MICRORNAs IN THE *Drosophila* EGG AND EARLY EMBRYO

by

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Abstract

Posttranscriptional regulation plays a very important role in animal oocytes and embryos. Maternally synthesized mRNAs and proteins control early animal development up until the maternal-to-zygotic transition (MZT). This is the point when the zygotic genome takes control. The maternally deposited mRNAs are posttranscriptionally regulated right from the time they are produced during oogenesis, through egg activation, and in the embryo. microRNAs (miRNAs) are posttranscriptional regulators that have been shown to play a role in both RNA stability and translation. I examined miRNA abundance in Drosophila stage 14 oocytes, activated unfertilized eggs, and embryos and have grouped all the then known Drosophila miRNAs into four distinct temporal classes. Class I and III appear to be maternally deposited, while Class II appears to be both maternally and zygotically transcribed, and Class IV appears to be strictly zygotically transcribed. Follow-up experiments validated three of the four classes. miRNAs of Class IV have previously been shown to play a role in maternal transcript destabilization, and I further show that this is through a SMAUG (SMG) dependent mechanism.
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CHAPTER 1
INTRODUCTION

Posttranscriptional regulation plays a very important role in early embryos. Early embryonic development is controlled by maternally deposited proteins and mRNA. Maternal mRNAs are posttranscriptionally regulated right from the time they are produced during oogenesis, through egg activation, and in the embryo at the level of stability and translation. miRNAs are posttranscriptional regulators that function in both the destabilization of RNA and translational repression of RNA.

1.1 Mechanisms of Posttranscriptional Regulation

1.1a Control of RNA Stability

Deadenylation dependent

Several mechanisms of eukaryotic mRNA decay exist (as reviewed by 2004; Day and Tuite, 1998; Semotok and Lipshitz, 2007). A major mechanism of mRNA decay is deadenylation dependent decay. For deadenylation dependent decay, degradation of mRNA is initiated by the removal of the poly(A) tail (Muhlrad and Parker, 1992; Shyu et al., 1991). Three deadenylases have been identified in eukaryotes: the CCR4-NOT deadenylase, the PAN2/PAN3 deadenylase, and the PARN deadenylase. The major cytoplasmic deadenylation activity in yeast is from the CCR4-NOT deadenylase, consisting of CCR4 and POP2/CAF1 the catalytic subunits. Downregulation of the CCR4-NOT complex and its components has been shown to have global effects on the length of poly(A) tails of bulk mRNA in Drosophila, and also the deadenylation of specific transcripts such as Hsp83 and nanos (nos) mRNAs during embryogenesis resulting in
increased poly(A) tail length (Semotok et al., 2005; Semotok et al., 2008; Temme et al., 2004; Zaessinger et al., 2006).

After deadenylation, there are two exoribonucleolytic pathways that can target transcripts for degradation (as reviewed by Coller and Parker, 2004; Semotok and Lipshitz, 2007). The first pathway occurs via decapping, followed by 5’-to-3’ exonucleolytic degradation. The major decapping enzyme in yeast is DCP1 and it is conserved in other eukaryotes (as reviewed by Tucker and Parker, 2000). The decapping enzyme DCP2 works along with DCP1 as the catalytic subunit (as reviewed by Coller and Parker, 2004). In yeast, DCP1 cleaves the m7GpppX cap of mRNA to produce both an m7GDP and a 5’-monophosphate mRNA (as reviewed by Tucker and Parker, 2000). Efficient decapping of mRNA also requires LSM1-7, PAT1, DHH1 and also the enhancers EDC1, EDC2, and EDC3. The 5’-monophosphate mRNA is rapidly degraded by the 5’-to-3’ exonuclease known as XRN1. In yeast, it has been shown that eIF4E inhibits the decapping activity of DCP1, and must be disassociated from the cap structure before decapping is initiated by DCP1 (Schwartz and Parker, 2000). PAB1 also acts as an inhibitor of decapping (as reviewed by Coller and Parker, 2004; Tucker and Parker, 2000).

The second exoribonucleolytic pathway involves 3’-to-5’ decay of mRNA by the exosome after deadenylation (Coller and Parker, 2004; Semotok and Lipshitz, 2007). The exosome includes core subunits RRP4, RRP40, RRP41, RRP42, RRP43, RRP44, RRP45, RRP46, MTR3, and CSL4. The exosome also functions with the SKI complex, consisting of SKI12, SKI3, and SKI8 RNA helicases. Mammalian mRNAs containing AU-rich destabilization elements are mostly degraded by the 3’-to-5’ exoribonucleolytic pathway.

Deadenylation Independent Decay

Degradation of mRNA can also occur via endonucleolytic cleavage without deadenylation (as reviewed by Day and Tuite, 1998). Endonuclease cleavage sites have been
identified within coding regions and 3’ untranslated regions (UTRs) of mRNA. The mammalian proto-oncogene \textit{c-myc} mRNA and \textit{Xenopus laevis albumin} mRNA contain cleavage sites within coding sequence, and the mammalian transferrin receptor (TfR) contains a cleavage site within its 3’UTR (Bernstein et al., 1992; Binder et al., 1994; Dompenciel et al., 1995). In many cases, the endonucleolytic cleavage of mRNA is regulated by RNA binding proteins that bind close to the cleavage site(s). \textit{Drosophila} utilizes an mRNA surveillance system that requires an endonucleolytic cleavage that is both poly(A) tail and cap independent (reviewed by Semotok and Lipshitz, 2007). This endonucleolytic cleavage occurs near the stop codon of an aberrant premature stop codon-containing mRNA (Gatfield and Izaurralde, 2004). The 5’ fragment is then degraded by the exosome, while the 3’ fragment is degraded by PACMAN (PCM), which is an XRN1 homologue.

\textbf{1.1b Control of RNA Translation}

Two general modes of translational control exist: first, global control, which occurs by the modification of translation initiation factors, and second mRNA specific control, which occurs via the recognition by regulatory proteins of particular elements present in the 5’ or 3’ UTRs of target mRNA (as reviewed by Gebauer and Hentze, 2004).

\textit{Regulation of Translation Initiation}

The closed loop model describes a mechanism in which translation is regulated at the step of initiation. The closed loop model proposes that mRNA molecules that are undergoing translation are circularized through interactions between trans-acting factors, which bind to the 5’ and 3’ ends of an mRNA (Johnstone and Lasko, 2001; Vardy and Orr-Weaver, 2007). The addition of a poly(A) tail stabilizes transcripts and is believed to cause the circulization of the mRNA and stabilize the eIF4F initiation complex. A component of the eIF4F complex, eIF4E,
binds to the 5’cap (m7G) of mRNA, which then binds to eIF4G. Then eIF4G interacts with the Poly(A) binding protein (PABP), which then binds to poly(A) tails and allows for translational activation of mRNA. Circularization stabilizes the preinitiation complex, and allows for the recruitment of the 40S ribosome subunit.

Inhibition of the Preinitiation Complex

The association of the 40S ribosomal complex with an mRNA can be regulated by RNA binding proteins that regulate specific mRNAs (as reviewed by Gebauer and Hentze, 2004). This regulation can be achieved through interference with the eIF4F complex. During vertebrate oocyte maturation and early embryo development, the cytoplasmic-polyadenylation element-binding protein (CPEB) regulates the translation of maternal mRNA by binding to the cytoplasmic polyadenylation element (CPE) located in the 3’UTR of specific mRNAs (as reviewed by Gebauer and Hentze, 2004; Mendez and Richter, 2001). This results in either translational activation or translational repression by a mechanism called masking. CPEB represses translation by binding to a protein called Maskin (Stebbins-Boaz et al., 1999). Maskin possesses an eIF4E-binding domain and competes with eIF4G for binding with eIF4E (the m7G cap-binding protein). In Drosophila, the eIF4E-binding protein, CUP, blocks the binding of eIF4G to eIF4E (Nelson et al., 2004). In Drosophila, SMG has been shown to bind to a cis element in the 3’UTR of unlocalized nos mRNA called the SMG Recognition Element (SRE). SMG then recruits CUP, which causes translational repression by competitively inhibiting the interaction of eIF4G with eIF4E. CUP has also been shown to be recruited by BRUNO (BRU) in Drosophila oocytes to prevent the translation of oskar (osk) mRNA (Nakamura et al., 2004; Wilhelm et al., 2003). BRU binds to a cis element called the BRU Response Element (BRE) in the 3’UTR of osk. Also in Drosophila, BICOID (BCD) has been shown to prevent the translation direct binding to eIF4E (Niessing et al., 2002).
In addition to CUP, d4EHP is a novel cap binding protein that has the ability to translationally repress mRNA (Cho et al., 2006; Cho et al., 2005; Vardy and Orr-Weaver, 2007). This protein has the ability to bind to the 5’ cap of mRNA and block the formation of the preinitiation complex. In order to translationally repress cad mRNA, BCD directly binds to the BCD Binding Region (BBR) in the 3’UTR of cad and recruits d4EHP (Cho et al., 2005; Niessing et al., 2002). d4EHP binds to both the 5’cap of cad and BCD, resulting in the deadenylation independent translational repression of cad mRNA.

Regulation of Poly(A) Tail Length

Poly(A) tail length also controls the translation of mRNA. For example, nos mRNA is translationally repressed by a deadenylation dependent mechanism, in which reduction of the poly(A) tail results in translational repression. It has been shown that SMG has the ability to recruit the CCR4-NOT deadenylase to nos mRNA (reviewed by Vardy and Orr-Weaver, 2007; Zaessinger et al., 2006). This recruitment results in the deadenylation, and therefore the translational repression of nos. OSK relieves this translational repression by preventing the binding of SMG in the posterior of the embryo, and therefore allowing translation to take place.

Cyclin B translation is also repressed in the pole cells of Drosophila embryos by a deadenylation dependent mechanism (Asaoka-Taguchi et al., 1999; Kadyrova et al., 2007; Vardy and Orr-Weaver, 2007). PUMILIO (PUM) has been shown to recruit NOS to cyclin B mRNA, which then recruits the CCR4-NOT deadenylase.

Translational Repression at the 60S Subunit Joining Step

Translation can also be repressed at the 60S subunit joining step. Although most translational regulatory events occur at the cap-binding step, there is evidence that shows that the 60S subunit joining step can also be regulated (Ostareck et al., 2001). In mammals, the RNA-binding proteins hnRNP K and hnRNP E1 repress the translation of 15-lipoxygenase (LOX)
mRNA by binding to a CU-rich element in the LOX 3’UTR known as the differentiation-control element (DICE) (Ostareck-Lederer et al., 1994; Ostareck et al., 1997). This translational repression has been shown to occur downstream of the 43S scan and during 60S subunit joining.

1.2 Posttranscriptional Regulation of Maternal mRNA in *Drosophila*

1.2a In the Ovary

Very early *Drosophila* development is controlled by maternally synthesized mRNAs and proteins (as reviewed by Tadros and Lipshitz, 2005). These maternally synthesized materials remain in control until the MZT, in which the zygotic genome is activated and takes control. The maternally deposited mRNAs are posttranscriptionally regulated from the moment they are produced during oogenesis. This posttranscriptional regulation allows for the coordinated control of development.

The female *Drosophila* reproductive tract consists of two ovaries containing approximately a dozen ovarioles each. Each ovariole contains maturing egg chambers that are arranged in an assembly line. The maturing egg chambers consist of both germline and somatically derived cells. The germline cells consist of an oocyte and nurse cells. The somatically derived cells, known as the follicle cells, surround the germline cells in a single layer. During oogenesis, the nurse cells undergo a large amount of transcription producing most of the maternal products that are deposited into the developing oocytes during oogenesis (as reviewed by Semotok and Lipshitz, 2007; Tadros and Lipshitz, 2005). About 55% of the entire protein coding genome is present in maternal mRNAs in mature oocytes (Tadros et al., 2007a).

In many species, including *Drosophila*, maternal mRNAs have been found to be associated with messenger ribonucleoprotein (mRNP) complexes (as reviewed by Johnstone and Lasko, 2001; Tadros and Lipshitz, 2005). In *Drosophila*, maternal mRNPs are seen both in nurse
cells and in the oocyte cytoplasm, in electron dense sponge bodies. EXUPERANTIA (EXU), YPSILON (Yps), the fly CPEB homologue Oo18 RNA binding protein (ORB), and maternal expression at 31B (Me31B) all have been shown to be part of the mRNP complexes (as reviewed by Tadros and Lipshitz, 2005). ORB has been shown to be involved in RNA localization, posterior-anterior patterning, and dorsal-ventral patterning during *Drosophila* oogenesis (Christerson and McKearin, 1994; Lantz et al., 1994). Furthermore, the loss of ORB in the oocyte prevents the polyadenylation and translation of maternal mRNA such as *osk*.

Localization-dependent translation is a commonly used mechanism in early development (as reviewed by Johnstone and Lasko, 2001; Piccioni et al., 2005). For example, *osk* mRNA is localized to the posterior pole of the oocyte, where OSK protein is translated in order to direct posterior cell fates (Ephrussi and Lehmann, 1992; Smith et al., 1992). *Osk* mRNA is translationally repressed before localization, and the localization of *osk* is necessary for its translation. ORB has been shown to directly bind to the 3’UTR of *osk* mRNA and activate its translation in the posterior region of the oocyte (Chang et al., 1999). In an ORB mutant, *osk* mRNA will have shorter tails. It is likely that in *Drosophila* oocytes, ORB activates the translation of *osk* by activating its cytoplasmic polyadenylation.

miRNAs are posttranscriptional regulators that affect both RNA stability and translation (as reviewed by Filipowicz et al., 2008). Recent evidence shows that miRNA dependent translational repression may occur in *Drosophila* ovaries (Reich et al., 2009). Reich et al. have shown that miR-312 is present in *Drosophila* ovaries in both germline cells and the somatic follicle cells. Synthetic miR-312 binding sites in GFP mRNA reporters reduce translation of the reporters during mid to late stages of oogenesis. Because they did not see the localization of ARGONAUTE 1 (AGO1), a protein part of the RNA-induced silencing complex (RISC) that mediates translational repression by miRNAs (Okamura et al., 2004), in sponge bodies, but
rather many small foci throughout the cytoplasm of germline cells, Reich et al. suggest that sponge bodies may not be the primary sites of miRNA activity in *Drosophila* ovaries. Previous evidence had also suggested that miRNAs are active during *Drosophila* oogenesis (Yang et al., 2007). When AGO1 is mutated during oogenesis, germline stem cells (GSC) are lost, and when AGO1 is overexpressed, GSC overproliferate. Another study by Nakahara et al. (2005) compares the proteome of wild-type oocytes and oocytes lacking the *dicer-1* gene. Their data suggests that about four percent of the genes examined are subject to miRNA repression during oocyte maturation.

### 1.2b During Egg Activation

Immature oocytes are arrested in prophase I of meiosis (as reviewed in Tadros and Lipshitz, 2005). The final stage of oogenesis is known as maturation, and is initiated by the release of the oocyte from the prophase I arrest ending with a second arrest at metaphase I. This second arrest persists until egg activation. Egg activation in *Drosophila* occurs internally and can occur without fertilization (Doane, 1960). Egg activation triggers the completion of meiosis and posttranscriptional changes (reviewed in Semotok and Lipshitz, 2007).

Egg activation in *Drosophila* triggers both translational activation and repression (as reviewed by Tadros and Lipshitz, 2005). Transcripts that are translationally activated in *Drosophila* upon egg activation include *bcd, nos, Toll (Tl), hunchback (hb), cad, smg, torso (tor), and string (stg)*. Translational activation requires cytoplasmic polyadenylation and certain maternal mRNAs such as *bcd, tor, Tl*, and *hb* have been shown to be polyadenylated during egg activation. Many of the mRNAs that are translated into protein upon egg activation, repress the translation of other maternal transcripts, spatially and temporally restricting protein expression
(Tadros and Lipshitz, 2005). For example, BCD represses CAD, so that CAD is expressed in a posterior to anterior gradient in the embryo after egg activation (Niessing et al., 2002).

Egg activation also activates the destabilization of maternal transcripts in *Drosophila* (as reviewed by Semotok and Lipshitz, 2007; Tadros and Lipshitz, 2005), and is both necessary and sufficient to trigger the destabilization of maternal transcripts (Tadros et al., 2003). Over 1600 maternal mRNAs are destabilized after egg activation (Tadros et al., 2007a). Some transcripts that are destabilized after egg activation include *Hps70, Hsp83, nos, stg, cyclin B, twine*, and *Pgc* (as reviewed by Tadros and Lipshitz, 2005). Deadenylation plays an important role in transcript destabilization, which in turn allows for spatially regulated zygotic transcription. Also, destabilization allows for the specific localization of maternal transcripts by degrading them in one region while they remain protected in another (as reviewed in Lipshitz and Smibert, 2000).

**1.2c In the Early Embryo**

In *Drosophila*, the joint action of two RNA degradation pathways control the timing of maternal transcript destabilization (Bashirullah et al., 1999). The first is the “maternal” degradation pathway that is driven by maternally encoded factors. The second pathway known as the “zygotic” degradation pathway becomes active two hours after fertilization and requires one or more zygotically transcribed factor(s). While each pathway alone is sufficient to destabilize maternal transcripts, it is the joint action of both that allows for the elimination of transcripts prior to the midblastula transition (MBT).

About 20% of maternal transcripts are destabilized in activated unfertilized eggs, while in embryos, 33% of maternal transcripts are destabilized (De Renzis et al., 2007; Tadros et al., 2007a). Two thirds (712 of 1069) of these unstable maternal transcripts are destabilized in a SMG-dependent manner because they are stabilized in *smg* mutant embryos. SMG is an RNA
binding protein, which has previously been shown to direct degradation of maternal transcripts such as *Hsp83* by recruitment of the CCR4/POP2/NOT deadenylase (Semotok et al., 2005; Semotok et al., 2008). SMG also has the ability to translationally repress maternal transcripts such as *nos* in the embryo (Dahanukar et al., 1999; Smibert et al., 1999; Smibert et al., 1996). SMG binds to SREs in the *nos* 3’UTR recruiting the eIF4E-binding protein CUP, and therefore blocking translation (Nelson et al., 2004; Smibert et al., 1999).

Computational analysis indicates that the 3’UTRs of maternal transcripts are enriched for various sequence elements such as miRNA target sites and stem-loop structures known as SREs, while unstable maternal transcripts in particular are enriched further for PUF-domain protein binding sites and AU-rich elements (AREs) (De Renzis et al. 2007, Tadros et al., 2007a). miRNAs, SMG protein, PUF proteins, and ARE binding proteins are posttranscriptional regulators at the level of stability and translation.

1.2d During the Maternal-to-Zygotic Transition

The zygotic genome begins to be expressed at high levels between 2 and 3 hours after fertilization, when 18% of the genome is transcriptionally activated during the MZT (Benoit et al., 2009; De Renzis et al., 2007). The zygotically transcribed mRNAs fall into three different categories. The first class (I) consists of purely zygotically transcribed mRNAs. These transcripts are not present in stage 14 oocytes, but are expressed in 2-3 hour embryos. The second class (II) of transcripts is seen in stage 14 oocytes and remains stable through the MZT, indicating that they are maternally deposited. Once zygotic transcription begins they increase in abundance, and therefore they are both maternally and zygotically transcribed. The third class (III) of transcripts is present in stage 14 oocytes, degraded during the MZT, but is re-expressed zygotically.
SMG is a key activator of the zygotic genome (Benoit et al., 2009). It has been shown that SMG expression is required during the MZT for the expression of 85% of the Class I genes and 90% of the Class II genes. The smg mutant was found to have little effect on the Class III genes, which may be related to the fact that SMG is required for the destabilization of 75% of the maternal pool of Class III transcripts at MZT (Benoit et al., 2009; Tadros et al., 2007a). SMG protein is also required for the transcription of zygotically transcribed miRNAs such as the miR-309 cluster (Benoit et al., 2009). The miR-309 cluster has been shown to play a role in the destabilization of maternal transcripts during MZT (Bushati et al., 2008). Benoit et al. hypothesize that MZT is triggered by the destabilization of maternal mRNAs by SMG, resulting in the downregulation of maternal proteins that repress zygotic transcription and the replication checkpoint.
Figure 1-1. Proposed model for SMG-dependent control of MZT.
Benoit et al. propose that SMG destabilizes maternal mRNAs, which allows for zygotic transcription and the replication checkpoint to occur. Zygotic transcription allows for the production of the miR-309 cluster, which further aids in the destruction of maternal mRNAs, and also allows for the production of transcriptional activators that also aid in the progression of MZT. (Reproduced with permission from Development, Benoit et al., 2009)

Zelda (Zld), a zinc-finger protein also has been shown to play a crucial role in activating the early zygotic genome in *Drosophila* (Liang et al., 2008). In *Drosophila*, the early zygotic genes are transcribed between 1 and 2 hours after fertilization. Many of the pre-cellular genes contain TAGteam DNA motifs that control the timing of the transcription of these genes (ten Bosch et al., 2006). Zld has been shown to bind the TAGteam sites and mediate transcriptional activation of zygotic genes (Liang et al., 2008). In *zld* mutants, many zygotic genes are downregulated and many maternal genes are upregulated. The miR-309 cluster contains two TAGteam sites, and miR-309 expression is absent in a *zld* mutant. Because the upregulated transcripts in a *zld* mutant do not overlap with the direct SMG targets (Liang et al., 2008; Tadros
et al., 2007a), Liang et al. suggest that Zld and SMG work in parallel pathways of RNA degradation.

1.3 Role of miRNAs in Posttranscriptional Regulation

1.3a Biogenesis of miRNAs

miRNAs are approximately 21 nucleotide long noncoding RNAs predicted to control approximately 30% of all protein-coding genes (Filipowicz et al., 2008). Many miRNA genes are transcribed by RNA polymerase II producing what is known as the primary miRNA (pri-miRNA), which can be hundreds of nucleotides to tens of kilobases in length (as reviewed by Bushati and Cohen, 2007; Cai et al., 2004; Lee et al., 2004). miRNAs can be transcribed from separate miRNA genes or can be located within the intron of protein coding genes (as reviewed by Filipowicz et al., 2008). The pri-miRNA can encode several different miRNA sequences, which are referred to as a miRNA family. The pri-miRNA contains a 5’cap structure, is polyadenylated, and may also be spliced (Bracht et al., 2004; Cai et al., 2004). The pri-miRNA is processed into an approximate 70 nucleotide precursor miRNA (pre-miRNA) in the nucleus by the nuclear RNase III DROSHA (as reviewed by Bushati and Cohen, 2007; Filipowicz et al., 2008; Lee et al., 2003). The DROSHA partner in Drosophila is pasha, a double-stranded RNA-binding-domain protein. The pre-miRNA is transported to the cytoplasm by EXPORTIN-5, in a Ran-GTP-dependent mechanism.

Once in the cytoplasm the pre-miRNA is cleaved a second time to produce the approximate 22 nucleotide mature miRNA by another RNase III-related enzyme, DICER, which works along with LOQUACIOUS in Drosophila (as reviewed in Bushati and Cohen, 2007; Filipowicz et al., 2008). The mature miRNA strand is incorporated into the RNA-induced silencing complex (RISC), a key component of which is the AGO protein. Once in the RISC
complex, miRNAs attack their targets. For cases in which miRNAs display perfect complementarity to their target, the result is normally cleavage, and therefore degradation. This mechanism is most commonly observed in plants. In animals, miRNAs usually display incomplete complementarity to their targets, resulting in translational repression and/or destabilization due to deadenylation and decapping.

**Figure 1-2. Biogenesis of a miRNA.**
Transcription by RNA Pol II produces the pri-miRNA, which consists of a 5’ cap and 3’ tail. The pri-miRNA is then cleaved by the nuclear RNase III DROSHA to produce the pre-miRNA. The pre-miRNA is transported into the cytoplasm by EXPORTIN-5. Once in the cytoplasm, the premiRNA is cleaved a second time by the RNase III enzyme DICER along with LOQUACIOUS to produce the mature miRNA. The mature miRNA is incorporated into the RISC, which consists of an AGO protein. (Reprinted, with permission, from the Annual Review of Cell and Developmental Biology, Volume 23 (c) 2007 by Annual Reviews  www.annualreviews.org)
1.3b miRNA Target Identification

The first animal miRNA targets were discovered in *C. elegans* by genetic methods (as reviewed by Bartel, 2009; Bushati and Cohen, 2007). It was discovered that *lin-4* RNA had some sequence complementarity to conserved sites in *lin-14* mRNA (Lee et al., 1993; Wightman et al., 1993). A little later on it was discovered that *lin-4* and *let-7* had complementarity to sites in the 3’UTR of *lin-28* and *lin-41*, which are in fact targets of *lin-4* and *let-7* miRNAs (Moss et al., 1997; Reinhart et al., 2000). miRNA target sites can be grouped into two broad categories (as reviewed by Bushati and Cohen, 2007). The first category are target sites that display 5’ dominant sites. These targets show complete base pairing to the “seed” sequence (nucleotides 2-8) of the miRNA and may or may not have significant pairing in the 3’ region. The second category shows strong pairing to the miRNA's 3’ region, which compensates for weaker pairing in the 5’ region.

The miRNA target prediction programs that exist today are very diverse, but a few conclusions from the various programs can be agreed upon (as reviewed by Bartel, 2009). The first conclusion is that conserved Watson-Crick pairing to the “seed” sequence of the miRNA is important and reduces false positives. The second conclusion is that when predicting conserved targets, looking at conserved pairing to the seed region is sufficient. And the third conclusion is highly conserved miRNAs will have many conserved targets. Bartel (2009) summarizes the various target prediction programs that exist today and their criteria for predicting targets. For example, TargetScan looks at conserved sites with stringent seed pairing (Kheradpour et al., 2007; Ruby et al., 2007), while PITA does not consider site conservation and predicts target sites based on site accessibility and moderately stringent seed pairing (Kertesz et al., 2007). This thesis uses the overlap of TargetScan and PITA to predict miRNA targets.
1.3c miRNAs as Posttranscriptional Regulators

miRNA Mediated Decay

miRNA-mediated decay has been shown to require many different components such as: a member of the AGO protein family, the P-body component GW182, CAF1-CCR4-NOT deadenylase complex, decapping enzyme DCP2 and decapping activators DCP1, Ge-1, EDC3, and RCK/p54 also known as Me31B (Behm-Ansmant et al., 2006a; Behm-Ansmant et al., 2006b; Eulalio et al., 2008; Eulalio et al., 2007b; Rehwinkel et al., 2005). P-body’s are sites of posttranscriptional regulation and have been shown to contain the necessary proteins for mRNA degradation and translational repression. P-body formation may be a result of miRNA-mediated gene silencing (Eulalio et al., 2007a). GW182 protein, a P-body component, is required for both miRNA-mediated translational repression and mRNA decay in *Drosophila* (Behm-Ansmant et al., 2006a). In *Drosophila*, mRNA degradation by miRNAs, and not translational repression, requires CAF1, NOT1 and the DCP1:DCP2 complex, possibly suggesting that miRNA-mediated decay is independent of mRNA translational repression. Indeed, the PIWI domain of AGO1 interacts with the N-terminal of GW182 (Behm-Ansmant et al., 2006a), while GW182 and the DCP1:DCP2 complex are essential for miRNA mediated gene silencing (Rehwinkel et al., 2005).

It has been hypothesized that miRNAs increase the accessibility of general degradation enzymes to the mRNA 5’ cap structure and poly(A) tail, and do so by changing the messenger ribonucleoprotein (mRNP) composition and/or structure rather than directly interfering with the binding of ribosomal subunits (Eulalio et al., 2009). These authors show that in *Drosophila*, deadenylation is a widespread effect of miRNA regulation; 60% of AGO targets are also regulated by CAF1 and/or NOT1. In zebrafish, it has been shown that miR-430 promotes the deadenylation, and therefore the degradation of maternal mRNA (Giraldez et al., 2006). As of
now, many studies indicate that miRNAs mediate mRNA decay, but it has not been determined if this decay is a result of translational repression or not. This is because the various studies give different results (as reviewed by Filipowicz et al., 2008). Studies in Drosophila S2 cells, indicate that the repression of reporters by miRNAs could be due to mRNA degradation, translational repression or a combination of the two (as reviewed by Filipowicz et al., 2008).

miRNA Mediated Translational Repression

The mechanisms of miRNA translational repression on mRNA can be divided into three categories: repression at the initiation step, repression by preventing the 60S subunit joining, and repression post-initiation (as reviewed by Filipowicz et al., 2008). In human cells, miRNAs repress the translation of m\(^7\)G-capped mRNAs, but not mRNAs containing an internal ribosome entry site (IRES) or a nonfunctional ApppN cap (Humphreys et al., 2005; Pillai et al., 2005). These studies suggest that miRNAs interfere with the initiation step of translation by interfering with the function of the cap-binding factor eIF4E. Along with the 5’cap, the poly(A) tail is also necessary for translational repression by miRNAs (Humphreys et al., 2005). Human AGO2 contains an mRNA m\(^7\)G cap-binding motif similar to eIF4E (Kiriakidou et al., 2007), suggesting that AGO2 represses the initiation of mRNA translation by binding to the m\(^7\)G cap of target mRNA, therefore preventing the recruitment of eIF4E. In Drosophila, it has also been shown that miRNAs inhibit translation initiation (Thermann and Hentze, 2007). In a cell free system, miR-2 inhibits the translation of mRNA with a GpppG cap but not with a non-functional ApppG cap structure. Furthermore, miR-2 induces the formation of “pseudo polysomes” (heavy microribonucleoproteins that are non-polysomal) even though polysome formation and 60S ribosomal subunit joining are blocked. In this case, miR-2 inhibits translation initiation without affecting mRNA stability.
There is also evidence that miRNAs inhibit translation by preventing the 60S ribosomal subunit from joining to the 40S initiation complex (Chendrimada et al., 2007; Filipowicz et al., 2008). Chendrimada et al. (2007) isolated a miRNA RISC-containing complex that includes (1) MOV10, which is a homologue of the *Drosophila* translational repressor ARMITAGE, (2) the 60S ribosome, and (3) the anti-association factor eIF6, which is a ribosome inhibitory protein that prevents the assembly of the 80S ribosome. In human cells, deleting the eIF6 protein terminates miRNA-mediated translational repression and increases mRNA levels, while deleting eIF6 in *C. elegans* terminates lin-4 miRNA mediated repression of endogenous LIN-14 and LIN-28 target protein and again also increases mRNA levels. Chendrimada et al. propose a model in which miRNA-directed complexes use eIF6 to assist in the disruption of productive polysome formation and therefore expose the target mRNA for degradation.

It has also been shown that miRNA-mediated translational control occurs post initiation (Filipowicz et al., 2008; Maroney et al., 2006; Nottrott et al., 2006; Petersen et al., 2006). Petersen et al. (2006) used small siRNAs to show that small RNAs translationally repress mRNAs that are associated with polyribosomes engaged in translational elongation. The inhibition occurs through a cap-independent process. Human let7a has also been shown by Nottrott et al. (2006) to translationally repress mRNAs that are associated with actively translating ribosomes. These authors hypothesize that miRNAs are possibly involved in the proteolytic cleavage of nascent polypeptides. Maroney et al. (2006) have shown that in Hela cells, miR-21, miR-16 and let-7a are associated with mRNAs in polysomes that are actively being translated.
1.3d miRNA Spatial and Temporal Expression Patterns in Early Development

In *Drosophila*, miRNAs display diverse temporal and spatial expressions (Aboobaker et al., 2005; Aravin et al., 2003; Leaman et al., 2005). Northern blot analysis reveals that many miRNAs in *Drosophila* are likely to be maternally deposited, displaying signal in 0-1 hour embryos. Other miRNAs are expressed after zygotic transcription begins, and many miRNAs can

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**Figure 1-3. Summary of the possible mechanisms of miRNA-mediated posttranscriptional gene repression.**

Binding of the micro-ribonucleoprotein (miRNP) to its target can result in both mRNA decay and translational repression. The picture on the top left hand side indicates that mRNAs that are deadenylated, decapped and degraded are moved to p-bodies. mRNAs that are repressed at the step of translational initiation are also moved to p-bodies as indicated by the bottom left picture. miRNAs are also proposed to translational repress mRNA post translation initiation. miRNA repression can possibly block elongation or result in the degradation of nascent peptides, which is indicated by the pictures on the right. (Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Genetics, Filipowicz et al., 2008, copyright 2008)
be detected in larvae, pupae and adults. *In situ* hybridization analysis done by Aboobaker et al. (2005) revealed that miRNAs in *Drosophila* also display diverse spatial expression in early embryos, suggesting that in *Drosophila*, specific miRNAs regulate specific aspects of developmental patterning. For example, neural and musculature-specific miRNAs could function in the development and/or physiology of the nervous system and musculature respectively.

The temporal and spatial expression patterns of miRNAs has been examined in zebrafish by Wienholds et al. (2005). Examining the expression pattern of 115 conserved miRNAs using microarrays and *in situ* hybridization, they discovered that most miRNAs are expressed in a highly tissue-specific manner during later stages of development. Giraldez et al. (2005) showed that zebrafish mutant for both maternal/zygotic *dicer*, which lack the ability to process pre-miRNAs into mature miRNAs, display defects in later stages of development rather than the earlier stages. Maternal/zygotic *dicer* mutants still undergo axis formation and differentiation of multiple cell types, but display abnormal morphogenesis during gastrulation, brain formation, somitogenesis and heart development.

Wantanbe et al. (2005) looked at the expression of various *Xenopus* miRNAs in oocytes, ovulated eggs, and different stages of development until the tadpole stage. miRNA expression in *Xenopus* can be categorized into three groups: group one consists of miRNAs that are detected in oocytes with abundance decreasing in ovulated eggs and then increasing after the tadpole stage; group two miRNAs are expressed at temporally defined stages such as the neurula stage and the MBT; group three miRNAs consists of miRNAs expressed after the MBT up until the tadpole stage. Interestingly, miR-427 is expressed just after the MBT in stage 9 embryos; it has been hypothesized that miR-427 might play a role in developmental transition at the MBT.

In the mouse, maternal miRNAs have been shown to be essential for early development (Murchison et al., 2007; Tang et al., 2007), playing crucial roles in the oocyte to allow for the
normal development of the mouse embryo. This was shown by removing all mature miRNAs in the oocyte by depleting maternally inherited dicer.

1.3e miRNAs as Developmental Switches

In *C. elegans*, both *lin-4* and *let-7* act as developmental switches during early development (Lee et al., 1993; Reinhart et al., 2000; Wightman et al., 1993). *Lin-4* negatively regulates the production of LIN-14 protein (Lee et al., 1993; Wightman et al., 1993). Since *Lin-4* is not expressed during late embryogenesis and the early part of larval stage 1, this allows the production of LIN-14 protein. During larval stage 1, *lin-4* is transcribed, thus preventing the production of LIN-14 protein. In the absence of *lin-4*, the animals are unable to transition from the first to second larval stages. Likewise absence of *let-7* results in the failure of the larval-to-adult transition in *C. elegans*, with larval cell fates persistent during adult stages. (Reinhart et al., 2000).

miR-430 in zebrafish has been show to play an important role in the fine tuning of developmental programming in zebrafish (as reviewed by Bushati and Cohen, 2007; Giraldez et al., 2005; Giraldez et al., 2006). In zebrafish, miR-430 is transcribed at the onset of zygotic transcription in embryos, and has been shown to promote the deadenylation and clearance of maternal mRNAs at the MZT (Giraldez et al., 2006). In maternal/zygotic *dicer* mutant embryos, several hundred transcripts that are enriched for miR-430 target sites are upregulated compared to wild-type. 40% of these misregulated targets are maternally expressed. This study shows that a miRNA can target several hundred transcripts for elimination during the MZT.

In *Drosophila* a similar mechanism has been shown to exist. The miR-309 cluster, which encodes six different miRNAs, is zygotically transcribed at the onset of zygotic transcription. The six different miRs of this cluster, miR-6, miR-5, miR-4, miR-286, miR-3 and miR-309
contribute to the clearance of maternally encoded mRNAs (Bushati et al., 2008). Comparing transcript levels in wild-type 2-3 hour embryos to miR-309 mutant embryos revealed that 410 maternal transcripts are upregulated (stabilized) in the miR-309 mutant. While a role for miRNAs in maternal transcript elimination is conserved, because miR-430 and miR-309 are unrelated, this may have occurred by convergent evolution.

1.4 Goals of this Thesis

The overall goal of this thesis is to examine the possible function of miRNAs in the posttranscriptional regulation of maternal transcripts in *Drosophila*. The first step of my project was to examine the temporal expression profile of all the then known *Drosophila* miRNAs through microarray analysis and high resolution Northern blot analysis by using locked nucleic acid (LNA) modified oligonucleotides. Since in *Drosophila*, maternal mRNAs are posttranscriptionally regulated from the time they are produced during oogenesis, through egg activation, and in the embryo, I examined miRNA abundance in stage 14 oocytes, activated unfertilized eggs, and fertilized embryos. The microarray analyses revealed four distinct temporal classes of miRNAs in early *Drosophila* development: Class I is loaded maternally and rapidly eliminated upon egg activation; Class II is maternal and eliminated in 3-6 hour activated eggs but replaced by zygotic transcription in embryos; Class III is maternal and stable; Class IV is strictly zygotic and transcribed at high levels in 2-4 hour embryos.

Follow-up experiments validated three of the four classes (I, III, and IV), and showed that Class I miRNAs are expressed in follicle cells while Class III are in the oocyte itself. The profile of Class II miRNAs could not be validated. I went on to show that zygotic synthesis of Class IV miRNAs is SMG-dependent. Thus, destabilization of a subset of maternal mRNAs by Class IV miRNAs represents an indirect effect of SMG in maternal transcript decay. Expressing Class IV
miRNAs during oogenesis when this class is not normally expressed did not reveal any phenotypic effect on the ovaries, thus, it would be interesting to determine if expressing Class IV miRNAs during oogenesis effects oogenesis at the level of mRNA destabilization.
CHAPTER 2
MATERIALS AND METHODS

2.1 Egg, Embryo, Ovaries and Oocyte Collections
Activated unfertilized eggs and embryos were collected on grape juice plates after two 45 minute pre-lays. Ovaries and stage 14 oocytes were dissected in Ringer’s solution from young female flies that had been kept in bottles containing wet yeast for 3-5 days. For analyses of Class I miRNAs, the cytoplasm of stage 14 oocytes was sucked out using a micropipette. For analyses of the chorion and follicle cell RNA, the cytoplasm of stage 14 oocytes was extruded using forceps and the “carcass” was used for RNA isolation.

2.2 Fly Stocks
Wild-type stocks were w^{1118} (Tadros et al., 2007a). In order to obtain activated unfertilized eggs, sterile males with the genotype T(Y;2)bw^{DRev#1}, cn bw^{Drev#11} mr2/SM6a (Reed and Orr-Weaver, 1997) were crossed to virgin female w^{1118} flies. The smg line used was smg^{l} NGV, which is a recombinant chromosome carrying the smg^{l} mutation and NGV (Tadros et al., 2007a). The nanos-Gal4-VP16 (NGV) refers to P[GAL4::VP16-nos.UTR] (Van Doren et al., 1998).

2.3 RNA Isolation
RNA was extracted from ovaries, stage 14 oocytes, embryos, activated unfertilized eggs, and the cytoplasm or carcass of stage 14 oocytes using a modification of the TRIzol (Invitrogen) method (Tadros et al., 2007a). RNA samples used for the microarray analysis were further purified with RNeasy Mini Kits (50) (Qiagen) using a protocol provided by Exiqon, Inc.
2.4 Gene Expression Profiling

To profile the expression of *Drosophila* miRNAs, total RNA samples were sent to Exiqon for miRCURY LNA Array microRNA Profiling. The Exiqon microarrays used consisted of LNA probes for all registered and all known miRNAs from miRBase 9.2. This includes over 2000 unique LNA capture probes covering 54 organisms (68 from *Drosophila*), and also unpublished human miRs referred to as miRPLUS. Exiqon tested the RNA samples for quality control and miRNA enrichment before hybridization to the arrays. Two technical replicates were done for each sample. The histograms represent the average of the two arrays for each sample and the error bars show the standard deviation.

The expression of 406/410 transcripts upregulated in the miR-309 cluster mutant (Bushati et al., 2008) were examined from gene expression profiling data of Chun Hua He, which looked at 2-3 hour wild type embryos and 2-3 hour *smg* mutant embryos relative to wild-type stage 14 oocytes (Benoit et al., 2009). Details of the mRNA expression profiling experiment can be found in Benoit et al. (2009).

2.5 miRNA Northern Blots

miRCURY LNA Detection Probes were purchased from Exiqon for miRNA detection. Radioactive Northern blotting was performed as described by a protocol obtained from Exiqon’s website: http://www.exiqon.com/MicroRNA_northern_blot, except that a 15% polyacrylamide gel was used. For most samples, 12 μg of total RNA was loaded per lane with the exception that RNA was extracted from the cytoplasm of about 100 oocytes and about 200 oocyte carcasses for the Northern blot verification of the Class I miRNAs. The hybridization temperature was 40°C for all miRCURY LNA probe-to-membrane hybridizations. Blots were stripped in a boiling solution of 0.1% SDS in dH₂O, which was repeated 2-3 times and then reprobed.
For nonradioactive Northern blots, miRCURY LNA Detection Probes were purchased from Exiqon with 5’-end DIG labeling. The same methods as the radioactive Northern blots were used for the nonradioactive Northern blots, except the probe-to-membrane hybridization temperature was 47°C and the DIG Luminescent Detection Kit (ROCHE) was used to detect miRNA signal according to the manufacturer’s instructions. Anti-digoxigenin-AP was used in a concentration of 1:10,000 and CSPD was used in a concentration of 1:100. The variability in the quality of the miRNA Northerns seemed to depend on miRNA abundance. Abundantly expressed miRNAs were easier to detect.

2.6 Whole Mount in situ Hybridization

miRCURY LNA Detection Probes were purchased from Exiqon. These probes were labeled on the 5’-end with DIG. Ovaries were fixed for 20 minutes on a nutator in a fix solution consisting of 10% formaldehyde, 10% DMSO, 50mM EGTA, 1X PBS and heptane. The fix solution and heptane were removed and the ovaries were washed in PBST. The ovarioles were separated by pipetting the whole ovaries onto the frosted part of one slide and then rubbing a second slide gently over the first. The ovarioles were treated with 2.67,ug/mL of proteinase K for 3 minutes. Hybridization with miRCURY LNA Detection Probe occurred overnight in a 1:500 concentration with hybridization buffer (50% formamide, 5x SSC, 100,ug/mL heparin, 100,ug/mL sonicated salmon sperm DNA and 0.1% Tween-20) and a hybridization temperature of 48°C was used. Ovarioles were blocked in a 1% milk and PBST. The primary antibody staining consisted of a 1:2,000 concentration of preabsorbed anti-DIG-AP in blocking solution. Signal was detected using 20,uL of BCIP/NBT per mL of staining buffer (100 mM Tris pH 9.5, 100mM NaCl, 50). Once signal was detected ovarioles were washed in PBST and mounted in 70% glycerol.
Stage 4 embryos were used to detect nascent transcripts of the miR3-6 cluster. The miR3-6 cluster DIG-RNA- probe was made from a genomic clone (Bushati et al., 2008). It was detected with Bio mouse anti-DIG, Strep-HRP, and Try-Cy3/DAPI (http://www.utoronto.ca/krause).

2.7 Dot Blot
DNA oligos consisting of entirely complementary sequences and two nucleotide mismatches to probes miR-13b and miR-306 were transferred to a nylon membrane using a dot blot apparatus. 10 ug of DNA were applied to each dot. The hybridizations to LNA probes were done as mentioned above for the Northern blots.

2.8 Generation of Transgenic Flies
The vector containing miR-309 genomic DNA in pUASp was provided by the Cohen lab and was injected using random P-element insertion by Genetic Services, Inc. Expression was driven using nanos-Gal4-VP16 (NGV) (Tadros et al., 2007a).

2.9 Computational Analysis
The web based programs TargetScan (Kheradpour et al., 2007; Ruby et al., 2007) and PITA (Kertesz et al., 2007) were used to predict the putative targets of the various miRNAs in each of the four defined classes of miRNAs. The overlap of predicted targets between TargetScan and PITA were used for the analysis. To determine if miRNA target sites of the four classes of miRNAs were enriched in the various lists of maternal transcripts defined by our lab (Benoit et al., 2009; Tadros et al., 2007a), the hypergeometric values were calculated by using a modified version of MATLAB. If a value is below the Bonfferoni threshold (q= 0.05/20= 0.0025), it is
considered significant enrichment. The value is divided by 20 because that is the number of hypergeometric values that were calculated in total, and this ensures that the values calculated that are below the Bonferroni threshold are significant.
CHAPTER 3

RESULTS

3.1 Gene Expression Profiling Reveals Distinct Temporal Classes of miRNAs in Early Development

I conducted a microarray analysis to look at the abundance of *Drosophila* miRNAs in activated unfertilized eggs, early embryos and stage 14 oocytes. Previously, Aravin et al. (2003) had described the small RNA profile of various stages of *Drosophila melanogaster*. In their study, they identified 62 unique miRNAs that were encoded by 68 genes. Twenty of the miRNAs they discovered were verified by Northern blots in 0-1, 1-2, 2-4, 4-6, 6-12, and 12-24 hour embryos, L1, L2, L3 stage larvae, pupae, and also adult male and female flies. Similarly, Leaman et al. (2005) looked at the expression of all the previously known *Drosophila* miRNAs in the same embryo time points and stages. Both studies indicate that *Drosophila* miRNAs are in fact temporally regulated throughout development and adulthood. These authors did not examine miRNA abundance in mature oocytes or in activated unfertilized eggs.

For my microarray analysis, I examined miRNA abundance in 0-3 hour and 3-6 hour activated unfertilized eggs, 0-2 hour and 2-4 hour embryos. I used stage 14 oocytes as my reference. Thus, I was able to identify miRNAs that are temporally regulated during very early development in *Drosophila*, to distinguish those that are maternally deposited, those that are zygotically expressed, and those both maternally deposited and zygotically expressed. This was made possible by the fact that there is no zygotic transcription in unfertilized eggs, while in 2-4 hour embryos zygotic transcription begins. RNA isolated from our various time points were hybridized to arrays consisting of LNA probes for 68 *Drosophila* miRNAs. LNA probes have
been shown to be ideal for the detection of small RNAs and this is due to a modification of the ribose ring (Valoczi et al., 2004).

A heat map displaying the results from my microarray analysis suggests that four temporally distinct classes of miRNAs are present in *Drosophila* during very early development (Figure 3-1). Class I ("highly unstable") miRNAs appear to have low abundance in activated, unfertilized eggs and early embryos relative to the stage 14 oocytes, which are indicated in green. Class II ("unstable") miRNAs decrease in abundance in 3-6 hour activated unfertilized eggs but not 2-4 hour embryos relative to stage 14 oocytes. Class III ("stable") display no change in abundance in activated, unfertilized eggs and early embryos relative to the stage 14 oocytes. Class IV ("zygotic") miRNAs display significantly higher abundance in 2-4 hour embryos relative to the stage 14 oocytes. miRNAs that are part the same family mostly fall in the same class, but there are some instances in which miRNAs in the same family fall into different classes. This is seen with the miRNAs of the miR-310, 312, 313 family, in which miRNAs in this family fall in Classes II and III. The gene expression profiling studies suggest that *Drosophila* miRNAs are temporally regulated during very early development. Each group of miRNAs will be further discussed and analyzed in detail below.
Figure 3-1. Microarray analysis reveals four temporally distinct classes of miRNAs in activated unfertilized eggs, early embryos and stage 14 oocytes.

Activated unfertilized eggs were collected 0-3 and 3-6 hours after egg laying. Embryos were collected 0-2 and 2-4 hours after egg laying. Each miRNA is indicated by a horizontal rectangle, with black indicating no change, green a decrease, and red an increase in miRNA abundance relative to stage 14 oocytes. The miRNAs are sorted according to their expression profiles. Class I consists of miRNAs that display low signal in activated unfertilized eggs and embryos relative to stage 14 oocytes. Class II consists of miRNAs that display a low signal in 3-6 hour activated unfertilized eggs relative to stage 14 oocytes. Class III consists of miRNAs that display similar abundance in activated unfertilized eggs and embryos relative to stage 14 oocytes. Class IV miRNAs display high signal in 2-4 hour embryos relative to stage 14 oocytes. The *Drosophila* miRNAs on the array that display background levels in all samples are not included in the heat map.
3.2 Analysis of Class I: miRNAs Eliminated Immediately After Egg Activation

3.2a Results of Array Experiment

Class I miRNAs: miR-318, miR-276b, miR-276a, miR-34, miR-317, let-7, and miR-284 display high signal in stage 14 oocytes, but only background levels in both the 0-3 hour and 3-6 hour activated unfertilized egg samples, representing approximately a 3-to-20 fold decrease (Figure 3-2A). Figure 3-2A’ shows a similar 3-to-30 fold decrease in 0-2 hour and 2-4 hour embryos.
Figure 3-2. Class I miRNAs display high signal in mature oocytes but not activated unfertilized eggs or embryos.
Looking at the raw signal from the microarray indicates miR-318, miR-276b, miR-276a, miR-34, miR-317, let-7 and miR-284 are abundant in stage 14 oocytes but not activated unfertilized eggs or embryos and appear to be degraded upon egg activation. (A) Signal intensity of miRNAs in stage 14 oocytes, 0-3 hour, and 3-6 hour activated unfertilized eggs. (A') Signal intensity of the same miRNAs in stage 14 oocytes, 0-2 hour, and 2-4 hour embryos.
3.2b Verification Experiments

To verify the microarray data, I carried out Northern blots of miR-318, miR-276a and miR-276b, all of which were confirmed as present in the stage 14 oocyte samples, but not detected in 0-2 hour embryos (Figure 3-3A). Figure 3-3B, which examined 20 minute time intervals after egg activation, shows that there is a sharp drop in the amount of miR-318 from the stage 14 oocyte sample to 0-20 minute embryos once egg activation occurs. These results are consistent with those of Leaman et al. (2005), which indicate that miRNAs in this first group are not detected in embryos after egg activation.

Our stage 14 oocyte sample contains RNA present in the oocyte as well as any RNA that remains in the carcass of the follicle cells. To assess whether Class I miRNAs are in the cytoplasm of stage 14 oocytes, or the follicle cell carcass, I conducted Northern blots on cytoplasm sucked out of stage 14 oocytes (Figure 3-3B) as well as carcasses left after extrusion of oocyte cytoplasm (Figure 3-3C). miR-318 signal was not detectable in the sucked out cytoplasm (Figure 3-3B, lane 1), but was found in the RNA extracted from carcasses (Figure 3-3C, lane 1). Because the Northern blot in figure 3-3B was done only once, and the background is high, the experiment should be repeated. To confirm the previous observation, I looked at the expression of miR-318 in stage 10 and stage 14 oocytes via in situ hybridization (Figure 3-3D). miR-318 is expressed in the somatic follicle cells that surround the developing stage 10 oocyte as well as the carcasses of the follicle cells found in the outer shell surrounding the stage 14 oocyte. miR-312 is an example of a miRNA that has been detected in ovaries in both the germline cells (nurse and oocyte) and somatic cells (Riech et al., 2009), and remains present in embryos after egg activation (Leaman et al., 2005). It would have been beneficial to use this miRNA as a negative control along with my experiments.
In summary, Class I miRNAs (using miR-318 as the representative) are expressed in follicle cells and are not detected in the oocyte. This result is consistent with the fact that, for activated eggs or embryos, bleach treatment removes the carcasses of the follicle cells. It is interesting that RNA remains in the carcasses of the follicle cells that surround the stage 14 oocyte as these have been reported to die after secretion of the egg shell (Nezis et al., 2002).
Figure 3-3. Verification of the microarray data indicates Class I miRNAs are present in the chorion surrounding the stage 14 oocyte rather than the cytoplasm of the oocyte. *Drosophila* embryos, oocytes and ovaries were collected to verify the temporal and spatial expression of miR-318, miR-276a and miR-276b through Northern blot and *in situ* hybridization analysis. Ethidium bromide staining of the 2S rRNA is used as a loading control (Aravin et al., 2003; Leaman et al., 2005). (A) Northern blots verify the presence of miR-318, miR-276a and miR-276b in whole stage 14 oocytes, while miR-318 is not detected in 0-2 hour embryos. (B) Northern blot showing that miR-318 is present in whole oocytes (lane 2), but not detected in the cytoplasm of stage 14 oocytes (lane 1) or in embryos after egg activation (lanes 3-5). (C) Northern blot revealing that miR-318 is present in whole ovaries (lane 3), whole stage 14 oocytes (lane 2) and the outer shell of stage 14 oocytes (lane 1). Again miR-318 is not detected after egg activation (lane 4). (D) *In situ* hybridization indicating the presence of miR-318 in follicle cells surrounding a developing stage 10 oocyte (indicated by the red arrows). miR-318 is also detected in the outer egg shell of a stage 14 oocyte where the nuclei of the dead follicle cells have fallen out (indicated by the red arrows). There does not appear to be signal in the cytoplasm of the stage 14 oocyte.
3.2c Computational Analysis

Consistent with the fact that Class I miRNAs are present in follicle cells rather than oocytes, the target sites of these miRNAs are not significantly enriched in any of our lists of maternal transcripts (Figure 3-4).

<table>
<thead>
<tr>
<th>Class I</th>
<th>P (Hypergeometric test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal vs. Nonmaternal</td>
<td>0.0845</td>
</tr>
<tr>
<td>Unstable vs. Stable maternal (UFE)</td>
<td>0.3799</td>
</tr>
<tr>
<td>SMG-dependent vs. SMG-independent (UFE)</td>
<td>0.956</td>
</tr>
<tr>
<td>Unstable vs. Stable maternal (FE)</td>
<td>0.8485</td>
</tr>
<tr>
<td>SMG-dependent vs. SMG-independent (FE)</td>
<td>0.8926</td>
</tr>
</tbody>
</table>

Bonferroni threshold $q = 0.05/20 = 0.0025$

**Figure 3-4. Target sites of the Class I miRNAs are not enriched in maternal transcripts.**
In order for a hypergeometric value to indicate significant enrichment of miRNA target sites, the value must be below the Bonferroni threshold. We looked at target site enrichment in maternal vs. nonmaternal transcripts, unstable vs. stable maternal transcripts in unfertilized eggs (UFE), SMG-dependent vs. SMG-independent unstable maternal transcripts in UFE, unstable vs. stable maternal transcripts in fertilized embryos (FE), SMG-dependent vs. SMG-independent unstable maternal transcripts in FE (Benoit et al., 2009; Tadros et al., 2007a).

3.3 Analysis of Class II: Maternal miRNAs Eliminated in Activated Eggs but Replaced in Embryos

3.3a Results of Array Experiment

Class II miRNAs displayed an approximate 1.5-to-9 fold decrease 3-6 hours after egg activation (Figure 3-5A), while in embryos levels remained high in both 0-2 hour and 2-4 hour
samples (Figure 3-5A’). In several cases, such as miR-13b, the signal in 2-4 hour embryos surpasses that in the stage 14 oocytes and 0-2 hour embryos (Figure 3-5A’), consistent with the possibility that some of these miRNAs are also zygotically transcribed, replenishing the eliminated maternal transcripts. It is also possible that zygotic transcription allows for the protection of the maternally loaded Class II miRNAs, although this cannot fully account for the large increase in abundance of my Class II miRNAs.
Figure 3-5. Microarray analysis indicates that Class II are maternally deposited but do not remain stable in activated unfertilized eggs.

Graphs from the microarray analysis showing the expression of the Class II miRNAs. The raw signal of the various miRNAs in stage 14 oocytes, activated unfertilized eggs and embryos are shown. (A) Signal intensity of miRNAs in stage 14 oocytes, 0-3 hour and 3-6 hour activated unfertilized eggs. (A’) Signal intensity of miRNAs in stage 14 oocytes, 0-2 hour and 2-4 hour embryos.
3.3b Verification Experiments

I carried out Northern blots of miR-2a, miR-306 and miR-13b, which displayed an approximate 5-to-9 fold decrease from the oocyte to 3-6 hour unfertilized egg samples according to the array data. In contrast to the microarray data, the Northern blots suggest that these miRNAs are stable in activated unfertilized eggs (Figure 3-6A). I extended my analysis of miR-13b to 6-8 hour activated unfertilized eggs to ensure that the failure to detect decay was not an artifact of females having held their eggs in the sample used for microarrays. I also analyzed the expression of miR-184 and miR-310 via Northern blots (Figure 3-6B). Although the microarray data revealed an approximate 1.5-to-3 fold decrease in signal from the stage 14 oocytes to the 3-6 hour activated unfertilized eggs, my Northern analysis indicated that miR-184 and miR-310 remained stable.

To assess whether the discrepancy between the microarray and Northern data might be a result of an incorrect LNA sequence, I tested the specificity of the miRNA probes, that had been hybridized to miR-13b and miR-306 by hybridizing these to membranes that contained DNA oligos fully complementary to each probe (match) or DNA oligos containing a two nucleotide mismatch to each probe (mismatch) (Figure 3-6C). The experiments indicated that both miR-13b and miR-306 probes are specific to their targets. At present the reason for the discrepancy between my microarray and Northern results is unknown. For most of the Class II miRNAs Leaman et al. (2005) also found that these miRNAs are present in embryos and that several displayed an increase in expression after zygotic transcription initiates, consistent with my data. However, since Leaman et al. did not examine activated, unfertilized eggs, their data cannot be used to assess my unfertilized egg time-courses.
Figure 3-6. Northern blot analysis suggests that Class II miRNAs remain stable in activated unfertilized eggs, which contrasts the microarray data. The signal of miR-2a, miR-306, miR-13b, miR-184, and miR-312 was observed in stage 14 oocytes, embryos and activated unfertilized eggs in order to verify the microarray data. (A) Northern blot reveals that miR-2a, miR-306, and miR-13b from Class II are present in stage 14 oocytes, embryos and remain stable in activated unfertilized eggs and embryos. (B) Northern blot showing that miR-184 and miR-312 are present in stage 14 oocytes and embryos and remain stable in activated unfertilized eggs and embryos. (C) Dot blot probing for DNA oligos completely complementary to (match) or containing two nucleotide mismatches (mismatch) to miR-13b and miR-306 probes.
3.3c Computational Analysis

Computational analysis indicated that miRNA target sites of the Class II miRNAs are significantly enriched in maternal transcripts relative to nonmaternal transcripts (Figure 3-7), but not enriched in any of the defined subclasses of maternal mRNAs. Tadros et al. (2007) has also shown that miRNAs in this class have target sites enriched in maternal transcripts.

<table>
<thead>
<tr>
<th>Class II</th>
<th>P (Hypergeometric test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal vs. Nonmaternal</td>
<td>4.26E-09</td>
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<td>Unstable vs. Stable maternal (UFE)</td>
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</tr>
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<td>SMG-dependent vs. SMG-independent (UFE)</td>
<td>0.9977</td>
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<tr>
<td>Unstable vs. Stable maternal (FE)</td>
<td>0.8519</td>
</tr>
<tr>
<td>SMG-dependent vs. SMG-independent (FE)</td>
<td>0.803</td>
</tr>
</tbody>
</table>

Bonferroni threshold $q = 0.05/20 = 0.0025$

Figure 3-7. Shows the enrichment of miRNA target sites for Class II miRNAs in the previously defined lists of transcripts. Target sites of miRNAs in Class II are significantly enriched in maternal transcripts relative to nonmaternal transcripts, but not any of the subclasses of maternal mRNAs.

3.4 Analysis of Class III: Maternal miRNAs that Remain Stable Early in Development

3.4a Results of Array Experiment

Class III miRNAs consist of miR-92a, miR-275, miR-92b and miR-311. These miRNAs display high signal in stage 14 oocytes, and the signal remains relatively stable in both the activated unfertilized egg time points (0-3 and 3-6 hour) and embryo time points (0-2 and 2-4 hour) (Figure 3-8).
Figure 3-8. Microarray analysis indicates that Class III miRNAs are maternally deposited in stage 14 oocytes and remain stable in activated unfertilized eggs and embryos. Graphs from the microarray analysis showing the expression of Class III miRNAs. The raw signal of the various miRNAs in stage 14 oocytes, activated unfertilized eggs and embryos are shown. (A) Signal intensity of miRNAs in stage 14 oocytes, 0-3 hour and 3-6 hour activated unfertilized eggs. (A’) Signal intensity of miRNAs in stage 14 oocytes, 0-2 hour and 2-4 hour embryos.
3.4b Verification Experiments

I examined expression of miR-275 on Northern blots and confirmed the miR to be present in stage 14 oocytes, activated unfertilized eggs and embryos. I conclude that miR-275 remains stable in activated unfertilized eggs, consistent with the hypothesis that Class III miRNAs are maternally deposited and remain stable during early development. Leaman et al. (2005) also found that the miRNAs in this group are present in early embryos.

Figure 3-9. Class III miRNAs are present in stage 14 oocytes and remain stable in activated unfertilized eggs and embryos.

I looked at the expression of miR-275 in stage 14 oocytes, activated unfertilized eggs (0-2, 2-4 and 4-6 hour) and embryos (0-2 and 2-4 hour) to verify the microarray data.

3.4c Computational Analysis

As indicated by Figure 3-10 Class III miRNA binding sites are significantly enriched in maternal transcripts relative to nonmaternal transcripts, but not in any of the subclasses of maternal mRNAs relative to the others. Tadros et al. (2007) has also shown that miRNAs in this group contain target sites enriched in maternal transcripts.
Figure 3-10. Shows the enrichment of miRNA target sites for the Class III miRNAs in the various lists of maternal transcripts previously defined. miRNA target sites for the Class III miRNAs are significantly enriched in maternal transcripts relative to nonmaternal transcripts. Target sites are not significantly enriched in any of the subclasses of maternal transcripts.

3.5 Analysis of Class IV: miRNAs Zygotically Transcribed in Early Embryos

3.5a Results of Array Experiment

The Class IV miRNAs display low signal in stage 14 oocytes, 0-3 hour, 3-6 hour activated unfertilized eggs and 0-2 hour embryos (Figure 3-11A), suggesting that these miRNAs are not maternally deposited into the oocyte or follicle cells during oogenesis like the Class I to III miRNAs. In 2-4 hour embryos, however, there is a 2-to-60 fold increase in signal compared to stage 14 oocytes (Figure 3-11A’). The miRNAs that display the highest signal in the 2-4 hour embryos are miR-286, miR-3, miR-4, miR-6, miR-309, and miR-5 as indicated by the red stars in Figure 3-11A’. These miRNAs are all part of the miR-309 cluster (Bushati et al., 2008).
Figure 3-11. Microarray analysis indicates that Class IV miRNAs are zygotically transcribed.

Class IV miRNAs display low signal in stage 14 oocytes and activated unfertilized eggs, but high signal in 2-4 hour embryos, thus indicating they are zygotically transcribed. (A) miRNA signal in stage 14 oocytes, 0-3 hour and 3-6 hour activated unfertilized eggs. (A’) signal of the same miRNAs in stage 14 oocytes, 0-2 hour and 2-4 hour embryos. The miRNAs in this group reveal an approximate 2-to-60 fold increase in expression from the stage 14 oocytes to the 2-4 hour embryo samples. miRNAs part of the miR-309 cluster are indicated by the red stars (Bushati et al., 2008). Our results are consistent with the Bushati et al. data, which also indicates that the miR-309 cluster miRNAs are zygotically transcribed.
3.5b Verification Experiments

miR-6, miR-286 and miR-3 were used on Northern blots to verify my microarray data (Figure 3-12A) and as our representatives of the miR-309 cluster. Consistent with both my data and the analyses by Bushati et al. (2008), 2-4 hours after egg activation high levels of mature miR-6, miR-286, and miR-3 signal was observed ranging between 20 and 25 nucleotides. The pre-miRNAs were also detected for each miRNA (ranging between 60 and 90 nucleotides) in the 2-4 hour embryo samples. Both the mature and the pre-processed miRNAs were not detectable prior to 2-4 hours. Using tyramide signal amplification fluorescence in situ hybridization (TSA FISH), it was possible to detect the nascent transcripts of the miR-3-6 cluster in stage 4 embryos using DIG-RNA probe made from the genomic clone (Figure 3-12B, data provided by Eric Lecuyer). These pri-miRNAs are located in the nucleus as expected.
Figure 3-12 Verification of the zygotically transcribed miR-309 cluster.
The primary, precursor and mature miRNAs of the miR-309 cluster can be detected in embryos. Northern blots and in situ hybridization experiments validate the microarray data and are consistent with the Bushati et al. data (2008). (A) The precursor and mature transcripts for miR-6, miR-286, and miR-3 of the miR-309 cluster are highly abundant in 2-4 hour embryos, but not in stage 14 oocytes or 0-2 hour embryos. (B) Nascent transcripts of the miR3-6 cluster are detected in stage 4 embryos with a Dig-RNA-probe made from genomic clone (Eric Lecuyer). The green staining indicates the pri-miRNA, which can be observed in the nucleus (red) of the embryo.
3.5c Regulation of miR-309 by SMG

miRNAs have been shown to play a role in transcript destabilization. In zebrafish, the zygotically transcribed miRNA, miR-430, has been shown to regulate several hundred maternal mRNAs by facilitating the deadenylation and clearance of these maternal mRNAs during the MZT (Giraldez et al., 2006). In *Drosophila*, the miR-309 cluster (Figure 3-13A), which encodes six miRNAs, has been shown to play a role in destabilization of more than 400 maternal mRNA targets during the MZT (Bushati et al., 2008). Since two thirds of unstable maternal transcripts are SMG-dependent, and much of the zygotic genome is not transcribed in a *smg* mutant (Benoit et al., 2009; Tadros et al., 2007a), I examined expression of the miR-309 cluster miRNAs in *smg* mutant embryos. By assaying miR-6, miR-286 and miR-3 (members of the miR-309 cluster) in *smg* mutant embryos, I observed that the expression of the miR-309 cluster is absent in *smg* mutants (Figure 3-13B). Not only are the mature miRNAs of the miR-309 cluster not expressed in a *smg* mutant, but also the precursor miRNAs are absent (data not shown). This indicates that the transcription of the miR-309 cluster is SMG-dependent.

Because the zygotic transcription of the miR-309 cluster requires SMG, I predicted that transcript destabilization by the miR-309 cluster would also be SMG-dependent. Comparing the 406 of the 410 transcripts destabilized in the miR-309 cluster mutant (from Bushati et al., 2008) in wild-type and *smg* mutant embryos, I determined that about 85% of the miR-309 dependent transcripts are stabilized in the *smg* mutant (Figure 3-13C, data from Chun Hua He). A hypothesis for the mechanism of miR-309 destabilization is shown in Figure 3-13D. SMG is responsible for the destabilization of many maternal transcripts that act as repressors of zygotic transcription, including of the miR-309 cluster. Once these maternal transcripts are eliminated, the miR-309 cluster is transcribed and destabilizes additional maternal transcripts.
A

2R ← miR-309 cluster CG15125 ← miR-6-5 miR-6-3 miR-6-2 miR-6-1 miR-5 miR-4 miR-286 miR-3 miR-309 → CG11018

B

wt  smg

ooocyte  0-2hr E.  2-4hr E.  ooocyte  0-2hr E.  2-4hr E.

miR-6

miR-286

miR-3

28S RNA

C

wt  smg

2-3hr E.

2-3hr E.

-3.0  1:1  3.0

D

SMAUG protein

maternal mRNAs

miR-309 cluster transcription

additional maternal mRNAs destabilized
Figure 3-13. miR-309 expression is SMG-dependent and therefore maternal mRNA destruction by the miR-309 cluster requires SMG.

(A) Schematic drawing of the miR-309 cluster (developed from Bushati et al., 2008). The different colours represent miRNAs with distinct seed sequences. (B) Northern blot comparing miR-6, miR-286, and miR-3 levels in wild-type (left) and smg mutants (right). The expression of all three miRs increases greatly in 2-4 hour wild-type embryos. The three miRs are not present in the smg mutant embryos. (C) Heat map showing 406 of the 410 miR-309 dependent transcripts (from Bushati et al., 2008) in 2-3 hour wild-type and smg mutants relative to stage 14 oocytes. Most of the transcripts are degraded in wild-type embryos indicated by the green, but almost 85% of the transcripts are stabilized in smg mutants indicated by the black. (D) Model for miR-309 destabilization. SMG protein represses maternal transcripts that repress the zygotic transcription of the miR-309 cluster, which plays a role in the maternal transcript destabilization.

3.5d Effects of miR-309 Expression in Oogenesis

To assess whether the miR-309 cluster RNAs are sufficient to destabilize maternal mRNAs, I expressed miR-309 during oogenesis. miR-309 genomic DNA was cloned into pUASp and expression was driven by nanos-Gal4-VP16 (NGV). To access whether mature miRNAs of the miR-309 cluster were being processed in the transgenic ovaries I carried out Northern blot analysis of miR-6 in two different transgenic lines (Figure 3-14A, lane 2 and 3). The presence of mature miR-6 suggests that the machinery needed to process the mature miRNAs of the miR-309 cluster is present during oogenesis; miR-6 was not detected in the negative control wild-type ovaries (Figure 3-14A, lane 1), but was detected in the positive control, 2-4 hour embryos (Figure 3-14A, lane 4).

Driving the expression of SMG during oogenesis results in a small ovary phenotype (Semotok et al., 2005). Expressing miR-309 during oogenesis had no affect on the ovaries (Figure 3-14B); the ovaries in which miR-309 genomic DNA is overexpressed appear phenotypically the same as wild type ovaries. Twenty ovaries from two different transgenic lines were examined. I conclude that expression of miR-309 during oogenesis is not sufficient to
induce a detectable phenotype. Future experiments will need to assess whether any of the 410 miR-309 target mRNAs are destabilized.

Figure 3-14. Expressing miR-309 genomic DNA during *Drosophila* oogenesis has no phenotypic affect on oogenesis.

(A) Northern blot showing the expression of mature miR-6 miRNA of the miR-309 cluster. Lane one indicates that miR-6 is not expressed in wild-type stage 14 oocytes. Mature miR-6 is expressed in two transgenic lines, in which genomic DNA of the miR-309 is driven by nanos-Gal4-VP16 (lanes two and three). miR-6 is expressed in the positive control 2-4 hour embryos. This northern was only completed once, and since the 2-4 hour embryo sample displays such low signal compared to the other Northern in figure 3-13A, it should be repeated. (B) Dissected ovaries showing genomic miR-309 overexpression during oogenesis (left) and wild-type ovaries (right). The black lines indicate 1mm. There does not appear to be any phenotypic difference between the two sets of ovaries. In the miR-309 overexpression ovaries, the oocytes develop into mature oocytes similar to the wild-type oocytes.
3.5e Computational Analysis

The target sites of the Class IV miRNAs are significantly enriched in maternal transcripts relative to nonmaternal mRNAs. Although the miR-309 cluster has been shown to play a role in maternal transcript destabilization, target sites of these miRNAs are not significantly enriched in unstable maternal transcripts relative to stable ones in either activated unfertilized eggs or in embryos.

<table>
<thead>
<tr>
<th>Class IV</th>
<th>P (Hypergeometric test)</th>
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</thead>
<tbody>
<tr>
<td>Maternal vs Nonmaternal</td>
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<td>Unstable vs. Stable maternal (UFE)</td>
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<td>SMG-dependent vs. SMG-independent (UFE)</td>
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<td>Unstable vs. Stable maternal (FE)</td>
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<td>SMG-dependent vs. SMG-independent (FE)</td>
<td>0.911401</td>
</tr>
</tbody>
</table>

Bonferroni threshold q = 0.05/20 = 0.0025

Figure 3-15. Shows the enrichment of miRNA target sites for the Class IV miRNAs in the various lists of maternal transcripts defined by the lab. Target sites for miRNAs in this group are significantly enriched in maternal transcripts relative to nonmaternal transcripts. Target sites of Class IV miRNAs are not significantly enriched in any of the subclasses of maternal transcripts.
CHAPTER 4
DISCUSSION AND FUTURE DIRECTIONS

4.1 Expression of miRNAs During Early Drosophila Development

4.1a Microarray Verification Issues

Our gene expression profiling experiment was designed to assess temporal changes in Drosophila miRNAs indicative of a possible role in maternal mRNA translational repression in oocytes, eggs and embryos, and/or in maternal mRNA destabilization via either the “maternal” or “zygotic” pathways. Evidence shows that miRNAs play a role in the latter pathway in both zebrafish and Drosophila (Bushati et al., 2008; Giraldez et al., 2006). Also, recently it has been shown that miRNA dependent translational control occurs during Drosophila oogenesis (Reich et al., 2009).

Previous studies of miRNAs in embryos using Northern blots and in situ hybridization (Aboobaker et al., 2005; Aravin et al., 2003; Leaman et al., 2005) showed both temporal and spatial regulation. Our microarray analysis has extended these studies to temporal regulation upon egg activation. Gene expression profiling looking at miRNA abundance in activated unfertilized eggs, early embryos, and stage 14 oocytes allowed us to group miRNA expression into four distinct classes. I have shown that Class I represents a group of miRNAs that are maternally expressed in the follicle cells around the oocyte, but are not detected in the oocyte itself. While my gene expression profiling experiments suggest that Class II consists of a group of miRNAs that are maternally deposited and eliminated in unfertilized eggs but either replaced or stabilized in embryos. My follow-up experiments suggest that this group is stable in unfertilized eggs. Class III consists of miRNAs that are maternally deposited during oogenesis and remain stable early in development, as confirmed by Northern blot data. Both the array data
and follow-up experiments have verified that Class IV consists of miRNAs that appear to be zygotically transcribed. *Drosophila* miRNAs that displayed background levels in all the samples on the arrays are not included in my analysis. This suggests that these miRNAs are not present in any of the selected time points. Because my study looked at mature oocytes and activated, unfertilized eggs in addition to embryos, my analyses provide a more in depth perspective on the temporal expression of miRNAs during early *Drosophila* development than the previous studies.

Three of the four classes of miRs were validated. The exception, Class II, appeared to be unstable in activated unfertilized eggs in the microarray study, but my Northern blot analysis suggests that these miRNAs are in fact stable. One possibility is that the RNA degraded before hybridization to the array. However, the same samples identified Class III miRs at the same time point, so degradation appears unlikely. I also showed that the LNA probes for miR-13b and miR-306 were specific for detecting their targets. Examination of pictures of the spots on the arrays after hybridization with the samples revealed that they were clean with no marks or smudges. Furthermore, the sequences of miRNAs from Class II (miR-13b, miR-2a, miR-306, miR-184, miR-312, and miR-310) are not homologous to any others in the *Drosophila* sequence (BLAST analysis), thus cross-hybridization of such sequences cannot be the case either. As of now, I have no plausible explanation for Class II miRs as to why the microarray data and the Northern blot data contrast.

**4.1b Future Experiments**

Because of expenses, each time point in the activated unfertilized egg and embryo samples included two technical and no biological replicates. It is therefore necessary to redo the microarrays with fresh biological replicates. Since Northern blots indicate that Class II miRNAs
are stable, it is possible that Class II and Class III miRNAs should be combined into a single class. More arrays and Northerns need to be done to verify this possibility.

Another future experiment is to do in situ hybridization of Class II, III and IV miRNAs, which would provide spatial information on the distribution of the miRNAs. Due to the fact that mature miRNAs are so small, their detection has been very difficult through high resolution in situ methods. Previous studies in Drosophila, have looked at the pri-miRNA because it is so much longer than the mature miRNA, and therefore easier to detect (Aboobaker et al., 2005; Stark et al., 2005). In Drosophila embryos, pri-miRNAs have been detected through both the enzymatic alkaline phosphatase method and high resolution tyramide signal amplification fluorescence in situ hybridization. Highly abundant, mature miRNAs have also been detected in Drosophila embryos using LNA probes. For example, mature miR-1 has been detected in Drosophila early embryos (Sokol and Ambros, 2005). In zebrafish, LNA modified oligonucleotide probes have been shown to be ideal for detecting mature miRNAs compared to regular DNA oligos (Kloosterman et al., 2006). Abundantly expressed mature miRNAs in frozen tissue sections have also been successfully detected using LNA modified probes combined with tyramide signal amplification fluorescence in situ hybridization (Silahtaroglu et al., 2007).

Although many methods have been developed to detect the spatial expression of mature miRNAs, there are still technical difficulties in detecting low abundantly expressed miRNAs due to the release or diffusion of miRNAs during early steps of the in situ protocol (Pena et al., 2009). A very recent study has shown that conventional formaldehyde fixation results in substantial miRNA loss from mouse tissue sections (Pena et al., 2009). However, adding an additional fixation step with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) prevents the loss of mature miRNAs into the buffers, thus allowing for the detection of miRNAs that are
not abundantly expressed and miRNAs in subcellular compartments (Pena et al., 2009). These methods should be applied to *Drosophila in situ*.

Since I was able to verify the Class III miRNAs, I propose to do follow-up experiments to further determine the function of these miRNAs during oogenesis and early embryogenesis. I propose to create mutants of the miR-92a and miR-92b cluster, miR-275 (clustered with miR-305) and miR-311 (clustered with miR-310 and miR-312). Because these miRNAs are maternally deposited, I would first look to see if mutating these miRNAs display any type of phenotype during oogenesis or even during early embryogenesis. If the mutants are viable, I would also like to see if there is an affect on transcript destabilization similar to the miR-309 mutant (Bushati et al., 2008). I would compare transcript levels of wildtype ovaries, and embryos to transcript levels of the miRNA mutant ovaries and embryos through microarray analysis to determine if transcripts are upregulated in the miRNA mutants. I also propose to look at the effect of the miRNA mutants at the level of maternal transcript translation. This could be done using the DIGE technique used by Nakahara et al. (2005) to compare the proteome of wildtype oocytes to *dicer* mutant oocytes. If protein levels are upregulated in the miRNA mutant ovaries or embryos compared to wildtype ovaries or embryos, it would suggest that the miRNAs of Class III control maternal transcripts at the level of translation.

4.2 The miR-309 Cluster During the Maternal-to-Zygotic Transition

4.2a Regulation of miR-309 Transcription

The MZT is the time during development when the maternal control of development is transferred to the zygotic genome. The MZT is driven by two processes: the degradation of a subset of maternal transcripts and the transcriptional activation of the zygotic genome (as reviewed by Tadros et al., 2007b). The process of cellular blastoderm formation is the first
developmental event that requires the function of the zygotic genome, at 2.5 hours after fertilization, and this is known as the MBT. Recently, miRNAs have been shown to play a role during the MZT in both zebrafish and *Drosophila* (Bushati et al., 2008; Giraldez et al., 2006). In *Drosophila*, the zygotically expressed miR-309 cluster miRs play a role in targeting maternal mRNAs for turnover via what has been termed the “zygotic” degradation pathway (Bashirullah et al., 1999; Bushati et al., 2008).

My data verifies the miR-309 cluster is expressed at the onset of zygotic transcription along with other miRNAs. I have also shown that the zygotic expression of the miR-309 cluster is dependent upon SMG protein; in *smg* mutants, the miR-309 cluster is not expressed, consistent with the fact that SMG has been shown to be required for high-level zygotic transcription during the MZT (Benoit et al., 2009). In *smg* mutants, there appears to be a block in the full activation of the transcriptional machinery during the MZT, which is indicated by the lack of the accumulation of the phosphorylated form of RNA polymerase II, which is normally observed in wild-type embryos (Benoit et al., 2009). SMG has also been shown to be required for the degradation of many maternal transcripts before the MZT (Tadros et al., 2007a). Benoit et al. (2009) hypothesize that SMG is needed for the destruction of maternal transcripts that encode transcriptional repressors, which allows for an initial wave of transcription that produces the miR-309 cluster. The miR-309 cluster then targets additional maternal transcripts, helping to complete clearance of maternal mRNAs during MZT.

The zing-finger transcription factor Zld has been shown to play a role in activating the early zygotic genome in *Drosophila* (Liang et al., 2008). Zld binds to TAGteam sites, which are *cis*-regulatory heptamer motifs that many of the early transcribed genes in *Drosophila* possess (De Renzis et al., 2007, ten Bosch et al., 2006), to activate transcription. The miR-309 enhancer contains two TAGteam sites, and in the *zld* mutant, the miR-309 primary transcripts are absent.
Therefore, Zld, along with SMG, is required for the expression of the miR-309 cluster. It is likely that SMG eliminates transcriptional repression of zygotic genes while Zld is required for transcriptional activation of zygotic genes. Thus SMG and Zld would act in parallel.

4.2b Destabilization of Transcripts by miR-309 Family miRNAs

In the miR-309 mutant, 410 transcripts are upregulated (stabilized) 2-3 hours after fertilization (Bushati et al., 2008). We have shown that destabilization by the miR-309 cluster is SMG dependent since approximately 85% of the miR-309 dependent transcripts are stabilized in a smg mutant (Benoit et al., 2009). Of the 410 transcripts that are upregulated in the mutant, 96 contain 7-mers complementary to the seed of one or more of the miRNAs of the cluster (Bushati et al., 2008). This would indicate that many of the miR-309 cluster dependent transcripts may in fact be indirectly regulated. In addition, I compared the predicted targets of my Class IV miRNAs (including the miR-309 cluster) to the 410 transcripts upregulated in the miR-309 cluster mutant. What I discovered is that only 30 of the 410 upregulated transcripts in the miR-309 cluster mutant overlapped with my list of predicted targets of the Class IV miRNAs (data not shown). This would indicate that miR-309 miRNAs may not directly target all of the 410 transcripts for destabilization and other cofactors may be needed. For example, the miR-309 cluster may directly target certain transcripts that repress maternal transcript destabilization.

Mutant flies with a deletion of the entire miR-309 cluster are viable and fertile (Bushati et al., 2008). My data indicates that driving the expression of genomic miR-309 cluster DNA during oogenesis, when the miR-309 cluster is not normally expressed, has no phenotypic affect on the ovaries. My data also indicates that the mature miRNAs of the miR-309 cluster can be processed in the ovaries during oogenesis. This suggests that the machinery needed to process the pri-miRNA into the mature miRNA is in fact present in the ovaries during oogenesis.
Consistent with this result, both DICER and AGO have been shown to affect the fate of germline stem cells during oogenesis, suggesting that the miRNA pathway is present and functioning during oogenesis in *Drosophila* (Hatfield et al., 2005; Yang et al., 2007). In addition, mature miR-312 has been detected in the ovaries and shown to have the ability to translationally repress reporters (Reich et al., 2009). Although I was able to detect the mature miR-6 in the ovary, I did not detect a phenotype upon driving the expression during oogenesis. This would suggest that either destabilization by the miRNAs of the miR-309 cluster has no affect during oogenesis, or that other cofactors are necessary for destabilization by the miR-309 cluster, and these are not present in the ovary.

### 4.2c Future Experiments

Because expressing genomic miR-309 cluster DNA during oogenesis has no phenotypic affect, it would be interesting to see whether there is an effect at the level of transcript destabilization. For a future experiment, I propose to do a microarray analysis comparing transcript levels in ovaries in which the miR-309 cluster is expressed as compared to wild-type, non-transgenic ovaries. This would allow me to determine if the mature miRNAs of the miR-309 cluster have the ability to destabilize transcripts during oogenesis as they do in the embryo after zygotic transcription. Specifically, I would look at the 410 transcripts that are upregulated in the miR-309 mutant in 2-3 embryos, and see if they are destabilized in the ovaries in which miR-309 is expressed compared to wild-type. Alternatively, it is possible that the miR-309 cluster cannot destabilize transcripts during oogenesis because certain cofactors that work with the miR-309 cluster are not present. For example, certain RNAs or proteins that are strictly zygotically encoded will not be present. Because the misexpression of SMG in the ovaries results in a rudimentary ovary phenotype (Semotok et al., 2005), it would be interesting to see if combining
the misexpression of SMG in the ovary, along with the misexpression of the miR-309 cluster, alters the severity of the SMG misexpression phenotype.
References


