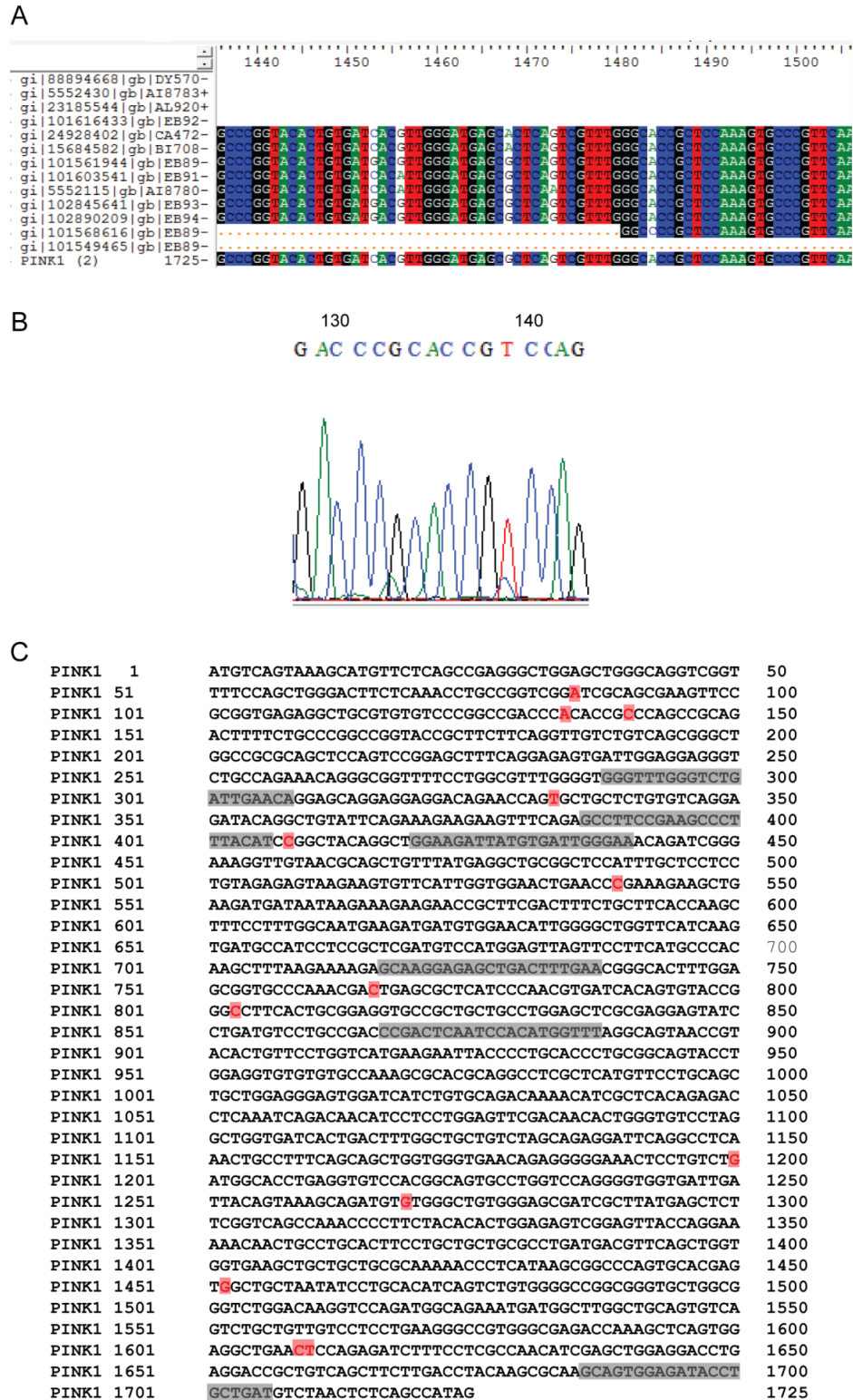


**Figure 4.2: PINK1 mRNA Levels Throughout Development and Across Tissues.**

Relative fold changes in mRNA levels of PINK1 compared to 1 dpf levels. Transcript levels were determined by real-time qPCR and normalised against the average of three housekeepers *ef1α*, *rpl13α* and  $\beta$ -actin. Quantification is based on three individual time courses and tissue banks. Results were analysed by One-way ANOVA followed by the Dunnett test using 1 dpf values as the control. \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ .

### 4.3.2 Identification of Putative PINK1 Polymorphisms

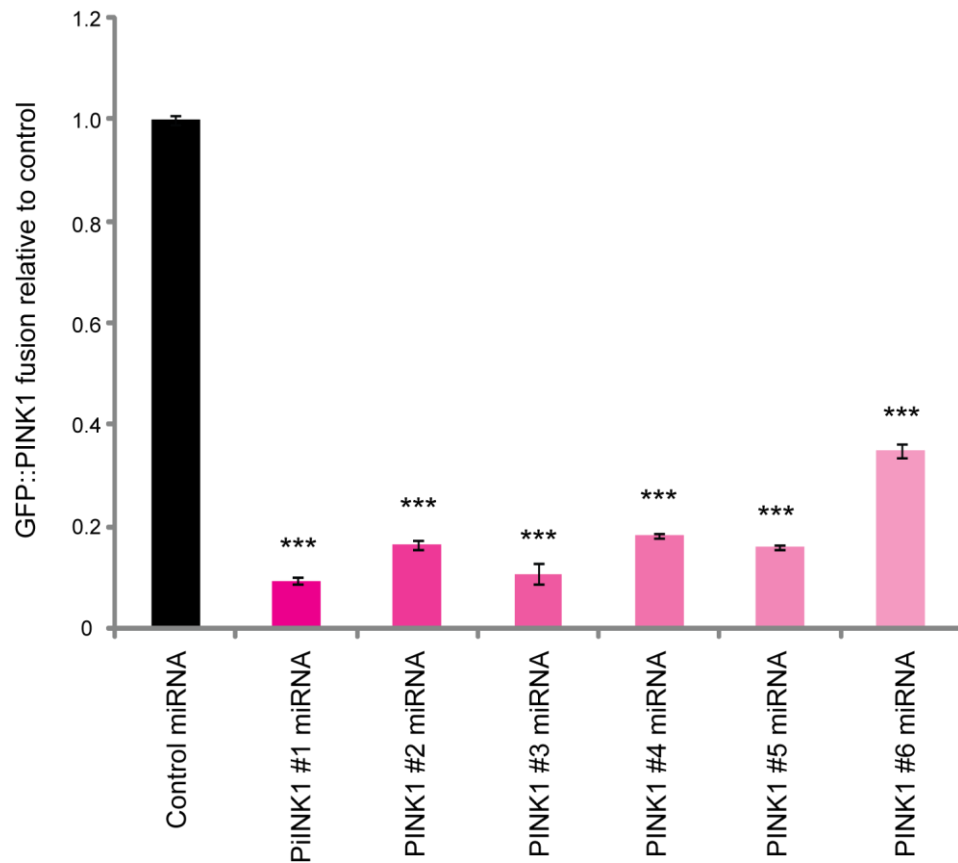
In order to design effective miRNAs against zebrafish PINK1, it was important to avoid polymorphic regions within the gene. To identify potential polymorphisms the open-reading frame (ORF) of the identified PINK1 cDNA was compared to pubmed-deposited EST sequences with high sequence homology to PINK1. A region of alignment between PINK1 cDNA and the ESTs can be seen in Figure 4.3 A. Conserved bases are highlighted (G=black, C=blue, T=red, A=green), non-conserved bases are non-highlighted. Base changes verified by two or more EST sequences were considered bona fide polymorphisms. In order to establish whether there were any additional polymorphisms in our own AB fish stocks, embryos were collected from 8 breeding pairs, RNA extracted, cDNA synthesised and sequenced (see section 4.2.2). Sequencing reactions were carried out in duplicate and only polymorphisms detected in both reactions were treated as bona fide. Figure 4.3 B shows an example of a sequencing polygraph, which clearly shows dual peaks at both position 133 and 139, indicating the presence of polymorphisms at these positions. Results of the EST and sequencing analysis were combined and a full sequence of PINK1 showing all identified putative polymorphisms can be seen in Figure 4.3 C. miRNAs were then designed using BLOCK-IT™ RNAi designer (Invitrogen) to avoid overlapping with potential polymorphic positions and cloned into the RNAi silencing vectors using methods described in section 2.5 and 3.2.1. The positioning of the six miRNAs designed against PINK1 is shown in grey (Figure 4.3 C).



**Figure 4.3: Analysis of Polymorphisms in PINK1.** A: Alignment of PINK1 cDNA (accession number: NM\_001008628) with deposited EST sequences. Conserved bases are highlighted (G=black, C=blue, T=red, A=green), non-conserved bases are non-highlighted. B: Example of a sequencing polygraph showing evidence for polymorphisms at positions 133 (G→A) and 139 (T→C) as indicated by multiple peaks at these positions. C: PINK1 cDNA sequence with potential polymorphic bases highlighted in red. Positions of miRNA sequences designed are highlighted in grey.

### **4.3.3 Validating PINK1 miRNAs in HEK 293 Cells**

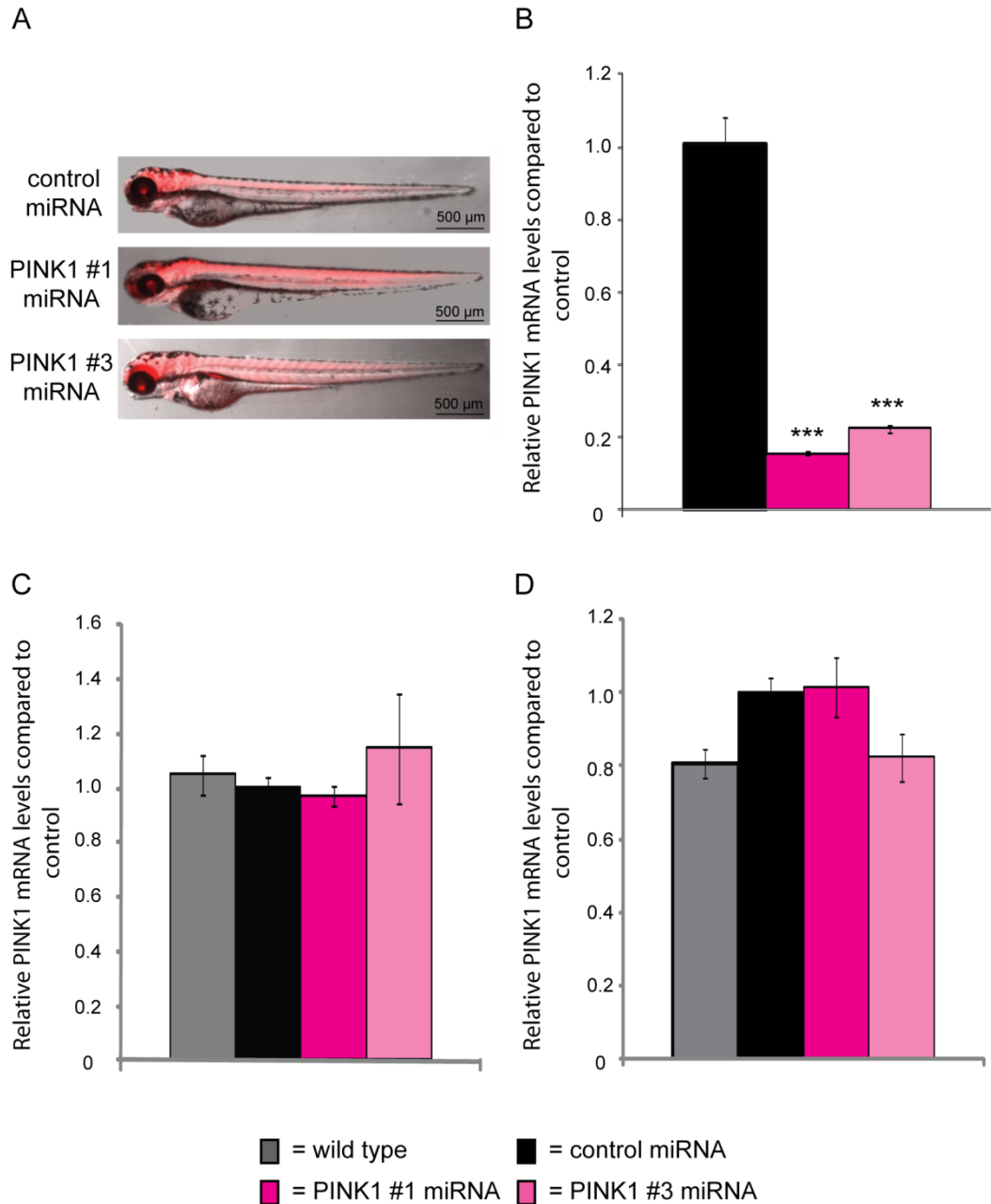
In order to determine the miRNAs which induce the most efficient knockdown of PINK1, a GFP:PINK1 fusion plasmid was created (see section 4.2.3) and co-transfected into HEK 293 cells along with either the control or one of the six PINK1 miRNAs under the control of the H2A.Zf promoter. The amount of GFP present in the mcherry positive cells was then recorded using flow cytometry (see section 2.8.1). Figure 4.4 shows the level of GFP:PINK1 knockdown achieved by each of the miRNAs. PINK1 #1 and PINK1 #3 miRNA were the most efficient resulting in approximately 90% knockdown of the GFP:PINK1 fusion. The PINK1 #6 was the most inefficient, only inducing 70% knockdown in HEK 293 cells.



**Figure 4.4: Efficiency of PINK1 miRNAs in HEK 293 Cells.** Relative knockdown of GFP:PINK1 fusion by six distinct PINK1 miRNAs compared to control. GFP and RFP measured by flow cytometry. For each sample  $n=3$ . Results were analysed by One-way ANOVA followed by the Dunnett test. \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ .

#### 4.3.4 PINK1 Knockdown in F1 Transgenic Embryos

Gal4-VP16-responsive transgenic zebrafish expressing the control and the two most efficient PINK1 miRNAs, PINK1 #1 and PINK1 #3 were generated using *To2*-mediated transgenesis methods (see section 2.3.1.2). F1 transgenic zebrafish which give rise to zebrafish embryos that express the transgene ubiquitously and uniformly were identified and out bred to wild type AB zebrafish. Examples of the expression patterns of the resulting zebrafish embryos expressing the control and two PINK1 miRNAs are shown in Figure 4.5 A. F2 transgenic and wild type zebrafish were harvested at 3 and 5 dpf to analyse PINK1 mRNA levels. Firstly however, to check that the PINK1 qPCR assay validated above in section 4.3.1.1 is sensitive enough to detect knockdown of PINK1, the assay was used on HEK 293 cells which had been transiently transfected with ef1a::PINK1 and either the control or PINK1 miRNAs. In this assay the house-keeper used was human ef1 $\alpha$  (gradient = -3.298,  $R^2 = 0.985$ ). In HEK 293 cells, PINK1 #1 and PINK1#3 miRNAs achieved 85% and 78% knockdown compared with the control miRNA respectively, as detected by qPCR (Figure 4.5 B). The same assay, this time normalised to zebrafish ef1a, was used to quantify PINK1 levels in wild type AB and control miRNA, PINK1 #1 miRNA and PINK1 #3 miRNA expressing transgenic zebrafish. For each sample 30 zebrafish embryos were used and three samples were measured for each group. Figure 4.5 C and D show the qPCR results at 3 and 5 dpf respectively. There is no statistically significant difference in PINK1 transcript levels relative to ef1 $\alpha$  levels across any of the groups at either time point.

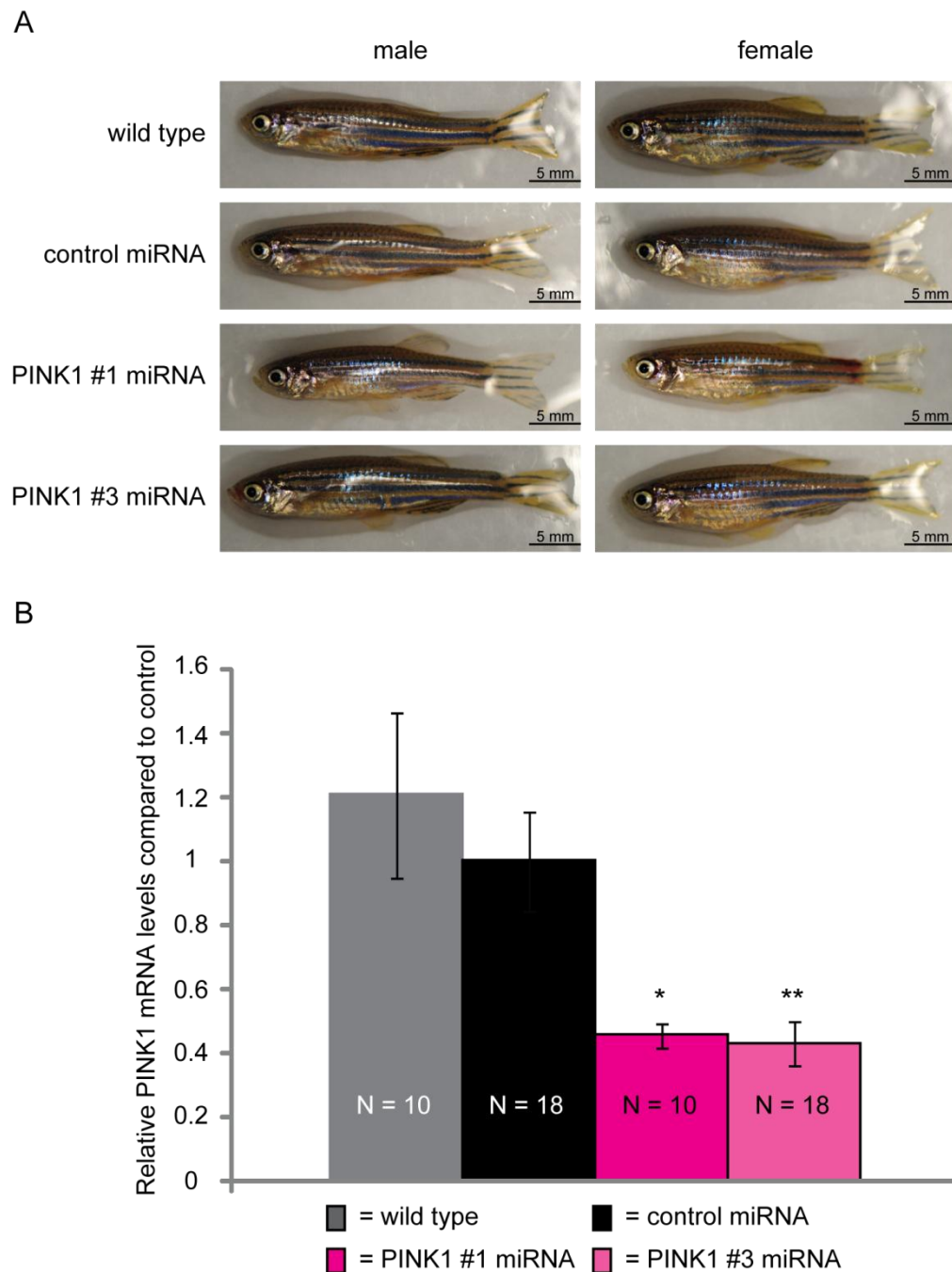


**Figure 4.5: Lack of Knockdown of PINK1 in Zebrafish Embryos.** A: Example of the expression of the RNAi transgene in transgenic F2 fish expressing either the control or PINK1 miRNAs. B: Validation of the PINK1 qPCR assay in detecting knockdown of zebrafish PINK1 in HEK 293 cells. 85% and 78% knockdown of zebrafish PINK1 can be seen relative to the control in HEK 293 cells with the PINK1 #1 miRNA and PINK1 #3 miRNA respectively. C and D: knockdown of PINK1 in transgenic F2 zebrafish expressing PINK1 miRNAs (pink bars, n=3) compared to control (black bars, n=3) and WT type (grey bars, n=3) zebrafish. C: 3 dpf. D: 5 dpf. Results were analysed using One-way ANOVA followed by Dunnett test. \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ .



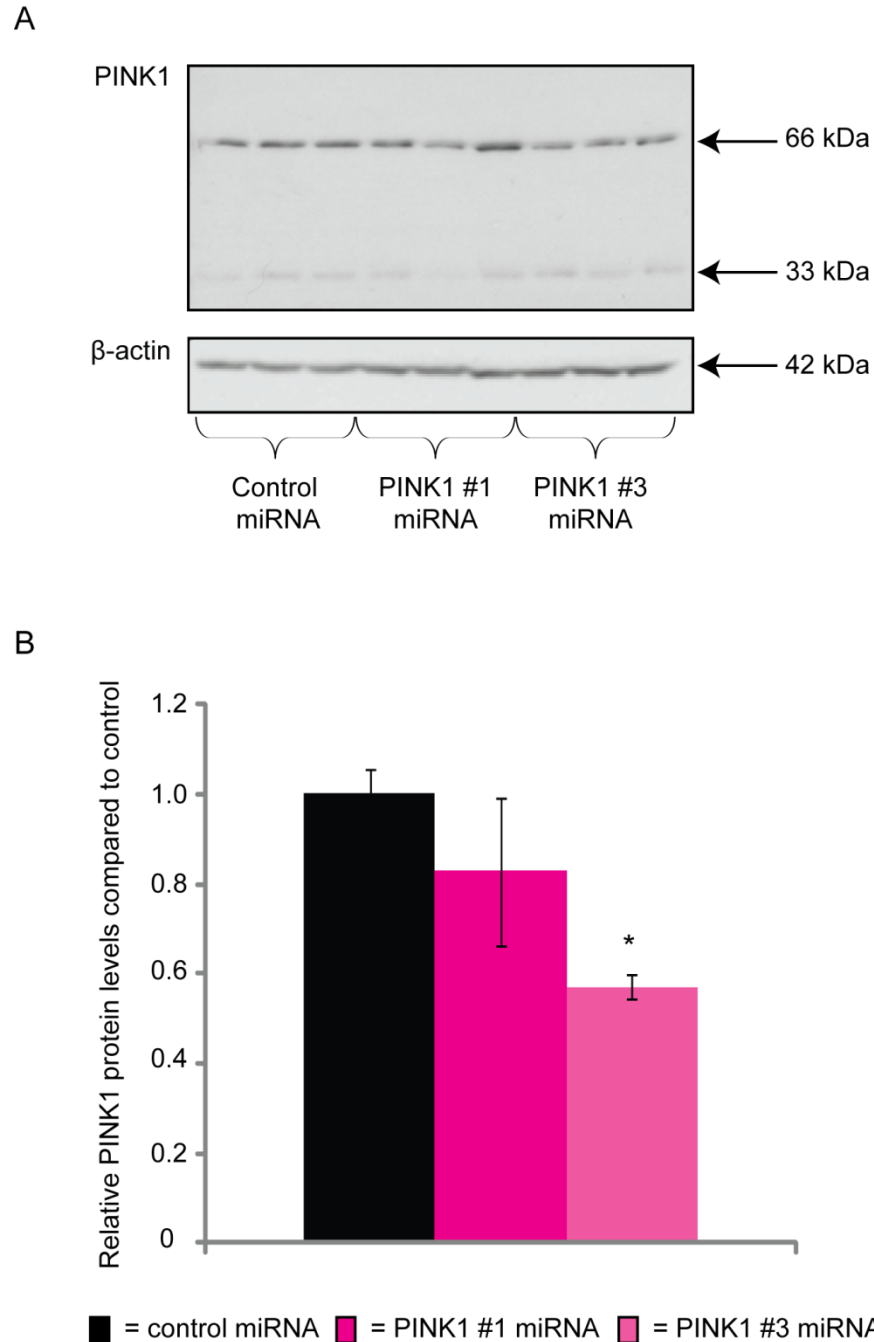
#### **4.3.5 PINK1 Knockdown in Adult Zebrafish Brains**

To test whether there is any PINK1 knockdown in the brains of adult zebrafish, brains from size- and aged-matched, 6 months old zebrafish (Figure 4.6 A) that were wild type or expressing either a control miRNA or Pink1 miRNAs were dissected out. Following RNA extraction and cDNA synthesis, RT- qPCR was performed to quantify PINK1 transcript levels relative to the house-keeper, ef1 $\alpha$ . A slight, but not statistically significant reduction in PINK1 was seen in zebrafish expressing the control miRNA compared to wild type zebrafish. However, a statistically significant reduction of 55% and a highly statistically significant reduction of 57% compared to control miRNA levels was seen in zebrafish expressing the PINK #1 and PINK1 #3 miRNAs respectively (Figure 4.6 B).



**Figure 4.6: PINK1 mRNA Knockdown in Adult Zebrafish Brain.** A: Images of age- and sized- matched zebrafish used in the analysis of brain PINK1 transcript levels. B: PINK1 transcript levels in PINK1 #1 miRNA and PINK1 #3 miRNA brain samples compared to control miRNA and wild type brain samples, as determined by RT-qPCR. There is a 55% and 57% reductive in relative PINK1 transcript levels in zebrafish expressing PINK1 #1 and PINK1 #3 miRNAs respectively compared to the control miRNA. Results were analysed by One-way ANOVA followed by the Dunnett test. \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ .

To check that this loss of PINK1 mRNA is also detected at the protein level, protein extracts from the brains of three control and PINK1 #1 and PINK1 #3 miRNA zebrafish were analysed by western blot (Figure 4.7 A) and quantified relative to the house-keeper  $\beta$ -actin. Quantification of PINK1 levels relative to  $\beta$ -actin levels can be seen in Figure 4.7 B. PINK1 levels in zebrafish expressing the PINK1 #1 miRNA are reduced by 28%; however this difference is not statistically significant due to the high variation in PINK1 levels across the PINK1 #1 miRNA expressing zebrafish brains. Zebrafish brains expressing the PINK1 #3 miRNA show a 43% reduction in PINK1 protein levels which is statistically significant at the 5% significance level and similar to the reduction in transcript levels (see Figure 4.6 B).



**Figure 4.7: PINK1 Protein Knockdown in Adult Zebrafish Brain.** A: Western blot analysis of three independent brain extracts expressing control, PINK1 #1 and PINK1 #3 miRNAs. Top panel is probed with anti-PINK1 antibody. Bottom panel is probed with the house-keeper anti- $\beta$ -actin antibody. B: Densitometry analysis of PINK1 protein levels relative to the house-keeper  $\beta$ -actin. PINK1 protein levels in PINK1 #1 miRNA expressing zebrafish are 28% lower than in control miRNA expressing zebrafish, though this difference is not statistically significant. PINK1 protein levels in PINK1 #3 miRNA expressing zebrafish are 43% lower than in control miRNA expressing zebrafish. Results were analysed by One-way ANOVA followed by the Dunnett test. \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ .

## 4.4 Discussion

Preliminary results of chapter one and research of others (Su *et al.*, 2008; Wang *et al.*, 2007) suggested that vector-mediated RNAi could be used as an efficient means to knockdown genes in zebrafish. Loss of function mutations in the *PINK1* gene are the second most common form of autosomal recessive Parkinson's disease. It was hoped that targeting zebrafish *PINK1* by vector-mediated RNAi might result in the development of a zebrafish Parkinson's disease model, which could be used to further characterise *PINK1* induced Parkinsonism and in high-throughput screening for potential therapeutic agents.

### 4.4.1 Identification and Characterisation of *PINK1*

The zebrafish *PINK1* gene has been identified and is currently assigned to chromosome 23. It encodes a 574 amino acid protein with a highly conserved protein kinase domain with close to 100% conservation at the active site. The identity and location of *PINK1* has in the meantime also been confirmed by Anichtchik *et al.*,(2008) and Xi *et al.*,(2010). *PINK1* mRNA is expressed at low and increasing levels throughout development up to three weeks, but is expressed more strongly in adult (6 month) tissues, with particularly high expression in the brain and muscle (Figure 4.2). Consistent with this Anichtchik *et al.*,(2008) showed increased *PINK1* mRNA signal intensity in the central nervous system and muscle of larval zebrafish by *in situ* hybridisation.

### 4.4.2 Designing and Validation of miRNAs Targeting *PINK1*

In order to design effective miRNAs against target genes, it is important that the target region is not polymorphic. Polymorphisms within the *PINK1* gene were identified by analysis of deposited EST sequences and direct sequencing of our wild type AB zebrafish cDNA. Where potential polymorphic sequences were identified these regions were avoided when designing miRNAs (Figure 4.3). Six potential miRNAs were identified, produced and cloned into the RNAi vector under the control of the H2A.Zf promoter, and validated by their ability to knockdown a zebrafish *PINK1*: GFP fusion in HEK 293 cells as determined by fluorescent flow cytometry

(Figure 4.4). This technique is similar to that used by Zhou *et al.*, (2007), to identify miRNAs which were later effectively used in a vector-mediated RNAi approach to knockdown mouse PINK1 by more than 95% in mouse brain. All the miRNAs effectively knocked down the PINK1:GFP fusion, the best being miRNAs 1 and 3 which both achieved approximately 90% knockdown. These two miRNAs were then used in the generation of H2A.Zf driven Gal4-VP16-responsive RNAi transgenic lines. Although this method is a convenient and effective means of determining the ability of miRNAs to target the PINK1 transcript, validating miRNAs in HEK 293 cells may fail to take into account some of the potential off-target effects in zebrafish. In future, validation in zebrafish cell lines may be a more reliable method of validation. The use of a PINK1:GFP fusion may also alter the efficacy of miRNAs by potentially altering the folding of the mRNA and hence the accessibility to miRNAs. To reduce these affects, in contrast to Zhou *et al.*, (2007) who used a mouse PINK1 partial sequence, we used full-length PINK1 in our fusion constructs. It should also be noted that while this system worked well for the validation of PINK1 miRNAs, other GFP fusions created failed to express efficiently in HEK 293 cells (data not shown), so the use of this technique may be limited in a target dependent manner.

#### **4.4.3 Lack of PINK1 Knockdown in Transgenic Zebrafish Larvae**

Transgenic F1 zebrafish displaying ubiquitous and robust expression of the H2A.Zf driven Gal4-VP16-responsive RNAi transgene with either control or PINK1 miRNAs were identified and their off-spring analysed by RT-qPCR for PINK1 expression relative to the house keeper *ef1 $\alpha$* . Despite this PINK1 qPCR assay being shown in HEK 293 cells to efficiently detect the knockdown of PINK1 by PINK1 #1 and #3 miRNAs compared to the control (Figure 4.5 B), no knockdown of PINK1 was detected in transgenic zebrafish expressing either PINK1 miRNA compared to the control at 3 or 5 dpf (Figure 4.5 C and D). This is consistent with the lack of knockdown of GFP detected in transgenic zebrafish in chapter one.

#### **4.4.4 PINK1 Knockdown in Adult Brain**

In order to see whether any knockdown was being achieved in adults, the brains of transgenic zebrafish expressing either PINK1 miRNA or the control miRNA were analysed for PINK1 message. The brain was chosen as the tissue of choice due to the consistent high level and uniform expression seen in transgenic zebrafish throughout development and also due to its relevance in the generation of a Parkinson's disease model. Brains taken from 6 month old adult zebrafish expressing the PINK1 miRNAs did show similar and efficient knockdown of about 50% of PINK1 compared to controls, as determined by qPCR. Interestingly, control miRNA expressing zebrafish brains also had slightly reduced levels of PINK1 compared to wild type AB zebrafish brains, though this was not significantly different. This may suggest that the Gal4-VP16 RNAi vector may have a slight "squenching" affect on the transcription of other genes.

In order to check that the reduction in PINK1 mRNA had a corresponding reduction on PINK1 protein levels, western blot analysis was performed on brain protein extracts. Brains expressing the PINK1 #3 miRNA had a 50% reduction in level of PINK1 protein levels, however the extracts from PINK1 #1 miRNA brains had no significant decrease in PINK1 protein despite having reduced levels of PINK1 mRNA. There was however, a large amount of variation in the protein levels detected in PINK1 #1 miRNA brains, and so this discrepancy may simply be an anomaly brought about by the small sample number of brains analysed by western blot. Further individual brain extracts or a pool of brain extracts would need to be analysed to address this.

The fact that knockdown of PINK1 mRNA was detected in zebrafish brains expressing two independent miRNAs is quite compelling evidence of RNAi-mediated knockdown. However, in order to prove categorically that the knockdown detected is due to vector-mediated RNAi, further controls should be considered. In larvae, the ideal experiment to validate knockdown would have been to knockdown part of the RNAi machinery by means of morpholino, in order to demonstrate that knockdown is dependent on the

production of mature miRNAs. Effective Dicer morpholinos have already been described (Wienholds et al., 2003) and could have been used in this type of analysis. However, it is currently not possible to knockdown genes in adult zebrafish, so this could not be used to control for knockdown seen in adult brains. An alternative control, which could be used on adults, is to generate a transgenic line expressing an RNAi resistant form of PINK1. This is a good control for demonstrating that any phenotypes observed are due to loss of the target gene, as replenishment with an RNAi resistant version should eliminate or alleviate any phenotype. However, the flaw with this technique is that it is not possible to ensure physiological levels of the transcript/protein are put back and so other phenotypes may emerge as a result of over-expression.

#### **4.4.5 An Adult Model of Parkinson's Disease?**

Recent morpholino approaches to knocking down PINK1 have revealed conflicting results. Although Anichtchik (2008) reported severe developmental phenotypes and substantial loss of dopaminergic neurons, Xi et al., (2010) and Sallinen et al., (2010) report much more modest effects, such as disorganised patterning of the neurons in the ventral diencephalon and shortened axonal projections. The lack of a severe larval phenotype induced by PINK1 knockdown is also consistent with the unpublished data from the Bandmann lab who have generated a *pink1<sup>-/-</sup>* stable line by use of TILLING (Bandmann and Burton, 2010). Furthermore, *pink1<sup>-/-</sup>* medaka (*Oryzias latipes*) also had no larval phenotypes (Matsui et al., 2009). Adult medaka however, had a slightly shortened life span, developed a late-onset reduction in spontaneous swimming and displayed deregulated dopamine metabolism by 4 months, although dopaminergic neuron survival was unaffected. These late-onset effects, together with the fact that Parkinson's disease is a progressive disease affecting humans in adulthood, suggests that effects of PINK1 knockdown in adults may be more apparent and biologically relevant. It would therefore be interesting to see whether the knockdown of PINK1 achieved here in adult zebrafish brains is sufficient to bring about any of the changes associated with PINK1 loss and Parkinson's disease, such as disrupted mitochondrial function, deregulated dopamine



metabolism or perturbed swimming behaviour. Analysis of the proteome of these transgenic zebrafish may also help elucidate other pathways affected by loss of PINK1. However, as PINK1 mutations are responsible for an autosomal recessive form of Parkinsonism, it is conceivable that 50% knockdown of PINK1 is insufficient to have any pathological affect. It should be pointed out, that this 50% knockdown of PINK1 in adult brains was seen in fish heterozygous for the RNAi transgene. Breeding the fish to homozygosity may therefore improve the levels of knockdown detected.

#### **4.4.6 Conclusions**

The zebrafish PINK1 homolog has been identified, characterised and analysed for PINK1 polymorphisms. Based on this PINK1 miRNAs were designed and validated in HEK 293 cells. Transgenic zebrafish larvae expressing the two most potent miRNAs did not have reduced levels of PINK1 mRNA compared to control. However, brains from adult 6 month old zebrafish expressing the PINK1 miRNAs did have a 50% reduction in PINK1 mRNA levels. Consistent with this, protein levels were also shown to be down by about 50% in PINK1 #3 miRNA expressing brains.

#### **4.4.7 Future Work**

Over the course of this and the previous chapter it has been apparent that although vector-mediated RNAi has been demonstrated in a G0 setting by this and other research, knockdown does not appear to be heritable as no knockdown of GFP or PINK1 were detected in transgenic zebrafish larvae. Future work should therefore focus on why this is the case. In particular, it would be interesting to look at the level of expression of RNAi components and the production of customised mature miRNA in zebrafish larvae and adult tissues. In addition, it would be interesting to see whether over-expression of components of the RNAi machinery is able to induce knockdown. Finally, future research may look at whether altering the type of RNAi vector used or the miRNA backbone can improve knockdown. In addition, the effect of concatemerisation of the miRNA so that each miRNA transcript gives rise to multiple mature miRNAs or breeding the fish to homozygosity so that each fish expresses twice the amount of miRNA could

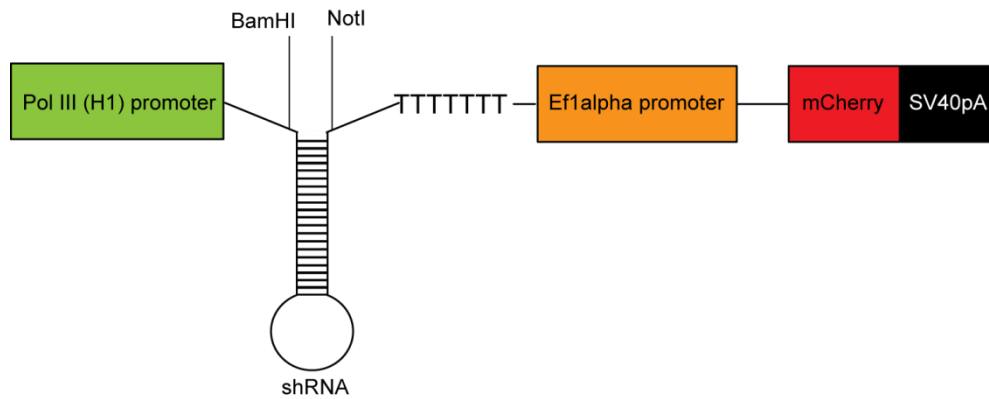
be investigated. If increasing the amount of primary miRNA transcript improves knockdown then it would suggest that the levels of transcript miRNA are what is limiting knockdown in zebrafish. However, if increasing levels of primary miRNA transcript has no effect then knockdown must be limited by factors downstream of miRNA transcription.

## **5 Analysis and Over-expression of the RNAi Machinery**

### **5.1 Introduction**

The previous two chapters have demonstrated that stable and heritable knockdown by vector-mediated RNAi do not seem to be feasible in zebrafish embryos. However, possible knockdown has been detected in adult brains, suggesting that zebrafish are capable of processing the RNAi vector. Lack of knockdown in embryos despite the presence of functional RNAi machinery and the importance of endogenous miRNAs during zebrafish development (Giraldez et al., 2005; Wienholds et al., 2003), suggests that something must be limiting RNAi in embryos. The limiting factor could be at the transcriptional or posttranscriptional level.

Though seemingly high level expression of the RNAi vector has been achieved in zebrafish, especially through combining pol II-responsive promoters with a Gal-4-VP16/UAS system it is possible that this still does not result in high enough expression of customised miRNA transcripts. Pol III promoters drive much higher levels of transcript than most pol II promoters, and therefore it is possible that use of a zebrafish pol III promoter to drive shRNA may be more efficient at knockdown. A vector suitable for pol III-responsive delivery of shRNA by use of a zebrafish H1 promoter has been created by Dr. Paul Walker, the Hurlstone laboratory, and is depicted below in Figure 5.1.



**Figure 5.1: Pol III RNAi vector.** The zebrafish H1 pol III promoter drives expression of shRNA. Termination of the transcript is determined by a run of Ts at the end of the shRNA. shRNAs can be easily cloned in using *Bam*HI and *Not*I sites. A separate cassette contains the ef1 $\alpha$  promoter which drives expression of mcherry, allowing for easy identification of transfected cells/transgenic zebrafish.

As well as containing a zebrafish H1 promoter which drives expression of a shRNA with stem loop based on that of hsa-miR-30a, the vector also contains an ef1 $\alpha$ ::mcherry cassette to enable identification of transfected/transgenic cells (for a full vector map see appendices section 8.1.6).

Instead, or in addition to transcriptional limitations, it is also possible that components involved in the processing of customised pri- and pre-miRNA or in the functioning of mature miRNAs could be limiting. In various organisms, over-expression of components of the RNAi machinery has improved levels of knockdown. For example, over-expression of Dicer2 enhances RNAi in *Drosophila* (Dietzl *et al.*, 2007). While over-expression of Ago2 and Xpo5 have been shown to significantly improve RNAi in mammalian cells (Diederichs *et al.*, 2008; Yi *et al.*, 2005). Ago2 has also been shown to enhance specificity of RNAi for targets with complete complementarity, thereby reducing off-target effects (Diederichs *et al.*, 2008).

Another potential limit to RNAi in zebrafish may be the backbone of the miRNA being used. At present the backbone miRNA is that of human miR-30a with the miRNA placed in the 3' arm, which was shown by Zeng *et al.*, (2005a; 2002) to produce efficient knockdown in human cells. Whether or not it is efficient in zebrafish cells however is unknown, and so it is

possible that changing the backbone of the miRNA may improve knockdown in zebrafish. In particular, it would be interesting to know what effect changing the strand orientation of the guide strand or using a zebrafish miRNA backbone has on the efficiency of RNAi.

### **5.1.1 Aims**

The aims of this chapter are therefore to:

- check for the expression of the major components of the RNAi machinery, Ago2, Dicer, Drosha and Xpo5 and see how they change throughout development and across tissues.
- see whether mature miRNA, indicative of correct processing of pri- and pre- miRNA can be detected and how levels of mature miRNA change throughout development and across tissues.
- determine whether pol II- or pol III-responsive vectors are capable of delivering RNAi in zebrafish tissue culture.
- see whether over-expression of Ago2, Dicer, Drosha or Xpo5 can induce RNAi in zebrafish cells
- see whether changes to the miRNA backbone enhances RNAi in zebrafish cells.
- test the effect of any beneficial factors to RNAi detected in zebrafish cells *in vivo*.

## 5.2 Materials and Methods

For general material and methods see chapter 2.

### 5.2.1 Ago2, Dicer1 and Xpo5 RT-qPCR/ Drosha PCR

Primers used for the detection of zebrafish Ago2, Dicer, Xpo5 and Drosha mRNA transcripts are given in Table 5.1 below, as are the primers for house-keepers  $ef1\alpha$ ,  $\beta$ -actin and  $rpl13\alpha$ . For sybr-green based qPCR and normal PCR methods see sections 2.9 and 2.4.1 respectively.

Gene	Accession Number		Primer sequence (5'- 3')
<i>Ago2</i>	XM_694134	Forward	CTCTCTGGACGGCTACCAAAC
		Reverse	CTCCACCAAGAGGGTTAGAGC
* $\beta$ -actin	NM_131031.1	Forward	CGAGCTGTCTTCCCATCCA
		Reverse	TCACCAACGTAGCTGTCTTTCTG
<i>Dicer</i>	XM_678382	Forward	GGACAAAAGCAGCACAGACA
		Reverse	TAGATGGCTCGACCTCTGCT
<i>Drosha</i> (722 bp fragment)	NM_001110472.1	Forward	GGAGACCCGCAGTATCAAAA
		Reverse	TGGGCATATAGCATGAAACG
* $ef1\alpha$	NM_131263.1	Forward	CTGGAGGCCAGCTCAAACAT
		Reverse	ATCAAGAAGAGTAGTACCGCTAGCATTAC
$ef1\alpha$ (448 bp fragment)	NM_131263.1	Forward	CTGGAGGCCAGCTCAAACAT
		Reverse	CAGGGTGGTTCAGGATGATG
<i>Xpo5</i>	XM_001921387.1	Forward	ACCTTCTGGCACTCATCAGG
		Reverse	TGAGCCGGGTGATGTTCT
* $rpl13\alpha$	NM_212784	Forward	TCTGGAGGACTGTAAGAGGTATGC
		Reverse	AGACGCACAATCTTGAGAGCAG

**Table 5.1: RT-qPCR/PCR Primer Sets used in Gene Transcript Analysis.** Primers used for qPCR and PCR analysis are listed in a 5' to 3' manner. Reference genes used are highlighted in grey. Accession numbers of genes analysed are also given. \* indicates primers published by Tang *et al.*,(2007)

### 5.2.2 Detecting Production of Mature miRNAs

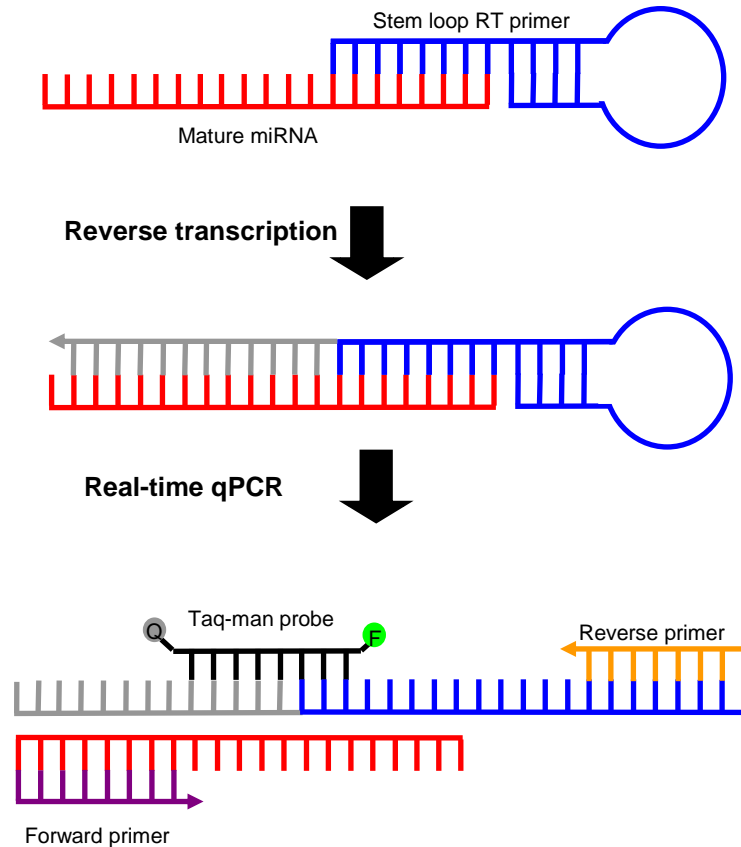
#### 5.2.2.1 Overview of Technique

In order to detect production of mature miRNA, a qPCR based assay which detects mature miRNA with sequence corresponding to the Arabidopsis miRNA ath-miR-162a was used. Ath-miR-162a was chosen due to the availability of an assay and lack of similar zebrafish endogenous miRNAs. Transgenic zebrafish expressing ath-miR-162a under the control of the H2A.Zf Gal4-VP16/UAS promoter were generated and the resulting offspring were analysed for the expression of mature miRNA relative to the levels of mcherry expression. Primers used to generate the ath-miR-162a miRNA were as follows:

**Fwd:**GCGGGGTACCGCGGGGATCCTGCTGTTGACAGTGAGCGACTGGATGCAGA  
GGTTTATCGATAGTGAAGCCACAGATG,

**Rev:**CCGCGAATTCCCGCAGATCTCCTTGAAGTCCGAGGCAGTAGGCAGCTGGA  
TGCAGAGGTTTATCGATACATCTGTGGCTTCAC.

To quantify mature miRNA production, mature ath-miR-162a miRNA was reverse transcribed from total RNA using a stem loop RT primer whose stem binds to the first 8 bases of the Dicer cut end of the mature miRNA. During reverse transcription the mature miRNA serves as a template for the addition of bases to the end of the stem loop primer. The resulting template is then used in a TaqMan based qPCR assay. During PCR the reverse transcribed product unfolds and a forward primer binds to the mature miRNA, a reverse primer binds to the stem loop primer and an appropriate TaqMan probe binds in between the two. Polymerase catalyses the addition of new complementary dNTPS to the 3' ends of the primers and as it moves along the template DNA the fluorescent dye, FAM is released from the TaqMan probe. Once released from the proximity of the quencher, FAM emits a fluorescent signal which can be detected by a qPCR machine (Figure 5.2).



**Figure 5.2: Schematic Representation of the Mature miRNA Assay from Applied Biosystems.** During reverse transcription the stem loop primer binds to the last 8 bases of the Dicer cut end of the mature miRNA. The mature miRNA then serves as a template for the addition of dNTPs to the stem loop primer. During RT-qPCR a forward primer binds to the mature miRNA (red), a reverse primer binds to the stem loop primer (blue) and an appropriate TaqMan probe (black) binds in between the two. Polymerase catalyses the addition of new complementary dNTPs to the 3' ends of the primers and as it moves along the template DNA releases the fluorescent dye, FAM from the TaqMan probe. Once released from the proximity of the quencher, FAM emits a fluorescent signal which can be detected by a qPCR machine.



### 5.2.2.2 RNA Extraction for miRNA Detection

#### 5.2.2.2.1 *From Adult Tissues and Pools of Embryos/Larvae*

RNA was extracted from transgenic zebrafish expressing the ath-miR-162a miRNA using Trizol<sup>®</sup> (Invitrogen) reagent and following manufacturers' guidelines. Adult tissue RNA was extracted from 6 (3 male and 3 female) 6 month old zebrafish. Embryo/larval RNA was extracted from a pool of 30 transgenic zebrafish using the same methods. Samples were diluted to 100 ng/ $\mu$ l, aliquoted into 1  $\mu$ g samples and stored at -80°C

#### 5.2.2.2.2 *From Individual Embryos/Larvae*

For the analysis of ath-miR-162a mature miRNA production in single embryos/larvae, a Cells-to-CT kit (Applied Biosystems) was used. Embryos/larvae were lysed in 50  $\mu$ l lysis buffer containing 0.5  $\mu$ l DNase and incubated at rt for 8 mins with regular agitation. After the incubation period 5  $\mu$ l of stop solution was added and the sample incubated at rt for a further 2 mins. Samples were then stored at -80°C.

### 5.2.2.3 Reverse Transcription of Mature miRNA

Mature ath-miR-162a miRNAs were reverse transcribed in a reaction consisting of 1 x RT buffer (Applied Biosystems), 1 mM dNTP mix, 0.2 u RNase Inhibitor, 1  $\mu$ l Multiscribe reverse transcriptase (Applied Biosystems) and either 1  $\mu$ g RNA or 5  $\mu$ l Cells-to-CT RNA (see section 5.2.2.2.2) and made up to 15  $\mu$ l in DEPC H<sub>2</sub>O. The reaction was then incubated at 16°C for 30 mins (annealing), 42°C for 30 min (extending) and 85°C for 5 min (inactivation of enzyme). mcherry mRNA, used as the normaliser was reverse transcribed as described in section 2.9.2.

### 5.2.2.4 Real-time TaqMan qPCR

#### 5.2.2.4.1 *For Detection of mature miRNA*

Real-time TaqMan qPCR reactions for miRNA detection consisted of 1 x TaqMan Master Mix (Applied Biosystems), 1  $\mu$ l Ath-mir 162a TaqMan MicroRNA assay (Applied Biosystems), 1  $\mu$ l RT reaction and made up to 20  $\mu$ l with DEPC H<sub>2</sub>O. A Peltier Thermal Cycler (PTC-200) connected to a Chromo4 Continuous Fluorescence Detector (MJ Research) was used with

the following cycling conditions: 95°C for 10 mins and then 40 cycles of 95°C for 15 secs and 60°C for 1 min. After each cycle the fluorescent signal from the fluorescent dye FAM was recorded.

#### 5.2.2.4.2 For Detection of mCherry

Real-time TaqMan qPCR reactions consisted of 1 x TaqMan Master Mix (Applied Biosystems), 0.2 µM forward (5'-AGGGCGAGATCAAGCAGAG-3') and reverse primers (5'-TTGACCTCAGCGTCGTAGTG-3'), 0.5 µl TaqMan assay #161 (Roche Applied Science), 2 µl cDNA and made up to 20 µl with DEPC H<sub>2</sub>O. The same qPCR machine as above was used with the following cycling conditions: 95°C for 10 mins and then 40 cycles of 95°C for 15 secs and 60°C for 1 min. After each cycle the fluorescent signal from the fluorescent dye FAM was recorded.

The amount of mature miRNA relative to the amount of mcherry production was then calculated using the  $\Delta\Delta$ CT method described in section 2.9.5.

### 5.2.3 Dicer Morpholino Injection in Transgenic Zebrafish

Single-cell transgenic embryos expressing the ath-miR-162a miRNA under the control of a H2A Gal4-VP16/UAS promoter were injected with 5 ng Dicer morpholino (5'-CTGTAGGCCAGCCATGCTTAGAGAC-3') (Wienholds et al., 2003) or standard control (5'-CCTCTTACCTCAGTTACAATTTATA-3') morpholino as described in section 2.3 and animals were assessed for mature miRNA production as described above in section 5.2.2

### 5.2.4 Cloning shRNA into the Pol III-responsive RNAi vector

A list of the primers used to make sequence encoding GFP and control shRNAs are listed in Table 5.2 below. shRNAs sequences were generated by annealing 200 ng of each primer pair in 98 µl annealing buffer (10 mM tris pH 8.0, 50 mM NaCl, 1 mM EDTA) and heating to 100°C for 5 mins and then allowing to cool to rt. Annealed primers have asymmetric ends complementary to those generated through *Bam*HI, *Not*I digestion of the pol III-responsive vector. Once annealed, primers were diluted 10-fold and ligated into the *Bam*HI, *Not*I digested pol III-responsive vector using methods described in section 2.4.

shRNA	Primer Sequence (5' to 3')	
Control	Fwd	GATCCAGTCTGTGCGCAGAAATATATGTGAAGCCACAGATGAGTCTGTG CGCAGAAATATATTTTTT
	Rev	GGCCAAAAAAGTCTGTGCGCAGAAATATATGTGAAGCCACAGATGAGTC TGTGCGCAGAAATATATG
GFP	Fwd	GATCCGCACAAGCTGGAGTACAACACTAGTGAAGCCACAGATGTAGTTGTA CTCCAGCTTGTGCTTTTTT
	Rev	GGCCAAAAAGCACAAGCTGGAGTACAACACTACATCTGTGGCTTCAC TAGT TGTACTCCAGCTTGTGCG

**Table 5.2: Primer used to make control and GFP shRNAs.** Sequences used to generate passenger and guide strands are highlighted in grey and red respectively.

## 5.2.5 Cloning Ago2, Dicer1, Xpo5 and Drosha into Expression

### Cassettes

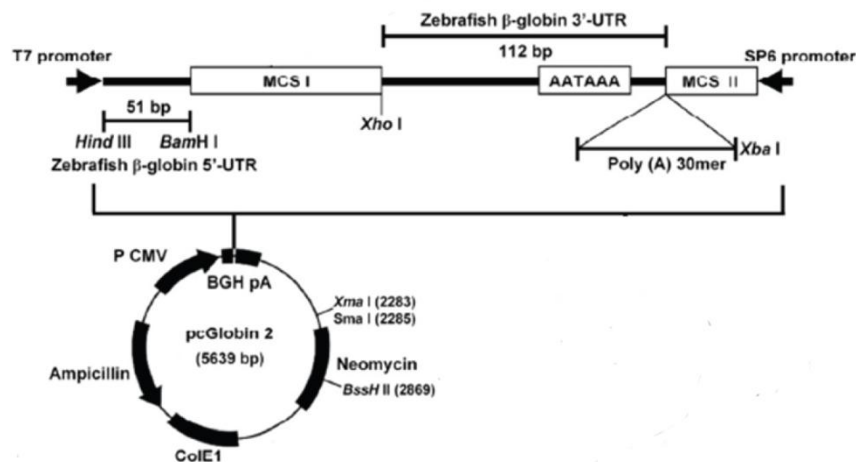
Ago2 and Xpo5 cDNA were amplified from pIRESneo-FLAG/HA Ago2 corrected (Addgene: 10822) and pQE60-Exp5 (Addgene: 12553) respectively, using methods described in section 2.4.1 and primers listed in

Table 5.3. Zebrafish Drosha cDNA was amplified from 3 dpf zebrafish cDNA using the same methods.

cDNA		Primer sequence (5' to 3')	Restriction site
Ago2 (human)	Fwd	AGGAGGATCCACCATGTACTCGGGAGCCGGCCCC	<i>Bam</i> HI
	Rev	ATGAGCGGCCGCCTCAAGCAAAGTACATGGTGCGCAGAGTGTCTTGG	<i>Not</i> I
Drosha (zebrafish)	Fwd	AGGACCGCGGACCATGTCTTTCCATGCTGGCCGTGGATG	<i>Sac</i> II
	Rev	AGGAGCGGCCGCCTATCCTTCATCACTATCTCTCTC	<i>Not</i> I
Xpo5 (human)	Fwd	AGGACCGCGGACCATGGCGATGGATCAAGTAAAC	<i>Sac</i> II
	Rev	AGTAGCGGCCGCCTCAGGGTTCAAAGATGGTGGC	<i>Not</i> I

**Table 5.3: Primers used in the amplification of human Ago2 and Xpo5 and zebrafish drosha cDNA.** Restriction enzymes incorporated into the primer sequence for ease of subsequent cloning are indicated in yellow. An adaptor sequence of ACC was used in the forward primers to introduce a KOZAK sequence (highlighted in blue).

PCR products were cloned into pGEM-T before sub-cloning into the pcGlobin 2 vector (Figure 5.3) using restriction sites incorporated into the amplified product by the primers. Dicer cDNA, a kind gift of Dr. Patrick Provost, Quebec, Canada, was sub-cloned from pCMV6 –XL4 Dicer (accession number: AJ132261) by cutting out Dicer with *Kpn*I/*Not*I restriction digest and inserting into the pcGlobin2 vector linearised with *Kpn*I/*Not*I.



**Figure 5.3: pcGlobin Vector.** The pcGlobin vector has been modified from pcDNA3 to contain zebrafish 5' and 3'  $\beta$ -globin UTRs. Cloned cDNAs are transcribed *in vitro/in vivo* under the control of CMV promoter or *in vitro* through the use of a T7 primer. Diagram modified from Ro *et al.*, (2004).

Human Dicer, Ago2 and Xpo5 were used due to very high sequence similarity between the human and zebrafish proteins, particularly in the functional domains. For a full alignment and comparison of sequences see appendices section 8.3.

#### 5.2.5.1 Detection of Over-expression of Ago2, Dicer, Xpo5 and Drosha

Over-expression of Ago2, Dicer and Xpo5 were detected by western blot using methods described in section 2.10. The rat monoclonal anti-Ago2 (11A9) antibody was a kind gift of Dr. Gunter Meister, the Max Planck Institute of Biochemistry, Munich, Germany (Rudel *et al.*, 2008). It was used at a 1:50 dilution, followed by an anti rat IgG HRP-conjugate used at 1:10 000. Rabbit polyclonal anti-Dicer (ab13502) and anti-Xpo5 (Abcam: ab31351) were used at 1 µg/ml followed by an anti rabbit IgG HRP-conjugate used at 1:10 000. For detection of full-length zebrafish Drosha mRNA forward (5'-ATGTCTTTCCATGCTGGCCGTGGATG-3'), and reverse, (5'-CTATCCTTCATCACTATCTCTCTC-3') primers were used along with general PCR methods described in section 2.4.1.

#### 5.2.5.2 Mutating the RNase H Domain of Ago2

The RNase H domain of Ago2 in pGEM T was destroyed by mutating the amino acid residue 597 from a aspartic acid residue to an alanine residue using a Quikchange® site directed mutagenesis kit (Stratagene), the forward primer (5'-CTTTCTGGGAGCCGCGGTCCTCACC-3') reverse primer (5'-GGTGAGTGACCGCGGCTCCAGAAAG-3') and following manufacturers' instructions. As well as mutating the aspartic acid (D) residue at position 597 to an alanine (A) the primers also introduce a *Sac*II site for the easy identification of positive clones. The mutated Ago2 was then subcloned into pcGlobin using *Bam*HI and *Not*I restriction digest of Ago2 D597A pGEM T and pcGlobin using methods described in section 2.4.

### 5.2.6 Cloning miRNAs with Different Stem Loops

To test whether altering the stem loop or strand orientation of the miRNA alters the efficiency of knockdown, additional miRNAs were made based on the human hsa-miR-30a and the zebrafish dre-miR-30a backbone using

methods described in sections 2.5 and 3.2.1. Primers used to clone the new miRNAs are listed in

Table 5.4.

miRNA Backbone	Target	Primer Sequence (5' to 3')
hsa-mir-30a (miRNA in 5' arm)	Control	Fwd GCGG <b>GGTACC</b> TGCTGTTGACAGTGAGCGAC <b>TCGATAAACCTCTG</b> <b>CATCCAG</b> CTGTGAAGCCACAGATG
		Rev CCGC <b>GAATTC</b> CCTTGAAGTCCGAGGCAGTAGGCAGT <b>TCGATAAAC</b> CTCTGCATCCAGCCCATCTGTGGCTTAC
	GFP	Fwd GCGG <b>GGTACC</b> TGCTGTTGACAGTGAGCGAC <b>TAGTTGTA</b> <b>CTTGTGC</b> CUGTGAAGCCACAGATG
		Rev CCGC <b>GAATTC</b> CCTTGAAGTCCGAGGCAGTAGGCAGT <b>TAGTTGTAC</b> TCCAGCTTGTGCCCATCTGTGGCTTAC
dre-mir-30a (miRNA in 5' arm)	Control	Fwd GCGG <b>GGTACC</b> TGCTGTTGACAGTGAGGCTCCTTGCAGT <b>TCGATA</b> <b>AACCTCTGCATCCAG</b> TTGTAATGCAGAAAAATCTCAG
		Rev CCGC <b>GAATTC</b> CCTTGAAGTCCGAGGCAGTAGGGCCACCAG TAGCACGATAAACCTCTGCATCCAGCTGAGATTTTCTGCA TTACAA
	GFP	Fwd GCGG <b>GGTACC</b> TGCTGTTGACAGTGAGGCTCCTTGCAGTT <b>AGTTG</b> <b>TACTCCAGCTTGTGC</b> TTGTAATGCAGAAAAATCTCAG
		Rev CCGC <b>GAATTC</b> CCTTGAAGTCCGAGGCAGTAGGGCCACCAGTAGC AAGTTGTACTCCAGCTTGTGCCCTGAGATTTTCTGCATTACAA

**Table 5.4: Primers used to make miRNAs with Different Stem Loops.** The sense sequence of the target is shown in grey on the forward primer. The anti-sense sequence of the target (equivalent to the mature miRNA sequence) is shown in red on the reverse primer. *KpnI* and *EcoRI* sites are indicated in turquoise and yellow respectively.

## 5.3 Results

### 5.3.1 Ago2, Dicer, Xpo5 and Drosha Transcript Levels in Zebrafish

To assess whether zebrafish have the necessary machinery to process vector embedded miRNAs levels of Ago2, Dicer, Xpo5 and Drosha were detected and quantified across zebrafish time-courses, tissue banks and cell types (Figure 5.4). For Ago2, Dicer1 and Xpo5 levels of transcript were assessed via RT-qPCR and normalised against the average of three house-keepers, *ef1α*, *β-actin* and *rpl13α* and three tissue banks and time courses were used. Before selecting primers for use in RT-qPCR they were validated for efficiency as described in sections 2.9.4 and 4.3.1.1. Table 5.5 shows the relevant gradient and correlation coefficient ( $R^2$ ) values for the primers used. A slope with a gradient of -3.32 indicates that the primers are 100% efficient. -3.1 is equivalent to 90% efficiency and -3.58 110% efficiency. Only primers which fell between these ranges and gave rise to a correlation coefficient ( $R^2$ ) of at least 0.97 were accepted for use in cDNA quantification.

Zebrafish Gene	Gradient	$R^2$ value
<i>Ago2</i>	-3.319	0.986
<i>β-actin</i>	-3.331	0.981
<i>Dicer</i>	-3.322	0.989
<i>ef1α</i>	-3.321	0.993
<i>rpl13α</i>	-3.317	0.993
<i>Xpo5</i>	-3.464	0.971

**Table 5.5: Gradient and  $R^2$  values of validated primers used in the detection of zebrafish Ago2, Dicer and Xpo5 mRNA.** House-keepers used were *β-actin*, *ef1α* and *rpl13α* mRNAs. A slope with a gradient of -3.32 indicates that the primers are 100% efficient. -3.1 is equivalent to 90% efficiency and -3.58 110% efficiency. Only primers which fell between these ranges and gave rise to a correlation coefficient ( $R^2$ ) of at least 0.97 were accepted for use in cDNA quantification.

Transcript levels of Ago2, Dicer and Xpo5 all start off extremely significantly higher in single-cell and 4 hpf embryos relative to the housekeepers and in comparison to 1 dpf embryos. After 1 dpf Ago2, Dicer and Xpo5 transcript levels remain significantly unchanged across the rest of the time course (Figure 5.4 A, B and C).

Across the tissue banks and zebrafish cells Ago2 levels in brain were increased extremely significantly by 6 fold on 1 dpf levels. Levels in skin,

testes, PAC.2 cells and ZFL cells were significantly increased by 5-6-fold on 1 dpf levels. Ago2 levels in fin, heart, intestine, kidney, liver, muscle and AB.9 cells remained unchanged (Figure 5.4 A).

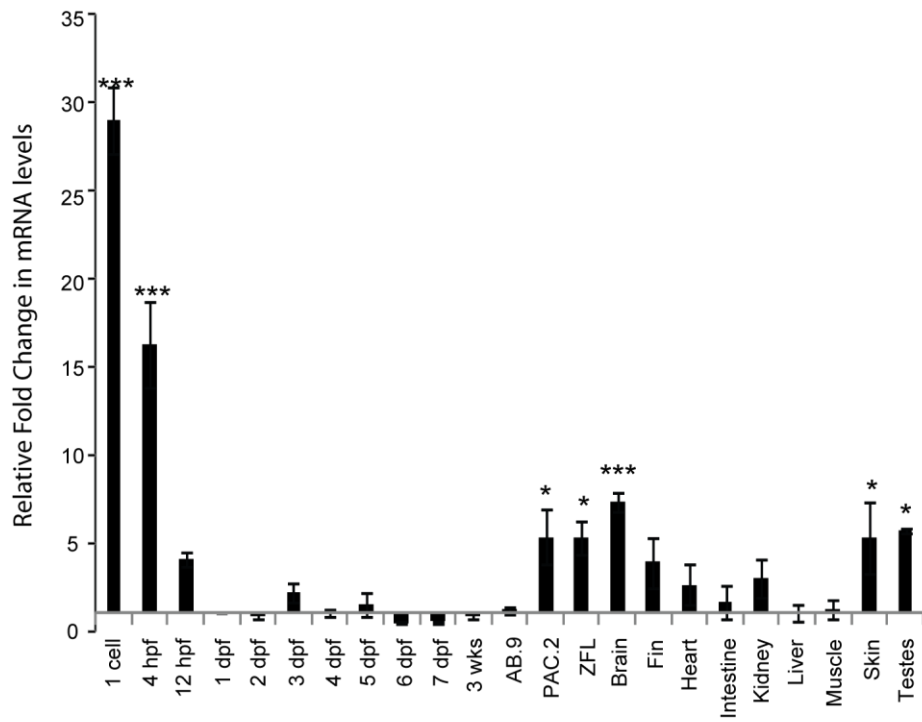
Dicer levels are slightly, but not significantly increased in brain, skin, testes, PAC.2 and ZFL cells. In the case of brain, transcript levels were very nearly significantly different ( $p < 0.051$ ). Across all other tissues Dicer transcript levels remained unchanged (Figure 5.4 B).

Xpo5 transcript levels showed the greatest fluctuations in mRNA relative to housekeepers. In PAC.2 cells transcript levels were extremely significantly increased by 25-fold compared to 1 dpf levels. In ZFL and skin there was a 13-fold increase. There were no other significant differences in Xpo5 transcript levels across the remaining tissues, although slight increases were observed in brain and intestines (Figure 5.4 C).

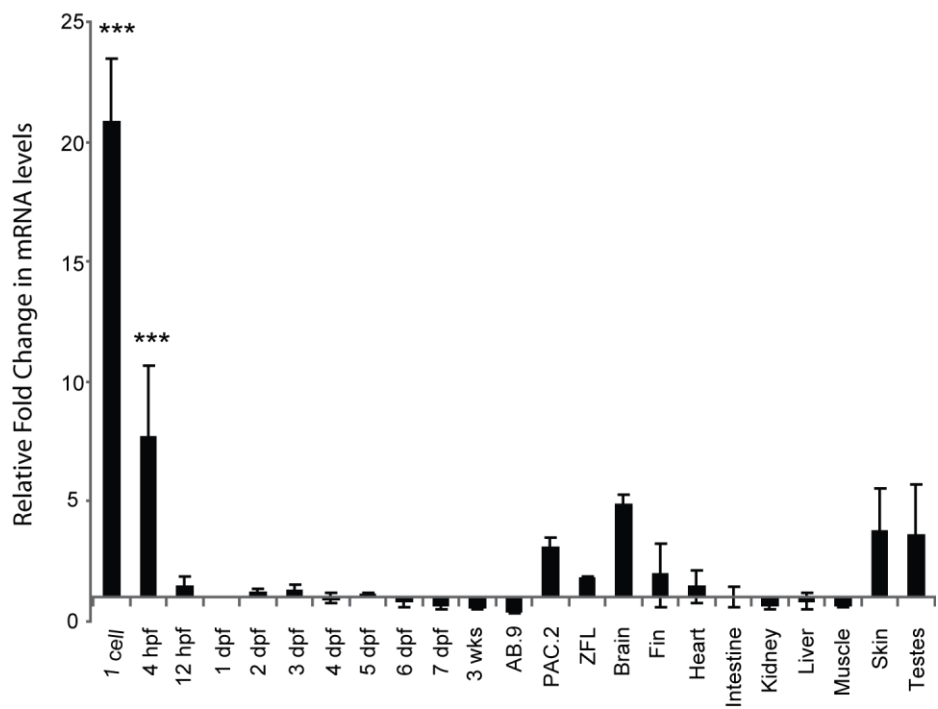
Drosha transcripts were detected by normal gel based PCR methods as an appropriate qPCR assay could not be identified. Drosha could be detected across all time points and in all tissues and cell types. Drosha levels were fairly consistent, across the time course but seemed up-regulated in all three cell types and especially in PAC.2 cells. Across the tissue bank, brain and kidney have slightly higher levels of Drosha, whereas in intestine and liver Drosha mRNA levels were slightly reduced. The housekeeper used was ef1 $\alpha$  which seemed consistent across the tissue bank, cell types and across the time course apart from at the single-cell embryo stage where levels of the housekeeper were considerably lower. Therefore, Drosha levels relative to the housekeeper at this stage are considerably higher (Figure 5.4 D).

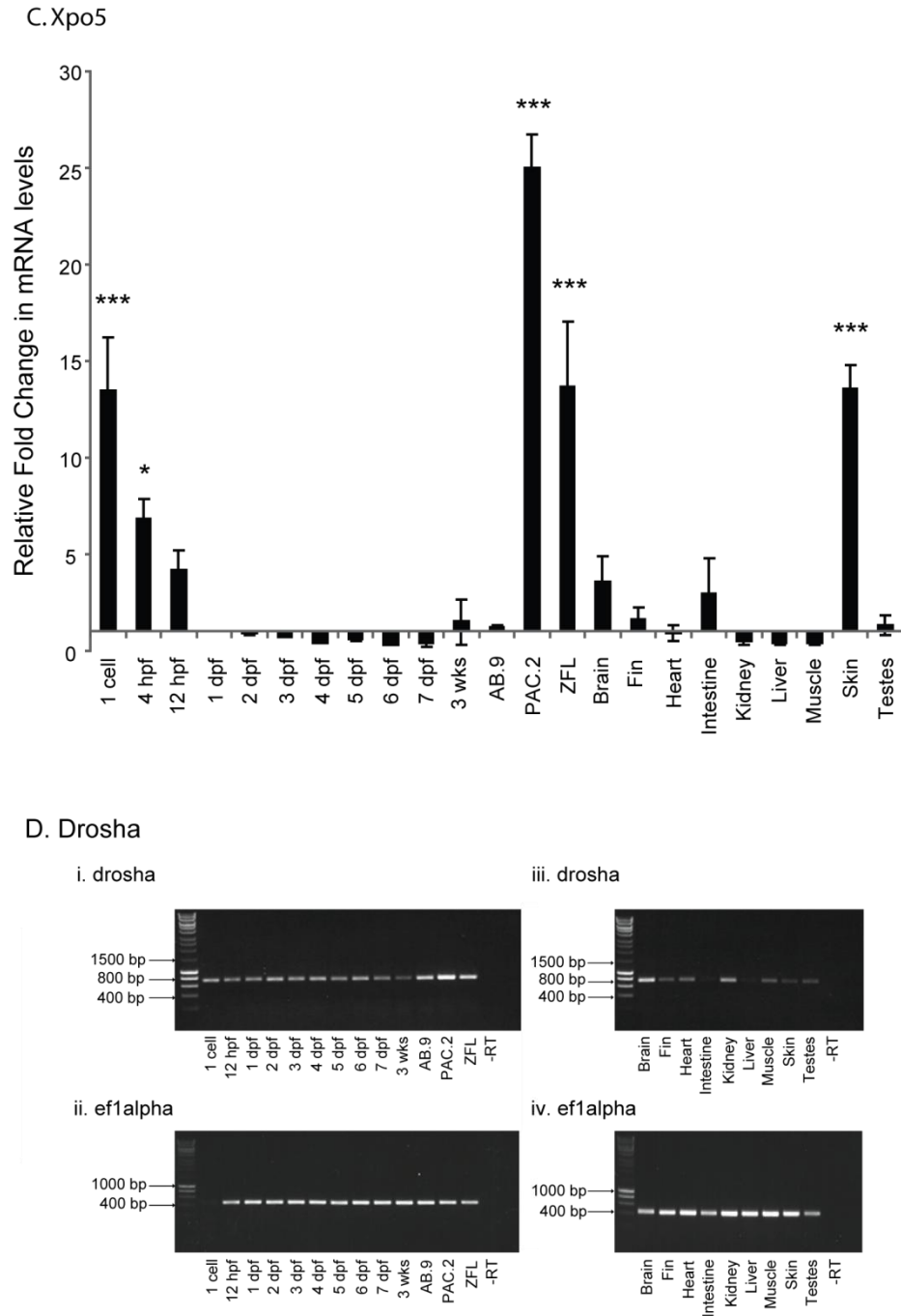


A. Ago2



B. Dicer





**Figure 5.4: Detection and Quantification of Zebrafish Ago2, Dicer, Xpo5 and Drosha mRNA levels throughout Development and across Tissues.** A-C: Relative fold changes in mRNA levels of Ago2 (A), Dicer1 (B) and Xpo5 (C) compared to 1 dpf levels. Transcript levels were determined by real-time qPCR and normalised against the average of three housekeepers (ef1 $\alpha$ , rpl13 $\alpha$  and  $\beta$ -actin). Quantification is based on three individual time courses and tissue banks. Results were analysed by One-way ANOVA followed by the Dunnett test using 1 dpf values as the control. \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ . D: Detection of a 722 bp fragment of Drosha mRNA across a time course (i) and a tissue bank (iii). Transcript levels of the housekeeper ef1 $\alpha$  (448 bp fragment) across the time course and tissue bank are shown in ii and iv respectively. A minus RT reaction was used as a negative control to check successful digestion of genomic DNA.

### 5.3.2 Detection and Quantification of mature miRNA

To investigate whether the efficiency of mature miRNA synthesis varied in a time- or tissue-specific fashion, and whether this correlated with expression of components of the RNAi machinery, a transgenic line expressing an miRNA with sequence corresponding to the Arabidopsis ath-miR-162a was established and assessed for mature miRNA production relative to the amount of mcherry (a measure of RNAi vector expression). Gradient and R<sup>2</sup> values for validated TaqMan qPCR assays are shown in

Table 5.6.

Transcript	Gradient	R <sup>2</sup> value
ath-miR-162a	-3.148	0.998
mcherry	-3.224	0.982

**Table 5.6: Gradient and R<sup>2</sup> values of validated TaqMan assays used in the detection of mature ath-miR-162a and mcherry mRNA.** A slope with a gradient of -3.32 indicates that the primers are 100% efficient. -3.1 is equivalent to 90% efficiency and -3.58 110% efficiency. Only primers which fell between these ranges and gave rise to a correlation coefficient (R<sup>2</sup>) of at least 0.97 were accepted for use in cDNA quantification.

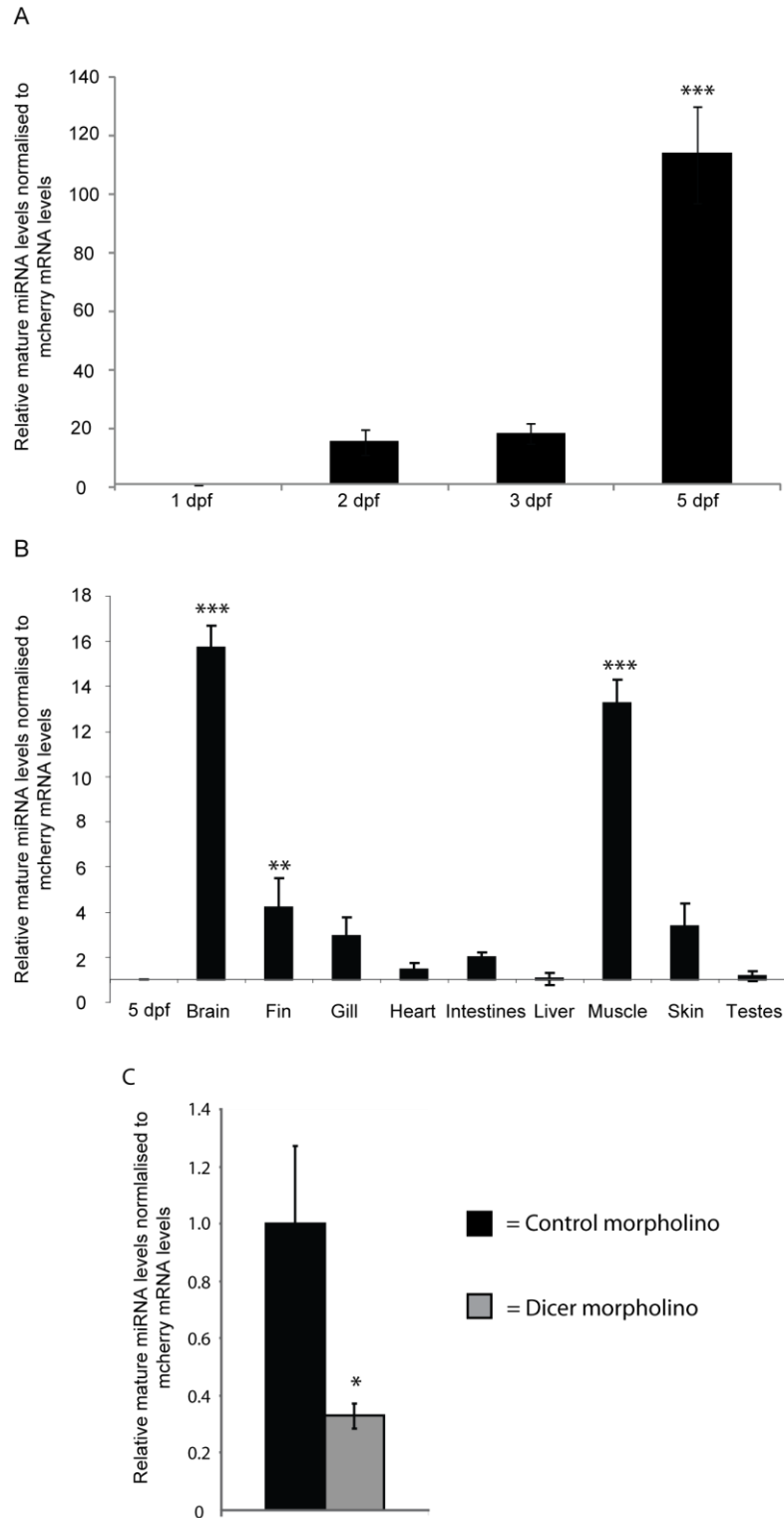
The amount of mature miRNA relative to the amount of mcherry mRNA produced was measured in individual embryos at 1,2,3 and 5 dpf and is shown in Figure 5.5 A as a relative fold change above 1 dpf levels. The amount of miRNA per mcherry mRNA increases (though non-significantly) by approx. 23-fold between 1 and 2 dpf at which point it remains stable to 3 dpf before increasing extremely significantly by 5 dpf to approx. 180 times 1 dpf levels. Though the general trend is towards increased miRNA production, there is a large amount of variation among individual embryos in the amount of mature miRNA produced.

Figure 5.5 B compares mature miRNA relative to mcherry production between that detected in a pool of 30 5 dpf embryos to a pool of 6 month old adult tissues. Relative miRNA production is extremely significantly increased in brain and muscle and highly significantly increased in fin. Gill and skin also show slight increases, though these are not significant. As a negative control this assay was also performed on zebrafish embryos/larvae and adult tissues expressing the control miRNA. In these samples no ath-miR-162a

mature miRNA was detected. Unfortunately, the amount of mature miRNA produced in AB.9, PAC.2 and ZFL cells could not be assessed due to the extremely poor transfection efficiencies of these cells.

#### 5.3.2.1 Mature miRNA Production is Dicer Dependent

To validate that the ath-miR-162a TaqMan assay was specific for mature miRNA and does not detect primary or precursor forms of the miRNA, transgenic fish expressing the ath-miR-162a miRNA were injected with a standard control or Dicer morpholino and individual zebrafish were assessed at 2 dpf for mature miRNA production. Figure 5.5 C shows that injection with Dicer morpholino leads to a statistically significant 67% reduction in mature miRNA relative to mcherry mRNA.



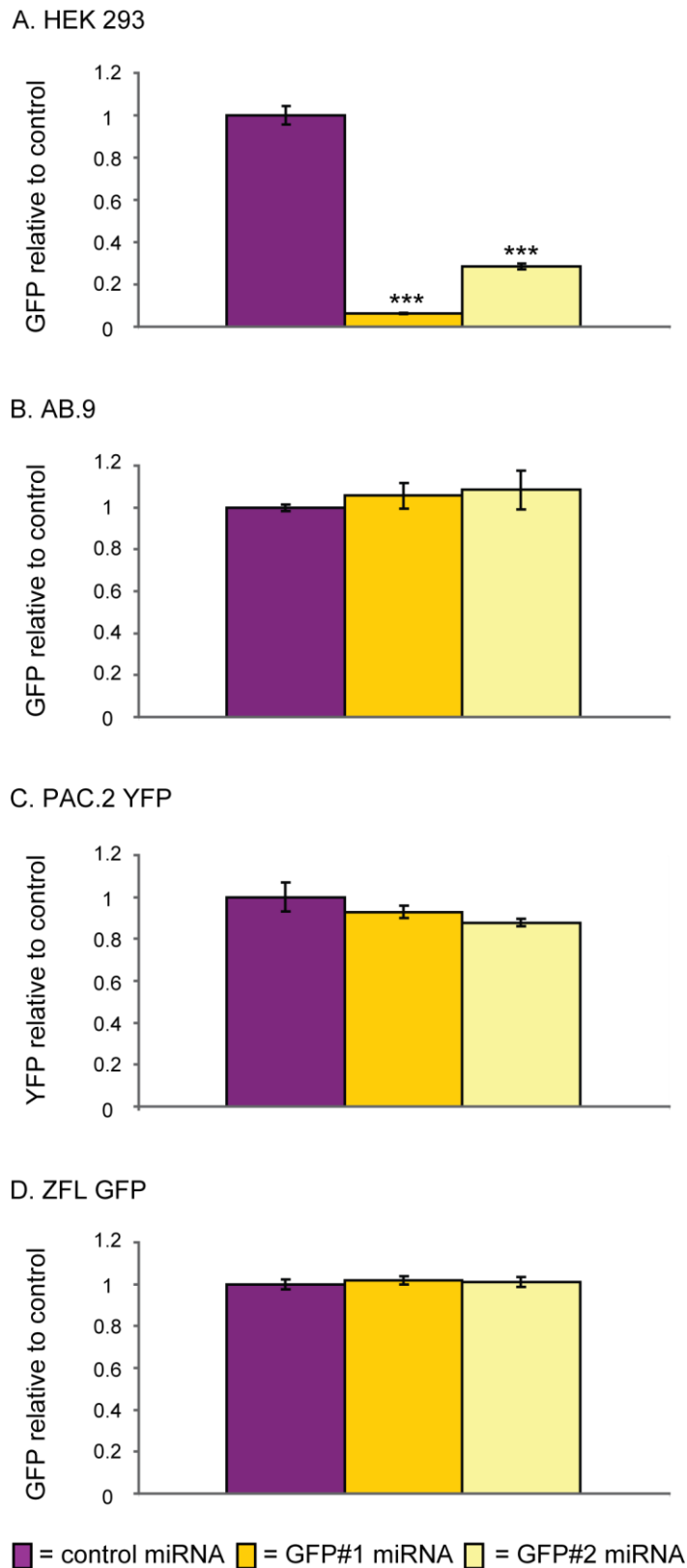
**Figure 5.5: Detection and Quantification of Mature miRNA Production.** Mature miRNA production in individual embryos at 1,2,3 and 5 dpf normalised to mcherry mRNA levels and relative to 1 dpf levels. One-way Anova followed by Dunnett test was carried out using 1 dpf values as the control (n=3). B: Mature miRNA production across tissues normalised to mcherry mRNA levels and relative to 5 dpf levels. One-way Anova followed by Dunnett test was carried out using 5 dpf values as the control (n=3). C: Mature miRNA production normalised to mcherry mRNA levels in individual 2 dpf embryos injected with a standard control or Dicer morpholino. Independent samples T test was carried out (n=5). \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ .

### **5.3.3 RNAi Mediated Knockdown in Zebrafish Cells**

In order to test whether zebrafish cells were capable of vector-mediated RNAi, and whether pol III-responsive vectors were more efficient than pol II-responsive vectors, both pol II- and pol III-responsive non-gal4-VP16/UAS vectors were tested for their ability to knockdown GFP/YFP in AB.9, stable PAC.2 YFP (a kind gift from Ewa Snaar-Jagalska, University of Leiden, Netherlands) and stable ZFL GFP cells.

#### **5.3.3.1 Pol II-responsive Vector-mediated Gene Knockdown**

Pol II-responsive, non-gal4 RNAi vectors under the control of the H2A.Zf promoter containing either the control or GFP miRNAs were transiently transfected into human HEK 293 cells (co-transfected with ef1 $\alpha$  GFP in a 3:1 molar ratio of RNAi vector:GFP), zebrafish AB.9 cells (co-transfected with ef1 $\alpha$  GFP in a 3:1 ratio of RNAi vector:GFP), stable PAC.2 YFP cells and stable ZFL GFP cells. After transfection cells were grown for three days and then analysed via flow cytometry for GFP/YFP content. As before, Figure 5.6 A shows the extremely significant level of GFP knockdown achieved in HEK 293 cells with GFP#1 (95% knockdown) and GFP#2 (72% knockdown) miRNAs. In AB.9 cells and ZFL GFP cells no knockdown with either miRNA was detected (Figure 5.6 B and D). In PAC.2 YFP cells a slight but non-significant level of YFP knockdown was detected with both miRNAs (Figure 5.6 C).

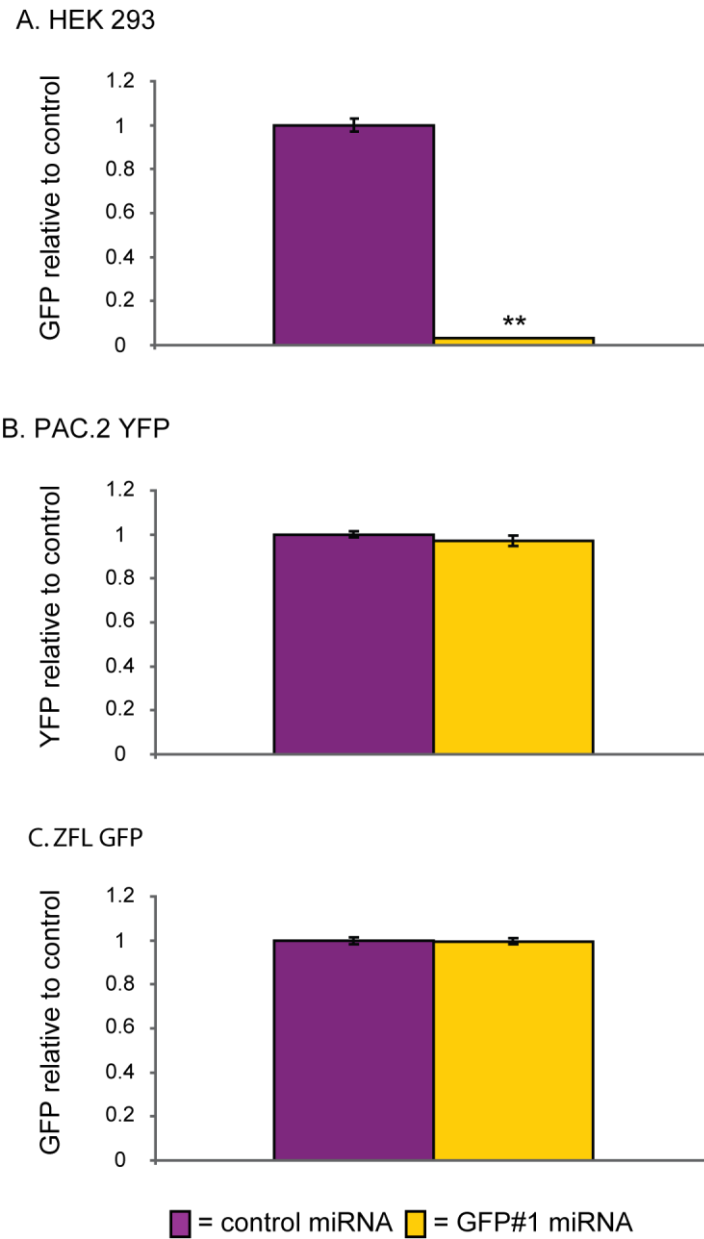


**Figure 5.6: Pol II-mediated GFP/YFP Knockdown in Human (HEK 293) and Zebrafish (PAC.2 YFP and ZFL GFP) Cell Lines.** Relative GFP/YFP levels after transfection with vectors containing a control miRNA (purple bars), GFP#1 miRNA (gold bars) or GFP#2 miRNA (yellow bars). A: HEK 293 cells (n=9). B: PAC.2 YFP cells (n=9). C: ZFL cells (n=9). GFP/YFP levels measured using fluorescent flow cytometry. Results were analysed by One-way ANOVA. \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ .

### 5.3.3.2 Pol III-responsive Vector-mediated Gene Knockdown

Pol III-responsive RNAi vectors containing control or GFP shRNAs were transiently transfected into human HEK 293 cells (co-transfected with ef1 $\alpha$  GFP in a 3:1 molar ratio of RNAi vector:GFP), stable PAC.2 YFP cells and stable ZFL GFP cells. Three days post transfection the cells were analysed for GFP/YFP content via flow cytometry. In HEK 293 cells, cells transfected with GFP#1 shRNAs have an extremely significant 97% reduction in GFP compared to control shRNA transfected cells. However, in PAC.2 YFP and ZFL GFP cells the same GFP#1 shRNA was unable to produce any knockdown of YFP/GFP relative to the control shRNA.

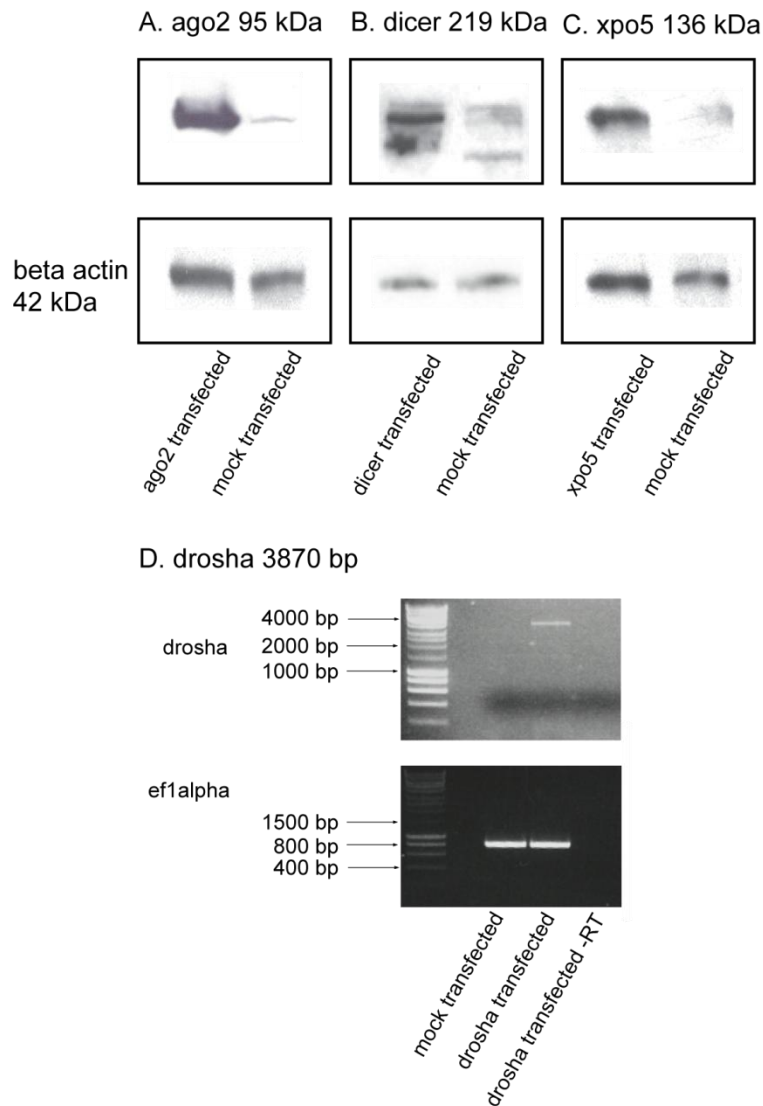




**Figure 5.7: Pol III-mediated GFP/YFP Knockdown in Human (HEK 293) and Zebrafish (PAC.2 YFP and ZFL GFP) Cell Lines.** Relative GFP/YFP levels after transfection with vectors containing a control miRNA (purple bars) or GFP#1 miRNA (gold bars). A: HEK 293 cells (n=3). B: PAC.2 YFP cells (n=9). C: ZFL cells (n=3). GFP/YFP levels measured using fluorescent flow cytometry. Results were analysed by One-way ANOVA. \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ .

### **5.3.4 Improving Knockdown by Over-expressing Components of the RNAi Machinery**

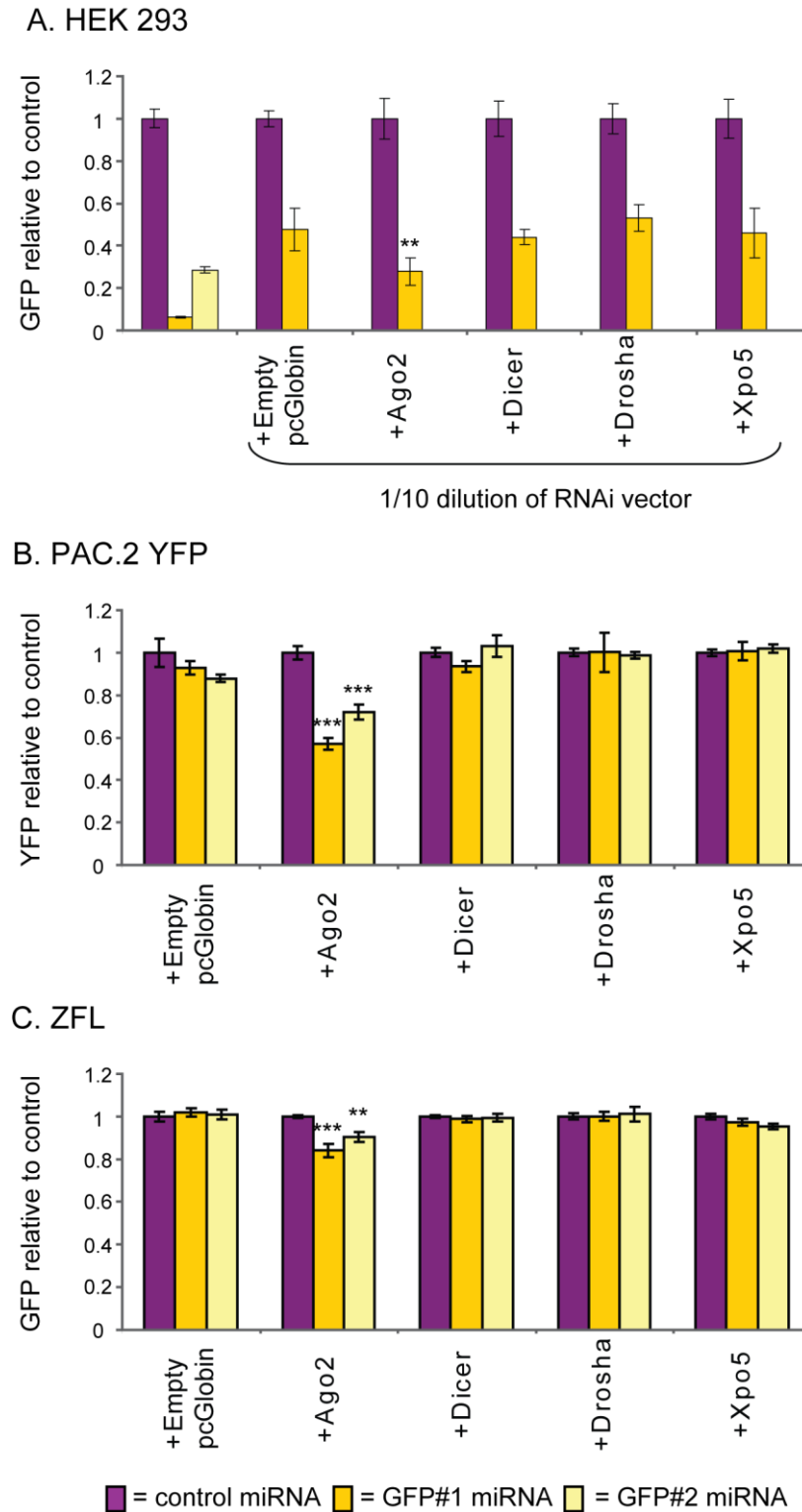
In order to test whether knockdown could be achieved by over-expressing components of the RNAi machinery, human Ago2, Dicer and Xpo5 and zebrafish Drosha were cloned into pcGlobin vector (see section 5.2.5), and correct expression of the vectors was confirmed by over-expression in HEK 293 cells (Figure 5.8). Over-expression of Ago2, Dicer and Xpo5 above background levels was confirmed by western blot using  $\beta$ -actin as a house-keeper to control for equal loading (Figure 5.8 A,B and C). Over-expression of pcGlobin Drosha was confirmed by RT-PCR of the full-length (3070 bp) Drosha transcript from cDNA synthesised from transfected cells. Mock-transfected cells were used as a negative control as primers only recognise the zebrafish Drosha and not endogenous human Drosha. A minus RT reaction was also carried out using mRNA from pcGlobin Drosha transfected cells to confirm that the amplicon originated from cDNA and not transfected plasmid DNA (Figure 5.8 D).



**Figure 5.8: Confirming Expression of pcGlobin Ago2, pcGlobin Dicer, pcGlobin Xpo5 and pcGlobin Drosha in HEK 293 cells.** A: (top panel) Detection of Ago2 in pcGlobin Ago 2 transfected cells compared to mock transfected cells by western blot. B: (top panel) Detection of Dicer in pcGlobin Dicer transfected cells compared to mock transfected cells by western blot. C: (to panel) Detection of Xpo5 in pcGlobin Xpo5 transfected cells compared to mock transfected cells by western blot. A-C: (bottom panels) Detection of the house-keeper  $\beta$ -actin by western blot to check equal loading of proteins. D: (top panel) Detection of full-length (3870 bp) Drosha in pcGlobin Drosha transfected cells compared to mock transfected cells by RT-PCR. A minus RT reaction was used as a negative control. D: (bottom panel) Detection of an 800 bp fragment of the house keeper ef1 $\alpha$  in both mock transfected and pcGlobin Drosha transfected cells, but not in the minus RT control by reverse transcription PCR.

Equal ratios of pcGlobin vectors containing either Ago2, Dicer, Xpo5 Drosha or no (empty pcGlobin) cDNA and either the pol II-responsive (Figure 5.9) or pol III-responsive (Figure 5.10) RNAi vectors containing control and GFP miRNAs/shRNAs were co-transfected into PAC.2 YFP cells and ZFL GFP cells (pol II-responsive vector only). HEK 293 cells were co-transfected with a 10-fold dilution of the RNAi vector and a 3:1 ratio of RNAi component: GFP (a 10-fold dilution of the RNAi vector was necessary due to the high efficiency of RNAi in HEK 293 cells). GFP/YFP levels were then measured in mcherry positive cells using fluorescent flow cytometry.

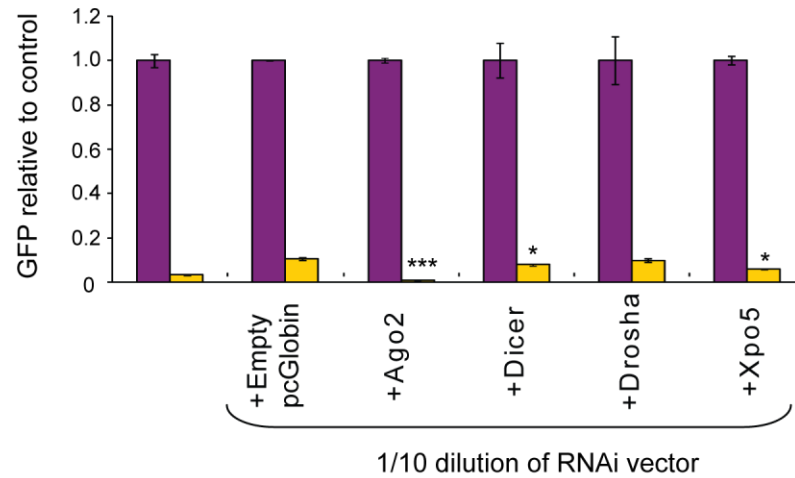
Using a pol II-responsive RNAi vector (Figure 5.9) in HEK 293 cells a 10-fold decrease in RNAi vector diminished knockdown efficiency from approx. 95% to 50% using the GFP#1 miRNA. Over-expression of Ago2 significantly improved this knockdown to 72% (a 1.5-fold improvement). None of the other components (Dicer, Xpo5 or Drosha) had any significant affect on knockdown efficiency (Figure 5.9 A). In PAC.2 YFP cells GFP#1 and GFP#2 miRNAs result in a slight, but non-significant reduction in YFP levels. Over-expression of Ago2 significantly improved this knockdown to approx. 43% and 28% with GFP#1 and GFP#2 miRNAs respectively. As in HEK 293 cells, over-expression of Dicer, Xpo5 and Drosha did not improve knockdown (Figure 5.9 B). In ZFL GFP cells GFP#1 and GFP#2 miRNAs result in no knockdown of GFP. Over-expression of Ago2 resulted in a slight but significant GFP knockdown of approx. 16% and 10% with GFP#1 and GFP#2 miRNAs respectively. As in HEK 293 cells and PAC.2 YFP cells, over-expression of Dicer1, Xpo5 and Drosha had no affect on GFP knockdown (Figure 5.9 C).



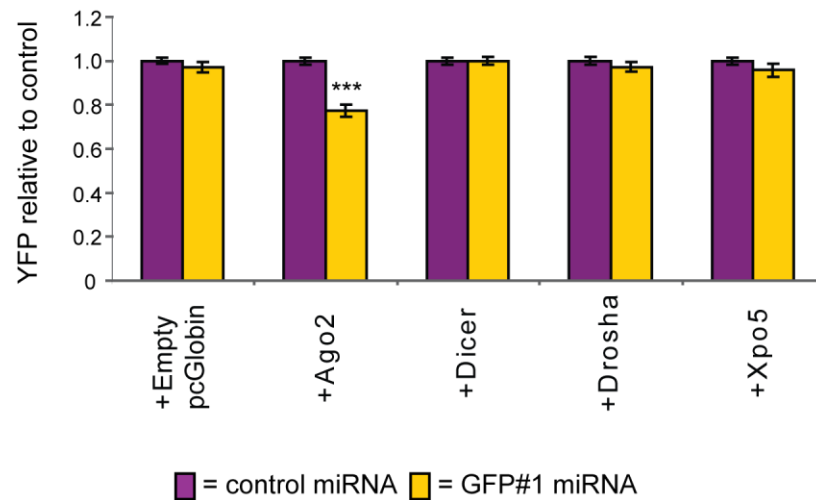
**Figure 5.9: Enhancing Pol II-driven Knockdown of GFP/YFP in Human and Zebrafish Cells Through Over-expression of RNAi Components.** Relative GFP/YFP levels after co-transfection with vectors containing a control miRNA (purple bars), GFP#1 miRNA (gold bars) or GFP#2 miRNA (yellow bars) plus RNAi components (Ago2, Dicer, Xpo5 or Drosha in pcGlobin). A: HEK 293 cells (n=9, due to extremely high knockdown efficiencies in HEK 293 cells, RNAi vectors were diluted 10-fold). B: PAC.2 YFP cells (n=9). C: ZFL cells (n=9). GFP levels measured using fluorescent flow cytometry. Results were analysed by One-way ANOVA. \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ .

Figure 5.10 shows the results of knockdown using the pol III-responsive vector and over-expressing RNAi components. In HEK 293 cells a 10-fold dilution of the pol III-responsive RNAi vector still leads to a 90% knockdown in GFP. As with the pol II-responsive vector over-expression of Ago2 results in an extremely significant improvement in knockdown. The extent of this improvement however, is smaller than with the pol II-responsive vector as knockdown efficiency even with a 10-fold dilution of the pol III-vector was already extremely high. With Dicer and Xpo5 over-expression there was also a very slight, but significant improvement in knockdown efficiency. No such improvement was seen with Drosha over-expression. In PAC.2 YFP cells over-expression of Ago2 led to a 23% reduction in YFP levels in cells expressing the GFP #1 shRNA compared to the control. No knockdown was seen in cells over-expressing Dicer, Xpo5 or Drosha.

## A. HEK 293



## B. PAC.2 YFP



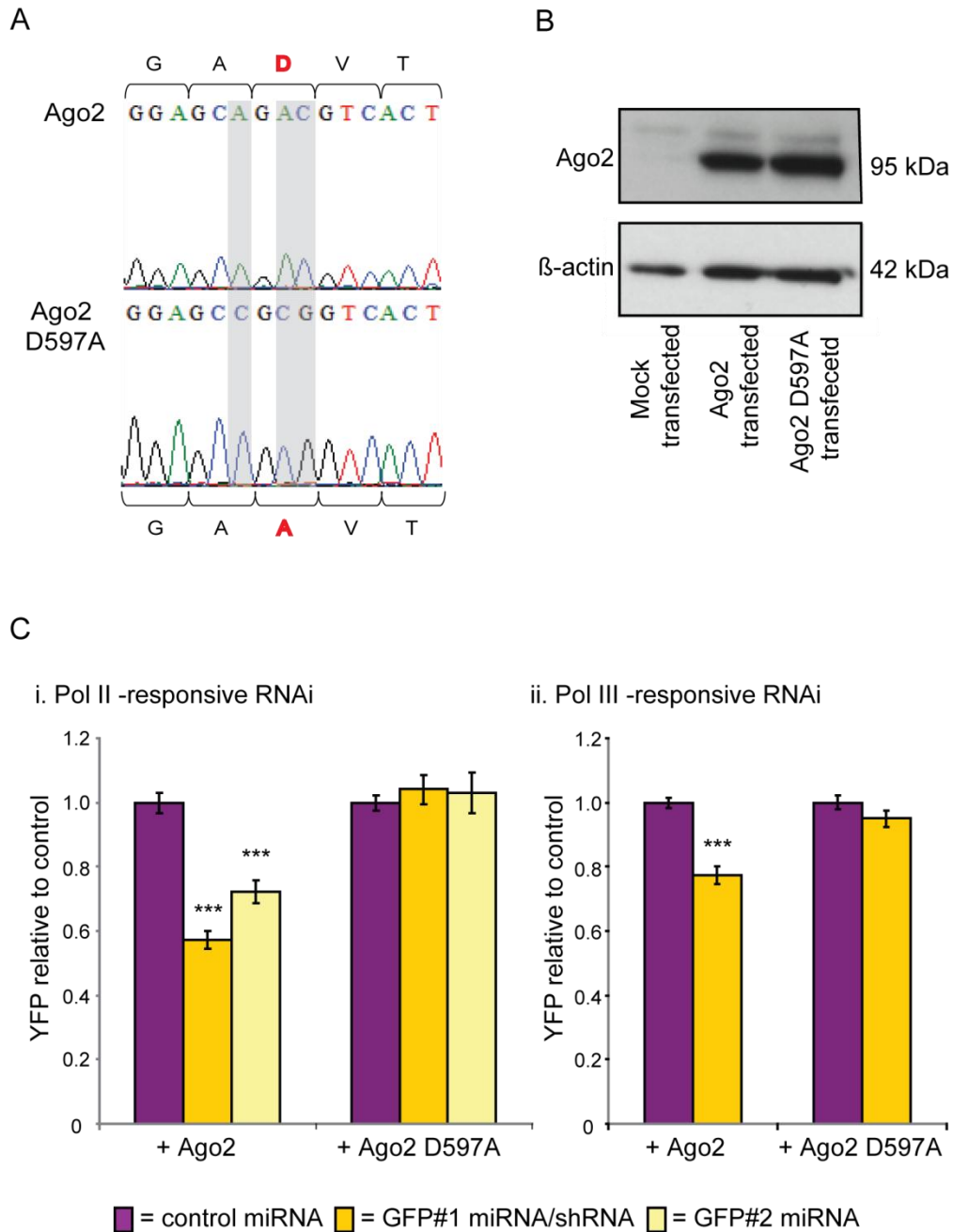
**Figure 5.10: Enhancing Pol III-responsive Knockdown of GFP/YFP in cells through Over-expression of RNAi components.** Relative GFP/YFP levels after co-transfection with vectors containing a control miRNA (purple bars) or GFP#1 miRNA (gold bars) plus RNAi components (Ago2, Dicer, Xpo5 or Drosha in pcGlobin). A: HEK 293 cells (n=3, due to extremely high knockdown efficiencies in HEK 293 cells, RNAi vectors were diluted 10-fold). B: PAC.2 YFP cells (n=9). GFP/YFP levels measured using fluorescent flow cytometry. Results were analysed by One-way ANOVA. \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ .

#### 5.3.4.1 Ago2 Enhanced Gene Knockdown is Dependent of Functional RNase Domain

In order to ascertain whether the improved knockdown detected with Ago2 over-expression is mediated through its intrinsic slicer activity, Ago2 cDNA was mutated to create an RNase dead version, Ago2 D597A by changing bases A and C at position 1790 and 1791 to C and G respectively. Base 1788 was also changed from A to C to introduce a *SacII* restriction site which aided in the identification of mutagenised clones (Figure 5.11 A). After sub-cloning into the pcGlobin vector the expression of Ago2 D597A was checked by transfecting into HEK 293 and western blotting for Ago2. Figure 5.11 B shows the detection of Ago2 and Ago2 D597A in transfected cells. A very faint band was also detectable in mock transfected cells and represents the endogenous Ago2 expression.

Co-transfection of PAC.2 YFP cells with Ago2 D597A and either the pol II - (Figure 5.11 C.i) or pol III - (Figure 5.11 C.ii) responsive RNAi vectors containing GFP#1 and GFP#2 miRNAs and GFP#1 shRNA respectively led to a loss of the Ago2 induced knockdown of YFP relative to control miRNA/shRNA.





**Figure 5.11: Ago2 Enhanced Gene Knockdown is due to a Functional RNase Domain.**

A: Schematic showing the mutagenesis of an aspartic acid (D) residue at position 597 to an alanine (A) residue by mutating bases AC at position 1790 and 1791 to CG to create an endonuclease dead Ago2, Ago2 D597A. Base 1788 was also changed from A to C to introduce a *Sac*II restriction site, used for identifying mutagenised clones. B: Western blot for Ago2 in Ago2 and Ago2 D597A transfected HEK 293 cells.  $\beta$ -actin was used as control for equal loading. C. Relative YFP levels in PAC.2 YFP cells after co-transfection with vectors containing a control miRNA/shRNA (purple bars), GFP#1 miRNA/shRNA (gold bars) or GFP#2 miRNA (yellow bars) plus either pcGlobin Ago2 or pcGlobin Ago2 D597A. C. i: pol II- responsive vector-mediated YFP knockdown (n=9). C.ii: pol III-responsive vector-mediated YFP knockdown (n=9). YFP levels measured using fluorescent flow cytometry. Results were analysed by One-way ANOVA followed by the Dunnett test. \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ .

### 5.3.5 Testing Different Stem Loops for Knockdown Efficiencies

Up until this point the backbone of the miRNA being used was the hsa-mir-30a backbone (miRBase accession number: MI0000088, Figure 5.12 A i) and the designed miRNAs were placed in the 3' arm (Figure 5.12 A ii). In order to test whether placement of the of the mature miRNA in the 5' arm or using an endogenous zebrafish backbone could further improve the knockdown efficiency achieved in PAC.2 YFP cells co-transfected with Ago2, the hsa-mir-30a backbone was adapted to contain the miRNA encoding sequence in the 5' arm (Figure 5.12 A iii) and the zebrafish dre-mir-30a (miRBase accession number: MI0001940, Figure 5.12 B i) was also likewise adapted to contain the miRNA sequence in the 5' arm (Figure 5.12 B ii).

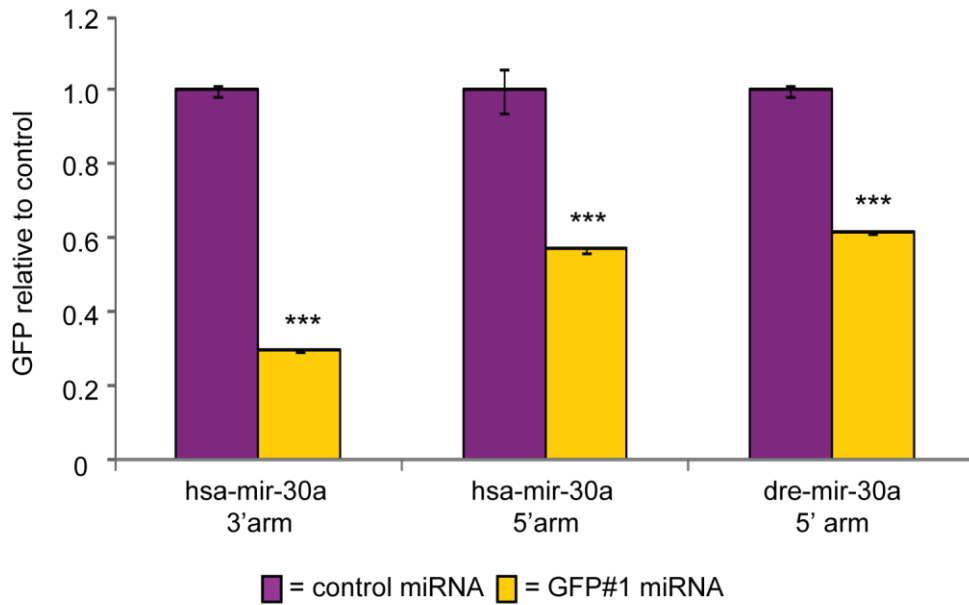
miRNA strand selection is based upon the thermodynamic stability at each end of the miRNA, with the strand with the least thermodynamically stable 5' end being preferentially unwound and incorporated into RISC. The Invitrogen BlockIT® miRNA designer used to design miRNAs throughout this thesis selects miRNAs based on this principle. Therefore, the 5' end of the miRNA are always more AT rich and the 3' end are more GC rich. Simply moving the mature miRNA sequence from the 3' arm to the 5' arm but keeping their 5' to 3' orientation the same should therefore change which strand is incorporated into RISC, but the sequence of the mature miRNA will remain the same.

Co-transfection of the silencing vectors with ef1 $\alpha$ ::GFP into HEK 293 cells resulted in silencing by each of the miRNAs. However, the most efficient silencing was induced by the original miRNA backbone, hsa-mir-30a with miRNA encoded in the 3' arm (~70%). Placement of the miRNA in the 5' arm of both the hsa-mir-30a and the dre-mir-30a backbone lead to similar less efficient silencing of between 40 and 50% (Figure 5.12 C).

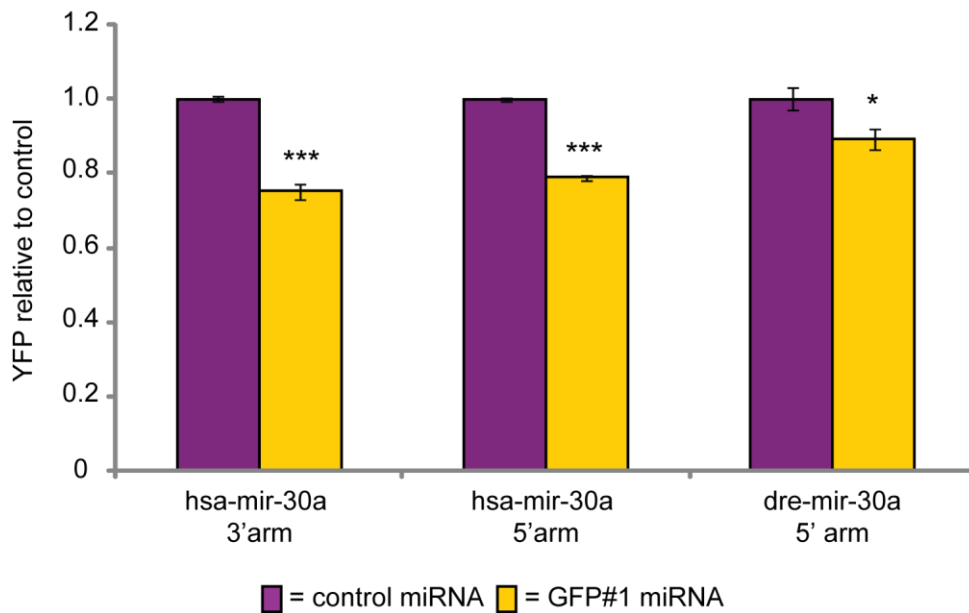
Transfection of the silencing vectors containing the hsa-mir-30a backbone with the miRNA in either the 5' or 3' arm led to similar and extremely significant silencing in PAC.2 YFP cells co-transfected with Ago2 pcGlobin. The dre-mir-30a backbone miRNA also induced significant



## C. HEK 293



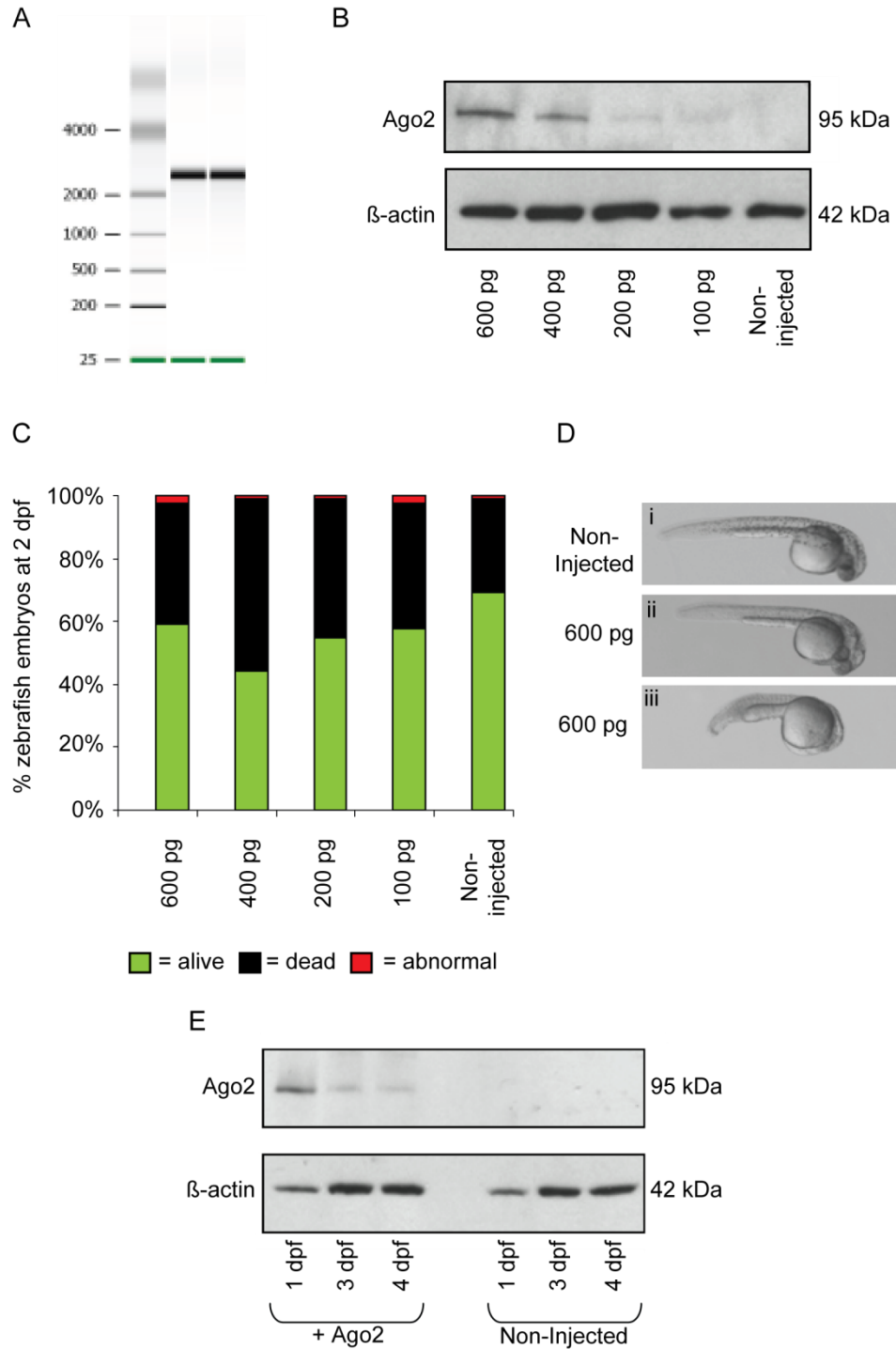
## D. PAC.2 YFP



**Figure 5.12: Testing Different Stem Loops for Knockdown Efficiencies.** A. i: the original has-mir30a stem loop showing the position of the mature miRNA in the 5' arm (red) and the 3' arm (purple) (miRBase accession number: MI0000088) A. ii and iii: adaptation of the has-mir30a stem loop to contain the GFP #1 miRNA in the 3' and 5' arms respectively. ii is the miRNA sequence used up until now. B. i: the original dre-mir-30a (miRBase accession number: MI0001940) stem loop showing the position of the mature miRNA in the 5' arm (red). ii: Adapted dre-mir-30a to contain the GFP #1 miRNA sequence in the 5' arm (red). C. Level of GFP knockdown achieved in HEK 293 cells co-transfected with the original vector containing the miRNA in the 3' arm of hsa-mir-30a or in the 5' arm of hsa-mir-30a or the 5' arm of dre-mir-30a and ef1 $\alpha$ :GFP (n=3). D. Level of YFP knockdown achieved in PAC.2 YFP cells co-transfected with the original vector containing the miRNA in the 3' arm of hsa-mir-30a or in the 5' arm of hsa-mir-30a or the 5' arm of dre-mir-30a and Ago2 pcGlobin (n=6). Results were analysed by One-way ANOVA followed by the Dunnett test. \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ .

### 5.3.6 Over-expression of Ago2 in Zebrafish Embryos

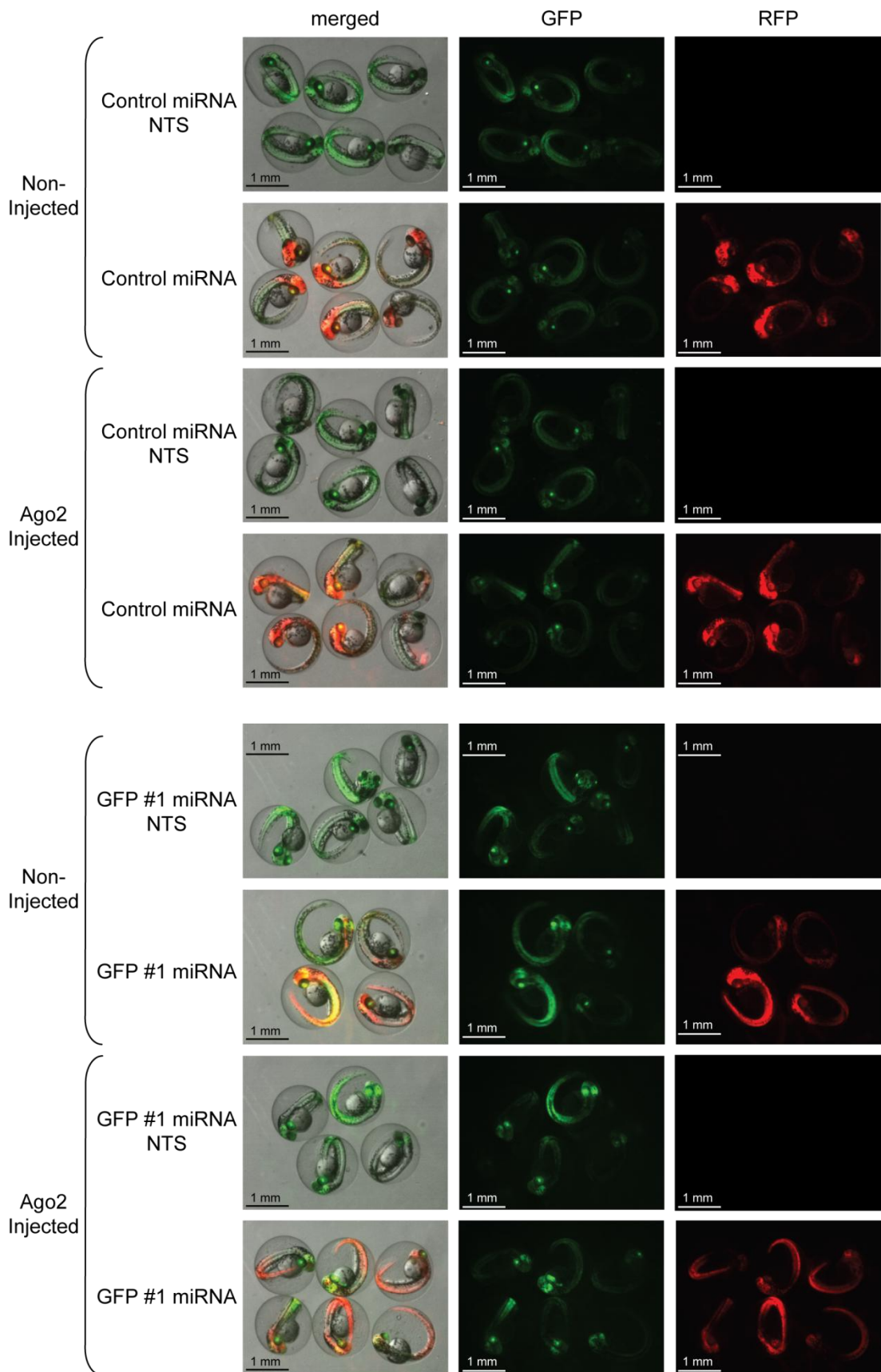
As over-expression of Ago2 enhanced RNAi in PAC.2 and ZFL cells, next I investigated whether Ago2 over-expression is tolerated and is sufficient enough to induce silencing *in vivo*. To do this, Ago2 and Ago2 D597A mRNA was transcribed *in vitro* using T7 primers from the Ago2 and Ago2 D597A pcGlobin vectors respectively. The RNA was then analysed on a bio-analyser to check the integrity of the RNA (Figure 5.13 A). The presence of a single band of approximately 3000 nt suggested that the RNA was intact. Ago2 mRNA was then injected into single-cell stage embryos at doses from 100 pg up to 600 pg RNA. Figure 5.13 B shows a western blot for Ago 2 protein. Endogenous Ago2 is not detectable in non-injected embryos. However, injection of Ago2 mRNA leads to detectable levels of Ago2 protein and the Ago2 signal becomes increasingly strong with increasing amounts of mRNA injected. The house-keeper  $\beta$ -actin remains unchanged. Compared to non-injected zebrafish, injection of mRNA led to between 10-15% increase in embryo mortality. However, amongst embryos injected with Ago2 mRNA there was no significant association between dose of mRNA injected and lethality ( $X^2 = 7.338$ , 3 d.f,  $p > 0.05$ ). Injection of mRNA into embryos did lead to a slight developmental delay as can be seen in Figure 5.13 D. However, despite the delay, embryos developed normally and by 5 dpf were indistinguishable from non-injected controls. A small percentage around 3% did develop abnormally, the majority of these abnormalities involved under development of the head and curvature of the spine (Figure 5.13 D iii). Such abnormalities however did also arise in non-injected zebrafish. Due to little apparent toxicity of Ago2 mRNA, an injection concentration of 600 pg was chosen for future injections. In order to see how long protein generated from Ago2 mRNA injection is detectable, Ago2 was injected at 600 pg and embryos/larvae were harvested and protein extracted at 1, 3 and 4 dpf. Figure 5.13 E shows the western blot for the detection of Ago2 across the time course. Levels in injected embryos are highest at 1 dpf, but can still be detected out to 4 dpf. Endogenous Ago2 protein levels in non-injected zebrafish are barely detectable.



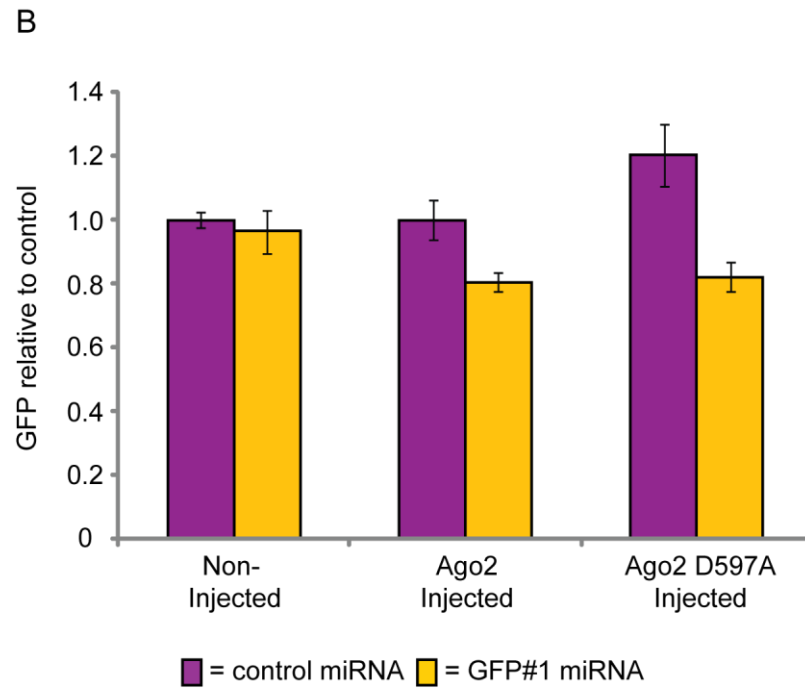
**Figure 5.13: Over-expression of Argonaute 2 in Zebrafish Embryos.** A: Electrophoresis of *in vitro* transcribed Ago2 and Ago2 D597A mRNA. A single band at 3300 nt indicates the presence of intact mRNA. B: Western blot for Ago2 in embryos injected with Ago2 mRNA ranging from 100 pg to 600 pg. β-actin was used to control for equal loading. C: % survival among embryos injected with varying doses of Ago2 mRNA compared to non-injected embryos. There is no association between Ago2 mRNA dosage and lethality ( $X^2 = 7.338$ , 3 d.f.,  $p > 0.05$ ). D: Non-injected (i) and Ago2 mRNA injected embryos (ii and iii) at 2 dpf. Injection of Ago2 mRNA leads to a slight developmental delay (ii) and in a minority of cases (<3%) more severe developmental defects (iii), such as malformation of the head and truncation and curvature of the spine. E: Western blot of Ago2 protein expression after injection with 600 pg Ago2 mRNA at 1, 3 and 4 dpf. β-actin was used to control for equal loading.

To test whether Ago2 mRNA injection could lead to knockdown of GFP, the highest tolerated dose (600 pg) of Ago2 or Ago2 D597A mRNA was injected into doubly transgenic zebrafish expressing both ubiquitous GFP and either the control or GFP #1 miRNA. At 2 dpf zebrafish over-expressing either the control or GFP #1 miRNA were analysed for GFP expression relative to their non-transgenic siblings (NTS). Figure 5.14 A shows a selection of the control and GFP #1 expressing embryos either non-injected or injected with Ago2. Although GFP levels vary, there was no obvious knockdown of GFP in Ago2 injected fish expressing the GFP #1 miRNA compared to their NTS. To analyse this further batches containing between 10-15 zebrafish embryos were trypsinised and the GFP content was analysed by flow cytometry. This analysis can be seen in Figure 5.14 B. Although there was some knockdown of GFP in zebrafish injected with Ago2 mRNA and expressing the GFP #1 miRNA this was not statistically significant, and a similar effect was also seen with injection of the RNase dead version of Ago2.

A







**Figure 5.14: GFP Knockdown in Zebrafish Injected with Ago2 mRNA.** A: Images of transgenic zebrafish expressing either the control or GFP #1 miRNA next to their relative NTSs and either non-injected or injected with Ago2 mRNA B: GFP levels relative to control in transgenic zebrafish expressing either the control or GFP #1 miRNA and either non-injected (control miRNA  $n=4$ , GFP miRNA  $n=3$ ) injected with Ago2 mRNA (control miRNA  $n=4$ , GFP miRNA  $n=4$ ) or injected with Ago2 D597A mRNA (control miRNA  $n=3$ , GFP miRNA  $n=3$ ). Results were analysed by One-way ANOVA followed by the Dunnett test using control non-injected as the reference data set. There is no significant difference between any of the experimental groups compared to the control.  $*$  =  $p < 0.05$ ,  $**$  =  $p < 0.01$ ,  $***$  =  $p < 0.001$ .

## 5.4 Discussion

The lack of knockdown detected in zebrafish embryos, as demonstrated in the previous two chapters, came as a surprise given the importance of the RNAi machinery in zebrafish development and the efficiency of our RNAi vectors at gene knockdown in HEK 293 cells. In this chapter I have therefore taken a closer look at the expression of the RNAi machinery and the ability of our vector to drive expression of mature miRNAs in embryos and across adult tissues. This chapter has also explored the possibility that use of a pol III- rather than a pol II-responsive vector or over-expression of components of the RNAi machinery might induce RNAi in zebrafish cells and embryos.

### 5.4.1 Expression of RNAi Components and Mature miRNA

Analysis of the expression of zebrafish Droscha, Dicer, Xpo5 and Ago2 revealed that all four components are expressed throughout development and in all tissues and cell types tested. Transcript levels of all components were especially high compared to house-keepers at the very early time points tested and decreased rapidly upon the onset of zygotic transcription (around 4 hpf), suggesting that high levels of the transcript of each of the components are maternally inherited and play an important role in early development. By 1 dpf the levels of all components are substantially reduced relative to the house-keepers and remain fairly constant throughout development. In contrast, levels of customised mature miRNA, as measured via a TaqMan qPCR against the miRNA ath-miR-162a, relative to the amount of mcherry produced, increases approx 180-fold on 1 dpf levels by 5 dpf (although a high amount of variation was seen here). One possible explanation for this may be low turn-over of the RNAi component proteins, causing an accumulation of RNAi components and hence more efficient processing of pri- and pre-miRNAs over time. Indeed the half life of Ago2 has been shown to vary from between 1-12 hrs in MDA-231 cells dependent on the presence of epithelial growth factor (EGF) (Adams *et al.*, 2009). Alternatively, the delay in mature miRNA production, despite high levels of maternally contributed RNAi component mRNAs, may be due to the RNAi machinery being otherwise occupied with miRNAs essential for the early

zebrafish development. One such miRNA is miR-430 which has been shown to be essential for normal gastrulation and brain morphogenesis and whose over-expression rescues defects associated with loss of maternal and zygotic Dicer (Giraldez *et al.*, 2005) and over-saturation of the RNAi machinery by siRNAs (Zhao *et al.*, 2008). Finally, the build up in mature miRNA could be due to low turnover of the miRNA leading to a rapid build up.

In the majority of tissues, transcript levels of RNAi components remain statistically unchanged from 1 dpf levels. The exception to this is the brain, which has elevated levels of all of the components analysed and the skin which has elevated levels of Dicer and Ago2 and extremely high levels of Xpo5. Relative levels of the mature miRNA ath-miR-162a however, were consistently at least as high as levels in 5 dpf larvae and therefore substantially higher than 1 dpf levels. Similar levels of RNAi component transcripts but increased amounts of mature miRNA produced in adult tissues, may suggest a decreased demand of the RNAi machinery by endogenous miRNAs in fully developed organs, or the stable build-up of customised miRNA. Mature miRNA expression levels were particularly high in the brain and muscle of 6 month old adult zebrafish. An increased level of mature miRNA in the brain is consistent with increased transcript levels of the RNAi components. However, muscle did not show increased transcript levels of any of the components tested, so seems to suggest a lower load on the RNAi machinery in muscle. Conversely, the skin which showed high level expression of Dicer, Xpo5 and Ago2 did not show increased levels of mature miRNA production, suggesting high demand on the RNAi machinery. Increased RNAi component expression and production of mature customised miRNAs in the brain compared to zebrafish embryos/larvae may explain why PINK1 knockdown was detected in the brain, but no knockdown of GFP or PINK1 was detected in zebrafish embryos/larvae up to 5 dpf.

Transcript levels of components of the RNAi machinery were also analysed in a panel of zebrafish cell lines, including AB.9 cells from adult caudal fin, PAC.2 cells, derived from 24 hpf embryos and ZFL cells, an adult liver epithelial cell line. AB.9 cells showed consistently similar transcript

levels to 1 dpf levels. However, PAC.2 cells and ZFL cells had consistently higher transcript levels for all the components tested, levels of Xpo5 being particularly high and levels of all components being higher in PAC.2 cells than in ZFL cells. Unfortunately however, it was not possible to determine the amount of mature miRNA produced from our vector in these cells, as the transfection efficiencies were too low for quantification of the mature miRNA. In future, to try to quantify miRNA expression in these cells, cells could be transfected and then FAC sorted for mcherry positive cells. RNA extractions could then be harvested from the sorted cells and analysed for mature miRNA production.

The validity of the TaqMan assay to detect only fully processed, mature miRNA was also demonstrated by the fact that morpholino knockdown of Dicer resulted in a significant decrease in mature miRNA detected. Residual detection of mature miRNA might reflect incomplete knockdown of Dicer and/or the presence of maternally contributed Dicer protein.

The measurement of mature miRNA production is extremely valuable as it tells us if, when and where mature miRNA is being expressed. However, it would also be informative to know the level of expression of customised mature miRNA in comparison to that of endogenous miRNAs such as miR430 which have been shown to extremely efficiently knockdown its targets. Absolute quantification of customised mature miRNA compared to miR430 could now be achieved using the TaqMan assay described in section 5.2.2.

#### **5.4.2 Knockdown in Zebrafish Cell Lines**

Both PAC.2 and ZFL cells have high levels of RNAi components. This combined with the fact that the RNAi vector being used has been shown to be fit for purpose by driving expression of mature miRNA throughout zebrafish development and in a variety of zebrafish tissues and achieves good levels of knockdown in HEK 293 cells, suggests that if zebrafish are at all capable of vector-mediated RNAi, it would be most likely detected in these cells. Despite this, no knockdown of GFP/YFP was detected in AB.9, PAC.2

or ZFL cells using the pol II-responsive RNAi vector with H2A.Zf promoter (Figure 5.6). A pol III-responsive vector, utilising a zebrafish H1 promoter, was also tested. In HEK 293 cells this produced highly efficient knockdown of GFP, but in zebrafish PAC.2 cells no knockdown was detected (Figure 5.7). Pol III-responsive promoters are known to give rise to very high levels of transcript and have been shown to be extremely efficient in the delivery of shRNA for gene silencing (Paddison *et al.*, 2004). Indeed, in these experiments a 10-fold dilution of the pol III-responsive RNAi vector was able to produce similar levels of knockdown as the undiluted pol II-responsive vector in HEK 293 (Figure 5.9 and Figure 5.10), clearly demonstrating how efficient this vector is at producing shRNA. The fact that the pol III vector also failed to produce any knockdown in these cells, may suggest that the production of primary miRNA or shRNA is not the limiting factor to achieving knockdown in zebrafish cells. Instead, factors downstream of miRNA/shRNA transcription must be limiting. Equally, it is unlikely that levels of Drosha are limiting, or at least it is probably not the only limiting factor, as pol III-driven shRNAs enter the RNAi pathway downstream of Drosha processing, but are still incapable of producing knockdown in zebrafish cells.

#### 5.4.2.1 Co-expression of Ago2 Results in Knockdown in Zebrafish Cells

In order to see whether over-expression of Dicer, Drosha, Ago2 or Xpo5 enhances RNAi in zebrafish cell lines, Ago2, Dicer, Drosha and Xpo5 cDNAs were cloned into the pcGlobin vector, a vector based on pcDNA3 which has been modified for efficient expression in zebrafish (Ro *et al.*, 2004). After confirming correct expression of these vectors in HEK 293 cells (Figure 5.8), their effect on RNAi in HEK 293 and zebrafish cells was assessed (Figure 5.9, Figure 5.10). Of all 4 components tested only Ago2 had any significant positive effect on RNAi efficiency and this effect was seen in both HEK 293 and zebrafish PAC.2 and ZFL cells. Within the zebrafish cells, PAC.2 cells, which also had the higher transcript levels of RNAi components, the effect was most pronounced. In PAC.2 cells, the pol II-responsive vector also achieved slightly better knockdown than the pol III-

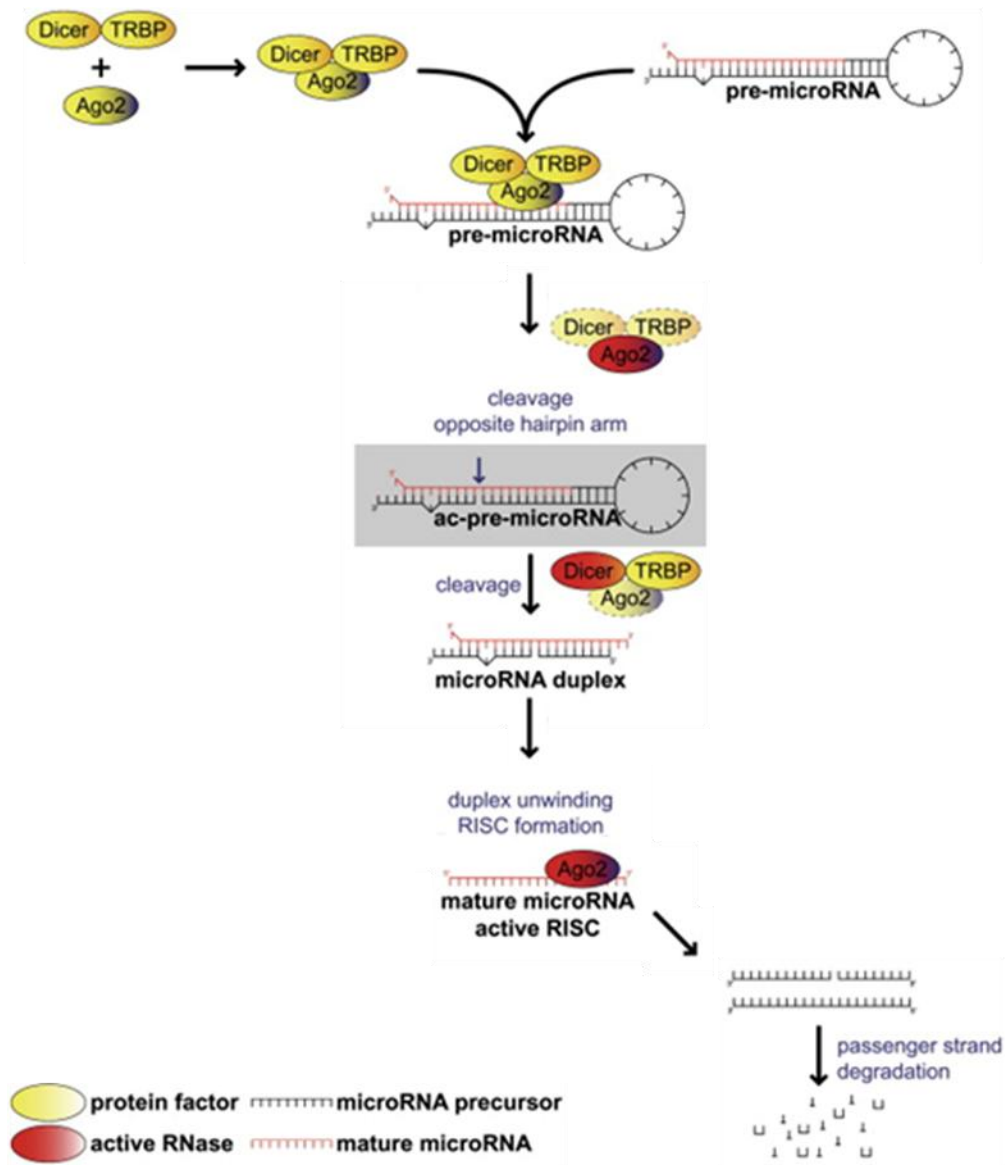
responsive vector, perhaps suggesting that the pol III vector needs further optimisation for use in zebrafish.

In contrast to previous reports (Diederichs *et al.*, 2008; Yi *et al.*, 2005), I found no evidence of Xpo5 induced enhancement of RNAi in zebrafish PAC.2 or ZFL cells, and only slight enhancement in HEK 293 cells when using the pol III-responsive vector, suggesting that in our hands, Xpo5 is not the limiting component to effective RNAi. Like Diederichs *et al.*, (2008) we also saw no enhancement of RNAi by over-expression of Dicer in PAC.2 or ZFL cells and again only slight enhancement in HEK 293 cells when using the pol III-responsive vector. This is in contrast to previous reports in *Drosophila* in which over-expression of Dicer2 substantially improved the potency of RNAi (Dietzl *et al.*, 2007).

That over-expression of Ago2 results in increased knockdown in cells is in line with the work undertaken by Diederichs *et al.*, (2008), who showed a 7-fold enhancement in RNAi in HEK 293 cells over-expressing Ago2. The same group had also previously demonstrated that, over-expression of each of the four Ago proteins enhances levels of mature miRNA, possibly through the stabilisation of the miRNA (Diederichs and Haber, 2007). They went on to show however, that improvements in RNAi brought about by Ago2 over-expression were not due to increased miRNA expression, as over-expression of each of the other Ago proteins do not improve RNAi efficiency. Instead, the enhancement of RNAi brought about by Ago2 was shown to be due to its intrinsic RNase activity as mutations in the RNase domain abrogate Ago2-mediated enhancement of RNAi. Likewise, by creating the D597A mutation of Ago2, I have demonstrated that the enhancement detected here in zebrafish cells is due to the catalytic RNase domain of Ago2 (Figure 5.11). In addition to demonstrating that Ago2 enhancement of RNAi was dependent on RNase activity of Ago2, Diederichs *et al* (2008) also demonstrated that the enhancement was only toward perfectly matched binding sites, as mutation of the base pairs either side of the cleavage site and mutation of 2 bases in the seed region of the miRNA inhibited any effect of Ago2. Furthermore, they reported no deleterious effects of over-expression of Ago2 on endogenous RNAi activity. This is most likely due to

the fact that by over-expressing Ago2 there is less competition with endogenous miRNAs for assembly into RISC. Moreover, as Ago2 can function both as a slicer and in translational repression (Wu *et al.*, 2008) where endogenous miRNA are incorporated into RISC containing Ago2, silencing via translational repression/mRNA destabilisation may still ensue.

Recently, truncated forms of pre-miRNAs have been detected in cell lysates and their presence shown to be dependent on Ago2 expression. This has led to a further role of Ago2 being proposed in the biogenesis of miRNAs (Diederichs and Haber, 2007). In this proposed model Ago2 binds to pre-miRNA along with Dicer and TRBP and makes a cut in the passenger strand miRNA to produce an Ago2-cleaved precursor miRNA (ac-pre-miRNA). This ac-pre-miRNA is then further processed by Dicer and the guide strand is loaded into RISC (Figure 5.15). Cleavage of the passenger strand by Ago2 may help in strand selection and the unwinding of the duplex and hence loading into RISC. At the same time, cleavage of the passenger strand of customised miRNAs would prevent passenger strand loading into RISC and its ensuing off-target effects. Thus, if Ago2 interacts with customised miRNAs embedded in the hsa-miR-30a backbone in the same way, this is further advantage to the over-expression of Ago2 to induce knockdown.



**Figure 5.15: Proposed Role of Ago2 in miRNA Biogenesis.** Ago2 binds to the pre-miRNA along with Dicer and TRBP and cleaves the passenger strand of the pre-miRNA. The pre-miRNA is then processed by Dicer to create the miRNA duplex and then the guide strand is loaded into RISC, while the passenger strand is degraded. Cleavage of the passenger strand prevents its incorporation into RISC and also aids in the unwinding of guide and passenger strands (modified from Diederichs *et al.*, (2007)).



#### 5.4.2.2 Strand Orientation and Other Changes to Stem-loop

In all experiments up to this point, the miRNA backbone used was that of hsa-miR30a described by Zeng *et al.*, (2002) with the guide strand orientated in the 3' arm. To see whether the efficiency of RNAi could be further enhanced by changing the orientation of the guide strand or by using a zebrafish miRNA backbone, the efficiencies of RNAi with the guide strand orientated in the 5' or the 3' arm and the efficiency of the zebrafish as opposed to human miR30a backbone were tested in both HEK 293 cells and PAC.2 cells over-expressing Ago2. In HEK 293 cells the original miR30 backbone with the miRNA located in the 3' arm was clearly the most efficient at inducing silencing. In PAC.2 cells over-expressing Ago2 however, there was no such strand preference; the human miR-30a backbone with miRNA orientated in either the 5' or 3' arms had similar knockdown efficiencies. Surprisingly, however the zebrafish miR30a backbone, which you might expect to be more suited to expression in zebrafish cells, was slightly less efficient than the human versions at inducing silencing (Figure 5.12). In future, the efficiencies of other zebrafish backbones might also be tested to see whether any of these are capable of improving knockdown. In particular, it would be interesting to see whether using the backbone of the dre-miR-430 miRNA, an early acting and highly expressed miRNA could improve knockdown in zebrafish embryos.

#### 5.4.3 Over-expression of Ago2 in Zebrafish Embryos

As over-expression of Ago2 in zebrafish cells enables RNAi, the next step was to see whether its over-expression *in vivo* is feasible and sufficient to bring about silencing. Injections of increasing doses of Ago2 mRNA resulted in dose-dependent protein production *in vivo* and had very little effect on mortality or development, suggesting that over-expression of Ago2 is feasible and non-toxic (Figure 5.13). However, injection of Ago2 mRNA failed to significantly reduce GFP levels in double transgenic zebrafish expressing GFP and the GFP miRNA (Figure 5.14). There are several potential reasons for this discrepancy between the knockdown seen in zebrafish cell lines expressing Ago2 and lack of knockdown in zebrafish embryos expressing

Ago2. Firstly, the amount of mRNA injected may not have been sufficient to produce high enough levels of Ago2 to significantly improve knockdown. Secondly, mRNA injected into single-cell embryos will become increasingly diluted as the embryo grows, and may not be uniformly inherited throughout all cells of the embryo causing very high levels of variation in the level of Ago2 expression in each cell. Thirdly, mRNA is highly susceptible to degradation, and despite the use of the pcGlobin vector which contains zebrafish  $\beta$ -Globin 5' and 3' UTRs which have been shown to increase the stability of mRNAs injected into zebrafish embryos, there may be a high level of decay of mRNA, and variation in the amount of decay of mRNA from embryo to embryo. Finally, in order to assess the effects of over-expression of Ago2, embryos were assessed at 2 dpf so that miRNAs had time to function on their target, but the levels of Ago2 would still be reasonably high. 2 dpf may be too early a time point to achieve/detect knockdown, particularly as expression of customised mature miRNA produced from our constructs were shown to increase dramatically by 5 dpf. Therefore, to assess properly whether over-expression of Ago2 can induce knockdown in zebrafish embryos/larvae and adults, it will be necessary to create a transgenic line over-expressing Ago2. Work on generating a 5 x UAS::Ago2 vector which leads to over-expression of Ago2 when combined with animals expressing Gal4 has already begun.

Recently vector-mediated co-expression of Ago2 and shRNA in intact xenopus was shown to moderately improve RNAi in xenopus optic tectal neurons to a similar extent as that observed here in PAC.2 and ZFL cells (Chen *et al.*, 2009). They also noticed no morphological or functioning defects in neurons over-expressing Ago2. The ability of Ago2 to improve knockdown in xenopus lends further support to the suggestion that stable expression of Ago2 may be the key to inducing RNAi in zebrafish embryos. However, it is important to note, that this study only looked at one type of neuron, and so it remains to be seen whether over-expression of Ago2 will enhance RNAi in other xenopus cell types. In addition, while moderate levels of knockdown were achieved in some cells, other cells did not appear to respond to Ago2 over-expression at all, suggesting that in some cells

Ago2 expression may not be, or at least is not, the only limiting factor to RNAi (Chen *et al.*, 2009). Other potential limiting factors could be Dicer, Drosha or Xpo5, but equally it could also be any one of the many accessory proteins involved in pri- and pre-miRNA production and RISC assembly and function. Due to potential cell type-dependent differences in the RNAi ability of cells over-expressing Ago2, it may be more informative to look at the effect of Ago2 over-expression in individual zebrafish cell types.

#### **5.4.4 Targeting the 3'UTR Causes Gene Knockdown in Zebrafish Cells and *in vivo***

The majority of research utilising RNAi to knockdown genes in zebrafish to date has been carried out by creating miRNAs/shRNAs with complete complementarity to the open reading frame of the gene (Blidner *et al.*, 2008; Chang and Nie, 2008; Dodd *et al.*, 2004; Gruber *et al.*, 2005; Liu *et al.*, 2005c; Su *et al.*, 2008; Wang *et al.*, 2007; Zhao *et al.*, 2008). It was/is believed that by targeting open reading frames (ORFs) with miRNAs/siRNAs which are completely complementary to their targets rather than targeting the 3' UTRs, where targeting relies on only a seed region within the miRNA, off-target effects would be reduced. Indeed, targeting the ORF is extremely efficient in cells such as HEK 293 cells and in many other systems. However, the work carried out for this thesis has highlighted the difficulty of this approach in zebrafish. A report recently by Dong *et al.*, (2009) also confirmed lack of knockdown achieved when targeting ORFs. However, they also showed that targeting the 3' UTRs of exogenous GFP and the endogenous gene *gata1* resulted in the stable and heritable knockdown of about 50%. To confirm and evaluate the effect of targeting the 3' UTR, I used the vectors and miRNA sequences against the GFP ORF and SV40pA described by Dong *et al.*, (2009) to knockdown YFP in PAC.2 YFP cells (see appendices section Figure 8.12). Indeed targeting the ORF resulted in very little knockdown, but by targeting the 3' UTR two independent miRNAs achieved around 50% knockdown. The difference in the ability of miRNAs against the ORF and miRNAs against the 3' UTR to induce silencing reflects the differences in mechanisms employed. All four argonaute proteins are

able to bind to the 3' UTR of their targets and induce knockdown either by translation repression or mRNA destabilisation. However, when targeting the ORF knockdown is largely dependent on Ago2, the only argonaute which can induce mRNA decay via mRNA cleavage, as binding of miRNA/siRNA to the ORF at best only weakly effects translation (Wu *et al.*, 2008). In animals the majority of endogenous miRNAs silence their targets by binding to a seed region of the 3' UTR and inducing translational repression or mRNA destabilisation. The only known exception to this is miR196, which is completely complementary to its target and induces cleavage (Yekta *et al.*, 2004). It is therefore perhaps not surprising that targeting the 3' UTR should be more efficient. However, caution should be taken in utilising Ago1,3 and 4 for targeted gene knockdown, as recognition of the target relies only on a seed region (usually bases 2-8 of the miRNA). The greater probability of finding at 7-8 bp match as opposed to a 21 bp match therefore increases the potential for off-target effects. Indeed most of the off-target targets of completely complementary miRNAs/siRNAs targeted against ORFs have been shown to be on targets which share seed region complementarity in their 3'UTRs (Birmingham *et al.*, 2006; Jackson *et al.*, 2003). However, as customised miRNAs/shRNAs targeted to the ORF are equally as likely to be incorporated into RISCs containing Ago1,3 or 4 as miRNAs/shRNAs targeted to the 3' UTR, the potential for off-target effects will be similar. Therefore rather than the position of target being the important determining factor, relative levels of Ago2 are likely to have greatest effect on the possible off-targeting of customised miRNA/shRNAs. Indeed, work by Wu *et al.*, (2008) has demonstrated that reduced off-target effects of siRNAs correlate with higher levels of Ago2 expression relative to the other Ago proteins and vice versa. Therefore, although targeting the 3' UTR does indeed give rise to moderate silencing, due to the enhancing effect of Ago2 towards perfect-match binding sites, a system in which Ago2 is over-expressed may be more desirable and could alleviate off-target effects both when targeting the ORF and the 3' UTR.

### 5.4.5 Conclusion

The major RNAi components Ago2, Dicer, Drosha and Xpo5 are expressed throughout development and across tissues. In addition, the RNAi vector used has been shown to be capable of driving expression of customised mature miRNAs and eliciting knockdown in HEK 293 cells. However, despite this zebrafish embryos and cells remain resistant to vector-mediated RNAi. Over-expression of Ago2, the major component of RISC enables vector-mediated RNAi in cells, and this has been shown to be dependent on its intrinsic RNase activity. Results *in vivo* however have been less promising, but this may be due to the variable and unreliable effects of mRNA injections.

#### 5.4.5.1 Future Work

In order to determine whether Ago2 over-expression will enable vector-mediated RNAi targeted against ORFs in zebrafish, an Ago2 expressing transgenic zebrafish needs to be created and assessed for its RNAi ability. As the potential for different cell types to perform RNAi may vary, it may be better to assess the RNAi ability in individual cell types/tissues. The success of RNAi targeted against 3' UTRs opens another potential possibility to achieving good knockdown; however, the potential for off-target effects should not be overlooked. Therefore, combining targeting of the 3' UTR with Ago2 over-expression may be beneficial. The efficiency of silencing both by targeting the 3' UTR and by targeting the ORF while over-expressing Ago2 are very similar and knockdown seems to be limited to around 50%. This suggests that at this point other factors become limiting. Therefore, future experiments may look at the effect of over-expression of multiple components of the RNAi machinery.

## 6 General Discussion

At present the use of zebrafish to model diseases which may then be used to further understand the biology of the disease and for use in drug discovery is hampered by a lack of an efficient and reliable means for knocking down target genes. The work of this thesis has therefore focused on trying to develop a method of vector-mediated RNAi which would achieve stable and heritable knockdown. The efficacy of vector-mediated RNAi in zebrafish was first addressed through knocking down GFP. However, I was also interested in knocking down PINK1, a gene whose loss of function has been associated with early onset Parkinson's disease.

Initial results of vector-mediated RNAi in a G0 setting shown here and reported by Su *et al.*, (2008) and Wang *et al.*,(2007) were encouraging. However, in stable transgenic lines, with strong expression of the RNAi vector made possible through the insertion of a Gal4-VP16/UAS amplification cassette, no knockdown of GFP or the Parkinson's disease associated gene, PINK1 was detected in zebrafish embryos. This lack of knockdown was despite the expression of the main components required for mRNA cleavage-induced silencing and the production of customised mature miRNAs throughout development and across all tissues tested.

Although RNAi appears to not be working in zebrafish embryos, evidence of moderate knockdown of PINK1 was detected in the adult zebrafish brains of F2 transgenic zebrafish. Importantly, it was the brain which had the highest transcript levels of RNAi components and produced the highest amounts of customised mature miRNA relative to the expression of the RNAi vector. With this in mind it would now be extremely interesting to see whether knockdown of GFP is detectable in doubly transgenic fish brains expressing both GFP and the RNAi silencing vector. Detection of GFP knockdown in zebrafish brains would lend further support to the results of PINK1 knockdown. Much lower expression of the RNAi components and mature miRNA was seen in embryos and may explain the lack of knockdown detected. Although in the brain higher levels of RNAi components correlated with higher levels of mature miRNA production, the correlation between

component expression and mature miRNA production did not hold true in all tissues, and may reflect differences in the endogenous load levels of miRNA and differences in post-transcriptional regulation of RNAi. It remains to be tested whether any other adult tissues are amenable to RNAi. Whether or not partial knockdown of PINK1 in the brain leads to any Parkinson's-like pathology also need to be addressed.

Despite modest but successful knockdown in the brain, a method of gene silencing that functions in embryos and throughout development is the ultimate goal if RNAi is to be the method of choice in knocking down genes in zebrafish. In other systems the efficiency of RNAi has been improved by the over-expression of various components of the RNAi machinery (Diederichs *et al.*, 2008; Dietzl *et al.*, 2007; Yi *et al.*, 2005). Here, I have demonstrated that over-expression of Ago2 but not Dicer, Drosha or Xpo5 induces RNAi-mediated knockdown in zebrafish cell lines which prior to over-expression of Ago2 were resistant to RNAi. This induction is dependent on the RNase domain of Ago2 which cleaves RNA with fully complementary sequences to the miRNA/siRNA. The amount of knockdown detected however, was at best in PAC.2 cells only approximately 50%, suggesting that although Ago2 may be the primary limiting factor to RNAi, other factors must soon also become limiting. These other limiting factors may be proteins such as Drosha, Dicer or Xpo5, but equally could be one of the many accessory proteins involved in processing pri- and pre-miRNA or in the silencing complex RISC itself. Despite the induction of RNAi in zebrafish cells through over-expression of Ago2, no knockdown of GFP was detected in double transgenic zebrafish expressing GFP and the RNAi vector injected with Ago2 mRNA. This may reflect problems with the delivery of Ago2 or may suggest that factors other than Ago2 are limiting in embryos. In future, it may be more appropriate to look at the knockdown capabilities of individual cell types as the ability of different cell types is likely to vary a lot and cells which are amenable to RNAi may be missed when looking at global levels.

Throughout this research and indeed in most published attempts at RNAi in zebrafish to date, efforts at RNAi have focussed on targeting the ORF of the target gene with complete complementarity. However, whilst

undertaking this research another group have demonstrated that although targeting the ORF is not effective at delivering RNAi, by targeting the 3' UTR effective RNAi can be achieved in a stable and inheritable manner (Dong *et al.*, 2009). That targeting the ORF doesn't work but targeting the 3' UTR does, suggest that the Ago2-mediated cleavage part of the RNAi pathway is limiting in zebrafish, but that silencing via translational repression/mRNA destabilisation mediated through Ago1,3 and 4 is fully functional and can be manipulated. However, silencing mediated by RISC containing Ago1, 3 and 4 is dependent on only seed region complementarity between the miRNA/siRNA and the target mRNA, which means that miRNA/siRNAs incorporated into RISC containing Ago1, 3 or 4 are much more likely to have off-target effects due to the increased probability of finding seed region matches compared to completely complementary matches. Thus, while this method does seem to be effective, it is important to be aware of potential off-target effects. Recently, off-target effects of miRNAs/siRNAs targeted to both the ORF and 3' UTR have shown to be reduced with increasing levels of Ago2 (Diederichs *et al.*, 2008; Wu *et al.*, 2008), suggesting that a system in which Ago2 is over-expressed would be advantageous both when targeting ORFs or 3' UTRs.

Although I have been unable to demonstrate effective vector-mediated RNAi of target genes in zebrafish embryos in this thesis, I have demonstrated that the RNAi vectors described herein are able to deliver mature miRNA in zebrafish. Therefore, the vectors described in this thesis could be used to over-express endogenous miRNAs. Indeed, Nicoli *et al.*, (2010) used the original RNAi vector created by Dr. Paul Walker, the Hurlstone lab, to tissue-specifically over-express miR-126. This over-expression successfully rescued the effects of loss of its upstream activator, the mechano-sensitive zinc finger transcription factor *klf2a*.

In plants and invertebrates such as *C. elegans* and *Drosophila* targeted Ago2-mediated cleavage of mRNAs is highly efficient. However, the efficiency of Ago2-mediated cleavage of targets in mammalian and other vertebrate systems has proved to be extremely variable. This suggests that there may have been some evolutionary change in RNAi during the



invertebrate to vertebrate transition. The Ago2-mediated cleavage part of the RNAi pathway is proposed to have originally evolved as a defence mechanism against viruses and transposable elements (Obbard *et al.*, 2009). dsRNA from viruses is recognised by Dicer and cut up into siRNAs. One strand of the siRNA is then incorporated into Ago2-containing RISC which binds to complementary viral sequences and cleaves the viral RNA. While this form of defence has been demonstrated as important in *Drosophila C. elegans* and fungi, there is a lack of evidence suggesting RNAi plays an important role in vertebrate systems (Cullen, 2006; Obbard *et al.*, 2009). Instead, in these systems contact with dsRNA results in an interferon response resulting in global mRNA degradation and translational inhibition (Stark *et al.*, 1998; Williams, 1997). This transition between RNAi playing an important role in antiviral defences to the more general interferon response, may explain why Ago2-mediated cleavage of targets is less efficient in vertebrate systems. However, despite this several vertebrate cell types are capable of efficient Ago2-mediated RNAi. Most notably, HEK 293 cells which have been used throughout this thesis and extensively by the wider scientific community in RNAi experiments.

In conclusion, the inefficiency of RNAi in zebrafish, both when targeting the ORF or the 3' UTR suggests that its use to model diseases which result from loss of function of a gene is not at present a viable option. It is possible however, that this technology could be used to model the effects of haploinsufficiency of target genes. For development of animal models resulting from complete loss of gene function, mutagenesis, zinc fingers and in a transient setting morpholinos, despite their relative disadvantages, are much better suited.

## 7 References

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## 8 Appendices

### 8.1 Vector Maps

#### 8.1.1 Pol II-responsive RNAi Vector

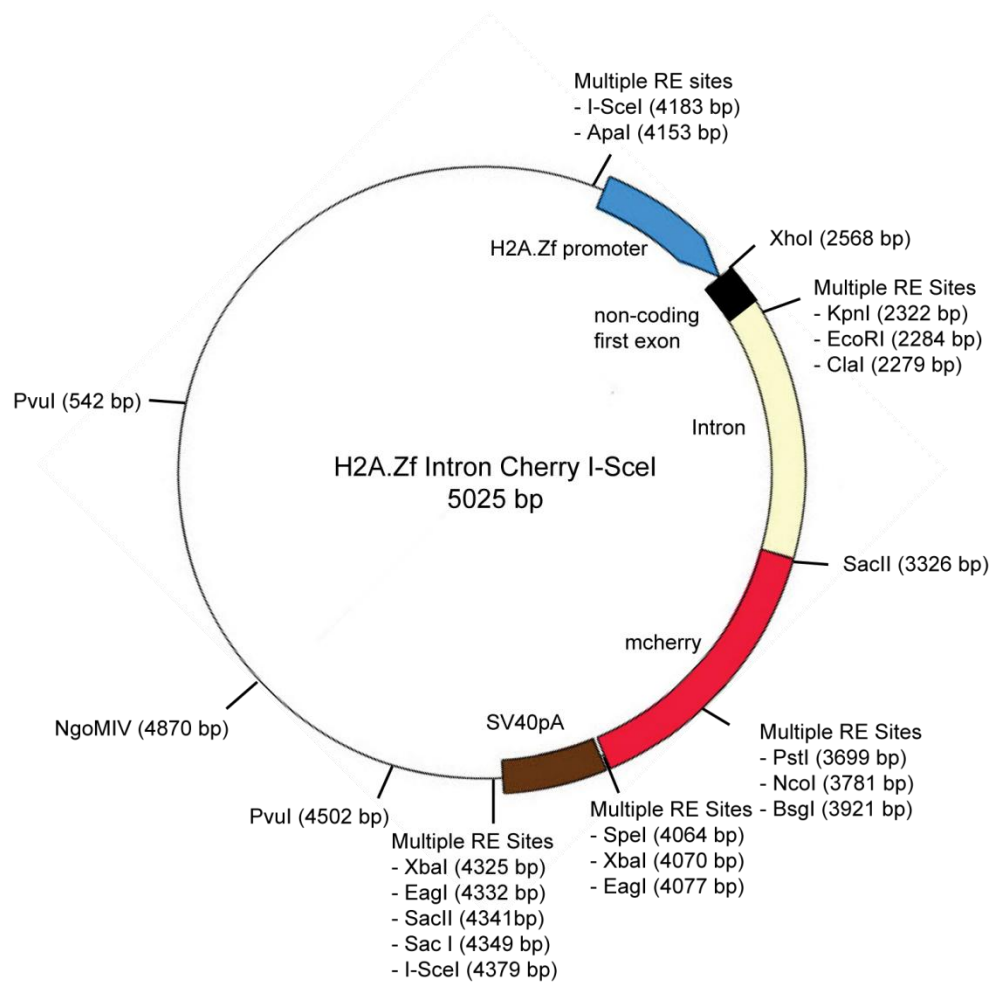
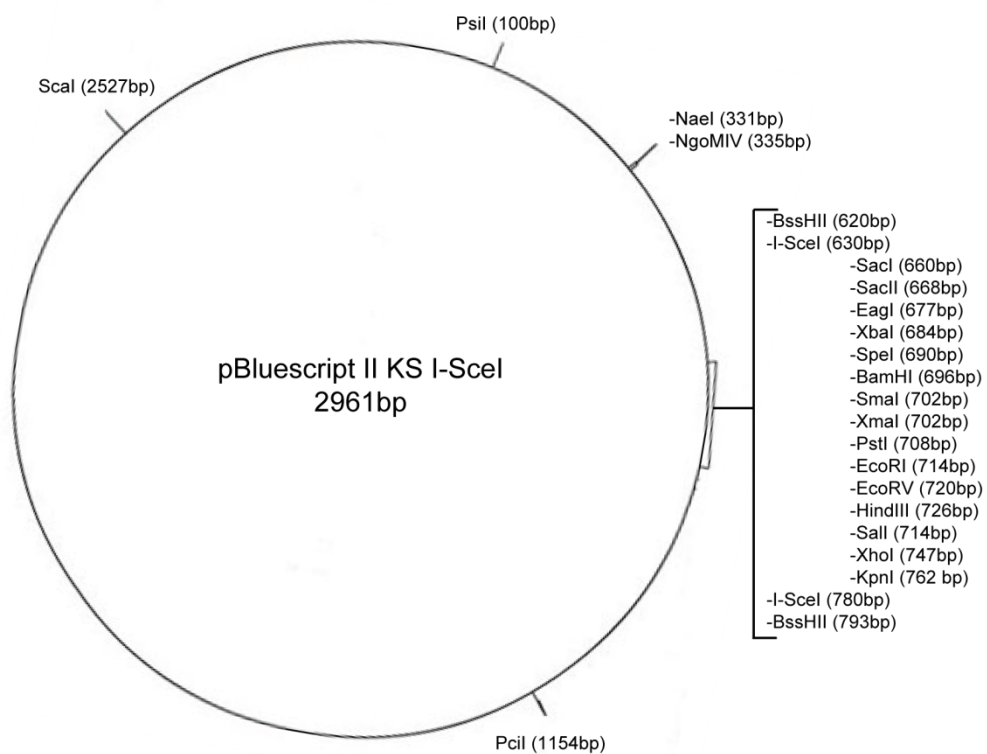


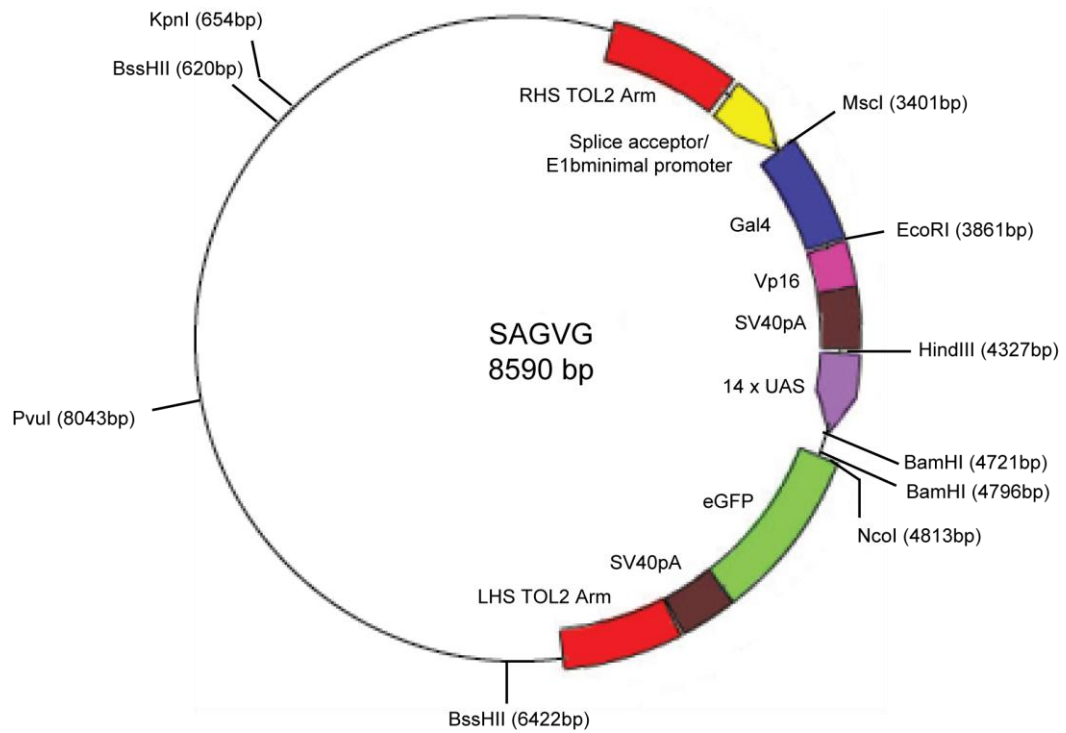
Figure 8.1: Pol II-responsive RNAi Vector Map Depicted Here with H2A.Zf Promoter.

### 8.1.2 pBluescript I-SceI Vector Map



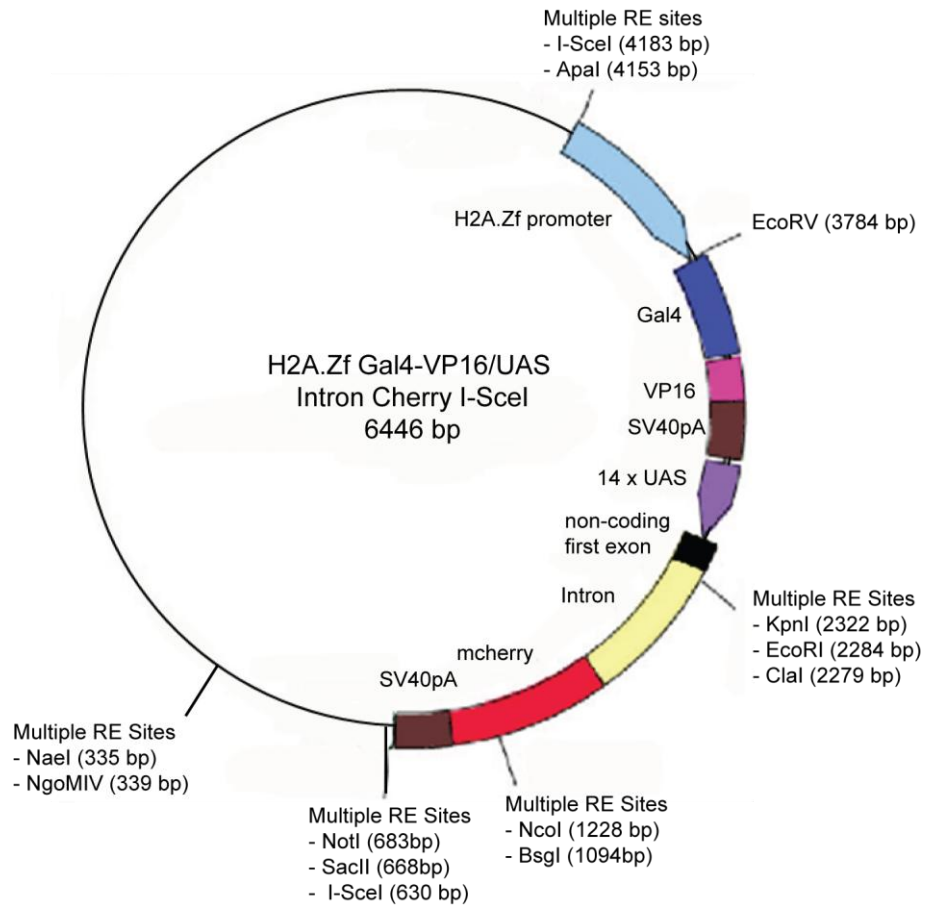
**Figure 8.2: pBluescript I-SceI Vector Map.** Multicloning site (MCS) of pBluescript is flanked by I-SceI meganuclease restriction site.

### 8.1.3 SAGVG Vector Map



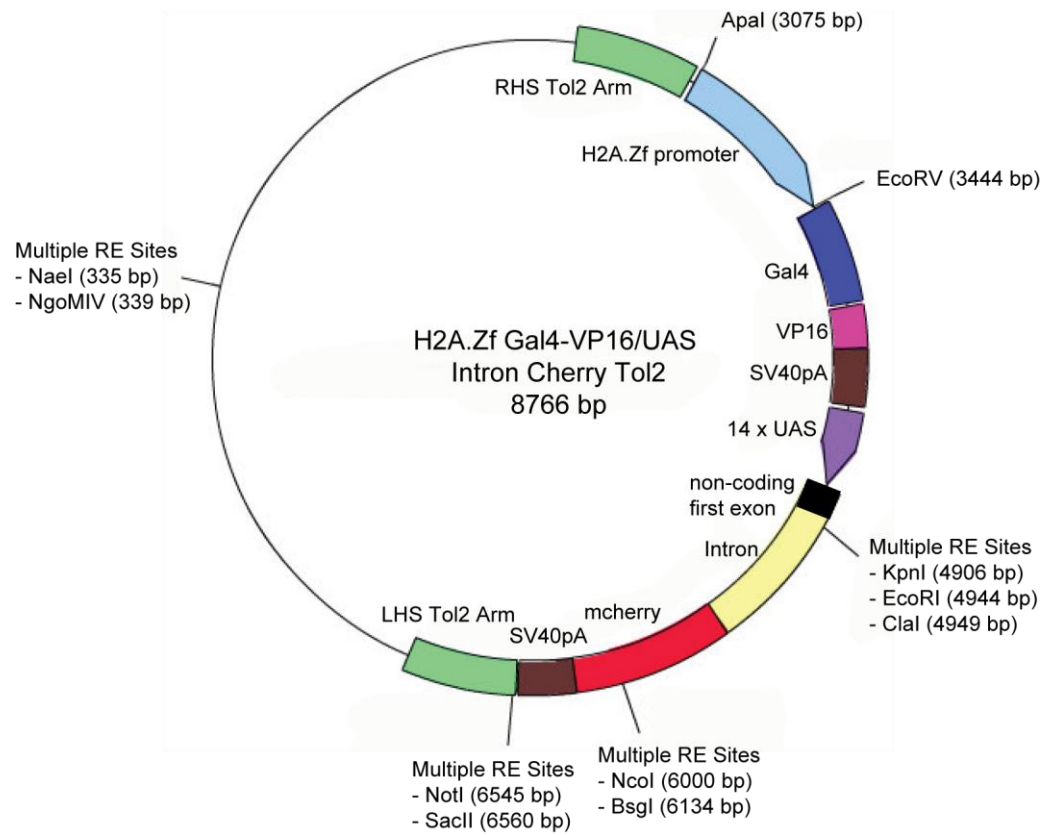
**Figure 8.3: SAGVG Vector Map.** Tol2 vector containing splice acceptor followed by Gal4-VP16 and 14 UAS sequences and GFP.

### 8.1.4 *I-SceI* Gal4-VP16-responsive RNAi Vector



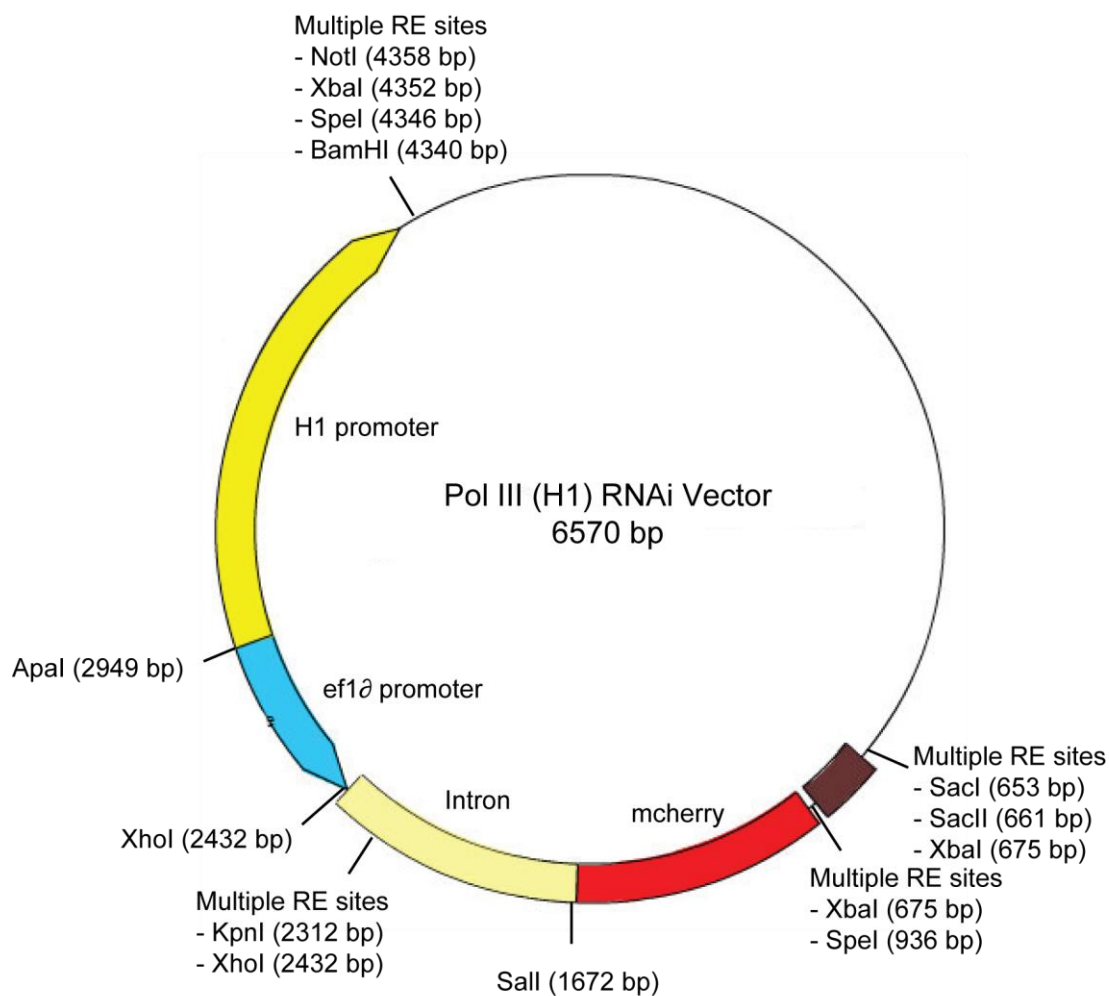
**Figure 8.4:** *I-SceI* Gal4-VP16-responsive RNAi Vector Depicted Here with H2A.Zf Promoter.

### 8.1.5 Tol2 Gal4-VP16-responsive RNAi Vector



**Figure 8.5:** Tol2 Gal4-VP16-responsive RNAi Vector Depicted Here with H2A.Zf Promoter.

### 8.1.6 Pol III-responsive RNAi Vector



**Figure 8.6: Pol III-responsive RNAi Vector.** The zebrafish H1 promoter drives expression of an shRNA. In a separate tandem cassette, ef1 $\alpha$  drives expression of mcherry, allowing for easy identification of transfected/transgenic cells.







Domain name	Corresponding amino acid (Human)	Function	Identity	Similarity
DUF1785	175 – 227	Unknown	98.1%	98.1%
PAZ_argonaute_like; PAZ domain, argonaute_like subfamily	227 – 347	Nucleic acid binding domain, with a strong preference for single-stranded nucleic acids (RNA or DNA) or RNA duplexes with single-stranded 3' overhangs.	95.9%	100%
Piwi_ago-like; Piwi_ago-like: PIWI domain, Argonaute-like subfamily	392 – 783	Two sub-domains. One provides the 5' anchoring of the guide RNA and the other, the catalytic site for slicing.	92.3%	96.9%
Piwi domain	517 – 784	dsRNA guided hydrolysis of ssRNA.	93.7%	97.4%

**Table 8.1: Comparison of Functional Domains of Argonaute 2.**

### 8.3.2 Dicer

HuDicer1	-----	
ZfDicer1	MAGLQLVTPASSPMGPFGLPWQQEAIHNDNIYTPRKYQVELLEAALEHNTIVCLNTGSGK	60
HuDicer1	TFIAVLLTKELSYQIRGDFSRNGKRTVFLVNSANQVAQQVSAVRTHSDLKVG EYSNLEVN	60
ZfDicer1	TFIAVLLIKELSHQIRGE--NGKRTVFLVNAASSVAQQASTVRTHSDLQVGDYMS EDMI	117
	***** :***:***: :*****:*. .***. :*****:***: . :.:	
HuDicer1	ASWTKERWNQEFTHQVLIMTCYVALNVLKNGYLSLSDINLLVFDECHLAILDHPYREIM	120
ZfDicer1	S-WPEEMWNREMIENQVLVMTCHIFLHVLKNGVLP LSKINLLVFDECHLAITGHYREIM	176
	:* .:* **::: :***:***:: :***** * .** ***** ***** *****	
HuDicer1	KLCENCPSCPRIILGLTASILNGCDPEELEEKIQMLEKILKSNAETATDLVLDYTSQP	180
ZfDicer1	KICEGCPSCPRIILGLTASILNGCDPCDLEEKIQNLEKILQSN AETATDLVLDYTSQP	236
	*:*. ***** ***** ***** ***** ***** ***** ***** *****	
HuDicer1	CEIVVDCGFPTD SGLYELLMLELEALNFINDCNISVHSKERDSTLISKQILSDCRAVL	240
ZfDicer1	REEVLDGCGYQDQSGLEHLLNLEDEALNFINDCNLSVHREDRDP TFIISKQVLD CRAVL	296
	* :*** : * :*** ** ** * :***** :***** :***** :***** :*****	
HuDicer1	VVLGPWCADKVGMMVRELQKYIKHEQEELHRKFLLFTDTFLRKIHALCEEHFS PASL DL	300
ZfDicer1	TVLGPWCADKAAGIMVRELQKYIKHEQEELNRKFLLFTDTILRKIHALCEEHFS PASL DL	356
	. ***** . * :***** ***** ***** ***** ***** ***** *****	
HuDicer1	KFVTPKVIKLEILRKYKPYERQQFESVEWYNNRNQDNVSWSDSEDDDEDEEIEEKEKP	360
ZfDicer1	KFVTPKVIKLEILRKYKPYERQQFESVEWYNNRNQDNVSWSDSEDDDEDEEIAEKEKT	416
	***** :***** :***:***** ***** ***** ***** ***** ***** *****	
HuDicer1	ETNFPSPTNILCGIIFVERRYTA VVLNRLIKEAGKQDPELAYISSNFITGHGIGKNQPR	420
ZfDicer1	EANFPSPTNILCGIIFVERRYTA VVLNRLIKEAGKQDPELAYISSNFITGHSIGKNQPR	476
	* :***** ***** ***** ***** ***** ***** ***** ***** *****	
HuDicer1	NKQMEAEFRKQEEVLRKFR AHTNLLIATSIVEEGVDIPKCNLVVRFDLPT EY SYVQS	480
ZfDicer1	NKQMEAEFRKQEEVLRKFR AHTNLLIATSIVEEGVDIPKCNLVVRFDLPT EY SYVQS	536
	***** . ***** ***** ***** ***** ***** ***** ***** *****	
HuDicer1	GRARAPISNYIMLADTDKIKSFEEDLKYKAI EKILRNCSKSVDTGETDIDPVMDDDDV	540
ZfDicer1	GRARAPVSNYIMLADSEKTKTFQEDLKYKAI EKILRNCSKSAECNDFELEPVTDDDNV	596
	***** :***** :***:***** ***** ***** ***** ***** ***** *****	
HuDicer1	FPPYVLRPDDGGPRVINTAIGHINRYCARLPSPDFTHLAPKCRTR ELPDGT FYSTLYLP	600
ZfDicer1	LPPYVLRSEDGGPRVMTAIGHVNR YCARLPSPDFTHLAPKCR T VEMNTGGYRSTLFLP	656
	:***** .:*****:*****:*****:*****:*****:*****:*****:*****	
HuDicer1	INSPLRASIVGPPMSCVRLAERVALICCEK LKIGELDDHMPVGK ETVKYEELDLHD	660
ZfDicer1	INSPLRVVPTGPMNCARLAEKAVALLCCEK LKIGELDDHMPVGK ETVKYEELDLHD	716
	***** .: .** * . * .***** : * :***** ***** ***** ***** *****	
HuDicer1	EEETSVPGRPGSTKRRQCYPKA IPECLRDSYPRPDQPCYLYVIGMVLTTPLPDELNFRFR	720
ZfDicer1	EEETSVPGRPGSTKRRQCS PKA IPECLRGCPVPEQPCYLYVIGMVLTTPLPDELNFRFR	776
	***** ***** ***** ***** ***** ***** ***** ***** *****	
HuDicer1	KLYPPEDTRRCFGILTAKPIPIPHFPVYTRSGEVTISIELKKS GFMSLQMLELITRLH	780
ZfDicer1	KLYPPEDTRRCFGILTAKPIPIPHFPVYTRSGEVTISIELQKSGFSLSAEQLELITRLH	836
	***** ***** ***** ***** ***** ***** ***** ***** *****	
HuDicer1	QYIFSHILRLKPALEFKPTDADSAYCVLP LNVDSS TLDIDFKFMEDIEKSEARIGIP	840
ZfDicer1	QYIFSHILRLKPALEFKPVEADSAYCVLP LNIVEDSNTL DLDLFKFMEDIEKSEARIGIP	896
	***** ***** ***** ***** ***** ***** ***** ***** *****	
HuDicer1	STKYTKETPFVFKLEDYQDAVIIPRYRNFDPHRFYVADVYTDLTPLSKFSP EYETFAE	900
ZfDicer1	NTQYTKQNPFIFKLEDYQDAVIIPRYRNFDPHRFYVADVYTDLTPLSKFSP EYETFAE	956
	. * :***: . * :***** ***** ***** ***** ***** ***** ***** *****	
HuDicer1	YYTKYNLDLTNLNQPLLDV DHTSSRLNLLTPRHLNQK G KALPLSSAEKRKAKWESLQNK	960
ZfDicer1	YYTKYNLDLSNVNQPLLDV DHTSSRLNLLTPRHLNQK G KALPLSSAEKRKAKWESLQNK	1016
	***** :* :***** ***** ***** ***** ***** ***** ***** *****	
HuDicer1	QILVPELCAIHPIPASLWRKAVCLPSILYRLHCLLTAEELRAQTASDAGVGVRS LPADFR	1020
ZfDicer1	QILVPELCAIHPIPASLWRKAVCLPSILYRLHCLLTAEELRSQTAIDAGVGAQTLPPDFR	1076
	***** ***** ***** ***** ***** ***** ***** ***** *****	
HuDicer1	YPNLDFGWKKSIDSKSFISISNSSSAENDNYCKHSTIVPENAAHQGANRTSSLENHDQMS	1080
ZfDicer1	YPNLDFGWKKSIDSKSFISCPSCMEEDDHCKLGTSS---DSNHTAPESCSMEVS-QPP	1132



Domain name	Corresponding amino acid (Human)	Function	Identity	Similarity
DEXDc; Dead-like helicase	58-209	ATP-dependent RNA and DNA unwinding	74.3%	84.2%
HELICc; Helicase superfamily c-terminal domain	444-553	ATP-dependent RNA and DNA unwinding	80.9%	92.7%
double stranded RNA binding domain	630-722	Binding double stranded RNA	94.6%	95.7%
PAZ-domain	886-1008	nucleic-acid binding domain, with a strong preference for single-stranded nucleic acids (RNA or DNA) or RNA duplexes with single-stranded 3' overhangs	96.5%	99.1%

**Table 8.2: Comparison of Functional Domains of Dicer1.**



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*****: ** : *** : *****
HuRNASEN F I H F P D H H E G H L T L L R S S L V N N R T Q A K V A E E L G M Q E Y A I T N D K T K R P V A L R T K T L A D L L E 1185
ZfRNASEN F I H F P D H H E G H L T L L R S S L V N N R T Q A K V A E E L G M Q E F A I T N D K T K R P V A L R T K T L A D L L E 1142
*****: ** : *** : *****

HuRNASEN S F I A A L Y I D K D L E Y V H T F M N V C F F P R L K E F I L N Q D W N D P K S Q L Q Q C C L T L R T E G K E P D I P 1245
ZfRNASEN S F I A A L Y I D K D L E F V H T F M N V C F F P R L K E F I L N Q D W N D P K S Q L Q Q C C L T L R T E G K E P D I P 1202
*****: ** : *** : *****

HuRNASEN L Y * T L Q T V G P S H A R T Y T V A V Y F K G E R I G C G K G P S I Q Q A E M G A A M D A L E K Y N F P Q M A H Q K R 1305
ZfRNASEN L Y K T L Q T V G P S H A R T Y T V A V Y F K G E R I G C G K G P S I Q Q A E M G A A M D A L E K Y N F P Q M A H Q K R 1262
*****: ** : *** : *****

HuRNASEN F I E R K Y R Q E L K E M R W E R E H Q E R E P D E T E D I K K 1337
ZfRNASEN F I E R K Y R Q E L K E M R R E R E R Q E R D S D E G ----- 1289
*****: ** : *** : **

```

**Figure 8.10: Comparison of Human (NP\_001093882.1) and Zebrafish (predicted from cDNA image clone:3815032) Drosha (RNASEN) proteins.** Red indicates small, hydrophobic amino acids (A,F,I,L,M,P,V,W). Blue indicates acidic amino acids (D,E). Pink indicates basic amino acids (K,R). Green indicates hydroxyl+amine+basic amino acids (C,G,H,N,Q,S,T,Y). Identical amino acids are indicated with a \*. : indicates a conservative substitution and . indicates a semi-conservative substitution. Human and zebrafish drosha are 77.3% identical and 83.0% similar.

Domain name	Corresponding amino acid (Human)	Function	Identity	Similarity
rnc; ribonuclease III domain	1068 – 1296	Double stranded RNA-specific endonuclease.	97.8%	99.6%
RIBOc. Ribonuclease III C terminal domain.	1085 – 1217	Double stranded RNA-specific endonuclease.	98.5%	100%
DSRM; Double-stranded RNA binding motif.	1223 – 1294	Highly specific binding of double stranded RNA.	100%	100%

**Table 8.3: Comparison of Functional Domains of Drosha.**



```

****:*****:* .:*.:*: **:* ** *:* :*:*:*: * :*:**
HuXpo5 DCKLLNPSLQKVADKRRKDQFKRLIAGCIGKPLGEQFRKEVHIKKNLPSLFKKTTPMLETE 1189
ZfXpo5 DQKIQ-PGASKLGEKKKKEQFRRLIAGTVGKPLAQQFKKEVHIRNLPSLFKPKPKTKD-L 1160
* * : * . *.:*:*:*:**:*:***** :*****:***:*****:*****_* * :
HuXpo5 VLDNDGGGLATIFEP----- 1204
ZfXpo5 LENNEDATLISLFTPDHDC 1180
: :*:.. * ::* *

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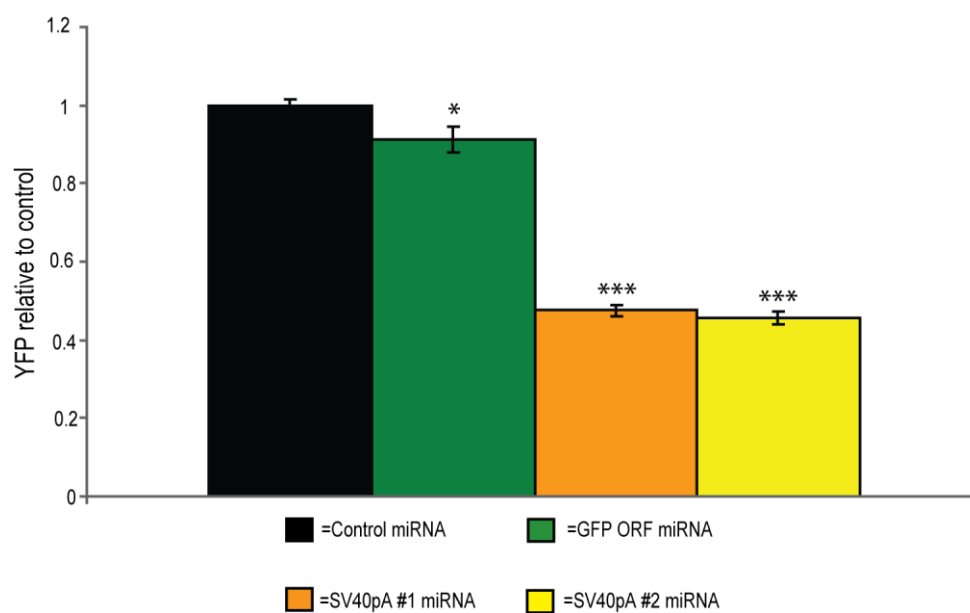
**Figure 8.11: Comparison of Human (NP\_065801.1) and Zebrafish (XP\_001921422.1) Exportin 5.** Red indicates small, hydrophobic amino acids (A,F,I,L,M,P,V,W). Blue indicates acidic amino acids (D,E). Pink indicates basic amino acids (K,R). Green indicates hydroxyl+amine+basic amino acids (C,G,H,N,Q,S,T,Y). Identical amino acids are indicated with a \*. : indicates a conservative substitution and . indicates a semi-conservative substitution. Human and zebrafish protein sequences are 58.6% identical and 76.2% similar.

Domain name	Corresponding amino acid (Human)	Function	Identity	Similarity
CRM1; Importin beta-related nuclear transport receptor domain	56-353	mediates the transport of protein and RNA macromolecules containing nuclear import and export signals between the nucleus and cytoplasm	67.8%	82.9%
Xpo1; Exportin 1-like protein domain	109-243	nuclear export receptor that interacts with leucine-rich nuclear export signal (NES) sequences, and Ran-GTP, and is involved in translocation of proteins and RNA out of the nucleus.	69.1%	80.9%

**Table 8.4: Comparison of Functional Domains of Exportin 5.**



## 8.4 Targeting the 3' UTR



**Figure 8.12: Open-reading frame miRNA compared to 3'UTR miRNA mediated gene knockdown in PAC.2 YFP cells.** Relative YFP levels in PAC.2 YFP cells after transfection with vectors containing a control miRNA (black bars), a YFP ORF miRNA (green bars) or SV40pA miRNAs (gold and yellow bars). YFP levels measured using fluorescent flow cytometry. Results were analysed by One-way ANOVA. \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ .