Different modes of regulation govern miRNA expression levels in *Drosophila melanogaster*

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**NANYANG
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**DIFFERENT MODES OF
REGULATION GOVERN MIRNA
EXPRESSION LEVELS IN
*DROSOPHILA MELANOGASTER***

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SCHOOL OF BIOLOGICAL SCIENCES

A thesis submitted to the Nanyang Technological University
in partial fulfilment of the requirement for the degree of
Doctor of Philosophy

2019

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research done by me except where otherwise stated in this thesis. The thesis work has not been submitted for a degree or professional qualification to any other university or institution. I declare that this thesis is written by myself and is free of plagiarism and of sufficient grammatical clarity to be examined. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

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Authorship Attribution Statement

This thesis contains material from two papers published in the following peer-reviewed journals where I was the first co-author.

Section 3.1 is published as Lim MYT, Ng AWT, Chou Y, Lim TP, Simcox A, Tucker-Kellogg G, and Okamura K. 2016. The *Drosophila* Dicer-1 partner Loquacious enhances miRNA processing from hairpins with unstable structures at the dicing site. *Cell Rep*, 15, 1795-1808.

The contributions of the co-authors are as follows:

- Chou Y, Simcox A, and Okamura K established the *loqs* mutant cell line.
- Ng AWT and Lim TP performed bioinformatics analyses, under supervision of Tucker-Kellogg G.
- I performed all other experiments.
- Okamura K conceptualized the project, provided supervision, and wrote the manuscript.

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The contributions of the co-authors are as follows:

- Zhou L performed bioinformatics analyses, under supervision of Tucker-Kellogg G and Okamura K.
- I performed all other experiments that are presented in the thesis.
- Okamura K conceptualized the project, provided supervision, and wrote the manuscript.

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Abbreviations

3'UTR: 3' untranslated region
A: adenine
AGO: Argonaute
AMP: adenosine monophosphate
Apaf1: apoptosis protease-activating factor 1
BCS: bathocuproinedisulfonic acid disodium salt
C: cytosine
C. elegans: *Caenorhabditis elegans*
cDNA: complementary DNA
CMP: cytidine monophosphate
CS: Canton S
Dcr: Dicer
DCS: decapping scavenger enzyme
dFmr1: *Drosophila* fragile X mental retardation 1
DGCR8: DiGeorge Syndrome Critical Region 8
G: guanine
GMP: guanosine monophosphate
DMSO: dimethyl sulfoxide
dsRBD: dsRNA-binding domain
dsRNA: double-stranded RNA
DTT: dithiothreitol
EDTA: ethylenediaminetetraacetic acid
Efn: Ephrin
FAF1: fas associated factor 1
HCV: hepatitis C virus
HDAC3: histone deacetylase 3
IP: immunoprecipitation
Kb: kilobase
LNA: locked nucleic acid
Loqs: Loquacious
mRNA: messenger RNA
NaCl: sodium chloride
NaOH: sodium hydroxide
NP-40: nonidet P-40
Nt: nucleotide
PACT: protein activator of PKR
PAZ: Piwi-Argonaute-Zwille
PBS: phosphate buffered saline
PCR: polymerase chain reaction
PEG: polyethylene glycol
PIWI: P-element-induced wimpy testes

PNPase: polynucleotide phosphorylase
Pre-miRNA: precursor miRNA
Pri-miRNA: primary miRNA
PTBP1: polypyrimidine tract binding protein 1
RISC: RNA-induced silencing complex
RNAi: RNA interference
rRNA: ribosomal RNA
SCP1: small C-terminal domain phosphatase 1
SDN-1: small RNA degrading nuclease 1
TBE: Tris/Borate/EDTA
TNRC6: trinucleotide repeat containing 6
TRBP: TAR RNA Binding Protein
tRNA: transfer RNA
TSN: Tudor staphylococcal nuclease
U: uracil
UAS: upstream activating sequence
UMP: uridine monophosphate
Wt: wild-type
XRN: exoribonuclease

Abstract

MiRNAs are a class of small non-coding RNAs that play important roles in post-transcriptional regulation of complementary target mRNAs.

Loquacious (Loqs) is a dsRNA-binding protein that binds Dicer-1 to cleave pre-miRNAs into short duplexes. The maternally deposited Loqs protein in *Drosophila* could potentially complicate their study in zygotic mutants. Here, Loqs function was investigated in the complete absence of maternal and zygotic contribution by using a cell line established from *loqs*-null fly embryos. Genome-wide analysis of pre-miRNA structures showed that Loqs enhances processing of sub-optimal substrates with unstable base-pairing at the dicing site. Post-transcriptional mechanisms that regulate miRNA expression during fly embryogenesis are not well understood. Here, distinct half-lives of individual miRNAs and identity of terminal 5' nucleotides were found to influence miRNA expression profiles. Overall, the results illustrate roles for dsRNA-binding proteins and miRNA stability in regulation of miRNA expression, demonstrating the complexity that underlies these processes.

1. Introduction

Non-coding RNAs refer to RNA molecules that are not translated into proteins. A large fraction of eukaryotic genomes is non-coding: up to 80% and 98% in *Drosophila* and humans, respectively (Alexander et al., 2010). The genome size of humans is ~30 times larger than that of worms; however, given that both worms and humans share a similar number of protein coding genes (~21,000) (Hillier et al., 2005, Ezkurdia et al., 2014), much of the complexity of eukaryotes may be directed by the non-coding genome and RNA regulatory networks. Even though they lack protein-coding capacity, many non-coding RNAs have dedicated functions in the cell. For instance, ribosomal RNAs (rRNAs) and transfer RNAs (tRNAs) are important in protein translation, small nuclear RNAs play a role in splicing events, and small nucleolar RNAs are involved in chemical modifications of mainly rRNAs (Mattick and Makunin, 2006).

In general, non-coding RNAs are categorized into several classes based on their length, biogenesis mechanisms, and functions (Brosnan and Voinnet, 2009). Long non-coding RNAs (lncRNAs) are defined as those longer than 200 nucleotides in length, many of which are biologically important and play key roles in developmental processes (Kung et al., 2013). One of the better-studied lncRNAs is Xist, which mediates transcriptional inactivation of the mammalian X chromosome by excluding RNA polymerase II on the X chromosome through binding to SHARP and subsequent activation of the histone deacetylase HDAC3 (McHugh et al., 2015). Small non-coding RNAs, on the other hand, are processed from longer precursors into mature species of about 20 to 30 nucleotides. In animals, they can be broadly classified into three major classes: microRNAs (miRNAs), short interfering RNAs (siRNAs), and Piwi-interacting RNAs (piRNAs). These small silencing RNAs are important in post-transcriptional gene regulation and function as key regulators of diverse cellular processes (Ghildiyal and Zamore, 2009, Cech and Steitz, 2014). A common theme among all three classes of

regulatory small RNAs is that they provide the specificity determinant to guide Argonaute (AGO) proteins to complementary messenger RNAs (mRNAs) to exert effector functions. Despite some similarities between the three classes of small RNAs, their biogenesis mechanisms, precursor structures, and modes of gene regulation are distinctly different. MiRNAs and siRNAs bind to the AGO clade of AGO proteins, and act in both somatic tissues and the germline to negatively regulate their cognate mRNAs. PiRNAs are a class of small RNAs that are distinctly different from siRNAs and miRNAs. They are slightly longer at about 26 to 30 nucleotides, and bind to the PIWI clade of AGO proteins (Kim, 2006). PiRNAs are gonad-specific, and function to protect germline integrity by silencing transposable elements (Siomi et al., 2011).

The following sections give an introduction to siRNAs and miRNAs, with an emphasis on miRNAs. PiRNAs will not be discussed as they fall beyond the scope of this thesis.

1.1 Small interfering RNAs (siRNAs) and the RNAi pathway

Post-transcriptional repression of complementary transgenes by antisense RNAs was first reported in plants (Baulcombe, 1996). The RNA-RNA base-pairing between complementary sequences resulted in RNA degradation through endonucleolytic cleavages (Metzlaff et al., 1997). By studying infiltration of GFP signals in leaves of transgenic plants, the phenomenon of sequence-specific gene silencing was found to be non-cell-autonomous (Voinnet and Baulcombe, 1997). It was later found to be mediated by 25-nucleotide antisense RNA synthesized from an RNA template (Hamilton and Baulcombe, 1999).

These initial reports in plants are believed to have laid the groundwork for later findings from the Mello laboratory for work performed in *Caenorhabditis elegans* (*C. elegans*). In their work, they describe potent and specific RNA interference (RNAi) elicited by double-stranded RNAs

(dsRNAs) injected into adult *C. elegans*, which can act systemically by migrating to areas away from the site of injection (Fire et al., 1998). This delivery of exogenous dsRNAs to induce genetic interference serves as a powerful experimental tool to target specific genes and study their functional significance. The phenomenon of RNAi is widespread among many eukaryotes including plants, flies, and mammalian cells (Elbashir et al., 2001a, Kennerdell and Carthew, 1998, Waterhouse et al., 1998).

Integral to the RNAi pathway, the core processing machinery consists of the proteins Dicer (Dcr) and Argonaute (AGO). Even though the RNAi machinery is evolutionarily conserved, different organisms can possess varying numbers of Dicer and AGO proteins. Dicer belongs to the class III RNase III family, consisting of a helicase domain, domain of unknown function (DUF283), Piwi-Argonaute-Zwille (PAZ) domain, two tandem RNase III domains, and a dsRNA-binding domain (dsRBD) (MacRae et al., 2007). Dicer makes two staggered cuts in dsRNA substrates to generate duplexes with each strand containing 5' phosphate and 3' hydroxyl termini, with 2-nucleotide overhangs at the 3' ends (Elbashir et al., 2001b). The *Drosophila* genome encodes two *dicer* genes, *dcr-1* and *dcr-2*, each playing distinct roles in the miRNA and siRNA pathways, respectively (Lee et al., 2004b). The two Dicer proteins differ slightly in terms of structure, with Dcr-1 lacking a functional helicase domain and Dcr-2 lacking a PAZ domain (Lee et al., 2004b). AGO proteins were first discovered in plants, and are structurally characterized by PAZ, MID (middle), and PIWI (P-element-induced wimpy testes) domains (Bohmert et al., 1998, Cerutti et al., 2000, Song et al., 2004). The structural similarity between the PIWI domain and RNaseH (an endoribonuclease that cleaves RNA-DNA hybrids) suggested that AGO proteins possess catalytic cleavage activity (Song et al., 2004). The *Ago* gene family in *Drosophila* consists of five members that can be divided into the AGO clade (AGO1 and AGO2) or PIWI clade (AGO3, PIWI, and AUB) based on sequence alignments (Williams and Rubin, 2002). AGO1 and AGO2 are functionally distinct, with roles in miRNA- and siRNA-mediated gene silencing, respectively (Okamura et al., 2004, Lee

et al., 2004b). AGO1-loaded miRNAs direct translational repression and decay, whereas AGO2-loaded siRNAs result in target gene cleavage (Forstemann et al., 2007). Both AGO1 and AGO2 possess catalytic “Slicer” activity that can mediate mRNA cleavage, but AGO1 is generally regarded as a poor Slicer with weak endonucleolytic cleavage capability (Miyoshi et al., 2005, Forstemann et al., 2007).

In RNAi, Dicer initiates the production of short duplexes in an ATP-dependent process (Bernstein et al., 2001). These short duplexes are loaded onto AGO proteins and subsequently unwound to form the functional RNA-induced silencing complex (RISC). Due to the perfect symmetry of highly base-paired siRNA duplexes, strand selection is determined by thermodynamic differences, with the strand possessing the less stably paired 5' end selected as the guide strand (Tomari et al., 2004). The active mature RISC then specifically drives degradation of complementary or near-complementary target mRNAs (Hammond et al., 2000, Hammond et al., 2001). The target mRNAs are cleaved once in the middle of the region bound to the siRNA, between residues base-paired to nucleotides 10 and 11 of the guide siRNA (Elbashir et al., 2001b, Martinez et al., 2002). After cleavage, the 5' and 3' mRNA fragments are degraded by the exosome and XRN1 respectively (Orban and Izaurralde, 2005). Both target recognition and cleavage reactions take place independently of ATP requirement (Nykanen et al., 2001). The complex can direct multiple cycles of target cleavage, ensuring high efficiency of the silencing pathway (Haley and Zamore, 2004).

1.2 MicroRNAs (miRNAs)

The first miRNA gene, *lin-4*, was discovered in nematodes and shown to post-transcriptionally down-regulate the levels of *lin-14* mRNA based on an antisense RNA-RNA interaction (Lee et al., 1993, Wightman et al., 1993). Seven years since the recognition of the first small non-coding RNA, the second miRNA gene, *let-7*, was found to regulate several heterochronic genes to control the timing of larval transitions in *C.*

elegans (Reinhart et al., 2000). Detection of let-7 homologs across animal phylogeny suggested that these 21-nucleotide small RNAs are evolutionarily conserved, and represent an intriguing class of regulatory RNAs that were previously uncharacterized (Pasquinelli et al., 2000). This brought about intense cloning efforts by various laboratories, and resulted in identification hundreds of new miRNA genes (Bartel, 2004). Since then, the number of miRNA genes identified has grown exponentially. To date, there are about 250 miRNA genes annotated in *Drosophila melanogaster* (Kozomara and Griffiths-Jones, 2014).

1.2.1 Biogenesis of miRNAs

MiRNAs derive from endogenous transcripts that fold back on themselves to form irregular hairpin-like structures that often harbour internal mismatches, loops, and bulges (Bartel, 2004, Krol et al., 2004). Depending on their genomic location, miRNAs can be classified as intergenic, intronic, or exonic (Lagos-Quintana et al., 2001, Lau et al., 2001, Lee and Ambros, 2001, Aravin et al., 2003, Rodriguez et al., 2004, Weber, 2005). Animal miRNA genes are also often organized in clusters on the genome and are co-transcribed as a single polycistronic transcript that is later spliced into individual miRNAs (Lau et al., 2001, Lagos-Quintana et al., 2001, Mourelatos et al., 2002, Altuvia et al., 2005). Most miRNA clusters contain two to three miRNA genes (Figure 1.1), but larger clusters containing up to 40 members have also been described (Altuvia et al., 2005).

Figure 1.1. Genomic organization of a typical miRNA cluster.

MiRNA clusters contain two or more miRNA genes. Clustered miRNAs are typically co-transcribed, with gene expression driven by a common promoter.

The majority of eukaryotic miRNA genes are transcribed by RNA polymerase II to give primary miRNA (pri-miRNA) transcripts containing 5' cap structures and poly(A) tails (Lee et al., 2004a, Cai et al., 2004) (Figure 1.2). Transcription by RNA polymerase III has also been reported for miRNAs that are interspersed among repetitive Alu elements on the human genome (Borchert et al., 2006). In mammals, pri-miRNAs are cleaved by Drosha/DGCR8 (Pasha in flies) in the nucleus to give a stem-loop structure of approximately 60 to 80 nucleotides in length, known as precursor miRNAs (pre-miRNAs) (Lee et al., 2003, Denli et al., 2004, Han et al., 2004). Each arm of the precursor has the potential to produce a mature miRNA, denoted as -5p or -3p depending on the strand from which they are derived. Pre-miRNAs are exported to the cytoplasm by Exportin-5 in a Ran-GTP-dependent manner (Yi et al., 2003, Bohnsack et al., 2004, Lund et al., 2004). Exportin-5 reportedly acts in a sequence-independent manner, and can recognize a variety of dsRNA substrates for transport (Bohnsack et al., 2004).

In the cytoplasm, pre-miRNAs undergo a second cleavage step catalysed by Dicer (Dcr-1 in flies) to produce short duplexes (Hutvagner et al., 2001, Ketting et al., 2001). Crystal structure of full-length Dicer in *Giardia intestinalis* provided first insights into the “molecular ruler” mechanism by which Dicer family proteins produce small RNA duplexes of discrete size (Macrae et al., 2006). Later, extensive biochemical data from work in human and fly Dicers provide further evidence on its mode of action (Park et al., 2011, Tsutsumi et al., 2011). The helicase domain of fly Dcr-1 specifically recognizes the terminal loop of pre-miRNAs to inspect the loop size, measures the distance between the 3' overhang and the loop through its PAZ and helicase domains, and finally cleaves the substrate at a fixed distance from the 3' overhang (Tsutsumi et al., 2011).

These asymmetrical small RNA duplexes are loaded onto AGO (AGO1 in flies), and subsequent unwinding leaves one strand (guide or mature

strand) selectively retained to form the mature RISC (Tomari et al., 2007). Relative thermodynamic stabilities of the two ends of the duplex influences guide strand selection; the strand with a less stable 5' end is selected as the guide strand, and the other strand (passenger or miRNA*) is discarded (Khvorova et al., 2003, Schwarz et al., 2003). In addition, fly AGO1 has been shown to prefer guide miRNAs bearing a terminal 5'U (Czech et al., 2009, Okamura et al., 2009, Ghildiyal et al., 2010).

Figure 1.2. Canonical miRNA processing pathway in *Drosophila*. Stepwise cleavage steps mediated by ribonucleases Drosha and Dicer produces a miRNA-miRNA* duplex. After loading to AGO1, one strand is selectively retained to form the mature RISC and directs it to its target mRNA to mediate downstream silencing events.

1.2.2 Structural insights into miRNA-processing machinery

Pri-miRNA transcripts are often over 1 kb in length, and possess an imperfect stem structure approximately 35 base-pairs in length, which is flanked by basal single-stranded RNA segments and an apical loop (Han et al., 2006). High-throughput analysis of human pri-miRNA variants by the Bartel laboratory revealed key features of transcripts that promote processing by the Microprocessor complex. These include (1) a UG motif at the basal end, (2) an apical UGU motif, (3) a CNNC motif at approximately 17 nucleotides downstream of the Drosha cleavage site, and (4) a mismatched GHG motif (where H is any nucleotide other than G) on the lower stem (Auyeung et al., 2013, Fang and Bartel, 2015).

Structural and biochemical studies showed that the Microprocessor is a heterotrimeric complex comprising of one Drosha and two DGCR8 molecules (Nguyen et al., 2015, Herbert et al., 2016, Kwon et al., 2016). Recent resolution of the crystal structure of Drosha in complex with the C-terminal helix of DGCR8 revealed key information into its mode of action (Kwon et al., 2016). Further modeling of Drosha-DGCR8 in complex with pri-miRNA showed that several modules of Drosha act together to recognize the basal junction of pri-miRNAs for precise measurement of 11 base-pairs to the cleavage site (Kwon et al., 2016). DGCR8 consists of a central RNA-binding heme domain (Rhed, two dsRBDs, and a C-terminal tail (CTT). It plays multiple roles in processing: (1) Rhed domain is important for dimerization, and required for efficient and accurate cleavage, (2) dsRBDs bind the stem region of pri-miRNAs, and (3) CTT tail stabilizes Drosha (Nguyen et al., 2015). Specific interaction of the basal UG and apical UGU elements by Drosha and DGCR8, respectively, allows proper orientation of the complex on its substrate. More recently, extensive analysis of close to 40,000 human pri-miRNA variants by deep sequencing, biochemical assays, and structural modeling showed that following initial binding, the mismatched GHG motif is recognized by the dsRBD of Drosha, which

then precisely positions the catalytic center to mediate cleavage with single-nucleotide accuracy (Kwon et al., 2019).

Owing to the large and complex structure of Dicer, resolution of the full-length protein is challenging. As a result, understanding of its structure is mainly restricted to lower eukaryotes or of particular domains. Dicer architecture is highly conserved among eukaryotes, and the first crystal structure of full-length Dicer was solved in *Giardia intestinalis*, containing only the PAZ and RNase III domains (Macrae et al., 2006). Early electron microscopy reconstructions of human Dicer revealed that it has an L-shaped structure, and provided first insights into its mode of action (Lau et al., 2009, Wang et al., 2009a). Later, the MacRae group utilized an improved method of site-specific tagging in combination with single-particle electron microscopy to clearly define and visualize the three-dimensional positions of individual domains in Dicer (Lau et al., 2012). Here, it was suggested that upon recognition of dsRNA by the PAZ domain, the helicase domain shifts to form a clamp-shaped structure and facilitate translocation of the substrate into the catalytic core of Dicer.

In humans, Dicer can associate with two different dsRNA-binding proteins known as trans-activation response RNA-binding protein (TRBP) and protein activator of PKR (PACT) (Haase et al., 2005, Chendrimada et al., 2005, Lee et al., 2006b). Crystal structure of the Dicer-TRBP binding interface showed that both TRBP and PACT binds to a conserved surface on Dicer, and their binding is mutually exclusive (Wilson et al., 2015). Both TRBP and PACT are structurally similar, each containing three dsRBDs; the first and second dsRBDs bind to dsRNAs and the third dsRBD participates in protein-protein interactions (Wilson et al., 2015). More recently, a high-resolution reconstruction of the Dicer-TRBP complex bound to pre-let-7 was obtained by single-particle cryoelectron microscopy (Liu et al., 2018). Here, the authors proposed that the Dicer-TRBP complex stabilizes and primes pre-let-7 in a pre-

dicing state, and subsequent conformational changes in the helicase domain help position the substrate for precise cleavage.

Early structural studies on AGO proteins were limited to prokaryotes (Yuan et al., 2005, Yuan et al., 2006, Wang et al., 2008a, Wang et al., 2008b, Wang et al., 2009b) and individual domains in eukaryotes (Lingel et al., 2003, Song et al., 2003, Ma et al., 2004, Frank et al., 2010). Despite being evolutionarily distant, prokaryotic and eukaryotic AGO proteins share remarkable similarity in their structures and mechanisms of action (Elkayam et al., 2012, Schirle and MacRae, 2012). Recent resolution of full-length human AGO2 bound to a heterogeneous population of guide RNAs helped to establish the mode of binding to substrates and accessory proteins such as GW182 (Schirle and MacRae, 2012). Another study reported the structure of human AGO2 in complex with miR-20a, which described stabilization of AGO2 upon small RNA binding, and illustrated the binding of the 5' and 3' ends of miRNA to the MID and PAZ domains, respectively (Elkayam et al., 2012).

Overall, resolution of the structures of miRNA-processing machinery has provided valuable insight into their mechanism of action, and may prove useful for future design of siRNAs for human RNAi.

1.2.3 MiRNA-mediated target recognition and repression

Early studies have shown that mature miRNAs bind to multiple sites within the 3' untranslated regions (3' UTRs) of target mRNAs through imperfect base pairing (Lee et al., 1993, Reinhart et al., 2000). Target recognition predominantly depends on Watson-Crick base-pairing between the miRNA 5' region (positions 2 to 8, commonly referred to as the "seed" sequence) and 3' UTRs of the target gene (Bartel, 2004). Apart from the seed region, pairing to the 3' region of the miRNA can supplement seed pairing to enhance target recognition, or to compensate for a mismatch in the seed region (Bartel, 2009, Broughton

et al., 2016). Owing to the relatively short complementarity required for target recognition, a single miRNA can potentially regulate hundreds of transcripts.

The degree of complementarity between the guide miRNA and its target mRNA determines its mode of action; target mRNAs possessing perfect or near-perfect complementarity to the miRNA can be cleaved and degraded, whereas those with imperfect complementarity promote translational repression, mRNA deadenylation and decay (Bartel, 2009). Translational repression can be achieved by inhibition of initiation or elongation, premature termination, or co-translational protein degradation (Huntzinger and Izaurralde, 2011). In addition, AGO1 can directly interact with TNRC6 (also known as GW182) to recruit the deadenylation complex and decapping enzymes, ultimately leading to mRNA degradation by cytoplasmic exonucleases (Chen et al., 2009, Chekulaeva et al., 2011, Huntzinger and Izaurralde, 2011). Similar to siRNA-directed mRNA cleavage, the mature miRNA RISC is also a multiple turnover complex capable of regulating multiple transcripts (Baccarini et al., 2011).

1.2.4 Biological functions of miRNAs

MiRNAs have widespread roles in many biological processes, including development, proliferation, differentiation, apoptosis, etc (Tufekci et al., 2014). They exert their functions in complex gene networks by spatial and temporal regulation of their target genes. A few examples are discussed below.

In the nervous system, miRNAs play roles in patterning, differentiation, and maturation, ensuring its proper development (Cochella and Hobert, 2012). Early studies on *dicer* knockout mutants in zebrafish first reported the importance of miRNAs in the development of the nervous system (Giraldez et al., 2005). Several brain-specific miRNAs including miR-101, -127, -128, -132, and -132 have been documented, with the highly

abundant miR-124 accounting for 25% to 50% of all brain miRNAs (Lagos-Quintana et al., 2002, Krichevsky et al., 2003). By targeting PTBP1, miR-124 triggers a switch from general to neuron-specific alternative splicing patterns in a wide range of transcripts, thereby promoting development (Makeyev et al., 2007). Levels of the polycomb group protein Ezh2 and density of Ezh2-mediated Histone 3 Lysine-27 trimethylation are down-regulated upon neuronal differentiation (Boyer et al., 2006, Bracken et al., 2006, Lee et al., 2006a). A more recent study reported that miR-124 negatively regulates Ezh2 expression to promote neuronal differentiation (Neo et al., 2014). Studies on miR-124 in various model systems have identified other targets of miR-124, including EfnB1, SCP1 and BAF (Cochella and Hobert, 2012).

A major role for let-7 lies in cell differentiation. Levels of let-7 are undetectable in human and mouse embryonic stem cells, and they increase upon differentiation (Boyerinas et al., 2010). The involvement of let-7 in various cancers has been demonstrated in numerous studies (Yu et al., 2007, Johnson et al., 2007, Brueckner et al., 2007, Iliopoulos et al., 2009, Sung et al., 2013). In humans, let-7 can regulate the oncogene Ras to promote tumorigenesis (Johnson et al., 2005). The loss or decreased levels of miR-34 in malignancies has also been relatively well explored, and has been linked to cancers of the lung, breast and prostate, among others (Liu et al., 2011, Rokavec et al., 2014, Okada et al., 2014, Li et al., 2013). Following DNA damage, an up-regulation of miR-34 is induced upon p53 (a tumour suppressor gene) activation, which triggers apoptosis (Tarasov et al., 2007, Chang et al., 2007, Raver-Shapira et al., 2007). Down-regulation of miR-34 attenuates p53-dependent apoptosis, and the ability to suppress proliferation in cancerous cells is lost.

In *Drosophila*, the miRNA gene *bantam* promotes cell proliferation and possesses anti-apoptotic activity. A computational search for targets of *bantam* identified the pro-apoptotic gene *hid*, and experimental data showed that *bantam* could suppress *hid*-induced apoptosis (Brennecke

et al., 2003). Besides its role in apoptosis, bantam associates with the dFmr1 (*Drosophila* fragile X mental retardation 1) protein in fly ovaries to maintain germline stem cell maintenance (Yang et al., 2009). Several other anti-apoptotic miRNAs have also been identified, that function via a similar mechanism of action by inhibiting pro-apoptotic genes. In *Xenopus laevis*, miR-24a down-regulates Caspase-9 and Apaf1, to repress apoptosis and promote development of the retina (Walker and Harland, 2009). The myocardial-specific miR-1 and miR-133a similarly targets Caspase-9 to inhibit apoptosis (He et al., 2011). In cancer cells, miR-24 regulates apoptosis by targeting FAF1 (Qin et al., 2010).

Based on differential expression profiles in normal and disease states, miRNAs can serve as important biomarkers for clinical diagnostic purposes. They can serve as indicators for various malignancies, viral infections, cardiovascular diseases, nervous system disorders, and metabolic diseases, among others (Wang et al., 2016a).

1.2.5 MiRNA-based therapies

An increasing number of studies have demonstrated a link between miRNAs and the development of various pathologies. The expression levels of miRNAs are often dysregulated with disease, and their functional roles in certain diseases have been well studied. This has led to the recent emergence of miRNAs as novel therapeutic targets.

Depending on their roles in the manifestation of diseases, miRNA-based therapeutics can be classified into miRNA mimics and miRNA inhibitors (Rupaimoole and Slack, 2017). Synthetic miRNA mimics resemble endogenous miRNAs in structure and sequence, and are aimed at restoring down-regulated miRNAs in disease. MiRNA inhibitors, in contrast, are complementary in sequence to the target miRNA, binding to and blocking its function. The first miRNA-targeted drug discovered makes use of locked nucleic acid-modified antisense oligonucleotides to target liver-specific miR-122 and reduce viral titer in the treatment of

hepatitis C virus (HCV) infection (Lindow and Kauppinen, 2012). The first to enter clinical trials, this miRNA inhibitor (Miravirsen) is currently in phase II of clinical testing to evaluate its safety and effectiveness in chronic HCV patients, and is showing promising results.

The field of miRNA-based therapeutics is still in early stages of development but is progressing rapidly. It faces several challenges, particularly in the delivery of these drug molecules. The expression and distribution of miRNAs is often dynamic and heterogeneous in a particular microenvironment, which complicates the identification of relevant target miRNAs (Rupaimoole and Slack, 2017). RNA molecules are inherently less stable than DNA molecules. Furthermore, due to their small sizes, miRNA inhibitors are envisioned to be degraded rapidly *in vivo*. Even with modifications to increase their stabilities, specific delivery to the target site might not be always achievable (Simonson and Das, 2015). Strategies to evade degradation include addition of chemical modifications and design of delivery vehicles to encapsulate and protect the drugs from RNases (Rupaimoole and Slack, 2017). On the other hand, creating highly stable drugs that persist in the body and have long-lasting effects can result in undesirable consequences, especially if there is local accumulation at healthy tissues (Bernardo et al., 2015). The ability of a miRNA to regulate multiple transcripts can either be a pro or a con in terms of therapeutics. The disadvantage is that the miRNA inhibitor can result in off-target effects through the inhibition of unknown genes, leading to toxicity. On the other hand, the ability to target several genes overcomes the potential desensitization of a single target to the drug, thereby maximizing efficacy (van Rooij and Olson, 2012).

In spite of the challenges in development of miRNA-based therapeutics, these molecules offer immense potential to develop into next generation therapeutic agents.

1.2.6 DsRNA-binding partners in miRNA processing

RNase III enzymes often recruit dsRNA-binding proteins to promote substrate processing, and much of their specificity is conferred by their partners (Tomari and Zamore, 2005). Together with the dsRNA-binding protein DGCR8 (in mammals) or Pasha (in flies), Drosha and its partner protein form a complex known as the Microprocessor to cleave pri-miRNAs into shorter pre-miRNA hairpins (Denli et al., 2004, Gregory et al., 2004, Han et al., 2004, Landthaler et al., 2004). Drosha recognizes basal elements and serves as a molecular ruler to generate staggered cleavage of the two strands at approximately one helical turn away (~11 base-pairs) from the basal junction. Besides stabilizing Drosha, DGCR8 interacts with the stem and apical elements to ensure fidelity of the cleavage (Nguyen et al., 2015).

The second step of the miRNA biogenesis pathway involves cleavage mediated by Dicer to process pre-miRNAs into short miRNA duplexes. The protein structure of Dicer is highly conserved among eukaryotes, with the tandem RNase III domains making up the catalytic core of the enzyme (Macrae et al., 2006). The binding partners for human Dicer are known as TRBP and PACT (Haase et al., 2005, Chendrimada et al., 2005, Lee et al., 2006b). Even though both TRBP and PACT do not regulate the rate of Dicer processing and have no influence over miRNA abundance, TRBP have been shown to affect the accuracy Dicer cleavage for a subset of pre-miRNAs (Kim et al., 2014, Wilson et al., 2015). Using an *in vitro* reconstituted system, the Doudna laboratory revealed that differences in the N-terminal RNA-binding domains of TRBP and PACT confer cleavage and substrate specificities to Dicer activity (Lee et al., 2013). Furthermore, the Dicer-PACT complex selects against pre-siRNA substrates as compared to Dicer-TRBP. A more recent study using single-molecule fluorescence for real-time observations reported a role for TRBP in ensuring accurate and efficient pre-miRNA processing by Dicer in RNA-crowded cellular environments (Fareh et al., 2016). The distinct activities of Dicer-TRBP or Dicer-PACT

complexes may be a consequence of their differential expression levels and cellular localization, which may in turn play important roles under certain cellular conditions or in different cell types. However, these possibilities remain to be investigated.

The *Drosophila* genome encodes two Dicer proteins, Dcr-1 and Dcr-2, that function in the miRNA and siRNA silencing pathways, respectively (Lee et al., 2004b). Fly Dcr-1 is capable of distinguishing between pre-miRNA substrates and long dsRNAs, with the latter being funneled to the Dcr-2 processing pathway. To achieve substrate specificity, the helicase domain of Dcr-1 specifically recognizes the single-stranded terminal loop region of miRNA precursors, measures the distance between the 3' overhang and terminal loop, finally producing duplexes of discrete size (Tsutsumi et al., 2011).

Using chromatographic fractionation to purify siRNA-generating activity, Liu and colleagues identified the dsRNA-binding protein R2D2 (named for possessing two dsRNA-binding domains (R2) and binding to Dcr-2 (D2)) as the interaction partner of Dcr-2 (Liu et al., 2003). Even though Dcr-2 alone is capable of efficient production of siRNAs, R2D2 enhances the loading of siRNAs onto the effector complex (Liu et al., 2003, Liu et al., 2006). In the presence of R2D2 and inorganic phosphate, cleavage of pre-miRNAs is blocked and Dcr-2 acquires its specificity for long dsRNAs as substrates (Cenik et al., 2011). In *r2d2* mutants, misdirection of siRNAs to AGO1 was reported, indicating a role for R2D2 in sorting of small RNAs to different AGO complexes (Okamura et al., 2011).

Using RNAi-based screening and bioinformatics approaches, three laboratories independently identified Loquacious as the dsRNA-binding protein that binds to Dcr-1 (Forstemann et al., 2005, Jiang et al., 2005, Saito et al., 2005). Together, they demonstrated the formation of a functional Dcr-1-Loqs complex that is important for pre-miRNA processing both *in vivo* and *in vitro*. Dcr-2 and R2D2 have a reciprocal

stabilization effect, and either protein alone is unstable (Liu et al., 2003). On the contrary, a previous study on TRBP found that it does not stabilize mammalian Dicer (Kim et al., 2014). Similarly, depletion of *loqs* by RNAi did not significantly reduce protein levels of Dcr-1, suggesting that the role of Loqs in pre-miRNA processing extends beyond stabilization of Dcr-1 (Forstemann et al., 2005, Saito et al., 2005).

1.2.7 Loquacious isoforms and their functions

The *loqs* gene produces three alternatively spliced mRNA isoforms: *loqs-RA*, *-RB*, and *-RD*. These mRNAs encode for the respective proteins Loqs-PA, -PB, and -PD (Figure 1.3). Loqs-PB is the longest isoform containing three dsRBDs. Loqs-PA also consists of three dsRBDs, but lacks a section of amino acids that is present in the third dsRBD of Loqs-PB. Loqs-PD contains only two dsRBDs, and has a unique sequence at the C-terminus (Fukunaga et al., 2012).

Figure 1.3. Protein domains of Loqs isoforms.

Loqs-PA and -PB both contain three dsRBDs. Loqs-PA lacks 46 amino acids that are present in the third dsRBD of Loqs-PB. Loqs-PB also contains a longer linker region between the second and third dsRBDs as compared to Loqs-PA. Loqs-PD contains only two dsRBDs, with the canonical third dsRBD replaced by a unique sequence. DsRBDs are illustrated as black boxes (aa: amino acids).

Both Loqs-PA and -PB co-precipitate with Dcr-1, but Loqs-PB constitutes the majority of Loqs isoform associated with Dcr-1 *in vitro* (Forstemann et al., 2005). *In vitro* dicing assays have shown that even though Loqs-PB enhanced Dcr-1 cleavage, it was dispensable for substrate recognition (Tsutsumi et al., 2011). Interestingly, binding of Dcr-1 to Loqs-PB can alter pre-miRNA cleavage sites, producing mature miRNA isoforms with distinct seed sequences and thus different target

specificities (Fukunaga et al., 2012). Loqs-PD, on the other hand, relies on its unique C-terminus to bind Dcr-2 and promotes production of endogenous siRNAs (endo-siRNAs) (Hartig et al., 2009, Zhou et al., 2009).

Loqs-PB binds to pre-miRNA using the second dsRBD and to the helicase domain of Dcr-1 using the third dsRBD (Ye et al., 2007). A recent structural study further showed that the extended linker region preceding the third dsRBD of Loqs is important for efficient Dcr-1 interaction (Jakob et al., 2016). A comparison of binding activities of the third dsRBD alone or of the third dsRBD together with the linker region showed that the linker region provided a 10-fold increase in binding affinity to Dcr-1. With this, the authors propose a two-step binding model, where initial binding of low affinity between the third dsRBD and Dcr-1 provides a specificity platform to allow for establishment of stronger interactions between the linker region and Dcr-1, thereby serving as a proof reading mechanism.

Initial studies that characterized Loqs utilized a fly strain in which the piggyBac transposon was inserted upstream of the *loqs* transcription start site, creating a partial loss-of-function mutation (Forstemann et al., 2005, Jiang et al., 2005). Homozygous mutant *loqs* male flies are incompletely fertile, while females flies are completely sterile and are defective in oogenesis due to the disruption in germline stem cell maintenance (Forstemann et al., 2005). Loqs-PA is the major isoform expressed in the testis, while Loqs-PB is predominantly expressed in the ovary, possibly hinting at their distinct roles during development (Forstemann et al., 2005, Park et al., 2007). Through a combination of genetic and biochemical experiments, Park and co-workers showed that Loqs-PB, but not Loqs-PA, is necessary and sufficient for *Drosophila* female germline stem cell maintenance and normal miRNA processing (Park et al., 2007).

Although zygotic *loqs*^{KO} mutants are pupal lethal, those embryos and larva harbour wild-type levels of Loqs proteins, indicating that Loqs

proteins are maternally deposited in the zygote (Park et al., 2007). Depletion of maternal and zygotic *loqs* resulted in embryonic lethality, which can be rescued by the transgenic expression of Loqs-PA or Loqs-PB (but not Loqs-PD) in a *loqs*^{KO} background (Park et al., 2007, Fukunaga et al., 2012). Studies in *loqs*^{KO} flies reported that Loqs is essential for the production of only a subset of miRNAs (Liu et al., 2007, Fukunaga et al., 2012). However, in the absence of maternal contribution, the role of Loqs in mature miRNA production remains elusive. Furthermore, past studies have yet to address the substrate specificity of Loqs that resulted in only certain miRNA genes being Loqs-dependent.

1.2.8 Mature miRNA stability

Levels of mature miRNAs in the cell must be precisely controlled. Degradation processes serve as an additional mechanism to regulate target gene expression through modulation of miRNA levels. Despite the importance of degradation in maintaining homeostasis, studies on miRNA stability in living organisms are generally lacking.

Mature miRNA stability is governed by a myriad of regulatory mechanisms that can vary widely between individual miRNAs and across species. They are perceived to be extremely stable molecules that can persist up to several days; however, this was drawn from isolated studies on few tissue-specific miRNAs (e.g. heart-specific miR-208 and liver-specific miR-122) (van Rooij et al., 2007, Gatfield et al., 2009). By using genetic ablation of *Dicer1* in mouse embryonic fibroblasts to study global miRNA decay, Gantier and colleagues estimated miRNAs to be approximately 10 times more stable than mRNAs, with an average half-life of 119 hours (~5 days) (Gantier et al., 2011). Since miRNAs play important roles in an array of developmental processes (e.g. developmental timing, embryo patterning, differentiation, apoptosis, etc) (Alvarez-Garcia and Miska, 2005, Wienholds and Plasterk, 2005), it appears conceivable that their levels should be highly

dynamic and fluctuate rapidly in order to facilitate their functional roles. In fact, neuronal miRNAs have been reported to decay considerably faster than non-neuronal miRNAs (Rajasethupathy et al., 2009, Sethi and Lukiw, 2009, Krol et al., 2010). However, the significance and mechanisms underlying rapid decay of neuronal miRNAs and its regulation remain unknown.

Even though mature miRNAs are short sequences of ~22 nucleotides, certain sequence elements embedded within specific miRNAs can contribute to decay kinetics. AU-rich elements have long been recognized as a predictor of unstable mRNAs (Chen and Shyu, 1995). Brain-enriched miRNAs have relatively short half-lives of ~1 to 3.5 hours, and were found to be enriched in AU-rich elements as well (Sethi and Lukiw, 2009). Using an *in vitro* pre-miRNA processing and miRNA decay system, Bail and colleagues found that the seven nucleotides (GGAUUCG) at the 3' terminal end of miR-382 drives its instability (Bail et al., 2010). Using a pulse-chase assay to measure half-lives in mammalian cell cultures, it was reported that even though most miRNAs possess half-lives of 12 hours or longer, certain miRNAs exhibit shorter half-lives of several hours (Zhang et al., 2011). In the same study, mammalian miR-29b was reported to have a half-life of 7 hours, and further examination of sequence determinants revealed that a uracil-rich motif at positions 9 to 11 drives rapid decay of this miRNA. Levels of miR-503 is regulated by cell cycle progression, and its instability was found to be dependent on nucleotides in the seed region and at the 3' end (Rissland et al., 2011). In a comprehensive deep sequencing analysis of miRNA turnover across eight mammalian cell lines, Guo and colleagues identified multiple sequence features that differentiate between stable and unstable miRNAs (Guo et al., 2015). Overall, a variety of sequence elements have been implicated in the stability of specific miRNA species, but there does not appear to be a general sequence specificity feature that defines unstable miRNAs.

Several studies have also investigated ribonucleases involved in mature miRNA degradation. Even though homologs of certain factors can be found across species, whether they play conserved roles in miRNA degradation remains unclear. Furthermore, a general miRNA-degrading nuclease has yet to be identified in flies. Among the first to be discovered, the 3'-to-5' exonuclease small RNA degrading nuclease (SDN-1) in plants was found to mediate degradation of single-stranded miRNAs, but not of miRNA duplexes and longer RNA species, in a sequence-independent manner (Ramachandran and Chen, 2008). In *C. elegans*, the 5'-to-3' exoribonucleases XRN-1 and XRN-2 were found to mediate miRNA degradation (Chatterjee and Grosshans, 2009, Chatterjee et al., 2011). Follow-up studies further identified decapping scavenger enzyme (DCS-1) as a novel player that interacts with XRN-1 in the cytoplasm to promote miRNA release from RISC and subsequent degradation of AGO-free miRNAs (Bosse et al., 2013). These miRNA-degrading enzymes act in a sequence-independent manner, thus it remains unclear how specific targeting of miRNAs is achieved.

On the other spectrum, factors that only act on specific miRNAs have also been identified. In flies, the XRN-1 homolog known as Pacman was found to specifically down-regulate mature miR-277 (but not pri- and pre-miR-277) in a tissue-specific manner (Jones et al., 2013). However, the authors did not demonstrate a direct role for XRN-1 in degradation of miR-277, and how specific targeting of this particular miRNA during maturation or degradation remains unresolved. The Newbury laboratory used genome-wide profiling on *Drosophila* wing imaginal discs to investigate the effect of 3'-to-5' ribonuclease Dis3 on mature miRNA expression profiles (Towler et al., 2015). In general, Dis3 can influence the expression of a small subset of miRNAs. More interestingly, together with Rrp40, the Dis3-Rrp40 complex can specifically degrade miR-252 to promote normal wing development (Towler et al., 2015). The underlying mechanism of action remains unclear at present. In mammals, polynucleotide phosphorylase (PNPase), a type I interferon-inducible 3'-to-5' exonuclease, can degrade specific oncogenic miRNAs

in human melanoma cells (Das et al., 2010). This was speculated to be clinically important due to its potential therapeutic applications in targeting certain human malignancies. In cultured HEK293 cells, the exosomal component Rrp41 was found to play a major role in degradation of miR-382, with a minor contribution by XRN-1 (Bail et al., 2010). MiR-382 is involved in HIV-1 provirus latency, therefore the regulation of miR-382 stability might be beneficial in disease intervention. Human Tudor staphylococcal nuclease (TSN) is another recently identified factor that mediates endonucleolytic decay of specific miRNAs at CA and/or UA dinucleotides that are preferentially positioned at least five nucleotides away from the miRNA ends (Elbarbary et al., 2017b). Experiments using recombinant TSN by itself was able to degrade both AGO2-free and AGO2-loaded synthetic miRNAs. However, in cell lysates, the RNA helicase UPF1 stimulates the activity of TSN by promoting the release of AGO2-bound miRNAs from their targets in an ATP-dependent manner (Elbarbary et al., 2017a).

Steady-state miRNA levels are determined by their rates of transcription, processing, loading, and decay. Dysregulation of miRNA levels is often implicated in various diseases, including cancer (Iorio and Croce, 2012). Analysis of global miRNA stability and degradation processes during animal development is generally lacking. Fly embryogenesis is a short but precise process that completes within 24 hours. During this embryogenesis, miRNAs have to be synthesized and degraded in a highly controlled manner, presenting as an ideal model system to study miRNA degradation in the physiological state.

1.3 Aims of the project

This study aimed to address a few open questions pertaining to miRNA biogenesis and regulation of their turnover in *D. melanogaster*. There are fly homologs for approximately 75% of known human disease genes, making *D. melanogaster* a powerful model organism (Reiter et al., 2001). Their relatively cheap and easy maintenance in the

laboratory, short generation time, and availability of a powerful genetic toolkit, have made the fruit fly the model organism of choice for more than a century.

The first part of the thesis aimed to elucidate the role of Loqs in the pre-miRNA processing. Since previous studies on Loqs function were performed using partial loss-of-function *loqs*^{KO} flies, the present study sought to address this question in the complete absence of Loqs protein, by using a newly generated mutant *loqs* cell line derived from fly embryos. The structural features of miRNA precursors that contribute to Loqs-dependency of particular genes were further examined on a genome-wide scale.

The second part of the thesis aimed to examine post-transcriptional mechanisms governing miRNA stability. Here, differential regulation of miRNA stabilities and degradation mechanisms during fly embryogenesis were investigated through a combination of bioinformatics and biochemical analyses.

Overall, these findings will provide a better understanding of dsRBPs in modulating the activity of their binding partners, and offer a clue to differential miRNA turnover rates in general.

2. Material and methods

2.1. Generation of *loqs* mutant cells

The *loqs* mutant cell line was generated by Prof. Amanda Simcox (Ohio State University), from embryos lacking the *loqs* gene locus according to the previously described protocol (Simcox et al., 2008a). Meiotic recombination was used generate recombinant chromosomes whereby the *loqs* null mutation (*loqs*-KO, Park et al., 2007) was combined with *Act5C-Gal4* or *UAS-Ras^{V12}* transgenes, to generate *loqs*KO, *Act5C-Gal4/CyO* or *loqs*KO, *UAS-Ras^{V12}/CyO* heterozygous flies. *Act5C* is a broadly expressed actin gene that can direct expression of Gal4 in many cell types. *Ras^{V12}* is a constitutively activated form of Ras locked in the GTP-bound state that can promote survival and proliferation in the establishment of *Drosophila* cell lines. The heterozygous parental flies of the above genotypes were crossed and embryos collected to establish primary cultures. Out of the four possible genotypes generated from this cross, only embryos of the *loqs*KO, *Act5C-Gal4/loqs*KO, *UAS-RAS^{V12}* genotype express Ras^{V12}, allowing for a selective proliferation advantage. Following overnight collection at 17°C on grape juice plates supplemented with yeast paste, embryos were collected and dechorionated in 50% bleach (v/v) for 3 minutes. They were then washed extensively in buffer containing 0.7% NaCl and 0.02% Triton X-100. Embryos were rinsed once with water, and once with 3 mL of culture medium (Schneider's medium (Sigma) supplemented with 10% heat-inactivated fetal bovine serum and 1:100 diluted streptomycin penicillin (Invitrogen)). The embryos were homogenized in the culture medium, pelleted by centrifugation and rinsed with new aliquots of culture medium for 3 times. The final pellet was re-suspended in a small volume of culture medium and plated on T25 flasks containing 3 mL of culture medium and grown at 22°C. Primary cultures were checked periodically and the medium was changed 10 days after the first culture, and thereafter every 2 weeks. Once the primary cultures reached ~70%

confluency, they were subcultured by trypsinization and seeded at 1:2 to 1:4 dilution in new culture flasks.

2.2. Stable transfection of *loqs* mutant cells

Confluent *loqs* mutant cells were diluted at 1:10, seeded on a 6-well plate, and incubated overnight at 25°C. 400 ng of rescue plasmid and 40 ng of helper pCoHygro selection plasmid were used for transfection using Effectene transfection reagent (Qiagen). The rescue plasmids used were pRmHa-3XFLAG-EGFP or pRmHa-3XFLAG-Loqs-PB that are under control of a copper sulphate-inducible metallothionein promoter (described in (Saito et al., 2005)). 4 days after transfection, cells were trypsinized and grown in selection medium containing 200 µg/ml hygromycin (iDNA). Cell growth was monitored closely and the culture medium was replaced with fresh selection medium every 3 to 4 days until viable colonies started to grow.

2.3 Maintenance of cell cultures

S2-R+ cells and *loqs* mutant cells (and the respective stable cell lines established from these cells) were grown in Schneider's *Drosophila* medium (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (iDNA) and 1% penicillin/streptomycin (Gibco). Kc167 cells (*Drosophila* Genomics Resource Center) were grown in Shields and Sang M3 insect medium (Sigma) supplemented with 0.25% Bacto Peptone, 0.1% Yeast Extract, and 10% fetal bovine serum. BG3-c2 cells (*Drosophila* Genomics Resource Center) were grown in Shields and Sang M3 insect medium (Sigma) supplemented with 10% fetal bovine serum (iDNA) and 10 µg/ml insulin (Sigma). All cultures were maintained at 25°C in a thermostatically controlled incubator.

2.4 Collection of *Drosophila* embryos

Wild-type Canton S (CS) flies were kept in embryo collection cages (Genesee Scientific) with grape juice agar plates supplemented with yeast paste. Embryos were dislodged from plates with deionized water,

dechlorinated in 50% (v/v) bleach for 2 minutes, washed extensively with deionized water, blotted dry, and collected in RNase-free tubes (Fisher Scientific).

2.5 Northern blotting

10 µg of heat-denatured total RNA was separated on a 15% denaturing polyacrylamide gel (National Diagnostics) at 300 V for 3 hours, and stained with 1:10,000 SYBR green I nucleic acid gel stain (Invitrogen). Using the Transblot SD semi-dry transfer cell (Bio-Rad), RNA was transferred onto Hybond-N+ membrane (GE Healthcare) in 0.5X TBE at 10 V and 300 mA in the cold room. The membrane was cross-linked using a UV crosslinker. LNA or DNA probes were labeled with gamma-³²P-ATP (PerkinElmer, Cat#NEG502A250UC) at 37°C for 1 hour using T4 polynucleotide kinase (NEB). The labeled probes were purified using microspin G-25 columns (GE Healthcare) according to manufacturer's instructions. Hybridization was performed in a hybridization oven at 45°C overnight in LNA or DNA hybridization buffer, depending on the type of probe used. Following overnight hybridization, membranes were washed with 2X SSC, 0.1% SDS twice and 0.2X SSC, 0.1% SDS thrice (for LNA probes) or with 2X SSC, 0.1% SDS for five times (for DNA probes). All washing steps (10 minutes per wash) were performed at 45°C in a hybridization oven with rotation. Membranes were exposed using hypercassette autoradiography cassettes and screens (GE Healthcare) and analysed on Typhoon phosphorimager (GE Healthcare). Compositions of stock solutions used to prepare the respective buffers are listed in Table 2.1.

Table 2.1. Compositions of buffers used for Northern blotting.

Stock solution	Composition
10X TBE	1 M Tris base, 1 M boric acid, 0.02 M EDTA
LNA hybridization buffer	50% formamide, 5X SSPE, 5X Denhardt's solution, 0.5% SDS, 20 µg/ml salmon sperm DNA
DNA hybridization buffer	5X SSC, 1 mM EDTA, 2x Denhardt's solution, 1% SDS, 2% dextran sulphate (Sigma), 30 µg/ml salmon sperm DNA
20X SSPE	3 M NaCl, 0.2 M sodium phosphate monobasic, 0.02 M EDTA
50X Denhardt's solution	1% (w/v) Ficoll 400, 1% polyvinylpyrrolidone (w/v), 1% (w/v) bovine serum albumin
20X SSC	3 M NaCl, 0.3 M trisodium citrate

2.6 Western blotting

Gel electrophoresis and blotting were carried out using the Mini-PROTEAN Tetra Cell system and Mini Trans-Blot cell blotting module respectively (Bio-Rad). Protein samples were denatured at 95°C before being separated on a 10% polyacrylamide/SDS gel (prepared from 30% acrylamide/bis solution, 29:1, Bio-Rad) in 1X SDS-PAGE running buffer at 160 V for 60 minutes or until the gel front reached the bottom of the gel. Proteins were transferred to PVDF membrane (Merck) at 50 V for 90 minutes in the cold room. After transfer, the membrane was blocked using 5% skim milk (Nacalai Tesque) on a shaker for 1 hour at room temperature. Overnight incubation with primary antibody was performed at 4°C with rotation. The membrane was washed 5 times, 10 minutes each, with 1X TBST on a shaker. Incubation with secondary antibody was performed at room temperature for 1 hour, after which the membrane was washed again with 1X TBST for 5 times, 10 minutes each. The membrane was then incubated with 1 mL of luminol solution and 1 mL of peroxide solution from the ECL Prime detection kit (GE Healthcare) for 5 minutes at room temperature with rotation. Imaging was performed with Amersham Hyperfilm ECL (GE Healthcare) combined with KODAK processor, or with the ChemiDoc Touch Imaging System (Bio-Rad). Dilutions of primary and secondary antibodies are summarized in Table 2.2.

Table 2.2. Antibodies used for Western blotting.

All dilutions were in 1X TBST.

Primary antibody	Secondary antibody
Mouse anti-FLAG (Wako chemicals, Cat#012-22384) 1:1000	anti-mouse IgG, HRP linked (Invitrogen, Cat#F21453) 1:5000
Rabbit anti-Loqs (gift from the Siomi lab) 1:1000	anti-rabbit IgG, HRP linked (Invitrogen, Cat#A10547) 1:5000
Rabbit anti-AGO1 (Abcam, Cat#ab5070) 1:1000	anti-rabbit IgG, HRP linked (Invitrogen, Cat#A10547) 1:5000

2.7 Small RNA library construction

Small RNA libraries were constructed using a modified method (Brennecke et al., 2007). Total RNA was extracted from stable cell lines or developmentally staged embryos (0-2h, 2-4h, 4-6h, 6-8h, 8-10h, 10-12h, 12-18h, and 18-24h) using Trizol (Invitrogen). 40 µg or 30 µg of total RNA from stable cell lines or fly embryos, respectively, was spiked with ³²P-labeled 19-28mer oligos (Table 2.3). The size markers contain a PmeI digestion site that facilitates their removal by a PmeI digestion step after PCR amplification (Hafner et al., 2008). RNA was separated on a 15% denaturing gel (National Diagnostics), directly exposed using hypercassette autoradiography cassettes and screens (GE Healthcare), and analysed on Typhoon phosphorimager (GE Healthcare). The small RNA corresponding to 19 to 28 nt was excised from the gel and manually homogenized. RNA was eluted in 400 µL of 0.4 M NaCl, and incubated overnight with rotation at room temperature. The following day, samples were passed through Pierce centrifuge columns (Thermo Scientific) to remove residual gel slices from the eluted RNA. Samples were transferred to new tubes and ethanol precipitated.

3' linker ligation was performed in a reaction mixture containing 1X ATP-free T4 RNA ligase buffer (NEB), 10% DMSO (Bio-Rad), 30% PEG 8000 (NEB), 1 µM 3' linker (Table 2.3), and T4 RNA ligase 2, truncated K277Q (NEB). The reactions were incubated at room temperature for 1 hour. Following incubation, 20 µL of RNA loading buffer was added to each reaction and samples were purified on a 15% denaturing gel. The bands corresponding to ~36 to 41 nt were excised, homogenized, and

eluted as described above. After ethanol precipitation, 5' linker ligation was performed in a reaction mixture containing 1X T4 RNA ligase buffer (Thermo Scientific), 10% DMSO (Bio-Rad), 30% PEG 8000 (NEB), 1 μ M 5' linker (Table 2.3), and T4 RNA ligase (Thermo Scientific). The reactions were incubated at 37°C for 1 hour. Following incubation, 20 μ L of RNA loading buffer was added to each reaction, and samples were purified on a 8% denaturing gel. The bands corresponding to ~68 to 73 nt were excised, homogenized, eluted, and ethanol precipitated as described above.

Reverse transcription was performed by first adding 4.2 μ L of 5 μ M reverse transcription primer (Table 2.3) to 6.3 μ L RNA samples and incubated at 72°C for 2 minutes. A reverse transcription mix containing 1X first strand buffer (Invitrogen), 2 mM dNTP (Promega), 2 mM DTT (Invitrogen), 2 μ L Superscript III reverse transcriptase (Thermo Scientific), and 0.5 μ L RNaseOUT recombinant ribonuclease inhibitor (Thermo Scientific) was then added to each tube and incubated at 50°C for 1 hour. Extension was carried out at 70°C for 15 minutes.

The cDNA was amplified by PCR using iProof High-Fidelity DNA polymerase (Bio-Rad) with a PCR forward primer (Table 2.3) and indexed reverse primer (Illumina) for multiplexing. PCR products were purified using Phenol/Chloroform/Isoamyl alcohol (Sigma), and ethanol precipitated. Samples were digested with PmeI (NEB) to prevent accumulation of marker sequences, and then gel purified on a 6% native TBE PAGE gel. High-throughput sequencing was performed on Illumina HiSeq2000 at Duke-NUS Genomics facility (for cell lines stably transfected with EGFP or Loqs-PB) or Beijing Genomics Institute (for staged *Drosophila* embryos).

Table 2.3. Oligos used for small RNA library construction.

Oligo name	Sequence
Solexa standard 19mer	rGrUrCrGrUrArCrGrGrUrUrUrArArArCrUrU rCrGrArArArUrGrUrArU
Solexa standard 28mer	rCrGrUrArCrGrGrUrUrUrArArArCrUrUrCrG rA
3' linker	TGGAATTCTCGGGTGCCAAGG
5' linker	GUUCAGAGUUCUACAGUCCGACGAUC
Reverse transcription primer	GCCTTGGCACCCGAGAATTCCA
Forward primer for PCR	AATGATACGGCGACCACCGAGATCTAC ACGTTTCAGAGTTCTACAGTCCGA

2.8 Quantitative RT-PCR

10 µg of total RNA from EGFP or Loqs-PB stable cell lines was extracted by Trizol (Invitrogen) and subjected to DNase I treatment using Turbo DNA-free kit (Thermo Scientific) After incubation at 37°C for 30 minutes, 5 µL of DNase inactivation reagent was added and the reaction mix was incubated at room temperature for 2 minutes. Samples were centrifuged at 10,000 g for 1.5 minutes and the supernatant collected. Reverse transcription was performed using iScript Reverse Transcription Supermix (Bio-Rad) according to manufacturer's protocol. The following thermal cycling protocol was used: 5 minutes at 25°C, 30 minutes at 42°C, and 5 minutes at 85°C. The cDNA product was subsequently diluted to a concentration of ~100 ng/µl by adding 180 µL of sterile water. Quantitative PCR was performed using iTaq Universal SYBR Green Supermix (Bio-Rad) with primers listed in Table 2.4. Four technical replicates were made for each sample. Values were normalized to the reference housekeeping gene rp49 and expressed relative to the EGFP control sample.

Table 2.4. Oligos used for qRT-PCR.

Oligo name	Sequence
dcr-1-4223p	GCCTTCCATCTCCCAGTTTT
dcr-1-4308-	GACTGCTCTTTGGACCCTTG
AGO1_F	GCACTCTACGGTCTGTCCGT
AGO1_R	CAAGTATCGTGTGTGCAACG
rp49_F	CGCACTCTGTTGTCGATACC
rp49_R	ACTTCATCCGCCACCAGTCG

2.9 β -elimination

β -elimination was performed as described previously (Horwich et al., 2007). 10 μ g of total RNA extracted from *loqs* mutant cells stably expressing EGFP and Loqs-PB was incubated with 25 mM sodium periodate in borax/boric acid buffer and incubated in the dark at room temperature for 30 minutes. This was followed by NaOH treatment in the dark at 45°C for 90 minutes. Samples were ethanol precipitated and Northern blotting performed as described above. The membrane was sequentially probed for bantam (AGO1 sorted), miR-277 (both AGO1 and AGO2 sorted), and hp-CG4068B (AGO2 sorted).

2.10 Immunoprecipitation

Wild-type CS embryos were collected as described above and homogenized in RIPA buffer (1X PBS pH 7.4, 0.1% SDS, 0.5% deoxycholate, 0.5% NP40, 1X complete mini EDTA free (Roche)). Samples were cleared by centrifugation. Protein concentration was measured by Bradford's assay using bovine serum albumin (Promega) as a protein standard. 400 μ L of lysates was added to 32 μ L of Dynabeads (Thermo Scientific) bound to 5 μ g rabbit IgG or anti-AGO1 (Abcam), and incubated for 1.5 hours at 4°C. After binding, 10% of the sample was used for protein extraction and 90% used for RNA extraction. Protein samples were analyzed by Western blotting as described above and incubated with anti-AGO1 antibody (Abcam, Cat#ab5070). RNA samples were extracted with 0.4 M NaCl and Phenol/Chloroform/Isoamyl alcohol (Sigma), ethanol precipitated and analyzed by Northern blotting as described above.

For sequential immunoprecipitation of AGO2 and AGO1 complexes, S2-R⁺ cells stably expressing FLAG-HA-AGO2 seeded at a density of 1 x 10⁶ cells/ml on a T150 culture flask were transfected with 11.25 μ g of wild-type or 5'C mutant constructs using Effectene transfection reagent (Qiagen). 1.5 mM CuSO₄ was added to the culture medium 3 days after transfection to induce gene expression. The following day, cells were

collected by centrifugation, lysed in RIPA buffer and cleared by centrifugation. Protein concentration was measured by Bradford's assay and was approximately 15 mg/ml. 400 μ L of cell lysates were incubated with 32 μ L of Dynabeads (Thermo Scientific) bound to 3 μ g mouse anti-IgG or anti-FLAG antibody (Wako chemicals, Cat#012-22384) for 1.5 hours at 4°C. The supernatant from the first immunoprecipitation was used for the second immunoprecipitation with 5 μ g rabbit anti-IgG or anti-AGO1 antibody (Abcam, Cat#ab5070) bound to 32 μ L of Dynabeads (Thermo Scientific). RNA was extracted with 0.4 M NaCl and Phenol/Chloroform/Isoamyl alcohol (Sigma) and the aqueous phase was ethanol precipitated. Co-precipitated RNA was analyzed by Northern blotting as described above.

2.11 *In vitro* loading assay

In vitro loading assay was performed as previously described (Okamura et al., 2013). Cell lysates were prepared from FLAG-EGFP or FLAG-Loqs-PB cultures according to a protocol as described previously (Okamura et al., 2008a). Cells were re-suspended in hypotonic buffer (30 mM HEPES-KOH, pH. 7.4, 2 mM magnesium acetate, 5 mM DTT, 1X complete mini EDTA free (Roche)) and lysed by passing through a 25-gauge needle for five times. The lysate was cleared by centrifugation at 14,600 g for 25 minutes at 4°C. Protein concentration was measured by Bradford's assay, and was approximately 10 mg/ml. One strand of synthetic miR-10 was radiolabeled with γ -³²P and annealed with its partner strand to form duplex structures, then added to 40 μ L of FLAG-EGFP or FLAG-Loqs-PB cytoplasmic extracts and incubated for 1 hour at room temperature. 620 μ L of RIPA buffer (1X PBS, 0.5% NP-40, 0.5% deoxycholate, 0.1% SDS, 1X complete mini EDTA free (Roche)) was added to the reaction, and the *in vitro* loaded complex was purified by AGO1-IP as described above, using 3 μ g AGO1 antibody (Abcam, Cat#ab5070) bound to 18 μ L of Dynabeads (Invitrogen). RNA was extracted using Phenol/Chloroform/Isoamyl alcohol (Sigma). The RNA was ethanol precipitated in the presence of excess cold RNA

corresponding to the labelled strand, to prevent re-annealing. Total RNA was separated on a 15% native polyacrylamide gel and Northern blotting was performed as described above. Oligos used are listed in Table 2.5.

Table 2.5. RNA oligos used for *in vitro* loading.

Oligo name	Sequence
mir-10-3p	rCrArArArUrUrCrGrGrUrUrCrUrArGrArGrArGrGrUrUrU
mir-10-5p	rArCrCrCrUrGrUrArGrArUrCrCrGrArArUrUrUrGrUrU

2.12 Cloning, mutagenesis, and transfection

The *mir-283* or *mir-277* hairpin was cloned from *loqs* mutant genomic DNA and inserted into the 3' UTR of UAS-DsRed (Stark et al., 2003). Dicing site mutants were generated by site-directed mutagenesis using primers listed in Table 2.6, followed by DpnI (NEB) digestion. The *mir-92b*, *mir-263a*, or *mir-283* genomic fragments were individually cloned into a plasmid driven by a copper sulphate inducible metallothionein promoter. 5'A-to-U, or 5'A-to-C, or 5'A-to-G mutants were generated by site-directed mutagenesis using primers listed in Table 2.6, followed by DpnI (NEB) digestion. Sequence mutants disrupting particular motifs of miR-3 were generated in a similar manner, using primers listed in Table 2.6.

Table 2.6. Oligos used for cloning and site-directed mutagenesis.

Oligo name	Sequence
NotI_dme_mir283_251F	AGAGGCGGCCGCTGGGAGCGAGAGAGA GAGAG
XhoI_dme_mir283_721R	AGAGCTCGAGCGGTGAGTACTTCCGTT TCG
mir-283-UtoC_F	TAAATATGAAACACcCGGAATTTTCAGTT GGTATCGAC
mir-283-UtoC_R	CAACTGAAATTCCGgGTGTTTCATATTTA GGCTTAGCT
mir-283_GtoA_F	CAGCTGGTAATTCTGAGAGCTAAGCCTA AATATG
mir-283_GtoA_R	CAGCTGGTAATTCTGAGAGCTAAGCCTA AATATG
mir283_uppermut3_F	AGCTAAGCCTAAATATGAAACACTCTAT ATTTTCAGTTGGTATCGACTTTTTTTGAATT

mir283_uppermut3_R	TGTTTCATATTTAGGCTTAGCTCCCTATA TTACCAGCTGATATTTACCTTTGA
mir277_CtoU_F	GTCAGGAGTGCATTTGTACTGAAACTAT CTGAAG
mir277_CtoU_R	CTTCAGATAGTTTCAGTACAAATGCACT CCTGAC
EcoRI_dme_mir283c_F	AGAGGAATTCCACGTAACATACATATTCC CAC
Sall_dme_mir283c_R	AGAGGTGACCACTGACCACATAGCCA ATG
mir92b_genespecificF	GATGACGATGACAAGGAATTCCAGCTT CTCGCATTTGGGAG
mir92b_genespecificR	CTTGCATGCCTGCAGGTCGACCGCACC ACATTATGGCCATG
mir263a_genespecificF	GATGACGATGACAAGGAATTCGCTGAC GGATTCATGAACTC
mir263a_genespecificR	CTTGCATGCCTGCAGGTCGACCTTCAAT TCTCTTCTCAGC
dme_mir283_5pAtoU_F	CGATTCTCAAAGGTTAATATCAGCTGGT AATTCTGGGAGC
dme_mir283_5pAtoU_R	CCAGAATTACCAGCTGATATTAACCTTT GAGAATCGTGTGAGG
mir92b_5pAtoU_F	CATTTTCGAAATACATATTGCACTAGTC CCGGCCTGCAATG
mir92b_5pAtoU_R	CATTGCAGGCCGGGACTAGTGCAATAT GTATTTGAAAATG
mir263a_5pAtoU_F	GGTATATAGATCTCGGCACAGTTTATGG CACTGGAAGAATTC
mir263a_5pAtoU_R	GAATTCTTCCAGTGCCATAAACTGTGCC GAGATCTATATACC
mir92b_5pAtoC_F	CATTTTCGAAATACACATTGCACTAGTC CCGGCCTGCAATG
mir92b_5pAtoC_R	CATTGCAGGCCGGGACTAGTGCAATGT GTATTTGAAAATG
mir92b_5pAtoG_F	CATTTTCGAAATACAGATTGCACTAGTC CCGGCCTGCAATG
mir92b_5pAtoG_R	CATTGCAGGCCGGGACTAGTGCAATCT GTATTTGAAAATG
mir263a_5pAtoC_F	GGTATATAGATCTCGGCACAGTTCATGG CACTGGAAGAATTC
mir263a_5pAtoC_R	GAATTCTTCCAGTGCCATGAACTGTGCC GAGATCTATATACC
mir263a_5pAtoG_F	GGTATATAGATCTCGGCACAGTTGATG GCACTGGAAGAATTC
mir263a_5pAtoG_R	GAATTCTTCCAGTGCCATCAACTGTGCC GAGATCTATATACC
dme_mir283_5pAtoC_F	CGATTCTCAAAGGTCAATATCAGCTGGT AATTCTGGGAGC
dme_mir283_5pAtoC_R	CCAGAATTACCAGCTGATATTGACCTTT

	GAGAATCGTGTGAGG
dme_mir283_5pAtoG_F	CGATTCTCAAAGGTGAATATCAGCTGGT AATTCTGGGAGC
dme_mir283_5pAtoG_R	CCAGAATTACCAGCTGATATTCACCTTT GAGAATCGTGTGAGG
mir3_seedmut1_F	TTTCAATCTCACATTTGTGGGCAAAGTG TGTCTCAAGATC
mir3_seedmut1_R	TGTGAGATTGAAACATGTGTGCACAAGA TGCATCCCAGGATC
mir3_seedmut2_F	TTTCAATCTCACATCACACGTCAAAGTG TGTCTCAAGATC
mir3_seedmut2_R	TGTGAGATTGAAACATAACACCCCAAGA TGCATCCCAGGATC
mir3_variant1_F	TTTCAATCTCACATCACTGGGCTTCTAG TGTCTCAAGATC
mir3_variant1_R	TGTGAGATTGAAACATAACTGCACTTTC AGCATCCCAGGATC
mir3_variant2_F	TTTCAATCTCACATCACTGGGCAAAGTG GTAGTCAAGATCCTGGCCAC
mir3_variant2_R	GAAACATAACTGCACAAGATGACAGCC AGGATCGGGACCTTAGGTG

For analysis of dicing site mutants, confluent *loqs* mutant cells were seeded at 1:10 dilution on a 6-well plate and incubated overnight at 25°C. Transfection of 0.4 µg DNA (0.15 µg of miRNA over-expression plasmid, 0.15 µg of miR-312 over-expression plasmid, 0.1 µg of rescue plasmid) was performed using Effectene transfection reagent (Qiagen). A *Loqs*-independent miRNA miR-312 was co-transfected and used as an internal control. Total RNA was extracted after 4 days using Trizol (Invitrogen), and Northern blotting was performed as described above.

2.13 Analysis of miRNA half-lives

S2-R+ cells seeded at a density of 1×10^6 cells/ml on a T75 culture flask were transfected with 5.625 µg of copper sulphate inducible plasmids using Effectene transfection reagent (Qiagen). Cells were washed thrice with fresh medium and plated onto new flasks 24 hours following transfection. 1.5 mM CuSO₄ was added to induce expression or 50 µM bathocuproinedisulfonic acid disodium salt (BCS) was added (as control). After 24 hours of induction, cells were washed twice with fresh medium containing 500 µM BCS and re-suspended in medium

containing 50 μ M BCS. Cells were washed with 1X PBS prior to RNA extraction using Trizol (Invitrogen) at indicated time points after BCS addition. Northern blotting was performed as described above and degradation curves generated by normalization against the control miRNA bantam.

2.14 Purification of mature-RISC

Mature-RISC purification was performed as described previously (Flores-Jasso et al., 2013). 2-6h wild-type CS embryos were collected as described above. Embryos were lysed with lysis buffer (30 mM HEPES-KOH, pH 7.4, 100 mM potassium acetate, 2 mM magnesium acetate, 5 mM DTT, 1X complete mini EDTA free (Roche)) and then cleared by centrifugation. The supernatant was transferred to a new tube and total protein concentration was measured by Bradford assay and was in the range of 20 to 30 mg/ml. 100 μ L of MagnaBind streptavidin beads (Thermo Fisher) was washed with 1X PBS for three times and incubated with 500 pmol of 5' biotinylated, 2'-O-methyl capture oligonucleotides (IDT) with rotation at room temperature for 30 minutes. After coupling, the beads were re-suspended in 100 μ L of embryo lysate and incubated with rotation at room temperature for 30 minutes. After binding, the beads were washed five times with lysis buffer containing 2 mM DTT and 0.01% (v/v) Triton X-100, followed by five times with lysis buffer containing 2 M potassium acetate, DTT, and Triton X-100. Mature RISC was eluted in lysis buffer containing 1 M potassium acetate, DTT, Triton X-100, and 1 nmol competitor oligo, and incubated for 2 hours at room temperature. RNA from input and supernatant samples were extracted by Trizol. RNA was extracted from eluate with 0.4 M NaCl and Phenol/Chloroform/Isoamyl alcohol (Sigma), and the aqueous phase was ethanol precipitated. Northern blotting was performed as described above. Modified biotin-tagged RNA oligos and competitor DNA oligos used are listed in Table 2.7.

Table 2.7. Modified antisense RNA oligos and competitor oligos used for mature RISC purification.

Oligo name	Sequence
mir286_2O_seednsupp	/5Biosg/mUmCmUmGmAmCmAmAmCmG mUmUmGmUmGmUmAmAmCmCmUmCm UmAmGmUmCmCmAmUmCmU
mir286_2O_perfect	/5Biosg/mUmCmUmGmAmAmGmCmAmC mGmAmGmUmGmUmUmCmGmGmUmCm UmAmGmUmCmAmAmUmCmU
mir286_2O_scrambled	/5Biosg/mUmCmUmGmGmUmCmAmGmU mAmUmGmAmCmCmGmGmUmAmCmGm CmUmUmAmGmAmAmUmCmU
mir286_DNAcompetitor	AGATGGACTAGAGGTTACACAACGTTGT CAGA
mir286_perf_comp	AGATTGACTAGACCGAACACTCGTGCTT CAGA
mir286_scramb_comp	AGATTCTAAGCGTACCGGTCATACTGAC CAGA

3. Results

3.1 Role of Loqs in pre-miRNA processing

Previous studies on Loqs function were performed using partial loss-of-function *loqs*^{KO} flies (Forstemann et al., 2005, Jiang et al., 2005, Liu et al., 2007), where residual maternally deposited Loqs may mask some of its functions. Here, the function of Loqs was systematically studied in the complete absence of maternal and zygotic Loqs protein by using a cell line established from *loqs*-null embryos. The underlying structure feature defining Loqs-dependence of selected miRNAs was further elucidated through a combination of computational methods and experimental validation.

3.1.1 Establishment of stable cell lines from *loqs* mutant cells

The Simcox lab generated the *loqs* mutant cell line from *loqs*-null mutant embryos by using oncogenic *Ras*^{V12}, where it is constitutively activated and promotes proliferation of *Drosophila* primary culture cells (Simcox et al., 2008a, Simcox et al., 2008b). Cell lines stably transfected with FLAG-tagged EGFP or Loqs-PB plasmids driven by a copper sulphate inducible metallothionein promoter were subsequently generated. This allowed expression to be conditionally induced upon copper sulphate addition. The stable expression of EGFP and Loqs-PB were verified by Western blotting (Figure 3.1A). Western blotting was performed to detect EGFP (~30 kDa) or Loqs-PB protein (~50 kDa) by using anti-FLAG (upper panel) or anti-Loqs antibodies (lower panel), respectively. The anti-Loqs antibody was generated against full-length Loqs, and is capable of detecting various Loqs protein isoforms (Miyoshi et al., 2009). It is of note that gene expression under the metallothionein promoter is leaky, since there was still detection of the proteins even when the inducer was not added (Figure 3.1A, CuSO₄ “-” lanes).

The level of AGO proteins in cells is influenced by miRNA abundance (Martinez and Gregory, 2013, Smibert et al., 2013). Consequently, a deficiency in miRNA processing factors (thus a decrease in mature miRNA levels) can result in reduction of AGO protein levels. It may also be possible that the loss of Loqs function is compensated by the up-regulation of other miRNA processing factors. Quantitative RT-qPCR was performed to check the levels of miRNA processing factors, such as AGO1 and Dcr-1. The results indicated that AGO1 and Dcr-1 levels were similar between EGFP and Loqs-PB cells (Figure 3.1B), indicating that levels of miRNA processing factors were not dramatically affected by the loss of Loqs function.

It has been reported that the loss of miRNA processing factors, such as Drosha and Dicer, can slow down the growth of *in vitro* mammalian cell cultures (Hackl et al., 2014, Kim et al., 2016). In order to test if cell division was affected in the *loqs* mutant cells, growth rates of EGFP and Loqs-PB cells were monitored for five days. Both cell lines displayed similar growth rates (Figure 3.1C), suggesting that loss of Loqs-PB function did not negatively affect cell growth.

Figure 3.1. Utility of stably transfected *loqs* mutant cells.

(A) Western blotting of stably transfected *loqs* mutant cells expressing FLAG-tagged EGFP or Loqs-PB. Protein samples were prepared from cell line stably transfected with EGFP or Loqs-PB, either with or without CuSO₄ induction. Samples were separated on denaturing SDS-PAGE. EGFP (~30 kDa) and Loqs-PB (~50 kDa) were detected using anti-FLAG antibody (top panel). Leaky expression of both proteins can be detected with anti-FLAG, even in the absence of the copper sulphate inducer. Loqs-PB was detected with anti-Loqs antibody (bottom panel). Anti-tubulin antibody was used as a loading control.

(B) Levels of AGO1 and Dcr-1 remain unchanged in *loqs* mutant cells with or without Loqs-PB expression. Total RNA was extracted from stably transfected EGFP or Loqs-PB cells (with copper sulphate induction) and qRT-PCR was performed to detect AGO1 and Dcr-1. Expression values were normalized against the housekeeping gene *rp49* and expressed relative to EGFP control. Levels of AGO1 and Dcr-1 were not significantly different between EGFP control and Loqs-PB rescue cells (AGO1: N.S: p=0.229, n=3; Dcr-1: N.S: p=0.465, n=3).

(C) Growth curves of *loqs* mutant cells with or without Loqs-PB rescue. Cells were grown under standard conditions and cell growth was monitored for five days. Cell counts were normalized to that on day one. Normalized cell count was plotted on a log scale. Loss of Loqs function did not have any effect on cell doubling time.

3.1.2 Deep sequencing of small RNA libraries and genome-wide analysis of Loqs-dependence

Previous studies performed using *loqs*^{KO} flies reported Loqs-dependence of only a subset of miRNAs (Fukunaga et al., 2012, Liu et al., 2007). In order to examine Loqs-dependence on a genome-wide scale, small RNA libraries were generated from *loqs* mutant cells stably transfected with EGFP or Loqs-PB plasmids (with or without copper sulphate induction). The library data was independently validated by Northern blotting analysis (Figure 3.2). Previous studies using *loqs*^{KO} flies showed that Loqs-PB alters the cleavage site of pre-miR-307a. Without Loqs-PB, the major mature species is 21 nucleotides, while presence of Loqs-PB generates the canonical 23 nucleotide mature miR-307a (Fukunaga et al., 2012). Since miR-307a resides in the 3p arm of its pre-miRNA hairpin, the 5' end of the mature strand is defined by Dcr-1 cleavage. The miR-307a 21-mer and 23-mer differ at their 5' ends, resulting in a shift in seed sequences and thus distinct target specificities (Fukunaga et al., 2012). Figure 3.2 shows the read densities of *mir-307a*, *mir-305*, and *mir-283* from EGFP and Loqs-PB small RNA libraries. There was a shift in the 5' end of *mir-307a* in EGFP control libraries compared to Loqs-PB rescue libraries. There was also an increase in read densities for *mir-305* and *mir-283*, suggesting that production of these two miRNAs are strictly Loqs-dependent. In order to validate the library data, cells were transiently transfected with *mir-307a*, along with EGFP or isoform-specific *loqs* rescue plasmids, and analyzed by Northern blotting to examine if the expected cleavage products were produced. Figure 3.2 shows that with Loqs-PB rescue, the major mature miR-307a species was 23 nucleotides, as expected. An enhanced production of miR-305 and miR-283 mature miRNAs upon Loqs-PB rescue was also observed (Figure 3.2).

Figure 3.2. Library data is highly consistent with Northern blotting data.

Read densities of *mir-307a*, *mir-305* and *mir-283* from EGFP and Loqs-PB small RNA libraries with or without copper sulphate induction are shown. Even though pMT genes were under control of a metallothionein promoter that required gene induction by the addition of copper sulphate, similar effects were observed in both “-” and “+” CuSO₄ libraries. The 5' end of *mir-307a* was shifted in EGFP libraries compared to Loqs-PB libraries. Production of *mir-305* and *mir-283* was strongly enhanced with Loqs-PB rescue, as seen in the increase in read densities. Overexpression of plasmids encoding respective miRNAs along with EGFP control or isoform-specific *loqs* rescue plasmids showed that Loqs-PB expression in *loqs* mutant cells produced the expected cleavage products of mature miR-307a and enhanced production of miR-305 and miR-283 mature miRNAs.

Northern blotting was further performed on an additional seven randomly chosen miRNA genes to validate the library data, including *mir-1*, *let-7*, *mirtron mir-1003*, among others. Northern blotting results were found to be highly consistent with small RNA library analysis (Figure 3.3). The processing of some miRNAs, such as miR-1003 and miR-989, were strongly enhanced by Loqs-PB overexpression in a *loqs*

mutant background. Other miRNAs, such as miR-79 and miR-125, were similarly well-processed regardless of the presence of Loqs.

Figure 3.3. Validation of small RNA library data by Northern blotting analysis of an additional seven miRNA genes.

Plasmids encoding for selected miRNAs were overexpressed along with EGFP control or isoform-specific *loqs* rescue plasmids. Total RNA was analyzed by Northern blotting. Bar charts show read counts after normalization against spike-in sequences in stably transfected EGFP (blue) or Loqs-PB (red) cells. Overall, library data was highly consistent with Northern blotting results.

Clustered miRNAs are believed to be co-transcribed as a single polycistronic transcription unit. The *mir-310* cluster encodes four individual miRNAs (*mir-310*, *-311*, *-312*, and *-313*). Even though they are co-transcribed, miRNAs of this cluster showed different sensitivities to Loqs-PB for processing, reflected in both the library data and verified by Northern blotting (Figure 3.4). MiR-311 is Loqs-dependent, where there is an up-regulation of mature species only when Loqs-PB is overexpressed. MiR-313 is more mildly sensitive to Loqs-PB overexpression. MiR-310 and -312, on the other hand, are Loqs-independent. The varied responses of clustered miRNAs in control and rescue cells supported the notion that differential Loqs-PB sensitivities stemmed from differential dicing efficiency.

Figure 3.4. Clustered miRNAs exhibit distinct responses to Loqs-PB rescue.

Library data (upper panel) showed that individual miRNAs within the *mir-310* cluster varied in responses to Loqs-PB rescue (green: mature; yellow: star). Comparison between pMT-EGFP and pMT-Loqs-PB tracks is indicative of differential Loqs-dependence. Individual miRNA genes from this cluster were co-expressed along with EGFP or isoform-specific *loqs* rescue plasmids. Total RNA was analyzed by Northern blotting (lower panel). Consistent with the library data, miR-310 and miR-312 were Loqs-independent whereas miR-311 and miR-313 were Loqs-dependent.

Expression levels of individual miRNAs were analyzed next. Expression of 55 out of 129 miRNA genes (40%) was up-regulated upon Loqs-PB rescue (Figure 3.5). Expression levels of a majority of miRNA genes were unchanged, and few genes showed a down-regulation in expression levels with Loqs-PB rescue (analysis was performed by Alvin Ng, Greg Tucker-Kellogg's lab, NUS). This confirmed previous results from other groups showing that loss of Loqs function does not dramatically affect the production of majority of miRNA genes (Liu et al., 2007, Fukunaga et al., 2012).

Figure 3.5. A subset of miRNA genes is Loqs-dependent.

MiRNA genes were classified into three groups (“no reads”, “detected”, and “expressed”) based on read counts from the small RNA libraries. Based on the ratio of read counts in Loqs-PB and EGFP libraries, the 129 genes that met the expression cutoff were further divided into three categories (“increased”, “unchanged”, and “decreased”). A total of 55 out of 129 (~40%) genes showed up-regulation upon Loqs-PB overexpression; few miRNA genes showed down-regulation, consistent with the known roles of Loqs-PB in enhancing miRNA processing.

3.1.3 Loqs is dispensable for miRNA duplex loading

In flies, loading of siRNAs onto effector AGO2 complexes requires Dcr-2 and its dsRNA binding protein R2D2 (Liu et al., 2003, Tomari et al., 2004, Liu et al., 2006). A previous study suggested an indirect role for Dcr-1 in miRNA loading since *dcr-1* null mutant eggs exhibited a RISC assembly defect which could not be rescued by recombinant Dcr-1 (Liu

et al., 2007). Later, depletion of >95% of endogenous Dcr-1 in S2 cells was shown to have no effect Ago1-RISC assembly, suggesting that Dcr-1 is dispensable for duplex loading (Kawamata et al., 2009). However, requirement for partner proteins of mammalian Dicer or fly Dcr-1 in miRNA loading is less clear. TRBP and PACT bind to mammalian Dicer to modulate its activity in a non-redundant manner (Lee et al., 2013, Wilson et al., 2015). However, the role of these proteins in small RNA loading has remained controversial (Daniels and Gatignol, 2012, Betancur and Tomari, 2012).

Using synthetic *mir-10* duplexes, the requirement for Loqs-PB in duplex loading was directly tested by using cytoplasmic lysates from *loqs* mutant cells stably transfected with EGFP or Loqs-PB (Figure 3.6A). The *mir-10* duplex was chosen because accumulation of the 5p and 3p species is highly symmetric (Okamura et al., 2008b), and thus any potential shift could be detected more easily in the loading assays. The AGO1 complex was purified using anti-AGO1 antibody, and co-precipitated small RNAs were analyzed on a native gel, which can separate both duplexes and single-stranded RNA species. Unwinding of miRNA duplexes occurs within the AGO complex, after successful loading (Kawamata et al., 2009). Therefore, in order to examine loading efficiency, the focus was placed on unwound single-stranded species (Figure 3.6B). As a control, the same experiment was performed using synthetic single-stranded *mir-34* loop (Figure 3.6C). The loading of these single-stranded loop species onto AGO1 allowed estimation of the amount of AGO protein available for loading (Okamura et al., 2013). After normalization against *mir-34* loading, no significant difference was detected in the loading efficiencies of miR-10-5p and miR-10-3p in EGFP control and Loqs-PB rescue cells (Figure 3.6D), suggesting that Loqs-PB is dispensable for miRNA duplex loading.

Here, the data demonstrated that Loqs is not required for loading of miRNAs onto AGO1, but it was of interest to check whether miRNAs are mis-sorted to AGO2 in the absence of Loqs. In *Drosophila*, small RNA

duplexes are sorted either to AGO1 or AGO2 according to their intrinsic structures. Duplexes with a central unpaired region are preferentially AGO1-loaded, whereas those duplexes with strong double-stranded character are AGO2-loaded (Tomari et al., 2007). The structure of *mir-277* duplex resembles that of a siRNA with a paired central region, and can be sorted to both AGO1 and AGO2 (Forstemann et al., 2007). AGO2-loaded small RNAs are 2'-O-methyl modified by Hen1 at their 3' termini, thus AGO1 and AGO2-loaded species can be distinguished by their distinct gel mobility after periodate oxidation and β -elimination treatment (Horwich et al., 2007). Total RNA was extracted from EGFP or Loqs-PB expressing cells and subjected to oxidation and β -elimination. AGO1-loaded species are sensitive to β -elimination and migrate one nucleotide faster on denaturing gels, whereas AGO2-loaded species are resistant to β -elimination treatment. The membrane was probed for AGO1-enriched (Bantam), intermediate (*miR-277*), or AGO2-enriched (*hp-CG4068B*) small RNAs and no difference was observed in the sorting between EGFP and Loqs-PB cells, suggesting that miRNA sorting is normal in a *loqs*-null background (Figure 3.6E).

Figure 3.6. Loqs is dispensable for miRNA duplex loading.

(A) Experimental workflow for *in vitro* loading of synthetic *mir-10* duplexes. ³²P-labeled synthetic *mir-10* duplex was incubated with cell lysates prepared from *loqs* mutant cells stably transfected with EGFP or Loqs-PB. The RISC complex was precipitated using anti-AGO1 antibody and co-precipitated small RNAs were analyzed on a native gel.

(B) *In vitro* loading of *mir-10* duplex. After incubation of labeled duplexes in EGFP or Loqs-PB cytoplasmic extracts, the effector complex was precipitated with anti-AGO1 antibody and small RNAs (duplexes and single-stranded unwound RNAs) separated on a native PAGE.

(C) *In vitro* loading of single-stranded *mir-34* loop, serving as a control for data normalization.

(D) Normalized loading efficiencies of miR-10-5p and miR-10-3p in EGFP or Loqs-PB cells showed no significant difference between EGFP

and Loqs-PB cells ($p > 0.15$), suggesting that miRNA duplex loading onto AGO1 occurs independently of Loqs-PB.

(E) Total RNA extracted from EGFP or Loqs-PB cells were subjected to oxidation followed by β -elimination. The AGO1-loaded miRNA bantam is sensitive to β -elimination, migrating one nucleotide faster on the gel. MiR-277 partitions to both AGO1 and AGO2, thus both β -elimination resistant and sensitive species could be detected by Northern blotting. The AGO2-loaded siRNA hp-CG4068B is resistant to β -elimination treatment. These expected migration patterns of small RNAs demonstrate that sorting to the two AGO proteins is unaffected in the absence of Loqs.

3.1.4 Unstable base pairing at Dicer-1 cleavage site defines Loqs-dependent miRNAs

The genome-wide analysis revealed that processing of majority of miRNAs is Loqs-independent, whereas a subset of miRNAs (e.g. miR-283) is strictly dependent on Loqs-PB for their accumulation. The project next sought to identify structural elements present in miRNA hairpins that may contribute to the observed Loqs-dependency.

For this analysis, the first 5' nucleotide on the miRNA 3p arm was defined as position 1, and all other nucleotides on the 3p arm were numbered accordingly (Figure 3.7A). MiRNAs were grouped based on the base-pairing status in two-nucleotide windows. If one of the two nucleotides in the window was unpaired, it was grouped under the “mismatch/GU” category. If both nucleotides in the window were paired, it was grouped under the “paired” category. This analysis showed that only the unpaired group at position “-1/0” had an effect on processing efficiency (Figure 3.7B, underlined in red). This position coincided with the Dcr-1 cleavage site, and suggested that an unstable base-pairing at this position is a potentially important feature that distinguishes Loqs-dependent and Loqs-independent miRNA hairpins (analysis performed by Alvin Ng, Greg Tucker-Kellogg's lab, NUS).

Figure 3.7. Global analysis of base-pairing status.

(A) A standard miRNA hairpin is shown. The first 5' nucleotide of the 3p species was numbered as position 1, and all other nucleotides numbered accordingly as shown. The staggered line represents Dcr-1 cleavage site.

(B) MiRNAs were grouped in each two-nucleotide window based on their base-pairing status ("mismatch/G:U pair" or "paired"). The ratio of read counts in Loqs-PB/EGFP libraries for each group was plotted. MiRNAs with an unpaired base-pairing at the -1/0 position were more Loqs-dependent (underlined in red). Base-pairings at all other positions did not affect Loqs-dependency.

According to the library data, *mir-283* showed one of the strictest dependencies on Loqs-PB for processing, and this was selected as the model miRNA to test the hypothesis. The *mir-283* wild type hairpin contains a G:U wobble pair at the Dcr-1 cleavage site, and this was thought to contribute to Loqs-dependency. In order to test the hypothesis, the G:U pair was mutated to a G:C canonical base pair (Figure 3.8A). In the wild-type, Northern blotting analysis showed that mature miR-283 was only produced when Loqs-PB was overexpressed. In miR-283 U-to-C mutant, mature miR-283 can also be produced in the absence of Loqs-PB (GFP lane) (Figure 3.8A). However, it is of note that the mutant was not completely Loqs-independent, suggesting that other features may also contribute to Loqs-dependency.

It was possible that this change in Loqs-dependency is sequence-specific instead of structure-specific. In order to demonstrate that unstable base-pairings in general, instead of specific sequences, contribute to Loqs-dependency, a double mutant was generated that

reverts the G:C canonical pair to an A:C mismatch (Figure 3.8B, U-to-C, G-to-A mutant). This mutation restored Loqs-dependency, suggesting that the contribution of unstable base-pairing was structure, and not sequence specific (Figure 3.8B). Furthermore, a mutation changing the unstable G:U pair to a canonical pair at a position located in the miRNA stem, and not at the Dcr-1 cleavage site, had no effect on processing (Figure 3.8B, mutant A), suggesting that the contribution of unstable base-pairing to Loqs-dependency is position-specific and only occurs at the Dcr-1 cleavage site.

Figure 3.8. Unstable base-pairing at the Dcr-1 cleavage site is a feature of Loqs-dependent miRNAs.

(A) Structures of *mir-283* wild-type and U-to-C mutant are shown (green: mature strand; yellow: star strand). *Mir-283* wild-type has a G:U pair at the Dcr-1 cleavage site (highlighted in red). This was mutated to a G:C canonical pair in the U-to-C mutant (highlighted in red). *UAS-mir-283* wild-type or U-to-C mutant plasmid was over-expressed along with EGFP or isoform-specific *loqs* rescue plasmids and analyzed by Northern blotting. *UAS-mir-312* (a known Loqs-independent hairpin) was co-transfected as an internal control to account for variations in transfection efficiency. Mutation from G:U base-pairing to G:C canonical base-pairing decreased Loqs-dependency.

(B) Analysis of two additional *mir-283* mutants further supports the hypothesis that unstable base-pairing at Dcr-1 cleavage site determines Loqs-dependency. Structures of wild-type *mir-283*, U-to-C, G-to-A mutant, and mutant A are shown (green: mature strand; yellow: star strand). The U-to-C, G-to-A mutant reverted Loqs-dependency of *mir-*

(C) Quantification of data in (A). Asterisks represent a significant difference with respective p values indicated (n=3, except R2D2 n=2).
(D) Quantification of data in (B). Asterisks represent a significant difference with respective p values indicated (n=3).

A

mir-277 hairpin (wild-type)

5' 3'

mir-277 hairpin (C-to-U mutant)

5' 3'

B

UAS-*mir-277*

wild-type C-to-U mutant

Rescue construct: GFP Loqs-PB GFP Loqs-PB

75

30

20

miR-277

miR-312

2S rRNA

C

UAS-*mir-277*

■ wild-type ■ C-to-U mutant

mature miR-277 level (% relative to Loqs-PB rescue cells)

*** p<0.001

Rescue: GFP Loqs-PB

Rescue	wild-type	C-to-U mutant
GFP	~82	~22
Loqs-PB	100	100

(A) Structures of *mir-277* wild-type and C-to-U mutant are shown (green: mature strand; yellow: star strand). *Mir-277* wild-type has a C:G pair at the Dcr-1 cleavage site. This was mutated to a G:U unstable pair in the

C-to-U mutant. Base-pairings at the Dcr-1 cleavage site are highlighted in red.

(B) Northern blotting analysis showed increased Loqs-dependence in the C-to-U mutant (seen in the reduction of signal intensity in GFP lane of the mutant).

(C) Quantification of data in (B). Asterisk represents a significant difference with respective p value indicated (n=3).

In summary, the genome-wide analysis revealed that approximately 40% of fly miRNAs require Loqs for efficient processing. Furthermore, the data showed that Loqs-dependent miRNAs share a common structural feature with unstable base-pairings at the Dcr-1 cleavage site.

3.2 Role for miRNA stability in gene regulation during *Drosophila* embryogenesis

MiRNAs provide an important layer of post-transcriptional regulation during fly embryogenesis. Steady-state optimal miRNAs levels are determined by both its production and degradation. Owing to the extremely stable nature of miRNA molecules and the lack of experimental approaches to analyze global miRNA stability, miRNA turnover in living organisms is currently poorly understood. During fly embryogenesis, miRNA synthesis and degradation have to be precisely controlled in order to support rapid development of the embryo in 24 hours. Here, *Drosophila* embryogenesis was used as a model to study mature miRNA stability.

3.2.1 Identity of terminal 5' nucleotide influences mature miRNA stability

In order to examine expression patterns during fly embryogenesis, kinetics of miRNA production and degradation were profiled on a genome-wide scale by analyzing mature miRNA expression and miRNA gene transcription levels using newly generated small RNA libraries and published modENCODE total RNA-seq libraries (Westholm et al., 2014) from a series of developmentally staged embryos (analysis performed

by Zhou Li). Total RNA-seq analysis provided an insight into transcription levels of pri-miRNAs that is representative of miRNA production rates. The small RNA library analysis allowed an approximation of miRNA abundance, relying on the assumption that miRNA degradation is proportional to miRNA abundance. Using an integrated analysis of pri-miRNA and mature miRNA levels, a model was generated to estimate miRNA degradation rates based on transcriptional activity and changes in mature miRNA levels (analysis performed by Zhou Li).

The 5' nucleotide of mature miRNAs determines strand selection by AGO proteins, with fly AGO1 displaying a preference for those with 5'U (Okamura et al., 2009). This bias for 5'U is supported by structural work demonstrating that UMP binds to the MID domain of AGO with a high affinity that is crucial for subsequent target silencing (Frank et al., 2010). However, it is unclear if the identity of the 5' nucleotide also influences mature miRNA stability. Based on the prediction model used to estimate miRNA degradation rates, the relative stabilities of miRNAs bearing 5'U and non-5'U were compared by looking at the coefficients of mature miRNA abundance (analysis performed by Zhou Li). A higher coefficient value suggests that the miRNA is stable, and vice versa. The computational analysis revealed that miRNAs with 5'U were generally associated with higher mature miRNA abundance ($p=0.01$, one-tailed Kolmogorov-Smirnov test), suggesting that these miRNAs were more stable than those with non-5'U (Figure 3.10A).

In order to experimentally verify the direct role of 5' nucleotides in determining miRNA stability, miRNA hairpin mutagenesis candidates were selected based on several criteria. Firstly, their endogenous expression in S2-R+ cells should be low, so as to sufficiently over-express these miRNAs and monitor their degradation without interference from endogenous signals. The mature miRNAs should also derive from highly asymmetrically loaded duplexes so that the experiment would measure degradation rates of single-stranded species

present in the mature AGO complex. In the miRNA duplex, the strand with the less stably paired 5' end is selected as the mature miRNA, and the other strand is discarded (Khvorova et al., 2003, Schwarz et al., 2003). Due to the importance of the 5' nucleotide in strand selection, it is also crucial that any mutation would not have an effect on the structure and subsequent strand selection.

With these selection guidelines, *mir-283* was chosen as a model hairpin to test the hypothesis in a cell culture system (Figure 3.10B). The wild-type or 5'A-to-5'U mutant plasmid was over-expressed in S2-R+ cells and expression was induced with the addition of copper sulphate. A known copper chelator, known as bathocuproinedisulfonic acid disodium salt (BCS), was used to inhibit transcription (Djuranovic et al., 2012), and the degradation rates were monitored over a 48-hour period by Northern blotting (Figure 3.10C). The signal intensities from three independent biological replicates were quantified and degradation curves were plotted on a log scale. The respective half-lives of wild-type and mutant species were calculated based on the slope of the line. Figure 3.10D plots the relative half-lives of miR-283 wild-type and 5'A-to-5'U species after normalization against the endogenous miRNA bantam. There was a slight, but reproducible, stabilization of the 5'A-to-U mutant as compared to the wild-type ($p=0.023$, $N=3$).

MiRNA duplexes are first loaded onto the effector AGO protein, and subsequent unwinding occurs within the AGO complex (Kawamata et al., 2009). Unloaded miRNA duplexes and mature miRNAs loaded onto AGO1 may possess different degradation rates. It was possible that the experiment measured degradation rate from a mixed population of duplexes and single-stranded miRNAs. In order to verify that the degradation rate only reflected those from AGO1-loaded mature single-stranded miRNAs, the membrane was re-probed to detect miR-283 star strand. The results showed no detectable signals at around 21 nucleotides, but detection of the precursor signals (Figure 3.10C),

indicating that the degradation rate observed here originated from the mature miR-283 that is loaded to AGO1.

Figure 3.10. MiRNAs bearing a 5'U are more stable than those with non-5'U.

(A) Comparison of predicted stabilities of miRNAs carrying 5'U and those with other 5' nucleotides (C, G, or A). The y-axis shows coefficients of mature miRNA abundance that was estimated based on miRNA transcriptional activity and changes in the mature miRNA level. Higher and lower values denote stable and unstable miRNAs, respectively. MiRNAs carrying 5'U showed higher values, and were predicted to degrade more slowly ($p=0.01$, one-tailed Kolmogorov-Smirnov test).

(B) Experimental workflow to monitor degradation of miRNAs.

(C) Degradation of miR-283 wild-type and 5'A-to-5'U mutant was monitored over 48 hours after transcriptional inhibition. Total RNA was analyzed by Northern blotting. Probing for miR-283-5p (mature species) showed a slight, but reproducible, stabilization of the 5'A-to-5'U mutant as compared to the wild-type. There was no detectable signal from miR-

283-3p (star strand), indicating that the degradation rate only reflected those from AGO1-loaded mature single-stranded miR-283. Bantam signals serve as internal loading controls.

(D) Signals from three independent replicates were quantified and normalized against the endogenous miRNA bantam. The half-life of 5'A-to-5'U mutant was expressed relative to the wild-type, showing significant stabilization of the mutant ($p=0.023$, $n=3$).

In order to generalize these findings, another two miRNA hairpins, *mir-92b* and *mir-263a*, were selected for experimental validation. Both miRNAs possess a 5'A (Figure 3.11A), and respective 5'A-to-5'U mutants were generated to monitor their degradation on a timecourse as described above. Representative Northern blotting panels are shown in Figure 3.11B. The relative half-lives of wild-type and 5'A-to-5'U species were calculated and plotted in Figure 3.11C. Similar to that observed with miR-283 (Figure 3.10), mutating the terminal 5' nucleotide to a 5'U resulted in slight but reproducible stabilization for both miRNA hairpins (*mir-92b*: N.S: $p=0.222$, $n=2$; *mir-263a*: N.S: $p=0.089$, $n=2$).

Figure 3.11. The terminal 5'U confers mature miRNA stabilization.

(A) Structures of *mir-92b*, *mir-263a*, and *mir-283* hairpins. Green: mature strand; yellow: star strand; blue: terminal 5' nucleotide.

(B) Representative Northern blotting panels for miR-92b and miR-263a wild-type and 5'A-to-5'U mutants. A slight stabilization of 5'A-to-5'U species was observed for both miRNA hairpins.

(C) Bar charts plotting the relative half-lives of the respective wild-type and 5'A-to-5'U mutants. (*mir-92b*: N.S: $p=0.222$, $n=2$; *mir-263a*: N.S: $p=0.089$, $n=2$).

Using the three miRNA hairpins, additional 5'A-to-5'C and 5'A-to-5'G mutants were generated in order to study the contributions of each of the other non-5'U nucleotides towards mature miRNA stability. Over-expression of *mir-263a* 5'A-to-5'G mutant resulted in an altered Drosha cleavage site and a change in strand selection (Figure 3.12A), therefore this mutant was excluded for further analysis. Representative Northern blotting panels for wild-type and 5'A-to-5'G mutants of miR-92b and miR-283 are shown in Figure 3.12B, and bar charts plotting their relative half-lives are represented in Figure 3.12C. The data showed a slight destabilization effect conferred by a 5'G mutation (*mir-92b*: N.S: $p=0.331$, $n=3$; *mir-283*: N.S: $p=0.288$, $n=3$).

Figure 3.12. 5'A-to-5'G mutations resulted in mild destabilization. (A) Over-expression of *mir-263a* wild-type, 5'A-to-5'C, and 5'A-to-5'G mutants in S2-R+ cells. The 5'A-to-5'G mutant showed a reduction in signal intensity of the 5p mature species, and a corresponding increase in signal intensity of the 3p star species. A difference in pre-miRNA size was also observed, indicating that this mutation resulted in altered Drosha cleavage site. This mutant was excluded for further analysis. (B) Representative Northern blotting panels for *miR-92b* and *miR-283* wild-type and 5'A-to-5'G mutants. There was a slight destabilization of 5'A-to-5'G species compared to the wild-type. (C) Bar charts plotting the relative half-lives of the respective wild-type and 5'A-to-5'G mutants. (*mir-92b*: N.S: $p=0.331$, $n=3$; *mir-283*: N.S: $p=0.288$, $n=3$).

Similarly, 5'A-to-5'C mutants were generated and their degradation monitored over 48 hours after transcriptional inhibition. Representative Northern blotting figures and quantification data are shown in Figure 3.13. Analogous to what was observed with 5'U mutations, the respective 5'C mutants were also stabilized as compared to the wild-type (*mir-92b*: N.S: $p=0.076$, $n=3$; *mir-263a*: N.S: $p=0.220$, $n=3$; *mir-283*: N.S: $p=0.196$, $n=3$).

Figure 3.13. 5'A-to-5'C mutations resulted in stabilization.

(A) Representative Northern blotting panels for miR-92b/-263a/-283 wild-type and 5'A-to-5'C mutants.

(B) Bar charts plotting relative half-lives of the wild-type and mutant species. For all three miRNA hairpins, stabilization of 5'A-to-5'C species was observed (*mir-92b*: N.S: $p=0.076$, $n=3$; *mir-263a*: N.S: $p=0.220$, $n=3$; *mir-283*: N.S: $p=0.196$, $n=3$).

Overall, 5'U and 5'C mutants were stabilized to similar extents, while those bearing a 5'G were the least stable (Figure 3.14). However, due to the minor stabilization or destabilization effects observed with the series of mutagenesis experiments, this study proposed that the 5' nucleotide contributes to mature miRNA stability, with other factors also potentially playing a role.

Figure 3.14. Contribution of the terminal 5' nucleotide to mature miRNA stability.

A series of mutagenesis experiments using three miRNA model hairpins suggested that 5'U and 5'C conferred stabilization, while 5'G resulted in mild destabilization. Student's t-test p-values are shown in the chart.

Fly AGO proteins are known to display a preference for 5' terminal nucleotides, with AGO1 favoring 5'U and AGO2 favoring 5'C respectively (Czech et al., 2009, Ghildiyal et al., 2010). In order to test if 5'C species are indeed sorted to AGO1 and stabilized, or misdirected to AGO2, sequential AGO2/AGO1 immunoprecipitation was performed in S2-R+ cells stably transfected with a FLAG-HA-AGO2 genomic construct (Figure 3.15A), and co-precipitated small RNAs were analyzed by Northern blotting (Okamura et al., 2009). For miR-92b and miR-263a, the ratios of AGO1/AGO2 sorting were not influenced by the 5' nucleotide mutation. A shift in sorting pattern was observed for miR-283, whereby the 5'A-to-5'C mutant showed increased AGO2 sorting. However, there appeared to be no correlation between miRNA half-lives and AGO sorting (Figure 3.15B, C).

Figure 3.15. Analysis of relative AGO2/AGO1 sorting of wild-type and 5'A-to-5'C mutants.

(A) Experimental workflow for purification of AGO2 and AGO1 complexes from S2-R+ cells stably expressing FLAG-HA-AGO2.

(B) AGO2 and AGO1 complexes were sequentially purified by using anti-FLAG and anti-AGO1 antibodies respectively. Northern blotting showed similar loading patterns for both miR-92b/-263a wild-type and 5'A-to-5'C mutants. For miR-283, the 5'A-to-5'C mutant showed increased sorting to AGO2 as compared to the wild-type.

(C) Quantification of signals in (B), expressed as AGO1/AGO2 loading ratios. Signals for miR-92b were over-saturated and could not be accurately quantified.

3.2.2 Differential stabilities of miR-309 cluster miRNAs give rise to uncoordinated expression profiles

Clustered miRNAs typically display coordinated expression profiles (Cullen, 2004). However, expression analysis of miRNA clusters showed that some clusters (e.g. *mir-309* cluster) exhibit uncoordinated expression levels during embryogenesis (analysis performed by Zhou Li). This suggested that transcriptional activity is not the sole determinant of miRNA output, and that additional post-transcriptional regulatory mechanisms exist to modulate steady-state miRNA levels.

The *mir-309* cluster (hereafter referred to as *mir-309c*) is one of the earliest transcribed zygotic genes that plays a role in the degradation of maternally deposited mRNAs to promote maternal-to-zygotic transition in early fly embryos (Bushati et al., 2008, Thomsen et al., 2010). This cluster encodes for eight miRNA genes arranged in tandem: *mir-309*, *mir-3*, *mir-286*, *mir-4*, *mir-5*, *mir-6-1*, *mir-6-2*, and *mir-6-3* (Figure 3.16A). It is transcribed as a longer pri-miRNA transcript that is driven by a common promoter (Biemar et al., 2005). Analysis of total RNA-seq libraries from modENCODE showed that transcription of the *mir-309c* is activated transiently at 2-4h after egg laying (AEL), and no new production could be detected thereafter (Figure 3.16B, analysis performed by Zhou Li). Hence, the rate of decrease in mature miRNA levels would be reflective of degradation rates of individual miRNAs.

Fly embryos were collected from eight time windows over the 24-hour embryogenesis period, in 2-hour time windows between 0-12 h AEL and in 6-hour time windows between 12-24 h AEL. Northern blotting analysis of total RNA extracted from staged embryos revealed rapid degradation of miR-3 and miR-309 within 6 hours AEL (Figure 3.16C). On the other hand, other miRNAs in the cluster, such as miR-6 and miR-286, are highly stable and persist throughout the 24-hour embryogenesis period (Figure 3.16C). Since miRNAs loaded onto AGO1 are protected from degradation (Winter and Diederichs, 2011), it was unclear if the differential stabilities of *mir-309c* members could be attributed to varied AGO1 loading efficiencies. To this end, AGO1 immunoprecipitation was performed and co-precipitated small RNAs were analyzed by Northern blotting. The results showed that all members of the cluster were similarly well loaded onto AGO1 regardless of their differential stabilities (Figure 3.16D, E), and there was no obvious correlation between stability and loading efficiency. This also suggested that the rapid degradation of miR-3 and miR-309 occurs after the formation of miRNA-AGO1 complexes.

Figure 3.16. The *mir-309c* miRNAs exhibit distinct degradation profiles during embryonic development.

(A) The genomic organization of *mir-309c* is shown. This cluster encodes eight miRNA genes located in tandem, and are processed from the same primary transcript into distinct mature miRNAs. MiR-3 and miR-309 share an identical seed sequence, as shown in red.

(B) Levels of *pri-mir-309* during fly embryogenesis inferred from published total RNA-seq libraries deposited in modENCODE.

Transcription of this cluster is transiently activated in a specific time

window after the onset of zygotic transcription, and there is no new synthesis after 4-6 h AEL.

(C) Northern blotting analysis of staged embryos revealed distinct decay rates among *mir-309c* members. MiR-3 and miR-309 are highly unstable, while other members of the same cluster are relatively more stable and persist throughout the 24-hour embryogenesis period. Both left and right panels were from stripping and re-probing of the same membrane and thus share the same 2S rRNA panels.

(D) Using 2-4 h and 4-6 h wild-type embryos, AGO1 complex was purified with anti-AGO1 antibody and the co-precipitated small RNAs were analysed by Northern blotting. The results showed that distinct stabilities of miRNAs in this cluster did not stem from differential loading efficiencies to AGO1.

(E) Quantification of signals in (D), expressed as AGO1/IP ratios and normalized against signals from 2-4 h lysates.

The differential expression profiles of *mir-309c* prompted further investigation, but it is technically more challenging to work with fly embryos. Therefore, it would be ideal if further experiments could be performed in a cell culture system. The next experiment tested if the degradation dynamics of *mir-309c* could be recapitulated in S2-R⁺ cells, a late embryonic cell line. Since this cluster is not endogenously expressed in S2-R⁺ cells, they could be over-expressed at sufficiently higher levels than the background level. In contrast to the degradation patterns observed in embryos, miR-3 appeared to be more stable than miR-309 in S2-R⁺ cells (Figure 3.17A, left panel). In order to test if the disparity reflects a real difference in stabilities in different systems, or that the artificial over-expression of miRNAs in cell lines is an inaccurate reflection of how endogenous miRNAs behave in embryos, the same experiment was performed in Kc167 (early embryonic cell line) and BG3-c2 (larval central nervous system derived) cells. The degradation profile of miR-309c miRNAs in Kc167 cells was similar to that observed with S2-R⁺ cells (Figure 3.17A, middle panel). However, in BG3-c2 cells, degradation rates of miR-3 and miR-309 were more similar to that observed in embryos (Figure 3.17A, right panel). The signal intensities were quantified and represented in Figure 3.17B. This suggested that the degradation of the *mir-309c* miRNAs display potential cell-type

specificity, and the BG3-c2 cell line could potentially serve as a useful tool for the study of destabilization mechanisms. It is also plausible that different nucleases or factors may be present or active in different contexts that plays a role in regulation of *mir-309c* stability.

Figure 3.17. Degradation profiles of *mir-309c* members in various *Drosophila* cell lines.

(A) Degradation was monitored over 96 hours (for S2-R+ and Kc167 cells) or 48 hours (for BG3-c2 cells) after transcriptional inhibition. MiR-3 and miR-309 were used to represent unstable miRNAs, and miR-6 and miR-286 were used to represent stable miRNAs. Note that only signals from mature miRNAs are shown.

(B) The signal intensities were quantified and plotted on a bar chart as shown. Signals from miR-309 in Kc167 and BG3-c2 cells could not be accurately quantified and were omitted from the chart. The relative half-lives of each miRNA in the cluster were normalized against miR-286.

The molecular mechanisms underlying miR-3/-309 destabilization are currently unclear. The following two sections will discuss two approaches to investigate destabilization and present some preliminary

data. Further studies will be required in the future to allow a more thorough understanding of these mechanisms.

3.2.2.1 Intrinsic sequence elements that drive instability

Various destabilizing elements have been characterized in specific unstable miRNAs in certain cell types (Ruegger and Grosshans, 2012), but not much is known otherwise. MiR-3 and miR-309 share an identical seed sequence and high homology within their mature sequences (Figure 3.18A), but it is currently unclear if there are any specific motifs within that promote their rapid degradation. In S2-R⁺ cells, miR-3 is comparatively better processed than miR-309 (Figure 3.17A), therefore for simplicity, mutagenesis experiments were first performed using miR-3 as a template.

In order to identify potential destabilizing elements, four variants that systematically mutate a section of homology shared between miR-3 and miR-309 were designed by swapping nucleotides between the mature and star strand, so as to maintain the hairpin structure. The structures of wild-type and respective mutants are illustrated in Figure 3.18A. The wild-type and mutant constructs were first over-expressed in S2-R⁺ and BG3-c2 cells to check for potential processing defects as a result of the mutations introduced. Mutation of the region in “seed mutant 2” inhibited processing in both cell types, and this mutant was excluded from further analysis (Figure 3.18B).

Figure 3.18. Design of miR-3 sequence mutants.

(A) Structures of *mir-3* wild-type and mutant hairpins. Each mutant was designed by swapping nucleotides between the mature and star strands, so as to preserve the hairpin structure. “Seed mutant 1” and “seed mutant 2” disrupt respective regions within the 7-nt seed region. “Variant

1” and “variant 2” mutants disrupt the AAAGT and TGTC motifs, respectively. Green: mature strand; yellow: star strand; blue: region disrupted in mutant.

(B) *Mir-3* wild-type and mutant constructs were transfected in S2-R+ or BG3-c2 cells, and Northern blotting was performed using the respective probes that were perfectly complementary to the wild-type or mutant sequences. All mutants were efficiently processed into mature species, except for “seed mutant 2”. This mutant was excluded for further analysis.

The degradation rates of wild-type and each of the other three sequence mutants in S2-R+ and BG3-c2 cells were monitored by Northern blotting (Figure 3.19A, C). In both cell types, mutation of the AAAGT motif in the middle of the mature miRNA conferred a stabilization effect (Figure 3.19B, D).

Figure 3.19. Contribution of sequence elements to miR-3 destabilization in S2-R+ and BG3-c2 cells.

(A) Representative Northern blotting panels for miR-3 wild-type and sequence mutants in S2-R+ cells are shown. Bantam signals serve as internal loading controls.

(B) Signal intensities in (A) were quantified and represented in a bar chart as shown. All values were normalized against bantam signals, and expressed relative to the wild-type. MiR-3 “seed mutant 1” and “variant 2 mutant” resulted in a slight destabilization effect, while mutating the region in “variant 1 mutant” had a stabilization effect. Asterisks represent a significant difference with respect to wild-type (n=3).

(C) Representative Northern blotting panels for miR-3 wild-type and sequence mutants in BG3-c2 cells are shown. Bantam signals serve as internal loading controls.

(D) Signal intensities in (B) were quantified and represented in a bar chart as shown. All values are normalized against bantam signals, and expressed relative to the wild-type. Asterisks represent a significant difference with respect to wild-type (n= 3).

3.2.2.2 Factors important for selective degradation

Chemically modified antisense oligoribonucleotides have been shown to inhibit RISC function *in vivo and in vitro* (Hutvagner et al., 2004, Meister et al., 2004). These nuclease-resistant oligoribonucleotides have also been used for sequence-specific purification of mature RISC complexes containing a single specific guide RNA (Hutvagner et al., 2004).

Purification of endogenous RISC complexes from *Drosophila* extracts can be recovered under native conditions by using antisense oligonucleotides that are partially complementary to the guide RNA (Flores-Jasso et al., 2013). Figure 3.20A illustrates a brief schematic of the technique used to isolate miR-3/-309 mature RISC that can be used to identify putative interacting cellular factors by mass spectrometry.

MiR-286 is one of the most abundant miRNAs expressed in early embryos (Ruby et al., 2007), and successful purification of mature miR-286-RISC has been demonstrated previously (Flores-Jasso et al., 2013). Thus, initial establishment of the procedure was performed with miR-286 as a template. Based on previous literature which showed better recovery of active RISC using partial complementary oligos than fully complementary oligos (Wee et al., 2012, Flores-Jasso et al., 2013), three capture oligos were designed for purification: (1) complementary to the seed sequence and 3' supplementary region, (2) perfectly complementary to the mature miRNA sequence, and (3) a scrambled control. Figure 3.20B illustrates design of the capture oligos. Nucleotides that are underlined denote arbitrary bases at the 5' and 3' ends to increase binding and stability of the complex and improve pull-down efficiency (Jannot et al., 2011, Li and Zamore, 2018).

Western blotting to detect AGO1 showed a band at 100 kDa in all samples purified by the three different antisense oligos (Figure 3.20C). However, this was not consistent with results obtained from the concurrent RNA samples that were analyzed by Northern blotting (Figure 3.20D). In Northern blotting, miR-286 was detected for “seed and supplementary” and “scrambled control” purified samples, but not

for “perfect complementary” sample. Therefore, the AGO1 protein detected by Western blotting is likely background and not a real signal.

Figure 3.20. Purification of mature RISC.

(A) Schematic of mature RISC purification. Modified 2'-O-methyl antisense oligonucleotide bearing a 5' biotin tag (“capture oligo”) are captured by streptavidin coupled magnetic beads. The capture oligo can base-pair with mature RISC containing the guide miRNA of interest. Elution is achieved in the excess of a competitor oligonucleotide fully complementary to the capture oligo.

(B) Design of capture oligos used to purify miR-286-RISC.

(C) Western blotting detection of AGO1 protein (100 kDa) suggested the presence of AGO1 in all three samples purified by the three modified antisense oligos, which is not consistent with the Northern blotting result in (D).

(D) Analysis of total RNA by Northern blotting detected miR-286 in “seed and supplementary” and “scrambled control” lanes, but not the “perfectly complementary” lane.

4. Discussion

4.1 Loqs-PB enhances processing of a subset of miRNA genes

The presence of dsRNA-binding partners is a common theme among ribonuclease III enzymes, playing important roles in the modulation of activities of their binding proteins.

Sequence alignment analyses showed that Loqs shares approximately 34% sequence identity with human TRBP and PACT, with greatest similarity in the third dsRBD (Forstemann et al., 2005, Haase et al., 2005, Saito et al., 2005). In contrast, there is no clear human homolog for R2D2, which participates in the fly siRNA pathway. Human Dicer is more similar to fly Dcr-1 than Dcr-2, and recent structural studies showed that the interaction surfaces between human Dicer and fly Dcr-1 with their respective partner proteins are highly similar (Wilson et al., 2015, Jakob et al., 2016). Furthermore, all four human AGO proteins are more closely related to fly AGO1 than fly AGO2 (Leuschner et al., 2005, Tolia and Joshua-Tor, 2007). These similarities between the fly and human systems raise the possibility that findings in the *Drosophila* miRNA pathway can be extended to the mammalian system.

In this study, the function of Loqs was investigated in a *loqs*-null background in the complete absence of maternal and zygotic Loqs protein. Consistent with previous studies (Liu et al., 2007, Fukunaga et al., 2012), Loqs enhanced the processing of only a subset of miRNA genes (~40%). The molecular basis and substrate selectivity of Loqs were previously uncharacterized. Here, the data unraveled the underlying structural feature that defines Loqs-dependent miRNA hairpins. Pre-miRNAs with unstable base-pairing at the Dcr-1 cleavage site may present as sub-optimal substrates for dicing, and thus recruitment of Loqs is essential to enhance their processing efficiencies (Figure 4.1). In this way, Loqs serves to fine-tune the production of mature miRNAs and ensure dicing fidelity.

Figure 4.1. Loqs enhances processing of suboptimal pre-miRNAs. MiRNA hairpins with unstable base-pairings at the dicing site present as suboptimal substrates for Dicer cleavage, and require Loqs-PB as a cofactor for efficient processing.

The fact that one unstable base-pairing at the Dcr-1 cleavage site can result in detectable alteration in Loqs-dependency is an interesting phenomenon. Analogous to Loqs, human TRBP and PACT do not significantly influence global miRNA abundance or loading to AGO, but instead TRBP regulates the accuracy of Dicer cleavage for a subset of miRNAs (Fukunaga et al., 2012, Kim et al., 2014). The generation of a catalytically active Dicer with mutations to its TRBP interface provided further supporting evidence, and noted that strand selection can be altered in cases where the 5' nucleotide is changed (Wilson et al., 2015). A recent study elucidating the structure of two N-terminal dsRBDs of TRBP showed that it ensures fidelity of Dicer cleavage by binding to the stem region of pre-miRNAs (Masliah et al., 2018). However, these TRBP-regulated miRNAs did not appear to share any common sequence elements or secondary structures, and it was unclear how substrate specificity is achieved (Ding and Wang, 2015, Wilson et al., 2015).

In a study that analyzed the effect of different structural features of precursor small RNAs on dicing and silencing rates, the authors showed that dicing rates and silencing efficiency are correlated, and both parameters increase as a function of loop length (Koh et al., 2017).

Furthermore, it was also reported that mismatches in the stem region diminish efficiency of gene silencing without affecting the dicing rate (Koh et al., 2017), which was an interesting observation given that most pre-miRNAs harbour multiple internal mismatches and bulges. One possible explanation is that the mismatches create conformational changes that result in steric hindrance in the AGO binding pocket. A recent report showed that Loqs-PB specifically recognizes and directly binds base-mismatched nucleotides in the pre-miRNA stem to produce shorter or longer isoforms in a context-dependent manner (Zhu et al., 2018). The authors hypothesized that tuning of miRNA length is achieved by a change in the conformation of base-mismatched nucleotides to base-paired ones by Loqs-PB. However, structural evidence supporting this model is currently lacking. Structurally, base-mismatches within pre-miRNAs might confer a degree of flexibility that can influence Dicer processing efficiency. The next interesting point to look at would be from the perspective of Dcr-1/Loqs in complex with pre-miRNA substrate variants, in order to elucidate potential changes in protein conformations during substrate discrimination. Recent resolution of the third dsRBD of Loqs and associated biochemical analysis of a series of mutants (Jakob et al., 2016) have provided the first step towards a better understanding of Loqs function.

In contrast to the respective roles of Loqs-PB and Loqs-PD in modulating miRNA and siRNA processing, the function of Loqs-PA is less clear. Early studies showed that even though both Loqs-PA and -PB isoforms (but not Loqs-PD) can rescue the embryonic lethality of *loqs*^{KO} flies, Loqs-PA is dispensable for the maintenance of female germline stem cells (Park et al., 2007, Fukunaga et al., 2012). Loqs-PA is also incapable of rescuing miRNA processing defects; Northern blotting showed that while transgenic Loqs-PB expression can fully restore miRNA processing defects in *loqs*^{KO} flies, re-introduction of Loqs-PA had no effect on pre-miRNA and mature miRNA levels (Park et al., 2007). Consistent with this, Northern blotting analyses from the present study also showed a strong enhancement in miRNA processing

with Loqs-PB rescue, while the effects seen with Loqs-PA rescue were comparable to that of Loqs-PD rescue. Since Loqs-PD does not rescue the embryonic lethality phenotype observed in a *loqs* mutant background, the effects of Loqs-PA on miRNA processing is insufficient to account for the weak rescue effect. In order to understand the roles of Loqs-PA on small RNA processing on a genome-wide scale, it may be useful to construct and analyze small RNA libraries from Loqs isoform-specific rescue stable cell lines.

4.2 Challenges in measurement of miRNA degradation

Since the discovery of the first miRNA and recognition of the gene-regulating potentials of this new class of small RNAs, a large body of research has been devoted to understanding miRNA biogenesis pathways and mechanisms. One way of regulating the activity of miRNAs is by modulating their abundance through degradation. However, research in this area has been largely overlooked. In particular, analysis of global miRNA stability during normal development by using high-throughput techniques is generally lacking.

MiRNAs are widely perceived to be extremely stable molecules that can persist up to several days. However, this was derived from a small number of studies on specific miRNAs or in certain cell types (van Rooij et al., 2007, Gatfield et al., 2009). The experimental measurement of miRNA degradation is technically challenging, and approaches to study miRNA half-lives, in general, rely on the use of RNA polymerase II inhibitors or the depletion of miRNA processing factors. These methods perturb the normal physiology of cells and are based on an artificial system. Since measurement of degradation events is time-sensitive, a more striking caveat of these methods is notably their failure to capture and differentiate fast and slow decaying species. This is in part due to the long time frame (three days) required to achieve knockdown, and the cellular toxicity arising from usage of transcriptional inhibitors (Marzi et al., 2016). An early study in mammalian cells used a microarray

approach to study global miRNA stability following an 8-hour transcriptional shutoff by actinomycin D, and showed that a vast majority of miRNAs do not show any considerable decrease in expression levels (Bail et al., 2010). It should be noted that complete transcriptional inhibition is often difficult to achieve, and some degree of processing of mature miRNAs from pri- and pre-miRNAs are to be expected. In order to circumvent toxic effects, treatment time using transcriptional inhibitors has to be kept to the shortest effective time. As such, half-life measurements of slow-decaying miRNAs remain a challenge.

Pulse-chase experiments, which minimize cellular stress, have also been used widely to measure protein or mRNA stability. In pulse-chase analysis, proteins or mRNAs are labeled with radioisotopes or ribonucleoside analogs for a short duration (the pulse), which is followed by monitoring the rate of decrease of labeled molecules over time (the chase). A more recent approach based on combined metabolic RNA labeling and high-throughput sequencing was employed to measure global miRNA degradation rates in mammalian cells under physiological conditions (Marzi et al., 2016). In this study, a pulse-chase method was used to measure half-lives of AGO2-loaded mature miRNAs labeled with 4-thiouridine. High-throughput sequencing was performed with labeled RNA purified from several time points spanning 16 hours. An inherent feature of pulse-chase experiments is that new molecules synthesized during the chase period will not be labeled. Therefore, the decay rates of miRNAs that are processed less efficiently cannot be accurately measured, limiting the utility of this experimental set-up in some cases.

MiRNA degradation rates are intrinsically linked to their loading efficiencies. Stabilization of mature miRNAs by human AGO2 has been reported previously (Diederichs and Haber, 2007, Winter and Diederichs, 2011). In the current study, experiments were based on the artificial over-expression of miRNAs that are not endogenously produced in S2-R⁺ cells, so as to facilitate half-life measurements without interference from endogenous species. Regardless, these

exogenously introduced species can be loaded onto AGO1, and failure to detect miRNA star strands in Northern blotting experiments indicated that the degradation rates reflect only those from AGO1-loaded mature single-stranded species. Even though AGO1-free duplexes and AGO1-loaded single-stranded species may possess distinct degradation rates, this has often not been taken into account in half-life measurements from past studies. One study that measured miRNA turnover rates across eight mammalian cell lines reported that majority of miRNAs are stable, and a subset which are unstable; however, the majority of unstable miRNAs detected in the study are in fact miRNA* species (Guo et al., 2015). In another study, Zhang and colleagues identified a uracil-rich element at positions 9-11 that contributes to rapid decay of mammalian miR-29b (Zhang et al., 2011). However, the uracil-rich sequence was found to only impact stability of duplexes but not in single-stranded miR-29b. Since nucleotides at positions 9 and 10 influence sorting (Czech et al., 2009, Okamura et al., 2009), it was proposed that the uracils indirectly influence stability at the loading or unwinding step.

4.3 Outstanding questions on miRNA stability

Although miRNA biogenesis pathways have been intensely studied, open questions remain regarding degradation processes.

General and miRNA-specific degradation machinery are being continually discovered in various organisms. Crystal structures of AGO protein in complex with single-stranded guide RNAs showed that the 5' and 3' ends of miRNAs are embedded within the MID and PAZ domains of AGO respectively (Ma et al., 2004, Frank et al., 2010), suggesting that AGO release has to precede miRNA degradation by exoribonucleases (Park et al., 2017). On the other hand, enzymes such as methyltransferases (HEN1) and miRNA trimming factors (e.g. Nibbler) can act on single-stranded AGO-bound mature miRNAs, raising the possibility for miRNA-degrading enzymes to also gain access to

these AGO-loaded miRNAs to mediate decay. It is currently unclear how miRNAs are unloaded prior to degradation, or how ribonucleases are able to gain access to loaded miRNAs. In cases of targeted degradation of specific miRNAs, it is unclear if they possess unique modification marks to recruit downstream degradation machinery, and the biological consequences associated with this process.

The role of target binding on miRNA stability is also controversial. In *C. elegans*, association with mRNA targets protects and stabilizes miRNA (Chatterjee and Grosshans, 2009, Chatterjee et al., 2011). On the contrary, target binding results in destabilization in fly and human systems. Ameres and colleagues first showed in flies that high complementarity between a miRNA and its target exposes the guide miRNA to tailing and trimming events (Ameres et al., 2010). In humans, highly complementary targets, especially to the 3' end of mature miRNA, destabilize the AGO2-guide RNA complex to promote release of the guide RNA from AGO2 (De et al., 2013). The possibility for miRNA-degrading enzymes to gain access through a similar mechanism remains to be investigated.

Mature miRNAs can reside in multiple subcellular locations in the cytoplasm (such as P bodies, endoplasmic reticulum and mitochondria), and recent studies have also demonstrated the presence of miRNAs in the nucleus (Leung, 2015). Location-specific miRNAs could have specialized functions that are physiologically relevant. With recent advances in single molecule analysis (Pitchiaya et al., 2017), these techniques can be applied to study the trafficking and spatiotemporal distribution of miRNAs. These methods can also potentially be used to study the cellular localization and sequestration of specific miRNAs and the degradation machinery, perhaps offering an explanation for the specificity that governs turnover of specific miRNAs.

4.4 Importance of the terminal 5' nucleotide

A series of mutagenesis experiments was performed here to validate the observation through the genome-wide analysis that miRNAs with 5'U are more stable than those with non-5'U. The data also suggested that those with 5'C are stabilized as compared to 5'A and 5'G. However, it is of note that mutation of the 5' nucleotide only resulted in mild changes in degradation rates as detected by Northern blotting experiments, hence other determinants are also likely to be in play.

Strong preferences for 5'U of mature miRNAs have been consistently observed across plants, nematodes, and flies (5'U or 5'A in humans) (Ghildiyal et al., 2008, Ghildiyal et al., 2010, Hu et al., 2009, Lau et al., 2001, Frank et al., 2010, Mi et al., 2008). Structural studies using mammalian AGO2 revealed preferential base-specific interactions, with AMP and UMP binding to the MID domain of AGO2 with up to 30-fold higher affinity than other nucleoside monophosphates CMP and GMP (Frank et al., 2010). Upon target interaction, the 3' end of the guide strand is released from the PAZ domain of AGO, whereas the 5' end maintains its interaction with the MID domain of AGO (Wang et al., 2009b). In turn, maintaining a high binding affinity between the 5' end of guide miRNA (provided by 5'A or 5'U) and AGO protein becomes especially crucial since this contact persists after target binding to ensure proper target cleavage (Frank et al., 2010). In another report, the MacRae laboratory studied influence of the 5' nucleotide on guide miRNA unloading. In a time course experiment of unloading guide RNAs bearing each of the four nucleotides (U, A, C, or G), it was found that those carrying a 5'U or 5'A slowed dissociation of the guide RNA from human AGO2 by approximately 3-fold (De et al., 2013).

Fly miRNAs bearing a 5'U are known to favour AGO1 loading, and mature miRNAs loaded onto AGO proteins are stabilized and protected from degradation by nucleases (Czech et al., 2009, Okamura et al., 2009, Ameres et al., 2011, Winter and Diederichs, 2011). Preferential

loading of miRNAs with 5'U has been proposed to act as a purifying selection step to select against incorrectly processed species (Seitz et al., 2011). The Tomari laboratory analyzed *in vitro* RISC assembly using fly embryo extracts, and showed that a 5'U provides an additional anchoring interaction to facilitate loading of mismatch-containing miRNA duplexes (Kawamata et al., 2011). This is consistent with the consensus that AGO loading determines miRNA stability. Hence, it may not be unexpected that miRNAs bearing a 5'U possess extended half-lives. On the other hand, out of the ~200 naturally occurring mature miRNAs in *Drosophila*, only four of them possess a 5' terminal G (miRBase). A previous study using recombinant human Dicer showed that Dicer avoids cleaving at sites that produce miRNAs beginning with 5'G (Starega-Roslan et al., 2015). The low occurrence of 5'G mature miRNAs might be justified if this nucleotide confers destabilizing properties, thereby limiting their functionality.

The *Arabidopsis* genome encodes ten AGOs, and the sorting of small RNAs into distinct AGO complexes is largely influenced by the identity of the 5' terminal nucleotide (Mi et al., 2008). Likewise in *Drosophila*, a direct role for the 5' nucleotide identity has also been implicated in sorting between AGO1 and AGO2 (Ghildiyal et al., 2010). Nevertheless, mutation of the terminal 5' nucleotide has not been shown dramatically change AGO1/AGO2 sorting outcomes, therefore nucleotide bias at the 5' end only has a minor effect on sorting (Czech et al., 2009). In line with this, changes in sorting patterns were not consistently observed for 5'C mutants when sequential purification of AGO2/AGO1 complexes was performed in the present study (Figure 3.16).

Overall, the current study proposed that the 5' nucleotide plays a role in mature miRNA stabilization, even though the effects are mild. One study that used cell lysates prepared from brain tumor tissues showed that miRNAs that possess secondary structures are more stable (Belter et al., 2014). However, the conclusion was based on measurement of half-lives of a very small number of miRNAs in a specific microenvironment, therefore it is still unclear if the correlation between stability and

structure is a general rule. Future work should explore other potential determinants of miRNA stability.

4.5 Distinct stabilities of *mir-309* cluster miRNAs act as a post-transcriptional mechanism to regulate miRNA expression

Expression of miRNA genes is subject to transcriptional regulation by transcription factors and epigenetic marks such as DNA methylation and histone modification (Ha and Kim, 2014). Some early studies reported instances of poor correlation between pri-miRNA and mature miRNA abundances, hinting at post-transcriptional regulation of miRNA expression (Obernosterer et al., 2006, Wulczyn et al., 2007).

MiRNA clusters are defined as those residing within 10 kb of each other on the genome. About 30% of *Drosophila* miRNA genes are present in clusters on the genome, and are predicted to co-regulate functionally related target genes (Wang et al., 2016b). Members from the same cluster are typically transcribed as a single unit, and therefore are expected to possess similar expression profiles. Nevertheless, despite originating from a common precursor transcript, biogenesis of clustered miRNAs can be differentially regulated to produce varying levels of mature miRNAs (Truscott et al., 2016, Lee et al., 2002, Conrad et al., 2014, Lataniotis et al., 2017). A previous study that analyzed publicly available small RNA libraries from various fly tissues reported uncoordinated expression profiles of several miRNA clusters (Ryazansky et al., 2011). This suggests a role for post-transcriptional regulatory mechanisms that shape the tissue-specific expression of uncoordinated miRNA clusters. It should be noted that these small RNA libraries were generated by different laboratories, and might account for some of the differences in expression profiles that were observed. In general, post-transcriptional regulation has not been thoroughly investigated, especially during normal development. In the current study, expression profiles of clustered miRNAs were examined over the course

of fly embryonic development in order to better understand the underlying regulatory mechanisms.

Expression of the *mir-309* cluster stems from exclusive zygotic transcription with little maternal contribution (Bushati et al., 2008). Even though individual miRNAs of the cluster are predicted to target many genes involved in embryonic patterning, genetic ablation of the entire cluster in flies resulted in no discernable embryonic patterning defects with only modest reduction in viability (Bushati et al., 2008). Microarray analyses of control and mutant embryos during early embryonic development showed that miRNAs of this cluster mediate clearance of maternal transcripts to promote maternal-to-zygotic transition (Bushati et al., 2008). Mutants lacking both miR-6 and miR-11 (both of which share the same seed sequence) display a strong embryonic lethal phenotype with patterning defects in the embryonic central nervous system, due to their overlapping functions in regulation of pro-apoptotic genes (Ge et al., 2012). However, specific functions of other individual miRNAs of the cluster have not been investigated.

Results in the present study described a role for stability in modulating differential expression profiles of the *mir-309* cluster during embryogenesis. In embryos, miR-3 and miR-309 represent two miRNAs in the cluster that show fast degradation rates, while others in the cluster are relatively more stable. The fast turnover of miR-3 and miR-309 can be recapitulated in the BG3-c2 larval CNS cell line, but not in embryonic cell lines such as S2-R+ and Kc167. Diverse degradation mechanisms may be in play under various contexts, leading to the perceived cell-type specificity. Dissimilar expression levels of AGO proteins in different cellular backgrounds may also alter the protective effect on miRNAs, thereby influencing miRNA stabilities. Furthermore, the expression and/or activity of miRNA-degrading factors may be cell line specific.

The *mir-309* cluster is conserved across all Drosophilids and its expression restricted to early embryos (Mohammed et al., 2014). *D.*

melanogaster and *D. virilis* are evolutionarily distant; they are estimated to have diverged about 63 million years ago and represent the two major subgenera of Drosophilids (Tamura et al., 2004). However, differential stability of cluster members is conserved between *D. melanogaster* and *D. virilis*, suggesting biological significance (Zhou et al., 2018).

Even though various sequence motifs have been implicated in promoting decay (Sethi and Lukiw, 2009, Bail et al., 2010, Rissland et al., 2011, Zhang et al., 2011, Guo et al., 2015), these rules often only apply to single or a small subset of genes. Using S2-R⁺ and BG3-c2 cells to recapitulate degradation in embryos, mutagenesis experiments designed to mutate a section of homology shared between mature miR-3 and miR-309 revealed that the AAAGT motif in the middle of the mature miRNA sequence might be an important element that characterizes destabilization. Since nucleotide identities in this region influence sorting profiles, it is currently unclear if the sequence element identified here has an effect on AGO loading kinetics.

4.6 Elucidation of destabilization mechanisms

To date, general nucleases responsible for degradation of animal miRNAs remain unidentified. Furthermore, it is plausible that specialized mechanisms exist to regulate turnover of specific miRNAs. Thus, identification of these miRNA-degrading factors remains a major question to be tackled.

Human RISC purification using streptavidin affinity purification with biotin-tagged siRNAs has been reported in early studies (Martinez et al., 2002, Martinez and Tuschl, 2004, Ameres et al., 2007). A more recent method using modified antisense oligonucleotides, allows purification of endogenous complexes containing a single guide sequence under native conditions (Flores-Jasso et al., 2013). The biochemical purification of mature RISC containing miR-3/-309 is one approach to dissect the underlying instability of these miRNAs, aimed at narrowing

down candidates involved in the rapid degradation of miR-3/-309 in fly embryos. These purified complexes are active and soluble, allowing this method to be coupled to downstream mass spectrometry applications. Compared to purification of exogenously programmed RISC, pull-down of endogenous miR-286-RISC from fly embryos was shown to be less efficient, with incomplete depletion of the miRNA from the lysate (Flores-Jasso et al., 2013). From the preliminary results presented above, western blotting detection failed to detect AGO1 from the pull-down samples, leaving much room for method optimization.

Besides the biochemical purification of mature RISC, large-scale RNAi screening using RNAi libraries publicly available from DRSC (*Drosophila* RNAi Screening Center) might also provide some useful information. In contrast to mammalian cells where long dsRNAs mount an interferon response, this is not present in *Drosophila* cells, and long dsRNAs can be spontaneously taken up by cells under serum-lacking conditions (Ramadan et al., 2007). These high-throughput screens using genome-wide libraries or sub-libraries for knockdown of functionally related gene sets allow systemic screening and can provide a preliminary list of factors that may be important in miR-3/-309 degradation. Each well of the assay plate contains dsRNAs against the same gene. As a read-out, plate readers usually rely on luminescent or fluorescent signals for the sensitive detection of positive hits. As with other assay systems, false positives and negatives should always be taken into account, and positive hits should be further validated by Northern blotting experiments. In order to test the feasibility and reproducibility of this method, luciferase reporter constructs should first be monitored for luminescence signals at two time points after transfection, in order to monitor degradation rates. However, an obvious disadvantage of *in vitro* screening methods lies in its bias in identifying non-physiological binding events.

In humans, TSN is overexpressed in various malignancies, including colon cancer and prostate cancer (Tsuchiya et al., 2007, Kuruma et al.,

2009). Owing to its miRNA-degrading activities, depletion of TSN resulted in up-regulation of selected miRNAs that control genes encoding for proteins important for cell cycle progression, thereby playing an important role in cell fate determination and function (Elbarbary et al., 2017b). This signifies the utility of targeting TSN activity in order to inhibit uncontrolled cell proliferation, for instance in cancer. Since miRNAs are frequently implicated in disease states, the identification of effectors and regulators of miRNA degradation may aid in the future development of therapeutics.

Preliminary data presented here should motivate further work in this area, to reveal a novel mechanism of differential regulation of the *mir-309* cluster, and allow further dissection of their biological functions.

5. Future work

Historically, large-scale purification of active Dicer protein has proved challenging. More recently, using an optimized baculovirus expression system, the Bass laboratory overcame previous limitations to purify Dicer and its partner proteins (Loqs-PD and R2D2) in high yields (Sinha and Bass, 2017). Extension of the protocol to purify Loqs-PB, and future studies using recombinant Loqs-PB can allow for a biochemical dissection of its function by exploring binding and processing kinetics (through measurements of substrate affinity and catalytic activity), and potentially yield mechanistic insights into its action.

Defects in the miRNA processing machinery would impair biogenesis and result in dysregulation of cellular miRNA levels. Aberrant miRNA expression profiles have been linked to pathogenesis of diverse diseases, including cancer (Ardekani and Naeini, 2010, Iorio and Croce, 2012). Intracellular TRBP-interacting partners were previously identified through mass spectrometry, where ~30% fall into the protein synthesis pathway (Chi et al., 2011). Even though the study implicated TRBP in a variety of cellular processes, many of these interacting factors have not been fully functionally characterized. To date, the link between dsRNA-binding proteins and diseases has not been thoroughly investigated. It will be interesting to establish the Loqs-proteome and identify interacting proteins, particularly in normal and disease states, in order to better understand its biological relevance.

Overall, the study of miRNA stability remains largely unexplored, and many questions remain unresolved. This is in part due to the variety of mechanisms that act in a miRNA-, tissue- or developmental stage-specific manner that may not be conserved across phylogeny. Future work should be aimed at understanding the molecular mechanisms underlying rapid degradation of miR-3/-309. It is unclear how miR-3/-309 are sequestered and/or marked for selective degradation, and how the decay machinery is subsequently recruited. Since miRNAs can

potentially serve as valuable therapeutic targets to modulate expression of their target genes, understanding the mechanisms of their expression and regulation is crucial and can provide insights for future work regarding their roles in various pathologies. In a broader sense, recognizing the underlying specificity of degradation may allow the manipulation of stability of individual miRNAs of interest, signifying potential use in therapeutic applications.

6. Conclusion

Binding partners of Dicer can expand the functionalities and modulate its activity in a variety of ways. Here, the results showed that Loqs can enhance the processing of a subset of miRNA genes, and that Loqs-dependent miRNAs share a common structural feature with an unstable base-pairing at the dicing site. Given that dsRNA-binding proteins are widespread in biological processes, it is crucial to understand how they impose specificity and functionality to miRNA biogenesis.

MiRNA stability is an important, yet understudied, aspect of regulation. The data demonstrated that miR-3 and -309 exhibit faster degradation profiles compared to other miRNAs from the same cluster, and this phenomenon is likely to be cell-type specific. Preliminary results presented here suggest an interesting possibility whereby particular sequence elements may influence turnover, and the specific recognition by RNA-binding factors or degradation machinery remains to be investigated. Furthermore, the results also suggested a role for the terminal 5' nucleotide in mature miRNA stability. Overall, the findings highlight the importance of miRNA stability and/or degradation in gene regulatory networks, as an additional mechanism to regulate target gene expression through the modulation of mature miRNA levels.

Even with a relatively short history, a combination of high-throughput sequencing, biochemical, and structural approaches has provided a comprehensive perspective into molecular events surrounding small RNA-mediated gene silencing. A better mechanistic understanding of these processes will hopefully lead to advances in RNAi research that will ultimately prove useful in the prevention and therapy of human diseases.

7. References

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