IDENTIFICATION OF COMPLEXES THAT INTERACT WITH tRNA-DERIVED SMALL RNAs by GENE EXPRESSION METHODS

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ABSTRACT

IDENTIFICATION OF COMPLEXES THAT INTERACT WITH tRNA-DERIVED SMALL RNAs by GENE EXPRESSION METHODS

tRNA is a vital molecule of life and has been a focus of biochemical research since long. New aspects of tRNA biology have been drawn to light recently. Accumulating data indicates that tRNA has many diverse biological functions other than its canonical role in coding for amino acids.

tRNA halves are produced by cutting the tRNA molecule from its anti-codon loop by angiognenin and even smaller RNA fragments (tRFs) produced from 5’ or 3’ ends of mature tRNA have been shown to have biological functions, most notable of which is the inhibition of the initiation of protein synthesis.

In our study, the aim was to show the interaction between tRFs and RNA binding proteins. For this reason, two proteins were determined: Rox8 and D-La which are Drosophila homologs of human TIA-1 and La. In the beginning of study, we overexpressed FLAG-tagged versions of these proteins in S2 cells. Then immunoprecipitation experiments were performed with the tagged proteins. At the end of our research the potential interaction between tRFs and tRF related proteins are demonstrated.

ÖZET

tRNA KÖKENLİ KÜÇÜK RNA'LARLA ETKİLEŞEN KOMPLEKSLERİN GEN EKSPRESYONU YÖNTEMLERİYLE BELİRLENMESİ

tRNA, hayatın yaşamsal bir molekülü ve bir süredir biyokimyasal araştırmaların hedefi olmuştur. Son zamanlarda tRNA biyolojisinin yeni yönleri açığa çıkarılmaktadır. Biriken datalar, tRNA'nın amino asit kodlamak gibi bilinen rolünün yanında farklı biyolojik fonksiyonlarda da rolleri olduğunu göstermektedir.

tRNA yarıları, tRNA molekülünün antikodon bölgesinin angiogenin tarafından kesilmesiyle üretilirler ve olgun tRNA'nın 5' ve 3' uçlarından üretilen daha küçük olan RNA fragmentleri (tRFs) de protein sentezinin inhibisyonu gibi en belli başlı biyolojik fonksiyonları da göstermektedirler.

Çalışmamızda, tRF'ler ve RNA'ya bağlanan proteinler arasındaki etkileşimin gösterilmesi amaçlandı. Bu sebeple, insanlardaki TIA-1 ve LA proteinlerinin Drosophila homologu olan iki proteine (Rox8 ve La) karar verildi. Çalışmanın başında bu proteinlerin FLAG-işaretli versiyonları S2 hücrelerinde aşırı-ifade edildi. Sonrasında bu işaretli proteinler sayesinde immunopresipitasyon deneylerini gerçekleştirildi. Çalışmamızın sonunda tRF'ler ve tRF'lerle ilişkili proteinler arasındaki potansiyel ilişki meydana çıkarıldı.

To my family

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CHAPTER 1

INTRODUCTION

1.1. Small RNAs

The evolution of gene expression profiling techniques has positive effect on clarifying the gene regulatory mechanisms that are mediated by variety of non-coding RNAs. Only 1-5% of total RNAs are mRNAs that are translated into proteins. Other major part comes from non-coding RNAs such as ribosomal RNAs (rRNAs), transfer RNAs (tRNAs), small nuclear RNAs (snRNAs) and small nucleolar RNAs (snoRNAs). These non-coding RNA groups are smaller than 200 nucleotides so named as small RNAs (Sobala, et al. 2011). Small RNAs have important roles in gene expression regulation and other uncovered roles. According to recent studies, small RNA molecules have found in diverse organisms. They have roles in translation repression and mRNA stability. Long non-coding RNAs are precursor of small RNAs. These long non-coding RNAs mostly overlap or run antisense to protein coding genes and directly have role on nucleosome assembly or provide a scaffold to recruit chromatin-modifying enzymes (Thebault et al. 2011; Hainer et al. 2011). In addition, they target the epigenetic modifications to specific regions of genome (Finnegan et al. 2003). The overlaps of genetic information cause the sequence similarity but different duty in RNA world. Most of them occur at the end of posttranscriptional cleavage then interact with Argonaute (Ago) proteins and mostly function as mRNA silencers and stimulate proliferation (Tuck et al. 2011).

Recent studies show us that small RNAs are mostly originated from well characterized classes of parent RNAs, such as tRNAs, rRNAs and small nucleolar RNAs (snoRNAs). These newly generated small RNAs simply reflect the parent RNA sequences, but differ in their function from the parents (Tuck et al. 2011).

1.1.1. Small RNA Fragments

1.1.1.1 snoRNA Fragments

Many eukaryotes possess snoRNA fragments, which are generated from small nucleolar RNA (snoRNAs) precursors. They function as miRNA-like RNAs. snoRNA fragments consist of two groups according to RNA modifications. One of them is box H/ACA snoRNAs directing pseudouridylation. Other one is box C/D snoRNAs directing *2'-O*-methylation. snoRNA fragments are approximately 12 to 35 nt in length and generally originate from 5', 3' and central part of snoRNAs. Their length, function and distrubition differs in different organisms. Their processing is like miRNAs and snoRNA fragments also need microprocessor complex for synthesis. However, only H/ACA snoRNAs are synthesized in this way, possibly C/D snoRNAs use another different mechanism which is independent from Drosha/ Dicer. Although their synthesis differs from miRNAs, they probably act like miRNAs (Tuck et al. 2011). The interaction between microRNAs and snoRNAs has been shown as one snoRNA produces a small RNA that functions as canonical microRNAs and has role in gene expression regulation (Ender et al. 2008). Another hypothesis about microRNAs and snoRNAs interaction is that probably microRNAs are affected by snoRNA-dependent small RNA production. According to recent studies, snoRNA ACA36b was annotated as Dicer-sensitive endogenous shRNA (short hairpin RNA) and able to generate microRNAs-like small RNAs (Taft et al. 2009). In addition to functional interactions, another observation about microRNAs and siRNAs is that many pri-microRNA structures include well-defined snoRNA elements (Scott et al. 2009)

1.1.1.2 mRNA Fragments

First discovered and best understood subclass of small RNAs is microRNAs. In 1993, *lin-4* was identified in *Caenorhabtidis elegans* by using genetic screening (Lee et al. 1993). MicroRNAs are produced as 21- 23 nucleotide-long RNAs and are mostly expressed from introns of protein coding genes or as long noncoding RNA transcripts. They are mainly coded in short hairpin structures in intronic sequences. miRNA are also encode from long noncoding RNA transcripts (Tuck et al. 2011). They are expressed

from origin, pri- miRNA forms. These pri-miRNAs are cleaved with Drosha enzyme, which is an RNase III enzyme. After cleavage, pri-miRNAs are processed into pre-miRNAs and they are transported travel from nucleus to cytoplasm with exportin 5. In cytoplasm, pre-miRNAs are further processed into miRNAs by a complex containing Dicer (2). Mature miRNAs regulate gene expression postranscriptionally as miRNA-induced silencing complexes (miRISC) by guide Argonaute (Ago) proteins to 3' untranslated region (3'UTR) of genes(Hee et al. 2004; Lee et al. 2009).

Different organisms have multiple Argonaute proteins that load microRNAs and siRNAs. Argonaute proteins have several domains which are N terminal domain, PAZ domain (contains the binding site for the 3' end of the small RNA), MID domain (contains the binding site for the 5' end of small RNA) and PIWI domain (contains the catalytic center for the cleavage reaction) (Djuranovic et al. 2010). Argonaute proteins have mainly two roles in microRNAs/siRNAs pathway. First is the endonuclease ('slicer') activity, second one is the sequence specific cleavage of complementary targets. In humans, there are four Ago proteins and only Ago2 has slicer activity. Without slicer activity, Ago proteins act as scaffold proteins and cause the recruitment of other proteins to target mRNA and translation inhibition, mRNA degradation and transcriptional silencing occur (Huntzinger et al. 2011). In *Drosophila*, there are three Ago proteins which are Ago1-2-3. The roles of *Drosophila* Ago proteins are similar to human Ago proteins, that they are the catalytic core of RISC that binds short RNAs and, in many cases, displays RNase H-like mRNA-cleaving activity. Mostly, Ago1 acts in miRNA pathway, Ago2 contributes to antiviral siRNA pathway. Lastly, Ago3 acts in piRNA-ping pong mechanism (Obbard et al. 2009).

According to miRBase depository, version 16.0, there are more than 17,000 entries. This huge microRNAs word has pivotal roles in gene expression regulation and almost all biological processes (Sobala et al. 2011). miRNAs induce silencing by inhibition of translation initiation or elongation and the promotion of mRNA decay (Wu et al. 2008). MicroRNAs are also important for developmental programs (Carrington et al. 2003).

1.1.1.3 siRNAs

Examining the RNA interference mechanisms led to the discovery of a second small RNA group called small interfering RNAs (siRNAs) (Hamilton et al. 1999). In addition to their exogenous/ synthetic double stranded roles in cells (from external origins; transgenes, viruses), they can form from transposable and repetitive elements endogenously (shown in plants and animals). Their targets are probably mRNAs and they can regulate the expression of transposons by RNAi pathway (Czech et al. 2008; Lee et al. 2009; Ghildiyal et al. 2009).

1.1.1.4 piRNAs

Another small RNA group is Piwi-associated small RNAs (piRNAs) that were first discovered in *Drosophila melanogaster* and mouse germline cells. They are longer family of small RNAs and approximately 27- 31 nucleotides in length (Cole et al. 2009). This group of small RNAs is generated with Drosha and Dicer independent way by Piwi proteins (Brennecke et al. 2007) and has roles in expression of transposable elements (Cole et al. 2009). They are responsible for germline integrity and fertility (Aravin et al. 2001; Aravin et al. 2006; Girard et al. 2006).

1.1.1.5 rRNA Fragments

rRNA fragments are produced mainly under stress conditions (Sobala et al. 2011). In *Neurospora crassa*, small RNAs derived from rRNAs that are processed by siRNA machinery and loaded into Argonaute homoloug proteins are produced by ultraviolet (UV) damage (Lee and Chang et al. 2009). After loading Ago like proteins, these fragments function in translation machinery inhibition as a response to UV damage.

1.1.1.6 tRNA Fragments

tRNA is the fundamental component of translation machinery. A tRNA has three stem loops: the D loop, anticodon loop and T loop. tRNAs go through some steps before being used in translation. The order of these steps differs in different organisms and tRNA isotypes. To accomplish this duty, primary transcripts (pre-tRNAs) have to be transcribed by RNA polymerase III. Pre-tRNAs contain 5' leader and 3' trailer sequences. For mature tRNA formation these sequences must be cleaved by RNase P/ Z. After removing trailer and leader sequences, a "CCA" sequence is added to most eukaryotic mature tRNAs (Lee et al. 2009). Also tRNAs undergo many modifications which affect the tRNAs' secondary and tertiary structures. When tRNAs are modified correctly, they become lower conformational flexible and higher thermal stabile than their unmodified forms (Helm et al. 2006). After the modifications, tRNAs are aminoacylated and exported from the nucleus to cytoplasm to perform their role in translation.

tRNAs play role not only in translation but also in some cellular processes such as reverse transcription (Lee et al. 2009). Additionally tRNAs play role in cell proliferation and stress response (Thompson et al. 2009).

In addition to miRNA subclass, another class of small RNA originated from tRNA has been reported in previous studies (Cole et al. 2009; Haussecker et al. 2010; Lee and Shibata et al. 2009). Cleavage of tRNAs are mostly occurs in the anticodon loop of tRNAs (Thompson et al. 2009). tRNA derived fragments are tRNA regulatory fragments (tRFs) and stress induced tRNA fragments (also known as tiRNA or half tRNAs). Stress induced fragments are mostly formed by functionary enzymes but unstressed conditions can also cause fragmentation (Thompson et al. 2008). tRFs contain three distinct categories depending on their origin: Dicer dependent (matching to 5' end of mature tRNA (tRF-5), matching to 3' end of mature tRNA (tRF-3)) and Dicer independent (matching to precursor tRNA transcripts (tRF-1)). Basso K. et al have shown that tRF-3s are more abundant than other categories (Basso et al. 2011). In recent studies it has been reported that tRNA fragments can induce transient translational arrest (Yamasaki et al. 2009).

Depending on organisms, the tRF biogenesis and tRF length differs. As an example, in mammals the length of tRFs is approximately 19 nucleotide but in yeast one tRF is observed as 23 nucleotide long (Cole et al. 2009).

tRF-3 class of tRNA-derived small RNAs are 13- 22 nt in length and originate from the 3' end of mature tRNAs. tRNA cleavage occurs within the T-loop of mature tRNA, so mostly tRF-3 group contains "CCA" (Figure 1.1). According to recent studies, the process of tRF-3 synthesis resembles Dicer dependent miRNA synthesis. In addition, Li Zhihua et al. advert the complementarities between human endogenous retroviral sequences and the 3' terminal tRFs but not the 5' tRFs in the genome (Li et al. 2012).

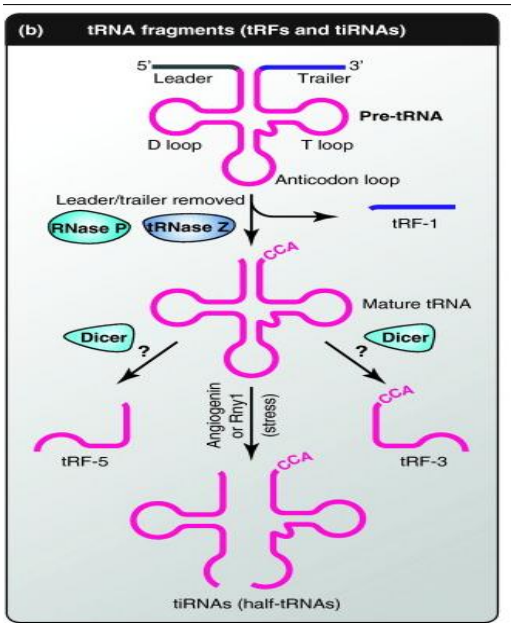

Figure 1.1. Single-stranded, short regulatory tRNA fragments (tRFs) are generated by precursor (pre)-tRNA processing, which releases tRF-1 fragments (trailers), and mature tRNA cleavage, which releases tRF-3/5 fragments. Additionally, stress-induced cleavage by angiogenin or Rny1 releases longer tRNA halves (Adapted from Alex C. Tuck and David Tollervey, 2011).

tRF-5 class is another mature tRNA fragments class. They occur via D-loop cleavage of mature tRNAs. Like tRF-3s, they are mostly Dicer dependent, cytoplasmic and Ago associated.

The cleavage of tRF-3 and tRF-5 from mature tRNAs is not random. With the aid of specific enzymes, there are certain cleavage sites of tRNAs according to circumstances (Lee and Shibata et al. 2009).

tRF-1 class be formed from precursor (pre-) tRNAs leader and trailer sites by the tRNase Z and RNase P (Figure 1.1). tRNA maturation takes place in nucleus, whereas tRF-1 fragments localize in cytoplasm. This situation shows an export mechanism from nucleus to cytoplasm for tRF-1s (Liao et al. 2010). In addition to tRF-1 export, alternative way for tRF-1 production is that pre-tRNAs may be transported from nucleus and cleaved by cytoplasmic RNase Z (Haussecker et al. 2010; Lee and Shibata et al. 2009). Some researches mention about miRNAs and tRFs competition (Babiarz et al. 2008). According to researchers, miRNAs and tRFs compete for Ago binding and this affects the silencing activity of miRNAs. For this reason, the positive effects of tRF-1s in proliferation are speculated (Tuck et al. 2011).

Recent studies suggest that Dicer affects the misfolded pre-tRNAs to generate tRFs. The generation of tRFs from misfolded tRNAs may be an intracellular signal mechanisms. Premature nuclear export or hypomodified tRNAs undergo the cleavage and this situation may stimulate the signaling (Sobala et al. 2011).

Stress induced tRNA halves (tiRNAs) are 30- 35 nt in length, found in diverse organisms and formed from anticodon loop of mature tRNAs. First tRNA halves were reported in *Tetrahymena thermophila*. Then, a wide variety of eukaryotes have been shown to produce a conserved response to oxidative stress (Lee et al. 2005). In yeast and human, especially, Rny1 and Angiogenin (ANG) were found as responsible enzyme for cleavage of tRNAs. In normal conditions, ANG is inhibited with RNH1 in human and Rny1 is found in a vacuole formation in yeast, where their activity is inhibited. However, in stress conditions, these enzymes become free and cleave the tRNAs with limited specificity (Tuck et al. 2011).

About stress induced tRNA fragments, there are some observations from different organisms (Thompson et al. 2009). First assumption is the cleavage of tRNAs are not specific, many tRNAs can undergo endonucleolytic cleavage. However, another assumption about cleavage is that different tRNAs undergo cleavage in different conditions (Sobala et al. 2011). This discovery points that tRNA fragments can be used as information signal. Secondly, because of the accessibility, cleavage mostly occurs in the anticodon loop of tRNAs. Another assumption is that only small proportion of tRNAs undergo cleave, the percentage of tRNA fragments is smaller than full-length tRNAs. Finally, tRNA fragments have no intronic regions, suggesting that fragments come from mature tRNAs (Thompson et al. 2008).

Not all stress conditions induce fragmentation. For instance, in human and yeast, studies show the cleavage occurs in amino acid or glucose starvation and UV radiation (Thompson et al. 2008), but γ-irradiation (Fu et al. 2009), etoposide treatment (Yamasaki et al. 2009) and caffeine treatment (Thompson et al. 2008) do not stimulate the tRNA cleavage. Under stress conditions, tiRNAs inhibit translation or target specific mRNAs for degradation (Thompson et al. 2009). Cleavage of tRNAs provides adaptation to stress conditions by producing more stable RNA products and warn neighboring cells against stress (Tuck et al. 2011; Li et al. 2012). This group of tRNA fragments plays role in eIF2α independent translational repression and stress granule formation (SG). SGs have roles in inhibition of translation initiation and disassembly of polysomes. In stress condition, SGs provide storage for non-translating mRNAs. New suggested role of SGs is the elevation of reactive oxygen species (ROS). In this situation, SG formation inhibits ROS dependent apoptosis (Takahashi et al. 2012). In addition, we know the effect of tRNA fragments on SG formation, but there is not enough information about mechanism between tRNA fragments and apoptosis yet. In yeast studies, Rny1p, which is the homolog of human ANG, promotes tRNA cleavage and activates the downstream pathways, which stimulates cell death. This can be model for yeast to classical example of cytochrome c release from mitochondria promoting apoptosis in human (Thompson and Parker, 2009).

According to today's literature, these tRNA fragments are cytoplasmic. They cofractionated and coimmunoprecipitated with some Ago proteins like miRNAs. This shows that tRNA-programmed RISCs also have some mRNA targets. Not only Ago protein interaction but also tRNA cleavage itself has biologically active roles.

1.2. tRF Related Proteins

In 2011, Ivanov group has observed that stress induced tRNA fragments (tiRNAs) are related with some RNA binding proteins in U2OS cells, when they use immunoprecipitation (IP) with biotinylated 5'-tiRNA^{Ala} and streptavidin antibody. As a reference their study, we examine some of probably related proteins in *Drosophila melanogaster* S2 cells (Ivanov et al. 2011).

1.2.1. Rox8 Protein

SGs are nonmembranous, dense cytoplasmic RNA granules and are composed of several proteins and RNAs. The composition of SGs include stalled initiation complexes which have translation initiation complexes (eIF4E, eIF2, eIF3, poly(A)-binding protein (PABP), small 40S ribosomal subunit) and SGs hallmark proteins (T-cell intracellular antigen 1 (TIA-1), TIA-1 related protein (TIAR) and GTPase binding protein G3BP) (49). The SGs composition differs depending on cellular stress types. Another key component of SGs is mRNA, but the only RNA composition of SGs is not mRNAs. According to reports, tRNA fragments also exist in SGs (Emara et al. 2010).

Rox8 protein is *Drosophila* homolog of human TIA-1 protein. In addition to its key roles in SG formation, TIA-1 protein is an RNA binding protein and has roles in splicing regulation and translational repression, such as by binding of TIA-1 protein to specific U-rich sequences in 3' non-coding region of TNF-α mRNA causes the translational block. TIA-1 is widely expressed in skeletal muscles, additionally (Kedersha et al. 1999; Kedersha et al. 2000; Izquierdo et al. 2007). Mutant TIA-1 is related with Walender Distal Myopathy (WDM) by affecting the stress granule dynamics (Hackman et al. 2012). WDM is a dominant, mostly late onset muscular dystrophy. WDM has mostly been shown in Finland and Sweden and disease starts at the 40 to 60 years with weakness atrophy of finger extensors (Welander et al. 1951; Borg et al. 1998).

1.2.2. D-La Protein

La/SBB is an RNA binding protein and one of the major antigens against autoantibodies that are targeted to human rheumatological autoimmune diseases. La protein is a part of ribonuclear protein (RNP) complex and localize in nucleus and cytoplasmic components of cells (Bai and Tolias, 2000). La shows binding affinity to the 3' end of small nuclear RNA (snRNA) precursors such as tRNA, 4.5S RNA, 5S RNA, 7S RNA and U6 RNA precursors that are transcribed by RNA polymerase III (Reddy et al. 1983; Mathews and Francoeurt, 1984). La is able to bind to the oligo(U)-tails that are formed by RNA polymerase III and in some circumstances such as cleavage process of RNAs, the interaction between RNAs and La is transient

(Rosenblum et al. 1998). Because of its localization, La plays roles in termination, processing and nuclear export of RNA polymerase III precursor transcripts; also according to cellular stage, La can regulate the transcript release and transcription reinitation by RNA polymerase III (Maraia et al. 1994; Iii and Maraia, 1996).

La acts with tRNA modifications and tRNA related proteins to maintain tRNA structural stability and efficient biogenesis of tRNAs (Wolin et al. 2006). The genetic analysis in *S. cerevisiae* La plays role in 3'-endonucleolytic cleavage of tRNA precursors in vivo (Yoo et al. 1997). Moreover, Fan group also shows the effect of La protein to 5' and 3' processing of tRNA precursors in HeLa cells (Fan et al. 1998).

Additional role of La protein is the acting as a molecular chaperone to stabilize the tertiary structure of RNAs (Pannone et al. 1998). Thanks to this stabilization, RNAs become more accessible for diverse cellular processes such as RNA transport, processing and translation.

D-La is the *Drosophila melanogaster* homolog of human La protein. As its human homolog, D-La has also RNA recognition motif (RRM) and this conserved La protein family activity is essential for proper development of high eukaryote organisms (Bai and Tolias et al. 2000).

According to cellular need, La is able to be at several places in cells. For its nuclear roles such as transcription and tRNA-processing activities and cytoplasmic activities such as translation and mRNA protection activities, La works as a shuttle between nucleus and cytoplasm (Bachmann et al. 1989).

1.3. Aim of the Project

The aim of our research is that to investigate potential interactions between endogenous tRFs and related proteins by using immunoprecipitation technique.

CHAPTER 2

MATERIALS AND METHODS

2.1. RNA Isolation and cDNA Synthesis

Total RNA was extracted from 8-hour *Drosophila melanogaster* embryos using Trizol reagent (Invitrogen). Isolated RNA was used in RT-PCR for cDNA synthesis according to manufacturers' instructions (Promega). The Rox8 cDNA was amplified with forward primer (5'-GGAGCTCATGGACGAGTCGCAACCG-3') that caries *SacI* restriction site and reverse primer (5'-CGAGCTCTCACTTGTCATCGTCGTCCTTGTAGTCTTGGGTCTGGTATTGTGG-3') that caries *SacI* restriction site and FLAG tag (5'-TCACTTGTCATCGTCGTCCTTGTAGTC-3') by using Phusion Hot Start II High Fidelity DNA Polymerase (Fermentas, F-549L).

The La cDNA was also amplified with forward primer (5'-GAGCTCATGGCCGAAGTTGCTGAAACC-3') and reverse primer (5'-CCTCGAGTTACTTGTCATCGTCGTCCTTGTAGTCATCCCCACGCGCCTTCT-3') that carry SacI restriction site and reverse primer also caries FLAG tag by using Phusion Hot Start II High Fidelity DNA Polymerase.

2.2. TA Cloning

The PCR-amplified products were run in 0,8% agarose gel and purified using QIAquick Gel Extraction Kit (QIAGEN). The purified PCR product was incubated with dATPs, Taq polymerase buffer, $MgCl_2$, dH_2O and Taq polymerase at 72° C to create sticky ends. At the end of incubation, the product was purified with gel purification (QIAquick Gel Extraction Kit, QIAGEN).

Purified 1413 bp Rox8 and 1173 bp D-La cDNA fragments were cloned into pGEM®-T Easy TA cloning vector (Promega) using DH5α *E.coli* strain. Transformant cells were selected using blue-white screening. White colonies were cultured in LB-

Broth medium containing 100 mg/ml ampicilin at 37 °C overnight in shaker incubator. Plasmids were isolated using alkaline lysis protocol and digested by SacI enzyme at 37 °C for confirmation.

Cloned plasmids were sequenced at Izmir Institute of Technology, Biotechnology and Bioengineering Research and Application Center as a service provider by different fluorescent dye labelled dideoxynucleotide chain terminating method by using SP6 and T7 primer. The sequences obtained from the sequencer were analysed using BLAST (Basic Local Alignment Search Tool).

After the sequences were verified, the cloning vectors (pGEM-T Easy/Rox8 and pGEM-T Easy/La) were propagated in *E. coli* and purified with QIAprep Spin Miniprep Plasmid Purification Kit (QIAGEN, 27106).

2.3. Propagation of pACT5C Expression Vector

The propagated cloning vectors were digested with *SacI* enzyme and purified by gel extraction method. At the same time, the expression vector (pACT5C) was also digested with same enzyme (*SacI*) and purified by the same method. After the purification, related genes (Rox8 and La) were cloned into expression vector (pACT5C) by using Dh5α *E. coli* strain and transformation was done. The colonies were selected and propagated in LB medium. After plasmid purification, transformation was verified by digestion with *SacI* enzyme. For high amount and quality plasmid, expression vectors were propagated with PureLink™ Plasmid DNA Purification Kit (Midiprep, Invitrogen, K2100-04) and confirmed using SacI enzyme (Fermentas) at 37 °C, 2 hour and loaded on 1% agarose gel.

2.4. Cloning of Rox8 and La cDNA into pACT5C Expression Vector

Concentrations of samples were measured with nanodrop (NanoDrop Technologies, Inc. ND-1000 spectrophotometer). Purified and digested Rox8 and La cDNA and pACT5C were ligated with T4 DNA Ligase (Fermentas, EL0014) according to manufacturer's instructions.

After ligation 5 µl of ligation mixture were transformed into chemically competent Dh5α *E.coli* cells using heat shock method. Cells were plated on 100 mg/ml

ampicilin LB-Agar plates and incubated at 37 °C for 16 hours. Following day, transformant cells were selected and inoculated in 100 mg/ml ampicilin LB-Broth. After overnight incubation, plasmid purification was performed using alkaline plasmid purification kit (QIAGEN). Plasmid was confirmed by digestion method with SacI restriction enzyme at 37 °C for 2 hours and digestion mixture was run on 1% agarose gel. Later, large scale inoculation was performed in 500 ml LB-Broth containing 100 mg/ml ampicilin at 37 °C for 16 hours. After incubation, plasmids were purified by plasmid purification kit (PureLink™ HiPure Plasmid Filter MidiPrep Kit K2100-04 (Invitrogen).

2.5. *Drosophila* S2 Cell Culture and Transfection

S2 cells were propagated in Schneider's *Drosophila* medium (Life Technologies) supplemented with 10% fetal bovine serum (FBS), 50 units ml^{-1} penicillin, and 50 $\mu g\ ml^{-1}$ streptomycin at 25 °C.

Transfection to S2 cells was performed by using FuGENE HD Transfection Reagent (Promega, E2311) according to manufacturers' instructions (DNA:FuGENE HD ratio, 1:3) in six-well plates. 1 μg plasmids per well was transfected. In addition to the expression vectors that carry expressed genes, pACT5C-GFP (expression vector which carries GFP protein) was also transfected for calculating transfection efficiency. The cells were incubated for 48 hour (optimum expression time for S2 cells with pACT5C vector). After 48 hours of transfection, cells were collected and washed with cold PBS 3 times. Cells were gently mixed with lysis buffer (RIPA) including protease inhibitor cocktail (Sigma) and RiboLock RNase inhibitor complex (Fermentas). Lysates were incubated 45 minute on ice and centrifuged on 14.000 rpm for 30 minutes at 4 °C. Supernatant was collected, aliquated and stored at -80 °C.

2.6. Quantification of Protein Amount

Bradford Assay was used to determine protein concentration. Firstly, different BSA concentrations was prepared, ranging between 10 and 200 µg/ml to draw standard graphic for absorbance-protein concentration diluted isolated proteins with Bradford

solution in ratio of 1:10. Absorbance was measured at 520 nm wavelenght and protein concentrations were determined using standart graphic.

2.7. Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blotting

The constructs pACT5C/Rox8 and pACT5C/La vectors were transfected to *Drosophila* S2 cells in 6 well plates to detect their expressions. Cells were lysed and total protein extract was collected. 15 µg of protein was loaded on 10% SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane using a transfer apparatus according to the manufacturer's protocols (Bio-Rad). After incubation with 5% nonfat milk in TBS-T (10 mM Tris, pH 8.0, 150 mM NaCl, 0.5% Tween 20) for 60 min, the membrane was washed once with TBST and incubated with antibodies against FLAG (Sigma, F3040) (1:10000) for 1 hour by shaking gently at room temperature. Membranes were washed three times for 10 min and incubated with a 1:20000 dilution of horseradish peroxidase-conjugated anti-mouse antibody for 1 hour by shaking gently at room temperature. Blots were washed with TBS-T three times. After last washing, 300 µl enhanced chemiluminescent substrate for HRP (Thermo) and 300 µl enhancer solution (Thermo) were spread on membrane and incubated for 2 minutes. Membranes releasing chemiluminescence were visualized using VersaDoc Imager in Izmir Institute of Technology, Biotechnology and Bioengineering Research and Application Center.

Another gel was prepared to stain proteins with 1 mg/ml Coomasie Blue (Sigma), 10% acetic acid, 30% methanol or ethanol. Gel was incubated in Coomasie Blue for 1 hour and incubated in the same solution without Coomasie Blue to visualize protein bands in order to confirm whether lysis method was worked or not.

2.8. Northern Blotting

Total RNA samples were run on 12% acrylamide denaturing urea gels and then transferred to PVDF membranes by semi-dry electrophoresis. Then membranes were baked at 80°C for 15 minutes for cross-linking. After cross-linking, membranes were transferred to hybridization bottles with 10 ml ULTRAhyb Ultrasensitive Hybridization

Buffer (Ambion) for 1 hour at 42˚C as pre-hybridization. After pre-hybridization step, the hybridization buffer were used as 10 ml hybridization buffer with 10 µl tRF^{Gly} probe and 5 µl microRNAs marker and incubated in hybridization oven for 16 hours at 37˚C. Following day, 2X SSPE 0,1 % buffer was warmed up to 37˚C. The membrane was washed at 10 minutes 3 times with SSPE buffer. Other step was washing the membrane with 50 ml 1X wash buffer at room temperature 2 times, 30 minutes in total. Before the blocking step, membrane was incubated with blocking buffer 2 times, 10 minutes in total and 30 minutes with 50 ml blocking buffer as blocking step. Afterwards, the membrane was treated with streptavidin HRP in 10 ml blocking buffer at 30 minutes. To remove the unbounded streptavidin HRP conjugates, the membrane was incubated with blocking buffer for 15 minutes and then washed with 1X wash buffer 3 times, 45 minutes in total. Before the monitoring results, membrane was washed with 50 ml 1X assay buffer for 2 times. At the end of these steps, membrane were visualized with 1 ml chemiluminesence for 5 minutes in dark by using VersaDoc Imager in Izmir Institute of Technology, Biotechnology and Bioengineering Research and Application Center.

2.9. Immunoprecipitation of tRF Related Proteins

After the protein isolation from transfected S2 cells, at least 500 µg of proteins were aliquoted and total volumes were completed to 500 µl with RIPA buffer. The protein lysates were incubated with 2 µl anti-FLAG antibody at +4˚C for 2 hours. In the meantime, beads were prepared for binding. For this reason, beads were washed with 500 µl with RIPA buffer for 3 times, 15 minutes in total.

At the end of incubation, protein-antibody mixes were added onto beads and second incubation was done for 1.30 hours. Following step, the mixes were centrifugated at 1000 g for 15 minutes and supernatant was removed. The pellet, which contained proteins and RNAs bounded beads, was treated with loading dyes and loaded into SDS-PAGE gel for Western and 12% denaturing gel for Northern Blotting.

CHAPTER 3

RESULTS

3.1. Cloning, Confirmation by Restriction Enzymes and Sequence Analysis

Drosophila Rox8 and La cDNA was cloned into pGEM-T Easy cloning vector and pACT5C expression vector, then purified by using miniprep plasmid purificaion kit (QIAGEN). Vectors were confirmed by digestion with SacI restriction enzyme (Figure 3.1, Figure 3.2).

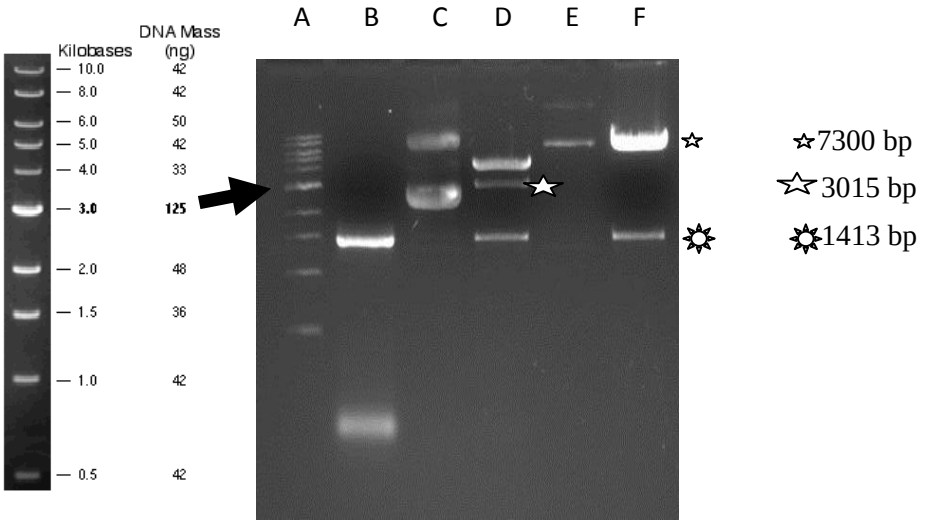

Figure 3.1. 1% Agarose gel electrophoresis of Rox8 cDNA. A) 1 kb DNA ladder B) Rox8 PCR product (1413 bp) C) Rox8 ligated pGEM-T Easy Vector D) SacI digested pGEM-T Easy/Rox8 (pGEM-T Easy 3015 bp, Rox8 1413 bp) E) Rox8 ligated pACT5C Vector F) SacI digested pACT5C/Rox8 (pACT5C 7300 bp, Rox8 1413 bp)

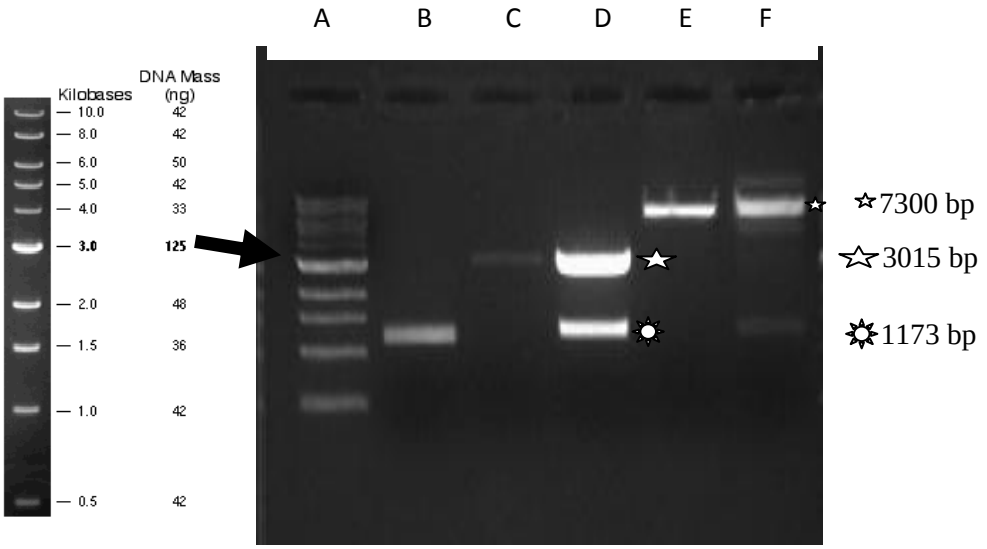

Figure 3.2. 1% Agarose gel electrophoresis of La cDNA. A) 1 kb DNA ladder B) La PCR product (1173 bp) C) linear pGEM-T Easy Vector D) SacI digested pGEM-T Easy/La (pGEM-T Easy 3015 bp, La 1173 bp) E) linear pACT5C Vector F) SacI digested pACT5C/La (pACT5C 7300 bp, La 1173 bp)

3.2. Transfection of S2 Cells with pACT5C/GFP

pACT5C/GFP vector was designed and used to optimize transfection. The best transfection efficiency was observed with 1:3 ratio of DNA:FuGENE HD. GFP was visualized by fluorescent microscope and cells that expressed GFP were observed as 25% ratio (Figure 3.3).

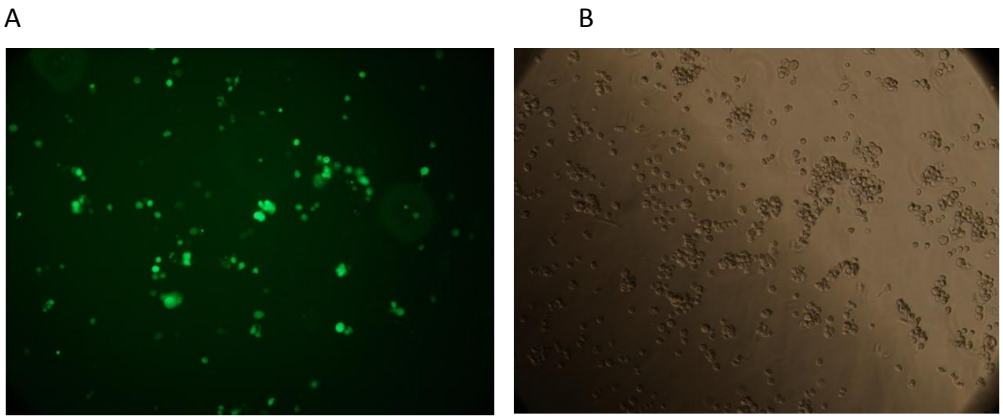

Figure 3.3. Transfection Optimization. 1/3 ratio of DNA/FuGene reagent was used to transfection to *Drosophila* S2 cells. A) Transfected *Drosophila* S2 cells (left) under fluorescent light. B) Same area of *Drosophila* S2 cells under white light.

3.3. Expression of Rox8 and La Protein in S2 Cells

To optimize Western Blotting, proteins from non-transfected, as well as empty pACT5C vector and Rox8 transfected *Drosophila* S2 cells was used according to time depended manner. We used anti-FLAG (Sigma) antibody and detected the 49 kDa Rox8 protein in different time scales (Figure 3.4).

Same procedures with Rox8 protein were used to optimize La protein that is 44 kDa (Figure 3.5).

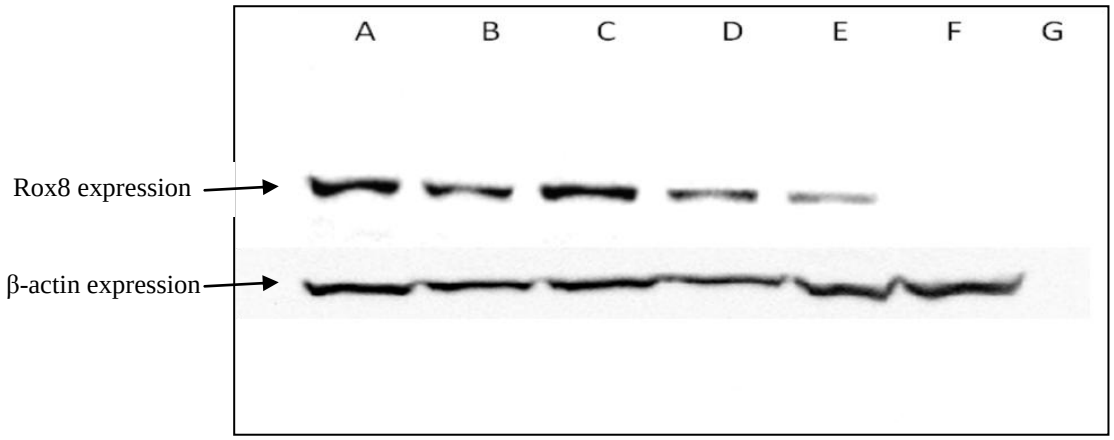

Figure 3.4. Detection of Rox8 protein in *Drosophila* S2 cells by Western Blotting. 10% SDS-PAGE gel transferred to PVDF membrane, primer antibodies (anti-FLAG and anti-β actin) was used 1/10000 dilution and seconder antibody (goat anti-mouse) was used 1/10000 dilution. Following blotting, 10 sec exposure was taken. A) 72-hours post transfection B) 48-hours post transfection C) 36-hours post transfection D) 24-hours post transfection E) 12-hours post transfection F) Empty pACT5C vector transfection G) Protein Ladder

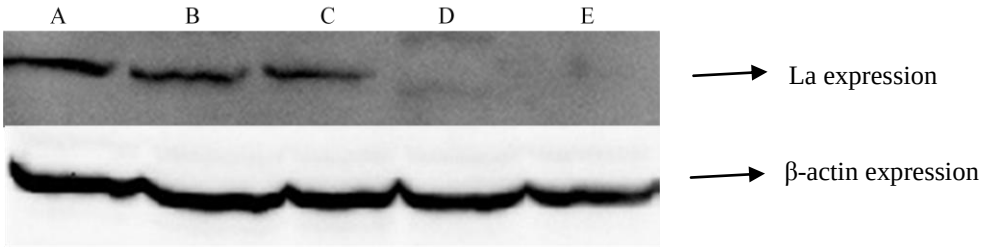

Figure 3.5. Detection of La protein in *Drosophila* S2 cells by Western Blotting. 10% SDS-PAGE gel transferred to PVDF membrane, primer antibodies (anti-FLAG and anti-β actin) was used 1/10000 dilution and sekonder antibody (goat anti-mouse) was used 1/10000 dilution. Following blotting, 10 sec exposure was taken. A) 48-hours post transfection B) 36-hours post transfection C) 24-hours post transfection D) 12-hours post transfection E) Empty pACT5C vector transfection

3.4. Interaction of tRFs with Rox8 and La Proteins in S2 Cells

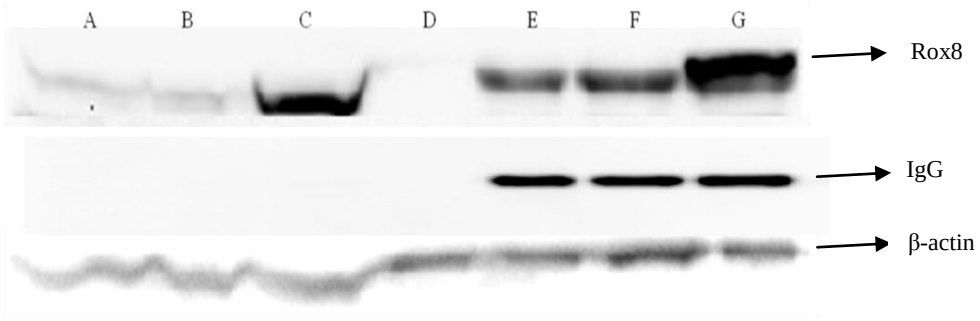

Figure 3.6. Detection and immunoprecipitation of Rox8 protein via anti-FLAG antibody in *Drosophila* S2 cells by Western Blotting. 10% SDS-PAGE gel transferred to PVDF membrane, primer antibodies (anti-FLAG and anti-β actin) was used 1/10000 dilution and seconder antibody (goat anti-mouse) was used 1/10000 dilution. Following blotting, 10 sec exposure was taken A) No transfected S2 cells for negative control B) pACT5C empty vector transfection C) Rox8 transfected cells D) No antibody used immunoprecipitation of Rox8 transfected cell lysates E) No transfected S2 cell lysates was precipitated F) Empty pACT5C vector transfection was precipitated G) Rox8 transfected lysates were immunoprecipitated.

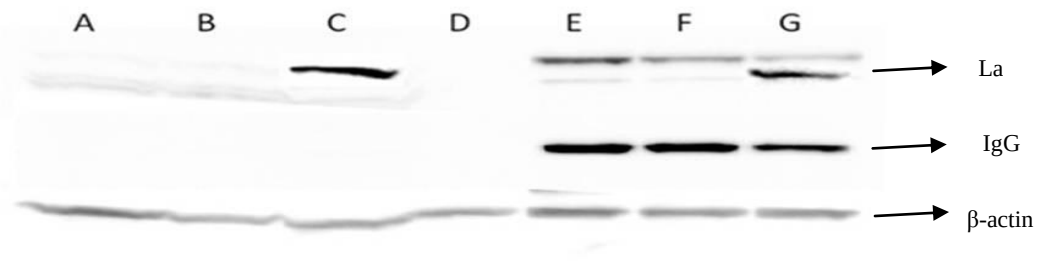

Figure 3.7. Detection and immunoprecipitation of La protein via anti-FLAG antibody in *Drosophila* S2 cells by Western Blotting. 10% SDS-PAGE gel transferred to PVDF membrane, primer antibodies (anti-FLAG and anti-β actin) was used 1/10000 dilution and seconder antibody (goat anti-mouse) was used 1/10000 dilution. Following blotting, 10 sec exposure was taken A) No transfected S2 cells for negative control B) pACT5C empty vector transfection C) La transfected cells D) No antibody used immunoprecipitation of La transfected cell lysates E) No transfected S2 cell lysates was precipitated F) Empty pACT5C vector transfection was precipitated G) La transfected lysates were immunoprecipitated.

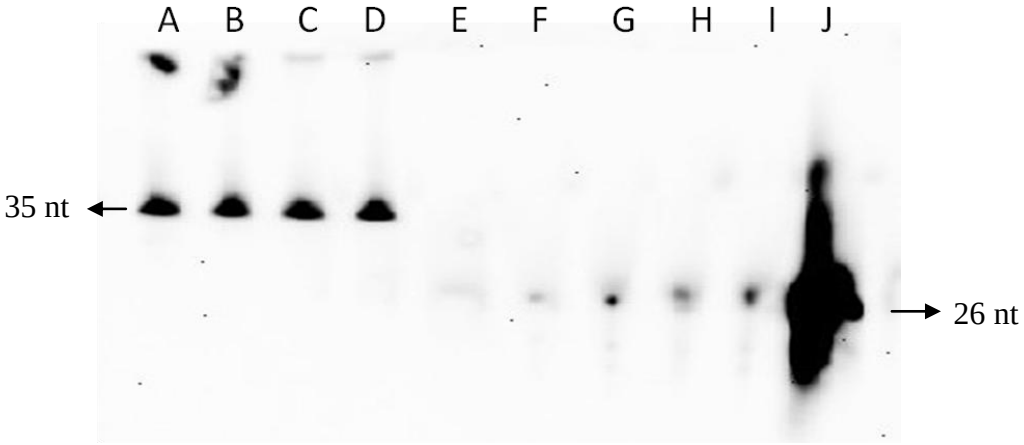

Figure 3.8. Detection and immunoprecipitation of La and Rox8 protein related tRFs in *Drosophila* S2 cells by Northern Blotting. 12% SDS gel transferred to cellulose membrane, $tRNA^{Gly}$ probes were used and HRP-conjugated streptavidin was used 1/10000 dilution. Following blotting, 250 sec exposure was taken A) No transfected S2 cells for negative control B) pACT5C empty vector transfection C) La transfected cells D) Rox8 transfected cells E) No antibody used immunoprecipitation of Rox8 transfected cell lysates F) No transfected S2 cell lysates was precipitated G) Empty pACT5C vector transfection was precipitated H) La transfected lysates were immunoprecipitated. I) Rox8 transfected lysates were immunoprecipitated J) Marker of 26 and 35 nucleotide tRNA fragments.

CHAPTER 4

DISCUSSION

tRNAs play fundamental roles in translation. In addition to this role, tRNAs are also serve as tRNA derived small RNA sources.

tRNA derived small RNAs are newly recognized group of small RNA world and the thinking is that they probably work as microRNAs in cellular events. tRNA halves are examined during the stress conditions and work in stress response in cells. The other tRNA derived small RNA group is tRFs. The studies about tRNA fragments are still going on and the knowledge is very limited.

According to literature, stress induced tRNA fragments interact with some RNA binding proteins in humans. In this study, the interaction between biological tRFs and *Drosophila* La and Rox8 proteins and their roles in *Drosophila* development want to be identified in *Drosophila.* For this reason, we try to catch tRFs by using our tagged and overexpressed proteins in S2 cells. The reason to work with S2 cells is that they mimic the 24^{th} hour of *Drosophila* biogenesis and the changes are mostly occurred in this quick development.

Immunoprecipitation results show that tRNA derived small RNA^{Gly} (26 nt) may potentially interact with Rox8 and La proteins which have RNA binding domains and play roles in stress granules and transcription steps.

CHAPTER 5

CONCLUSION

In this study, Rox8 and La associated tRFs were potentially identified by using immunoprecipitation and Northern methods.

pACT5C/Rox8 and pACT5C/La which were FLAG tagged transfected S2 cells were lysed and immunoprecipitation was performed by using anti-FLAG antibody. After immunoprecipitation, Northern blotting was carried out to detect proteins related tRFs. Therefore, tRF^{Gly} was used for detection.

Results have shown that RNA binding proteins are potentially associated with tRF^{Gly}. However, there are nonspecific bands detected both in Western and Northern blots. These nonspecific bands will be removed by using different washing conditions. As a further study, the expression frequency of tRFs will be analyzed in 1 and 8-h mutant *Drosophila* embryos.

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