Structural and functional analysis of the *miR-282* microRNA gene of *Drosophila melanogaster*

Ph.D. thesis

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Introduction

The role of proteins in the formation of dynamic gene expression patterns in cells has been known for a long time. However, only in the past few decades that RNA-based regulatory complexes were identified, which have shown to be involved in regulation of gene expression.

MicroRNAs (miRNAs), the endogen single-stranded regulatory RNAs are derived from a hairpin-loop precursor. The approximately 22-nucleotide long mature miRNAs are incorporated into a large protein complex and inhibit gene expression post-transcriptionally. miRNAs bind to complementary sequences in the 3'-untranslated regions of target mRNAs, resulting transcript degradation or translational suppression of the target genes. In animals, there is partial base pairing between miRNAs and target transcripts, so simple genome alignment cannot be used in target prediction, so experimental evidence is essential. The functional analysis of miRNAs is still in its infancy. Although it is clear now, that miRNAs are required for the fine-tuning of the regulation of very complex mechanisms, and their activity covers almost all biological processes. The functional analyses are hampered primarily by redundancy (different miRNAs share the same target) and by coexpression. Plus a single miRNA can regulate even hundreds of protein coding genes.

In our model organism, *Drosophila melanogaster* 256 miRNAs have been predicted to date (miRBase release 21, June 2014). There are over 12000 putative target genes which cover more than 90 percent of genes of fruit fly.

My Ph.D. dissertation presents the molecular characterization of the genomic region encoding a computationally predicted *Drosophila melanogaster* microRNA, *mir-282*, and presents phenotypic analysis of *mir-282* gene.

Aims

We have gained an understanding of the miRNA maturation process, and their role in gene regulation, the scope of gene regulation; however, there is still very little known about the miRNA coding genes. Our aim therefore was a detailed structural and functional analysis of *Drosophila melanogaster mir-282* microRNA gene.

- The aim of our study was first of all to confirm the existence of the putative *mir-282* miRNA gene of *Drosophila* experimentally, and find its othologs.
- To define the *mir-282* gene molecularly we intended to determine the length of the primary transcript of *mir-282*. We also checked if *mir-282* is a part of a neighbor gene as a splice variant, or an independent gene.
- In addition to the structural characterization of the *mir-282* gene, our other major goal was to elucidate the function of the gene. We aimed to analyze the phenotypes of transposon insertions in the *mir-282* locus, as well as to create null alleles and transgenic lines with ectopic expression.
- Furthermore, we contemplated to determine at least one potential target gene of *mir-282* whose expression is regulated by *mir-282* indeed.

Methods

- To find *mir-282* orthologs, the database of miRBase, Flybase and NCBI were searched. In addition in some cases homologs of the *D. melanogaster* genes were manually identified based on high sequence similarity and genomic position.
- To determine the primary transcript of *mir-282* we performed RACE (rapid amplification of cDNA-ends) experiments.
- Northern blot experiments were carried out to demonstrate the mature miRNA.
- The *mir-282* deletion was created by remobilizing a P element over an overlapping deletion.
- Transgenic *Drosophila* lines were created to rescue the null mutant phenotype, to overexpress *mir-282* and to test the target sequence of 3‘UTR of the *rutabaga* gene.
- To determine phenotypes of *mir-282* null mutants viability test, hatching-rate study, egg yield measurements, male fertility test and starvation test were carried out.
- Immunostaining experiments were carried out. Germline stem cells were stained by Hts monoclonal antibody.
- Apoptosis in the ovaries was detected by TUNEL assays (terminal deoxynucleotidyl transferase dUTP nick end labeling).
- Germline chimeras were constructed by transplanting pole cells to determine whether the mutant phenotype is brought about by altered functions of the germline or the soma.
- Quantitative real-time PCR experiments were performed to measure the expression level of the candidate target transcripts.

Results

We have confirmed the previous gene prediction of *Drosophila mir-282* by demonstrating its transcriptional activity and identify the primary miRNA.

The *mir-282* is an insect-specific gene. The *mir-282* orthologs can be found in all the members of the Drosophilidae group whose genome sequence is currently available, and the *miR-282* coding sequence was also found in all holometabolic species analyzed. To determine the degree of conservation in the *mir-282* region, we searched for synteny in the vicinity of *mir-282*. These data reveal that the *mir-282* gene is not associated with adjacent protein-coding genes, however, in all species analyzed: *mir-282* resides in a long region devoid of protein-coding genes, arguing for the presence of a *mir-282* promoter and important regulatory elements within this area.

The length of the primary transcript was determined by an enhanced version of the RACE technique. The primary *mir-282* transcript is 4.9 kb from its 5' CAP structure to the 3' terminal polyA tail. m*ir-282* is not connected to any exon of the adjacent protein coding gene (*cracked*), so there is a separate transcriptional unit that is transcribed into RNA.

To confirm that the transcript of the *mir-282* region is indeed the source of a mature microRNA Northern blot experiments were carried out on total RNA samples obtained from adult females. This experiment demonstrated that a mature microRNA of 23-24 nt is generated from the *mir-282* gene.

To investigate whether the *mir-282* transcript has a biological function, we first dissected the genomic region around *mir-282* by analyzing the phenotypes of transposon insertions in the *mir-282* locus. Although a great number of transposon-bearing lines were examined, no visible phenotype could be observed except for

homozygous lethality in some lines. We have found that decreased viability in these stocks is due to background mutations and not to the loss of *mir-282* activity.

To gain further insight into the activity of *mir-282*, transgenic lines overexpressing *miR-282* were created. The general, ectopic overexpression of *mir-282* leads to lethality in the second larvae stage. This observation suggests that the putative transcript encoded by the *mir-282* locus does have biological function.

Newly induced *miR-282* specific deletions were identified. The null mutant phenotype analysis demonstrated that the *mir-282* locus encodes a functional transcript that could affect viability, lifespan and female's egg yields:

Our viability experiments indicated shortened adult lifespan at 18, 25 and 29 ^{o}C among males and females, as well as semi-lethality before reaching adult stage. The average life span was found to be decreased by ~50% both in mutant males and females, while the number of hatching imagos decreased by 56% compared to that in the control line. While *mir-282* null mutant males displayed normal fertility, mutant females exhibited reduced egg production compared to the control: they laid a decreased number of eggs, on the average 55% less than the control females. It is known that *Wolbachia*, which are common intracellular gram-negative bacteria, can reduce egg production in insects by reproductive parasitism. We tested our stocks for the presence of *Wolbachia* by simple DAPI staining and by PCR amplifying the 16S RNA of *Wolbachia*. These experiments clearly confirmed the absence of *Wolbachia* in the *mir-282* mutant lines and excluded the possibility of the decreased egg production in *mir-282* mutants being due to *Wolbachia* infection.

Furthermore we have generated transgenic flies carrying a genomic fragment containing *mir-282* primary transcript. This genomic fragment was able to rescue both

the reduced viability and the decreased egg production phenotypes in the mutants confirming that these phenotypes are indeed caused by the loss of *mir-282* function.

The decreased egg production of *mir-282* mutants might be the consequence of germline stem cell (GSC) depletion in the adult ovary. We investigated this possibility by analyzing GSCs in the ovaries of mutant females via anti-Hts antibody staining. We found that the numbers of GSCs and cysts were normal, indicating that the decrease in egg production is not a result of low GSC number.

To determine whether the decrease in the egg production of *mir-282* mutants is germline or soma dependent, pole cell transplantation experiments were carried out. The egg production of chimeric females was found to be normal, suggesting that *mir-282* mutant's germ line is not impaired, whereas the somatic cells of the ovaries are unable to function properly.

In the background of the decreased egg yield in *mir-282* mutant flies, we have observed a remarkable increase in apoptotic activity in eggs after stage 8 with apparent accumulation of apoptotic debris in the proximal end of the oviduct via TUNEL assay. Starvation has been described to induce an increase in the apoptosis of *Drosophila* nurse cells. We inspected the possibility of malnutrition causing the increase of apoptotic activity in mutant ovaries. The kinetics of egg yield changes were the same in mutant and control groups, indicating that *mir-282* mutants show normal physiological reactions to temporary food deprivation and that altered energy homeostasis is not responsible for the enhancement of apoptotic activity in *mir-282* mutants.

We have investigated the potential target genes of *mir-282* as well. The list of computationally predicted targets for *mir-282* was manually screened, taking in account our findings about *mir-282*. Five candidates from the hundreds of potential *mir-282* target genes were selected. Gene expression studies were performed on these genes that

revealed the *rutabaga* gene, as a target of *mir-282*. The *rutabaga* gene codes a nervous system-specific adenylate cyclase. The rutabaga mRNA has predicted regulating sites for four different potential miRNAs and *mir-282* has many other potential targets, probably the regulation of *rutabaga* is not the only function of *mir-282*. However, based on our data, we hypothesize that one of the main functions of *mir-282* is the regulation of adenylate cyclase activity in the nervous system during metamorphosis.

Summary

The following conclusions can be stated based on the results described above:

- The *dme-mir-282* gene is transcribed as an independent transcription unit
- The *mir-282* primary transcript has a 5'CAP and a 3' polyA sequence with a length of 4.9 kb
- The ~23-24 nt mature *miR-282* is detectable
- The lack of *mir-282* affects viability, lifespan and female's egg yields
- The focus of the *mir-282* mutation is in the somatic cells
- *mir-282* negatively regulates the expression of *rutabaga* gene

Publications

Publication used in the Thesis:

Péter Vilmos*, Ágnes Bujna*, Milán Szuperák, Zoltán Havelda, Éva Várallyay, János Szabad, Lucie Kucerova, Kálmán Somogyi, Ildikó Kristó, Tamás Lukácsovich, Ferenc Jankovics, László Henn, Miklós Erdélyi. Viability, longevity, and egg production of *Drosophila melanogaster* are regulated by the *miR-282* microRNA (2013) *Genetics*, Vol. 195, 469–48. *These authors contributed equally to this work.
IF: 4,389

Other publications:

Ferenc Jankovics, László Henn, Ágnes Bujna, Péter Vilmos, Nóra Kiss, Miklós Erdélyi. A Functional Genomic Screen Combined with Time-Lapse Microscopy Uncovers a Novel Set of Genes Involved in Dorsal Closure of *Drosophila* Embryos (2011) *PLoS ONE* 6(7): e22229.
IF: 4,092

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IF: 3,730

Total IF:12,211