# GENOMIC PROFILING OF MicroRNAs REGULATING TRANSLATION IN *DROSOPHILA MELANOGASTER* EMBRYOS

**A Thesis Submitted to**
**the Graduate School of Engineering and Sciences of**
**İzmir Institute of Technology**
**in Partial Fulfillment of the Requirements for the Degree of**

**MASTER OF SCIENCE**

**in Molecular Biology and Genetics**

**by**
**Özge TÜNCEL**

**November 2008**
**İZMİR**

We approve the thesis of **Özge TÜNCEL**

_____
**Assist. Prof. Bünyamin AKGÜL**
Supervisor

_____
**Assist. Prof. Ayten NALBANT**
Committee Member

_____
**Assoc. Prof. Dr. Kemal KORKMAZ**
Committee Member

24 November 2008

_____
**Assoc. Prof. Dr. Sami DOĞANLAR**
Head of the Department of
Molecular Biology and Genetics

_____
**Prof.Dr. Hasan BÖKE**
Dean of the Graduate School of
Engineering and Sciences

# ACKNOWLEDGMENTS

First of all, I would like to thank and express my gratitute to my supervisor Assist. Prof. Dr. Bünyamin AKGÜL for giving me the chance of studying in this delightful project.

I would like to thank Assist. Prof. Dr. Ayten NALBANT for her guidance, understanding, motivation and excellent support.

I would like to thank my friends and lab mates İpek ERDOĞAN and Hatice YİĞİT, for their sincere help and their good friendship. I also wish to express my thanks to all my other friends working in Molecular Immunology and Gene Regulation Laboratory.

I also would like to thank Assist. Prof. Dr. Selçuk YURTSEVER in Trakya University, Biology Deparment for his support.

Finally, I would like to thank my family for both financial and moral supports they showed in every stage of my life.

# ABSTRACT


## GENOMIC PROFILING OF MicroRNAs REGULATING TRANSLATION IN *DROSOPHILA MELANOGASTER* EMBRYOS

Among the small RNAs, microRNAs are a particular class of 21 to 23 nucleotide RNAs that negatively regulate translation and play a pivotal role in post-transcriptional gene expression. microRNAs are found in many phyla that control such diverse events as metabolism, cell fate, cell death and development.

The aim of this study is to investigate molecular mechanism of miRNA-mediated translational regulation by profiling developmentally important microRNAs according to their translational status and to identify new microRNAs that have roles in translational regulation during the embryogenesis of *Drosophila*. Following RNA purification from different embryonal stages the fractionated RNAs were analyzed by microRNA microarray. Preliminary results show that 9 miRNAs were expressed in both stages whereas 60 miRNAs were accumulated in RNA fractions of 8 hour embryos. Also there are 2 miRNAs in all fractions of both stages in *Drosophila* embryos. It can be concluded that most of them were expressed in late embryonal development and there does not appear to be a switch in microRNA profiles in fractions for different stages of embryos. The preliminary results suggest that microRNAs may suppress protein synthesis at pre-initiation and initiation phases based on the microarray data. Further studies are required to solidify the preliminary findings.

# ÖZET

## *DROSOPHILA MELANOGASTER* EMBRİYOLARINDA TRANSLASYONU DÜZENLEYEN MikroRNALARIN GENOMİK PROFİLLENMESİ

mikroRNAlar protein sentezini negatif olarak kontrol eden ve transkripsiyon sonrası gen ifadelenmesinde öncül rol oynayan 21-23 nükleotid uzunluğunda olup küçük RNAlar sınıfında yer almaktadırlar. mikroRNAlar pekçok filumda metabolik olayları, hücrelerin kaderini belirleyen olayları, hücre ölümü ve gelişimi gibi olayları düzenlerler.

Bu çalışmanın amaçları arasında microRNAlar tarafından düzenlenen translasyonal regülasyon mekanizmasını, gelişim açısından önemli olan mikroRNAları translasyonal statülerine gore profilleyerek aydınlatmaya çalışmak ve *Drosophila* embryogenezini model alarak translasyonu regüle eden yeni mikroRNAlar tanımlamak yer almaktadır. Farklı embryonal dönemlere ait örneklerden RNA saflaştırma işlemi sonrasında fraksiyonlara ayrılmış RNAlar mikroRNA mikroerey yöntemi ile analiz edildi. Ön sonuçlar 9 adet mikroRNA'nın iki embriyonal zamanda da var olduğunu göstermekte iken 60 tanesi 8 saatlik dilimde yoğunlaşmış durumdadır. Ayrıca her iki zaman dilimine ait embriyoların tüm RNA fraksiyonlarında 2 adet mikroRNA bulunmaktadır. Çoğu mikroRNA'nın geç embriyonal zamanda ifade edildiği ve farklı zamanlara ait embryoların RNA fraksiyonlarında mikroRNA profillerinin değişim göstermediği çıkarımı yapılabilir. Mikroerey analizinden elde edilen ön sonuçlara dayanarak mikroRNAların protein sentezini başlangıçtan önce ve başlangıç sırasında etkileyebileceği öne sürülebilir. Daha sonra yapılacak analizler ön sonuçları doğruluğunu kuvvetlendirecektir.

# TABLE OF CONTENTS

# LIST OF FIGURES

# LIST OF TABLES

# ABBREVIATIONS

| | |
|---|---|
| DNA | Deoxyribonucleic acid |
| RNA | Ribonucleic acid |
| mRNA | Messenger ribonucleic acid |
| UTR | Untranslated region |
| ncRNA | Non-protein coding RNAs |
| tRNA | Transfer RNA |
| rRNA | Ribosomal RNA |
| snoRNAs | Small nucleolar RNAs |
| siRNAs | Small interfering RNAs |
| piRNAs | Piwi interacting RNAs |
| miRNA | MicroRNA |
| pri-miRNAs | Primary microRNAs |
| pre-miRNAs | Precursor microRNAs |
| bp | Basepair |
| RISC | RNA-induced silencing complex |
| miRNP | Microribonucleoprotein complex |
| P-bodies | Processing bodies |
| stRNAs | Small temporal RNAs |
| RIN | RNA integrity number |
| Nt | Nucleotide |
| TBE | Tris/Borate/EDTA electrophoresis buffer |
| PAGE | Polyacrylamide gel electrophoresis |
| RNase | Ribonuclease |
| RPA | Ribonuclease protection assay |
| eIF4E | Eukaryotic translation factor 4E |
| PABP | Poly-A binding protein |
| DEPC | Diethylene pyrocarbonate |

# CHAPTER 1

# INTRODUCTION

## 1.1. Translational Regulation

Molecular studies in genomic level of biology focus on flow of information from DNA (deoxyribonucleic acid) to mRNA at transcription step (messenger ribonucleic acid) and to protein at translation step. Both mRNA transcription and protein synthesis steps are regulated transcriptionally and translationally for maintenance of homeostasis during information transference (Garcia, et al. 2005). A typical eukaryotic mRNA, the functional unit of gene expression, is composed of UTRs (untranslated regions) at 5' and 3' ends, coding region, cap structure and poly-A tail parts (Shabalina, et al. 2006). mRNAs are first transcribed as precursor mRNA and modified by capping, poly-adenylation and splicing mechanisms. In the cytoplasm, mRNAs which are properly capped and poly-adenylated are efficiently translated (Figure 1.1) (Mignone, et al. 2002).

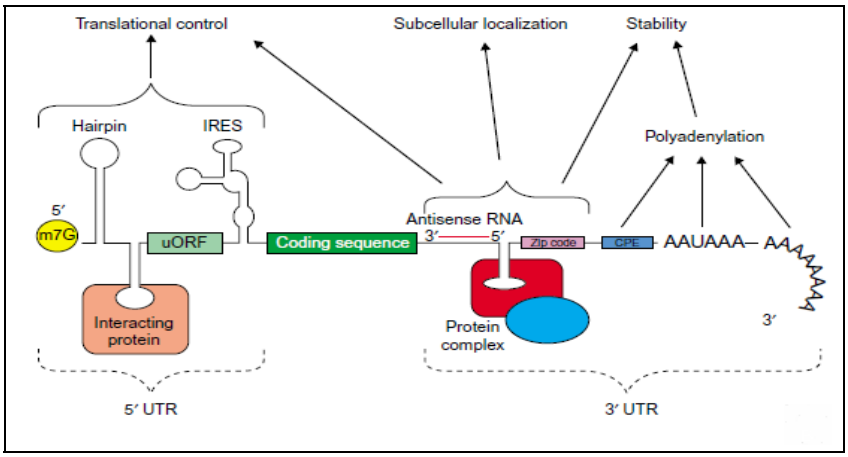


Figure 1.1. The structure of a eukaryotic mRNA includes some post-transcriptional regulatory elements that affect gene expression such as UTR, m7G (cap structure), coding region, poly-A regions (Source: Mignone, et al. 2002)

mRNA translation into proteins occurs in initiation, elongation and termination stages. As the most complex step, initiation is subject to large number of modifications. Initiation of translation starts with recognition of 5' cap by eIF4E (eukaryotic translation factor 4E). Interactions of eIF4E, eIF4G with eIF3 facilitate the recruitment of 40S subunit. Then AUG codon (initiation codon) is begun to search following the joining of 60S subunit for initiation of elongation. Elongation is regulated by phosphorylation of elongation factors. When ribosome encounters a termination codon (UAA, UAG, UGA), translation release factors mediate the termination process. At the end ribosomal subunits dissociate from both mRNA and from each other (Figure 1.2).

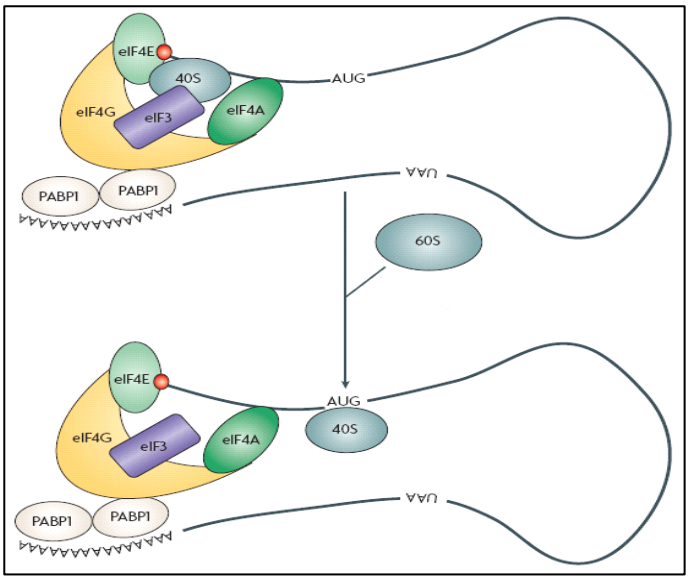


Figure 1.2. General view of translation mechanism is composed of initiation, elongation and termination stages. Translation requires several initiation factors. Many of them are multisubunit complexes. Initiation of translation starts with recognition of 5' cap by eukaryotic translation eIF4E. Interactions of initiation factors facilitate the recruitment of 40S subunit. After joining of 60S subunit and AUG codon elongation is initiated (Source: Filipowicz, et al. 2008).

Translational control is generally mediated by interactions between specific sequences on the mRNA and proteins present in the cytoplasm. In this concept, non-coding sequences on mRNA, 5' and 3' ends are key repositories for the regulation of cytoplasmic mRNAs (Cohen, et al. 2007). 3' and 5' UTR modifications are necessary for efficient translation in terms of specifically binding of diverse proteins. A number of RNA-binding proteins regulate translation through 3′ UTR elements (Hughes, et al. 2008). The importance of UTRs comes from having fundamental roles in control of gene expression at the post-transcriptional level especially during development of

organisms. This approach is underlined by the mutations that altered UTRs can lead to serious pathology (Mignone, et al. 2002). Interactions between sequences in UTRs and specific non-protein coding RNAs (ncRNA) which are any RNA molecule that is not translated into protein have been shown to play key regulatory roles in translation (Hughes, et al. 2008). Non-coding RNA genes include transfer RNA (tRNA), ribosomal RNA (rRNA), small RNAs such as small nucleolar RNAs (snoRNAs), small interfering RNAs (siRNAs) and piwi interacting RNAs (piRNAs).

Among these small RNAs, microRNAs are a particular class of 21 to 23 nucleotide RNAs that negatively regulate translation and play a pivotal role in post-transcriptional gene expression. microRNAs are found in many phyla that control such diverse events as metabolism, cell fate, cell death and development. It has been shown that they have unique tissue-specific, developmental stage-specific or disease-specific patterns (Frash, et al. 2008). Development in RNA silencing world is summarized in Table 1.1.

Table 1.1. Brief history of small RNAs. From 1960s to 2000s, researchers have discovered RNA silencing mechanism and identified small RNAs which regulate different processes in organisms (Source: Zamore, et al. 2005).

| History | Discoveries in RNA silencing world |
|---|---|
| **1960s** | It is proposed that RNA regulates gene expression |
| **1970s** | It is shown that human cells contain double-stranded RNA |
| **1990s** | - First microRNA, *lin-4*, was discovered<br>- RNA that regulate DNA methylation in plant viroids was found<br>- Sense and antisense RNAs that inhibit gene expression in *C. Elegans* were identified<br>- Argonaute proteins that are required for RNAi were characterized<br>- Double stranded RNA that trigger RNA interference (RNAi) and small interfering RNAs (siRNA) are detected. |
| **2000s** | - It is identified that Dicer make siRNAs and microRNAs<br>- MicroRNAs are implicated in cancer researches<br>- Animal viruses found to encode microRNAs<br>- Dicer's functions are discovered<br>- Argonaute defined as "Slicer" as a endonuclease<br>- microRNAs are shown to act as oncogenes<br>- Animal specific microRNAs are identified |

## 1.2. MicroRNA (miRNA) Biogenesis

MicroRNAs are expressed as 21-23 nucleotide RNA molecules initially transcribed by RNA polymerase II as long primary RNAs (pri-microRNAs) (He, et al. 2004). Pri-microRNAs are processed in the nucleus into ~70-80 nucleotide precursor microRNAs (pre-microRNAs) by the RNase III enzyme Drosha. Pre-microRNAs are exported from the nucleus to the cytoplasm by Exportin-5 which has a role in coordination of nuclear and cytoplasmic processing steps. (Gregory, et al. 2005a) Dicer cleaves the cytoplasmic pre-microRNA approximately 21-23 bp (basepair) from the Drosha cut site. In order to control the translation of target mRNAs, the double-stranded RNA produced by Dicer is separated by helicase enzymes. After Dicer processing, the miRNA duplex is unwound and the mature miRNA binds to an Argonaute (Ago) protein in a process that is referred to as miRNA loading or assembly, while the miRNA strand is degraded (Liu, et al. 2007). The single stranded mature microRNA is associated with the RISC (RNA-induced silencing complex). The core proteins of RISC appear to be Argonaute proteins which include PAZ and Piwi domains, Dicer and double-stranded RNA binding protein (TRBP) **(**Moor, et al. 2005**)**. It has been demonstrated that Argonaute 2 is the catalytic center of RISC (Gregory, et al. 2005). Piwi domains' function is unknown but PAZ domain forms a nucleic acid binding site for binding of RNA. In *Drosophila* it is identified that there are two Dicer proteins (Dcr-1 and Dcr-2) with distinct functions in miRNP complexes. Dcr-1 is responsible for microRNA production and is an essential protein that is required for fly development, whereas Dcr-2 is required for siRNA production from long dsRNA (Liu, et al. 2007). In addition to these molecules R2D2, Ago2, Loquacious and Ago-1 mediate microRNA silencing.

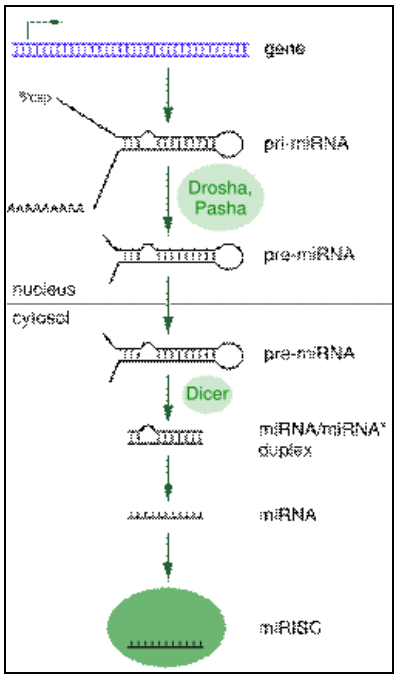


Figure 1.3. Step-wise processes involved in microRNA biogenesis. RNA Polymerase II transcribes the microRNA gene to a primary transcript before processing by Drosha to release precursors. Following precursor processing in nucleus, it is transported to cytoplasm by Exportin-5. Final stage of maturation is achieved by RNase III enzyme 'Dicer' (Source: Standart, et al. 2007).

Mature microRNAs are incorporated into a ribonucleoprotein complex (miRNP) (Figure 1.3). miRNP complexes that take part in P-bodies (processing bodies) are cytoplasmic components of RNA metabolism. P-bodies contain Dicer-TRBP-Argonaute 2 proteins, microRNAs and artificially bulged target mRNAs (Höck, et al. 2007). Degradation of several mRNAs occurs in P-bodies, which can also act as temporary storage depots for untranslatable mRNAs. Modes of microRNAs and their regulatory effects are different in several organisms.

## 1.3. Diverse Functions of MicroRNAs in Animals

The effects of microRNAs in animals have been shown to regulate several physiological processes including stem cell differentiation, haematopoiesis, cardiac and skeletal muscle development, neurogenesis, insulin secretion, cholesterol metabolism, and immune response (Williams A. E., 2007). Vertebrate animals encode hundreds of different microRNAs that have distinct functions in animal development and diseases.

MicroRNAs regulate not only developmental processes but also up or down regulate different cancer pathways in humans (Table 1.2). They constitute 1% of genes in humans, worms and flies (Robins, et al. 2005). In animal species 0.5-1.5% of total number of genes are microRNA genes. Estimates suggest that 200-300 unique microRNA genes are present in human genome. Also the worm and fly genomes are estimated to contain 100-120 distinct microRNA genes (Ventoso, et al. 2008).

Table 1.2. MicroRNAs have role in different regulatory mechanisms in several organisms. They regulate developmental events, haematopoiesis, and cancer pathways (Source: Wienholds, et al. 2005).

| **microRNA** | **Function(s)** |
|---|---|
| ***Ceanorhabditis elegans*** | |
| *lin-4* | Early developmental timing |
| *let-7* | Late developmental timing |
| *lsy-6* | Left/right neuronal asymmetry |
| *miR-273* | Left/right neuronal asymmetry |
| **Human and other vertebrate cell lines** | |
| *miR-16* | AU-rich element mediated mRNA instability |
| *miR-32* | Antiviral defence |
| *miR-143* | Adipocyte differentitation |
| **Cancer in humans** | |
| *miR-15, miR-16* | Downregulated in B-cell leukemia |
| *miR-143, miR-145* | Downregulated in colonic adenocarcinoma |
| *Let-7* | Downregulated in lung cell carcinoma |
| *miR-17, miR-92* | Upregulated in B-cell lymphoma |

In metazoans early developmental processes such as early embryonic patterning, organogenesis, differentiation, migration, proliferation and programmed cell death are regulated at mRNA transcription, translation and protein degradation steps of gene regulation. Members of microRNA family have been discovered as small temporal RNAs (stRNAs) which regulates larval-adult transition in *C.elegans* (He, et al. 2004). Also microRNAs have been identified in *Chlamydomonas reinhardtii* which is a unicellular alga. These studies indicate microRNAs are probably evolutionary older than originally thought (Filipowics, et al. 2008).

Until now studies indicate that 152 *Drosophila* microRNAs have been identified (Retrieved from www.sanger.ac.uk/Software/Rfam/mirna/ on April 2008). MicroRNAs control apoptosis, cell division, Notch signaling, neural development, and oogenesis in flies (Nilsen 2007). In *Drosophila*, two microRNAs, *bantam* and *miR-14*, were found to be involved in regulation of programmed cell death. Over-expression of *bantam* causes tissue overgrowth and inhibits proliferation-induced apoptosis whereas loss of *bantam* function is lethal. Notch signaling, which is essential for proper patterning and development of all multicellular organisms, is supposed to be regulated by several microRNAs. For instance *miR-7* is responsible for photoreceptor differentiation in the *Drosophila* eye. It is found that *miR-9a* is required for providing generation of sensory organs in *Drosophila* embryos and adults. Knock-out studies show that *miR-1* which is conserved from worms to mammals has a function in *Drosophila* muscle growth (Table 1.3) (Li, et al. 2006).

Table 1.3. List of biological roles of microRNAs in *Drosophila*. They regulate diverse functions especially in developmental processes (Source: Behura K. S., 2006).

| **Biological Role** | **microRNA** |
|---|---|
| Apoptosis | *bantam, mir-6, mir-2* |
| Cardiogenesis | *mir-1* |
| Cellularization | *mir-9* |
| Dorsal closure | *mir-311, mir-312* |
| Fat metabolism | *mir-14* |
| Muscle growth | *mir-1* |
| Notch signaling | *mir-7* |
| Segmentation | *mir-31, mir-313* |

The annealing of the microRNA to the mRNA inhibits protein translation, but on the otherside it facilitates cleavage of the mRNA. Although there are several microRNAs that regulate different events in diverse animal species, the mechanistic details of the functions of microRNAs in repressing protein synthesis are still poorly understood.

## 1.4. MicroRNA-Mediated Translational Regulation

In widely accepted models, binding of microRNA to an mRNA either triggers mRNA cleavage and decay or leads to inhibition of translation, without degrading the mRNA (Stefani, et al. 2008). Increase in the rate of mRNA degradation subscribes to decrease in protein expression (Jackson, et al. 2007). Perfect base pairing is required for mRNA degradation whereas repression mechanism requires imperfect complementarity between microRNA and mRNA transcript. It is speculated that target mRNA-microRNA recognition can be achieved by presence of 2-7 nucleotide seed region in 5' end of microRNA (Ambros 2003) (Figure 1.4).

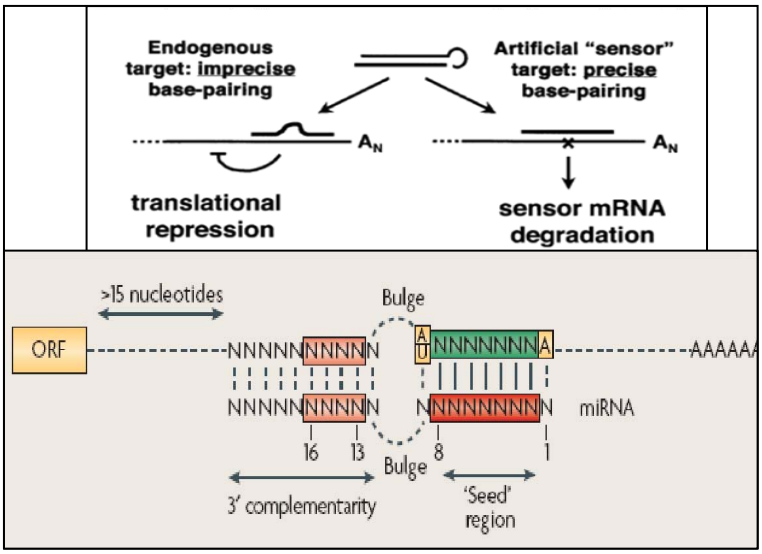


Figure 1.4. Gene silencing by microRNAs and microRNA-mRNA pairing principles in target mRNA-microRNA recognition module. Perfect base pairing is required for mRNA degradation. Imperfect complementarity in the 5' region of microRNAs results in repression mechanism. It has been discovered that mRNA-microRNA recognition can be achieved by 2-7 nucleotide seed region in 5' end of microRNA (Source: Wu, et al. 2006, Ambros 2003, Filipowics, et al. 2008).

In addition to degredation and translational inhibition, recently a third regulatory mechanism has been described in *Drosophila*, in which microRNAs guide the degradation of not perfectly complementary target mRNAs by recruiting deadenylation and decapping enzymes (Carthew R. W., 2006). Studies performed on mammalian cells indicate that reduction in mRNA level is a consequence of accelerated deadenylation which results in mRNA decay (Wu, et al. 2006). Furthermore it is suggested that several

thousand genes are potential targets for regulation by microRNAs in post-transcriptional and translational steps in gene expression.

Viewing gene expression in totality, translation takes part in complex pathway that begins with transcription, continues with RNA processing and transport out of nucleus and ends with protein translocation, modification, folding, assembly and degradation. MicroRNAs' regulatory effects on these steps are diversified. MicroRNAs regulate gene expression by controlling mRNA translation or stability in the cytoplasm. MicroRNAs also modify mRNA structure or modulate mRNA-protein interactions as chaperons (Filipowics, et al. 2008). They were firstly thought to down-regulate the gene expression by inhibiting mRNA at initiation of translation without effecting mRNA abundance.

Translational effects of microRNAs can be divided in 3 parts as initiation, elongation and termination stages. There are possible at least 4 mechanisms about regulatory effects of microRNA's in translation (Filipowics, et al. 2008). First mechanism indicates that the rate limiting step is initiation phase of translation and microRNAs frequently affect the initiation step. It is shown that microRNAs can affect translation initiation by inhibiting the roles of mRNA cap structure and poly-A tail in the absence of accelerated mRNA decay. The function of cap binding protein eukaryotic initiation factor 4E is accepted as a molecular target in initiation mechanism (Humphreys, et al. 2005). In addition it is found that cap structure and poly-A tail have central role in early initiation and both are required for microRNA mediated repression. Several translation factors may interact with Ago protein to inhibit translation machine including cap-binding factor, eIF4E; poly-A binding protein (PABP), interactive proteins between cap structure and poly-A tails and proteins associated with 40S ribosome subunit (Wang, et al. 2008) (Figure 1.5).

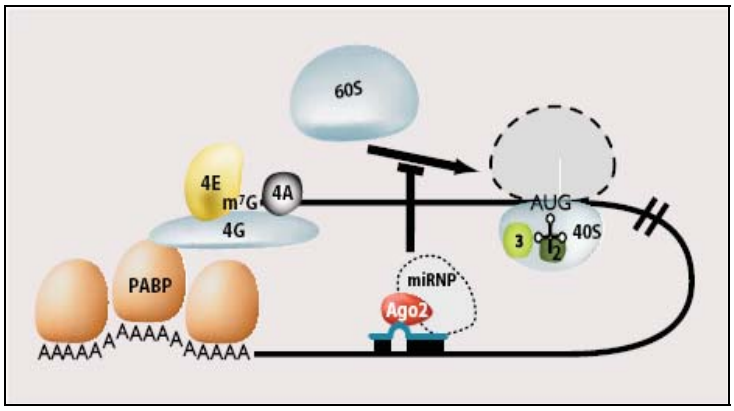


Figure 1.5. A model of microRNA-directed repression of translation initiation. Several translation initiation factors may interact with a recruited Ago protein to repress translation including the cap-binding factor, eIF4. Multicomponent proteins are associated with the 40S ribosome (Source: Wang, et al. 2008).

In the second proposed mechanism, researchers found that eIF6 and 60S ribosomal proteins coimmunoprecipitate with AGO2-Dicer-TRBP complex. eIF6 is defined as a protein which binds to 60S subunit to prevent the pre-interaction with 40S subunit. Binding of miRNP complexes to target mRNA result in repressed 60S subunit joining step. Third, repression may occur in post-initiation stage in translation. It is proposed that the repressed mRNA transcripts are associated with active polysomes. MicroRNAs cause ribosomes involve in slowed elongation or premature termination of translation. This mechanism is termed as ribosome drop-off model. Also the study on Hela cells showed that the vast majority of microRNAs are associated with polyribosomes that indicate the block to translation seemed to be after initiation of protein synthesis (Maroney, et al. 2006). Fourth, proteolytic cleavage of polypeptides was also proposed as a mechanism of microRNA-mediated repression of protein production. The repressed mRNAs at the initiation stage are moved to P-bodies for either degradation or storage (Figure 1.6).

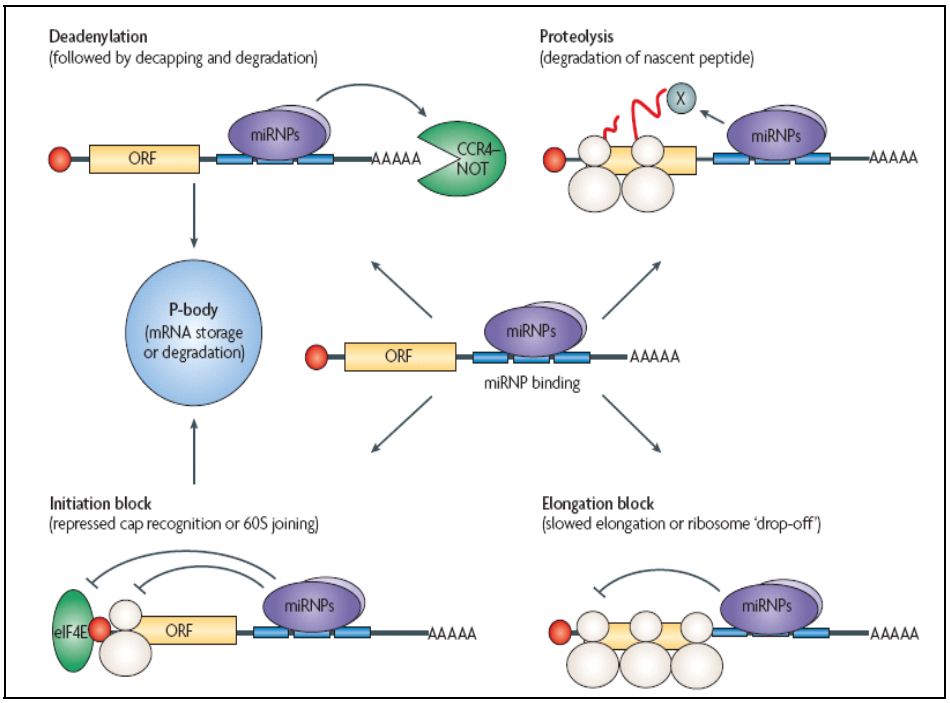


Figure 1.6. MicroRNA mediated repression mechanisms can be grouped in 4 ways as denadenylation, initiation block, elongation block and proteolysis (Source: Filipowicz, et al. 2008).

Until now, several microRNAs have been identified in both adult and embryo stages of development by using total RNA extracts of organisms. In addition, as it has been mentioned before regulatory mechanisms and translational effects of microRNAs have been speculated in studies. The most drastic question to arise from the discovery of the hundreds of different miRNAs is, what are all these noncoding 21-23 nt RNAs doing and where are they in translational machinery? MicroRNAs' location in translational apparatus has not been determined systemically by using *in vivo* models. There is gap in the literature about locations of microRNAs in translational machinery in order to shed light on the potential role of microRNAs in translational regulation at early stages of development. As a consequence, microRNAs need to be profiled based on their translational status.

In dynamism of translational events, an mRNA which is poorly translated or translationally inactive sediments are found in mRNP or monosome (80S) parts whereas translationally active mRNAs are found in polyribosomal (polysome) units of the cell. Monosomes contain one ribosome in contrast; (Therman, et al. 2007) polyribosome parts include more than one ribosome on mRNAs. Profiling microRNA expression in initiation step of translation composed of mRNP, 40S and monosome parts or

translationally active polyribosome parts can provide mechanistic details for determining regulatory functions of microRNAs in translational steps.

In consequence of the presence of extensive translational regulation, early embryogenesis of organisms has been used as an *in vivo* model mechanism in order to investigate translational regulation mechanisms (Sokol 2008). The first few hours of many animals' life proceed with little or no transcription due to the presence of maternal mRNA transcripts. In development of organisms, regulation of embryonic stages is dependent on maternal mRNAs found in the egg cell before fertilization and play prominent roles in early decisions (Prigent, et al. 2007). Maternal transcripts are translationally regulated in the beginning of development in order to manage early decisions. In this study early embryos of *Drosophila* were used as an *in vivo* model in order to understand regulatory mechanisms of translation meditated by microRNAs.

Depending on these criteria the aim of this study is to profile microRNAs according to their translational status by fractionating RNAs from embryos in a time-dependent manner. Fractionating total RNA populations into functional units such as mRNP, 40S, monosome and polysomal RNAs from different ages of fly embryos will provide enrichment for microRNAs that has not been identified systematically before.

# CHAPTER 2

# MATERIALS AND METHODS

## 2.1. Materials

A detailed list of commonly used chemicals, medium, buffers, solutions and their compositions are presented in Appendix B.

## 2.2. Methods

### 2.2.1. Growth of *Drosophila melanogaster*

Embryos of *Drosophila melanogaster* were used as an *in vivo* model organism in this study. The fruit fly *Drosophila* serves as a model system for the investigation of many developmental and cellular processes common to higher eukaryotes, including humans (Sandman, et al. 2007). The fruit fly is preferable because of inexpensive culturing condition, ease of manipulation, rapid life cycle, and large amount of progenies. In *Drosophila*, it is possible to investigate the function of genes in a way that is impossible in humans and impractical in mice (Roberts 1998). In this study adult flies were grown in Nihat Bozcuk media containing 104 gr cornflour, 94 gr sucrose, 19 gr yeast extract, 7 gr agar, 6 ml propionic acid in glass milk bottles covered with cheesecloths and cotton (Figure 2.1.).

Figure 2.1. Adult *Drosophilas* were grown in milk bottles with adult media. Nihat Bozcuk media which includes yeast exract, sucrose, corn flour, agar and acid mixture was used for growing adult flies.

### 2.2.2. *Drosophila* Embryo Collection

The developmental stages of *Drosophila* can be divided into 4 different stages as embryo, larvae, pupa and adult. Embryo stage takes 24 hours, larvae and pupa take 4 days and an embryo becomes an adult fly in 12 days. 0-2 hours and 8 hours embryos were collected by using embryo collection cages. Adult flies were transferred to the embryo collection cage with the embryo grape juice plates inside. Embryo grape juice plates were prepared as follow. Agar (20.25 g) was boiled in 700 ml water. The mixture was cooled down following the addition of 333 ml of grape juice and 135 ml molasses. 11.25 ml of fungicide consisting of 0.1% Nipagin (Applichem) and 1% propionic acid was added to medium. The mixture was poured into styrofoam plates (130-150 ml/plate). Approximately 10.000 flies were transferred to plastic containers for growing adult flies in large numbers. Then nearly $10^6$ flies were transferred from plastic containers to embryo collection cages. Following adult fly transfer the plates with embryos were collected in every 2 hours in a day. Embryos were collected with a solution composed of 0,7 % NaCl and 1% Triton-X 100 and by the aid of mesh and wash basket. Wash basket is prepared from a 50-ml Falcon tube. And the lid of tube was screwed back to hold a 100-250 μm mesh in place. The eggs are collected with a paint brush and transferred to the beaker. The collection materials are illustrated and cage is photographed in Figure 2.2. The embryos were stored in $-80^0$C until use.

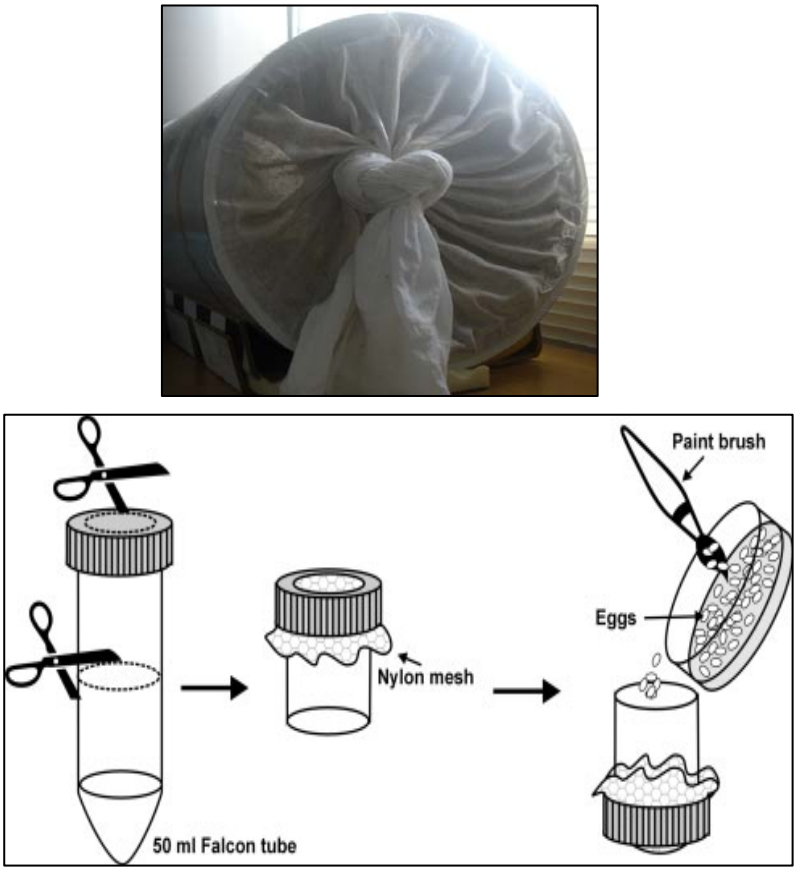


Figure 2.2. *Drosophila* embryo collection cage and embryo collection materials. After growing adult flies in milk bottles, flies were transferred to large cages. Nylon mesh and falcon set up were used for embryo collection as indicated in figure.

## 2.2.3. RNA Isolation

## 2.2.3.1. Fractionation of Microribonucleoproteins

Density gradient centrifugation system is used in order to separate total RNAs based on their molecular weight. Following embryo collection, miRNP complexes RNA samples were isolated by using density gradient fractionation system. The experiment was carried out essentially according to (Bagni, et al. 2000). This experiment can be divided into 3 parts as preparing sucrose density gradient, homogenization of samples and fraction collection steps.

### 1) Preparing Sucrose Density Gradient

In order to make a 5-70% sucrose gradient, firstly 5% and 70% sucrose solutions which contain 1M NaCl, 1M $MgCl_2$, 1M Tris-HCl, SUPERase-in RNase inhibitor (Ambion Inc.) were prepared. Sucrose gradients were prepared in order to analyze RNAs according to their sedimentation rate by using ISCO systems.

### 2) Homogenization of Samples

Embryo samples were homogenized in lysis buffer composed of 100 mM NaCl, 10 mM $MgCl_2$, 30 mM Tris-HCl (pH=7), 30 U/ml SUPERase-in RNase (ribonuclease) inhibitor (Ambion), 1% Triton-X 100, 1% sodium deoxycolate (NaDOC), 100 ug/ml cycloheximide (Applichem). In homogenization step ~0.15 g embryo samples frozen in liquid $N_2$, are homogenized in 5 ml lysis buffer. Followed by 8.minute incubation on ice, the homogenate was centrifuged for 8 min at 12.000 x g at $4^0$C. The supernatant was sedimented in 5%-70% (w/v) sucrose gradient for 2.55 h, at 27.000 RPM, $4^0$C in SW28 rotor (Beckman Coulter (Optima™ L-XP Ultracentrifuge System).

### 3) Fraction Collection

By using ISCO Tris pump while monitoring absorbance at 254 nm with an ISCO UA-6 monitor, 40 fractions were collected from the top of the gradient. The sensitivity and chart speed were set at 1 and 60 respectively. The RNP populations were grouped according to their sedimentation rate: mRNP, 40S, monosome and polyribosome fractions.

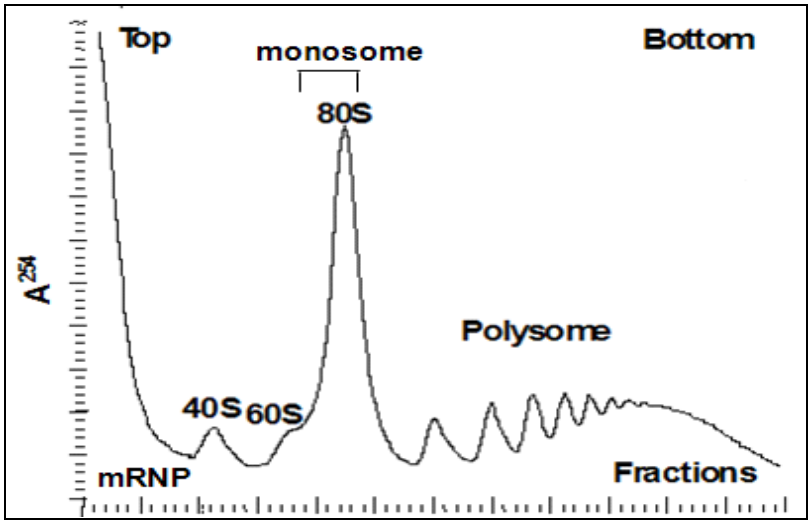


Figure 2.3. RNA fractionation according to translational status by sucrose gradient analysis. Fractions were grouped by their sedimentation rate as mRNP, 40S, monosome and polysome.

Following the addition of 10% SDS and 200 mM NaCl to fractions, phenol-chloroform extraction was applied twice in order to remove proteins. Aqueous phase was recovered after vortexing and centrifuging at 3000 RPM, at $24^0$C, for 5'. The aqueous parts containing RNA were ethanol precipitated in 1/10 NaOAC, 2X 96% ethyl alcohol and stored at $-20^0$C overnight.

Ethanol-presipitated RNA was centrifuged at 12.000 RPM for $4^0$C for 20'. The RNA pellet was washed twice with %70 cold ethyl alcohol and centrifuged at 12.000 RPM at $4^0$C for 5'. Finally the RNA pellet was resuspunded in 200-300 ul DEPC water. RNA concentration was measured by NanoDrop spectrophotometer. Samples were aliquoted and stored at -80C.

## 2.2.3.2. RNA Isolation by Guanidium Thiocyanate Procedure

RNA samples were isolated from adult flies by using guanidium thiocyanate procedure in order to use as a reference RNA while quantifying total RNA samples' quality (Ullrich, et al. 1977). At the first step, 5 g *Drosophila* frozen in $LN_2$ was homogenized in 7 ml denaturing solution with a baked Dounce homogenizor. The lysate was transferred to a baked beaker. Before transfer of solution to quick seal tubes (Beckman Coulter), 1 g CsCl/ml lysate was added and dissolved. Then cushion solution

was added to the tube by the amount of ¼ of the lysate solution and tube was filled with mineral oil. The quick-seal tube was closed by tube topper and centrifuged at 45.000 rpm, 22$^{o}$C for 25 hours in a 70.1 Ti rotor. The RNA flakes were taken by a 1 ml syringe. For precipitation 7 ml DEPC (DiethylenePyrocarbonate)-treated water and 16-20 ml ice-cold 96% EtOH were added to 1 ml RNA sample. After overnight incubation RNA was centrifuged at 12.000 rpm, +4$^{o}$C for 20 min. The pellet was washed in 2-3 ml of ice-cold 70% EtOH, air dried and dissolved in 300 µl DEPC-treated water.

### 2.2.4. RNA Quality Control

RNA quality was determined by Nanodrop spectrophotometer 260/280 ratio, running samples on agarose, polyacrylamide gel electrophoresis and Agilent 2100 Bioanalyzer.

#### 2.2.4.1. Nanodrop Spectrophotometer

Nanodrop spectrophotometer was used in order to measure RNA quality and RNA concentration of samples. Spectrophotometer gives 260/280 and 260/230 ratios. The acceptable RNA should have a 260/280 ratio of 1.8-2.1 and a 260/230 of 1.8-2.0. Also a ratio higher than 1.8 indicate phenol ethanol, phenol, thiocyanate and protein contamination from extraction steps.

#### 2.2.4.2. Agarose Gel Electrophoresis

Agarose gel electrophoresis was used for initial RNA quality control. Agarose gels (1-1.5%) were prepared in 0.5X TBE buffer (Tris/Borate/EDTA electrophoresis buffer). EtBr was added at the concentration of 0.5 µg/ ml from a 10 mg/ml stock and it was poured into a horizontal gel apparatus. RNA samples denaturated in RNA loading dye (Ambion) were loaded into the wells of the gel and they were exposed to an electric constant at 80V. The movement of the RNA molecules could be observed with bromophenol blue present in the gel loading dye. RNA samples were visualized in gel documentation system (Vilber Lourmat).

### 2.2.4.3. Denaturing Polyacrylamide Gel Electrophoresis

Denaturing polyacrylamide gel electrophoresis (PAGE) analysis provides higher resolution for detecting different RNA bands on denaturing gel. Denaturing polyacrylamide gel (12%) was prepared in 10X TBE electrophoresis buffer. Urea was used as a denaturing agent in order to denature RNA samples. After preparing gel mix containing 40% acrylamide, urea, 10X TBE buffer, 10% ammonium persulfate, TEMED, it was poured into vertical gel apparatus. Gel glass-ware and plastic-ware were cleaned by RNase ZAP solution with Kimberly-Clark paper towels (Ambion Inc.) to remove RNase contamination from surfaces. Before loading samples, the system was exposed to an electric constant at 350-400V in order to melt urea residues. Then samples were loaded with gel loading dye following cleaning the wells by a syringe. The gel was exposed to 300V electric constant and incubated at ethidium bromide (EtBr) 0.5 ug/ml solution for 30 min. Then RNA samples were visualized in gel documentation system (Sanger, et al. 1978)

### 2.2.4.4. RNA Quality Control by Agilent 2100 Bioanalyzer

Bioanalyzer was used to assess RNA quality and purity. RNA quality was analyzed and monitored by bioanalyzer RNA 6000 Nano kit and the small RNA kit. According to manifacturer's instructions (Agilent) RIN (RNA integrity number) was estimated and RNA degradation was checked by Agilent 2100 software. RIN higher than 7 indicates relatively intact RNA on a scale of 0-10 (Mueller, et al. 2004).

## 2.2.5. MicroRNA Array for Genomic Profiling

Genomic profiling was performed by Febit Inc. (Germany). 1 ug RNA samples are required to be enriched for small RNAs which are of 20-30-nt in size. These arrays are designed to be composed of oligonucleotide sequences complementary to known miRNAs. A microRNA microarray experiment consists of the following steps: 1) Total RNA isolation and small RNA enrichment or direct small RNA isolation; 2) microRNA

labeling and clean-up; 3) microRNA hybridization to arrays spotted with microRNA probes; 4) Washes and scanning (Liu, et al. 2008). Advantages of DNA microarray tests include high throughput in terms of obtaining several information with one test, and good coverage of the genome with the chips that have larger numbers of test spots (Helling, et al. 1974). Also another advantage of this system opposed to northern blot is that, all known miRNAs can be screened simultaneously.

### 2.2.6. Ribonuclease Protection Assay for Verification of Array Results

Nuclease protection assay is used for monitoring the mRNA levels in gene expression studies. In this study, Ribonuclease Protection Assay (RPA) will be used to validate the array results for detection and quantitation of RNA species. The method is based on hybridization of unknown samples and antisense RNA of known genes. In this study, nonisotopic labeled probes are used for hybridization. Samples are digested with a single strand specific RNase A/T1 and complementary of antisense RNA is quantified. RNase Protection Assay was preferred to northern blot because of accuracy and efficiency. It is also ~20-100 folds more sensitive than northern hybridization.

RPA is composed of an RNA probe synthesis through an in vitro transcription reaction, incubation with a sample total RNA, treatment with ribonuclease, electrophoresis of RNA samples steps. In order to prepare reference RNA to use in RPA, primers were designed according to expression profiles of microRNAs that were studied in 2006 by Aravin, et al. *miR-3, miR-4, miR-8, miR-1* and U1, U2, U3, U6 small RNAs were selected to be cloned. *miR-1* is highly expressed in skeletal and heart muscles in whole development stages of flies, *miR-4* is highly expressed in especially 2 to 12 hours in embryos of flies, *miR-8* is expressed in late larval stages, *miR-31* 1 to 6 hours of embryos of flies. And primers for small RNAs were designed in order to verify the data of sucrose gradient analysis. In order to confirm gradient analysis results U1 and U2 small RNAs which are present in cytoplasm, U3 and U6 small RNAs which are located in nucleus were chosen **(**Hopper A, 2006). They were chosen to indicate that whole cell content was homogenized for profiling of RNA populations. Sequences of these primers are given in Appendix A.

Genomic DNA was prepared and amplified with primers by using single fly PCR method (Ref). One or two fly were placed in squashing buffer containing 10 mM

Tris-HCl pH=8, 1 mM EDTA, 25 mM NaCl, 200 g/ml Proteinase K solutions (Bertucci, et al. 2001). Flies were homogenized by pipetting and incubated at 37$^{o}$C for 30 min. In order to inactivate the proteinase K, solution was heated to 95$^{o}$C for 3 min. The solid junk was spun down and the supernatant was recovered.

The recovered DNA was amplified by using a thermal cycler. PCR reactions contained 4 µl DNA (750 ng/µl), 10 µl 10X PCR buffer, 10 mM dNTP, 5 pmol of each forward and reverse primer, 6 µl of 25 mM $MgCl_2$, 6,5 µl *Taq* polymerase and 57,5 µl $dH_2O$ in a total volume of 100 µl. PCR was carried out according to the following programme:

Step1: 94$^{o}$C for 2 min

Step2: 94$^{o}$C for 45 sec (denaturation)
Step3: 55$^{o}$C for 20 sec (annealing) } 2 cycles
Step4: 72$^{o}$C for 1 min (elongation)

Step5: 94$^{o}$C for 15 sec (denaturation)
Step6: 58$^{o}$C for 30 sec (annealing) } 28 cycles
Step7: 72$^{o}$C for 1 min (elongation)

Step8: 72$^{o}$C for 10 min (final extension)

PCR products were run on 1% agarose gel. DNA was extracted from gel, ligated into pGEM T-Easy Vector (Promega) using TA cloning principle. pGEM T-Easy Vector contains a 3´ terminal thymidine to both ends and *Taq* polymerase amplifies DNA fragments by adenine extensioning. Following transformation, competent cells (DH5α) were spread on LB agar plates with ampicillin/IPTG/Xgal for blue-white colony selection. Selected white colonies were inoculated into 5 ml LB broth with ampicillin. Plasmid isolation was performed by using kit (Fermentas Gene Jet $^{TM}$ Miniprep Kit). Plasmid DNA was cut with EcoRI to control for insertion and sample was confirmed by sequence analysis.

Following the plasmid isolation, the next step was in vitro transcription. For in vitro synthesis, DNA sequence of *miR-3, miR-4, miR-8,* U3 to be transcribed has to be linearized with a restriction enzyme SacI. Also DNA sequence of U2 and *miR-1* to be transcribed has to be linearized with a restriction enzyme NcoI that cleaves downstream

from the region to be transcribed. Transcription of linearized plasmid DNA was performed with Ambion's T7/SP6 Maxiscript transcription kit. This procedure is established as follows:

0,5 ug DNA, 1 ul 10X transcription buffer, 0.5 ul 10 mM ATP, GTP, CTP and biotin labeled-UTP, 0,1 mM 1,5 ul UTP and according to the orientation of the probe 1 ul T7 or SP6 Enzyme mix is put into an RNase free eppendorf, after the volume is adjusted to 10 ul with nuclease free water supplied with the kit, the reaction was incubated at 37 C for 1 hour. In order to remove template DNA, after transcription reaction 0,5 ul DNase I was added and incubated at 37 C for 15 minutes. 7,5 ul of this reaction was taken and 7,5 ul ammonium acetate was added and incubated 15 minutes on ice. Then it was centrifuged at 12000 g at +4C for 15 minutes. Pellet was washed with 70% ethanol and dissolved with 100 ul nuclease free water. Now the labeled probes are ready to use for RPA reactions.

In hybridization step, 0,5 ul non-isotopic labeled probes and 5 ug total RNA samples were precipitated with ethanol and the pellet was dissolved in hybridization buffer (supplied with Ambion's AM1414 kit) and incubated overnight at 42C . Single stranded and unhybridized probes were digested by adding 150ul RNase digestion buffer+1,5ul RNase A/T1 and incubating 30 min at 37C Following RNase is inactivated by 225 ul RNAse inactivation buffer and this reaction is incubated at least 30 min at -20 so as to precipitate protected fragments. Then pellet was dissolved with 5 ul gel loading buffer and the samples was loaded into %12 denaturing polyacrylamide gel and run at 300V for 3 hours.

After running, bands were cut out and blotted on nylon membrane by Owl's semidry electroblotting. In this procedure 6 pieces of 3M paper and a membrane was cut according to size of the gel that will transfer and they were incubated 15 minutes in 0,5 X TBE which was at +4C. On the glass of the electroblotter three 3M paper was layered and the gel was aligned then the membrane was put. Over these three 3M paper was put again. In each step it is important to driving out all bubbles. After forming this sandwich, the blot was performed at constant 500 mA current for 1 hour by EC135 thermo power supply. After this, the RNA molecules were cross-linked to the membrane at 80C for 15 min in oven. Then the membrane was incubated 2 times for 5 min in 50 ml washing buffer containing 1X PBS, 0.5% SDS. Then it was incubated in 50 ml blocking buffer (2% BSA, 10X PBS, 10%SDS). After then the membrane was incubated in 10 ml blocking buffer+ 0.5 ug streptavidin HRP for 30 min. Afterwards,

the membrane was incubated three times in 50 ml washing buffer. Then it was incubated two times in 25 ml 1X assay buffer (supplied by Ambion's Biodetect kit) The last step was incubating the membrane 2.5 luminol enhancer (Biorad Immun-Star HRP) +2.5 ul peroxide buffer (Biorad Immun-Star HRP). Finally, the RNAs on the membrane were detected by imager at the chemiflourescence mode (Biorad).

# CHAPTER 3

# RESULTS AND DISCUSSION

## 3.1. Embryo Collection and Total RNA Isolation by Using Sucrose Density Gradient Centrifugation System

0-2 hour and 8 hour embryos were collected for total RNA isolation from four groups of fractions by using sucrose density gradient centrifugation system. The polysome profile of 0-2 hour embryos was shown in Figure 3.1. There were mRNP, 40S, monosome and polysome fractions as explained in Chapter 2. 40S includes only 18S rRNA components while monosome and polysome fractions include both 28S and 18S components.

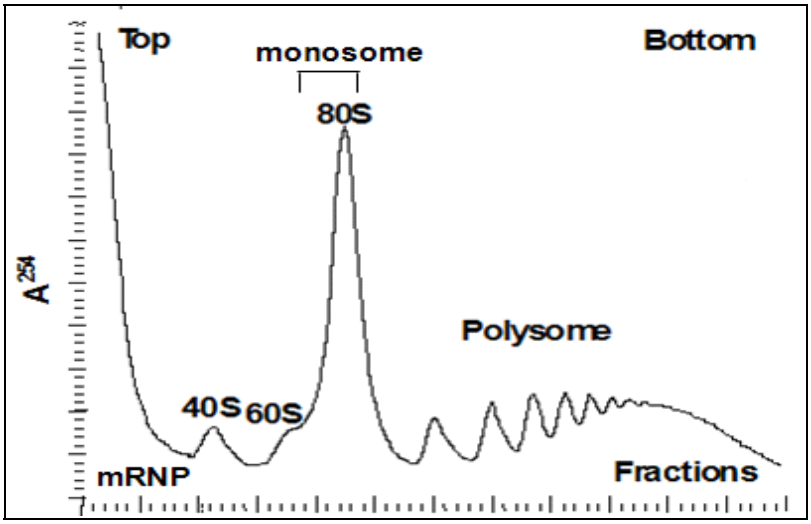


Figure 3.1. Total RNAs of 0-2 hour embryos were profiled according to translational status. Total RNA fractions were composed of mRNP, 40S, and monosome and polysome fractions. RNA quality of fractions was assessed by Bioanalyzer.

Total RNAs were isolated from 0-2 hour and 8 hour embryos as 3 replicates. Table 3.1 indicates average RNA yield and RNA enrichment percentages of each fraction. Approximately 1000 ug total RNAs were isolated from 0.15 g embryo samples for each 0-2 hour and 8 hour embryos. Total RNA samples were divided into fractions depending on their translational status to enrich RNA samples in terms of fraction

content. Using fractionation system provides to profile microRNAs depending on their location in translation apparatus. Enrichment of RNAs by fractionation analysis can provide determining and cloning of rarely expressed microRNAs in *Drosophila* development.

Table 3.1 RNA enrichment of fractions belonging to different developmental stages. Fractionation system provides enrichment in terms of total RNA content of each fraction.

| **RNA concentration in average** | **RNA enrichment of fractions** | **Fractions** |
|---|---|---|
| 25 ug | 40 X | mRNP |
| 15 ug | 66 X | 40S |
| 200 ug | 5 X | Monosome |
| 760 ug | 1,31 X | Polysome |

## 3.2. Polyacrylamide Gel Electrophoresis of Total RNA Samples

After RNA isolation from embryo samples, 3 replicates of 0-2 hour and 8 hour embryo RNA samples were visualized on 12% denaturing polyacrylamide gel (Figure 3.2).

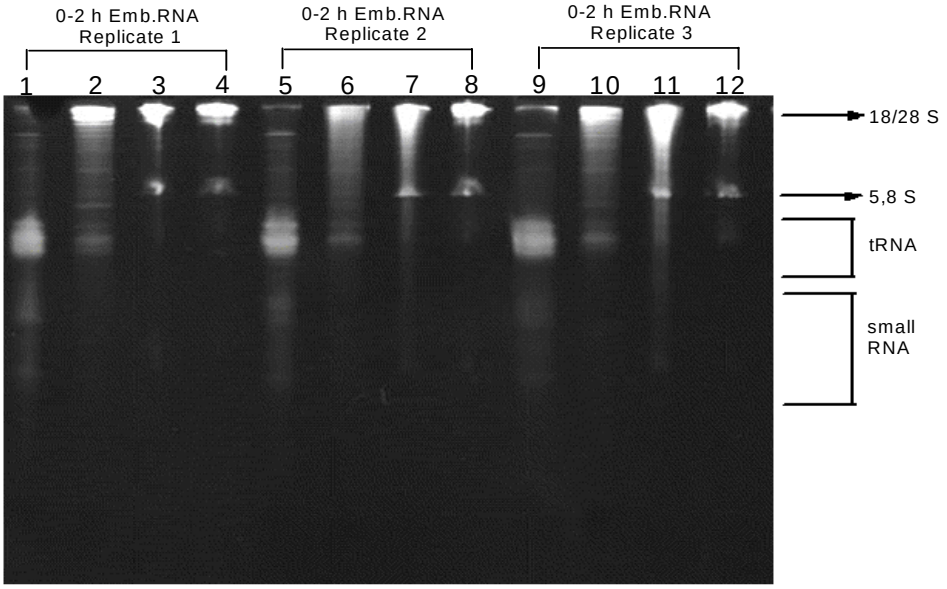


Figure 3.2. Total RNA quality of 0-2 hours fly embryos were visualized in polyacrylamide gel electrophoresis. Arrows indicate rRNA bands and quartets indicate replicates in Figure 3.2. Each replicates is composed of RNA samples that were isolated from mRNP, 40S, monosomal and polysomal fractions.

Gel photograph indicates total RNA samples of 28S, 18S, tRNA, small RNA populations without any degradation. Bioanalyzer results demonstrate that the quality of mRNP, 40S, monosome and polysomal RNA samples were acceptable (Figure 3.3).

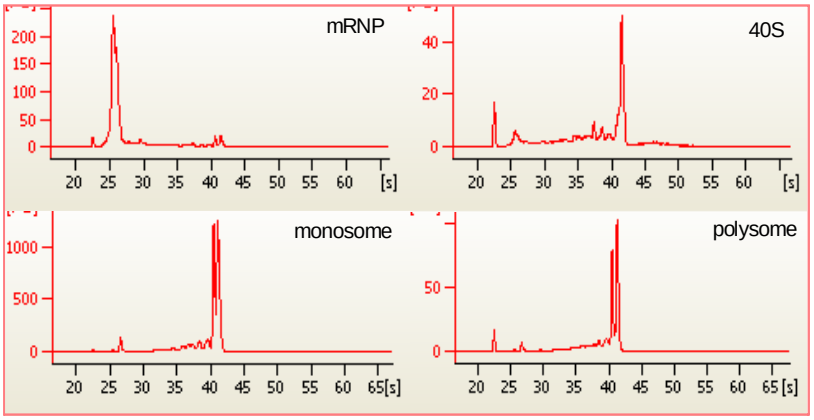


Figure 3.3 Electropherographic RNA quality of embryo samples. A sharp 28S and 18S peak indicates that isolated RNAs are intact and not degraded.

In order to compare RNA quality and test the system, yeast cells and Jurkat T-cell were used instead of *Drosophila* embryo samples for RNA isolation. After sucrose gradient experiment, their total RNA samples were isolated by phenol-chloroform extraction same as *Drosophila* RNAs. Then RNA samples were run on denaturing 12% polyacrylamide gel and bioanalyzer system. In figure 3.4., quartets indicate RNA samples that were isolated from in order of mRNP, 40S, monosomal and polysomal fractions.

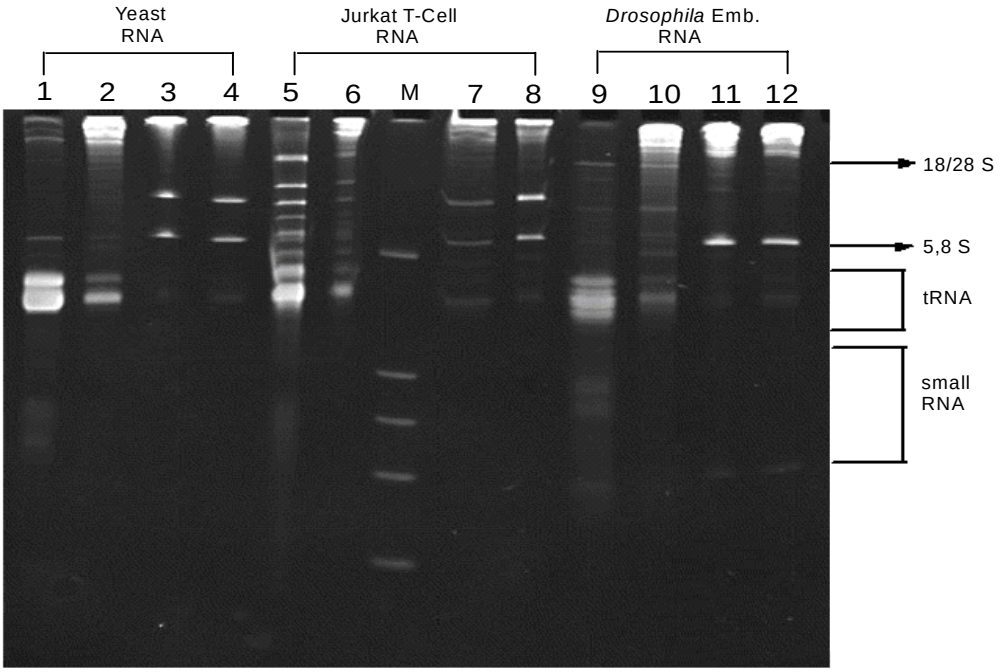


Figure 3.4. The total RNA quality of yeast, Jurkat T-cells and *Drosophila* embryo samples was analyzed in 12% polyacrylamide gel. Sharp bands indicate the acceptable RNA quality for further analysis. First quartet include fractionated yeast RNA samples, second one has Jurkat T-cell RNA samples and last one includes *Drosophila* embryo RNA samples. Each quartet is composed of mRNP, 40S, monosome and polysomal RNA samples.

## 3.3. MicroRNA Array Analysis

microRNA expression profiles of 0-2 hour and 8 hour embryos were analyzed due to expression changes (1,8-fold change cutoff) by miRNA microarray analysis (Febit Holding GMBH, Heidelberg). Microarray chips were composed of 1217 siRNAs, 152 *Drosophila melanogaster* (Dme) miRNAs and 837 non-*Drosophila melanogaster* (11 *Drosophila* species) miRNAs. For each of these groups of 3 biological replicates were analyzed except one of 8 hour's mRNP fractions (Figure 3.5). In the literature, it has been indicated that, diverse microRNAs are expressed in different developmental stages (Aravin, et al. 2006) (Table 3.2).

Table 3.2. Aravin, et al. has identified that microRNAs are expressed in different developmental stages (Source Aravin, et al. 2006).

| microRNA | Expression Time |
|---|---|
| dme-miR-8 | 6. hour-late larval stages |
| dme-miR-87 | 12-24h and L2 |
| dme-miR-9a | 2h-adult |
| dme-miR-1 | whole stages |
| dme-miR-10 | 12-24h to L2 |
| dme-miR-278 | 12-24h- adult |
| dme-miR-7 | 2-6h |
| dme-miR-3 | 1-6 h |
| dme-miR-6 | 2-12h |
| dme-miR-4 | 2 to 12h |
| dme-miR-312 | 0-12 h |
| dme-miR-311 | 0-2h |
| dme-miR-310 | 0-24h |
| dme-miR-2a | 2h-L2 |
| dme-miR-283 | 0-24 |
| dme-miR-124 | 2-4 h and 4-6h to L2 |
| dme-miR-133 | 12-24h, larvae and pupa |

But location of microRNAs in translation based on developmental time has not been pointed out yet. In this study we have aimed to understand translational status of microRNAs in order to shed light on their regulatory roles in protein synthesis. The microRNAs were profiled in microarray data and were presented as hierarchical clustering map. According to map, it can be said that there may be significant changes in miRNA expression profiles of 0-2 hour and 8 hour embryo samples.

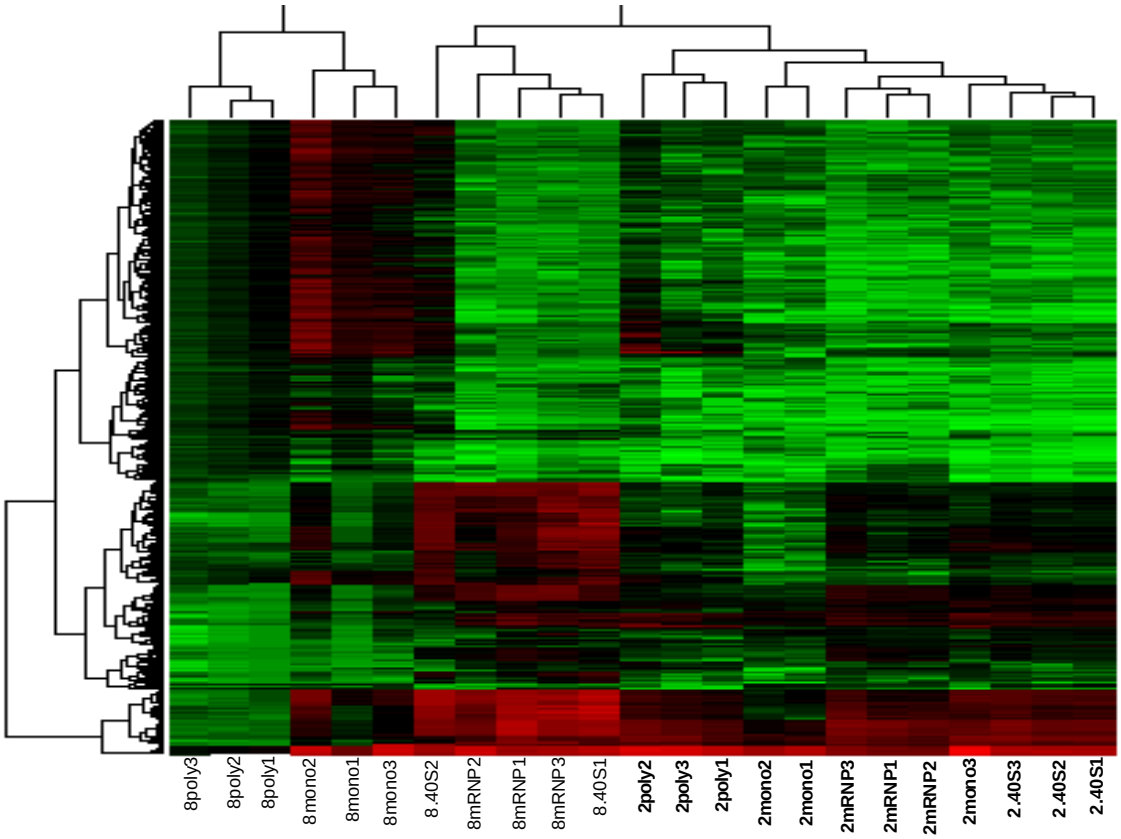


Figure 3.5. Hierarchical clustering map of microRNAs and siRNAs. Cluster of miRNAs that peak at different stages was indicated in the map. Downregulated microRNAs were indicated as red bands whereas unregulated ones were indicated as green bands. Also if there is no change in expression profiles of small RNAs it was indicated as black bands. The RNA samples were labeled at the bottom of hierarchical map. According to map, it can be said that there may be significant changes in miRNA expression profiles of 0-2 hour and 8 hour embryo samples.

According to microarray results, 9 miRNAs (dme-miR-8, dme-miR-275, dme-miR-1012, dme-miR-965, dme-miR-1, dme-miR-2b, dme-miR-1002, dme-miR-124, dme-miR-312) were expressed but in different RNA fractions in both stages of *Drosophila* among the 152 *Drosophila melanogaster* microRNAs. Also there are 2 miRNAs (dme-miR-184, dme-miR-289) in all fractions of both stages of *Drosophila* among the 152 *Drosophila melanogaster and* 60 miRNAs were accumulated in RNA fractions of 8 hour embryos. This distribution was summarized in Table 3.2.

Table 3.3. microRNA distribution was summarized based on their status. 9 miRNAs were expressed in both two stages of *Drosophila* among the 152 *Drosophila melanogaster* microRNAs. There are also 2 miRNAs in all fractions of both stages of *Drosophila* among the 152 *Drosophila melanogaster and* 60 miRNAs were accumulated in RNA fractions of 8 hour embryos.

| **miRNAs according to translational status** |
|---|
| **in all fractions** |
| *dme-miR-184, dme-miR-289* |
| **Mostly 8h** |
| 60 miRNAs |
| **Only 8D** |
| *78 miRNAs* |
| **In 0-2h and 8h** |
| dme-miR-8, dme-miR-275, dme-miR-1012, dme-miR-965, dme-miR-1, dme-miR-2b, dme-miR-1002, dme-miR-124, dme-miR-312 |

On the other side percentage analysis of microarray results indicates that there may not be significant changes in expression profiles of miRNAs in both stages. The fold of change percentages of 0-2 hour and 8 hour embryo samples were in same range. It can be deduced from the data as far as analyzed, the microRNAs may not jump out according to their translational status during embryogenesis. They may be stable during the embryogenesis of *Drosophila melanogaster*. Therefore it can be speculated that microRNAs may suppress protein synthesis at pre-initiation and initiation phases based on the microarray data. Among the mechanisms on regulatory effects of microRNAs in translation, initiational and elongational block proposals can be supported by our data. The microarray data have to be verified by different methods such as ribonuclease protection assay or quantitative polymerase chain reaction.

# REFERENCES


Aravin A.A., Quintana M.L., Yalcin A., Zavolan M., Marks D., Snyder B., Gaasterland T., Meyer J., Tuschl T. 2003. The Small RNA Profile during *Drosophila melanogaster* Development. *Developmental Cell* 5:337–350.

Bagni C., Mannucci L., Dotti C.G., Amaldi F. 2000. Chemical stimulation of synaptosomes modulates alpha -ca2+/calmodulin-dependent protein kinase II mRNA association to polysomes. *The Journal of Neuroscience* 20: 10.

Bertucci P., Lisa A., Mohamed A.F., Noor M.A. 2001. Single fly total RNA preparations for RT-PCR. *Dros. Inf. Serv* 84: 166-168.

Bozcuk A.N. 2000. *Genetik,* Palme Yayıncılık, Ankara.

Campbell and Reece. 2005. *International Biology.* Seventh Edition.

Cohen S. and Bushati N. 2007. MicroRNA functions. *Annual Review of Cell and Developmental Biology.* 23:175-205.

Roberts D.B. 1998. *Drosophila: A Practical Approach.* Oxford New York: Oxford University Press.

Filipowics W., Bhattacharyya N.S., Sonenberg N. 2008. Mechanisms of posttranscriptional regulation by microRNAs: are the answers in sight?. *Nature Reviews Genetics* 9: 102-114.

Frasch M. 2008. A Matter of Timing: MicroRNA-Controlled temporal identities in worms and flies. *Genes and Development* 22: 1572–1576.

Garcia I.A. and Miska E.A. 2005. MicroRNA functions in animal development and human disease. *Development* 132: 4653-4622.

Gilbert S.F. 2004. *Developmental Biology*. Fourth Edition.

Gregory R.I., Chendrimada T.P., Cooch N., Shiekhattar R. 2005a. Human RISC couples microRNA biogenesis and post-transcriptional gene silencing. *Cell* 123: 631-640.

Gregory R.I. and Shiekhattar R. 2005b. MicroRNA biogenesis and cancer. *Cancer Research* 65: 3509-3512.

He L. and Hannon G.J. 2004. MicroRNAs: Small RNAs with a big role in gene regulation. *Nature* 5: 522-531.

Helling R.B., Goodman H.M., Boyer H.W. 1974. Analysis of endonuclease R-EcoRI fragments of DNA from lambdoid bacteriophages and other viruses by agarose-gel electrophoresis. *Virology* 14: 1235-44.

Höck, J., Weinmann L., Ender C., Rüdel S., Kremmer E., Raabe M., Urlaub H., Meister G. 2007. Proteomic and functional analysis of argonaute-containing mRNA-protein complexes in human cells. *Embo Reports* 8: 1052-1060.

Hopper A. 2006. Cellular dynamics of small RNAs. *Critical reviews in Biochemistry and Molecular Biology* 41: 3-19.

Hughes T.A. and Jones P.F. 2008. Regulatory RNAs: have mRNA untranslated regions joined the party?. *Plos Med.* 5: 1-3.

Humphreys D.T., Westman B.J., Martin D.I.K., Preiss T. 2005. MicroRNAs control translation initiation by inhibiting eukaryotic initiation factor 4E/cap and poly(A) tail function. *PNAS* 102: 16961–16966.

Jackson R.J. and Standart N. 2007. How Do MicroRNAs regulate gene expression?. *Science* 2007: 1-14.

Li Y., Wang F., Lee J-A., Gao F-B. 2006. MicroRNA-9a ensures the precise specification of sensory organ precursors in *Drosophila*. *Genes & Dev* 20: 2793-2805.

Liu C.G., Calin A.G., Volinia S., Croce C.M. 2008. MicroRNA expression profiling using microarrays. *Nature Protocols* 4: 1-14.

Liu X., Fortin K., Mourelatos Z. 2007. MicroRNAs: biogenesis and molecular Functions. *Brain Pathology* 18: 113-121.

Maroney P.A., Yu Y., Nilsen T.W. 2006. MicroRNAs, mRNAs, and translation. *Cold Spring Harbour Symp Quant Biology* 71:531-5.

Mignone F., Gissi C., Liuni S., Pesole G. 2002. Untranslated regions of mRNAs. *Genome Biology* 3: 1-10.

Moor C.H., Meijer H., Lissenden S. 2005. Mechanisms of translational control by 3'UTR in development and differentiation. *Cell & Developmental Biology* 16: 49-58.

Mueller O., Lightfoot S., Schroeder A. 2004. RNA Integrity Number (RIN) – standardization of RNA quality control. Agilent Technologies Application Notes. Publication Number 5989-1165EN.

Nilsen T.W. 2007. Mechanisms of microRNA-mediated gene regulation in animal cells. *TRENDS in Genetics* 23: 5.

Prigent S.R. and Rajpurohit S. 2007. Genome, evolution, *Drosophila* and beyond: the new dimensions. *Fly (Austin)* 5:297-302.

Robins H., Li Y., Padgett R.W. 2005. Incorporating structure to predict microRNA targets. *PNAS* 102: 11.

Sambrook J. and Russell D.W. 2001. *Molecular Cloning*. New York, Cold Spring Harbour Laboratory Press.

Sandmann T. and Cohen S.M. 2007. Identification of Novel *Drosophila melanogaster* MicroRNAs. *Plos One* 2: 1-9.

Sanger F. and Coulson A.R. 1978. The use of thin acrylamide gels for DNA sequencing. *FEBS Letters* 87: 107-110.

Shabalina S.A., Ogurtsov A.Y., Spiridonov N.A. 2006. A periodic pattern of mRNA secondary structure created by the genetic code. *Nucleic Acid Res*. 34: 2428-37.

Sokol N.S. 2008. An overview of the identification, detection, and functional analysis of *Drosophila* microRNAs. *Methods Mol Biol*. 420: 319-34.

Standart N. and Jackson R. J. 2007. MicroRNAs repress translation of m7Gppp target mRNAs in vitro by inhibiting initiation and promoting deadenylation. *Genes and Dev.* 21: 1975-1982.

Stefani G. and Slack F.J. 2008. Small non-coding RNAs in animal development. *Nature Reviews*. 9-3:219.

Thermann R. and Hentze M.W. 2007. *Drosophila* miR2 induces pseudo-polysomes and inhibits translation initiation. *Nature* 447: 875-8.

Ullrich A. 1977. Rat insulin genes: construction of plasmids containing the coding sequences. *Science* 196: 1313.

Ventoso C.I. and Vora M. Driscoll M. 2008. Sequence Relationships among C. elegans, D. melanogaster and Human microRNAs Highlight the Extensive Conservation of microRNAs in Biology. *PLoS ONE* 3: 7.

Wienholds E. and Plasterk H.A. 2005. MicroRNA Function in Animal Development. *FEBS Letters* 579: 5911-5922.

Williams A.E. 2007. Functional aspects of animal microRNAs. *Cellular and Molecular Life Sciences* 65: 545-562.

Wu L., Fan J., Belasco J.G. 2006. MicroRNAs direct rapid deadenylation of mRNA. *PNAS* 103: 4034-4039.

Wang B., Yanez A., Novina C. D. 2008. MicroRNA-repressed mRNAs contain 40S but not 60S components. *PNAS* 105: 5343-5348.

Zamore P. D. and Haley B. 2005. Ribo-gnome: The big World of small RNAs. *Science* 309:1519-1524.

# APPENDIX A

## PRIMER SEQUENCES FOR REFERENCE RNA IN RPA PROCEDURE

1. miR-1 primer
   5’-GGGAGCTCGAGAGTTCCATGCTTCCTTGCA-3’ forward primer
   5’-CAGGGATTGTAGAAACGGGTTA-3’ reverse primer
2. miR-8primer
   5’-GAAACCAATGGAATACCGAATCTTGCTAATTAGC-3’ forward primer
   5’-TGCTGCTGTTGGTGGTACTGAAGGTT-3’ reverse primer
3. miR-4 primer
   5’-GGGAGCTCGCTCAGTAAAGTTGCGAGTG-3’ forward primer
   5’-GACTCGTGGTGCTGCTAATG-3’ reverse primer
4. miR-3 primer
   5’-GGGAGCTCTGTGAGCCACCTAAGGTCCC-3’ forward primer
   3’-GGCCACATCGTCGCAACTTC-5’ reverse primer
5. U1 primer
   5’-CATTCCCGGCTACCAAAAATTACACGC-3’ forward primer
   5’-AAGCATACTTACCTGGCGTAGAGGT-3’ reverse primer
6. U2 primer
   5’-ATGAGCTCTACTGCAATACCGGGCCAACCCGCG-3’ forward primer
   5’-CGGCCTTATGGCTAAGATCAAAGTGTAGTATCTGTTC-3’ reverse primer
7. U6 primer
   5’-GGGAGCTCGGAAAGATATCCGGGTGAACTTC-3’ forward primer
   5’-CGATTGTAGATGTGACAAAAAAATGTGGAACGC-3’ reverse primer
8. U3 primer
   5’-GGGAGCTCGGACGGTTGGATCTTCTGGTG-3’ forward primer
   5’-CCCAGAGGAGGAAGACGG-3’ reverse primer

# APPENDIX B

## EMBRYO COLLECTION, RNA ISOLATION AND RPA PROCEDURES

### A. Composition of *Drosophila* Growing Medium and Solutions

1. Adult Fly Growing Medium per liter
    - Cornflour 104 g
    - Agar 7 g
    - Yeast extract 19 g
    - Sucrose 94 g
    - Propionic acid 6 ml

2. Fly Embryo Collection Medium per liter
    - Molasses 25ml
    - Grape juice 50ml
    - Distiled water 1500ml
    - Agar 25g
    - Methyl paraben solution 1g % in 70% alcohol

### B. Solutions for Sucrose Density Gradient Analysis

1. 5% and 70% Sucrose Solutions
    - Sucrose
    - Tris-Cl 30mM pH=7,5
    - NaCl 100mM
    - $MgCl_2$10mM
    - SUPERase-in RNase inhibitor 10U/ml

2. Lysis Buffer for Homogenization of Embryo Samples
    - Tris-Cl 30mM pH=7,5

- NaCl 100mM
- $MgCl_2$10mM
- SUPERase-in RNase inhibitor 10U/ml
- Triton-X 1%
- NaDOC 1%
- Cycloheximide 100 ug/ul

## C. Solutions for Guanidium Thiocyanate Procedure

1. Guanidine HCl denaturing solution (for 100 ml)
    - 4 M Gu HCl (Applichem, A1106,1000)
    - 1 M β-Mercaptoethanol (Applichem, A1108,0100)
    - 0.1 M NaOAC pH 5.2
    - 0.01 M EDTA pH 8.0

2. Saturated CsCl solution (for 50 ml) (Applichem, A1098,1000)

3. CsCl cushion solution
    - 30 ml saturated CsCl solution
    - 0.6 ml 0.5 M EDTA
    - 0.3 ml 5 M NaOAC

4. Mineral oil (Applichem, A2135,0100)

## D. Solutions for Single Fly PCR Method

1. Squashing buffer
    - 10 mM Tris-HCl pH8
    - 1 mM EDTA
    - 25 mM NaCl
    - 200 g/ml Proteinase K