Expression analysis of *Drosophila melanogaster* microRNAs

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For Gizem

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Abbreviations

#	number
AGE	agarose gel electrophoresis
as	antisense
ATP	adenosine triphosphate
bp	base pair
BPB	bromophenol blue
Cat. #	catalog number
C. elegans	*Caenorhabditis elegans*
cDNA	complementary DNA
dNTP	deoxynucleotide triphosphate
DNA	deoxyribonucleic acid
D. melanogaster	*Drosophila melanogaster*
D. rerio	*Danio rerio*
dsRBD	double stranded RNA binding domain
dsRNA	double stranded RNA
E. coli	*Escherichia coli*
H. sapiens	*Homo sapiens*
ISH	*in situ* hybridization
LNA	Locked nucleic acid
mRNA	messenger RNA
miRNA	microRNA
miRNP	microRNA-containing RNP
M. musculus	*Mus musculus*
MW	molecular weight
nt	nucleotide
NTP	nucleotide triphosphate
ORF	open reading frame
PA	polyacrylamide
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction

pre-miRNA	precursor miRNA
pri-miRNA	primary precursor miRNA
PTGS	post transcriptional gene silencing
qRT-PCR	quantitative real time RT-PCR
PNK	polynucleotide kinase
rasiRNAs	repeat associated siRNA
RdRP	RNA dependent RNA polymerase
RISC	RNA induced silencing complex
RITS	RNA-induced transcriptional gene silencing
RNA	ribonucleic acid
RNAi	RNA interference
RNP	ribonucleoprotein
rRNA	ribosomal RNA
rpm	revolutions per minute
RT-PCR	reverse-transcription PCR
s	sense
SDS	sodium dodecylsulfate
shRNA	short hairpin RNA
siRNA	small interfering RNA
S. pombe	*Schizosaccharomyces pombe*
S. cerevisiae	*Schizosaccharomyces cerevisiae*
ss	single stranded
ssDNA	single stranded DNA
ssRNA	single stranded RNA
TGS	transcriptional gene silencing
UTR	untranslated region
UV	ultraviolet
WM	whole mount
wt	wild type

D. melanogaster gene names, synonyms, and abbreviations are used according to Flybase nomenclature. More information can be obtained from http://flybase.net/docs/nomenclature/lk/nomenclature.html

1. Abstract

miRNAs are tiny regulatory RNAs of 20-25 nucleotides (nt) in length that are endogenously expressed in cells. Hundreds of miRNA genes have been identified in plants and animals. Together they form a large family of regulatory molecules that control important cellular and developmental pathways. Most animal miRNAs regulate the translation of different cellular mRNAs via imperfect base pairing interactions. Due to the complexity of target mRNA recognition only a small subset of miRNAs is annotated with biological functions.

In order to contribute to their functional identification, I identified the temporal and spatial expression pattern of *D. melanogaster* miRNAs by Northern blot analysis at different developmental stages. Temporal expression patterns of miRNAs can be used for the verification of predicted miRNA targets and biological functions. Tissue specific expression patterns of *M. musculus* miRNAs were identified again by northern blot analysis. Several miRNAs are highly tissue specifically expressed in adult mouse suggesting that they may have important tissue specific biological functions. For the identification of spatial expression patterns of *D. melanogaster* miRNAs, I tried to optimize the whole-mount *in situ* hybridization protocol for fly embryos. Our efforts to identify an efficient method for the identification of spatial expression patterns of *D. melanogaster* miRNAs were not successful. Detecting fly miRNAs by *in situ* hybridization is challenging and only few miRNAs were detected so far.

I targeted miRNA processing factors by RNAi in order to determine the effects on miRNA biosynthesis in *D. melanogaster* cells. We identified dmDGCR8 (Pasha), which is a Drosha interacting protein, as a novel factor involved in nuclear processing of pri-miRNAs. I demonstrated that dmDGCR8 and Drosha depletions resulted in pri-miRNA accumulation in the cells. In order to completely deplete Drosha from the embryos I tried to knock out the Drosha gene with the P-element mobilization method. I could not delete Drosha gene by this method.

2. Introduction

2.1 Identification of miRNAs

Aiming to identify genes that are involved in cell lineage transitions during C. elegans development, the *lin-4* mutation was identified. In lin-4 mutant worms specific cells failed to complete the transitions between two larval stages because of unusual cell division iterations taking place (Chalfie et al., 1981). Later, the gene *lin-4*, which is associated with the phenotype, was cloned and characterized (Liu and Ambros, 1989). It was soon discovered that *lin-4* gene code for a pair of small RNA species (Arasu et al., 1991; Lee et al., 1993). One RNA was 21 nucleotides in length, and its precursor, being slightly longer (61 nucleotides), folds into a stem loop structure. The Ambros and Ruvkun laboratories both discovered that the *lin-4* RNA had antisense complementarity to multiple sites in the 3'-UTR of the *lin-14* mRNA (Lee et al., 1993; Wightman et al., 1993). The antisense complementarity was located in a region, which was previously identified to mediate the repression of *lin-14* by *lin-4* (Wightman et al., 1991). The pairing of the *lin-4* RNA to the 3'-UTR of *lin-14* causes a decrease in the LIN-14 protein, without a noticeable change in the mRNA levels. Together these discoveries led to a model, in which *lin-4* RNA pairing with the 3'-UTR of the *lin-14* mRNA and inducing a translational repression of proteins that regulate a series of cell divisions, which in turn trigger the transition of some cells from one larval stage to another (Lee et al., 1993; Wightman et al., 1993).

For several years *lin-4* RNA was thought to be a peculiarity of *C. elegans*, since *lin-4* homologs could not be identified in other organisms. When *let-7*, another gene which codes for a small RNA, was identified (Reinhart et al., 2000; Slack et al., 2000), it was realized that repression via small RNA mediated silencing is a common mechanism various multiple organisms. Similar to *lin-4*, *let-7* mediated the translational repression of another gene, *lin-41*. Unlike *lin-4*, *let-7* was involved in the late larval to adult stage transition (Pasquinelli et al., 2000). Being responsible for mediating the timing of developmental events, *lin-4* and *let-7* were both named *small temporal RNAs (stRNAs)*.

One year later, three different groups hoping to identify endogenous small interfering RNAs (siRNAs), identified over a hundred new small RNA gene products in worm, fly and human (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001). Like *lin-4* and *let-7* RNAs, the new sequences were approximately 22 nt in size and were processed from 60-80 nt foldback RNA precursors. The sequences were conserved in closely related species (e.g. *C. elegans* and *C. briggsae*) and some were even conserved in more distant species. Although the expression of some of the identified RNAs is temporally regulated like in the case of *lin-4* and *let-7* RNAs, many of them are ubiquitously present throughout development, hence instead of the previously coined *stRNA*, a more comprehensive name was sought and finally a decision for *microRNA (miRNA)* was made (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001).

Today, lin-4 and let-7 are considered the founding members of a large small RNA family, the miRNAs. Many more miRNAs have been identified in worms, insects, plants, chicken, fish, and some mammals. The number of identified miRNAs approaches 1% of all genes and miRNAs represent an important family of regulatory molecules in cells. Currently more and more functions of miRNAs are revealed. These functions include the control of cell proliferation, regulation of apoptosis, fat metabolism, hematopoietic differentiation, and the control of leaf and flower development in plants (Bartel, 2004).

Considerable efforts have been made to identify new miRNAs since the early discoveries. The methods, which were successfully used to identify new miRNA sequences, can be classified in three major groups. The least efficient approach is the identification of miRNAs through genetic screens. In addition to lin-4 and let-7 miRNAs of *C. elegans*, lys-6 miRNA was identified by forward genetic screens in worms. *lys-6* mutants have defects in neuronal left/right-asymmetry (Johnston and Hobert, 2003). In *D. melanogaster*, bantam miRNA was identified when a gene, which was responsible for the small body size phenotype, was discovered (Brennecke et al., 2003). While genetic studies provide well established information about the *in vivo* functions of miRNAs, they depend largely on the clear phenotypes. Not every miRNA mutation results in a visible phenotype. Thus not all miRNAs can be identified by genetic studies.

The most effective way of miRNA identification was through the, construction of cDNA libraries of endogenous small RNAs, as reviewed by (Ambros and Lee,

2004; Chen et al., 2005b). Briefly, size-fractioned RNA from different sources is ligated to 5' and 3' adapter molecules, which are then reverse transcribed in order to construct a cDNA library. Cloning miRNAs using this approach is a multi-step process for which different protocols have been developed by various groups, as reviewed by (Ambros and Lee, 2004; Aravin and Tuschl, 2005). Figure 1 outlines the process of a typical miRNA cloning procedure. First, RNA samples from different sources are size fractionated by polyacrylamide gel electrophoresis. RNAs of the desired sizes are excised from the gel and eluted. Small RNA species, which are produced by Dicer cleavage, have a 5' terminal phosphate and a 3'-hydroxyl group. The 5'-phosphate is then removed by an enzymatic dephosphorylation reaction in order to enable the ligation of the modified 3'-adaptor molecule. 3'-adaptor joining is followed by the re-addition of the phosphate group to the 5' end of the generated hybrid molecule, which facilitates the ligation of the 5'-adaptor molecule. The ligation product is reverse transcribed and the cDNA is then amplified by PCR. The PCR products are concatemerized via DNA ligation and cloned into the sequencing vectors for identification (Figure 1).

When a small RNA is discovered by cDNA cloning, it has to meet the following criteria to be classified as a miRNA. First, the small RNA sequence should be at the arm of a 60-80 nucleotide hairpin RNA structure. Second, if the small RNA was not cloned repeatedly, the expression of the small RNA should be confirmed experimentally, typically by Northern blotting. The sequence should also be phylogenetically conserved in related species. (Ambros et al., 2003a).

The procedure requires about 10 μg RNA as starting material. Hundreds of miRNAs from different organisms, tissues, cell lines, and viruses (Aravin et al., 2003; Brennecke et al., 2003; Chen et al., 2005b; Dostie et al., 2003; Houbaviy et al., 2003; Kasashima et al., 2004; Lagos-Quintana et al., 2002; Watanabe et al., 2005) were cloned using similar procedures. The limitation of the approach, however, is that a miRNA needs to be present in the starting RNA sample and it needs to be abundant in order to be identified. Low copy number miRNAs are statistically difficult to clone and sequence.

To overcome the limitations of the directional cloning approaches of miRNAs, several bioinformatic miRNA searching methods, depending on examining the genomic DNA, have been devised (Adai et al., 2005; Cui et al., 2006; Grad et al., 2003; Lai et al., 2003; Legendre et al., 2005; Wang et al., 2005; Wang et al., 2004).

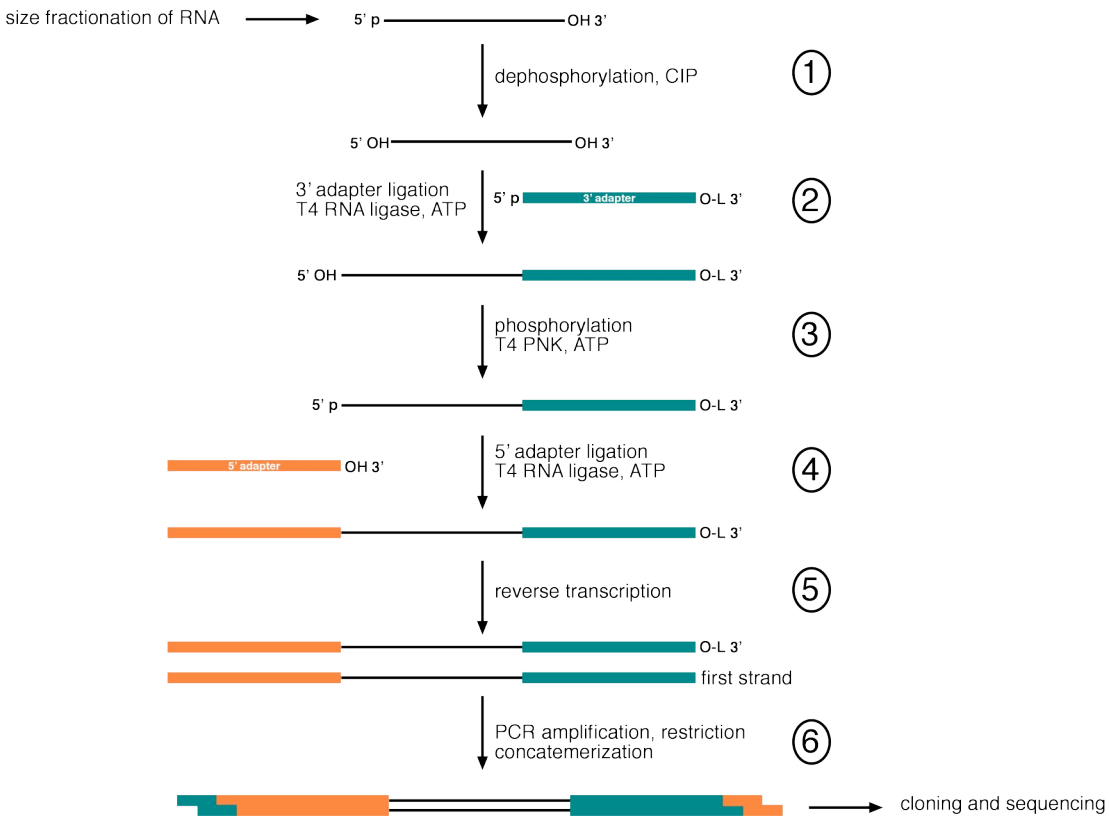


Figure 1. Small RNA cloning procedure.

Input RNA is dephosphorylated (1) for joining of the chemically modified 3'-adapter by T4 RNA ligase in the presence of ATP (2). The ligation product is re-phosphorylated by T4 PNK (3) and the 5'-adapter oligo is joined by another RNA ligation step (4). First strand cDNA is produced by reverse transcription (5) and amplified by PCR followed by restriction digestion, concatemerization and cloning into PCR vector. After transformation into bacteria, individual clones are sequenced.

Computational identification methods depend mainly on the phylogenetic conservation of miRNA genes. Simple homology searches of the known miRNAs can reveal homologs in different species. In order to identify novel miRNAs, search algorithms that take precursor hairpin RNA conservation into account have been used successfully (Lai et al., 2003; Lim et al., 2003). Mainly the search programs scan the intergenic regions of the genome to identify hairpin RNA structures, which are conserved in related species. Integrative approaches that combine the efficient computational prediction with fast expression data from microarray analysis have also been used (Bentwich et al., 2005). A limitation of bioinformatic approaches is the high number of the false-positive candidates. Predicted miRNAs need to be validated experimentally as true miRNAs if their hairpin precursor sequences are not conserved among related species. Most of the current miRNA identification searches concentrate

on the intergenic regions of genomic DNA. Although current computational identification approaches overlooked miRNAs located in the protein coding genes, it is widely accepted that a significant number of miRNAs reside in the intronic sequences of protein coding genes.

The cloning method is also used to identified other classes of small RNA species present in different organisms. In addition to miRNAs, repeat-associated small RNAs (rasiRNAs) that associate with the repetitive heterochromatic regions of the genome were cloned from *S. pombe* and *D. melanogaster* (Aravin et al., 2003; Reinhart and Bartel, 2002). Endogenous small interfering RNAs (siRNAs), which are processed from long dsRNA precursors produced by RNA-dependent RNA polymerase (RdRP) activity, were identified in plants (Allen et al., 2005; Vazquez et al., 2004). Small RNAs, which specifically bind to the Piwi family of proteins (PIWI, MIWI, MILI) were cloned from germline cells of mouse and rat (Aravin et al., 2006; Girard et al., 2006; Grivna et al., 2006; Lau et al., 2006; Vagin et al., 2006). Piwi is a subgroup of the Argonaute family of proteins, which are expressed in germline cells. Until recently, the biological functions of Piwi proteins remained unidentified. The expression pattern of Piwi proteins suggested that they may play a role in spermatogenesis. The novel small RNAs that were associated with Piwi proteins, were named Piwi-interacting RNAs (piRNAs). The biogenesis and function of germline piRNAs are yet to be identified. And finally, tiny non-coding RNAs (tncRNAs) were identified in *C .elegans* (Ambros et al., 2003b).

2.2 Genomics of miRNAs

Hundreds of miRNAs have been identified in plants and animals; and the number of the identified miRNA genes is still increasing. At the time of writing, the miRNA registry database reported 131 miRNAs in *A. thaliana*, 78 in *D. melanogaster*, 114 in *C. elegans*, 337 in *D. rerio*, 373 in mouse, and 474 in human. (Griffiths-Jones, 2004). It is estimated that miRNAs may account for as many as 2-3 % of the number of all genes (Kim and Nam, 2006). miRNA genes are dispersed all over the genome. In humans all chromosomes host miRNA genes except the Y chromosome. Many human miRNA genes are arranged as clusters of genes located very close to each other. In flies the organization of miRNA genes on the genome is similar. All 78 *D.*

melanogaster miRNA genes are scattered on X (7), second (42), and third (29) but not on Y chromosome. In Drosophila 35 out of 78 miRNAs are arranged in clusters. The *D. melanogaster mir-3* cluster is the largest miRNA cluster, which contains 8 miRNA genes located closely to each other (*mir-309*, *mir-3*, *mir-4*, *mir-5*, *mir-6-1*, *mir-6-2*, *mir-6-3*, and *mir-289*). It was suggested that clustered miRNAs are expressed from a longer transcription unit and processed to mature miRNAs (Lagos-Quintana et al., 2001).

miRNA clusters are often formed by miRNAs with related sequences, indicating that they originate from miRNA gene duplications during evolution. For instance, all three *mir-6* genes are located in the same cluster. However, sometimes unrelated miRNAs can also be found in the same cluster. For instance, *mir-125* and *let-7* miRNAs, which are located very close to each other in the *D. melanogaster* genome, do not have conserved sequences. It has been postulated that miRNAs on the same cluster may have related cellular functions. *mir-125* is an ortholog of *C. elegans lin-4*. In *C. elegans lin-4* and *let-7* are on separate chromosomes and regulate different developmental steps of worm development. However, in *D. melanogaster* these genes are located on the same cluster. It is possible that *mir-125* and *let-7* are functionally related unlike their *C. elegans* homologs (Aravin et al., 2003). Sequentially related miRNAs can also be found in different genomic locations. A striking example is the *D. melanogaster mir-2* family, which contains 8 members (*mir-2a-1, mir-2a-2, mir-2b-1, mir-2b-2, mir-2c, mir-13a, mir-13b-1,* and *mir-13b-2*) that are located in 4 different genomic loci.

It was originally thought that most of the miRNA genes are located in intergenic regions, but after careful exanimations it was soon realized that significant numbers of miRNA genes are located in the introns of protein coding genes. Many miRNA genes are located far apart from the protein coding genes suggesting that their expression is regulated by their own promoters. However, miRNA genes can also be located within protein coding transcription units with sense orientation. In a study concerning a large subset of mammalian miRNAs, it was observed that 161 out of 232 miRNA genes are located within defined transcription units. 90 of these 161 transcripts are protein coding mRNAs, whereas 71 are non-coding RNAs (Rodriguez et al., 2004). 27 of the latter are located in the introns of these non-coding transcripts. The rest are miRNA genes, which are located in the exons of non-coding RNA transcripts. It therefore seems like the majority of miRNA genes in the group of studied miRNAs is located in

the introns of the transcription units of protein coding genes or non-coding RNAs (Rodriguez et al., 2004). It remains to be determined whether splicing of the primary RNA transcript is required for the expression of miRNAs, which are located in introns. In *C. elegans*, let-7 miRNA synthesis is dependent on the splicing and polyadenylation of the primary transcript (Bracht et al., 2004) suggesting that miRNA biogenesis may involve splicing and polyadenylation of the primary transcripts in other species too. As expected, intronic miRNAs have similar expression profiles like their host mRNAs in human, mouse, and *C. elegans* (Baskerville and Bartel, 2005; Lau et al., 2001; Rodriguez et al., 2004).

In *D. melanogaster*, 21 out of 78 miRNAs are located in the introns of protein coding transcripts. In some cases, the genomic organization of miRNA clusters is conserved among different species. For instance *mir-307*, which is located in the intron of a protein coding gene, is also intronic in anopheles and honey bee genomes. The organization of the *mir-100/let-7/mir-125* cluster is also conserved during evolution in different organisms, although the distance between the genes has changed over time (Aravin et al., 2003). Another striking example is the *mir-7* gene, which is located in the intron of the *hnRNP K* gene in mammals. Fly *mir-7* gene is located in the intron of the *bancal* gene, which can be complemented by mammalian hnRNP K protein.

Some miRNA genes are located in interesting genomic locations. One example is the *mir-10* gene which is located within the *Hox* gene clusters of *D. melanogaster.* Another miRNA located in *Hox* clusters is the *mir-iab-4* gene. *mir-10* is located in the Antennapedia complex of *Hox* clusters whereas *mir-iab-4* is located in the Bithorax complex. Interesting miRNA loci include the *mir-15a/mir-16* cluster, which falls in a region of the human chromosome 13 that is related to both mantle cell lymphoma and B cell chronic lymphocytic leukemia (Calin et al., 2002; Lagos-Quintana et al., 2001). *mir-15/mir-16* are vertebrate miRNAs they do not have homologs in *D. melanogaster*.

Most miRNAs, which were identified by cloning and for which expression were verified experimentally, are conserved within closely related animals (e.g. *C. elegans* and *C. briggsae*). Many are also conserved in distant organisms, although the copy number of miRNA genes can vary among the orthologs. The most striking example is the *let-7* miRNA family, which has four members in *C. elegans*, 15 in human, but only a single member in *D. melanogaster* (Aravin et al., 2003; Lai et al., 2003; Lim et al., 2003; Pasquinelli et al., 2000). 27 of 78 *D. melanogaster* miRNAs are highly

conserved (e.g. mir-1). Orthologs of these conserved miRNAs can be found in vertebrate and invertebrate genomes (metazoans). 13 miRNAs are conserved in invertebrate genomes (e.g. *C. elegans*) and 17 are conserved only in insect species such as *D. melanogaster* and *A. gambiae* (arthropoda). The rest of the miRNAs have been identified only in Drosophilid genomes. Among them, 21 miRNAs are conserved in *D. melanogaster* and *D. pseudoobscura*. miR-303 was cloned only from *D. melanogaster* and does not have a homolog in *D. pseudoobscura* (Table1).

Table 1. Conservation of *D. melanogaster* miRNAs[1]

Metazoans	Invertebrates	Arthropoda	Drosophilae	*D. melanogaster*
let-7	*bantam*	*mir-12*	*mir-3*	*mir-303*
mir-1	*mir-2a-1*	*mir-14*	*mir-4*	
mir-7	*mir-2a-2*	*mir-263a*	*mir-5*	
mir-8	*mir-2b-1*	*mir-263b*	*mir-6-1*	
mir-9a	*mir-2b-2*	*mir-275*	*mir-6-2*	
mir-9b	*mir-2c*	*mir-276a*	*mir-6-3*	
mir-9c	*mir-13a*	*mir-276b*	*mir-11*	
mir-10	*mir-13b-1*	*mir-277*	*mir-274*	
mir-31a	*mir-13b-2*	*mir-278*	*mir-280*	
mir-31b	*mir-87*	*mir-279*	*mir-284*	
mir-33	*mir-281-1*	*mir-282*	*mir-286*	
mir-34	*mir-281-2*	*mir-283*	*mir-287*	
mir-79	*mir-307*	*mir-305*	*mir-288*	
mir-92a		*mir-308*	*mir-289*	
mir-92b		*mir-315*	*mir-304*	
mir-100		*mir-317*	*mir-306*	
mir-124		*mir-iab-4*	*mir-309*	
mir-125			*mir-314*	
mir-133			*mir-316*	
mir-184			*mir-318*	
mir-210				
mir-219				
mir-285				
mir-310				
mir-311				
mir-312				
mir-313				

1 *Genes belonging to the same miRNA family are indicated with identical colors.*

2.3 Biogenesis of miRNAs

Since flanking sequences near miRNAs can often be found in EST databases it was already obvious that miRNAs are expressed from long RNA transcripts, which are then processed to produce mature miRNAs. In mammals 80% of miRNA genes can be found among ESTs, so it is highly unlikely that RNA polymerase III (Pol III) play a role in their transcription. Later, it was confirmed that human miRNA genes are transcribed by Pol II (Cai et al., 2004; Lee et al., 2004a). The proof of Pol II mediated transcription of miRNAs came from several experiments. First, it was shown that primary miRNA transcripts contain both poly (A) tail and cap structures, signature of the Pol II transcription products (Cai et al., 2004; Lee et al., 2004a). Second miRNA transcription is sensitive to treatment with α-amanitin, a drug which specifically inhibits Pol II transcription but not Pol I or Pol III (Lee et al., 2004a). And finally Pol II mediated transcription was verified by demonstrating the physical association of Pol II with the miRNA promoters (Lee et al., 2004a). With the exception of some viruses, miRNA transcription is mediated by Pol II transcription. Several miRNA genes in the mouse γ-herpes virus 68 (MHV68) are transcribed by Pol III, suggesting that viral miRNAs may have evolved independent of the cellular miRNA genes (Pfeffer et al., 2005).

Pol II mediated transcription enables the temporal and spatial regulation of miRNA expression. Unlike the constitutively expressed Pol I or Pol III transcripts, Pol II transcription can be regulated by upstream regulatory elements in *cis*, and by *trans* acting transcription factors (O'Donnell et al., 2005). For instance, *c-myc* activates the transcription of *mir-17* cluster. MyoD and Mef2 transcription factors enable the muscle specific expression of *mir-1-1* and *mir-1-2* (Zhao et al., 2005).

Transcription of miRNAs yield long single stranded RNA molecules (pri-miRNAs) that fold into structures with local hairpins corresponding to the pre-miRNAs. The hairpin structures are cleaved by a nuclear endonuclease reaction catalyzed by Drosha RNase III enzyme (Lee et al., 2002). Drosha cleavage produces the 60-80 nucleotide precursor RNA (pre-RNA), with 5'-phosphate and 2-nt 3' overhangs, which is the signature of RNase III cleavage (Basyuk et al., 2003; Lee et al., 2003). The remaining RNA sequences are supposedly degraded.

Drosha is a 160 kDa protein conserved in animals (Filippov et al., 2000; Fortin et al., 2002; Wu et al., 2000). It contains two RNase III domains and a double stranded RNA binding domain (dsRBD) (Figure 2). The RNase III domains, dsRBDs, and the central region adjacent to the RNase III domain are essential for miRNA processing (Han et al., 2004). pri-miRNAs lack sequence motifs which can be recognized by the Drosha protein for processing. Tertiary structure of the pri-miRNAs are the most important feature for substrate specificity (Lee et al., 2003; Zeng and Cullen, 2003; Zeng et al., 2005). Double-stranded stem and the terminal loop (~10 nucleotides) are important structural features of pre-miRNAs which can be utilized by Drosha for recognition. It seems that Drosha can act as a molecular ruler for the length of the produced pre-miRNA, since the cleavage site is exactly two helical turns (~22 nucleotides) from the terminal loop structure (Zeng et al., 2005). The experiments to identify the sequence requirements of Drosha processing were done on vertebrate specific miRNA, miR-30, which has a highly conserved hairpin structure in vertebrate species. Although miR-30 is not conserved in invertebrates, hairpin sequences of *D. melanogaster* miRNAs are also highly conserved in different species suggesting that similar requirements may also be relevant for invertebrate miRNA processing.

DGCR8 was first identified as an interaction partner of Drosha protein in a yeast two hybrid screen for *D. melanogaster* proteins (Giot et al., 2003). DGCR8 is a 120 kDa protein that contains two dsRBDs and a WW protein interaction domain, which is specific to proline-rich domains of the proteins (Figure 2). It remains to be verified if WW domain of DGCR8 interacts with the proline-rich regions of the Drosha protein. HYL1 is a DGCR8 related protein, which assists DCL-1 in efficient and precise processing of pri-miRNAs in plants (Kurihara et al., 2006). HYL1 has the conserved dsRBD domains but lacks the WW protein interaction domain. DGCR8 may be involved in the assisting Drosha in target RNA recognition.

Following nuclear processing, the precursor miRNAs (60-80 nt) are transferred to cytoplasm for production of mature miRNAs. Nuclear export of pre-miRNAs is a Ran-GTP dependent event (Yi et al., 2003). The nuclear receptor involved in the recognition of the pre-miRNAs and their transport to cytoplasm is Exportin 5, a Ran-GTP dependent nuclear transporter (Bohnsack et al., 2004; Lund et al., 2004; Yi et al., 2003). Exportin 5 recognizes the ~14 nucleotide long dsRNA stem of the pre-miRNAs and the 2-nt 3' overhangs (Gwizdek et al., 2003; Zeng and Cullen, 2004).

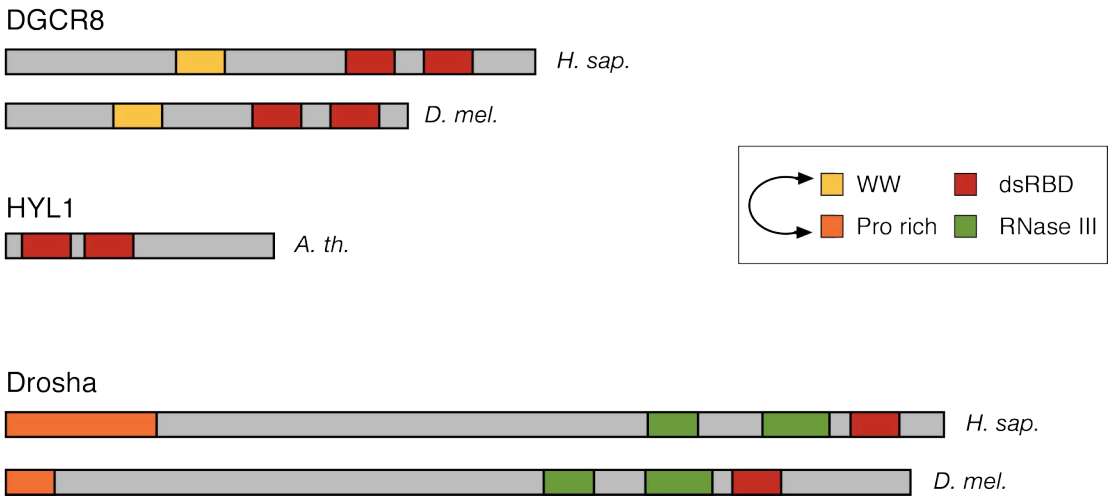


Figure 2. Domain structure of Drosha and DGCR8

Diagram above shows the protein domains of *D. melanogaster* and H. sapiens Drosha and DGCR8 proteins. DGCR8 related protein HYL1 in *A. thaliana* is also shown. The WW domain is a protein-protein interacting domain that bind to proline-rich peptide motifs (Pro-rich). The dsRNA binding domain (dsRBD) and RNase III catalytic domain (RNase III) are also indicated.

After transported to the cytoplasm the pre-miRNAs are processed by RNase III Dicer (Bernstein et al., 2001; Grishok et al., 2001; Hutvagner et al., 2001; Ketting et al., 2001; Knight and Bass, 2001). Drosha cleavage defines one end of the mature miRNA. The other end is determined by the Dicer cleavage. Dicer is a 200 kDa protein, which is conserved in all eukaryotes except *S. cerevisiae* (Bernstein et al., 2001). Some organisms have multiple Dicer analogs that mediate different enzymatic reactions. For instance *D. melanogaster* have two different Dicer homologs, Dicer1 and Dicer2. Dicer1 is required for miRNA cleavage but Dicer2 is essential for siRNA generation (Lee et al., 2004b). After Dicer cleavage a ~22 nucleotide dsRNA with 2-nt 3' overhangs is produced. The resulting molecule is very similar to siRNA duplexes that mediate double-stranded RNA-induced mRNA cleavage, except in miRNA duplexes there can be some nucleotide mismatches and bulges. The opposite strand to miRNA is called the miRNA* (Lau et al., 2001). miRNA* sequences can be find in the miRNA cloning libraries but their frequency of occurring is, by definition, much lower than miRNAs (Aravin et al., 2003; Lagos-Quintana et al., 2002; Lim et al., 2003). There are a few exceptional miRNA genes, from which two strands were cloned with comparable frequencies. In such cases two strands are denoted as 5p and 3p respectively (e.g. dme-mir-iab-4-5p and dme-mir-iab-4-3p).

The Dicer-cleaved miRNA duplex is then incorporated in protein complexes to form miRNPs. miRNPs are effector complexes similar to RISC (RNA-induced

silencing complex) that mediate dsRNA-induced targeted mRNA degradation and sometimes are referred to as miRISC (miRNA containing RISC). During RISC assembly miRNA duplexes are converted to single strands, possibly by a helicase activity. The strand, which forms the mature miRNA, is stabilized by proteins. The opposite strand is usually short lived and rapidly disappears. During RISC assembly the strand with relatively unstable base pair interactions at its 5' end is preferentially chosen for production of effector complexes (Khvorova et al., 2003; Schwarz et al., 2003). Drosophila R2D2 protein binds to the more stable end of the siRNA duplex and mediates the orientation of the protein complexes on the siRNAs (Tomari et al., 2004). R2D2 is associated with Dicer2 but not with Dicer1. dsRNA-binding protein counterpart of Dicer1, Loqs have recently been identified (Forstemann et al., 2005; Jiang et al., 2005; Saito et al., 2005). It is suggested that Loqs is involved in assembly of miRNA effector complexes.

2.4 Mechanism of miRNA-mediated gene silencing

The siRNA effector complex, RISC shares a lot of similarities with the miRNA complexes, miRNPs. First both types of effector complexes are produced by Dicer dependent mechanism. Besides the most prominent players in both RISC and miRNP complexes are Argonaute proteins. Different sizes of functional siRNA and miRNA effector complexes have been biochemically characterized. RISC complexes range from minimal 160 kDa up to ~80S macromolecular holo-RISC complex. Human miRNPs are ~15S in size. Biochemical studies have led to identification of several proteins as components of RISC and miRNP complexes. Some of the identified proteins are, Gemin3, Gemin4, vasa intronic gene (VIG), Fragile X mental retardation protein (FMRP), and a staphylococcal nuclease, Tudor-SN (Sontheimer, 2005). The biochemical functions of these proteins in RISC and miRNP complexes are not yet clear. The proteins, which were consistently identified in these complexes, are highly conserved Argonaute proteins. It is obvious that the most important players of both miRNP and RISC complexes are Argonautes. It is widely accepted that minimal effector complex contains only a small RNA and an Argonaute protein.

siRNA effector complexes mediate the endonucleolytic cleavage of the cellular mRNAs that have sequence complementarity with the siRNAs. Cleavage reaction is

the hydrolysis of a single phosphodiester bond on the backbone of the mRNA molecule corresponding to the 10th and 11th nucleotides to the 5' end of the siRNA (Elbashir et al., 2001). The cleavage is the result of "slicer" activity in the RISC. The core components of RISC, Argonaute proteins are ~100 kDa proteins with highly conserved PAZ and PIWI protein domains. PAZ is the RNA-binding domain of the Argonaute proteins (Lingel and Sattler, 2005). PIWI is a highly conserved protein domain, which can be found even in the Argonaute proteins of archea and eubacteria. PIWI domain of archeal proteins has a similar protein structure to catalytic center of RNase H, which cleave RNA/DNA hybrids (Parker et al., 2004; Song et al., 2004). It was later discovered that Ago2 is the catalytic subunit of mammalian RISC complex, which mediate the endonucleolytic mRNA cleavage (Liu et al., 2004b; Meister et al., 2004). Later it was confirmed that Ago2 is the only protein that was required for mRNA cleavage in human RISC complex, and the slicer activity is absent in human Ago1 (Rivas et al., 2005). Although mammalian Argonaute proteins have separate functions, in *D. melanogaster* there is a functional overlap between Ago1 and Ago2 proteins. It was suggested that both Ago1 and Ago2 proteins are involved in mRNA degradation and translational repression of cellular mRNAs (Rehwinkel et al., 2006). It was also biochemically confirmed that *D. melanogaster* Ago1 protein has slicer activity unlike its human homolog (Miyoshi et al., 2005).

Like siRNA effector complexes, miRNPs also target cellular mRNAs through base pairing interactions. However, the mode of action of miRNA effector complexes is more complex and varies between mRNA cleavage and translational repression depending on the complementarity between the miRNA and mRNA. Plant miRNAs, similar to siRNAs generally bind to a single perfectly complementary sequence on the coding sequences of their target mRNAs and trigger the endonucleolytic cleavage of the mRNAs (Bartel and Bartel, 2003). Animal miRNAs on the other hand bind to multiple partially complementary sequences preferentially on the 3'-UTR of mRNAs and cause downregulation of their translation, but it was demonstrated that sequences on the 5'-UTR region are also functional (Kloosterman et al., 2004).

Complementarity between the animal miRNAs and the mRNAs are higher at the first ~8 nucleotide of the miRNA (Lai, 2002; Lewis et al., 2003). Third region is often referred to as 'seed' sequence of miRNA. It was suggested that this seed sequence is required to nucleate the binding of miRNA to its target mRNA (Tomari and Zamore, 2005). The seed sequences of the miRNAs, which are phylogenetically

related are highly conserved indicating that they may be targeting similar sequences redundantly. An exception to this is between miR-16, and some cellular mRNAs, which is instead located at the central part of the miRNA. It was suggested that this interaction leads to downregulation of the half-life of mRNAs via AU rich element (ARE) mediated mRNA decay pathway (Jing et al., 2005).

Early studies with *C. elegans* miRNAs lin-4 and let-7, suggested that miRNAs downregulate the protein synthesis from targeted mRNAs without a significant change in the amounts of the mRNAs themselves (Reinhart et al., 2000; Wightman et al., 1993). It was later shown that imperfectly complementary siRNAs can act as miRNAs and downregulate protein synthesis from reporter constructs with multiple partially complementary binding sites (Doench et al., 2003; Zeng et al., 2003). miRNAs can act as siRNAs and mediate endonucleolytic degradation of reporter mRNAs with perfectly complementary binding sites (Hutvagner and Zamore, 2002). The mammalian miR-196 has a near perfect complementarity to target sequences on the Hoxb8 mRNA and leads to its cleavage (Yekta et al., 2004). These observations suggested that translational repression may not be the only mode of action of animal miRNAs as originally thought. Depending on the nature of the complementarity miRNAs may regulate the sequence specific degradation or mRNA decay of some of their targets. miRNAs can cause sequence specific degradation of their target mRNAs even when there is no perfect complementarity between them. In a study which tried to identify the off-target effects of siRNAs, it was discovered that imperfect complementarity between the siRNA and mRNA sometimes cause the decrease of cellular mRNAs (Jackson et al., 2003) suggesting that perfect complementarity is not necessary for mRNA degradation. Recently, it was reported that miRNAs mediate sequence specific degradation of their target mRNAs (Bagga et al., 2005).

The mRNA translation process can be regulated in many steps. It was suggested that miRNAs can downregulate the protein synthesis by inhibiting the initiation process. Alternatively they can also inhibit the transition of ribosomes from initiation stage to elongation. Careful examination of the mRNAs repressed by miRNPs indicated that these miRNPs could be found among polyribosomes, suggesting that the repression takes place after the ribosomes pass the initiation step (Nelson et al., 2004). *In vitro* transcribed dual reporter mRNAs with an m^7G cap dependent message and a cap-independent IRES controlled message, containing miRNA binding sites, were injected to mammalian cells in order to analyze the miRNA mediated repression

of cap-dependent and cap-independent translation. It was reported that only cap-dependent translation could be repressed by miRNAs and IRES-controlled protein expression can escape miRNA repression (Pillai et al., 2005). In a separate study, dual reporter mRNAs containing miRNA binding sites were introduced in mammalian cells by *in vivo* transcription. It was reported that when the reporter mRNAs were transcribed in the nucleus instead of being injected to the cytoplasm both cap-dependent and IRES-controlled translation could be downregulated by miRNAs (Petersen et al., 2006) suggesting that nuclear history is important for miRNP function, at least for cap-independent translation.

Mammalian GW182 protein, which is a major component of cytoplasmic P-bodies in mammalian cells, interacts with Argonaute proteins (Liu et al., 2005). GW182, which is also known as TNRC6A, is member of protein family with glycine-tryptophan (GW) repeats. Members of GW protein family are modular proteins composed of a central UBA domain, a C-terminal RRM RNA recognition domain, and highly conserved N-terminal GW repeats. Multiple sequence alignment of all proteins with these domains revealed that there are three paralogs (TNRC6A/GW182, TNRC6B, and TNRC6C) in vertebrates, a single ortholog in insects (GW182), and no orthologs in worms and fungi. TNRC6B also localizes to cytoplasmic P-bodies and is a component of purified Argonaute complexes, which contain several other proteins such as MOV10, PRMT5, and Gemin3/4 (Meister et al., 2005). P-bodies are cytoplasmic processing locations that contain mRNA decapping and degradation machinery. In order to investigate the functional significance of this interaction, several functional studies have been carried out. It was suggested that miRNP components localize to P-bodies in an RNA dependent manner (Sen and Blau, 2005). GW182 depletion interfered with the miRNA mediated translational repression in mammalian cells. GW182 depletion also disrupted the P-body formation and localization of the miRNPs to the P-bodies (Liu et al., 2005; Sen and Blau, 2005). TNRC6B and MOV10 are required for miRNA guided mRNA cleavage in mammalian cells (Meister et al., 2005). So it seems that localization of miRNP complexes to P-bodies is essential for both siRNA mediated mRNA cleavage and miRNA mediated translational repression pathways. Although the mechanism of P-body association is a subject of active research, it was speculated that GW182 (TNRC6A) and TNRC6B mediate targeting of Argonaute complexes to P-bodies,

while at the same time might act in assembly of the silencing complexes and/or in guiding target recognition (Liu et al., 2005; Meister et al., 2005).

GW182 bears similarity in the N-terminal region, where GW repeat elements are located, with *C. elegans* AIN-1 protein. Although AIN-1 does not contain UBA and RRM domains, it is considered as a functional homolog of GW proteins of vertebrates and insects. AIN-1 is localized to P-bodies, interacts with Ago1, and is involved in miRNA pathway in *C. elegans* (Ding et al., 2005). In *D. melanogaster*, GW182 interacts with Ago1 in vivo and in vitro. Interaction with Ago2 however, was not detected (Behm-Ansmant et al., 2006) suggesting that Ago2 may act independently of GW128 in *D. melanogaster*. The lack of GW182 interaction with Ago2 is consistent the observation that GW182 is dispensable for siRNA-mediated mRNA degradation in *D. melanogaster* (Rehwinkel et al., 2005).

In *D. melanogaster* it appears that miRNA-mediated silencing of mRNA targets through GW182-Ago1 pathway takes place by two distinct mechanisms: translational repression and mRNA degradation. mRNA degradation mechanism can be inhibited by depletion of proteins CAF1, NOT1, and DCP1:DCP2 complexes, which are involved in mRNA decay pathway (Behm-Ansmant et al., 2006), suggesting that GW182 may be involved in mRNA deadenylation and decapping. The requirement of decapping enzymes is consistent with the observation that mRNAs which are translated cap-independently are resistant to miRNA-mediated repression (Pillai et al., 2005). The mechanism of translational repression by GW182 remains to be elucidated.

2.5 Regulatory functions of miRNAs

For more than 500 identified miRNAs there are still a handful of them with the identified biological functions. Table 2 summarizes some of the functions of animal and plant miRNAs identified by various approaches.

As discussed before plant miRNAs are different from animal miRNAs in the way they target their cognate mRNAs. Plant miRNAs and cellular mRNAs pair with near-perfect complementarity, which is required for miRNA-mediated repression. Plant miRNAs act like siRNAs do and trigger cleavage of the cognate mRNAs. Perfect complementarity of the miRNA sequences and the corresponding sites on

mRNAs made the identification of the target mRNAs as easy as simple homology searching (Rhoades et al., 2002). Most of the identified proteins that are targeted by plant miRNAs are transcription factors (Table 2), which are involved in developmental patterning and cell differentiation (Rhoades et al., 2002). This bias partly explained the pleiotropic developmental phenotypes observed in mutants affecting the miRNA biogenesis, like DCL1, HEN1 and AGO1 (Bohmert et al., 1998; Jacobsen et al., 1999; Park et al., 2002; Schauer et al., 2002).

Because of several mismatches and bulges between the miRNAs and mRNAs, identification of the functions of animal miRNAs is more challenging. *lin-4* and *let-7* miRNA genes were identified through mutations affecting the developmental timing of the *C. elega*ns (Liu and Ambros, 1989; Reinhart et al., 2000). *lin-4* targets *lin-14* and *lin-28* and *let-7* targets *lin-41* and *hbl-1* were identified by genetic complementation studies. Generation of deletions in mRNA sequences revealed that target sites are located at the 3'-UTR of *lin-4* and *let-7* targets. Since the miRNAs identified this way were obtained from a phenotype of a mutation affecting the gene, the miRNAs were easily characterized and their biological function was discovered promptly. Another *C. elegans* miRNA gene, which was identified by a genetic mutation, was *lys-6*. It was identified from a mutation affecting the asymmetric expression of chemoreceptors in *C. elegans* embryos (Johnston and Hobert, 2003). *lin-28* is conserved in various species. Mouse and human homologs of *lin-28* contain miR-125a and let-7b target sites in 3'-UTR region (Moss and Tang, 2003). *mir-125a* is homolog of *C. elegans lin-4* in vertebrates. Although the expression profile of *D. melanogaster lin-28* is very similar to its *C. elegans* homolog, mir-125 target sequences could not be identified in 3'-UTR of *D. melanogaster lin-28* (Moss and Tang, 2003). Fly homolog of *lin-41* is *dpld* (*dme-lin-41*), which contain*s* let-7 target sequences its like *C. elegans* homolog in its 3'-UTR (Pasquinelli et al., 2000). Abrupt (ab) mRNA, which is involved in neuronal development, was predicted computationally to be the target of let-7 miRNA in *D. melanogaster* (Enright et al., 2003), and the prediction was experimentally confirmed by *in vitro* luciferase reporter gene assay (Burgler and Macdonald, 2005).

In *D. melanogaster* the functions of bantam, miR-14 and miR-278 were identified from mutations affecting these miRNAs. Screens for mutations affecting the cell death or cell proliferation in the developing fly led to the identification of *bantam* locus, which coded for a miRNA. It was identified that bantam miRNA

targeted *Hid* mRNA, which coded for a proapoptotic protein, Hid (Brennecke et al., 2003). The mutations indicated that miR-14 is involved in apoptosis and total body triglyceride amounts (Xu et al., 2003). miR-278 is also involved in fat metabolism and triglyceride levels in *D. melanogaster* (Teleman et al., 2006).

Table 2. Examples of miRNAs with identified functions

miRNA	Target Gene(s)	Biological Role	Ref
Nematodes			
lin-4	*Ce lin-14*	Cell differentiation in early larval stages	1
	Ce lin-28	Cell differentiation in early larval stages	2
let-7	*Ce lin-41*	Cell differentiation in late larval stages	3
	Ce hbl-1	Cell differentiation in late larval stages	4
lys-6	*Ce cog-1*	Left/right asymmetry of chemoreceptor expression	5
Insects			
bantam	*Dm hid*	Apoptosis and growth during development	6
let-7	*Dm lin-41 (dpld)*	Regulation of transcription	7
	Dm ab	Neuronal development	8
miR-1		Postmitotic muscle growth	9
miR-14		Apoptosis and fat metabolism	10
miR-278		Fat metabolism	11
Vertebrates			
miR-1	*Xl HDAC4*	Myogenesis	12
miR-29b		Amino acid catabolism	13
miR-122		Fat metabolism	14
miR-133	*Xl SRF*	Myogenesis	12
miR-143		Adipocyte differentiation	15
miR-181	*Mm Hox-A11*	Myogenesis and hematopoietic differentiation	16
miR-375		Insulin secretion	17
Plants			
miR165/166	*At REV*	Leaf development	18
miR159	*At MYB33*	Leaf development	18
miR172	*At AP2*	Flower development	19
miR-JAW	*At TCP4*	Leaf development	20

Species abbreviations: *Ce, C. elegans; Dm, D. melanogaster; Xl, X. laevis; Mm, M. musculus; At, A. thaliana*

References: 1 (Lee et al., 1993; Wightman et al., 1993); 2 (Moss et al., 1997); 3 (Reinhart et al., 2000; Slack et al., 2000); 4 (Abrahante et al., 2003; Lin et al., 2003); 5 (Johnston and Hobert, 2003); 6 (Brennecke et al., 2003); 7 (Pasquinelli et al., 2000); 8 (Burgler and Macdonald, 2005); 9 (Sokol and Ambros, 2005); 10 (Xu et al., 2003); 11 (Teleman et al., 2006); 12 (Chen et al., 2006); 13 (Mersey et al., 2005); 14 (Esau et al., 2006); 15 (Esau et al., 2004); 16 (Chen et al., 2004); 17 (Poy et al., 2004); 18 (Rhoades et al., 2002); 19 (Park et al., 2002); 20 (Palatnik et al., 2003)

Identification of miRNAs by forward genetic methods in worm and fly suggested that many interesting miRNA genes remains to be identified in other animals, too. An alternative to forward genetic approach is to start from the sequence of the known miRNAs and identify the function by reverse genetic experimental systems. Over-expression of the miRNA genes or depleting miRNAs by modified antisense oligonucleotides were used by several groups to identify the functions of already identified miRNAs. For instance over-expression of miR-181 in mouse bone marrow cells showed that miR-181 can modulate B-cell or T-cell development (Chen et al., 2004). On the other hand, liver-specific metabolic functions of miR-122 were identified by two different groups by targeting and inactivating the miR-122 with modified antisense oligonucleotides (Esau et al., 2006; Krutzfeldt et al., 2005).

In order to completely deplete miRNAs in the development, Giraldez et al. developed maternal zygotic mutants of Dicer in zebrafish embryos. The loss of global miRNA processing led to very severe abnormal brain morphogenesis. When the researchers tried to introduce different miRNAs exogenously to the embryos, they observed that miR-430, which has 71 copies in zebrafish genome but not conserved in other species, rescued the brain malformation. Since the loss of miR-430 responsible for the phenotype it was suggested that miR-430 was involved in brain morphogenesis in zebrafish embryos (Giraldez et al., 2005).

Any given miRNA can target different cellular mRNAs, as any mRNA can be targeted by many different miRNAs. These complex miRNA/mRNA interactions can lead to very complex regulatory pathways. In order to identify the mRNAs targeted by miRNAs, several computational target mRNA searching algorithms have been devised (Enright et al., 2003; Kiriakidou et al., 2004; Lewis et al., 2003; Rajewsky and Socci, 2004; Stark et al., 2003). These algorithms were based on the assumptions that hypothetical miRNA/mRNA interactions can be modeled on the genetically identified interactions (e.g. lin-4/lin-14). The common characteristics of the all known miRNA targets are, (1) miRNA target sites are located on the 3'-UTR region of the mRNAs, (2) complementary base pairing interactions are located on the 5' half of the miRNA sequences, (3) miRNA target sequences on the 3'-UTRs are phylogenetically conserved among different species. Approximately 200 mRNA target per miRNA was identified by computational target predictions, which are yet to be experimentally verified. Because of the assumptions made for the predictions, miRNA target

sequences which are not conserved or not located on the 3'-UTR can not be identified by these algorithms.

The function of such miRNAs can be correlated to the cell specific functions of the cells they are expressed. For instance miR-375, which is cloned and specifically expressed in pancreatic islet cells is involved in insulin secretion from pancreatic β cells (Poy et al., 2004). One striking example to the miRNA expression pattern that was used for target identification is vertebrate *miR-1*/*miR-133* cluster. It was previously shown that miR-1 was expressed in skeletal muscle and heart tissues suggesting that it is involved in the development of these tissues (Lagos-Quintana et al., 2002). Genetic knock-out of mir-1 in *D. melanogaster* (Sokol and Ambros, 2005) and over expression studies in *X. leavis* (Chen et al., 2006) verified that miR-1 has an important function in establishing and maintenance of the muscle cell identity, which is conserved among species. Microarray studies revealed that miR-1 is expressed preferentially in muscle tissues and the predicted mRNA targets, which contain miR-1 recognition sequences are also preferentially expressed in similar tissues, suggesting that miR-1 may be involved in muscle tissue differentiation and maintenance of the muscle tissue identity (Lim et al., 2005; Sood et al., 2006).

And finally analysis of the expression patterns of miRNAs in cancer tissues led to the discovery of a new class of miRNAs that are involved in tumorigenesis. For example it was noted that expression of miR-15 and miR-16 can be lost by genetic deletions in some B-cell chronic leukemia patients (Calin et al., 2002), *mir-143/mir-145* cluster is located in a tumor suppressor locus correlated with colon carcinomas (Michael et al., 2003), and let-7 expression is reduced in small lung carcinomas (Takamizawa et al., 2004). Systematic expression analysis of miRNAs in human tumor samples revealed that miRNAs are generally downregulated in human tumors, which can be classified according to miRNA expression profiles (Lu et al., 2005). The causal relationship of cancer and miRNA expression malfunctions is still debatable. But increasing evidence suggests that miRNA expression profiling could contribute to more precise tumor classifications and predict therapeutic outcomes in the future.

2.6 Detection of miRNAs

miRNA detection is an important part in miRNA studies. Hundreds of candidate miRNA sequences, which are identified by bioinformatic predictions, have to be experimentally verified by a miRNA detection assay. miRNA detection is also required for expression profiling of the known miRNAs for biological characterization. Although miRNA expression can be regulated in several steps of the maturation pathway (Obernosterer et al., 2006), transcriptional regulation is the major regulatory mechanism. Expression profiling of miRNAs suggested that miRNA genes have distinct tissue and cell specific expression profiles, which are suggestive to their biological functions.

Large scale cDNA cloning and sequencing provides information on the relative expression levels of miRNAs in different tissue samples. Expression patterns of miRNAs can also be identified by direct detection methods. All of the direct miRNA detection techniques are hybridization based. Northern blot hybridization is the most widely used technique for miRNA detection. As it can be used for verification of predicted miRNAs, it can also provide temporal and tissue specific expression information (Lagos-Quintana et al., 2001; Lagos-Quintana et al., 2002; Lai et al., 2003). Total RNA samples, which are prepared from different developmental stages and tissues are size fractioned by electrophoresis and transferred on to membranes for hybridization with 5' end labeled oligonucleotide probes. Northern blot can also be a quantitative technique with detection limits near 1 fmol. Absolute amounts of the miRNAs in the RNA samples are obtained by comparison to the signal strength of a dilution series of a synthetic miRNA of known concentration (Lim et al., 2003). The limitation of the technique is that, miRNAs with low expression levels require large amounts of total RNA (30-40 μg) for detection.

RNase protection assay (RPA) can be used to detect miRNA expression in various RNA samples. In this case the input material is not blotted to a membrane but rather hybridized in the solution to internally-labeled antisense probes that are longer than the miRNAs. After hybridization, unpaired labeled probes and single stranded segments of the paired molecules are removed by single-strand specific RNase treatment (Soutschek et al., 2004). The sensitivity of the RPA is comparable to

Northern blotting. Because large amounts of input RNA (>100 μg) can be used, less abundant miRNAs can be detected with better efficiency.

RT-PCR is probably the most powerful technique for miRNA detection. pre-miRNAs can be directly quantified by real-time qRT-PCR. Mature miRNAs can also be quantified by using a looped primer design to add a universal sequence to the miRNA cDNA. The obtained cDNA is then quantified by real-time qPCR amplification (Chen et al., 2005a). Nanogram range of starting RNA sample is sufficient for efficient quantification of abundant miRNAs. For less abundant miRNAs ~1 μg of input RNA is recommended.

The most widely used technique to determine the expression profile of hundreds of miRNAs are miRNA microarrays (Babak et al., 2004; Barad et al., 2004; Baskerville and Bartel, 2005; Krichevsky et al., 2003; Liu et al., 2004a; Miska et al., 2004; Sun et al., 2004; Wienholds et al., 2005). The conventional microarray systems are not suitable to detect short targets like miRNAs. Therefore most of the miRNA microarray systems targeted pre-miRNA expression instead of mature miRNAs (Barad et al., 2004; Liu et al., 2004a). However, miRNA processing can be regulated and pre-miRNA expression may not always correlate to the mature miRNAs (Obernosterer et al., 2006). Recent microarray studies detect mature miRNAs by specific probes, though closely sequence-related miRNAs may still cross-hybridize. Another limitation of the technique is the very low dynamic range of the expression data in microarrays that use signal amplification. Because of this microarray is considered to be a semi-quantitative technique although quantitative expression data can be obtained by using a universal standard for comparison. For absolute quantification of miRNA expression, Northern blot assay is still preferred over microarrays.

Spatial expression of miRNAs can be determined indirectly by reporter “sensor” transgenes. miRNA complementary sequences are inserted in the 3'-UTR region of constitutively expressed GFP transgenes (Brennecke et al., 2003). GFP sensor mRNA is degraded at the sites of miRNA expression, since miRNA/mRNA hybrid will induce dsRNA mediated mRNA degradation, RNAi. miRNA expression can be visualized by the loss of GFP expression.

The more direct method to identify spatial distribution of miRNAs in different tissues is *in situ* hybridization. I*n situ* hybridization is routinely used for detecting

mRNAs in different tissues but because of the small size of the miRNAs development of efficient *in situ* hybridization protocols for miRNA detection was challenging. The protocols should also be optimized for fixing small RNAs in the tissues efficiently without interfering hybridization. Locked nucleic acid (LNA) probes can be used to detect mature miRNAs in whole mount zebrafish embryos (Wienholds et al., 2005). However, the usage of the LNA probes in other types of tissues was not as successful except for the most abundant miRNAs. For instance, *D. melanogaster* miR-1, which is the most abundant miRNA in *D. melanogaster* embryos (Aravin et al., 2003), could be detected using LNA probes (Sokol and Ambros, 2005). miRNA expression can also be identified indirectly by targeting the pri-miRNA transcripts in stead of mature miRNAs. *D. melanogaster* miR-1 expression is identified by using long ssRNA probes complementary to the pri-miRNA sequence (Sokol and Ambros, 2005) and it was shown that the expression pattern of pri-mir-1 was similar to the mature miR-1.

2.7 *D. melanogaster* P-elements

P-elements are natural transposable elements that can be found on *D. melanogaster* genome. They can be used as genetic tools for manipulating *D. melanogaster* genomic DNA (Engels, 1992). Intact P-elements is a 3kb DNA featuring 31 bp inverted terminal repeat and 11 bp subterminal inverted repeat. These repeats are needed in *cis* for efficient transposition of P-element. Internally there are other repeats of unknown functions plus a transposase gene composed of four exons. this gene is required in *trans* for transposition process. Nonautonomous P-elements also exist. Such elements lack the transposase gene but retain the parts of the sequence required in *cis* for transposition. Artificial nonautonomous P-elements which contain a red eye marker w^+ gene have also been created.

P-element mobilization is an easy way of introducing site specific deletions on the *D. melanogaster* chromosomes (Preston et al., 1996). P-element insertion lines have been generated with insertions at thousands of genomic positions. Random insertional mutations generated this way can be used to screen the functions of the affected genes. Alternatively, mobilization of the P-elements can be used to generate flanking deletions at the sites of P-element insertions for generating site specific genetic mutations. In this method P-element with insertions at the targeted locations,

which can be obtained from public stock centers, are mobilized by a transposase activity in *trans* (Figure 3). Transposase activity is usually a chromosome with an intact transposase gene. Recombinants (jump-outs) are collected using the loss of red eye marker in the progeny. Then, the lines with flanking deletions can be identified among the jump-outs by PCR screens (Figure 3).

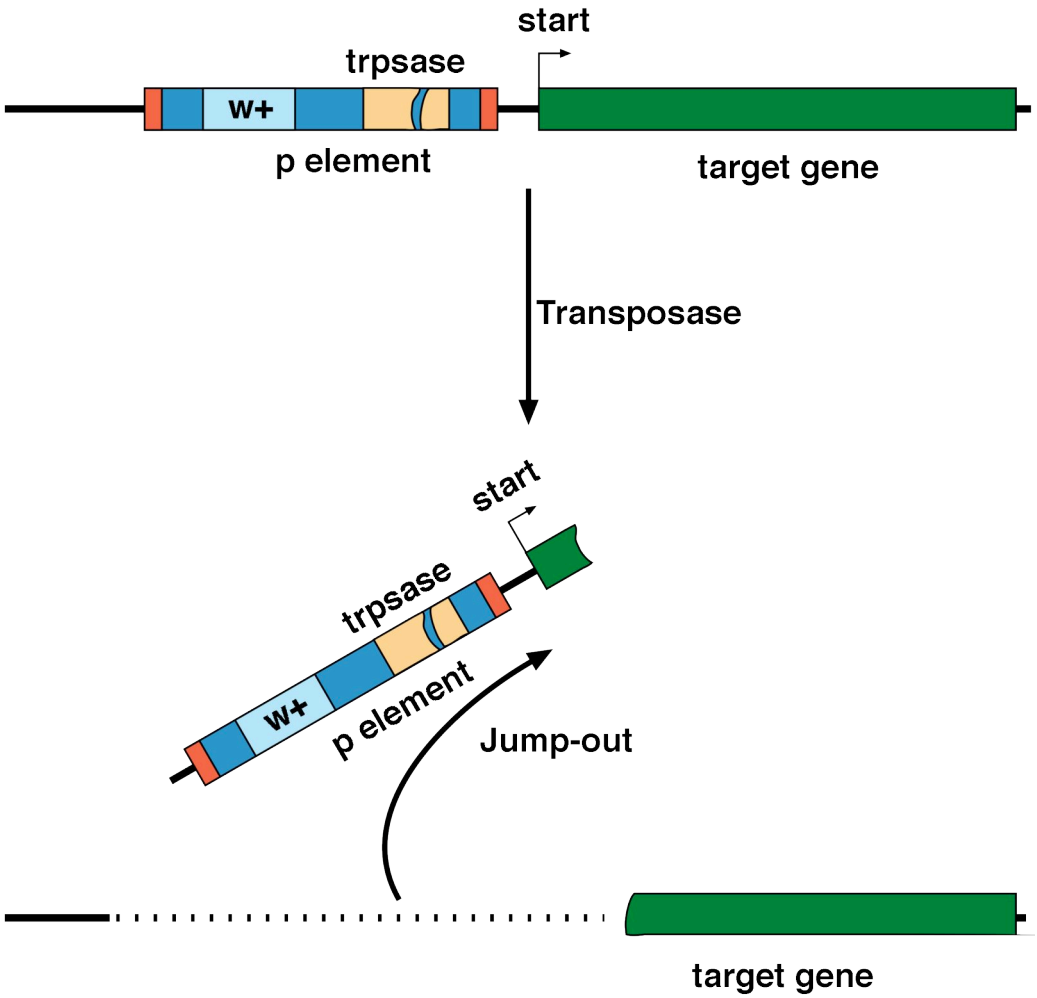


Figure 3. Generation of gene deletions by P-element mobilization

Deletions affecting targeted genes (green box) can be induced by mobilization of nearby P-elements. P-element lines, which lack transposase activity on the transposable element are readily available. Transposase activity can be introduced by a separate transposase source chromosome. P-elements are mobilized this way and random deletions affecting nearby genomic sequences are induced.

3. Rationale

By the time I joined the laboratory first miRNA genes were identified by cloning from cell and tissue samples. The functions of newly identified miRNAs were mostly unknown. The biological characterizations of miRNAs were not done and expression patterns of miRNAs were also undetermined. The characterization of novel gene products starts by determining their specific expression pattern and developmental regulations.

Better characterization of miRNA expression can contribute to the identification of novel miRNA functions and target mRNAs, which may be overlooked by large scale bioinformatic screens. In many cases very obvious biological correlations can be made solely by miRNA expression patterns. For instance any miRNA which is expressed exclusively in a specific tissue would necessarily be related to the biological functions of the specific cells they are located. They may also be involved in the cell type determination of the tissues they are expressed. Similar correlations can be derived for specific developmental events, if the developmental timing of the miRNA expressions can be characterized.

Methods for quantifying the miRNA expression levels and for identification of temporal and spatial miRNA expression patterns were needed. In my project I used Northern blot hybridization and *in situ* hybridization methods to identify the temporal and spatial expression pattern of *D. melanogaster* miRNAs during development.

D. melanogaster is a model organism useful to study miRNA function. It has a relatively simple life cycle and a short reproduction period which make Drosophila an excellent tool for genetic studies. *D. melanogaster* genome is completely sequenced and there are numerous stock centers with thousands of mutation lines open for researchers.

In summary the aim of this study was characterization of *D. melanogaster* miRNA expression patterns to provide a starting point for future functional analysis, and to guide identification of miRNA/target mRNA interactions.

4. Materials and Methods

4.1 Chemicals and equipment

4.11 Chemicals

All the chemicals were obtained from Applichem or Merck unless otherwise stated. Standard buffers were prepared as described in Molecular Cloning a Laboratory Manual (Sambrook and Russell, 2001).

4.1.2 Equipment

	Model	*Company*
Agarose gel electrophoresis	Sub-Cell GT System	Bio-Rad
Automated DNA sequencer	ABI PRISM 310	ABI
Automated thermal cycler	PTC-100	MJ Research, Inc.
Cell Counter system	CASY	Schärfe System
Laboratory Centrifuge	Evolution RC	Sorvall
Table Centrifuge	Megafuge 1.0R	Heraeus
Micro centrifuge	Biofuge fresco	Heraeus
Oligonucleotide synthesizer	Expedite 8909	ABI
PAGE apparatus	Model V15.17	Life Technologies
Phosphoimager	Typhoon 8600	Mol. Dynamics
Power supply	Power Pac 3000	Bio-Rad
Spectrophotometer	Ultraspec 3000 pro	Amersham PB
Lyophylizer	Concentrator 5301	Eppendorf
Gel documentation system	GelDoc system	Bio-Rad

4.2 Cloning and PCR

4.2.1 Primers

18-nt oligodeoxynucleotide primers were either chemically synthesized at 0.2 μM scale; in house using standard DNA phosphoramidites (Proligo) or purchased from MWG-Biotech. Chemically synthesized oligodeoxynucleotides were deprotected for 16 h at 55^{o}C in 1.5 ml 30% aqueous ammonia. After removal of the glass beads the oligodeoxynucleotides were precipitated by adding 12 ml absolute 1-butanol and collected by centrifugation. Residual 1-butanol was evaporated by using an Eppendorf Concentrator, and the pellets were suspended in 0.5 ml water.

4.2.2 PCR

PCR reactions were carried out in 20 mM Tris-HCl, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 200 μM dNTPs, pH 8.8 using 50 U/ml Taq polymerase (NEB, Cat. # M0267S) and 0.2 mM primers. 0.1-1 ng/ml plasmid DNA, and 1-10 mg/ml genomic DNA were used as templates for 30 successive cycles of denaturation, annealing and extension steps at 50 and 72 ^{o}C respectively, which were performed by automated thermal cycler.

For cloning purposes DNA fragments were amplified by Easy-A high fidelity cloning enzyme (Stratagene, Cat. # 600400). Reaction buffer was supplied by the manufacturer.

4.2.3 Cloning

PCR-produced DNA fragments were cloned in plasmid vector pCR 2.1-TOPO according to TOPO TA cloning kit (Invitrogen, Cat. # K4500-01) protocol. DNA fragments were then subcloned into P element transformation vector, pP{CaSpeR-4} (GenBank accession X81645) which was kindly supplied by Dr. Gerd Vorbrüggen. T4 DNA ligase and restriction enzymes were obtained from NEB and used as recommended by the manufacturer.

Culture of *E. coli*, plasmid isolation, and transformation, concentration measurement, and agarose gel electrophoresis of DNA fragments, were performed as described in Molecular Cloning a Laboratory Manual (Sambrook and Russell, 2001).

4.3 Total RNA isolation

Total RNA from different tissues was isolated as previously described (Lagos-Quintana et al., 2002). 3 to 5 g of *D. melanogaster* embryos from different stages of the development were collected on wire sieves and washed first using tap water and then with embryo wash buffer (120 mM NaCl, 0.05% Triton X-100). Embryos were dried using a paper towel and frozen at -80^{o}C until RNA extraction. Frozen embryos were placed in to a glass homogenizer before they thaw. 5 ml of extraction solution (50% acidic aqueous phenol, pH 4.0, 2 M guanidine isothiocyanate, 12.5 mM sodium citrate (trisodium citrate dihydrate), 0.25% N-lauroylisosarcosinate, 25 mM β-mercaptoethanol) was added per 1 g of embryo and then homogenized. 1/10 volume of chloroform/isoamyl alcohol (24:1) and 1/20 volume of 3 M sodium acetate (pH 5.2) were added to the mixture and homogenized further until a white and turbid suspension was obtained. The suspension was then transferred to a 30 ml glass centrifuge tube and centrifuged for 10 min at 10,000 rpm (Sorvall SS34 rotor), at 4^{o}C. The upper aqueous phase was transferred to a new glass tube and extraction was repeated once more by adding 1 volume of phenol (pH 4.0)/chloroform/isoamyl alcohol (25:24:1) and followed by centrifugation. The extraction cycles were repeated until clear aqueous solutions were obtained. After removal of the aqueous phase, the RNA was precipitated by the addition of 2.5 and incubated overnight at -20^{o}C. RNA was collected by centrifugation for 30 min at 12,000 rpm (Sorvall SS34 rotor), at 4^{o}C. The supernatant was removed and the RNA pellet was resuspended in 200 μl of formamide per 1 g of embryo.

Total RNA from cultured *D. melanogaster* Schneider S2 insect cells (Invitrogen, Cat. # 10831-014) was prepared as described above except cultured cells were collected and disrupted as follows; cells were resuspended in Ex-cell 420 serum-free culture medium (JRH Biosciences, Cat. # 14420-1000M) by vigorous pipeting. Cell pellets were collected by centrifugation for 3 min at 2,000 rpm (Megafuge 1.0R table centrifuge, Heraeus) and frozen at -80^{o}C after the removal of the medium. For

small amount of cells disruption was carried out by sucking in and pumping out the cell suspension several times using 2 ml syringes with 0.45x25 mm needles in stead of glass homogenizers.

Absorbance at 260 nm was measured and concentrations of RNAs were calculated according to (1 OD_{260} = 40 μg/ml). Typical yield of total RNA was 1 mg for 1 g of embryo and 5 μg for $1x10^6$ S2 Schneider cells.

4.4 Northern blot hybridization

4.4.1 Probes

Oligodeoxynucleotide probes of 20 to 25-nt of 1 μM concentration were 5'-labeled using 3.3 μM γ-^{32}P-ATP (Hartmann, Cat. # FP-301) and T4 Polynucleotide Kinase (NEB, Cat. # M0201S).

After 15 min, the labeling reaction was stopped by adding 30 μl of 30 mM EDTA (pH 8.0). Then the labeled probes were applied to Sephadex G25 spin columns (Roche, Cat. # 1273949) to remove unincorporated radioactive nucleotides and specific activity (Cherenkov count) was measured by Packard liquid scintillation counter. Typical specific activity of the labeled probes were 200,000 cpmA/μl.

Names, and nucleotide sequences of the oligodeoxynucleotide probes, which were used in Northern blot hybridization experiments are shown in table 3.

Table 3. Northern blot hybridization probes

probe	nucleotide sequence
as-dme-bantam	ATCAGCTTTCAAAATGATCTCA
as-dme-let-7	TACTATACAACCTACTACCTCAATTTGCC
as-dme-mir-1	CTCCATACTTCTTTACATTCCA
as-dme-mir-2a	GCTCATCAAAGCTGGCTGTGATA
as-dme-mir-3	TGAGACACACTTTGCCCAGTGA
as-dme-mir-4	TCAATGGTTGTCTAGCTTTAT
as-dme-mir-5	ATATCACAACGATCGTTCCTTT

as-dme-mir-6	AAAAAGAACAGCCACTGTGATA
as-dme-mir-7	AACAACAAAATCACTAGTCTTCCA
as-dme-mir-8	TAATGCTGCCCGGTAAGATGTGA
as-dme-mir-9a	TCATACAGCTAGATAACCAAAGA
as-dme-mir-10	AACAAATTCGGATCTACAGGGT
as-dme-mir-11	AGCAAGAACTCAGACTGTGATG
as-dme-mir-12	CCAGTACCTGATGTAATACTCA
as-dme-mir-13b	ACTCGTCAAAATGGCTGTGATA
as-dme-mir-14	ATAGGAGAGAGAAAAAGACTGA
as-dme-mir-31b	CAGCTATTCCGACATCTTGCCA
as-dme-mir-33	ACAATGCGACTACAATGCACCT
as-dme-mir-34	CAACCAGCTAACCACACTGCCA
as-dme-mir-79	ATGCTTTGGTAATCTAGCTTTA
as-dme-mir-87	CACACCTGAAATTTTGCTCAA
as-dme-mir-92a	ATAGGCCGGGACAAGTGCAATG
as-dme-mir-100	ACAAGTTCGGATTTACGGGTT
as-dme-mir-124	TGGCATTCACCGCGTGCCTTA
as-dme-mir-125	TCACAAGTTAGGGTCTCAGGGA
as-dme-mir-133	ACAGCTGGTTGAAGGGGACCAA
as-dme-mir-184	CCTCTTCTCATTTCTCCGTCCA
as-dme-mir-210	ATAGCCGCTGTCACACGCACAA
as-dme-mir-219	CAAGAATTGCGTTTGGACAATCA
as-dme-mir-263a	CGTGAATTCTTCCAGTGCCATT
as-dme-mir-263b	TGTGAATTCTCCCAGTGCCAAG
as-dme-mir-274	ATTACCCGTTAGTGTCGGTCACAAAA
as-dme-mir-275	GCGCGCTACTTCAGGTACCTGA
as-dme-mir-276	AGAGCACGGTATTAAGTTCCTA
as-dme-mir-277	TGTCGTACCAGATAGTGCATTTA
as-dme-mir-278	AAACGGACGAAAGTCCCACCGA
as-dme-mir-279	TTAATGAGTGTGGATCTAGTCA
as-dme-mir-280	ATCATTTCATATGCAACGTAAATACA
as-dme-mir-281	ACAAAGAGAGCAATTCCATGACA

as-dme-mir-282	CAGACAAAGCCTAGTAGAGGCTAGAT
as-dme-mir-283	AGAATTACCAGCTGATATTTA
as-dme-mir-284	ATTGCTGGAATCAAGTTGCTGACTTC
as-dme-mir-285	GCACTGATTTCGAATGGTGCTA
as-dme-mir-286	GCACGAGTGTTCGGTCTAGTCA
as-dme-mir-287	GTGCAAACGATTTTCAACACA
as-dme-mir-288	CATGAAATGAAATCGACATGAAA
as-dme-mir-289	AGTCGCAGGCTCCACTTAAATATTTA
as-dme-mir-303	CCAGTTTCCTGTGAAACCTAAA
as-dme-mir-304	TCACATTTACAAATTGAGATTA
as-dme-mir-305	CAGAGCACCTGATGAAGTACAAT
as-dme-mir-306	TTGAGAGTCACTAAGTACCTGA
as-dme-mir-307	CGCTCACTCAAGGAGGTTGTGA
as-dme-mir-308	CTCACAGTATAATCCTGTGATT
as-dme-mir-309	TAGGACAAACTTTACCCAGTGC
as-dme-mir-310	AAAGGCCGGGAAGTGTGCAATA
as-dme-mir-311	TCAGGCCGGTGAATGTGCAATA
as-dme-mir-312	TCAGGCCGTCTCAAGTGCAATA
as-dme-mir-313	TCGGGCTGTGAAAAGTGCAATA
as-dme-mir-314	GCCGAACTTATTGGCTCGAATA
as-dme-mir-315	GCTTTCTGAGCAACAATCAAAA
as-dme-mir-316	CGCCAGTAAGCGGAAAAAGACA
as-dme-mir-317	TGGATACCACCAGCTGTGTTCA
as-dme-mir-318	TGAGATAAACAAAGCCCAGTGA
as-dme-mir-iab4-3p	GTTACGTATACTGAAGGTATACCG
as-dme-mir-iab4-5p	TCAGGATACATTCAGTATACGT
as-dme-U2snRNA	CTGATAAGAACAGATACTACACT
as-dme-U6snRNA	GGGCCATGCTAATCTTCTCTGTA
as-mmu-mir-30a	GCTTCCAGTCGAGGATGTTTACA
as-mmu-mir-101	TCAGTTATCACAGTACTGTA
as-mmu-mir-122a	ACAAACACCATTGTCACACTCCA
as-mmu-mir-125a	CACAGGTTAAAGGGTCTCAGGGA

as-mmu-mir-127	AGCCAAGCTCAGACGGATCCGA
as-mmu-mir-128	AAAAGAGACCGGTTCACTCTGA
as-mmu-mir-131	ACTTTCGGTTATCTAGCTTTA
as-mmu-mir-132	ACGACCATGGCTGTAGACTGTTA
as-mmu-mir-143	TGAGCTACAGTGCTTCATCTCA

4.4.2 Blotting Procedure

30 μg of total RNA with equal volume of 2X bromophenol blue (BPB) gel loading buffer (8M urea, 50 mM EDTA, 0.2 mg/ml bromophenol blue) was loaded in each lane of a 20 well 1.5 mm thick, 15x17 cm large 12% denaturing polyacrylamide (PA) gel, which was prepared using a SequaGel Sequencing kit (National Diagnostics, Cat . # EC-833). The electrophoresis was carried out in 0.5X TBE buffer (Sambrook and Russell, 2001) at 30 W for 1-1.5 hours until BPB reached the bottom of the gel. Separation of the RNA was visualized by ethidium bromide staining (10 min in 1 ng/ml ethidium bromide/0.5X TBE) followed by UV geldoc analysis.

Hybond N+ nylon membranes (Amersham, Cat. # RPN303B) were used to transfer RNA using semidry gel transfer system (custom made) in TBE buffer at constant amperage for 1 hour (3 mA/cm^2). After membrane transfer, nylon membranes were air dried and RNA/membrane UV cross-linking was carried out in UV Stratalinker 2400 (Stratagene) at 1200 J for 30 seconds. The membranes were subsequently baked for 1 hour at 80^oC.

Before probe hybridization, membranes were pretreated with freshly prepared Northern blot hybridization solution (5X SSC, 7% SDS, 20 mM Na2HPO4, pH 7, 1X Denhardt's buffer, 100 ng/ml sonicated salmon sperm DNA) for 2 h at 50^oC. Hybridization with labeled ssDNA oligonucleotide probes was carried out overnight at 50^oC in 15 ml hybridization solution and 0.4 nM labeled probe.

After overnight hybridizations membranes were washed two times in first washing buffer (5% SDS, 5X SSC) for 10 minutes at 50^oC. After first washes membranes were washed one more time in second washing buffer (1% SDS, 1X SSC) again for 10 minutes at 50^oC. Finally membranes were exposed for 3 h to phosphoimager screens (Molecular Dynamics), which were then scanned in

phosphoimager for further analysis. Quantification of the Northern data was performed using ImageQuant (version 1.2) software from Molecular Dynamics.

4.5 RNAi in cultured *D. melanogaster* cell lines

4.5.1 Cell culture

The *D. melanogaster* Schneider (S2) insect cell line was derived from a primary cell culture of late stage (20-24 hours old) *D. melanogaster* embryos (Schneider, 1972). Many features of the cells suggest that they are derived from macrophage-like lineage. Commercially obtained *D. melanogaster* Schneider (S2) cells (Invitrogen, Cat. # 10831-014) were grown at 30°C without CO_2. according to the company protocol in Ex-cell 420 serum-free culture medium (JRH Biosciences, Cat. # 14420-1000M). S2 Schneider cells were semi-adherent monolayer, but when they were overgrown (more than 3 days) they became suspension cell cultures.

4.5.2 dsRNA synthesis and RNAi

Genomic DNA was used as template for PCR with primers containing T7 promoter sequences at their 5' end. The PCR product obtained from a 250 µl PCR reaction was used as template for a 500 µl transcription reaction (8 mM GTP, 5 mM ATP or CTP, 2 mM UTP, 26 mM $MgCl_2$, 1 mM spermidine, 10 mM DDT, 0.01% Triton-X 100, 5 µl recombinant T7 RNA polymerase (Davanloo et al., 1984), 40 mM Tris-HCl pH 7.9, 1 h, 37°C). The transcripts were fully annealed by incubating the transcription reaction mixture for 5 min at 95°C, followed by 15 min at room temperature. Annealing was verified by running an aliquot of annealing reaction on a native 1% agarose gel and visualization of dsRNA by ethidium bromide staining.

Sequences of primers which were used for preparation of dsRNA are listed in table 4.

Table 4. PCR primers of T7 transcription templates for dsRNA synthesis

primer	nucleotide sequence
5p-ago1	TAATACGACTCACTATAGGGAGAAGCCAAGCATAGAAGTTT
3p-ago1	TAATACGACTCACTATAGGGAGATTCATTTCGTATCGTGTA
5p-ago2	TAATACGACTCACTATAGGGAGAGCTACCAACAGCGTCCGC
3p-ago2	TAATACGACTCACTATAGGGAGAGACGGACGCTGTTGGTAG
5p-dcr1	TAATACGACTCACTATAGGGAGAAGGAGAGTTTGGGATACA
3p-dcr1	TAATACGACTCACTATAGGGAGACTCATCATGTTGCTATAT
5p-dcr2	TAATACGACTCACTATAGGGAGAGGTGGGCCGCAACAATAT
3p-dcr2	TAATACGACTCACTATAGGGAGAGGATCACAAAGCCAAAAG
5p-dgcr8	TAATACGACTCACTATAGGGAGAGCGGCACTGCGAAGAAAG
3p-dgcr8	TAATACGACTCACTATAGGGAGACATGGTTAGGACGCTCTC
5p-drsh	TAATACGACTCACTATAGGGAGAGCGCGCTTCTATTGGATG
3p-drsh	TAATACGACTCACTATAGGGAGACACGATCTTTGGTCTTCA
5p-exp5	TAATACGACTCACTATAGGGAGAGGTCAAGAGCGGCAACGT
3p-exp5	TAATACGACTCACTATAGGGAGAGCTTACCGGGCTCCTTGA
5p-gfp	TAATACGACTCACTATAGGGAGAGGGTGAAGGTGATGCAAC
3p-gfp	TAATACGACTCACTATAGGGAGATGTGGACAGGTAATGGTT

For RNAi experiments, S2 cells were spread on 75 cm^2 plates at a cell density of $3.5x10^6$/ml in Ex-Cell 420 insect serum-free medium containing 6 μg/ml dsRNA. The cells were grown for 5 days at 30°C and then collected by centrifugation. Total RNA from S2 cells was isolated as described above.

Number of living cells and cell viability were determined by CASY cell counter and analyzer system (Schärfe System, Germany).

4.5.3 Quantitative real time RT-PCR (qRT-PCR)

Total RNA was reverse transcribed using Superscript II Reverse Transcriptase (Invitrogen Cat. # 18064-014) 0.5 mM dNTPs, and 3.75 mM random decamer primer at 42°C. Quantitative PCR (qPCR) reactions were performed with 1 mM primer concentrations and the Quantitect SYBR Green PCR kit (Qiagen, Cat. # 204143) on a

DNA Engine Opticon (MJ Research) real-time cycler equipped with the manufacturer's software.

Every quantitative PCR reaction was performed in duplicate together with a series of control PCR using series of dilutions of a genomic DNA template. Fluorescence readouts were plotted automatically at every step and an amplification curve of *Fluorescence* vs. *PCR cycle* plot was obtained (Figure 4). Fluorescence is proportional with the quantity of PCR the product. Fluorescence readouts were collected at the *Reference points* where the PCR amplification was linear (dashed line in Figure 4) and the amplification curves for the dilution samples were evenly distributed.

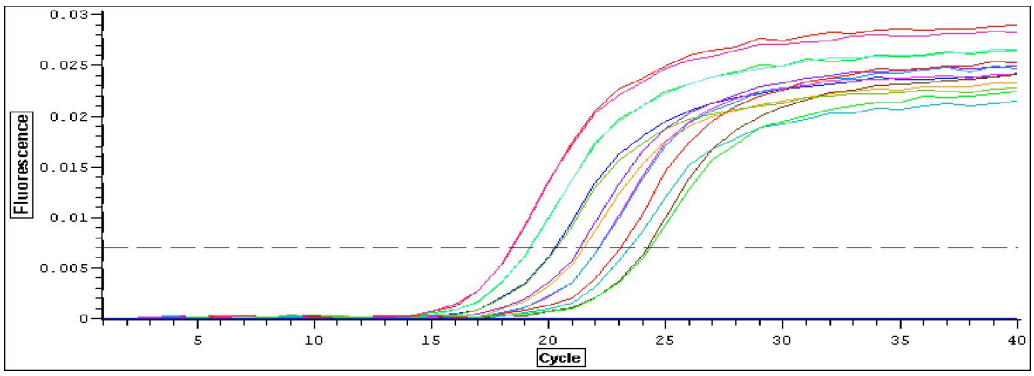


Figure 4. Amplification curve for control PCR series

Fluorescence readouts were plotted automatically at every step to obtain an amplification curve of fluorescence versus PCR cycle plot. Every dilution sample was performed as duplicate to minimize experimental errors. Horizontal dashed line indicates the chosen intensity range at which the regression analysis was performed.

Quantity readouts were converted to the *log(quantity)* vs. *cycle* plot by the software provided by the cycler (Figure 5). The linear plot indicates that the PCR amplification is linear at this point and linear correlations can be made between the quantities of the PCR products and templates. Standard curves with *Confidence values* (R^2) 0.995 and higher were used for calculations (Figure 5). R^2 values were obtained by regression analysis that is performed using the control PCR. The plot was used to obtain real time PCR *Efficiency constant* (E), which is unique to every qPCR reaction. *Efficiency constants* (E) for PCR reactions is a function of the slope of the *log(quantity)* vs. *cycle* plot, which is calculated by the formulae, ($E= 10^{-1/\text{slope of the line}}$).

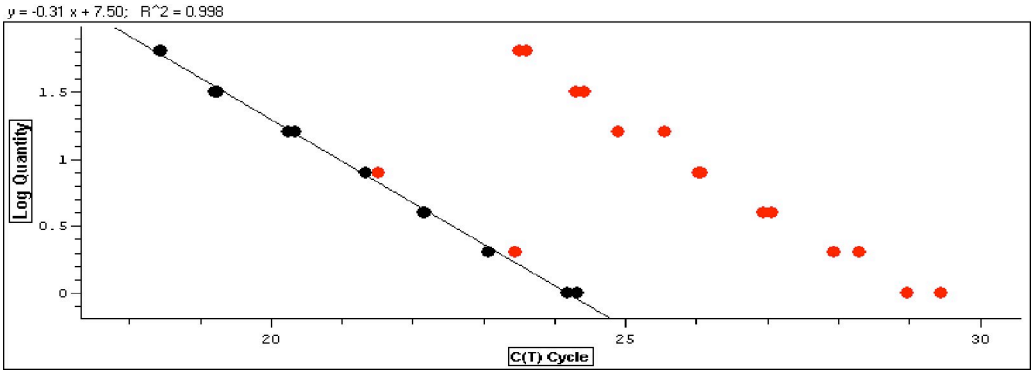


Figure 5. Regression analysis of control PCR series

A series of dilutions from genomic DNA were used as templates for quantitative real-time PCR to perform regression analysis. Standard curves with R values of 0.995 and higher were used. Slope of the line was used to calculate the E (Efficiency constant) values for quantitative PCRs. $E = 10^{-1/slope}$

Cycle points (CP) for the qPCR samples (control or knock-down) at the *Reference point* were obtained from the amplification curves of the reactions (*Fluorescence* vs. *PCR cycle*). The CP values were used to compare the quantities of the templates (cDNA) by calculating the Relative expression. Relative expression of the control vs. knock-down mRNAs were calculated by comparing the CP of the targeted mRNA and the *Reference mRNA* using the formula below. *Efficiency constant* (E) for the reference PCR was obtained the same way as it was done for the targeted mRNA PCR. Histone H1 housekeeping mRNA was used as the internal control. Histone H1 mRNA is a constitutively expressed mRNA. Relative expressions of the RNA in experimental samples with respect to control samples were obtained using duplicate samples and error bars were obtained from at least two independent RT-qPCR experiments.

RT-PCR primers, which were used to detect expression of different *mir-2/mir-13* clusters, and qRT-PCR primers, which were used to quantify the knock down efficiency of targeted mRNA and to quantify the accumulation of primary precursors of miRNAs, are listed in table 5.

$$\text{Relative expression} = \frac{E_{\text{targeted mRNA}}^{(CP_{\text{control}} - CP_{\text{knock-down}})_{\text{targeted mRNA}}}}{E_{\text{reference mRNA}}^{(CP_{\text{control}} - CP_{\text{knock-down}})_{\text{reference mRNA}}}}$$

Figure 6. Calculating relative expression from E values of qPCR

Relative expression of sample and control template was calculated according to the formulae. CP values are obtained at *Reference points* where the PCR amplification is linear. Multiple reactions were performed and mean values were used to obtain the relative expression ratios in order to minimize the experimental errors.

Table 5. RT-PCR and qRT-PCR primers

primer	nucleotide sequence
5p-ago1	GCACCCGATCCGTCAGTA
3p-ago1	GGCACCTGGCGTACGATC
5p-ago2	GTCAGGCCCTAAACCGCA
3p-ago2	GCAGCAAGTTCATAATCT
5p-dcr1	TGCATCAGTGAGGAGTAC
3p-dcr1	GGCACCTGCGATCGACTC
5p-dcr2	AAGAGACACAATTTACAG
3p-dcr2	AACTCTCCCAAACTGTAG
5p-dgcr8	GAGCGATCTGTCTGTTTT
3p-dgcr8	AGCAAGTCAGCAGAATGG
5p-drsh	CCAAGAGTCCAACAATGC
3p-drsh	GCGCTTGCACTTCGGAGA
5p-exp5	GGGCAAGCAGAGGTAGAC
3p-exp5	GCTGTGTCATCGGGTTCA
5p-clart	GCGACGCGATGCTCAAGG
3p-clart	CCACTTGATGAGGCTTAG
5p-clbrt	AGCTCTTCTCTGATGTTT
3p-clbrt	AAAATATCATCCACTTAG
5p-clcrt	GGGCTGAAGAAAGATATT

3p-clcrt	TAACGACATGGTATAAGA
5p-cldrt	CATCGTGCTAAACATCGT
3p-cldrt	GCACGCGCAACATTTCCA
5p-claqrt	ACGGTAACAAGTGTCTGA
3p-claqrt	ATTGAGGTTTCGGGATAC
5p-clbqrt	AAACCGCAACGCTCCTCA
3p-clbqrt	CTCTGTTAGCCGCGTTTA
5p-clcqrt	CCCGCTCGATGCGAATGC
3p-clcqrt	GAATTATGGCTTGGATCA
5p-cldqrt	TCGTCTACCTCTGCTGTC
3p-cldqrt	TCCAACAAACTCGTCAAA
5p-h1qrt	CCACCAGCGACAGTTGAG
3p-h1qrt	TGGCGGATGTGACGGCGT

4.6 Whole mount *in situ* hybridization

4.6.1 Preparation of synthetic 3'-end labeled oligonucleotide probes

For 3'-end labeling, short (20 to 25-nt) DNA or RNA oligonucleotide probes were either chemically synthesized as described before or commercially obtained from MWG Biotech. Pre-designed LNA (Locked nucleic acid) probes were also obtained commercially from EXIQON. The sequences of the probes are demonstrated in table 6.

Table 6. Oligonucleotide probes for 3' end labeling

oligo	nucleotide sequence	type
od-as-mir-1	CTCCATACTTCTTTACATTCCA	DNA
od-as-mir-6	CAAAAAGAACAGCCACTGTGATATA	DNA
od-as-mir-10	AAACAAATTCGGATCTACAGGGTAG	DNA
od-as-mir-124	CTTGGCATTCACCGCGTGCCTTA	DNA
ol-as-mir-1	CTCCATACTTCTTTACATTCCA	DNA/LNA[1]
ol-as-mir-124	CTTGGCATTCACCGCGTGCCTTA	DNA/LNA[1]

or-as-mir-1	CTCCATACTTCTTTACATTCCA	RNA
or-as-mir-6	CAAAAAGAACAGCCACTGTGATATA	RNA
or-as-mir-10	AAACAAATTCGGATCTACAGGGTAG	RNA
or-as-mir-124	CTTGGCATTCACCGCGTGCCTTA	RNA
or2'-as-mir-6	CAAAAAGAACAGCCACTGTGATATA	2'OMe RNA
or2'-as-mir-10	AAACAAATTCGGATCTACAGGGTAG	2'OMe RNA
1 *DNA/LNA probes are modified oligodeoxynucletides with every 3rd nucleotide replaces by an LNA nucleotide*		

Oligonucleotides were enzymatically labeled at their 3'-end with Terminal deoxynucleotidyl transferase by incorporation of a single Digoxigenin-labeled dideoxyuridine-triphosphate (DIG-ddUTP) using the DIG oligonucleotide tailing kit (Roche Cat. # 1 417 231). Alternatively, oligonucleotides were labeled using a mixture of the deoxynucleotide triphosphate dATP (1 mM) and DIG-dUTP (0.1 mM) (Roche, Cat. # 1209256) leading to addition of a long tail of labels. Labeling reactions were carried out as described within the kits manual. Typically, 100 pmol of oligonucleotide was used in a 20 µl labeling reaction. After the completion of the labeling reactions unincorporated nucleotides in the mixture were removed by using MicroSpin G-25 columns (Amersham Cat. # 27-5325-01) according to the manufacturer's protocol.

4.6.2 Preparation of internally labeled RNA probes

Single stranded RNA probes were synthesized by *in vitro* T7 RNA polymerase transcription. Digoxigenin uridine triphosphate (DIG-UTP) was supplied in the reaction mixture, together with all four unmodified NTPs to obtain DIG-labeled RNA probes. PCR-amplified DNA fragments corresponding to target genes were used as templates for transcription reactions. A T7 promoter sequence was introduced by PCR, adding the promoter sequence 5' to the reverse primer for antisense RNA probe transcription. *D. melanogaster* genomic DNA was used as template for PCR amplification of DNA fragments cognate to the target transcript. Primer sequences used in these reactions are shown in table 7.

Table 7. Primer sequences for internal labeling of ssRNA probes

primer	nucleotide sequence
5p-ftz	TAATACGACTCACTATAGGAGAATTCATTGCAAAGACTCGAAACG
3p-ftz	ATTTAGGTGACACTATAGGACCTTGGTCAAGACAGATGGTAGAGG
5p-ftz-e1.1	AAGGCCACCAAGCGCAAG
3p-ftz-e1.1	GCGTAATACGACTCACTATAGGGATTGGTGAGCAGAGC
3p-ftz-e1.2	GCGTAATACGACTCACTATAGAGCCCTCAGTGTGCTGG
5p-ftz-e1.3	AGGCCGAAGATGATGCTG
5p-ftz-e1.4	ATGCTGCTTCCATCATCG
5p-ftz-e1.5	ATCGCCGCCGTGGAGGAG
5p-ftz-e1.6	AGCGACCCAGCACACTGA
5p-ftz-e2.1	GCATGAAGTCGAAGAAGG
3p-ftz-e2.1	GCGTAATACGACTCACTATAGGTGCTTGTGGCCTCCAG
5p-ftz-e2.2	AGGATCGCACGCTGGACA
5p-ftz-e2.3	AGCTCCCCGGAGCACTGT
5p-ftz-e2.4	ACTGTGGTGCCGGCTACA
5p-ftz-e2.5	CTACACCGCGATGCTGCC
5p-ftz-e2.6	CCGCCACTGGAGGCCACA
5p-mir-1-las	CGAACACCGCACAACAAA
3p-mir-1-las	GCTAATACGACTCACTATAGGCGAAATGCGATCCGTA
5p-mir-2-as	TATCACAGCCAGCTTTGA
5p-mir-2p-as	GCTGGGCTCTCAAAGTGG
3p-mir-2p-as	GCGTAATACGACTCACTATAGGCTAAGCTCATCAAAGCT
5p-mir-3-as	ATCACTGGGCAAAGTGTG
5p-mir-3-ias	ACTCTGTGTGAAACGGGT
5p-mir-3p-as	GATCCTGGGATGCATCTT
3p-mir-3p-as	GCGTAATACGACTCACTATAGGATCTTGAGACACACTT
5p-mir-3p-s	GCGTAATACGACTCACTATAGATCCTGGGATGCATCTT
3p-mir-3p-s	GGATCTTGAGACACACTT
5p-mir-4-as	ATAAAGCTAGACAACCAT
5p-mir-4-ias	AGTTACCAACAGATCGAA
5p-mir-4p-as	TTGCAATTAGTTTCTTTG
3p-mir-4p-as	GCGTAATACGACTCACTATAGAATGCCACAACGAACTTC

5p-mir-10-as	AACCCTGTAGATCCGAAT
5p-mir-10p-as	CCACGTCTACCCTGTAGA
3p-mir-10p-as	GCGTAATACGACTCACTATAGGAACCACACAAACCTCTC

Transcribed 24-nt oligoribonucleotide probes were obtained from PCR amplified short DNA fragments. First, 42-to 44-nt synthetic DNA oligonucleotides were used as template for the PCR reactions. The sequences of the synthetic oligodeoxynucleotides, which were used in PCR reactions are shown in table 8. Antisense sequence of T7 promoter was included in 3' end of these oligonucleotide PCR templates to attach T7 promoter linker in the 5' end of the PCR products. T7 promoter sequence was used as reverse primer (3p-t7) in these PCR reactions. Amplified short DNA fragments were then used in transcription reactions as templates.

Table 8. Oligonucleotide templates for generation of 24 nucleotide ssRNA probes

oligo	**nucleotide sequence**
t-ftz-e1	AGCGACCCAGCACACTGAGGGCTCTATAGTGAGTCGTATTACGC
t-ftz-e2	CCGCCACTGGAGGCCACAAGCACCTATAGTGAGTCGTATTACGC
t-mir-2-as	TATCACAGCCAGCTTTGATGAGCCTATAGTGAGTCGTATTACGC
t-mir-3-as	ATCACTGGGCAAAGTGTGTCTCACTATAGTGAGTCGTATTACGC
t-mir-3-ias	ACTCTGTGTGAAACGGGTCACTACTATAGTGAGTCGTATTACGC
t-mir-4-as	ATAAAGCTAGACAACCATTGACTATAGTGAGTCGTATTACGC
t-mir-4-ias	AGTTACCAACAGATCGAAATACTATAGTGAGTCGTATTACGC
t-mir-10-as	AACCCTGTAGATCCGAATTTGTCTATAGTGAGTCGTATTACGC
3p-t7	GCGTAATACGACTCACTATAG

T7 transcription reactions were carried out as described before with the following modifications. Genomic DNA or synthetic DNA oligonucleotides were used as template for PCR reactions with only reverse primers containing T7 promoter sequences at their 5' end. The PCR product obtained from a 100 µl PCR reaction was used as template for 200 µl transcription reaction (1.6 mM GTP, 1 mM ATP or CTP, 0.65 mM UTP, 0.35 mM DIG-UTP, 26 mM $MgCl_2$, 1 mM spermidine, 10 mM DDT, 0.01% Triton-X 100. 1 µl recombinant T7 RNA polymerase (Davanloo et al., 1984),

40 mM Tris pH 7.9, 1 hour at 37°C). For synthesis 1 mM PCR amplified DNA template was used in transcription reactions.

Long RNA probes (>100-nt) were precipitated by adding 7.5 M LiCl (to a final concentration of 2.5 M LiCl) and incubating on ice for 30 minutes. Precipitated RNA probes were then collected by centrifugation at 13,000 rpm for 30 minutes at 4°C. The pellet was then resuspended in appropriate amount of formamide to achieve 0.5–5 μM final probe concentration.

RNA probes, which were shorter than 100 nucleotides were purified by PAGE. Transcription reactions were loaded on 15% denaturing polyacrylamide with equal volume of BPB gel loading buffer. PA gels were prepared as described before. PA gel was run in TBE buffer for 1 hour 15 minutes at 30W. Separation of RNA bands was visualized by UV shadowing on fluorescent material coated screens. RNA bands with expected sizes were cut from the gel and eluted in 0.3 M NaCl solution for 16 hours at 4°C. RNA probes were then precipitated by addition of 2.5 volume absolute ethanol at -20°C for 2 hours and collected by centrifugation for 30 minutes at 20,000 g, at 4°C. Finally the RNA pellet was suspended in appropriate amount of formamide to achieve 0,5–5 μM final probe concentration.

4.6.3 Membrane activity assay for ISH probes

In order to check the labeling efficiency of the *in situ* hybridization probes 1 μl probe was spotted on Hybond-N+ membrane (Amersham, Cat. # RPN303B) and air dried for 10 minutes. Blotted membrane was then washed for 2 minutes in maleic acid buffer (0.1 M maleic acid, 0.15 M NaCl, pH 7.5). Nonspecific binding sites were blocked by incubating membranes in blocking solution (1% Blocking reagent (Roche, Cat. # 1096176) in maleic acid buffer) for 30 minutes. Labeling was carried out in blocking solution containing 1:10,000 anti-Digoxigenin-AP antibody (Roche, Cat. # 1093274) for 30 minutes at room temperature.

Washing the membrane was carried out in maleic acid buffer with 0.3% Tween-20 for 30 minutes. Later the membrane was equilibrated in detection buffer (0.1 M Tris-HCl pH 9.5, 0.1 M NaCl, 50 mM $MgCl_2$) for 10 minutes. Finally the color reaction was carried out in detection buffer with 20 μl/ml AP substrate solution (18.75

mg/ml NBT, 9.7 mg/ml BCIP) for 30-60 minutes. Color reaction was terminated by washing the membrane in TE buffer (Sambrook and Russell, 2001).

4.6.4 *In situ* hybridization

The technique of *in situ* hybridization using nonradioactively labeled probes was originally described in Tautz and Pfeifle (1989). The protocol was adapted for *D. melanogaster* embryos with the following modifications. *D. melanogaster* (Oregon R strain) embryos were collected for 12 to 24 hours and transferred carefully on a fine plastic net, on which they were washed extensively in tap water. *D. melanogaster* embryos are surrounded by an eggshell, which is composed of an outer chorion layer and an inner vitellin membrane. Vitellin membrane, which is composed of proteins, contacts the embryo. The eggshell was removed from the embryos prior to *in situ* hybridization. After they were dried with paper towel, they were dechorionated in 50% commercial bleach (DanKlorix,, Germany) for 5 minutes. They were washed several times, dried, and then were then transferred to glass vial containing heptane. 2X fixation solution (100 mM HEPES, 2 mM $MgSO_4$, 1 mM EGTA, pH 6.9) was added together with formaldehyde (37% stock) to obtain a final concentration of 3.7% formaldehyde. Embryos were fixed for at least 16 hours in this mixture by vigorous shaking at room temperature.

Heptane sinks to the bottom of the suspension and contains the chorion that are detached from the embryos. Embryos were then devitellinized by removing the lower phase and adding equal volume of methanol on the mixture. This mixture was shaken for 1-2 minutes. After a few minutes of keeping the solution still, devitellinized embryos to sank to the bottom of the vial. The embryos were collected by pipeting into a fresh tube and washed a few times in methanol. They were stored in -80^{o}C until they were used for *in situ* hybridization.

On the day of *in situ* hybridization, embryos were first rehydrated in 50% methanol and 50% PBT (PBS buffer (Sambrook and Russell, 2001) with 0.1% Tween-20) for 10 min. Then RNA fixation was carried out in RNA fixation buffer (5% paraformaldehyde, 25 mM EGTA in PBT pH 7.0) for 20 minutes at room temperature. Embryos were washed in PBT several times and permeabilized by proteinase K (5 mg/ml in PBS) digestion for 5 minutes. Proteinase K was washed by

PBT and RNA fixation was repeated for 20 minutes at room temperature. Finally embryos were washed in PBT for several times prior to hybridization.

Embryos were equilibrated in increasing concentration of hybridization buffer (50% formamide, 5X SSC, 1X Denhardt's buffer, 50 mM Na2HPO4 pH 7.4, 5 μg/ml yeast RNA, 100 μg/ml Sonicated salmon sperm DNA) at room temperature. Then they were incubated for 2 hours in hybridization buffer at 55°C for prehybridization. Hybridization to DIG labeled probes carried out for 16 hours at 55°C in hybridization buffer containing 5-50 pmol/ml probe. the overall volume of the in situ hybridization mixture was 100 μl.

After hybridization embryos were washed several times, first in hybridization buffer and later in PBT buffer. Nonspecific binding sites were blocked by incubating embryos in ISH blocking solution (1% Blocking reagent (Roche, Cat. # 1096176), 5% Goat serum, in PBT buffer) for 1 hour. Labeling was carried out in ISH blocking solution containing 1:2,000 anti-Digoxigenin-AP antibody (Roche, Cat. # 1093274) for 16 hour at 4°C.

Embryos were extensively washed to remove unbound antibodies at room temperature with PBT buffer. They were then equilibrated in detection buffer (0.1 M Tris-HCl pH 9.5, 0.1 M NaCl, 50 mM $MgCl_2$) for 15 minutes. Finally the color reaction was carried out in detection buffer with 20 μl/ml AP substrate solution (18.75 mg/ml NBT, 9.7 mg/ml BCIP) for several hours at room temperature. Color reaction was terminated by washing the membrane in TE buffer (Sambrook and Russell, 2001).

Embryos were analyzed and photographed on an AXIOPHOT light microscope (ZEISS) equipped with a camera from Kontron Elektronik.

4.7 Genetic knock-out experiments

4.7.1 *D. melanogaster* handling

D. melanogaster stocks were handled and maintained according to protocols described in (Roberts, 1986) and (Ashburner, 1989). The food medium used was a mixture composed of dry yeast, soya flour, corn flour, beet sugar syrup, malt,

propionic acid, Nipagin and ethanol. Egg collections were done in cages using apple juice agar plates supplemented with live yeast paste as the support for egg disposition. Genotypes were as described in Lindsey and Zimm (1992).

Genomic DNA from *D. melanogaster* was isolated as described previously (Ashburner, 1989) with the following modifications. 5-to 10 flies were homogenized in a total of 100 µl homogenization buffer (20 mM Tris pH 7.5, 200 mM NaCl, 20 mM EDTA, 2% SDS) by crushing them with a pipette tip. After proteinase K was added to the mixture, the samples were incubated at 56^{o}C for 60 minutes. The was then extracted two times by 1 volume of phenol/chloroform/isoamyl alcohol (25:24:1), pH 7.0. The supernatant was treated with 1 µl of RNase A (1 mg/ml) and the phenol chloroform extraction was repeated. Then, 1/10 volume 3 M sodium acetate (pH 5) was added and the DNA was precipitated by 2.5 volume absolute ethanol for 2 hours at -20^{o}C. Precipitate was collected by centrifugation at 13,000 rpm for 30 minutes. Finally the pellet was suspended in 50 µl water.

4.7.2 Fly stocks

P-Element lines were obtained from Bloomington Drosophila stock center at Indiana University (http://flystocks.bio.indiana.edu/). P-element insertion lines were identified by searching the P-Screen/Gene disruption project database (Bellen et al., 2004) (http://flypush.imgen.bcm.tmc.edu/pscreen/).

Genotypes and Bloomington stock numbers of *D. melanogaster* stocks used during generation of genetic knock-outs are listed in tables 8 and 9.

Table 9. Genotypes and stock numbers of P-element Lines

P-Element line	Genotype	Stock #
rnh1^{k07624}	y w; P{w[+mC]=lacW}rnh1[k07624]/CyO	10677
pasha^{EY01325}	y w; P{w[+mC] y[+mDint2]=EPgy2}pasha[EY01325]	15509

y, Yellow; w, White; CyO, Curly of Oster

Table 10. Genotypes and stock numbers of deficiency lines

P-Element line	Genotype	Stock #
Df(2R)CA53	w; Df(2R)CA53/CyO	3364
Df(2R)Exel6055	w; Df(2R)Exel6055, P{w[+mC]=XP-U}Exel6055/CyO	7537
Df(3R)A113	Df(3R)A113/In(3R)C, Sb sprd cd Tb ca; Dp(3;1)34	2155

Sb, Stubble; sprd, Spread; cd, Cardinal; Tb, Tubby; ca, Claret

4.7.3 P-element-based transformation

DNA fragments corresponding to full-length rnh-1 and pasha genes were amplified by PCR using genomic DNA as template. Amplified fragments were then cloned into the vector pCR 2.1-TOPO, which were then subcloned into P-element transformation vector pP{CaSpeR-4} as described before. Primer sequences, which were used to amplify full length rnh-1 and pasha genes, are displayed in table 11.

Table 11. Primer sequences for cloning full length rnh-1 and pasha genes

primer	nucleotide sequence
5p-pasha	TTTCAAAATGGCCAATAG
3p-pasha	GCAGAGAGAAAGTCCGAG
5p-rnh-1	GAAGAATAGTTGTGAGAG
3p-rnh-1	CCCGCGCCTCCCAGTAAG

In order to rescue the deletion phenotypes of the targeted genes a P-element based germ line transformation technique was used as described by Rubin (1982) and Spradling (1982). A full length, PCR-amplified DNA fragment of the targeted gene was cloned in pP{CaSpeR-4} (GenBank accession X81645) P-element vector, downstream to a white^{+} gene flanked by the P-element terminal repeats. The rescue construct was then injected in w^{-} *D. melanogaster* embryos as described by Van Deusen (1977) together with Δ2-3 helper plasmid, which contains the P-element transposase gene but lacks the P-element terminal repeats (Laski et al., 1986).

G0 adults were backcrossed individually with w^- flies and w^+ progeny among G1 progeny was screened. Individual w^+ flies from G1 progeny were crossed with w^- flies. Heterozygote w^+/w^- flies from G2 progeny were crossed with each other to obtain homozygote flies in G3 generation. Homozygotes were crossed with 2nd chromosome balancer stock y w; wg[Gla-1]/CyO and 3rd chromosome balancer stock y w; Dr[1]/TM3, Sb in order to perform linkage analysis.

4.7.4 P-element based gene deletion of *Drosha*

Virgin females from P-element insertion stock of *Drosha* y w; P{w[+mC]= lacW}rnh1[k07624]/CyO was crossed with males from transposase source stock w; wg[Sp-1]/CyO; ry[506] Dr[1] P{Delta2-3}99B/TM6. Individual males with w; P{w[+mC]=lacW}rnh1[k07624]/CyO; ry[506] Dr[1] P{Delta2-3}99B/+ genotype were crossed with virgin females from 2nd chromosome balancer stock y w; wg[Gla-1]/CyO. White eyed G1 progeny from the crosses were back crossed individually with the 2nd chromosome balancer stock.

Heterozygote Jump-out flies were crossed with each other to obtain stable stocks either balanced or homozygote with respect to targeted chromosome. Stocks, which were balanced by a balancer chromosome indicated lethality caused by the jump-out event. These stocks were crossed with deficiency lines w; Df(2R)CA53/CyO and w; Df(2R)Exel6055, P{w[+mC]=XP-U}Exel6055/CyO with deletion on the targeted locus in order to rule out an independent insertional mutation by P-element mobilization. These flies were also crossed with rescue chromosome carrying flies with full length *rnh-1* gene in order to identify the exact cause of the lethality.

Rest of the flies, which were stably maintained as homozygotes, were screened for genomic deletions. PCR was performed by primers flanking the targeted genomic loci. Primer sequences for these PCR screens are displayed in table 12.

Table 12. Primer sequences for screening *Drosha* deletions in jump-out flies

primer	nucleotide sequence
5p-drosha	GAAGTTTAAGACACGCCA
3p-drosha	GCGCTTGCACTTCGGAGA

4.7.5 P-element based gene deletion of *Pasha*

Virgin females from P-element insertion stock of *Pasha* y w; P{w[+mC] y[+mDint2]=EPgy2}pasha[EY01325] was crossed with males from transposase source stock w; wg[Sp-1]/CyO; ry[506] Dr[1] P{Delta2-3}99B/TM6. Individual males with genotype w; +/CyO; P{w[+mC] y[+mDint2]=EPgy2}pasha[EY01325]/ ry[506] Dr[1] P{Delta2-3}99B were crossed with virgin females from 3rd chromosome balancer stock y w; Dr[1]/TM3, Sb. White eyed G1 progeny from the crosses were back crossed individually with the 3rd chromosome balancer stock.

Heterozygote Jump-out flies were crossed with each other to obtain stable stocks either balanced or homozygote with respect to targeted chromosome. Stocks, which were balanced by a balancer chromosome indicated lethality caused by the jump-out event. These stocks were crossed with deficiency line Df(3R)A113/In(3R)C, Sb sprd cd Tb ca; Dp(3;1)34 with deletion on the targeted locus in order to rule out an independent insertional mutation by P-element mobilization.

Rest of the flies, which were stably maintained as homozygotes, were screened for genomic deletions. PCR was performed by primers flanking the targeted genomic loci. Primer sequences for these PCR screens are displayed in table 13.

Table 13. Primer sequences for screening *Pasha* deletions in jump-out flies

primer	nucleotide sequence
5p-pasha	CCCACTATCGCTCAAAAG
3p-pasha	GCAGAGAGAAAGTCCGAG

5. Results

5.1 Temporal expression pattern of *D. melanogaster* miRNAs

There are 78 miRNAs identified in *D. melanogaster* and only a few of the identified miRNAs are annotated by a biological function. Various kinds of biological data can contribute to assigning function to miRNAs. Defining a specific expression pattern for a miRNA would provide important clues regarding its function. This data will also be valuable during verification of predicted mRNA targets of different miRNAs.

In order to obtain the temporal expression information for miRNAs during *D. melanogaster* development miRNAs were detected at different developmental stages by Northern blot hybridization technique. Total RNA was isolated from different stages of development of *D. melanogaster* was prepared and probed by in Northern blotting.

Total RNA from different embryonic stages of *D. melanogaster*, starting from egg laying until hatching was prepared. The embryonic phase of development was analyzed in six stages with respect to time after eggs were laid. Furthermore, three larval stages, pupae, and adult male and virgin females were included.

RNA samples were resolved by polyacrylamide gel electrophoresis followed by Northern blotting. Radioactively labeled antisense oligodeoxynucleotide probes were used to detect mature miRNAs together with their 70-to 80-nt stem-loop precursors (Figure 7A). Ethidium bromide staining of the polyacrylamide gels prior to membrane transfer served as loading control for Northern blots. Figure 7 shows one of the examples for Northern blot experiments, demonstrating the developmental expression of *D. melanogaster* miR-1. *D. melanogaster* miR-1 is expressed strongly in all the stages of development starting from egg laying until adulthood. Stem-loop precursor for this miRNA can also be detected by Northern blot as a higher molecular weight band. Detection of pre-miRNA was harder compared to the mature miRNA because of the lower abundance of the pre-miRNA in the total RNA.

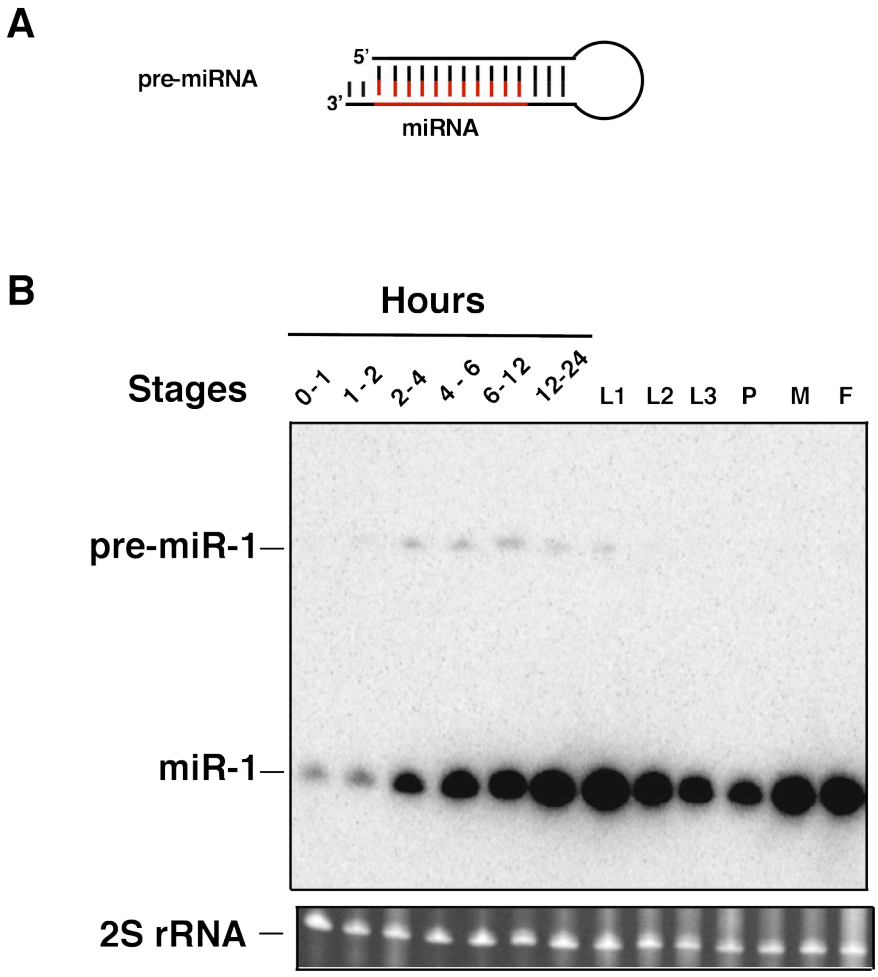


Figure 7. miR-1 expression during *D. melanogaster* development.

(A) A 22 nucleotide mature miRNA (red) within its hairpin precursor.
(B) Northern blot hybridization of miR-1. Development stages of embryos are indicated in hours after egg laying. Larval stages are indicated as L1, L2, and L3. P indicates pupal stage; M, adult male; F adult female. As control for loading 30-nt 2S rRNA band was visualized by ethidium bromide staining of the polyacrylamide gel before transfer of the RNA to the blotting membrane.

Unlike miR-1 some *D. melanogaster* miRNAs were detected in specific periods during fly development. miR-311 for instance was detected only during early embryonic stages e.g. during the first 24 hours after egg laying (Figure 8A). In contrast, miR-210 could be detected only in adult flies (Figure 8B). The pre-miRNAs were not detected.

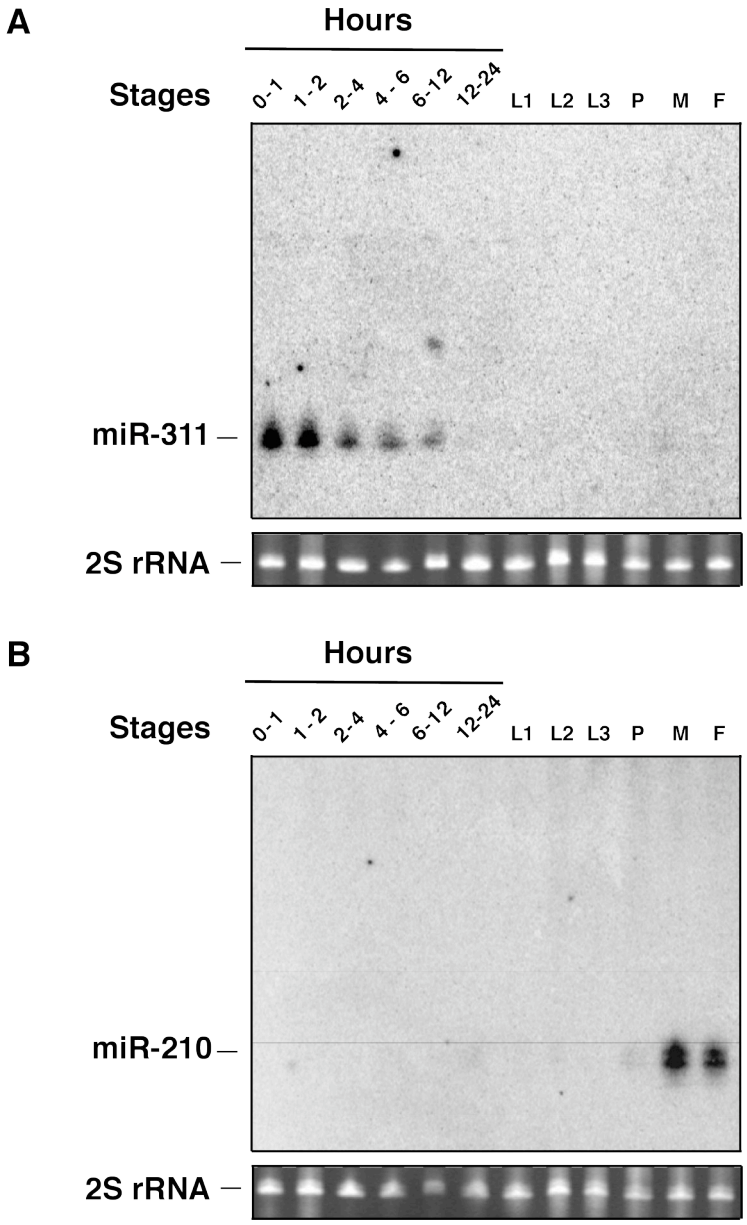


Figure 8. Early and late expressing miRNAs during *D. melanogaster* development

(A) Early expressing miRNA cluster, miR-310/miR-311/miR-312/miR-313 is expressed during the first 12 hours of embryonic stages.
(B) miR-210 expression is detected in after adulthood. miR-210 is an example of late miRNAs.

Nearly half of the *D. melanogaster* miRNA genes are arranged as clusters (35 miRNAs) on the genomic DNA (Altuvia et al., 2005; Aravin et al., 2003). The clustered D. melanogaster miRNA genes are listed in table 14. Northern blotting of individual miRNA gene clusters on genomic DNA, e.g. *mir-100*, *let-7* and *mir-125* genes, revealed that such miRNAs are co-expressed (Figure 9).

Table 14. miRNA gene clusters

miRNA Cluster	**intronic/intergenic**
let7/mir-100/mir-125	intergenic
mir-9b/mir-9c/mir-79/mir-306	intronic
mir-34/mir-277/mir-317	intergenic
mir-275/mir-305	intergenic
mir-12/mir-283/mir-304	intronic
mir-3/mir-4/mir-5/mir6-1/mir-6-2/mir-6-3/mir-286/mir-309	intergenic
mir-310/mir-311/mir-312/mir-313	intergenic
mir-2a-1/mir-2a-2/mir-2b-2	intronic
mir-2c/mir-13a/mir-13b-1	intergenic
mir-281a/mir-281b	intronic

The clustering of *mir-100*, *mir-125*, and *let-7* is conserved in *A. gambiae*, although the distance between the miRNA genes has increased (Figure 9C). In mouse and human, the gene cluster underwent duplication and the distance between miRNA genes was increased further (Figure 9C). In *C. elegans*, neither *lin-4* nor its paralog *mir-237* are spatially linked with *let-7* family members (Lim et al., 2003). The *lin-4* gene is expressed at an earlier stage than the *let-7* gene (Reinhart et al., 2000), but *mir-237* shows very similar expression pattern to *let-7* in *C. elegans* (Lim et al., 2003). Sequence alignment of *C. elegans* lin-4 miRNA with mouse miR-125a and miR-125b and the *D. melanogaster* miR-125 are shown below. Conserved nucleotides are highlighted by gray boxes.

C. elegans lin-4	**UCCCUGAGAC--CUC--AAGU-GUGA**
C. elegans mir-237	**UCCCUGAGAAUUCUCG-AAC-AGCUU**
D. melanogaster miR-125	**UCCCUGAGACC-CU---AACUUGUGA**
M. musculus/H. sapiens miR-125b	**UCCCUGAGACC-CU---AACUUGUGA**
M. musculus/H. sapiens miR-125a	**UCCCUGAGACC-CUUU-AACCUGUGA**

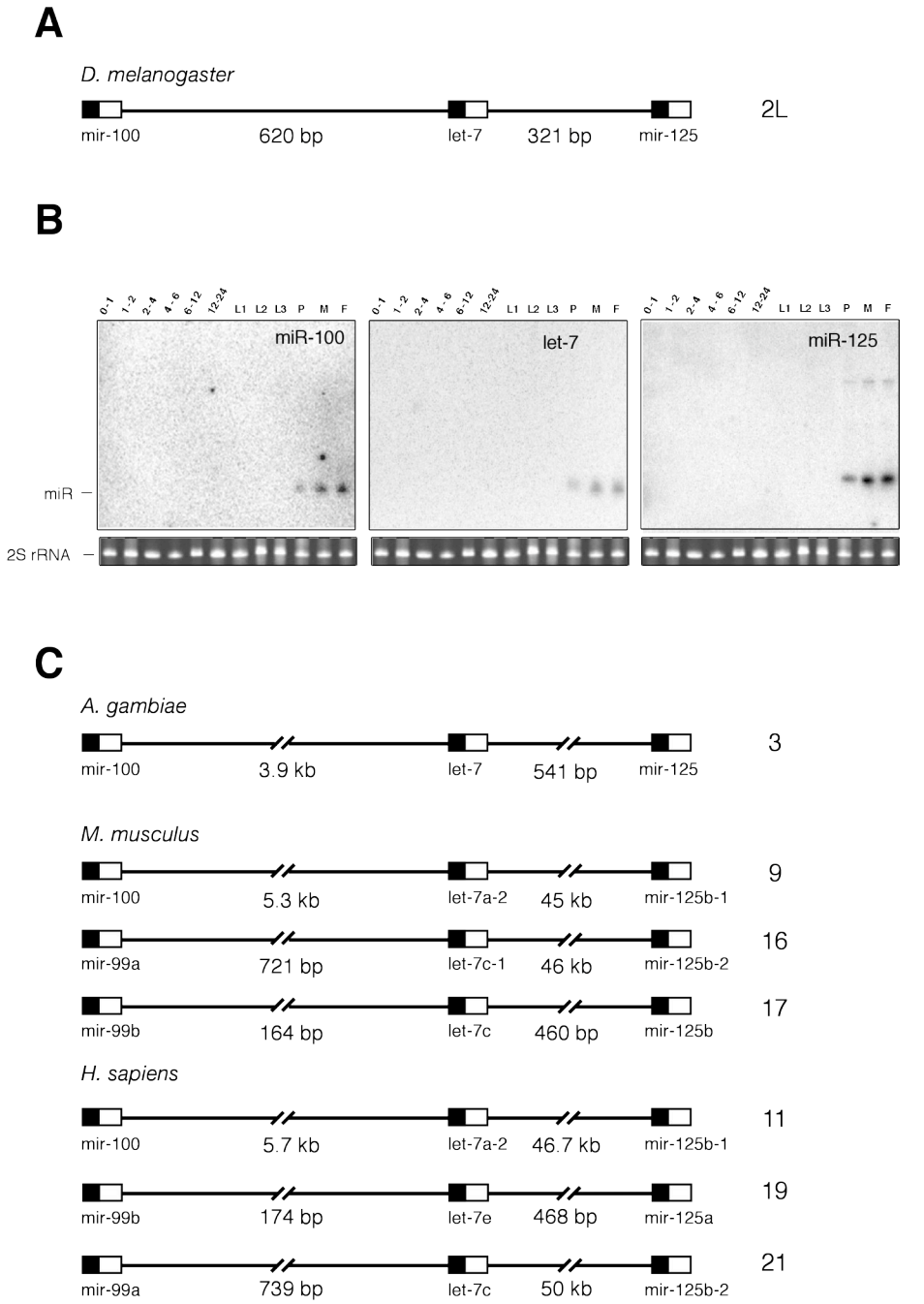


Figure 9. miRNA genes mir-100, let-7 and lin-4 homolog mir-125 are clustered

(A) Arrangement of the miRNA genes in *D. melanogaster*. The 70-80 nt fold-back precursor is indicated as box, the position of the miRNA in the precursor is shown black. The chromosome location is indicated to the right.

(B) Northern blots confirming the coexpression of miR-100, let-7, and miR-125 in pupa and adult. As control for loading 30 nt 2S rRNA band was visualized by ethidium bromide staining of the polyacrylamide gel before transfer of the RNA to the blotting membrane. Development stages of embryos are indicated in hours after egg laying. Larval stages are indicated as L1, L2, and L3. P indicates pupal stage; M, adult male; F adult female.

(C) The miRNA clustering is conserved between invertebrates and vertebrates but gene duplication occurred in mammals and spacing between miRNA precursors increased with increasing genome size. Numbers on the right indicate the chromosomes, on which miRNA genes are located.

In order to get the complete set of temporal expression information of *D. melanogaster* miRNAs 55 Northern blot hybridization experiments were performed with oligonucleotide probes against all the identified miRNA sequences (for probe sequences refer to Materials and Methods section) (Figure 10A). There are overall 55 Northern blot results displayed for miRNAs. Some of the miRNA sequences were nearly identical, differing only by one base with each other. Although we did not experimentally tested this, the identification of the specific expression pattern of such related miRNAs would be difficult by Northern blotting. Therefore Northern blot hybridization for only one member of such miRNA families was performed and displayed. One example is the *mir-2/mir-13* family, which is shown below. Only one miRNA (miR-2a) was examined by Northern blot. In this family there is also one single mismatch between two *mir-13* paralogs. However when compared to miR-2 sequence there are three mismatches and one bulge between miR-2a and miR-13b which makes it possible to detect the expression of these miRNA families specifically.

mir-2/mir-13 family

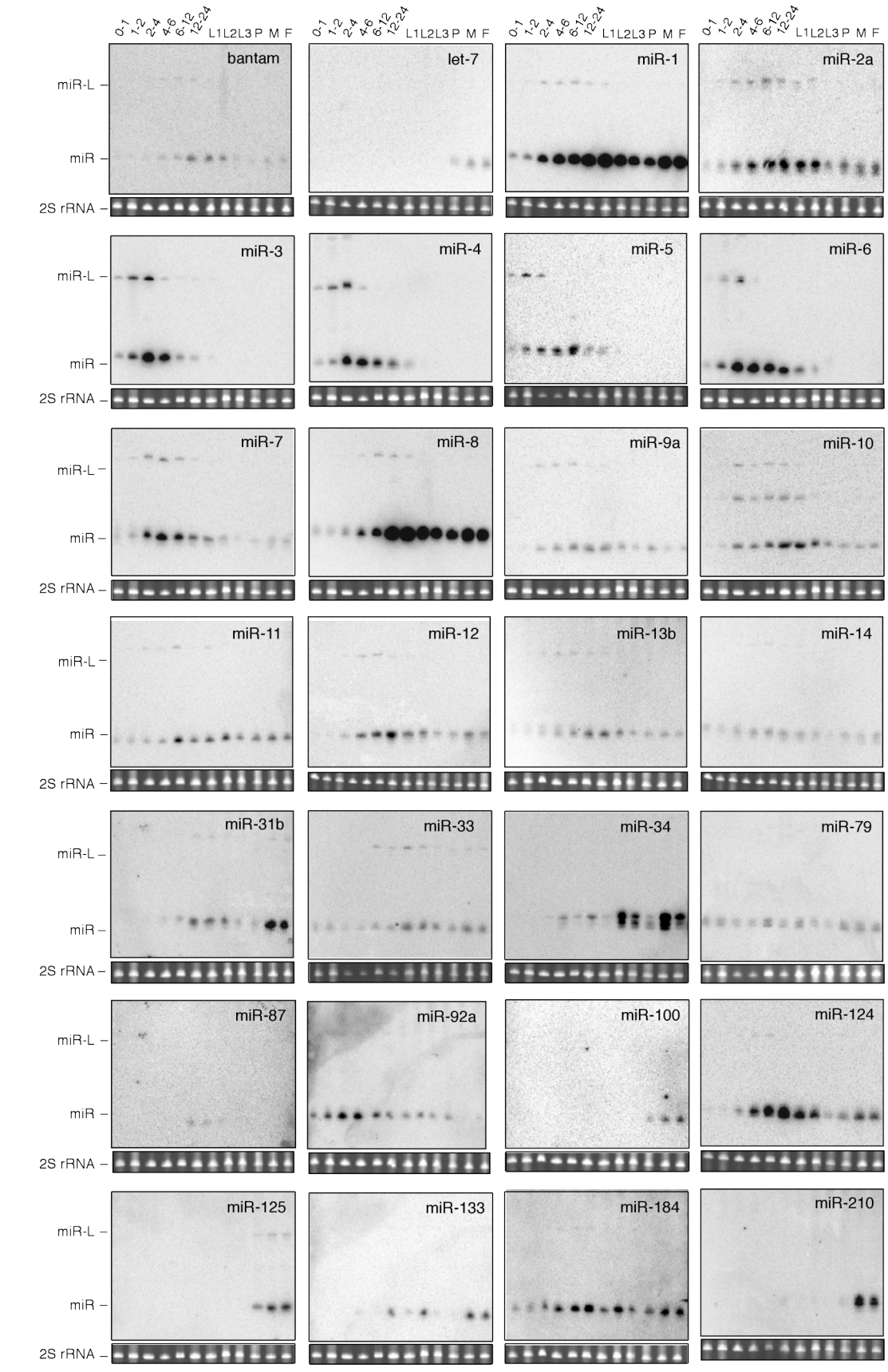
```
dme-miR-2a      UAUCACAGCCAGCUUUGAUGAGC
dme-miR-2b      UAUCACAGCCAGCUUUGAGGAGC
dme-miR-2c      UAUCACAGCCAGCUUUGAUGGGC
dme-miR-13a     UAUCACAGCCA-UUUUGAUGAGU
dme-miR-13b     UAUCACAGCCA-UUUUGACGAGU
```

Ten *D. melanogaster* miRNAs, which were identified by cloning, were not detected by Northern blot hybridization efforts. The results for these Northern blots are shown in figure 10B. Probably the expression of these miRNAs was not sufficient to be detected with this technique. Cloning frequencies of these miRNAs were lower compared to the abundant miRNAs (Aravin et al., 2003), which could be detected by Northern blotting. miR-308 was identified by cloning and the expression was confirmed by 5 clones, miR-318 with 3 clones, and miR-316 with 2 clones. miR-303, miR-iab-4-5p, and miR-iab-4-3p were cloned only one time during the identification. *mir-280* and *mir-282* are computationally predicted miRNAs, which are conserved in *D. pseudoobscura* genome. *mir-282* is also conserved in invertebrate species supporting that it is valid miRNA gene. Expression of miR-280 and miR-282 are

confirmed by Northern blotting (Lai et al., 2003). miR-280 is expressed at very low levels in adult flies. miR-282 expression was detected in larval stages. Although miR-288 and mir-289 were not detected by Northern blotting they are considered to be valid miRNAs because they are conserved in *D. pseudoobscura* genome (Lai et al., 2003).

In order to compare the relative expression of miRNAs in different stages of development expression levels were quantified by imaging, and the loading of the gel was corrected using the 2S rRNA levels from ethidium bromide stained gels. The values were translated into a crude quantity scale with 6 steps ranging from 'non detected' to 'very high expression' (Figure 11). The expression value for every single band is tabulated according to this scale to obtain a complete expression pattern of *D. melanogaster* miRNAs (Figure 11). In Figure 11 the miRNAs are arranged according to expression timing, early expressing miRNAs being on top and late expressing miRNAs at the bottom of the figure. Clustered miRNAs are indicated as vertical bars left to the names of miRNAs. All clustered miRNA families show identical expression pattern indicating that they are coexpressed during development. miRNAs which were not detected by Northern blot but identified by cloning are indicated as numbers of clones obtained.

miRNAs have various expression timing throughout development some of them expressing early in the development and some are expressed in later stages. All clustered miRNAs have demonstrated similar expression patterns suggesting that they are coexpressed from a common transcript. Northern data was transformed into semi-quantitative values to be able to compare relative expression of different miRNAs in different stages. This expression information will be a valuable tool to validate the prediction of targets, which are acquired by bioinformatics methods, and verify if the targets could make biological sense. It is expected that miRNAs and their target mRNAs have to be expressed in the same developmental stages.


A
0-1 1-2 2-4 4-6 6-12 12-24 L1 L2 L3 P M F
bantam
let-7
miR-1
miR-2a
miR-3
miR-4
miR-5
miR-6
miR-7
miR-8
miR-9a
miR-10
miR-11
miR-12
miR-13b
miR-14
miR-31b
miR-33
miR-34
miR-79
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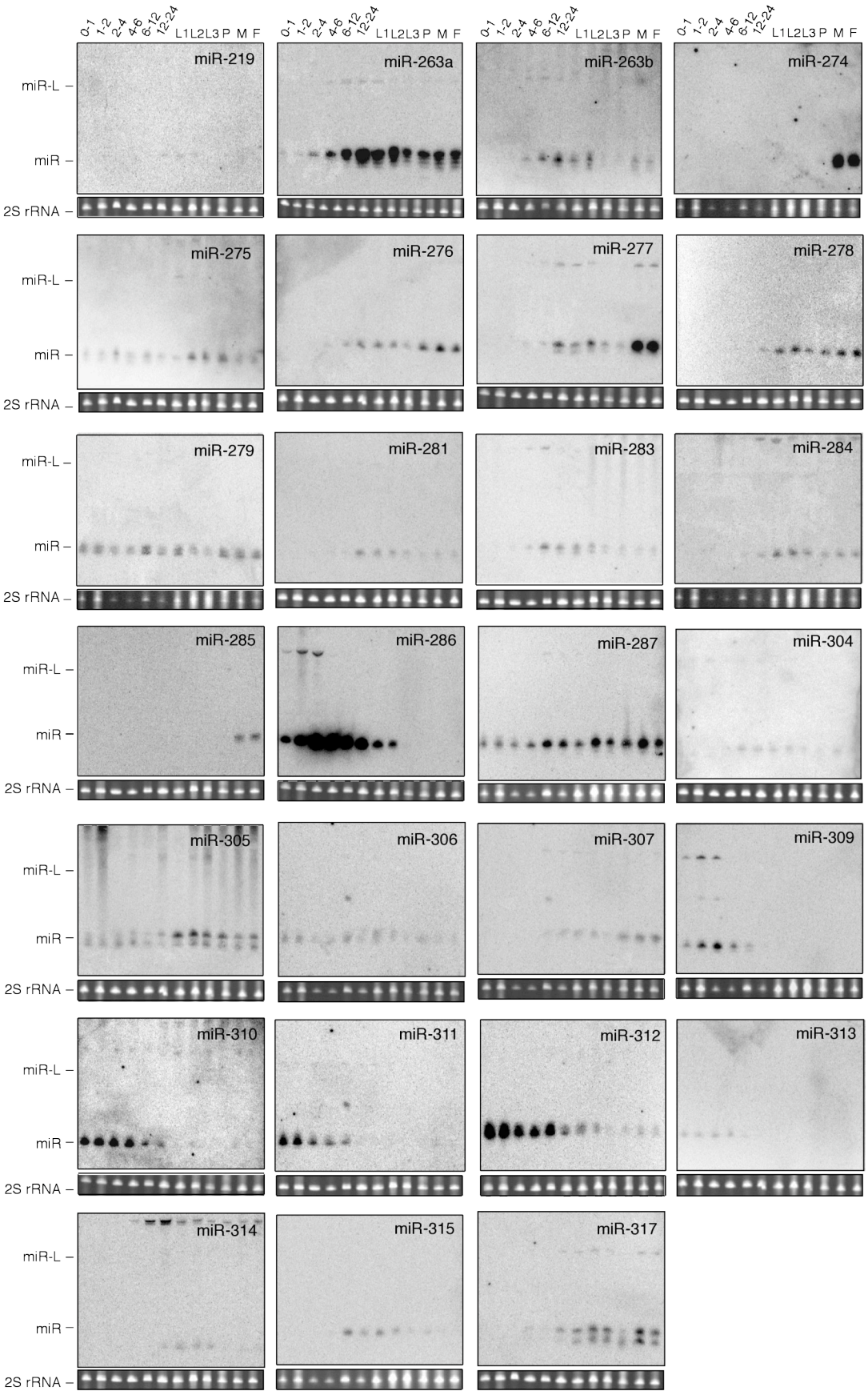
miR-184
miR-210
miR-L
miR
2S rRNA

A (continued)


0-1 1-2 2-4 4-6 6-12 12-24 L1 L2 L3 P M F
miR-219
miR-263a
miR-263b
miR-274
miR-275
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miR-277
miR-278
miR-279
miR-281
miR-283
miR-284
miR-285
miR-286
miR-287
miR-304
miR-305
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miR-314
miR-315
miR-317
miR-L
miR
2S rRNA

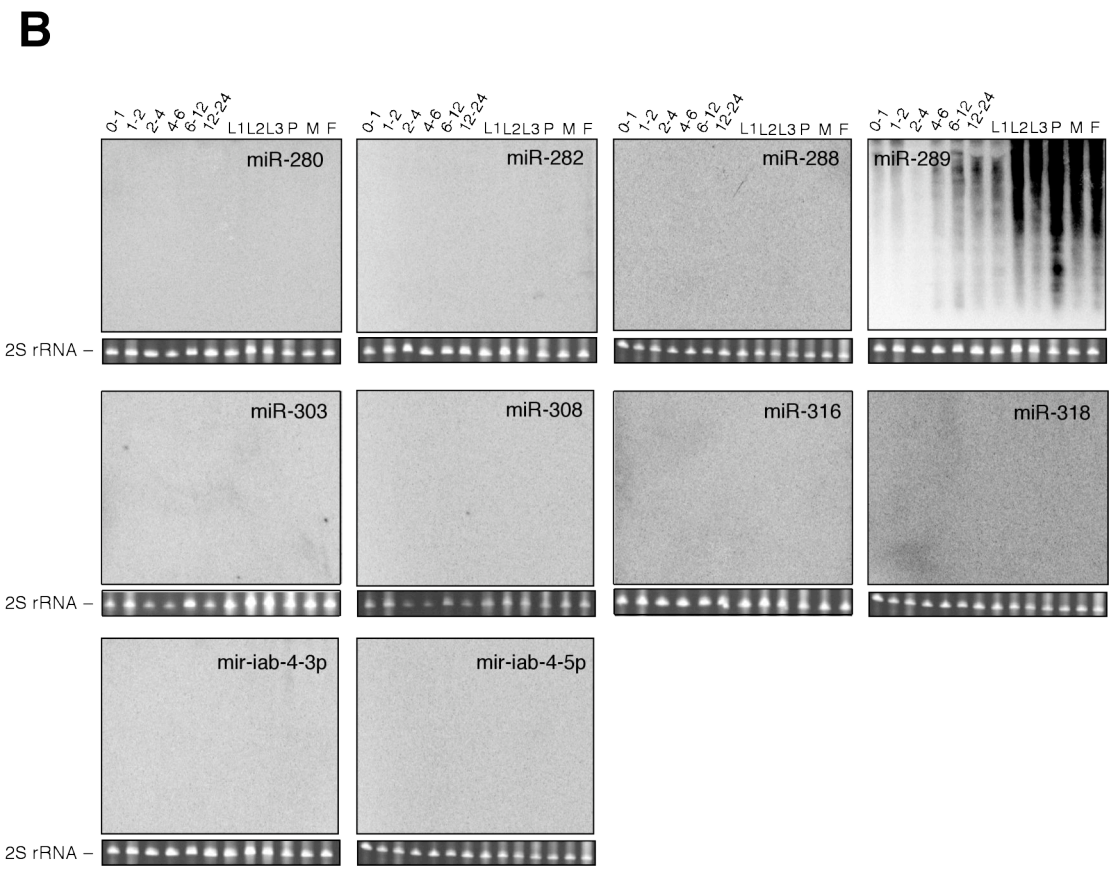


Figure 10. Temporal expression of all *D. melanogaster* miRNAs

(A) miRNAs with detected expression are shown alphabetically. Loading control pictures are shown below each Northern blot. Mature and precursor miRNA bands are indicated. Note that since miRNAs differing in only a few bases are expected to crosshybridize, only one miRNA per multicopy group was tested.

(B) Northern blots for undetected miRNAs. These miRNAs could not be detected by Northern blot.

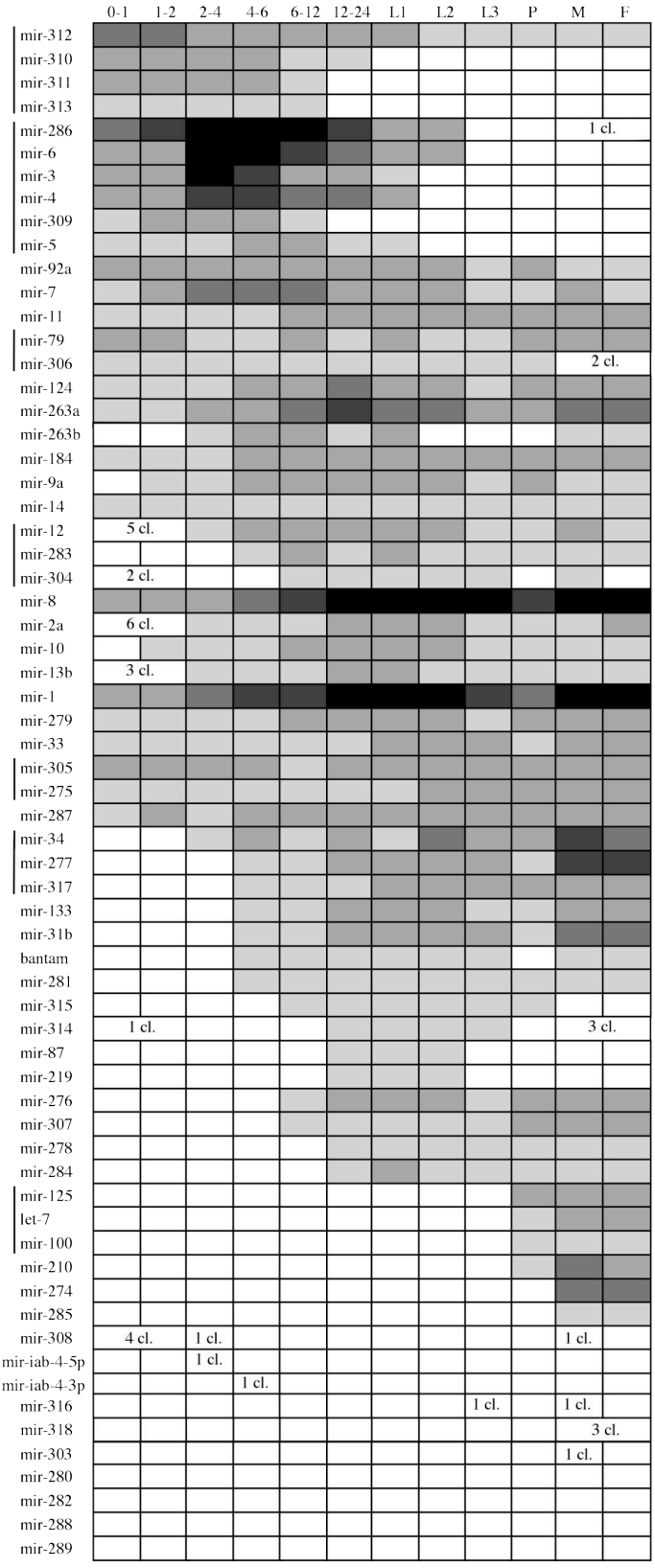


Figure 11. Graphical representation of expression patterns of *D. melanogaster* miRNAs

Graphical representation of expression patterns of all D. melanogaster miRNAs during development, obtained by Northern blot hybridization. Rows correspond to different miRNAs (e.g. miR-1) and the columns represent the developmental stage analyzed.
Vertical bars near the miRNA names indicate the clustered miRNAs. Development stages of embryos are indicated in hours after egg laying. Larval stages are indicated as L1, L2, and L3. P indicates pupal stage; M, adult male; F adult female. The color scale on the right shows the relative quantities of miRNA expression. miRNAs which were coned but not detected by Northern are indicated in boxes as numbers of clones obtained.

5.2 Tissue-specific expression of *M. musculus* miRNAs

To gain insights into the distribution and function of miRNAs in mammals, the tissue specific distribution of miRNAs in adult mouse was investigated. To assess the tissue specificity of 34 novel miRNAs, which were identified by tissue-specific cloning of approximately 21-nucleotide RNAs from various tissues of adult mouse (Lagos-Quintana et al., 2002), relative abundance of the identified miRNAs in different tissues was investigated by Northern blotting. The cloning frequencies of miRNAs alone do not give sufficient information regarding the absolute levels in different tissues.

The tissue-specific expression of mouse miRNAs was investigated by Northern blot analysis using 5'-radiolabeled antisense oligodeoxynucleotide probes. miRNA expression was examined in total RNA samples prepared from 14 different tissues of the adult mouse, and human HeLa SS3 cell lines.

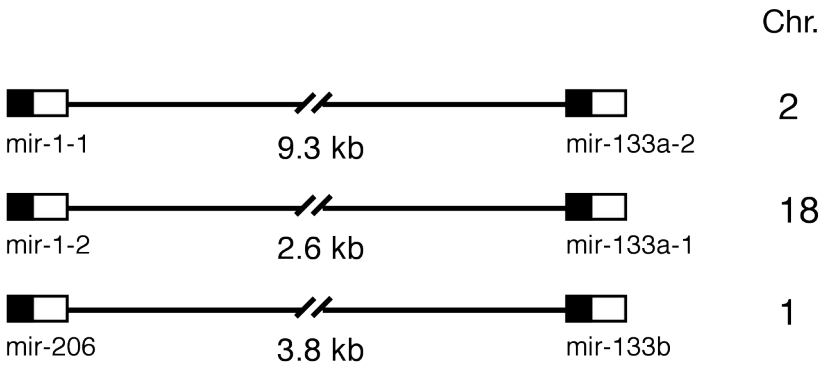


Figure 12. *M. musculus* mir-1 gene clusters

Conservation of genomic organization of the *mir-1/mir-133* gene clusters in M. musculus. Chromosomes are indicated on the right.

miR-1 was first identified in *D. melanogaster* and *C. elegans* (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001). miR-1 is conserved in vertebrate species and was shown by Northern blot analysis to be strongly expressed in human adult heart but not in human brain, liver, kidney, lung, or colon (Lee and Ambros, 2001). There are two copies of *mir-1* in *M. musculus* (*mir-1-1* and *mir-1-2*) and miR-206 is closely related to miR-1 with a single nucleotide change at the 3'-end. *mir-133* is located downstream to the *mir-1* and *mir-206* genes on the genomic DNA and they are coexpressed. The genomic organization of the *mir-1/mir-133* gene

clusters are similar in all three loci suggesting that they may be formed by gene duplications (Figure 11).

Like its human counterpart mouse mir-1 is also expressed explicitly in adult heart (Figure 12). miR-1 was cloned predominantly from heart tissue, although it was also cloned from liver and midbrain (Lagos-Quintana et al., 2002) at lower frequencies. miR-1 was undetectable by Northern blot analysis in these tissues. In liver, miR-122 accounts for 72% of all cloned miRNAs (Lagos-Quintana et al., 2002), and miR-122 was undetected in all other tissues of mouse in Northern blot assay (Figure 12).

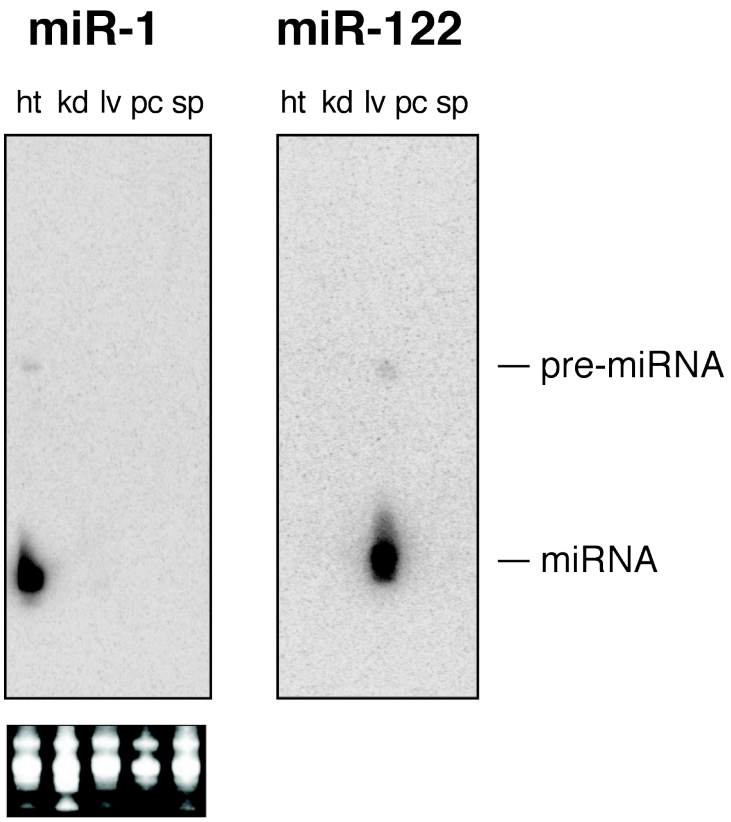


Figure 13. Northern Blot analysis of miR-1 and miR-122

Total RNA from different mouse tissues was blotted and probed with a 5'-radiolabeled oligodeoxynucleotide complementary to the miRNA. Equal loading of total RNA on the gel was verified by ethidium bromide staining prior to transfer; the band representing the tRNA is shown (picture below). The foldback precursors and mature miRNAs are indicated. Tissues are indicated as, ht (heart), kd (kidney), lv (liver), pc (pancreas), sp (spleen).

To gain insights in neural tissue miRNA distribution, RNA samples from cortex, cerebellum and midbrain were analyzed. The rest of the brain (rb) was also used for analysis. Similar to miR-1, which accounted for nearly half of the cloned miRNAs from adult mouse heart, miR-124 was represented dominantly in brain tissues and accounted for 25% to 48% of the cloned miRNAs in the brain samples (Lagos-Quintana et al., 2002). Several other miRNAs were expressed specifically in brain tissue, which is confirmed by Northern blotting (Figure 13). Besides miR-124, miR-127, miR-128, miR-131, and miR-132 were shown to be predominantly brain specific miRNAs (Figure 13). miR-9 was identified from brain samples suggesting it is brain specific, its tissue specific expression was also confirmed by Northern blotting (Krichevsky et al., 2003).

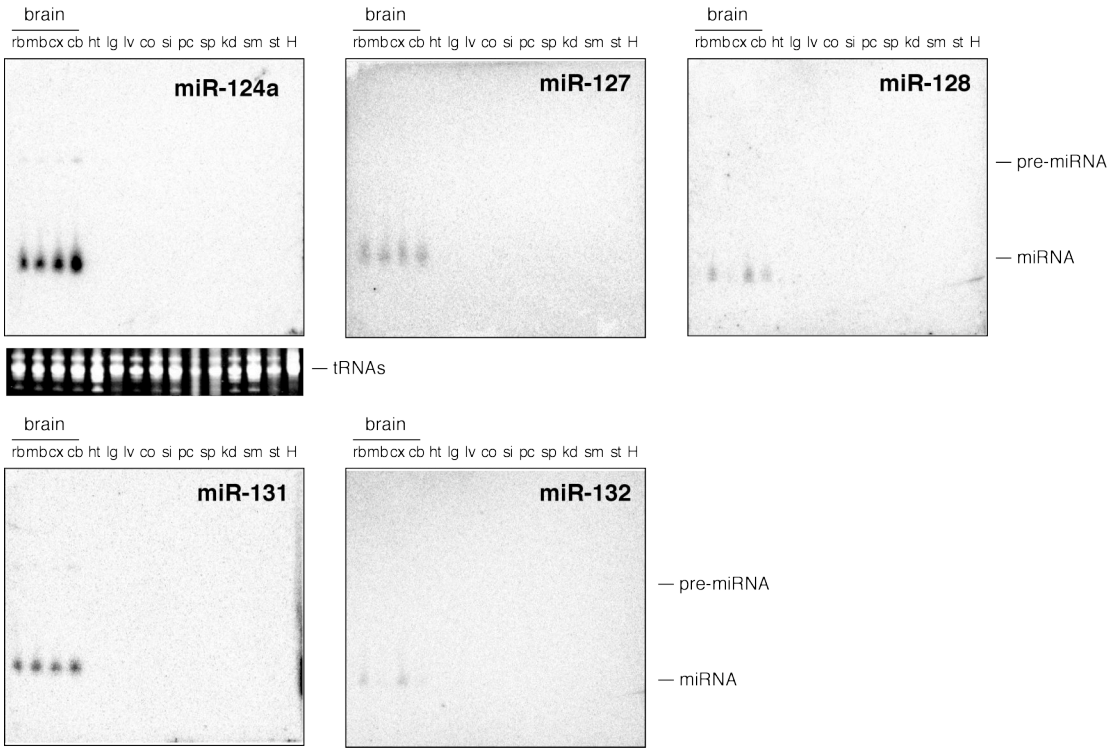


Figure 14. Brain-specific mouse miRNAs

miRNAs, which are expressed only in mouse brain are shown. Total RNA from different mouse tissues was blotted and probed with a 5'-radiolabeled oligodeoxynucleotide complementary to the indicated miRNAs. Equal loading of total RNA on the gel was verified by ethidium bromide staining prior to transfer; the band representing the tRNA is shown. The foldback precursors and mature miRNAs are indicated. Mouse brains were dissected into midbrain (mb), cortex (cx), and cerebellum (cb). The rest of the brain was also used (rb). Other tissues were heart (ht), lung (lg), lv (liver), colon (co), small intestine (si), pc (pancreas), sp (spleen), kidney (kd), skeletal muscle (sm), stomach (st), human HeLa S3 cells (H).

miR-101, which was initially cloned from brain tissue was also detected in liver, kidney, and skeletal muscle tissues in Northern blot assay (Figure 14). This is one of the examples, indicating that cloning data from about 100 clones can not be fully reliable to draw correlations with the tissue specific expression of low abundant miRNAs. miR-125a and miR-125b are very similar to the sequence of *C. elegans* lin-4 miRNA and represent its orthologs (section 5.1.2). miR-125a and miR-125b were cloned from brain tissue, but expression was also detected by Northern blot analysis in other tissues (Figure 14). In *D. melanogaster* miR-125 expression is developmentally regulated and was only detected in pupae adult fly but not in embryo or larvae (section 5.1.2).

miR-30 was cloned from RNA samples of spleen, cortex, and midbrain (Lagos-Quintana et al., 2002). When tissue specific expression of miR-30a was investigated by Northern blot analysis it was shown that it was present predominantly in brain it was also present in several other tissues like heart, lung, kidney, and skeletal muscles in detectable quantities (Figure 14).

Finally, miR-143 was represented significantly in spleen samples with several clones. However, Northern blot assay demonstrated that miR-143, which was also detected in heart, lung, small intestine, pancreas, spleen and brain was most abundantly found in colon (Figure 14).

Some miRNAs were expressed highly tissue-specifically. miR-1, miR-122, and miR-124 are the most striking examples of tissue specific miRNAs. miR-1 and miR-122 were heart and liver specific respectively. Several other miRNAs, such as miR124 appeared to be expressed exclusively or at least predominantly in brain. Several other miRNAs were detected in a variety of tissues but their expression seemed to be regulated dependent on the type of the tissue they are expressed. Appearance of miRNAs in specific adult organs suggests a complex regulation of miRNA expression.

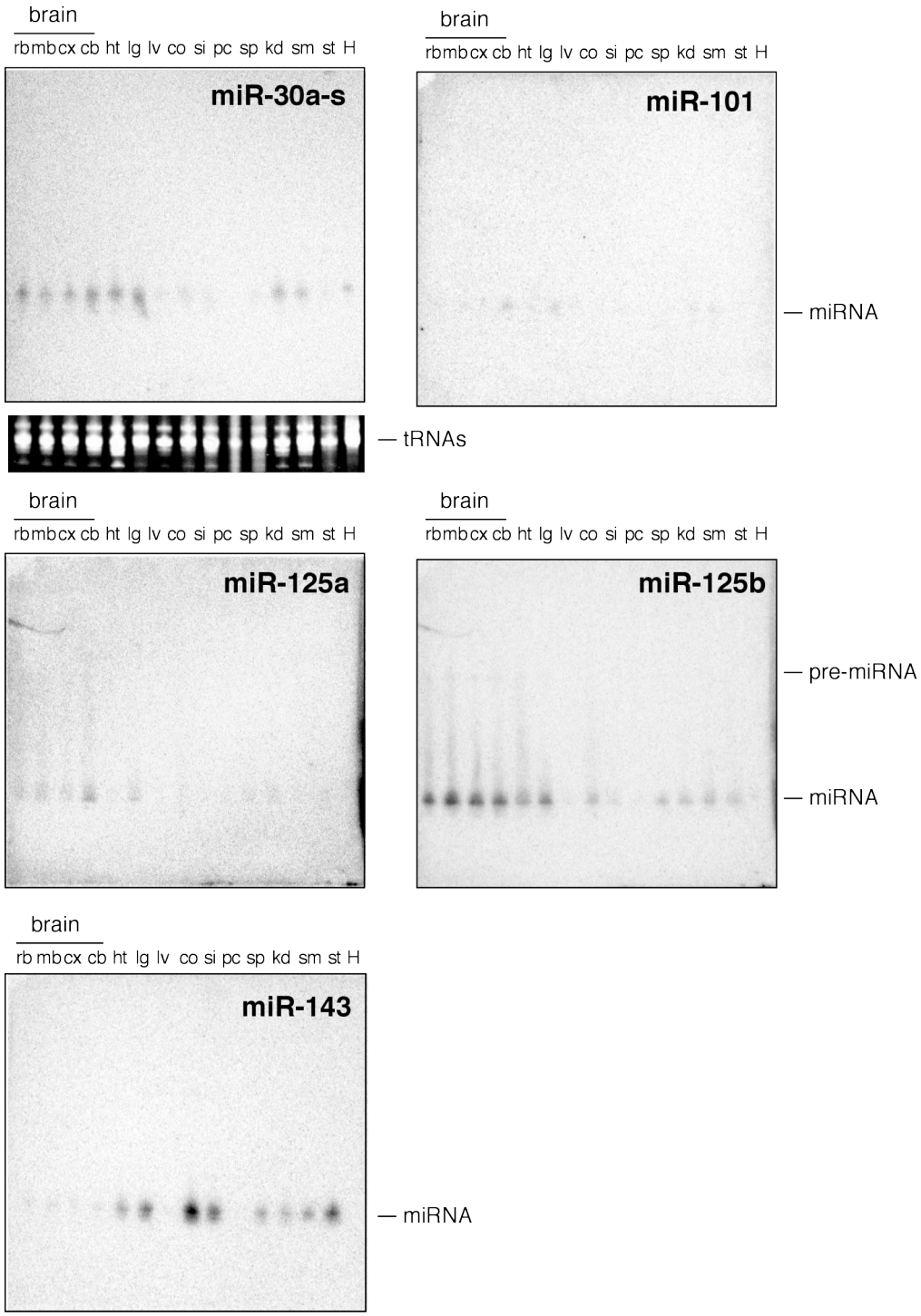


Figure 15. Northern blot analysis of miRNAs in various tissues of adult mouse

Total RNA from different mouse tissues was blotted and probed with a 5'-radiolabeled oligodeoxynucleotide complementary to the indicated miRNAs. Equal loading of total RNA on the gel was verified by ethidium bromide staining prior to transfer; the band representing the tRNA is shown. The foldback precursors and mature miRNAs are indicated. Mouse brains were dissected into midbrain (mb), cortex (cx), and cerebellum (cb). The rest of the brain was also used (rb). Other tissues were heart (ht), lung (lg), lv (liver), colon (co), small intestine (si), pc (pancreas), sp (spleen), kidney (kd), skeletal muscle (sm), stomach (st), human HeLa SS3 cells (H).

5.3 Spatial expression pattern of *D. melanogaster* miRNAs

In order to identify the spatial expression pattern of *D. melanogaster* miRNAs during embryonic stages, hybridization based *in situ* targeting strategies represents one of the possibilities. Whole mount *in situ* hybridization is an established method to identify embryonic locations of mRNAs during development (Tautz and Pfeifle, 1989). Digoxigenin (DIG) labeled nucleotide probes of different sizes are hybridized to immobilized endogenous RNAs followed by detection with anti-DIG antibodies. Due to the small sizes of miRNAs the natural choice of probes for detection are DIG-labeled antisense oligonucleotides, which are usually used for detecting RNAs on micro sections from different tissues. Usage of oligonucleotide probes in whole mount assays is not straightforward and optimizations of the standard technique or modifications in oligonucleotide chemistry and composition are required.

To determine the best suited oligonucleotide probe against miRNAs in embryos various kinds of probe molecules were tested. Single-stranded DNA (ssDNA), single-stranded RNA (ssRNA), and ssRNA with chemical modification (2'-O-Me) oligonucleotides were tested as probes. Various methods were also used to incorporate the DIG label into the probes. Probes were labeled by incorporating either DIG-UTP or DIG-ddUTP to the 3'-terminal (End labeling), or a poly(A)/DIG-U tail by *in vitro* T7 transcription (Tailing).

Digoxigenin-labeled probes were detected on membrane activity assay. RNA as well as DNA oligonucleotides were successfully labeled by end labeling or tailing methods (Figure 15).

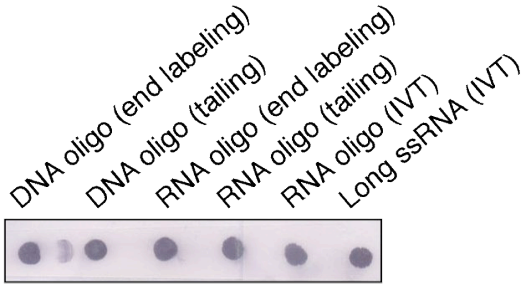


Figure 16. Membrane activity assay for miR-1 *in situ* hybridization probes

Antisense miR-1 oligonucleotide probes of 22-nt (DNA or RNA) and antisense miR-1 long RNA probes prepared for *in situ* hybridization were tested on membrane activity assay for labeling with DIG Probe sequences are listed in Methods section (4.6.1). 1 µl from the prepared probes (1 µM) were blotted on Nylon membrane and AP conjugated anti-DIG antibody was applied.

D. melanogaster embryos of 0 to 24 hours age were permeabilized and fixed. Digoxigenin labeled probes were used in whole mount *in situ* hybridization assays. As control antisense RNA probes 484-nt long against *D. melanogaster* fushi tarazu mRNA were tested. Fushi tarazu mRNA is composed of two exons separated by a single intron. Antisense probe against the second exon of Fushi tarazu was used for mRNA detection. Fushi tarazu mRNA is expressed in early *D. melanogaster* embryo in a seven-stripe pattern. The expression pattern of several miRNAs, which are expressed abundantly in early embryogenesis (miR-1, miR-2, miR-3, miR-4, miR-6, and miR-10) were investigated. Although fushi tarazu mRNA was successfully detected, miRNA expressions could not be readily identified using Digoxigenin labeled oligonucleotide probes (Figure 16). The usage of probes, which were labeled by different methods, did not show any improvements.

Figure 17. *In situ* hybridization of mir-1 by as RNA probes

Spatial distribution of mir-1 expression investigated by whole mount *in situ* hybridization using DIG labeled as RNA oligonucleotide probes. Picture on the left shows the expression of fushi tarazu mRNA in *D. melanogaster* embryos. In the middle picture *in situ* hybridization result using probes targeting mir-1 and on the right picture *in situ* hybridization using nonspecific probe are shown.

Since detecting miRNAs in *D. melanogaster* embryos with oligonucleotide probes was unsuccessful, DIG labeled RNA probes of approximately 1kb in length that are complementary to the genomic sequence of the *mir-6* cluster and the *mir-1* genes were prepared and tested in order to detect the primary precursor of miRNAs.

The *mir-6* locus contains 8 miRNA genes (*mir-3/mir-4/mir-5/mir6-1/mir-6-2/mir-6-3/mir-286/mir-309*) closely located to each other suggesting that these miRNAs are expressed from a common longer RNA transcript. *mir-1* locus encodes a single miRNA gene, and yield the most abundant miRNA during development. The RNA probe complementary to the *mir-6* gene cluster yielded no specific color signal,

compared to the non-specific sense probe. However, the Digoxigenin-labeled 992-nt long RNA probe complementary to the genomic locus of *mir-1* gene yielded a defined signal compared to the non-specific sense probe (Figure 17). miR-1 is expressed starting very early in the development starting in the first 2 hours and persists throughout embryogenesis. Early expression of miR-1 RNA is located at the ventral cells of the blastoderm. After gastrulation miR-1 expression continues in mesoderm cells, which form heart and skeletal muscle tissue in adult flies (Figure 17). This result is consistent with the expression of *M. musculus* miR-1, which is specifically expressed in heart tissue.

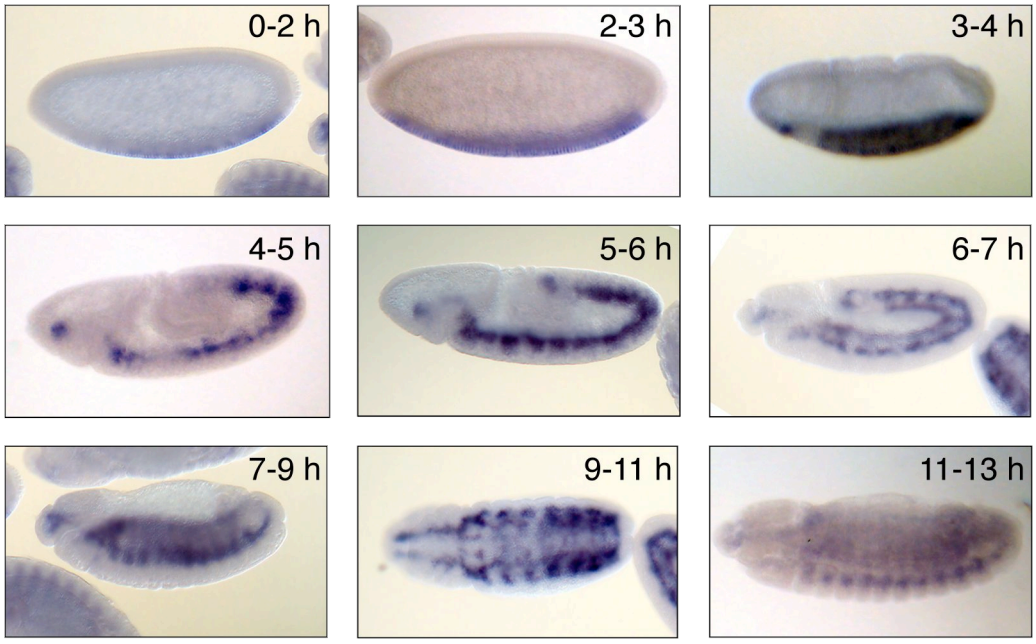


Figure 18. mir-1 expression in different stages of *D. melanogaster* embryos
mir-1 expression during *D. melanogaster* development was identified by in situ hybridization using long ssRNA probes labeled by Digoxigenin. Pictures above show the expression (blue color) of mir-1 in different developmental stages of D. melanogaster. Approximate ages are indicated in the upper right corners.

Not all pri-miRNA transcripts seem to be detectable and it was desirable to detect the accumulation of mature miRNAs. Figure 18 summarizes the various influencing *in situ* hybridization. The critical steps of (fixation, permeabilization, and hybridization) *in situ* hybridization assay were changed in order to optimize the assay for detecting miRNA expression. Although fixation of RNA in the embryos was sufficient to detect abundant miRNAs with long antisense probes in standard method, more severe fixation conditions were tried for miRNA fixation, by increasing the

fixative reagent concentration and duration of the fixation. Permeabilization is another important step, which can be optimized. Different pH and duration were tried to make the embryos permeable to probe molecules. Various hybridization conditions were also tried by changing the stringency of the hybridization mixture (salt and formamide concentration). Finally, the staining process was optimized in order to decrease the non-specific background signal and therefore obtain a specific color signal for miRNA expression. All the shown methods were applied in different combinations for different miRNAs but only the DIG labeled long ssRNA probes, which target primary transcript of miR-1 yielded specific signal.

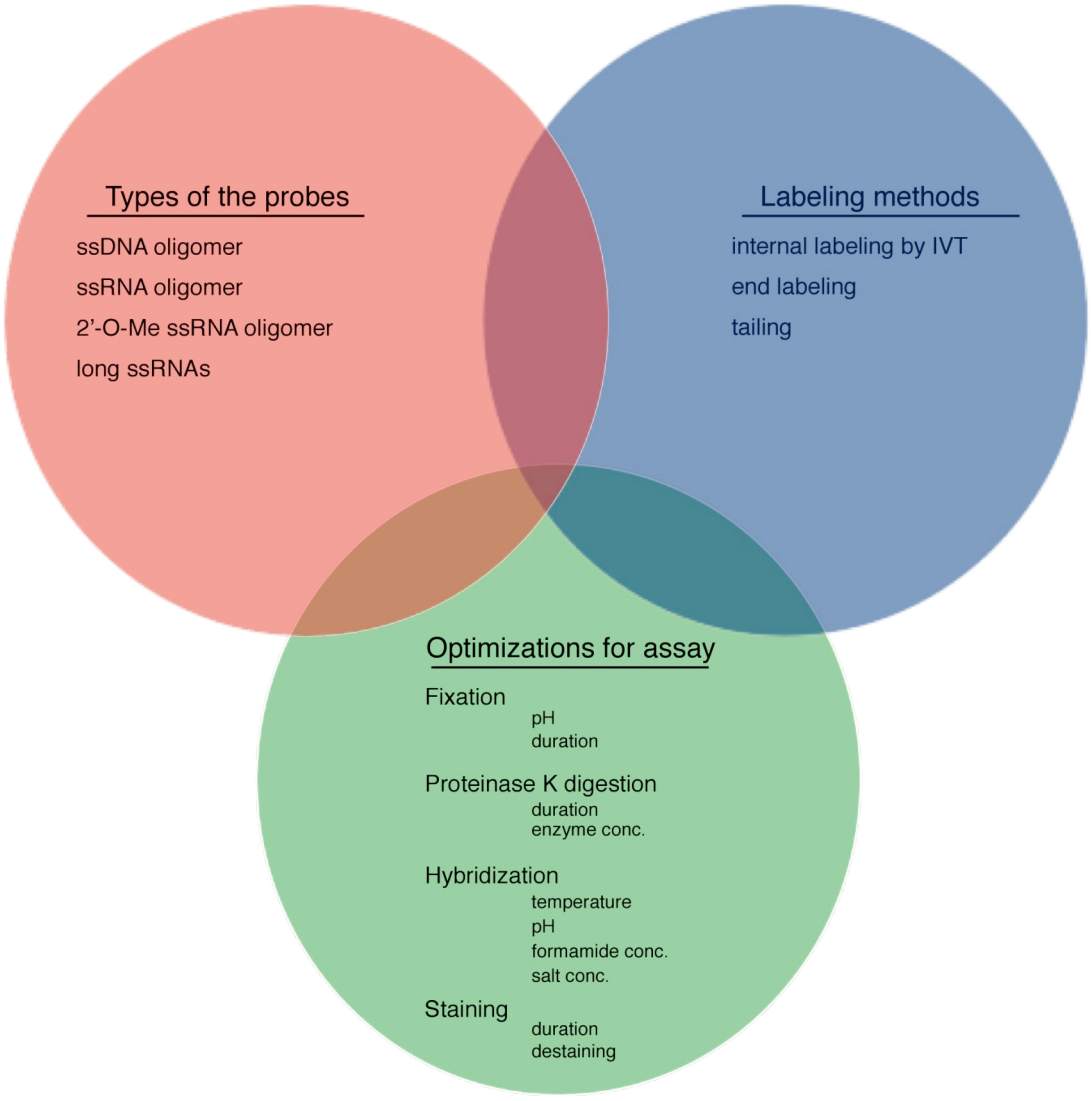


Figure 19. Optimizing *In situ* hybridization protocol for miRNA detection

In order to establish a detection method several different strategies were tried in different combinations.

I then tested if short probes, which contain fewer DIG residues per probe molecule, were still able to detect fushi tarazu mRNA. Digoxigenin labeled ssRNA probes with sizes from 24-to 98-nt were prepared from PCR products, which are complementary to either exons of fushi tarazu using *in vitro* transcription (Figure 19). The ratio of DIG-UTP to unmodified UTP was 1:6, indicating that the shortest 24-nt probes had about one DIG molecule per probe.

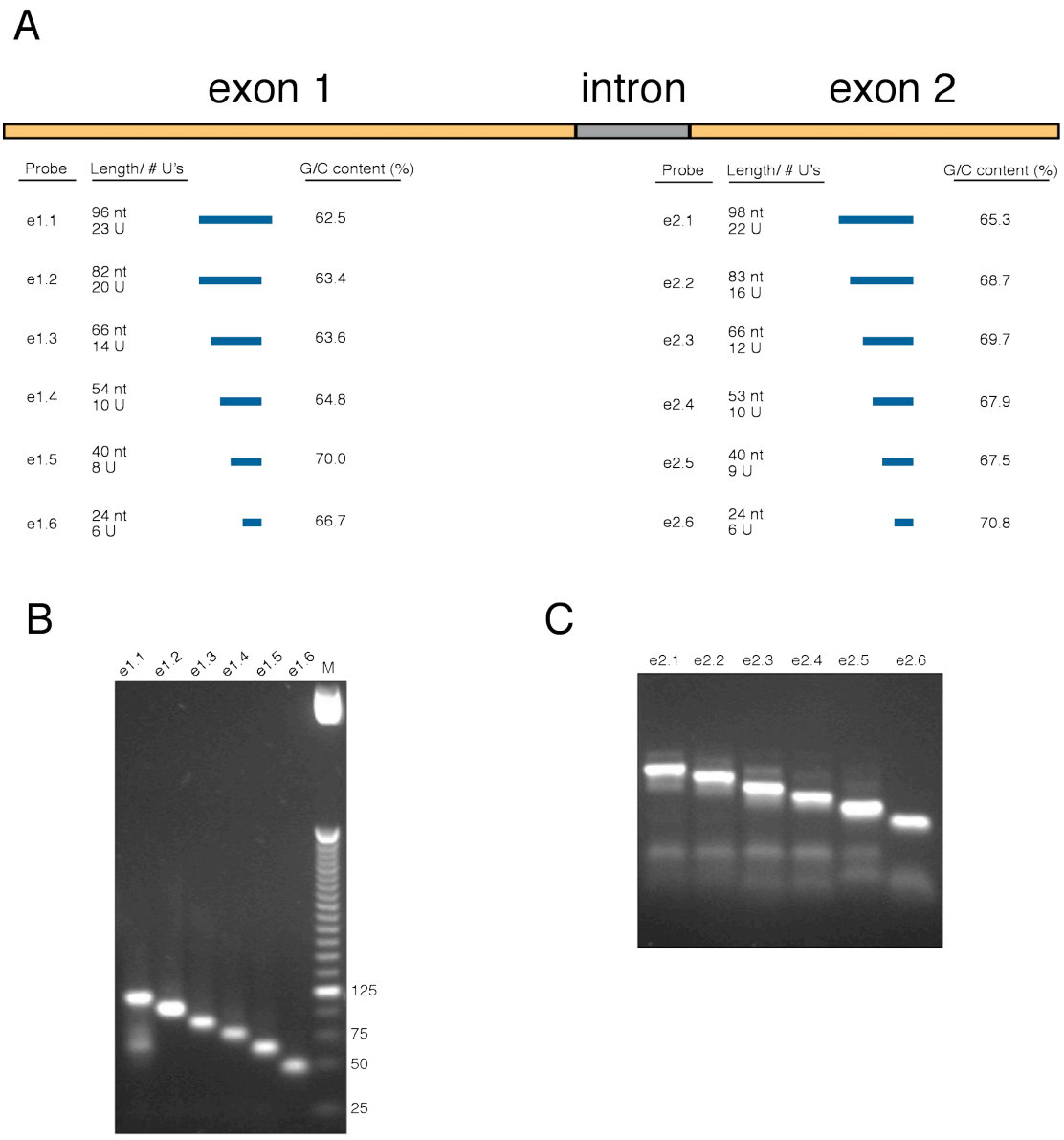


Figure 20. Short *in situ* hybridization probes for detecting fushi tarazu mRNA

Short *in situ* hybridization probes against the two exon of fushi tarazu mRNA prepared by *in vitro* transcription.
(A) Exon 1 and exon 2 of fushi tarazu mRNA is shown schematically. DIG labeled *in situ* hybridization probes and their targeting locations are shown below. The sizes of the exons and probes are on scale. Length in nucleotides and number of uridine (U) residues are also shown.
(B) PCR amplified *in vitro* transcription template of fushi tarazu exon 1 probes. Size marker is shown on the right. Numbers indicate the length in base pairs.
(C) PCR amplified *in vitro* transcription templates of fushi tarazu exon 2 probes.

The transcribed probes were then separated on 15% denaturing polyacrylamide gel and isolated from the gel (Figure 20). The specific activities of the Digoxigenin-labeled probes were assessed by the membrane activity assay. 1 µl from the probes (approx. 1-5 µM) were blotted on the Nylon membrane and AP-conjugated anti-DIG antibody detection was carried out (Figure 21). It was observed that specific activities of the probes decreased while the concentration determined by UV absorbance was constant. Consistent with probes containing less DIG residues per molecule with decreasing size.

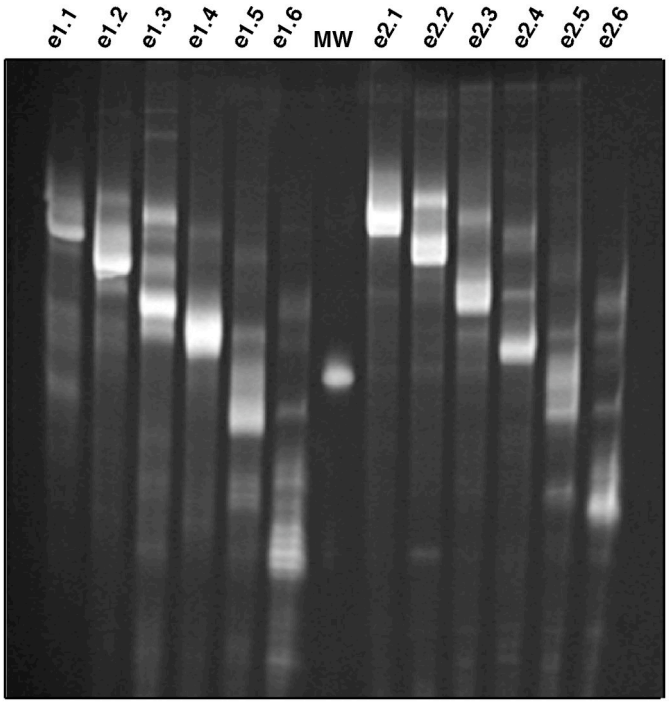


Figure 21. Amplification of short ssRNA transcripts for *in situ* hybridization targeting fushi tarazu mRNA

DIG labeled short ssRNA fragments probes targeting the two exon of fushi tarazu mRNA were prepared by *in vitro* transcription, separated on a 15% denaturing PA gel and ethidium bromide stained. RNA size marker of 50 nucleotide is shown on the middle band (MW).

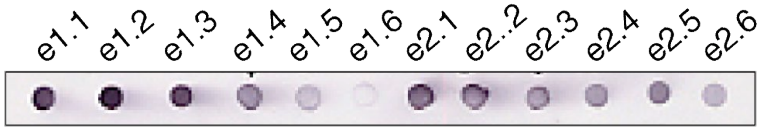


Figure 22. Membrane activity assay for short *in situ* hybridization probes.

Generated DIG labeled ssRNA probes were blotted on Nylon membrane and specific activities were determined by treating with AP conjugated anti-DIG antibody. Color reaction was carried out by AP substrate.

The in situ hybridization experiment is shown in Figure 22. While the 60-to 90-nt probes resolved the Fushi tarazu expression well, the shorter probes gave weaker signals with high non-specific background signals. Nevertheless, even the shortest probes (e1.6 and e2.6) yielded a recognizable seven-stripe Fushi tarazu expression pattern. The strong background could be resulted from either nonspecific binding of the short probes to embryonic RNAs or the long color reaction step, which was required for detecting the weak signal.

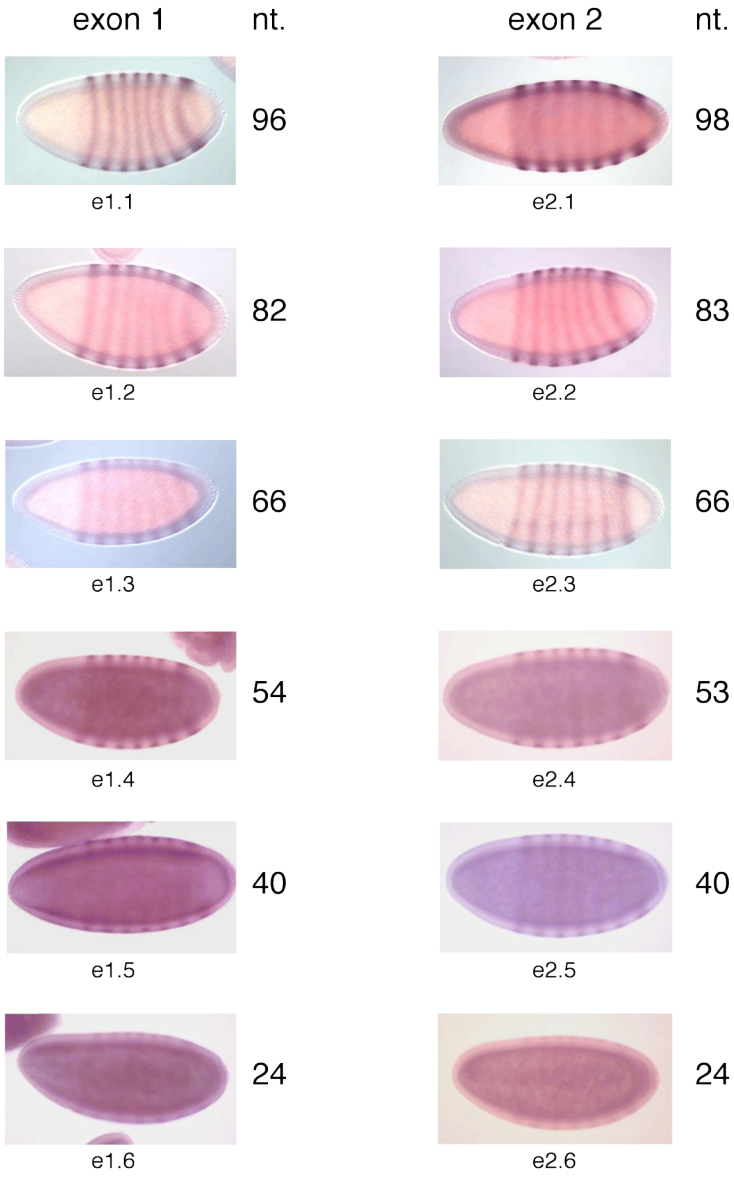


Figure 23. Whole mount *in situ* hybridization with short ssRNA probes

DIG-labeled short ssRNA fragments probes against the two exon of fushi tarazu mRNA constructed by *in vitro* transcription. Exon 1 probes are shown on the left column. Exon 2 probes are on the right column. Length of the probe in nucleotides are indicated on the right of each picture.

In an effort to increase the intensity of the specific signal of the short ssRNA probes (24-nt), labeling by poly-d(A)/DIG-U tailing was tested. During tailing reactions several DIG-UTP residues are added in the 3'-end of the RNA probes by terminal transferase (Figure 23). In order to test if the tailed probes really contained more than one DIGs per molecule the probes produced this way were loaded on 15% denaturing polyacrylamide gel for size determination. RNAs were then transferred on to Nylon membranes and membrane activity assay was carried out. It was shown that tailed 24 nucleotide probes were both longer and significantly had more specific activity compared to the end labeled and *in vitro* transcription labeled probes (Figure 23). The tailed probes were used in whole mount *in situ* hybridization assays and results were compared to the internally labeled and end labeled probes, which contain single digoxigenin molecule. The tailed probes yielded very strong background signal. Specific color signal could not be detected.

End labeled probe (Figure 23 lane 7) was seen as a single and weak band indicating that only single DIG-UTP was added to the end of the RNA. The tailed probe on the other hand (Figure 23 Lane 6) appeared as a strong smear stain indicating the uneven size of the probe and multiple DIG-UTP incorporation. Even though providing more DIG-UTP in the T7 transcription reaction yielded probes with higher specific activity (Figure 23 lane 4 and 5) the activities of the IVT labeled probes were not as high as the tailed probes.

Similar results were obtained when longer (96 nucleotide) probe, e1.1 was labeled with these methods. It was sown that end labeled probe yielded a single and weak band suggesting that a single DIG-UTP nucleotide was added to the end of the probes (Figure 23 lane 11). To track the progress of the rate of the tailing reaction two control reactions were carried out with different durations. It was shown that even after 1 hour the reaction was incomplete judged by the remaining reactants in the reaction (Figure 23 lanes 1 and 1).

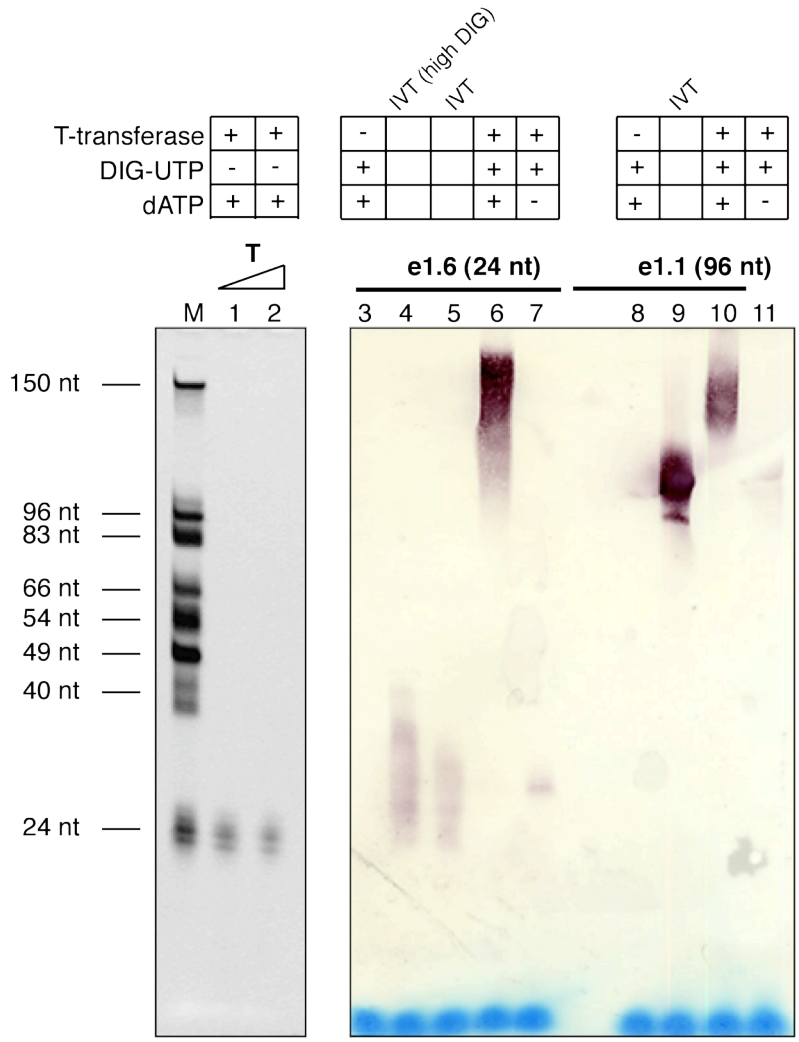


Figure 24. Quality and size control of DIG labeled *in situ* hybridization probes

Probes were labeled by different methods and run on 15% polyacrylamide gel for size separation. RNA bands were then transferred on to Nylon membrane and membrane activity assay was applied to detect the labeled probes on the blots. Left picture shows the ethidium bromide stained gel visualized by UV. RNA size marker (M) is shown with corresponding sizes indicated on the left. Lane 1 and 2 are labeling reactions of e1.6 with no DIG-UTP included in the mixture. Lane 1, 20 minutes reaction. Lane 2, 1 hour reaction.

The difficulty of detecting mature miR-1 whereas pri-mir-1 being successfully detected by whole mount *in situ* hybridization suggests that short mature miRNAs were diffusing out during the *in situ* hybridization assay. Although the RNA was formalin fixed before the *in situ* hybridization assay was carried out, the cross-links are reversible and can be reversed during long incubation at higher temperature. In order to find out if the mature miRNAs were retained during the *in situ* hybridization assay, total RNA was isolated from embryos at different steps of the protocol. Two cross-linking and permeabilization experiments were performed in parallel, one with 30 min and the other with 16 h formalin fixation. In order to determine the effect of

the duration of permeabilization step (Proteinase K digestion) embryos from different time points of digestions were compared. Before RNA isolation cross-linking was reversed by Proteinase K digestion at 65^{o}C for 5 hours. Then total RNA was isolated from the embryos. RNA was separated on a 15% denaturing polyacrylamide gel, and transferred to Nylon membrane for Northern blotting for miR-3. miR-3 was detected at all the steps both in short fixation and long fixation protocol (Figure 24). It was shown that miR-3 could be detected even after 10 minutes of Proteinase K digestion. Embryos prepared and fixed freshly or kept at -80 ^{o}C after formaldehyde fixation were compared. No significant difference in miR-3 content was detected between these embryos (Figure 24). There was a small decrease in the miR-3 amount in 16 h fixed embryos, which may be due to reduced recovery of total RNA from these embryos.

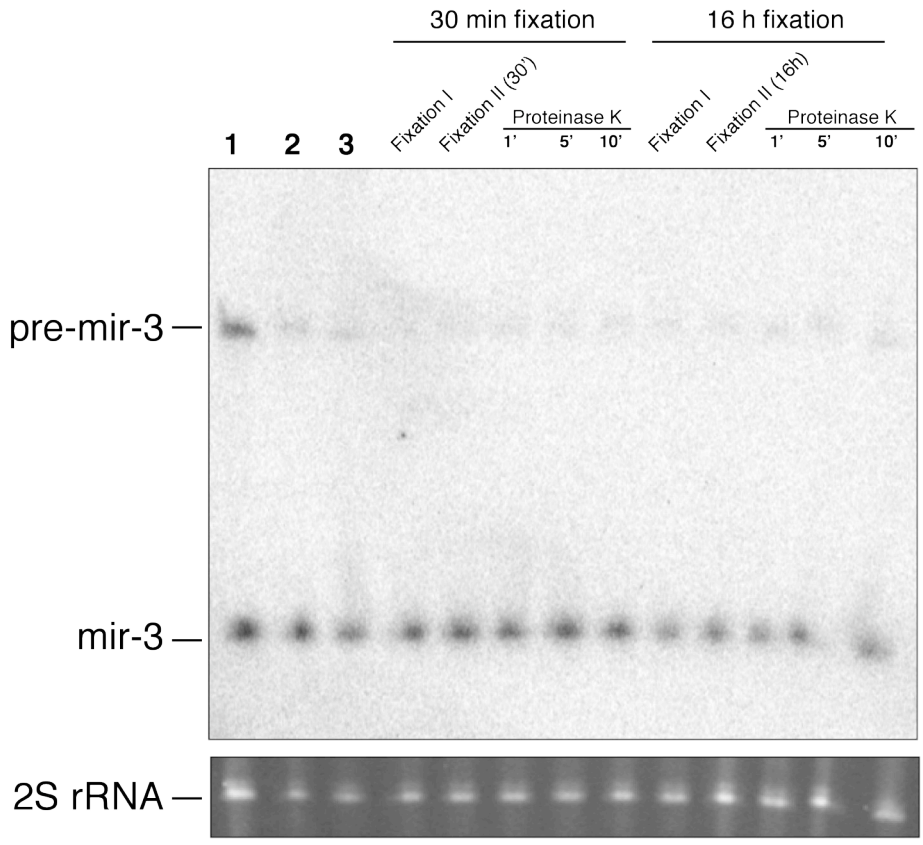


Figure 25. Northern blot from fixed embryos

Presence of miRNAs in the embryos after fixation and permeabilization steps tested by Northern blotting. Two fixation and permeabilization experiments were done in parallel with 30 min and 16 h formalin fixation. Control lanes are RNAs isolated from, 1- untreated, 2- freshly prepared, 3- embryos kept a few weeks at -80 ^{o}C. From ethidium bromide stained gel picture 2S rRNA band is shown as loading control at the bottom.

Next, presence of mature miRNAs after the probe hybridization steps was investigated. Two experiments, one with 30 min, and one with 16 h formalin fixation were compared. Total RNAs from embryos collected at different time points of probe hybridization at 50°C. RNA was isolated, separated on 15% denaturing polyacrylamide gel and transferred on a Nylon membrane for miR-3 Northern blotting. Mature miR-3 an precursor were detected at 3 h of hybridization at 50°C (Figure 25). However, after 3 h of hybridization the amount of miRNA and precursor of miRNA decreased rapidly presumably because of the reduced recovery of total RNA from fixed embryos. After 18 h at 50°C miR-3 was hardly detected in the embryos that were fixed for 30 min, while miR-3 was detected in the embryos fixed for 16 h (Figure 25). The miR-3 band in all cases was weaker compared to the untreated embryos.

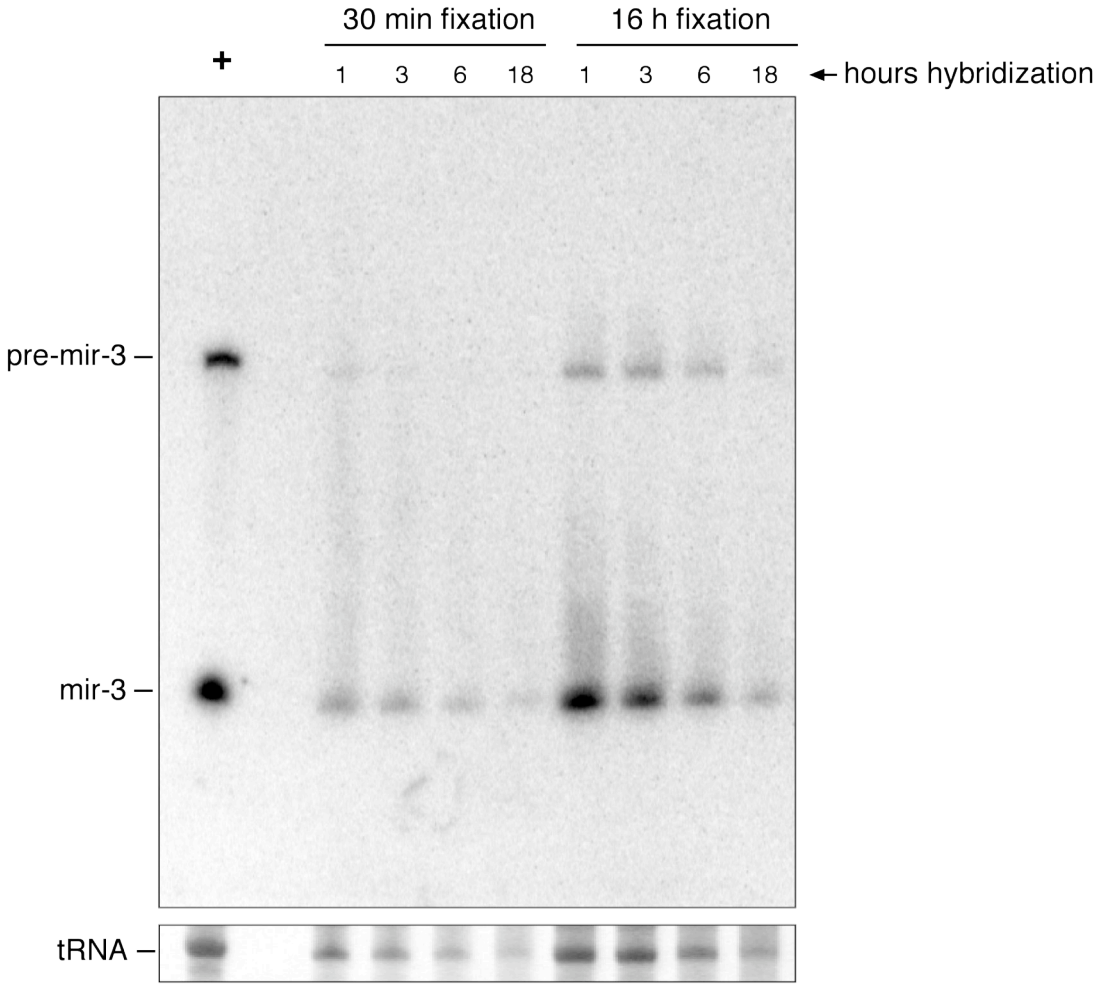


Figure 26. Northern blot for miRNAs during *in situ* hybridization

Presence of miRNAs in the embryos after 18 h incubation at 50 °C was tested by isolating total RNA from embryos and Northern blotting for miR-3. Two experiments were conducted in parallel using 30 min and 16 h formalin fixed embryos. + is positive control, embryos with no treatment. Equal amount of RNA was loaded on the lanes. tRNA band for each lane is shown on the lower picture, which is ethidium bromide stained gel visualized under UV.

The loss of miRNA was predominantly due to diffusion of the small RNAs. Prolonged incubation at 50^{o}C presumably reversed the sequential cross-links formed by formalin. In order to determine if the probe hybridization step was also affecting the presence of the longer RNAs the amount of 70-to 80-nt tRNA remaining under these conditions was examined. Like miRNAs and precursor of miRNAs, the amount of tRNA was also reduced during long periods of incubations (Figure 25). Also, the 30-nt 2S rRNAs could not be detected in the gel after ethidium bromide staining. This observation suggested that small RNAs vanish during high temperature incubation steps.

Together, these experiments suggest that formalin fixation was immobilizing miRNAs in the embryos. However, because the fixation is reversible and longer periods of incubation result in loss of small RNAs by diffusion. Extended formalin fixation keeps small RNA longer in the embryos. But even with the use of 16 h fixation mature miRNAs remained difficult to detect.

More recently, after the efforts for developing a suitable *in situ* hybridization protocol for *D. melanogaster*. The expression pattern of vertebrate miRNAs in the developing zebrafish embryo were determined using LNA-(locked nucleic acid) modified oligodeoxynucleotide probes (Wienholds et al., 2005). LNA is a bicyclic high-affinity RNA analog in which the furanose ring of the ribose sugar is chemically locked in an RNA-mimicking conformation, resulting in high hybridization affinity toward complementary DNA and RNA molecules (Vester and Wengel, 2004). In LNA oligodeoxynucleotide probes every 3rd residue is replaced by the LNA nucleotide analog.

In order to determine if LNA probes can be used to obtain the expression pattern of *D. melanogaster* miRNAs in developing embryos, LNA probes complementary to *D. melanogaster* miR-124 and miR-1 sequences were tested. LNA probes were labeled by tailing and end labeling as described before. Labeled probes labeled were used in whole mount *in situ* hybridization experiments using *D. melanogaster* and *D. rerio* (zebrafish) embryos and compared with DNA and RNA oligonucleotide probes prepared similarly.

Consistent with the previous reports (Wienholds et al., 2005), we also found miR-124 expressed in the central nervous system of developing zebrafish embryos starting from very early stages of development (Figure 26). miR-124 was detected as early as the 14 somite stage of zebrafish embryos and the expression followed until

the embryos were 5 days old. miR-124 was localized to central nervous system, brain and around the eyes of the embryo (Figure 26) suggesting that miR-124 functions in neural tissues.

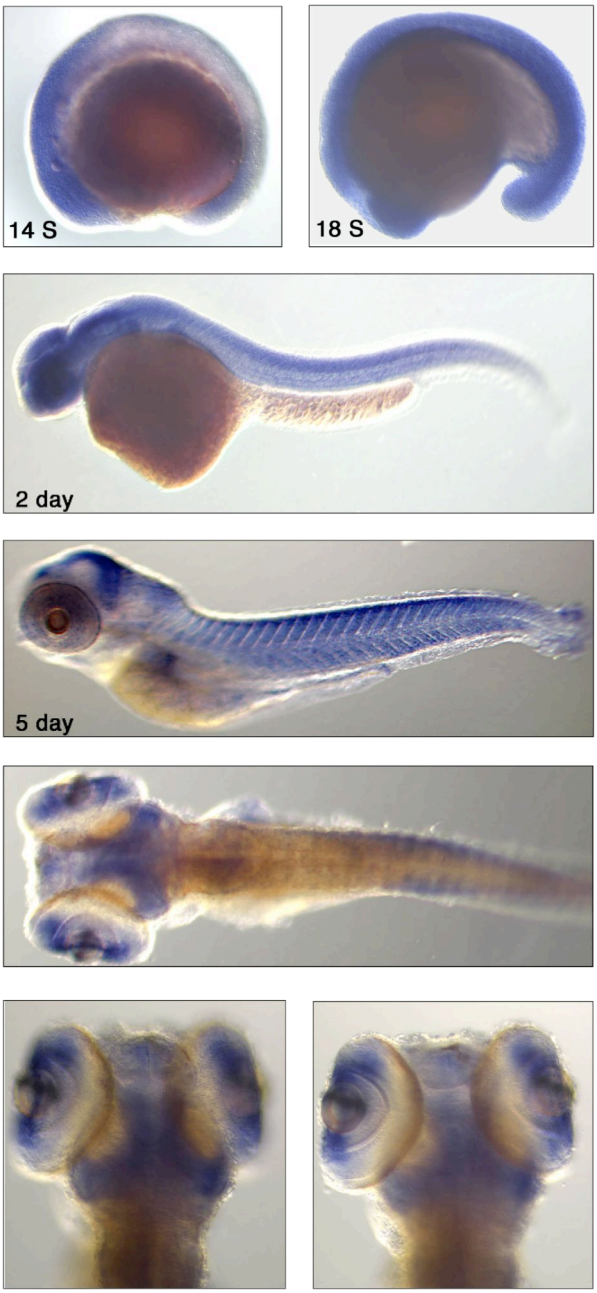


Figure 27. mir-124 expression in different stages of zebrafish embryos

mir-124 expression is shown in different stages (14 somite, 18 somite, 2 day, and 5 day) of zebrafish (*D. rerio*) embryos identified by in situ hybridization using locked nucleic acid (LNA) probes.

Although, the experiments were performed in parallel it was not possible to detect miR-124 or miR-1 expression in *D. melanogaster* embryos. The expression patterns of several *D. melanogaster* miRNAs including miR-124 and miR-1 were identified by using digoxigenin labeled LNA probes by other laboratories .(Sokol and

Ambros, 2005; Stark et al., 2005). *D. melanogaster* miR-124 expression was also identified by long RNA probes against pri-miRNA transcripts (Aboobaker et al., 2005). However I could not identify the expression of *D. melanogaster* miRNAs using LNA probes. The *in situ* hybridization protocol for miRNA detection in fly embryo is required to be optimized and perfected.

5.4 RNAi knock-down of *D. melanogaster* miRNA processing factors

A genome-wide, yeast two-hybrid screen for *D. melanogaster* proteins recently revealed an interaction between Drosha and the WW domain-containing protein dmDGCR8 (CG1800-PA, DNA Accession # NP-651879) (Giot et al., 2003) the ortholog of human DGCR8 protein (Shiohama et al., 2003). These observation led the investigation of this protein as a novel factor involved in processing of primary miRNA transcripts.

In order to test if dmDGCR8 is required for miRNA processing I used RNAi in *D. melanogaster* S2 Schneider suspension cells (Boutros et al., 2004) to target dmDGCR8 and other pri-miRNA processing factors. dsRNA of about 500 base pair (bp) in length, cognate to the coding region of processing factors and dmDGCR8, was added to the S2 cells suspension in culture medium. The growth and survival of the cells was monitored in a cell counter. In general there was a mild nonspecific effect of dsRNA treatment on the viability of the cells compared to the untreated S2 Schneider cells. GFP dsRNA served as unspecific control as it was not expressed in *D. melanogaster* cells. As positive control, dsRNA targeting the apoptosis inhibitor DIAP1 (CG12284) was used and caused a significant cell death (Boutros et al., 2004). Cell viability of the dsRNA-treated cells was determined 5 days after dsRNA introduced, by the percentage of the living cells in the medium and shown in figure 27. DIAP1 dsRNA had the most significant phenotype causing 60% apoptosis (Figure 27), indicating that RNAi assay was functioning. dsRNA targeting the miRNA processing factors, except for Ago2, did not show a significant growth and apoptosis phenotype. The overall effect was comparable to the effect of nonspecific GFP double stranded RNA. These observations indicated that knock-down of the miRNA processing factors did not affect the survival of the cells significantly.

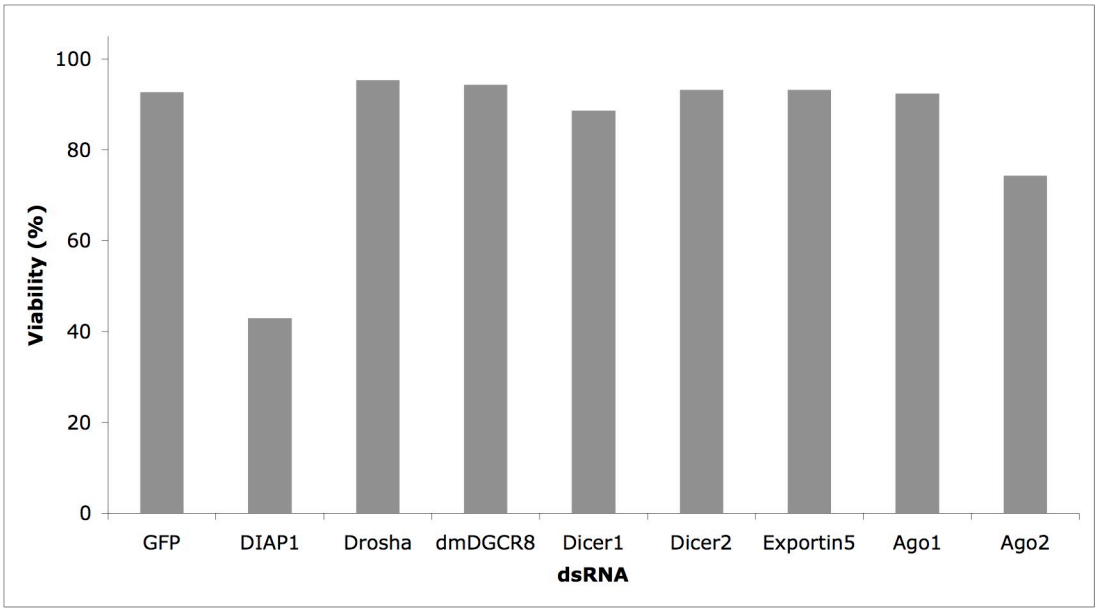


Figure 28. Viability of S2 Schneider cells after RNAi knock-down with dsRNA

Number of living cells were identified by cell counter based on the size of the particles in the cell suspension. apoptotic or dead cell appear as debris, which are recognized by their smaller size than the living cells. The ratio of living cells to whole cell number is calculated and the viability ratios of the samples are shown in graph. Bars indicate different dsRNAs administered to cells.

I first examined the processing of *mir-2* gene clusters that are expressed in S2 Schneider cells (Lagos-Quintana et al., 2001). *mir-2* genes are located on 4 different loci on the *D. melanogaster* genome (Figure 28). Among these genes *mir-2a-2*, *mir-2a-1*, and *mir-2b-2* are in the intron of the protein coding gene Spitz (CG10334). *mir-13b-2*, which is closely related to *mir-2,* is also located in the intron of a hypothetical protein coding gene, CG7033 on X chromosome. *mir-2b-1* and *mir-13b-1*/*mir-13a*/*mir-2c* clusters are located on the left arm of chromosome 2 and right arm of chromosome 3, respectively, and are controlled by their own promoters (Figure 28).

The efficiency of the RNAi assay was determined for a subset of the targeted genes by qRT-PCR using RNA isolated from dsRNA treated cells (Figure 33). Drosha and Dicer2 mRNAs were reduced about 5-fold whereas dmDGCR8 and Dicer1 mRNAs were reduced about 2.5-fold compared to the GFP dsRNA treated cells 5 days after dsRNA treatment (Figure 33).

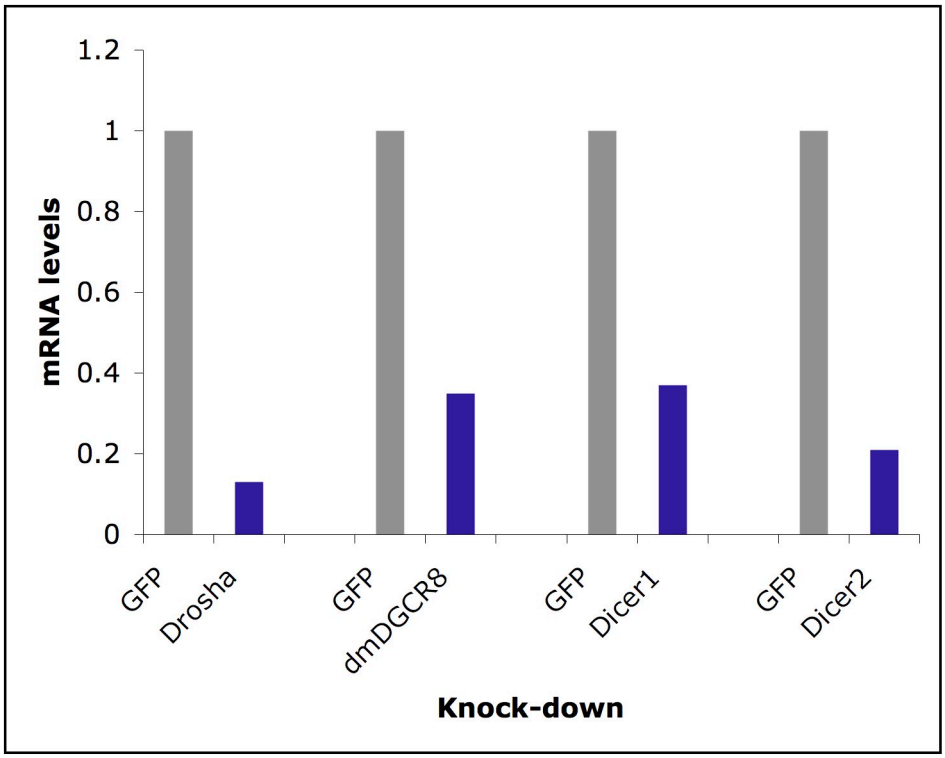


Figure 29. RNAi efficiency in dsRNA treated cells

Relative amount of mRNAs targeted by RNAi were determined by qRT-PCR using cDNA from the cells as template like described before. Efficiency of RNAi knock down in S2 Schneider cells was determined by the targeted mRNA levels in the cells compared to the internal control Histone H1 mRNA. Targeted mRNAs are shown below the bars. The relative amounts of mRNAs are shown after normalized to the amounts with the GFP dsRNA treated cells. Decreased mRNA levels indicate RNAi knock down of the indicated mRNA.

5 days after dsRNA was applied to the culture medium, total RNA was isolated from the cells and mature miR-2a was quantified by northern blotting (Figure 29). Since miR-2a and miR-2b sequences are very closely related to each other it was not possible to differentiate the expression of these miRNAs separately. Therefore the expression of these miRNAs and their precursors will be referred as mir-2 and pre-mir-2, respectively. Depletion of Drosha and dmDGCR8 proteins in S2 Schneider cells resulted in a significant reduction (5-to 10-fold) of mature mir-2 levels as well as the precursor of mir-2 (Figure 29) compared to the GFP dsRNA treated controls. RNAi against Dicer1, which is processes pre-miRNA in the cytoplasm, led to accumulation of pre-mir-2 and a small reduction in mature mir-2. Exportin 5 protein RNAi also showed some accumulation of pre-miRNA (Figure 29).

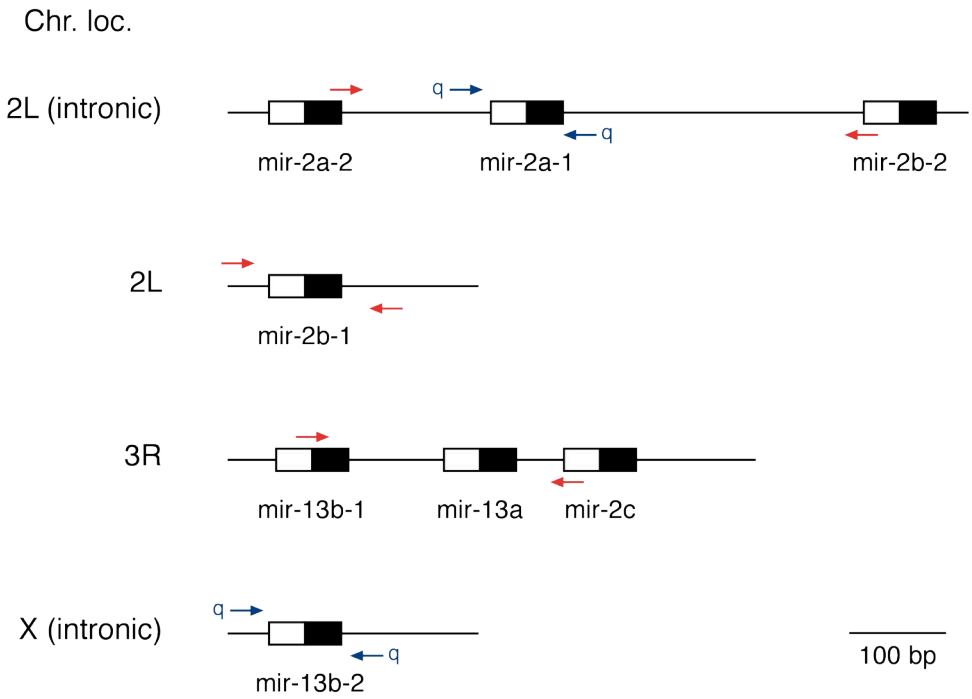


Figure 30. Genomic organization of mir-2 and mir-13 genes

The miRNA precursor structure is indicated as box, and the location of miRNA within the precursor is shown in black; the chromosomal location is indicated to the left. PCR primers for the amplification of primary transcripts are shown as red arrows. PCR primers used in quantitative RT-PCR are shown as blue arrows. The guide on the lower right corner shows the approximate size of a 100 nucleotide DNA fragment.

Dicer2, which is involved in dsRNA induced gene silencing pathway, was used in order to compare the effects of RNAi against different dicer enzymes. RNAi of Dicer2 did not have a significant effect on miRNA maturation. Knock-down of two Argonaute proteins Ago1 and Ago2 also did not change the miRNA and precursor levels significantly compared to control. It was suggested that Ago1 is an essential component of miRNA mediated gene silencing pathway and Ago2 is required for dsRNA induced mRNA cleavage. However, RNAi knock-down of these proteins did not significantly effected the expression of miRNAs in S2 cells suggesting that RNAi was incomplete.

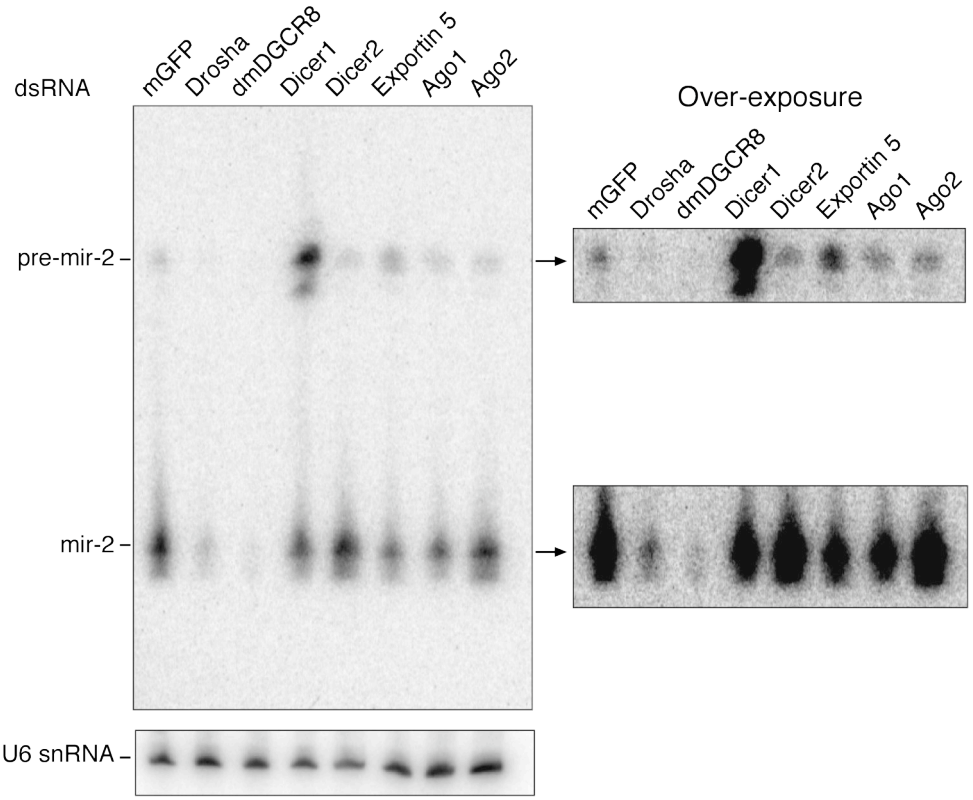


Figure 31. Knock-down of dmDGCR8 and Drosha reduces mature and pre-miRNA accumulation

Total RNA was isolated 5 days after initial dsRNA treatment of D. melanogaster S2 Schneider cells to the indicated target genes. The RNA was separated on 12% denaturing polyacrylamide gels, transferred to Nylon membranes, and probed with 5" radiolabeled mir-2a antisense oligodeoxynucleotide (upper left picture). The membranes were reprobed by radiolabeled antisense U6 snRNA probe (lower left picture) to show equal loading. mir-2a, pre-mir-2 and U6 snRNA bands are indicated on the left. dsRNAs, which were used during knock-down experiment are indicated as the name of the targeted genes on top of the lanes. Right panels show overexposure of the miRNA and pre-miRNA bands.

The effects of RNAi of miRNA processing factors was more obvious when Northern blot data was quantified relative U6 snRNA expression (Figure 30) and the values were normalized to the nonspecific GFP control cells. Drosha and dmDGCR8 knock-down decreased both mature and precursor miRNA levels 5-to 10-fold. Although it did not have a significant effect on the mature miRNA levels, Dicer1 knock-down caused a 6.5 fold increase of the precursor of mir-2. Dicer2 knock-down had no significant effect on mature or precursor miRNA expression.

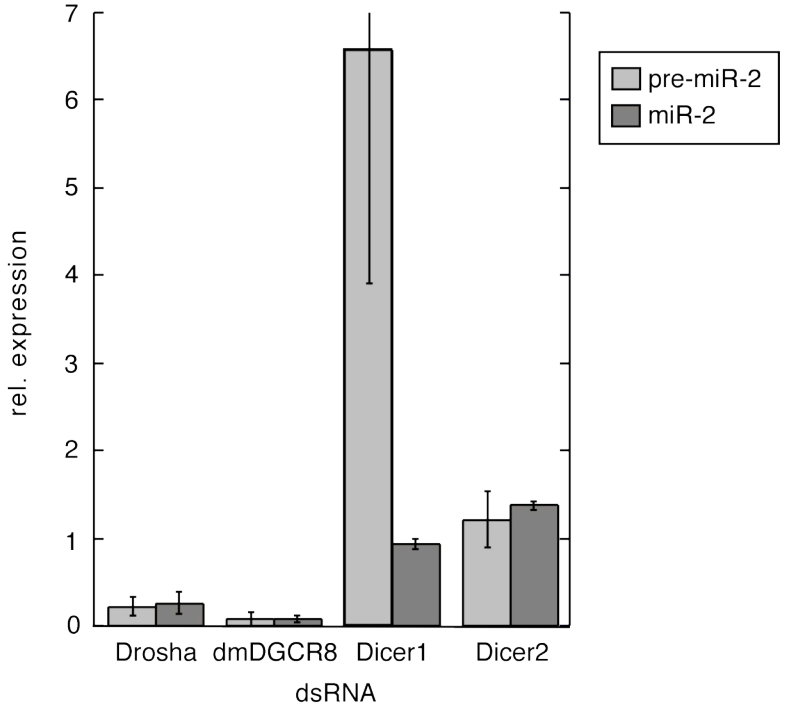


Figure 32. Quantification of mature mir-2a and pre-mir-2 levels in knock-down cells

miRNA and pre-miRNA bands are quantified after correction for background and normalization for loading relative U6 snRNA bands. Relative expression of the miRNAs (light gray) and pre-miRNAs (dark gray) in knock-down cells normalized to nonspecific GFP dsRNA treated control cells are shown on the graph. dsRNAs, which were used during knock-down experiments are indicated as the name of the targeted genes on the bottom. Error bars were obtained from two independent experiments.

In order to determine which *mir-2*/*mir-13* genes are expressed in S2 Schneider cells, cDNA from non-treated cells was prepared and used as template for PCR (Figure 31). Three out of the four genomic loci, 2L intronic, 3R, and X intronic were expressed (Figure 31).

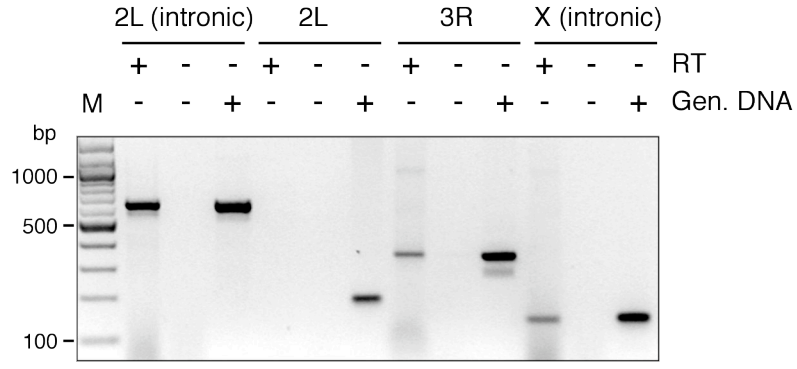


Figure 33. Expression of primary precursor of mir-2/mir-13

cDNA from total RNA of S2 Schneider cells were prepared by RT using random decamer primers followed by PCR-amplification using specific primers for primary precursors (Figure 28). Genomic location for each precursor RNA is indicated on top of the lanes.

For dmDGCR8 and Drosha, the accumulation of RNA transcripts from 2L intronic (pri-mir-2a-1) and X intronic (pri-mir-13b-2) loci was determined by quantifying the relative expression of the transcripts with respect to Histone H1 mRNA (Figure 32). Compared to GFP dsRNA treated cells, knock-down of dmDGCR8 resulted in a 5 and 23-fold accumulation of pri-miRNAs for intronic miR-2a-1 and miR-13b-2 transcripts, respectively (Figure 32).

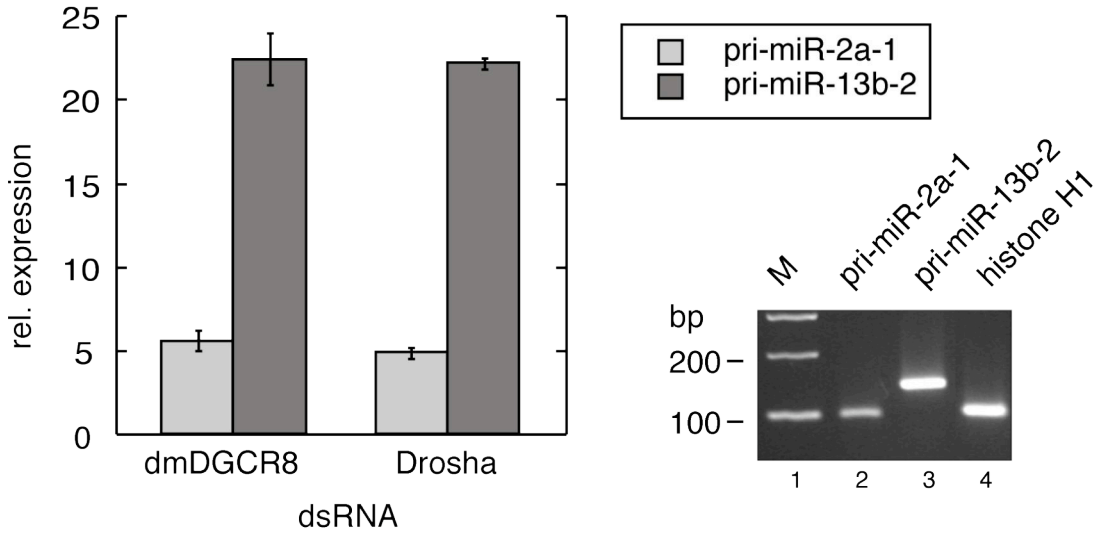


Figure 34. Accumulation of primary precursor of mir-2/mir-13 in knock-down S2 Schneider cells

Relative primary transcript accumulation, as determined by qRT-PCR with primer pairs shown previously. The gene knock-down source of RNAi is indicated on the x-axis. Relative pri-miRNA expression with respect to endogenous Histone H1 mRNA level was normalized to the control GFP-dsRNA treated S2 cells. The error bars are derived from two independent qRT-PCR experiments. Specific amplification of the DNA fragments during qPCR amplifications shown on the left as ethidium bromide stained agarose gel.

In summary, this analysis supports a function of dmDGCR8 in the first step of miRNA maturation process.

5.5 Attempts to genetically knock-out *D. melanogaster Drosha*

In order to facilitate *in situ* hybridization by pri-miRNA accumulation in the *D. melanogaster* embryos, I aimed to delete Drosha gene from the genome by P-element mobilization. The Drosha gene is located on the second chromosome of *D. melanogaster* genome at the 43F2 map location. P-element line with the closest insertion line is $rnh1^{k07624}$. In this line the P-element is closely located (134-nt) to the 5'-end of Drosha gene (Figure 35). P-element is located 95-nt downstream of the transcription start site of Rnh1 gene and causes a lethal insertional mutation possible by disrupting the promoter sequences of the essential Rnh1 gene.

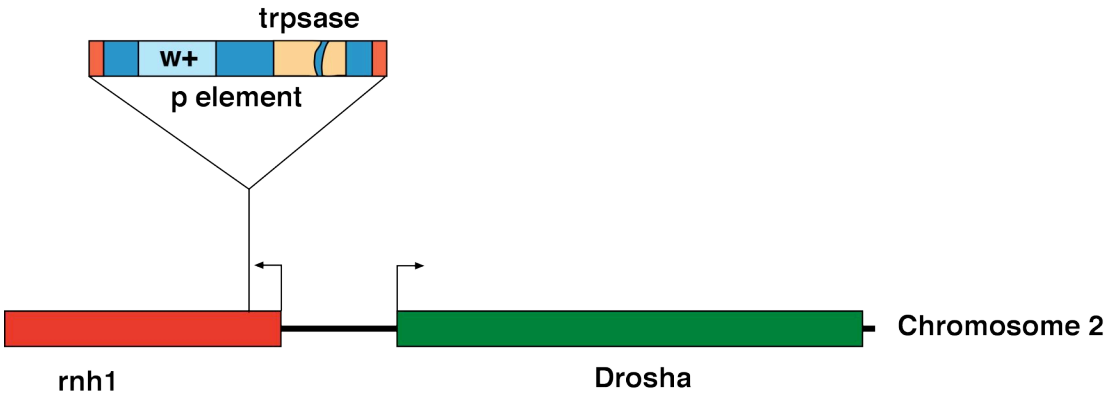
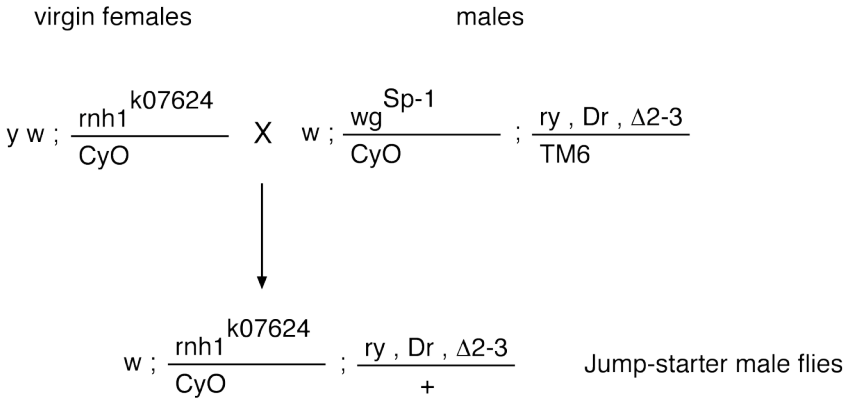


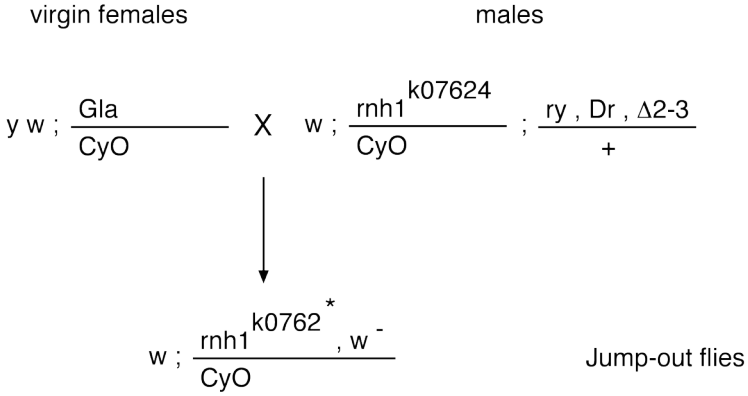
Figure 35. Drosha gene on D. melanogaster genome

Diagram above shows the Drosha gene on the second chromosome of *D. melanogaster*. P-element insertion is located 5'-end of Drosha. P-element disrupts the promoter sequences of the Rnh1 gene resulting with embryonic lethality.

This line was obtained from the stock center and P-element was mobilized by the following genetic cross. Symbols for individual chromosomes are as follows, y w and w are X chromosomes with white eyed mutation, $rnh1^{k07624}$ is P-element insertion line, ry, Dr, Δ2-3 is chromosome with transposase activity, wg^{Sp-1} is chromosome with visible genetic marker wingless, CyO and TM6 are second and third chromosome balancers, respectively. Names of the genetic markers are listed in method section.

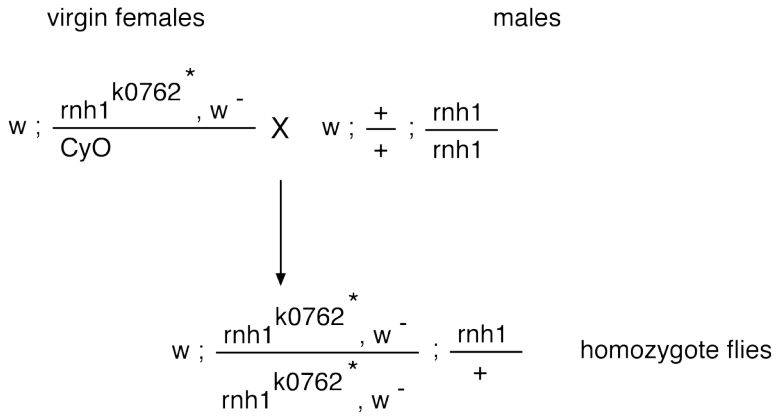


Individual jump starter males (81 males) were then crossed with virgin females with second chromosome balancer and white eye background. White eye progeny (jump-outs) were collected. Gla is chromosome with visible genetic marker wingless.



Jump-out flies (39 lines) were then crossed with flies of opposite sex having second chromosome balancer in order to obtain stable stocks. Most of the stocks were lethal linked to the modified second chromosome. The ones which produced homozygote for these chromosome were eliminated since this ones resulted from perfect mobilization of the P-element hence did not contain any deletion.

Lethal stocks (23 lines) were crossed with flies having an intact Rnh1 gene on their third chromosome. These flies were generated by P-element mediated transformation of the full length Rnh1 gene as described.



All 23 lines were rescued with the full length Rnh1 gene indicating that the original lethality was caused by the mutations in the Rnh1 gene instead of Drosha. Nevertheless, the homozygote flies were screened by PCR for deletions at the second chromosome by primers specific to the outside genomic sequences flanking the P-element at the Drosha locus. All the PCR products amplified were full length (1,312 bp) suggesting that there were no deletion generated in this region (Figure 36).

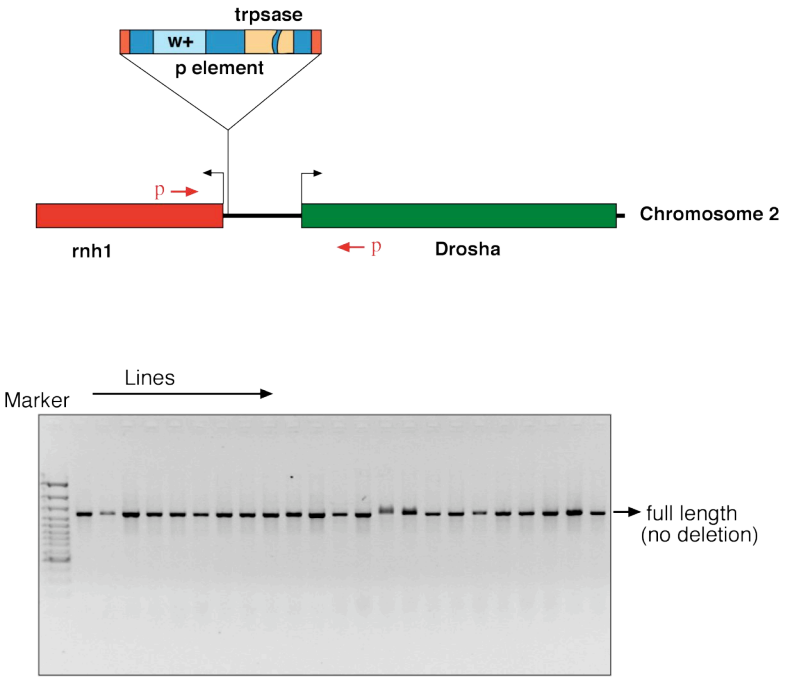


Figure 36. PCR screen for generated deletion on Drosha gene

Diagram above shows the Drosha gene on the second chromosome of *D. melanogaster*. P-element insertion is located 5' end of Drosha. PCR vectors used for screening for deletions that may be generated are indicated by red arrows. Below is the ethidium bromide stained gel picture for the PCR screen. All bands were full length indicating that there was no deletion in any fly line.

Generated deletion could be larger than the are that was covered by PCR. To cover a larger are by PCR screen, primers annealing to further apart were used for another PCR screen. In this screen 5,681 bp region was screened but again no generated deletion could be identified. Because the lethal lines could be rescued by full length Rnh1 gene and no deletion could be detected. All obtained lethal lines had mutations affecting the Rnh1 gene which is closer to the P-element insertion site.

6. Discussion

6.1 Temporal expression pattern of *D. melanogaster* miRNAs

Since the cloning does not yield a complete picture of miRNA expression, I performed Northern blot hybridization assays of developmentally staged RNA preparations. I obtained the temporal expression pattern for most of the cloned miRNAs except for miR-280, mir-282, miR-288, miR-289, miR-303, miR-308, miR-316, miR-318, miR-iab-4-5p, and miR-iab-4-3p, which were cloned from total RNA samples or predicted computationally by conservation in *D. pseudoobscura* genome. Cloning frequencies of these miRNAs were lower compared to the abundant miRNAs (Aravin et al., 2003) suggesting that their expression is too low to be detected by Northern blotting. Our Northern blotting method is sensitive enough to detect 1 fmol of miRNAs. I used 30 μg of total RNA per lane to analyze the miRNA expression. These miRNAs may be even less abundant than 1 fmol in 30 μg of total RNA. these rare miRNAs. The amount of total RNA could be increased to detect the rare miRNAs.

Another possibility is the low G/C content of the miRNA sequences required a lower annealing temperature than 50 ^{o}C for stable miRNA/probe duplexes. The G/C content of these miRNAs ranges from 27% to 50%, which is similar for other miRNAs, which could be detected by Northern blotting. For instance the most abundant miRNA, miR-1 have a G/C content of 36.4%. miR-304 has a significantly lower G/C content (22%) but could still be detected by our method. Although G/C content of the miRNA sequence may have an effect on the hybridization efficiency or stability of the miRNA/probe duplexes during washing steps, it is unlikely that it is the only reason why we could not detect these miRNAs.

Expression of miR-280 and miR-282 are confirmed by Northern blotting by another laboratory (Lai et al., 2003). miR-280 is expressed at very low levels in adult flies. miR-282 expression was detected in larval stages. Although the protocols they used was similar to our Northern blotting procedure their protocols differ from ours in some aspects. For instance they used a higher amount of total RNA sample per lane

(40 μg) then us, which can enable to detect rare miRNAs. Their blotting membrane and probe labeling system also differed from our protocol.

Nearly half of the *D. melanogaster* miRNAs (35 out of 78) are organized as gene clusters on the genomic DNA, suggesting that they are expressed from a common polycystronic transcript. Our Northern blotting confirmed that clustered miRNAs are coexpressed. For instance the expression profiles of miR-125, miR-100, and let-7 were identical. These miRNAs were only detected in pupal and adult stages. Coexpression of miR-125 and let-7 and induction by steroid hormone ecdysone at the onset of metamorphosis has been reported (Bashirullah et al., 2003; Sempere et al., 2003) as well as co-regulated expression of miR-100 (Sempere et al., 2003). miR-125 is the ortholog of *C. elegans* lin-4. In *C. elegans lin-4* and *let-7* genes are on separate chromosomes and are expressed at different developmental transitions (Reinhart et al., 2000). Homolog of *mir-100* was not identified in C. elegans. Clustering of. *mir-100*, *mir-125* and *let-7* in *D. melanogaster* is conserved in *A gambiae*, although the distance between the genes have been increased in mouse and human, the gene cluster underwent duplication and the distance between the genes was increased further.

Clustered miRNAs are usually from the same family of miRNAs with highly conserved seed sequences. Functional relationship of clustered miRNAs was verified by miRNA depletion study reported recently (Leaman et al., 2005). Embryos injected with antisense 2'-O-Methyl oligos targeting miR-310 family of miRNAs (miR-310/miR-311/miR-312/miR-313/miR-92) showed morphogenetic defects in later development. In cuticle preparations, all family members show head-involution defects. The similarity of the phenotypes suggested that miR-310 family miRNAs are functionally related. In addition to head-involution defects, miR-311 and miR-312 show mild dorsal closure defects; and miR-313 occasional germ-band retraction defects; miR-310 and miR-313 also show occasional segmentation defects. Despite the sequence identity at the seed region of these miRNAs, the members of the family show some differences in the depletion phenotypes. It was suggested that different miRNAs in the same family although being functionally related may have slight differences in their target mRNA repertoire and 3'-end of the miRNA contributes to the specificity of the miRNA/mRNA pairing (Leaman et al., 2005).

6.2 Tissue-specific expression of *M. musculus* miRNAs

A significant number of *D. melanogaster* miRNAs are conserved in the human genome and are also frequently found in other mammalian genomes, such as *M. musculus* genome. To gain insights into the distribution and function of miRNAs in mammals, the tissue specific distribution of miRNAs in adult mouse was investigated. 34 novel miRNAs were identified by tissue-specific cloning of approximately 21-nucleotide RNAs from various tissues of adult mouse (Lagos-Quintana et al., 2002). The tissue specific expression of a subset (119 miRNAs) of *M. musculus* miRNA were tested by Northern blotting and 30 miRNAs were identified as tissue or organ-specifically expressed or greatly enriched in a particular organ (brain, lung, liver or skeletal muscle) (Sempere et al., 2004). Today there are 373 registered *M. musculus* miRNA in the miRBase database (Griffiths-Jones, 2004) and among these miRNAs recent studies revealed other tissue-specifically expressed miRNAs (Chen et al., 2006; Kim et al., 2006; Poy et al., 2004; Tuddenham et al., 2006; Weston et al., 2006).

Although the cloning frequencies of the miRNAs can be used to identify the tissue specific regulation of miRNA expression, for less abundant miRNAs identification of tissue-specific expression pattern only from cloning frequencies is difficult. Thus to identify the complete tissue specific expression profiles of identified miRNAs Northern blot assay was performed.

miR-1 was previously shown to by Northern blot analysis to be strongly expressed in human adult heart but not in human brain, liver, kidney, lung, or colon (Lee and Ambros, 2001). There are two copies of *mir-1* in *M. musculus,* which are coexpressed with closely located *mir-133* genes. *mir-206* is a *mir-1* related gene and is coexpressed with *mir-133b* from a third locus on the genome. Like its human counterpart mouse mir-1 is also expressed explicitly in adult heart. Although it was possible to clone miR-1 from liver and midbrain (Lagos-Quintana et al., 2002) it remained undetectable by Northern blot analysis in these tissues indicating that experimental expression data is the most reliable information for relative abundance comparisons of miRNAs. In mouse liver, miR-122 account for 72% of all cloned miRNAs (Lagos-Quintana et al., 2002), and miR-122 was undetected in all other tissues of mouse in Northern blot assay. miR-1 and miR-122 are two striking examples of miRNAs, which are highly tissue specifically expressed.

Tissue specific expression of miR-1 in skeletal muscle and heart tissues suggests that it is involved in the development of these tissues. Genetic knock-out of mir-1 in *D. melanogaster* (Sokol and Ambros, 2005) and over expression studies in *X. leavis* (Chen et al., 2006) verified that miR-1 has an important function in establishing and maintenance of the muscle cell identity, which is conserved among species. Antisense mediated depletion of *D. melanogaster* miR-1 resulted in a severe phenotype, reminiscent of muscle dysfunction, also supporting the muscle specific function of miR-1 (Leaman et al., 2005). Likewise, the localization of miR-122 expression in the liver tissue indicated that miR-122 may have role in liver metabolism. Supporting this suggestion, liver specific metabolic functions of miR-122 were identified by two different groups by targeting and inactivating the miR-122 with modified antisense oligonucleotides (Esau et al., 2006; Krutzfeldt et al., 2005).

Identification of tissue specific expression of miRNAs is very important for functional identification of miRNAs. There are many examples where tissue specific expression pattern led to discovery of miRNA functions. For instance it was discovered that miR-375, which is cloned and specifically expressed in pancreatic islet cells is involved in insulin secretion from pancreatic β cells (Poy et al., 2004). Embryonic stem cells specific miRNA cluster (*mir-290*/*mir-295*) is suggested to have a role in the maintenance of the pluripotency of the embryonic stem cells (Houbaviy et al., 2003). It remains to be seen if this prediction is true or not.

Tissue-specific cloning led to discovery of several miRNAs, which are expressed explicitly in brain tissues, suggesting that they are involved in brain morphogenesis and function. Further analysis of these miRNAs by Northern blot confirmed that they were specifically expressed in brain tissues. Overall, miR-124, miR-127, miR-128, miR-131, and miR-132 were shown to be predominantly brain specific miRNAs. miR-125a and miR-125b were cloned from brain tissue, but expression was also detected by Northern blot analysis in other tissues. lin-4 the ortholog of miR-125 in C. elegans is involved with regulating neuronal remodeling by controlling lin-14 expression in *C. elegans* (Hallam and Jin, 1998). lin-14 do not have any homolog in mouse, still it is interesting that developmental functions of lin-4 and miR-125 are somehow connected.

In summary novel miRNAs, which were cloned from different tissues of adult mouse, were investigated by Northern blot assays to obtain relative tissue-specific expression profiles. Some of these miRNAs appeared to be highly tissue-specific. In

addition to miR-1 and miR-122, which were heart and liver specific respectively several other miRNAs appeared to be expressed exclusively or at least predominantly in brain. Several other miRNAs were detected in a variety of tissues but their expression seemed to be regulated dependent on the type of the tissue they are expressed. Tissue specific regulation of miRNA expression suggests that miRNAs may have critical roles in tissue specification and cell lineage decisions.

6.3 Spatial expression pattern of *D. melanogaster* miRNAs

In order to identify the spatial expression pattern of *D. melanogaster* miRNAs during development whole mount *in situ* hybridization technique was tested. Antisense oligonucleotide probes labeled by digoxigenin were applied but no specific expression pattern could be obtained.

I*n situ* hybridization is usually used to detect long cellular RNAs (e.g. mRNAs) by long antisense probes. Detection of miRNAs requires specific optimizations. Due to their short sizes and lower melting temperatures of the target/probe duplexes hybridization conditions for short antisense probes are different than the long probes. Besides short antisense oligonucleotide probes are labeled with fewer digoxigenin molecules hence have less specific activity, making the detection more difficult. Short RNAs can also be lost during the incubations and washing steps by diffusion and optimizations for the fixation conditions are required.

In order to optimize the *in situ* hybridization protocol we first tried different kind of molecules (DNA, RNA, 2'-O-Me RNA, LNA) as antisense oligonucleotide probes. I have also tried different probe labeling methods to increase the specific activity of the antisense oligonucleotide probes. However, in our experiments the property of the probe did not affect the outcome of the *in situ* hybridization.

It was reported recently that the expression pattern of vertebrate miRNAs in the developing zebrafish embryo can be determined using LNA (locked nucleic acid) modified DNA oligonucleotide probes (Wienholds et al., 2005). LNA is a new class of bicyclic high-affinity RNA analogs in which the furanose ring of the ribose sugar is chemically locked in an RNA-mimicking conformation, resulting in high hybridization affinity toward complementary DNA and RNA molecules (Vester and Wengel, 2004).

I tested LNA probes to detect miRNAs and compared the results of in situ hybridization using *D. melanogaster* and zebrafish embryos. miR-124 expression was identified in zebrafish embryos before (Wienholds et al., 2005). As reported before, miR-124 was detected in central nervous system of developing zebrafish embryos starting from very early stages of development. miR-124 could be detected as early as 14 somite stage of zebrafish embryos and the expression continued after the embryos were 5 days old. The expression was localized to central nervous system, brain and around the eyes of the embryo suggesting that miR-124 may have a function in development of neural tissues.

Although this kind of probes can successfully be used in zebrafish *in situ* hybridizations their usage in *D. melanogaster* embryos are inefficient. Only a few miRNAs could be detected by LNA probes. miR-1, which is the most abundant miRNA in D. melanogaster embryos, could be detected using LNA probes but it was reported that long probes yielded stronger signal compared to LNA probes (Sokol and Ambros, 2005). The differences in the tissue characteristics of the zebrafish and *D. melanogaster* embryos can account for such difference. It seems that in situ hybridization protocol should be optimized for *D. melanogaster* embryos.

In order to identify the reason why the short oligonucleotide probes are not suitable for whole mount *in situ* hybridization, I performed several control experiments. As a summary it was observed that short probes are very weak compared to the long probes, hence the specific signal obtained by short probes are difficult to detect. Although it was possible to detect control RNA target (fushi tarazu) with *in vitro* transcribed oligonucleotide probes, in this case the signal was weak and the background noise was very high. It seems that 50 nucleotide is the shortest length for *in vitro* transcribed probe for satisfactory results.

One of the major concerns when using short oligonucleotide probes for detecting miRNAs is the melting temperature (Tm) of the probe/miRNA hybrid duplexes. Tm of the duplexes are affected significantly by the salt and formamide concentration of the hybridization medium. Usually hybridizations were carried out in 5X SSC and 50% formamide concentrations. Under these conditions Tm of the RNA probe/miRNA duplexes are 10-to 15 ^{o}C lower than the Tm in the solutions without formamide. This makes the high hybridization temperatures (55-60 ^{o}C) unfeasible for the DNA and RNA oligonucleotide probes. In order to optimize the condition for short probes hybridizations were tested in a wide range of temperatures (37-65 ^{o}C).

Hybridization mixtures with different salt and formamide concentrations were also tested but an optimal hybridization condition could not be obtained for DNA or RNA oligonucleotide probes.

LNA probe/miRNA hybrid duplex have higher Tm, which enable the use of higher annealing temperatures for *in situ* hybridization. Typically LNA probes increased the melting temperature of the resulting probe/miRNA duplex by as much as 15 ^{o}C. This property can make LNA probes feasible for detecting miRNAs in zebrafish embryos.

The presence of mature miRNAs during *in situ* hybridization assay was determined. miRNAs diffuse from the embryos during long incubation periods even if they have been fixed. Stronger fixation methods like longer treatments with fixative are required to preserve miRNAs in their locations.

To address the specific activity issue with the short probes, oligonucleotide probes were compared with the long antisense probes targeting primary transcript of miRNAs. Long probes yield stronger signal because of the higher number of digoxigenin per probe molecule. I tried targeting miR-1 and miR-6 cluster miRNAs with long antisense probes. Only miR-1 probe yielded a specific and detectable signal. By using this method, spatial expression pattern of miR-1 was determined. It was shown that miR-1 is expressed in mesodermal tissues of embryos as reported before (Sokol and Ambros, 2005).

Long antisense RNA probes have been used to identify the spatial expression pattern of *D. melanogaster* miRNAs (Aboobaker et al., 2005; Stark et al., 2005). miR-1 is expressed in presumptive mesoderm at blastoderm stage, in visceral and somatic mesoderm at somatic mesoderm stage, in somatic and pharyngeal muscles at stage 17. miR-124 is expressed only in brain and central nerve cord. miR-9a is expressed in ectoderm but not presumptive mesoderm at blastoderm stage, in ectodermal cells at segment edges and in stomodeum but not in CNS at stage 12. miR-279 is expressed in head epidermis and in segmentally repeated groups of cells in the trunk at stage 11, in anterior spiracles gonads and in head segments at stage 17 (Stark et al., 2005). The expression pattern of 25 other miRNAs were also identified targeting pri-miRNAs (Aboobaker et al., 2005). However, not all miRNA genes could be visualized that were detectable by Northern blotting. Abundance of the primary transcript and its rate of processing can be responsible for the difficulties. RT-PCR amplification is usually required to detect very rare primary transcripts. It is also likely that the intron/exon

structure of miRNA primary transcripts, as it can be inferred by analysis of ESTs, has been ignored for probe design, and that the spliced and not further processed segments turn over faster than the miRNA containing sequence segments. One of the problems of detecting miRNA expression by long RNA probes is that it is not clear if the expression pattern identified by targeting pri-miRNAs is identical with the mature miRNA expression.

Primary miRNA transcripts are processed to pre-miRNAs and mature miRNAs in a sequential processing pathway. In order to make the detection of pri-miRNAs easier, miRNA processing pathway can be blocked at the first step, which would enable the accumulation of the pri-miRNA transcripts in the nucleus.

6.4 RNAi knock-down of *D. melanogaster* miRNA processing factors

Blocking pri-miRNA processing was tested by targeting miRNA processing factors in cultured *D. melanogaster* cells by RNAi. Drosha, nuclear RNase III that mediates the first processing step as well as Dicer enzymes and Argonaute proteins were depleted by dsRNA administration. Pre-miRNA nuclear export factor, Exportin 5 was also depleted by RNAi. Additionally dmDGCR8 was tested as a novel miRNA processing factor. dmDGCR8 was identified as a Drosha interacting protein in genome-wide yeast two hybrid screens (Giot et al., 2003).

Monitoring miRNA maturation pathway at various steps revealed that Drosha and dmDGCR8 are required for the nuclear processing of the pri-miRNAs. Knock-down of these proteins led to accumulation of the primary miRNA transcripts and loss of pre-miRNA and mature miRNAs. Exportin 5 knock-down did not have any significant effect on the accumulation of mature miRNA, although pre-miRNA levels were slightly increased. Dicer1 knock-down led to a significant accumulation of the pre-miRNAs, whereas RNAi of Argonaute proteins did not have significant effect on miRNA processing and maturation. It was anticipated that RNAi targeting Argonaute proteins would result with a decrease in the mature miRNA levels. Mature miRNA stability should significantly decrease without the interaction with Argonaute proteins. Inefficient knock-down of the Argonaute proteins or the semi-redundant

nature of the Ago1 and Ago2 proteins may be responsible for the absence of the phenotype.

Our RNAi experiments helped us to identify a novel dsRBD protein, dmDGCR8 (Pasha), which is involved in nuclear pri-miRNA processing through interacting with RNase III Drosha. RNase III class of proteins are commonly associated with a dsRBD protein that can modulate their function. For instance R2D2 protein is associated with Dicer2 protein, which mediates double stranded RNA mediated silencing in D. melanogaster. Although it interacts with Dicer2, R2D2 is not required for Dicer2 mediated dsRNA processing. Instead, R2D2 is involved in the orientation of the RISC complex on the siRNA duplex, assuring that the RNA strand whose 5'-end is at the less stable part of the duplex, is incorporated at the effector complex (Tomari et al., 2004). *D. melanogaster* Loquacious (Loqs) protein on the other hand is required for Dicer1 activity. Knock-down of Loqs had similar effect of Dicer1 knock-down and resulted with the accumulation of unprocessed pre-miRNAs in S2 Schneider cells (Saito et al., 2005). It remains to be seen if Loqs is also involved in the orientation of the miRNP complexes and the discrimination of different arms of the pre-miRNA hairpin. We have documented that Drosha/Pasha interaction is necessary for the action of the RNase III enzyme reminiscent of the Loqs/Dicer1 interaction. *D. melanogaster* dsRBD proteins and their proposed functions are listed in table below.

Table 15. *D. melanogaster* dsRBD proteins

dsRBD protein	Partner RNase III	Function
Pasha	Drosha	pri-miRNA processing
Loqs	Dicer1	pre-miRNA processing/miRNP formation
R2D2	Dicer2	Orientation of RISC on the siRNAs

6.5 Attempts to genetically knock-out *D. melanogaster Drosha*

Our data suggested that even partial depletion of Drosha and dmDGCR8 proteins led to significant accumulation of pri-miRNA transcripts in the *D. melanogaster* cells. Thus, genetic knock out of these proteins can enable to stable accumulations of pri-

miRNAs in the developing embryos. Increased amounts of pri-miRNAs can be detected easily by using long antisense RNA probes. Spatial expression pattern of miRNAs, whose pri-miRNA expression is not detectable by *in situ* hybridization can be identified.

miRNAs are essential for proper development in *D. melanogaster*. *mir-1* knock-out flies *mir-1* deficiency results in loss of larval growth and termination of the proper larval development. Mutants eventually die at larval transition stages. Thus miR-1 expression is required for *D. melanogaster* survival (Sokol and Ambros, 2005). Depletion of miRNA processing globally must therefore be lethal to Drosophila. Dicer1 and Dicer2 mutations have different phenotypes in *D. melanogaster*. Dicer2, which mediates RNAi is not essential for development, whereas Dicer1 knock-out flies exhibit severe developmental defects (Lee et al., 2004b).

In order to deplete pri-miRNA processing completely in the developing embryos, I attempted to genetically knock-out Drosha by P-element mobilization. P-elements located close Drosha and dmDGCR8 genes are mobilized by introducing transposase activity to the flies. Fly lines with mobilized P-elements were collected and screened for deletion in Drosha. No deletion have been found in Drosha lines. Homozygote lethal flies were investigated for a possible Drosha mutation. P-element mobilization in this location affected a nearby gene, Rnh1 causing a lethality in the homozygote flies. All of the mutant lines cold be rescued by full length Rnh1 gene. Since Drosha mutation is expected to be lethal it was concluded that Drosha gene was not affected in these stocks.

6.6 Outlook

Like Drosha knock-out, dmDGCR8 knock-out can be generated by P-element mobilization based gene deletion. dmDGCR8 gene is located on the third chromosome of *D. melanogaster* genome. Unlike Drosha, there is no genes closely located with the dmDGCR8. P-element line, with insertion site close to the dmDGCR8 gene is pasha^{EY01325} (Figure 37). Since there is no closely located gene nearby this knock-out is relatively less complicated than the Drosha knock-out. P-element line was obtained from Bloomington public stock center.

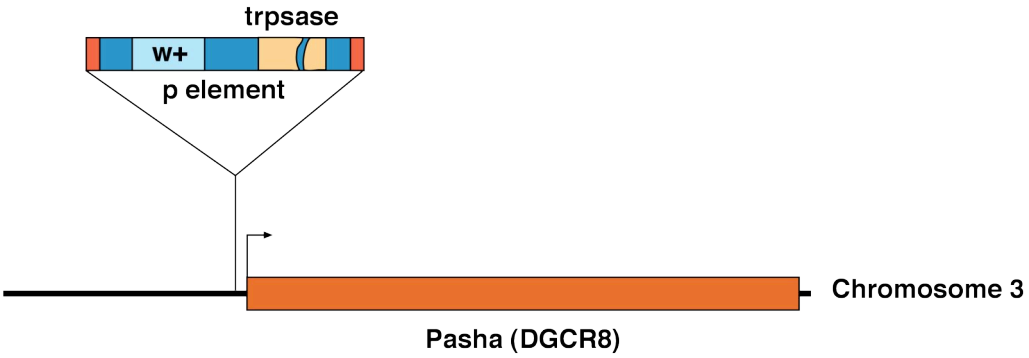


Figure 37. D. melanogaster Pasha gene

Diagram above shows the dmDGCR8 (Pasha) gene on the third chromosome of D. melanogaster. P-element line contains a very closely located P-element insertion near 5'-end of the Pasha.

A P-element present at the 5'-end of the dmDGCR8 gene was mobilized. Stocks with modified chromosomes were investigated for a lethal mutation. 292 jump-out lines could be obtained from the cross. 38 lines have been identified with mutations that cause lethality in homozygote flies. These lines will further be investigated by introduction of full length dmDGCR8 gene to determine a rescue of lethality. A rescue construct with full length dmDGCR8 gene was cloned in pP{CaSpeR-4} plasmid. The plasmid was prepared for embryo injections for P-element based transformation of the dmDGCR8 gene. Flies with full length dmDGCR8 gene introduced via P-element insertions will be obtained and the following cross will be done to identify lethal lines which will complement with the dmDGCR8 rescue.

6.7 Perspective

Expression profiling of the identified miRNAs is important for verifications of the predicted targets and correlations with the biological functions of miRNAs. Temporal expression pattern of all D. melanogaster miRNAs have been determined by Northern blot assay. Currently temporal expression pattern of only 7 out of 78 miRNAs were not determined (Table 16). Spatial expression pattern for some of the miRNAs were also identified (Aboobaker et al., 2005) by *in situ* hybridization targeting pri-miRNA. Among the 53 miRNA gene loci 24 were successfully targeted by long antisense RNA probes. The remaining 28 miRNA loci remains to be identified (Table 16). Rapid processing of the pri-miRNAs and insufficient expression most probably

account for the inability to detect the rest of the pri-miRNAs. Modifications for the probe design, such as using EST sequences as the antisense probes may increase the success rate further. Alternative approach is to enable the stable accumulation of the pri-miRNAs in embryos by genetic studies. Genetic knock out of dmDGCR8 in the embryos can enable us to detect pri-miRNAs during embryonic stages by whole mount *in situ* hybridization.

So far, among the 78 *D. melanogaster* miRNAs only 12 are annotated by a function or target mRNA (Table 16). Some of these functions were identified for homologous miRNAs in different species. The major concern of the miRNA studies is identification of the functions of the rest of the miRNAs.

Table 16. *D. melanogaster* miRNA expression patterns and functions

miRNA cluster/gene	**Temporal Expression**	**Spatial Expression**	**Function in**
mir-1	Northern	*in situ* (miRNA and pri-miRNA)	Muscle/Heart (1)
mir-2a-1/mir2a-2/mir-2b-2	Northern	*in situ* (pri-miRNA)	
mir-2b-1	Northern		
mir-2c/mir-13a/mir-13b-1	Northern	*in situ* (pri-miRNA)	
mir-3/mir-4/mir-5/mir-6-1/mir-6-2/mir-6-3/mir-286/mir-309	Northern	*in situ* (pri-miRNA)	
mir-7	Northern	*in situ* (pri-miRNA)	Mouse eye (2)
mir-8	Northern	*in situ* (pri-miRNA)	
mir-9a	Northern	*in situ* (pri-miRNA)	Mouse pancreas (3)
mir-9b/mir-9c/mir-79/mir-306	Northern		

mir-10	Northern	*in situ* (pri-miRNA)	
mir-11	Northern	*in situ* (pri-miRNA)	
mir-12/mir-283/mir-304	Northern	*in situ* (pri-miRNA)	
mir-13b-2	Northern	*in situ* (pri-miRNA)	
mir-14	Northern	*in situ* (pri-miRNA)	Fat metabolism (4)
mir-31a	Northern	*in situ* (pri-miRNA)	Tumorigenesis in human (5)
mir-31b	Northern		Tumorigenesis in human (5)
mir-33	Northern		
mir-34/mir-277/mir-317	Northern		
mir-87	Northern		
mir-92a	Northern	*in situ* (pri-miRNA)	Tumorigenesis in human (6)
mir-92b	Northern		Tumorigenesis in human (6)
mir-100/let-7/mir-125	Northern		
mir-124	Northern	*in situ* (pri-miRNA)	Mouse brain (7)
mir-133	Northern		Muscle differentiation in mouse (8)
mir-184	Northern	*in situ* (pri-miRNA)	Mouse eye (9)
mir-210	Northern		Oogenesis (10)
mir-219	Northern		
mir-263a	Northern		
mir-263b	Northern	*in situ* (pri-miRNA)	
mir-274	Northern	*in situ* (pri-miRNA)	
mir-275/mir-305	Northern		
mir-276a	Northern		

mir-276b	Northern		
mir-278	Northern		Energy homeostasis (11)
mir-279	Northern	*in situ* (pri-miRNA)	
mir-280	Northern		
mir-281-1/mir-281-2	Northern	*in situ* (pri-miRNA)	
mir-282	Northern		
mir-284	Northern		
mir-285	Northern		
mir-287	Northern		
mir-288			
mir-289			
mir-303			
mir-307	Northern	*in situ* (pri-miRNA)	
mir-308			
mir-310/mir-311/mir-312/mir-313	Northern		
mir-314	Northern		
mir-315	Northern	*in situ* (pri-miRNA)	
mir-316		*in situ* (pri-miRNA)	
mir-318			
mir-iab-4		*in situ* (pri-miRNA)	
bantam	Northern	sensor construct reporter	Apoptosis (12)
References: 1, (Sokol and Ambros, 2005); 2, (Li and Carthew, 2005); 3, (Plaisance et al., 2006); 4, (Xu et al., 2003); 5, (Bandres et al., 2006); 6, (Volinia et al., 2006); 7, (Smirnova et al., 2005); 8, (Chen et al., 2006); 9, (Ryan et al., 2006); 10, (Grun et al., 2005); 11, (Teleman et al., 2006); 12, (Brennecke et al., 2003)			

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8. Summary

miRNAs are small (20-to 25-nt) single stranded regulatory RNAs that regulate gene expression. Hundreds of miRNAs have been identified in plants and animals; and the number of the identified miRNA genes is still increasing. At the time of writing miRNA registry database reported, 131 miRNAs in *A. thaliana*, 78 in *D. melanogaster*, 114 in *C. elegans*, 337 in *D. rerio*, 373 in mouse, and 474 in human. miRNA genes are transcribed by RNA Pol II like protein coding genes. The initial transcript (pri-miRNA) is processed in the nucleus by Drosha to form 70-to 80-nt precursor hairpin RNAs (pre-miRNA). Mature miRNAs are excised from pre-miRNAs in the cytoplasm by Dicer. miRNAs regulate gene expression by base pairing interactions with cellular mRNAs and repressing the translation or mediating mRNA degradation. They regulate important cellular and developmental functions in animals and plants such as cell death, proliferation, differentiation, signal transduction, and metabolism.

My aim was to identify the expression patterns of miRNAs. The thesis can be divided into three sections. In the first part temporal and tissue-specific expression patterns of miRNAs are summarized. *D. melanogaster* was used as an experimental system to investigate the developmentally-regulated miRNAs. *M. musculus* miRNAs which are tissue or organ-specifically expressed were investigated. In the second chapter my attempts to develop an experimental system to examine the spatial regulation of miRNA expression during *D. melanogaster* development, are summarized. In the last section I summarized the experiments I have done to investigate the miRNA processing and maturation factors and my attempts to create a genetic knock-out of *D. melanogaster Drosha* gene.

There are 78 different miRNA genes identified in *D. melanogaster* genome. Northern blot hybridization assays were carried out using total RNA samples from different developmental stages of *D. melanogaster*. The expressions of most of the miRNAs were developmentally regulated and clustered miRNAs were coexpressed from a common long pri-miRNA transcript. The temporal expression pattern will be useful to interpret predicted mRNA targets for *D. melanogaster* miRNAs.

Some miRNAs are tissue-specifically expressed in mouse. Some of these

miRNAs were identified using total RNAs from different tissues of adult mouse. In order to examine tissue-specific expression of *M. musculus* miRNAs that were cloned from a few tissue samples in apparent tissue-specific manner, Northern blot hybridization assays were carried out using total RNA from various tissues of adult mouse. It was observed that some of the identified miRNAs were highly tissue-specifically expressed. A subset of these miRNAs seemed to be exclusively expressed in brain. Others were expressed in multiple tissues but their expression was highly regulated in a tissue-specific manner. These tissue-specific miRNAs may have critical roles in tissue specification.

It was shown that long single stranded RNA probes targeting primary transcripts of miRNAs, can be used to detect the expression of some abundant miRNAs. In order to identify the spatial expression pattern of *D. melanogaster* miRNAs during development whole-mount *in situ* hybridization techniques were evaluated for small probes cognate to miRNAs. Detecting miRNAs by this method is more challenging because of the small size of the mature miRNAs and insufficient quantities of the pre-miRNAs. Different types of oligonucleotide probes (DNA, RNA, 2'-O-Me RNA, LNA), which were digoxigenin labeled by various techniques, were tested for *in situ* hybridization under various conditions.

It was reported that locked nucleic acid probes (LNA) can be used to detect miRNA expression in vertebrate embryos. Although it was well documented that zebrafish embryos can easily and efficiently be detected by LNA probes labeled with digoxigenin, only very few *D. melanogaster* miRNAs could be detected by LNA probes so far. In line with this observation we have successfully detected miR-124 expression in zebrafish embryos. However we could not detect *D. melanogaster* miR-124 or miR-1 using LNA probes.

There were two problems for using short probes to detect miRNAs. The first one is the lower labeling efficiency due to fewer DIG labels per probe molecule. The second difficulty is the diffusion of short RNAs from the embryos because of the reversal of the cross-links during incubations at high temperatures. Probes shorter than 50 nucleotides in length were inefficient for detecting mRNAs by *in situ* hybridization. Other fixation and hybridization strategies specific to *D. melanogaster* embryos have to be developed.

In order to study the biochemical processing of miRNAs, I performed RNAi experiments targeting candidate miRNA processing proteins. I characterized a novel

protein, dmDGCR8, which interacts with Drosha RNase III, and showed that it was required for pri-miRNA processing to release pre-miRNA. When Drosha or dmDGCR8 protein was targeted by RNAi, mature miRNAs and their precursors disappeared. Dicer1 but not Dicer2 knock-down resulted in the accumulation of the miRNA precursors indicating it is involved in pre-miRNA miRNA processing. Exportin 5 knock-down had effects similar to Dicer1 knock-down but the accumulation of precursor of miRNAs was less severe.

Because of the difficulty of mature miRNA detection we thought of generating mutants that stably accumulate pri-miRNAs in *D. melanogaster* embryo. P-element mediated gene deletion was performed to obtain flies with Drosha deficiency. Because Drosha gene was closely located to another essential gene, deleting Drosha by random P-element mobilizations without affecting this essential gene is difficult. all generated deletions affected the unrelated essential gene without affecting Drosha.

dmDGCR8 gene, which is also responsible for miRNA maturation, was targeted next by the same strategy. We have successfully obtained 39 mutant lines with lethal mutation on the targeted chromosome. In order to verify that the lethality of the mutant chromosomes are due to dmDGCR8 deficiency a rescue construct with full length dmDGCR8 gene was prepared.

In summary, this study systematically examined the developmental *D. melanogaster* miRNA expression and characterized the maturation process of miRNAs in the nucleus. I systematically assessed the optimization of *in situ* hybridization in *D. melanogaster* and developed strategies for pri-miRNA accumulation in the embryo to facilitate embryonic whole-mount *in situ* hybridization analysis in the future.

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Appendix

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Statement of Originality

Erklärung

Ich versichere, daß ich die vorliegende Arbeit selbständig verfaßt und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe.
Die Arbeit hat in gleicher oder ähnlicher Form noch keiner Prüfungsbehörde vorgelegen.

Declaration

I herewith declare that I prepared the PhD thesis "Expression analysis of *Drosophila melanogaster* miRNAs" on my own and with no other sources and aids than those quoted.

Göttingen, den

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Publications

PAPERS

Leaman D, Chen PY, Fak J, **Yalcin A**, Pearce M, Unnerstall U, Marks DS, Sander C, Tuschl T, Gaul U. Antisense-mediated depletion reveals essential and specific functions of MicroRNAs in Drosophila development. *Cell.* 2005 Jul 1;121(7):1097-108

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Yalcin A, Aravin A, Tuschl T. Temporal and spatial regulation of *D. melanogaster* MicroRNAs during development. Poster presentation at 28th international FEBS (Federation of European Biochemical Societies) meeting, Istanbul Turkey. October 20-25, 2002

Yalcin A, Tuschl T. Regulation of *D. melanogaster* miRNAs in developing embryos. Poster presentation at German-Israeli Minerva School “Cytoplasmic fate of RNA” meeting, Berlin, Germany. April 19-21, 2002

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