Viability, Longevity, and Egg Production of *Drosophila melanogaster* Are Regulated by the miR-282 microRNA

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ABSTRACT The first microRNAs were discovered some 20 years ago, but only a small fraction of the microRNA-encoding genes have been described in detail yet. Here we report the molecular analysis of a computationally predicted *Drosophila melanogaster* microRNA gene, *mir-282*. We show that the *mir-282* gene is the source of a 4.9-kb-long primary transcript with a 5′ cap and a 3′-poly(A) sequence and a mature microRNA of ~25 bp. Our data strongly suggest the existence of an independent *mir-282* gene conserved in holometabolic insects. We give evidence that the *mir-282* locus encodes a functional transcript that influences viability, longevity, and egg production in *Drosophila*. We identify the nervous system-specific adenylate cyclase (*rutabaga*) as a target of miR-282 and assume that one of the main functions of *mir-282* is the regulation of adenylate cyclase activity in the nervous system during metamorphosis.

MICRORNAS (miRNAs) are 18- to 25-nt-long, single-stranded regulatory RNAs that bind to complementary sequences in the 3′-untranslated regions of target mRNAs, resulting in the negative regulation (transcript degradation and sequestering, translational suppression) and possibly the positive regulation (transcriptional and translational activation) of the target genes (Kim 2005). Although a significant group of the miRNA genes is found in introns (hence the mirtron denomination) or in exons of protein- and non-protein-coding genes, most microRNA genes lie in intergenic regions and contain their own promoters and regulatory components (Bartel 2004). Except for the mammalian miRNAs that are located among Alu repeats (Borchert *et al.* 2006), miRNA primary transcripts (pri-miRNAs) are synthesized by RNA polymerase II. Pri-miRNAs, which range from hundreds to a couple of thousands of nucleotides in length, are largely unstructured precursors, which have a 5′-m7G cap structure and are usually subjected to polyadenylation at their 3′ ends (Cai *et al.* 2004; Lee *et al.* 2004).

While hundreds of miRNAs have been predicted in animals, plants, and even viruses in the past decade (Griffiths-Jones *et al.* 2008), regulated target genes and biological functions have been assigned to only a few dozen of them. The functional analyses are hampered primarily by redundancy [different miRNAs share the same 5′-seed sequence or target(s)] and by coexpression (Abbott *et al.* 2005; Sokol 2008). In a genome-wide analysis, Miska and his colleagues found that the majority of miRNA genes are not essential for the viability or development of *Caenorhabditis elegans* and mutations in most miRNA genes do not result in grossly abnormal phenotypes (Miska *et al.* 2007). Despite these findings, it is clear now that miRNAs are required for the fine-tuning of the regulation of very complex mechanisms, and their activity covers almost all biological processes.

In *Drosophila*, 238 miRNAs have been predicted to date (miRBase release 19, in April 2013). It has been demonstrated that blocking different components of the miRNA pathway in the fly causes female sterility, abnormal cell

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division, segmentation defects, memory problems, and embryonic lethality (Bushati and Cohen 2007). Despite the wide range of phenotypic effects and the relatively large number of putative miRNAs in *Drosophila*, only a few (10) microRNAs have been analyzed in detail yet (Smibert and Lai 2008). Moreover, only 2 of them, the cell-death regulator *bantam* and the *mir-14* gene, have been identified by a forward genetic approach (Brennecke *et al.* 2003; Xu *et al.* 2003).

In this work, we present the molecular characterization of the genomic region encoding a computationally predicted *Drosophila melanogaster* microRNA, *mir-282*, and present evidence that argues for the presence of an independent *mir-282* gene. We demonstrate that the putative *mir-282* locus encodes a transcript that influences viability in *Drosophila* most likely through the regulation of the cAMP level and egg production.

Materials and Methods

Drosophila strains

The w^{1118}, e^s, *twist*>Gal4, *tub*>Gal4; *da*>Gal4; *elav*>Gal4; *sgs3*>Gal4; *act5C*>Gal4; *act5C*>Gal4/CyO-GFP; CyO-PDelta2-3 lines were obtained from the Bloomington Drosophila Stock Center. Stocks P(EP)1151, P(EP)3034, P(EP)3370, P(EP)3560, P(EP)3628, P(EP)3689, P(EP)3738, P(RS3)CB-5453-3, P(lacW)S147703, and Df(3L)ED208/TM2*Ubx* were from the Szeged Drosophila Stock Center. Stock *nos*>Gal4VP16 used in the ectopic expression experiments was a kind gift from R. Lehmann. ovo^{D1} [Fs(l)K1237] was described in Komitopoulou *et al.* (1983). The UAS-*mir-282* constructs were created using a 690-bp genomic fragment containing the pre-miRNA sequence. The *mir-282* genomic rescue construct was generated by PCR amplification of the entire 9.1-kb region between the upstream and downstream neighboring genes (CG43389 and *ckd*). The PCR product generated by the forward primer (mir_gen_Fw4Bgl) (GTCCGTACAAGATCTCCCACAATCTTGACCGCTAC) containing a *Bgl*II site and the reverse primer with a *Kpn*I site (mir_gen_Rev4Kpn) (GATGAGTCCTGAGGGTACCGACTTGGCCCAAACACTTGAC) was cloned into the pattB vector, which enabled site-specific integration into the fly genome. To generate flies expressing the EGFP transcript coupled with the 3′-UTR of *rutabaga* containing the single miR-282 target site under the control of the *actin5C* promoter, the EGFP-coding sequence together with the actin promoter was PCR amplified from the pAGW *Drosophila* Gateway vector with primers containing restriction sites *Hin*dIII and *Pst*I. The *rutabaga* 3′-UTR was PCR amplified using genomic DNA as template and primers with overhanging restriction sites *Sal*I and *Eco*RI. The PCR products were cloned into the pCaSpeR vector and the sequence was verified. Details are available upon request.

Database searches

To find *mir-282* orthologs, the database of miRBase (http://www.mirbase.org; release 16) was searched. For most of the analyzed genomes (*Drosophila* species, *Anopheles gambiae*, and *Tribolium castaneum*), data were collected from FlyBase (http://flybase.org; release FB2011_05) (Tweedie *et al.* 2009). In addition, *A. gambiae* homologs of the *D. melanogaster* CG14960 and CG12017 genes were manually identified based on high sequence similarity and genomic position. In *Apis mellifera*, the synteny information is based on sequence and homology data from the NCBI (http://www.ncbi.nlm.nih.gov). Data regarding gene expression and location of insulator sequences in the *mir-282* region of *D. melanogaster* were collected from FlyBase and modENCODE.

Generation of mir-282 mutants

The *mir-282* deletion was created by remobilizing the *P* element in line P(RS3)CB-5453-3. To enable the unambiguous identification of the chromosome carrying the transposon, the P(RS3)CB-5453-3 line was marked with the e^s marker mutation (RS5453, e^s). The *P* element was remobilized with the help of a Delta2-3 transposase source over an overlapping deletion, Df(3L)ED208. The candidates were screened for the loss of w^+ and confirmed molecularly by genomic PCR on single flies. The 5′-primer GCCGGTCCGAAATAGAAAGC (5vfor4) and the 3′-primer ACAAGCAGTGCAACCGTTGT (2rev) sequences were used to perform the PCR reaction, both hybridizing ~1 kb from the *P*-element insertion site. Subsequent sequencing of the PCR products was carried out with the same PCR primers.

RNA isolation

For total RNA purification, 30 females (3 days old) were homogenized in 1 ml of Trizol reagent, incubated for 5 min at room temperature, and centrifuged for 10 min (12,000 rpm, 4°). Two hundred microliters of chloroform was added to the supernatant. After centrifugation for 15 min (11,000 rpm, 4°), the supernatant was transferred to a new tube and the nucleic acids were precipitated by incubating the samples for 10 min with 500 μl isopropanol. The pellet was washed with 75% ethanol and dissolved in 100 μl of DEP-treated H_2O. The concentration and purity of the sample were measured at OD_{260} and OD_{280}. To identify the 3′ end of the primary transcript, RNA isolation was carried out with the RNeasy Mini kit (QIAGEN, Valencia, CA).

cDNA synthesis

A single-stranded cDNA collection was generated from total RNA extracts. To determine the 5′ end, the *mir-282*-specific 3′ primer TCGCCAAACCGACTCGCAAT (CO194054 rev2) was used, while for the amplification of the 3′ end a GeneRacer Oligo(dT) primer containing a 5′ adapter GCTGTCAACGATACGGCTACGTAACGGCATGACAGTG$(T)_{24}$ was used. Five micrograms of total RNA was incubated with the reverse primers for 5 min at 65° and subsequently chilled on ice. After adding 4 μl of 5× Reverse Transcriptase reaction buffer (Fermentas), 1 μl of 10 mM dNTP mixture, and 0,5 μl of RiboLock RNase inhibitor (Fermentas), 1 μl of RevertAid Premium Reverse Transcriptase enzyme (Fermentas;

20 units/μl) was added, followed by an incubation step at 50° for 30 min. The reverse transcription reaction was stopped by placing the sample at 85° for 5 min. Two microliters of the resulting cDNA sample was used as a template in the PCR reaction.

PCR experiments

The GeneRacer experiments were performed as described by the manufacturer (Invitrogen, Carlsbad, CA). In the 5′-RACE experiment, the GeneRacer 5′ and the gene-specific reverse primers (5v: GAGGGTCCGGGCTTATCAAGCAGT) were used in the first RACE reaction. To increase the specificity of the reaction, the GeneRacer 5′-nested and the gene-specific nested reverse primers (5v_nested: GTTCGCCTTGGTACACC TAACCTCTT) were used. To amplify the 3′ end of the primiRNA, the GeneRacer 3′ primer and the gene-specific 5′ primer (7for: TAGCTGGCACCTGCTGAAGT) were used, followed by the GeneRacer 3′ and the gene-specific forward nested (7for_nested: CGCGACTCTGTTGCAGTCAGCGCCAAT) primers.

For quantitative real-time RT-PCR experiments, RNA was extracted using RNA Blue reagent (Top-Bio, Prague) and cleaned with a NucleoSpin RNA II purification kit (Macherey-Nagel, Duren, Germany), including the on-column digestion step with DNase I. cDNA was synthesized from 1 μg of total RNA with oligo(dT) (17mer) and PrimeScript Reverse Transcriptase (Takara). qPCR was performed using the HOT FIREPol EvaGreen qPCR Mix Plus (Solis BioDyne). The PCR reaction volume was 20 μl, containing 5 μl of diluted cDNA and 250 nM primers. The amplification was carried out on the Illumina Eco Real-Time PCR System for 45 cycles (95° for 15 sec, annealing temperature depending on primer pair for 30 sec, and 72° for 20 sec), following an initial denaturation/Pol activation step (95° for 15 min). Each sample was analyzed in triplicate. Primers were designed with the Lasergene PrimerSelect Software (DNASTAR) to ensure the specificity of each amplicon. Melting analysis confirmed a single product for each primer pair reaction. The product size was verified by gel electrophoresis. Data were analyzed and quantified with the Illumina EcoStudy software. Relative mRNA levels were normalized to *Rack1* and *rp49* transcripts and standardized to the female or pupal control sample. All results are presented with means ± the standard errors of the mean (SEM) from four independent biological replicates.

Physiological tests and egg yield measurements

In all of these experiments, flies were kept at constant temperature (25°) and the density of eggs was checked. The line carrying the P(RS3)CB-5453-3 transposon, which was remobilized to generate the null mutant, was used as a control (RS5453, e^s). In the hatching-rate study, the number of eclosed adults and unhatched pharate adults was determined in a 2-week-old culture. When testing the viability of adult animals, males and females were tested separately in groups of 5. The viability of 30 virgin males and 30 virgin females was analyzed in this way. The flies were transferred to fresh medium every day, and the number of living flies was recorded. This experiment was performed at 18° and 29° as well. During the experiment designed to rescue the decreased viability of *mir-282* mutants, virgin females were crossed individually to five w^{1118} males and transferred to fresh medium every day, and the number of living flies was recorded.

During the starvation test, 30 mutant and 30 control females were reared individually together with 3 male siblings on normal medium. Flies were transferred to fresh medium every day, and the number of eggs deposited was counted. On the third and fourth days, sucrose starvation medium [1% agar, 0.5% propionic acid:phosphoric acid (9:1), 5% sucrose] was used (Terashima and Bownes 2004).

In the male fertility test, 30 freshly hatched males were collected and crossed individually to 5 w^{1118} virgin females. Males were transferred to new vials containing virgin females every second day. Offspring were counted on the 18th day. For egg yield measurements, 30 virgin females were crossed individually to w^{1118} males and transferred every day to new vials containing black fly media. The number of eggs deposited in the old vial was counted every day. This method was applied in the genomic rescue experiment as well.

For the statistical analysis of the data we calculated mean values and standard deviations and performed two-tailed *t*-tests to determine the significance of difference.

Detection of Wolbachia infection

The primers specific for *Wolbachia pipientis* 16S rRNA were 5′-TTGTAGCCTGCTATGGTATAACT, which is in the variable V1 region, and 5′-GAATAGGTATGATTTTCATGT, which is the reverse complement of the variable V6 region (O'Neill *et al.* 1992). Flies were reared on fly food containing 100 μg/ml ampicillin, 100 μg/ml streptomycin, and 0.25 mg/ml tetracycline antibiotics to exclude other types of infection.

Northern blot

Northern blot experiments were carried out as described in Várallyay *et al.* (2008) except for the following aspects: an LNA-modified, 28-bp-long oligonucleotide probe for *Drosophila* miR-282 mature miRNA was designed and purchased from Exiqon (Vedbaek, Denmark) (Exiqon's miRCURY LNA microRNA Detection Probes), and 10 pmol of the LNA-modified probe was radiolabeled. Sixty micrograms of total RNA isolated from wild-type, mutant, and *mir-282*-overexpressing adult females was loaded onto the lanes. The hybridization was carried out overnight at 48°, and the signals were visualized by exposing the membrane to a phosphorimage screen for 1 week.

Immunostaining

To stain germline stem cells, the monoclonal anti-Hts antibody 1B1 (1:50, Developmental Studies Hybridoma Bank) was used. For immunostaining experiments, ovaries were dissected in PBS and fixed in PBS with 4% formaldehyde for 15 min and then washed with PBS and 0.1% Triton X-100 (PBT) five times for 15 min. The ovaries were incubated in

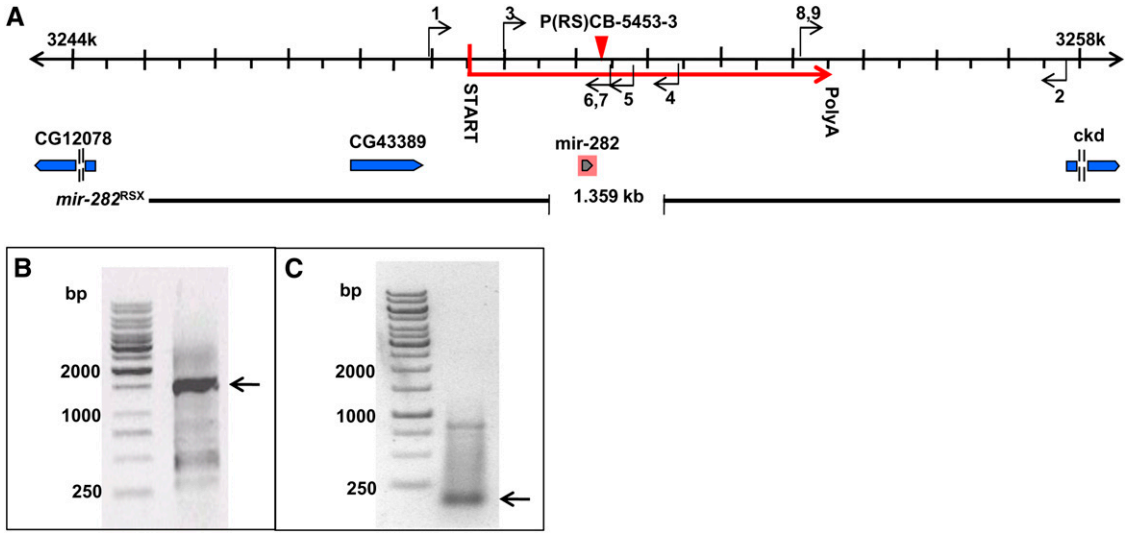


Figure 1 The organization, transcription, and deletions of the *mir-282* gene. (A) Map of the 63C1 region of chromosome 3. The *mir-282* primary transcript is represented by a red arrow. The size and position of the deletion *mir-282*^{RSX}, induced by the remobilization of transposon P(EP)CB-5453-3, are indicated by the gap in the thick horizontal line. Arrows with numbers symbolize the primers used: 1, *mir_gen_Fw4Bgl*; 2, *mir_gen_Rev4Kpn*; 3, *5vfor4*; 4, *2rev*; 5, *CO194054 rev2*; 6, *5v*; 7, *5v_nested*; 8, *7for*; 9, *7for_nested*. (B and C) Detection of the 5′ and 3′ ends of the mir-282 primary transcript by PCR amplification. The specific PCR products that were sequenced are indicated by arrows. Mw, molecular weight marker.

0.5% bovine serum albumin diluted with PBT (BBT) for 30 min. Primary antibodies were incubated at 4° overnight and then washed with PBT three times for 5 min and once for 30 min and incubated in BBT for 0.5 hr. Secondary antibody was added and incubated overnight at 4° and then washed with PBT three times for 10 min. Stained ovaries were mounted in ProLong Gold Antifade Mountant (Invitrogen; Molecular Probes, Eugene, OR). All micrographs were taken using an Olympus Fluoview FV1000 confocal microscope.

For the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays (BD Biosciences APO-BRDU Kit, material no. 556405), ovaries of 10-day-old, well-fed females were dissected in ice-cold PBS and ovarioles were teased apart. Samples were fixed for 15 min in PBS containing 4% paraformaldehyde. The staining was performed according to the manufacturer's instructions except for prolonged (overnight) DNA labeling and antibody incubation periods.

Transplantation experiments

Germline chimeras were constructed by transplanting pole cells (Lehmann and Nusslein-Volhard 1986) to determine whether the mutant phenotype is brought about by altered functions of the germline or the soma. Five to 10 embryonic pole cells from the *mir-282*^{RSX} null mutant and the control (RS5453, e^s) donor stocks were implanted into the posterior region of host embryos that originated from the cross between wild-type (Ore-R) females and ovo^{D1} males. ovo^{D1} is an agametic, strictly germline-dependent dominant female sterile mutation of *D. melanogaster* (Komitopoulou *et al.* 1983). Injected animals were kept at 18° for 2 days to recover, and then the larvae were raised at 25°. Eclosing virgin females were collected and individually crossed to w^{1118} males, and the egg production of the chimaeras was counted for 10 days.

Results

The mir-282 genomic region

The *mir-282* pre-miRNA was predicted by the Berkeley *Drosophila* Genome Project (http://www.fruitfly.org/) to be encoded on the third chromosome of *D. melanogaster* at cytological location 63C1, in a 9.4-kb-long genomic region between CG43389 and the *cracked* (*ckd*) genes (Figure 1A). A search for *mir-282* orthologs in eukaryotes in the miRBase database (http://www.mirbase.org) revealed that *mir-282* is an insect-specific gene present in all holometabolic (Endopterygota) species analyzed. In addition to all the members of the Drosophilidae group whose genome sequence is currently available, the miR-282 coding sequence was also found in mosquitoes (*A. gambiae* and *Aedes aegypti*), beetles (*T. castaneum*), butterflies (*Bombyx mori*, *Manduca sexta*, and *Heliconius melpomene*), and hymenopterans (*A. mellifera*, *Nasonia vitripennis*, and *N. longicornis*). With the single exception of *Strigamia maritime* (centipede), no homologs are predicted in other insects, arthropods, or any other metazoan phyla.

To determine the degree of conservation in the *mir-282* region, we searched for synteny in the vicinity of *mir-282* by analyzing the presence of neighboring annotated protein-coding genes both upstream and downstream from *mir-282* (Table 1). We found that in the *melanogaster* group the positions of all the genes analyzed are preserved, while the third closest upstream gene (CG17746) and the two adjacent downstream genes (*ckd* and CG14960) in *D. willistoni* are syntenic, assigning a minimal synteny group. Although both in the *obscura* and in the *repleta/virilis* subgroups inversion events occurred in this region, they did not fully abolish the synteny: at least two neighboring genes on both sides of *mir-282* retained their position (*D. persimilis*).

Table 1 Analysis of the synteny around the *mir-282* gene

Gene species	CG14967	*RpL28*	CG17737	CG11505	CG17746	CG12078	*CG43389*	*mir-282*	*ckd*	CG14960	CG12017	CG12009	CG14968	*ZnT63C*
Dmel	+	+	+	+	+	+	+[a]	+	+	+	+	+	+	+
mel. sg.	+	+	+	+	+	+	+[a]	+	+	+	+	+	+	+
Dana	+	+	+	+	+	+		+	+	+	+	+	+	
Dpse (i)		+	+	+	+			+	+	+	+	+	+	
Dper (i)		+			+			+		+	+			
Dwil					+			+	+	+				
Dmoj (i)		+	+	+	+	+		+	+	+	+			
Dvir (i)		+	+	+	+	+		+	+	+	+	+	+	
Dgri		+	+	+	+			+	+	+	+	+	+	
Agam	+							+		+	+	+	+	
Tcas								+		+	+	+	+	
Amel								+						

Dmel, *Drosophila melanogaster*; mel. sg., *melanogaster* subgroup; Dana, *D. ananassae*; Dpse, *D. pseudoobscura*; Dper, *D. persimilis*; Dwil, *D. willistoni*; Dmoj, *D. mojavensis*; Dvir, *D. virilis*; Dgri, *D. grimshawi*; Agam, *Anopheles gambiae*; Tcas, *Tribolium castaneum*; Amel, *Apis mellifera*. "+" marks the presence of the homologous gene in the mir-282 neighborhood compared to the chromosomal region of *D. melanogaster* (genes are listed according to their order in the *D. melanogaster* genome). (i) indicates inversion of the region. Homologous genomic sequences could be identified in the genome of the members of the *melanogaster* subgroup only.

[a] The gene CG43389 has been predicted in *D. melanogaster* by the most recent genome release (FB2011_06).

In the mosquito and the flour beetle, four upstream neighboring genes (from CG14968 to CG14960) were syntenic with *mir-282*, while in the honey bee no synteny was found. These data reveal that the *mir-282* gene is not associated with adjacent protein-coding genes, however, in all species analyzed: *mir-282* resides in a long region devoid of protein-coding genes, arguing for the presence of a *mir-282* promoter and important regulatory elements within this area.

Molecular definition of the mir-282 gene

Data from large-scale screens indicated the expression of miR-282 in pupae and female adults and a low-level maternal contribution in the embryos (Lai *et al.* 2003; Leaman *et al.* 2005; Fernandez-Valverde *et al.* 2010). According to miRBase, the predicted *mir-282* pre-miRNA is 97 nt long and gives rise to a single miRNA of 28 nt (Griffiths-Jones *et al.* 2008); however, deep sequencing data indicate a shorter miR-282 of 22 nt with additional nontemplate adenosine nucleotides at the 3′ end (Fernandez-Valverde *et al.* 2010; Kozomara and Griffiths-Jones 2011). In an effort to define the *mir-282* gene molecularly, we analyzed the RNAs transcribed in the *mir-282* locus. The 5′ and 3′ ends of the *mir-282* transcript were determined using the RACE technique (GeneRacer Kit; Invitrogen) (Figure 1, B and C). The 5′ end of the transcript was found to correspond to the genomic sequence location 3L: 3,249,590 (R5.35), which is 1444 bp upstream from the first nucleotide of the mature microRNA. The 3′-RACE technique using primers specific for the adapter sequence of the GeneRacer oligo(dT) primer revealed that the poly(A) tail at the 3′ end is in the 3L: 3,254,504 genomic position, which is 3446 bp downstream from the last nucleotide of the mature miR-282. These results suggest that the *mir-282* primary transcript has a 5′ cap and a 3′-poly(A) sequence and that it is 4918 bp long, which corresponds to the size predicted previously (Kadener *et al.* 2009) (Figure 1A).

The *mir-282* gene shows no evolutionarily preserved colocalization with neighboring protein-coding genes, but since the upstream and downstream adjacent genes (CG43389 and *ckd*) are in the same orientation as *mir-282* in *D. melanogaster*, it is possible that *mir-282* is in fact a part of a splice form they generate. To confirm or exclude this possibility in the case of the *ckd* gene, we screened a single-stranded cDNA pool generated both from pupae and from adult ovaries for a transcript that would contain sequences specific for both the *mir-282* region and the downstream *ckd* gene. PCR primers specific for all exons of *ckd* were used in these experiments. As controls, a region of ~600 bp covering the *mir-282* pre-miRNA sequence and a *ckd*-specific DNA of ~580 bp could be amplified from the cDNA pool, but no cDNA containing both *mir-282*- and *ckd*-specific sequences could be detected, indicating that *mir-282* and *ckd* form independent transcription units. To further confirm this finding, we defined the 5′ end of the *ckd* transcript with the RACE technique. We unambiguously mapped it to the genomic location 3L: 3,257,938, which is 217 bp upstream from the position currently predicted by the database (www.flybase.org) but still ~7 kb downstream from *mir-282* (data not shown).

The small upstream neighboring gene, CG43389, was predicted in *D. melanogaster* only in the most recent genome release (FB2011_06), which became available while preparing this article. Genomic sequences showing homology to different parts of the putative *D. melanogaster* CG43389 gene could be identified at the same genomic position but only in the members of the *melanogaster* subgroup. The search for a genomic sequence encoding a homolog of the CG43389 peptide revealed no hit at all; therefore, we believe it is unlikely that *mir-282* would be part of this newly annotated gene. Taken together with the other results, this strongly suggests that the ~9.4-kb region between the CG43389 and *ckd* genes contains an independent gene that is the source of the *mir-282* pri-miRNA transcript.

Analysis of the mir-282 region

To investigate whether the *mir-282* transcript has a biological function, we first dissected the genomic region around

mir-282 by analyzing the phenotypes of transposon insertions in the *mir-282* locus. Although a great number of transposon-bearing lines were examined, no visible phenotype could be observed except for homozygous lethality in some lines. Moreover, no relationship could be established between the site of insertion and the lethality observed in these lines. To unambiguously determine the link between the lethality and the *mir-282* region, we crossed the lethal *P*-element insertions [P(EP)1151, P(lacW)S147703, P(EP)3370, P(EP)3628, P(EP)3689, P(EP)3034, and P(EP)3738] to a deletion [Df(3L)ED208] covering the entire *mir-282* region. The chromosome containing the deletion complemented the lethality in all cases, confirming that decreased viability in these stocks is due to background mutations and not to the loss of *mir-282* activity.

In contrast to the phenotypic analyses, the *actin*>Gal4-dependent ubiquitous activation of the *EP* element oriented to drive expression of *mir-282* in the viable line EP3560 caused lethality, suggesting that the putative transcript encoded by the *mir-282* locus does indeed have a biological function. To gain further insight into the activity of *mir-282*, transgenic lines overexpressing miR-282 were created. For this purpose, a 690-bp genomic section containing the pre-miRNA sequence was PCR amplified and cloned after the UASt promoters with the help of the Gateway technology (Huynh and Zieler 1999). Ectopic expression of the miR-282-coding sequence was carried out by using embryonic (*twist*) and tissue-specific (*elav* for nervous system; *vg*, *ptc*, and A9 for wing disc expression) as well as ubiquitous (*Tub*, *act5C*, and *da*) Gal4 drivers. We observed 100% lethality at the second larval stage when ubiquitously expressing miR-282, while no phenotype could be detected when using any other driver. These experiments suggest that the putative transcript encoded by the *mir-282* locus does have biological function(s).

To gain insight into the function of *mir-282*, we generated mutants by remobilizing the P(RS3)CB-5453-3 transposable element, which is the *P* element most closely located to the core sequence of the mature miR-282. To increase the frequency of generating imprecise excisions, we mobilized the transposon over a deficiency [Df(3L)ED208] overlapping the *mir-282* region. The nature of the resulting *mir-282* mutations was determined by PCR amplifying and sequencing the region around the miR-282 sequence. In the deletion mutant designated as [RSX], 1359 nt were removed that cover the entire pre-miRNA region; thus, we consider this allele to be a physically verified null allele (Figure 1A). The *mir-282*^{RSX}, e^s line was used in the subsequent experiments, and the parental line marked with e^s was employed as a control to eliminate genetic background problems. Therefore, from this point on the terms "mutant" and "control" refer to the *mir-282*^{RSX} null allele and the P(RS3)CB-5453-3, e^s line, respectively.

The null mutant strain was homozygous viable and displayed no visible phenotype; however, it was a weak stock; thus, we analyzed its viability in detail. The viability experiments revealed a shortened adult life span as well as semilethality before reaching adult stage. The average life span was found to be decreased by ~50% both in mutant males (Figure 2A) and in mutant females (Figure 2B) while the number of hatching imagos decreased by 56% compared to that in the control line (Figure 2C). In these experiments, wild-type (w^{1118}) flies were also included to validate the control line (Figure 2C).

Systematic Northern blot experiments for all predicted miRNAs of *Drosophila* have detected the expression of *mir-282* with a particularly low intensity (Leaman *et al.* 2005). To confirm that the transcript of the *mir-282* region is indeed the source of a mature microRNA and to validate our null mutant and overexpression lines, Northern blot experiments were carried out on total RNA samples obtained from adult females. The comparison of wild-type, positive (miR-282 overexpressing), and negative (null mutant) controls has confirmed that the expression of miR-282 is abolished in the null mutant and elevated in the overexpressing line. This experiment demonstrated that a mature microRNA of ~25 nt is generated from the *mir-282* gene (Figure 2D), which together with the results of the phenotypic analyses strongly suggests that the *mir-282* gene is the source of a functional transcript.

Altered egg production in mir-282 mutants

Since mutant animals displayed a reduced hatching rate, we analyzed the fecundity of *mir-282* null mutant imagos as well. While *mir-282* null mutant males displayed normal fertility (Figure 2E), mutant females exhibited reduced egg production compared to the control: they laid a decreased number of eggs, on the average 55% less than the control females during the 10-day period examined (Figure 2F).

It is known that *Wolbachia*, which are common intracellular gram-negative bacteria, can reduce egg production in insects by reproductive parasitism (Clark *et al.* 2005). We tested our stocks for the presence of *Wolbachia* by simple DAPI staining of the cytoplasm in *mir-282* mutant ovaries and by PCR amplifying the 16S RNA of *Wolbachia*. A fly stock infected with *Wolbachia* was used as a positive control. These experiments clearly confirmed the absence of *Wolbachia* in the *mir-282* mutant lines and excluded the possibility of the decreased egg production in *mir-282* mutants being due to *Wolbachia* infection (data not shown).

To prove that the mutant phenotypes are indeed due to the loss of miR-282, we generated transgenic flies carrying a genomic fragment spanning the entire 9.1-kb region between the upstream and downstream neighboring genes (CG43389 and *ckd*) and containing the region that encodes the *mir-282* primary transcript. This genomic fragment was able to rescue both the reduced viability and the decreased egg production phenotypes in the mutants (Figure 2, G and H), confirming that these phenotypes are indeed caused by the loss of *mir-282* function.

Germline stem cells are not responsible for reduced egg production

The decreased egg production of *mir-282* mutants might be the consequence of germline stem cell (GSC) depletion in the adult

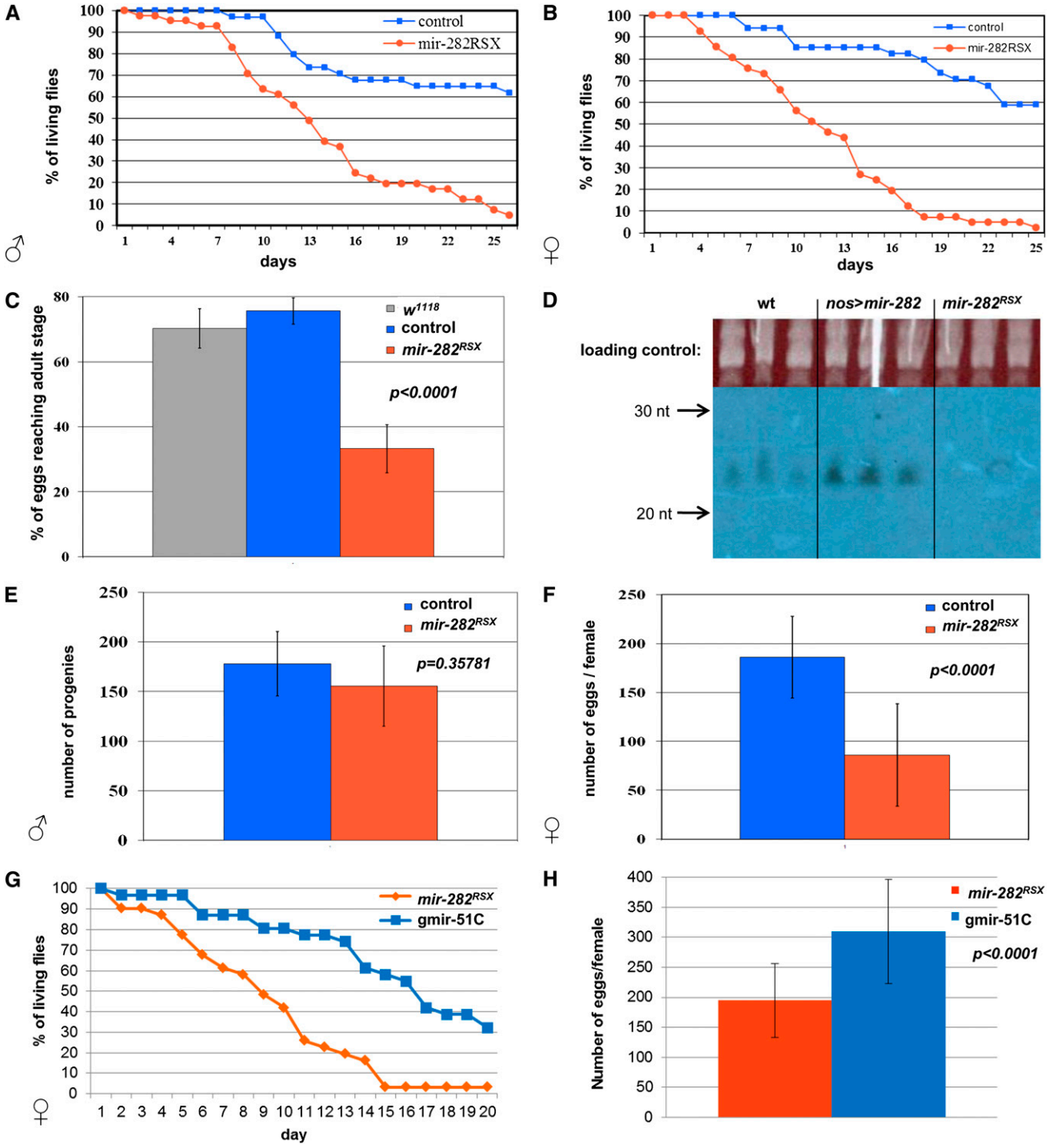


Figure 2 The *mir-282* region encodes a functional microRNA. (A–C) The lack of *mir-282* function results in a shortened life span for adult males (A) and females (B) as well as in a reduced hatching rate of pupae (C). (D) Northern blot experiment demonstrating that the *mir-282* region is the source of a microRNA. In this experiment, w^{1118} wild-type (wt) flies were compared to flies ectopically expressing miR-282 under the control of the *nanos* promoter (*nos>mir-282*) and to the null mutant, $mir\text{-}282^{RSX}$. (E and F) In addition to reduced viability, *mir-282* mutant females show decreased egg production (E) while the fertility of males (F) is unaffected. (G and H) The genomic fragment containing the region coding for the 4.9-kb mir-282 primary transcript (gmir-51C) is able to rescue both the viability (G) and the egg production (H) phenotypes of $mir\text{-}282^{RSX}$ null mutants.

ovary. We investigated this possibility by analyzing GSCs in the ovaries of mutant females via anti-Hts antibody staining (Figure 3, A and B). Since egg development takes ~8 days, 10-day-old females were used in these experiments to visualize the possible loss of GSCs. We found that although mutant germaria exhibited an elongated shape, the numbers of GSCs and cysts were normal, indicating that the decrease in egg production is not a result of low GSC number (Figure 3B).

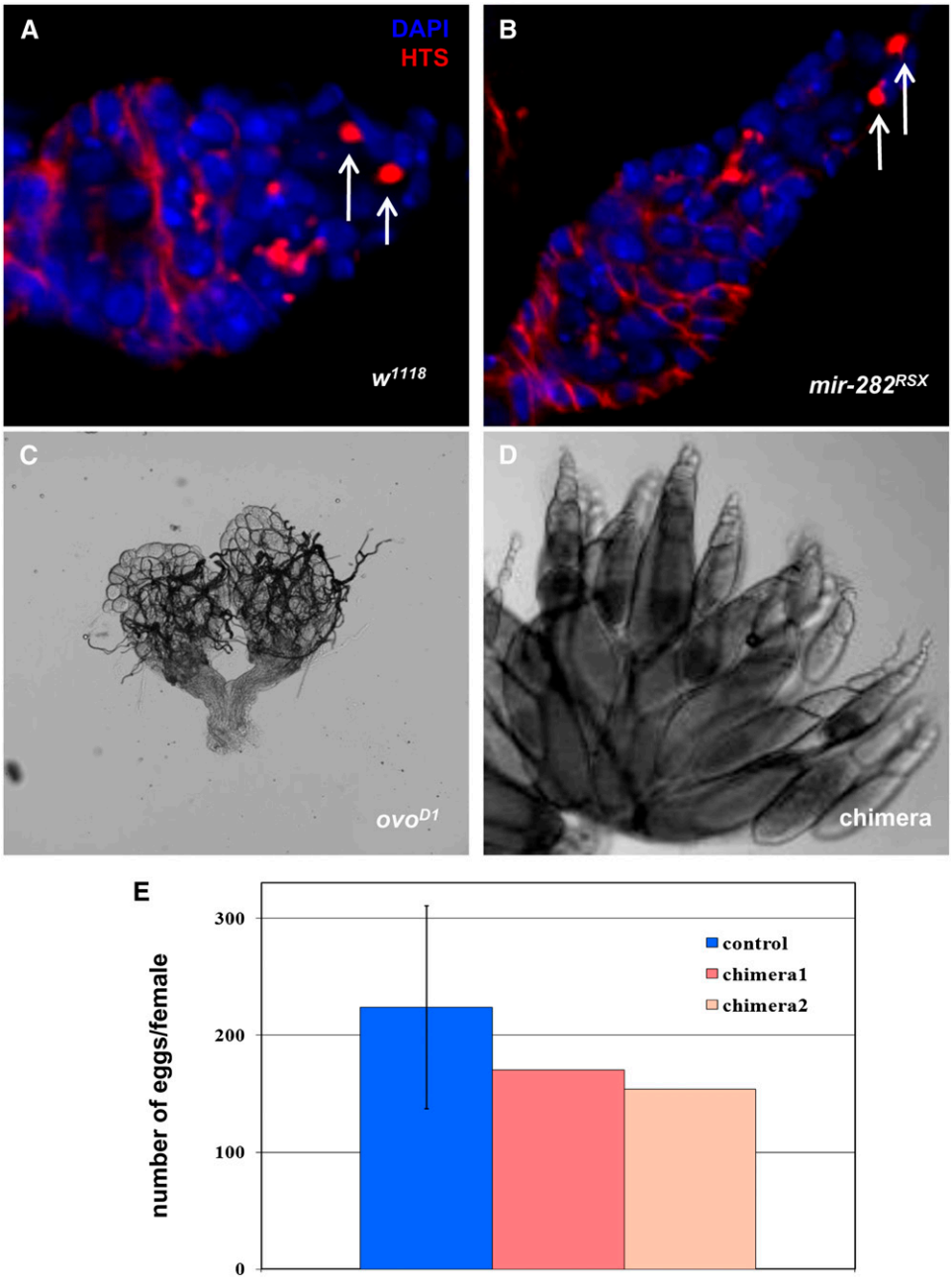


Figure 3 Germ cells are not responsible for the reduction in egg production. (A and B) The germaria of 10-day-old control (A) and *mir-282* mutant (B) females were stained with an antibody detecting a GSC marker (Hts). Antibody staining is revealed in red; DNA stained with DAPI is shown in blue. Arrows point to GSCs. (C–E) Pole cell transplantation experiments demonstrate that *mir-282* mutant GSCs are fully functional. Embryonic germ cells from *mir-282* null mutant embryos were transplanted into females carrying the dominant female sterile mutation ovo^{D1}, which has a strictly germline-dependent dominant female sterile phenotype (C). Chimeric females lay normal numbers of eggs compared to the control (E), and the Nomarski image of the ovary reveals that the number of functional ovarioles is normal in the chimeric females (D).

Reduction in egg yield may also be the consequence of improper GSC or somatic functions. To determine whether the decrease in the egg production of *mir-282* mutants is germline or soma dependent, pole cell transplantation experiments were carried out. Embryonic germ cells from *mir-282* null mutant embryos were transplanted into females carrying the dominant female sterile mutation ovo^{D1}, which has a strictly germline-dependent dominant female sterile phenotype and blocks the development of egg primordia at around the beginning of vitellogenesis (Figure 3C). The egg production of chimeric females was found to be normal (Figure 3E), suggesting that *mir-282* mutant GSCs are fully functional and that somatic cells but not germ cells require normal miR-282 function. This observation was further supported by the fact that the number of functional ovarioles was always higher than that of the transplanted mutant pole cells, indicating that pole cells lacking *mir-282* are able to divide and thus are fully functional at every developmental stage (Figure 3E). These experiments revealed that the decrease in egg production is not related to the germline but rather to the soma.

Elevated apoptotic activity in mir-282 mutant ovaries

As a next step, we examined whether reduced egg production in *mir-282* mutant females is caused by an increase in apoptosis during oogenesis. It is known that in *Drosophila* ovaries apoptosis-mediated cell death of egg chambers normally occurs at stage 8 (checkpoint of abnormal development)

and of nurse cells at stage 12 (after the completion of dumping) (Nezis *et al.* 2000). The analysis of programmed cell death via a TUNEL assay in ovaries of 10-day-old *mir-282* mutant females revealed that apoptotic activity is normal at checkpoints 8 and 12, but there is apoptosis in eggs after stage 8 (Figure 4, B and C) with apparent accumulation of apoptotic debris in the proximal end of the oviduct (Figure 4D).

Although *mir-282* mutant animals exhibit no apparent difference in body size and shape, since starvation has been described to induce an increase in the apoptosis of *Drosophila* nurse cells in stage 8 egg chambers (Terashima *et al.* 2005), we inspected the possibility of malnutrition/low energy homeostasis causing the increase of apoptotic activity in mutant ovaries. Therefore, we measured the egg production of mutant and control groups of females during starvation. Two days of food deprivation resulted in a dramatic decrease in egg production in both groups, followed by a rapid return to normal levels. The kinetics of egg yield changes were the same in both groups, indicating that *mir-282* mutants show normal physiological reactions to temporary food deprivation (Figure 4E) and that altered energy homeostasis is not responsible for the enhancement of apoptotic activity in *mir-282* mutants. Therefore, it is reasonable to assume that the accumulation of apoptotic debris after checkpoint 8 is a consequence of egg-laying defects rather than of abnormal egg development.

The level of adenylate cyclase transcript increases in mir-282 mutant pupae

To identify the possible molecular mechanism(s) leading to the decrease in viability and egg production in *mir-282* mutants, a selected group of the predicted targets of miR-282 was analyzed by quantitative RT-PCR. The list of computationally predicted targets for miR-282 by the MicroCosm Targets (v5) database at EMBL (Enright *et al.* 2003; Griffiths-Jones *et al.* 2008) comprises 358 genes, the majority of which are only predicted or uncharacterized to date. A thorough analysis of the putative targets (Gene Ontology term analysis) uncovered no functional categories that would be overrepresented among them. Therefore, to select possible target genes for further studies, the list was manually screened, taking into account our findings about miR-282 (null mutant and overexpression phenotypes, activity in pupae and females) and the specificity of the interaction with the target sequence (*e.g.*, number of miRNAs regulating the given gene). Based on these criteria, five predicted targets were selected for real-time PCR experiments: *Hippo* (*hpo*), *Target of rapamycin* (*Tor*), *Cyclin C* (*CycC*), *Autophagy-specific gene 12* (*Atg12*), and *rutabaga* (*rut*). A quantitative measurement of the transcription of the selected target genes revealed that the expression of the *rut* gene increased significantly in *mir-282* mutant pupae while the transcript level of the other four genes examined did not change (Figure 5A). No significant changes in the expression activity of any of the target genes were detected in mutant adults, indicating that *mir-282* regulates *rut* expression during metamorphosis.

miR-282 regulates the activity of the adenylate cyclase gene

To verify that the *rutabaga* transcript is a target of miR-282, we measured the transcript level of *rutabaga* in pupae overexpressing miR-282 in the nervous system with the help of the *elav* promoter (Figure 5B). As controls, *mir-282* mutant pupae and the four other predicted targets were also included in the experiment. The ectopic overexpression of miR-282 in the nervous system during metamorphosis resulted in a significant decrease of the *rutabaga* mRNA level while the transcript level of the four other targets examined did not change significantly.

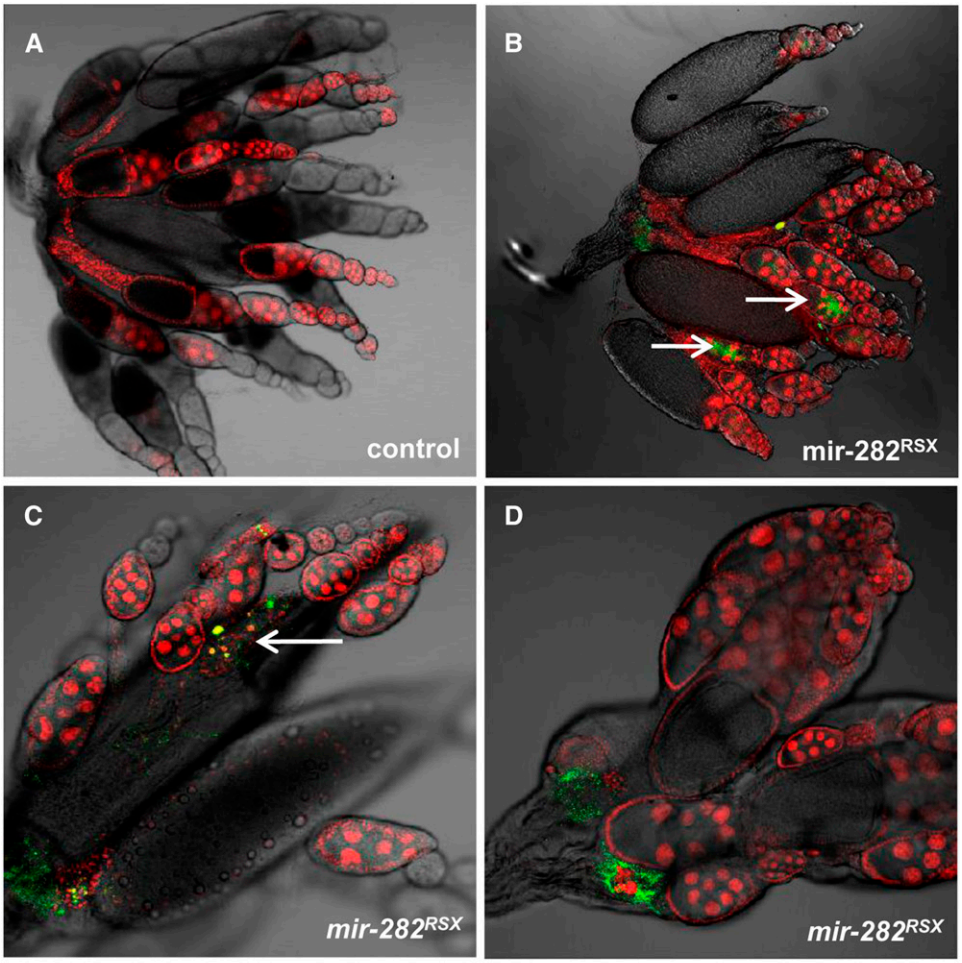
Next, we expressed the EGFP transcript coupled with the 3′-UTR of *rut* (Figure 5C). The GFP signal was strongly reduced in larval salivary gland cells when miR-282 was simultaneously overexpressed (Figure 5E) using a salivary gland-specific driver (*sgs3*>Gal4), confirming that miR-282 regulates the activity of the adenylate cyclase gene, *rutabaga*, in *Drosophila*.

Discussion

In this article, we report the molecular characterization of a predicted microRNA gene of *Drosophila melanogaster*. We demonstrate that the predicted *mir-282* gene is actively transcribed and that the lack of miR-282 leads to pupal semi-lethality and reduced egg production while ubiquitous, ectopic overexpression of miR-282 causes lethality at the larval stage. Our results, together with a finding that the knockdown of Drosha results in the accumulation of *mir-282* pri-miRNA (KADENER *et al.* 2009), provide molecular evidence for the existence of the predicted *mir-282* gene in *Drosophila melanogaster*.

Our computational analysis of the *mir-282* region showed that the *mir-282* gene is not associated with adjacent protein-coding genes in holometabolic insects, but the existence of an intergenic region of 9.4–27 kb surrounding the *mir-282* gene is conserved, suggesting the presence of a promoter and important regulatory elements in this area. Although it is still possible that the *mir-282* transcript is a splice form of a distant gene, the well-defined 5′ end of *mir-282* and the class I insulator-defined regulatory boundary predicted upstream from *mir-282* by the modENCODE database (Négre *et al.* 2010), as well as a recent report on *mir-282* being the target of the Drosha-Pasha complex (instead of the splicing machinery) (Kadener *et al.* 2009), all argue for an independent *mir-282* transcription unit.

The lack of *mir-282* function leads to a significant reduction in egg production. We found that the decrease in egg production is characterized by elevated apoptotic activity in the mutant ovaries, and we show that it is caused by the soma most likely through neurological defects. The somatic origin of the egg-laying phenotype and the presence of miR-282 in the ovary and the early embryo (Fernandez-Valverde *et al.* 2010) strongly suggest that *Drosophila mir-282* represents a maternal-effect microRNA gene whose function is



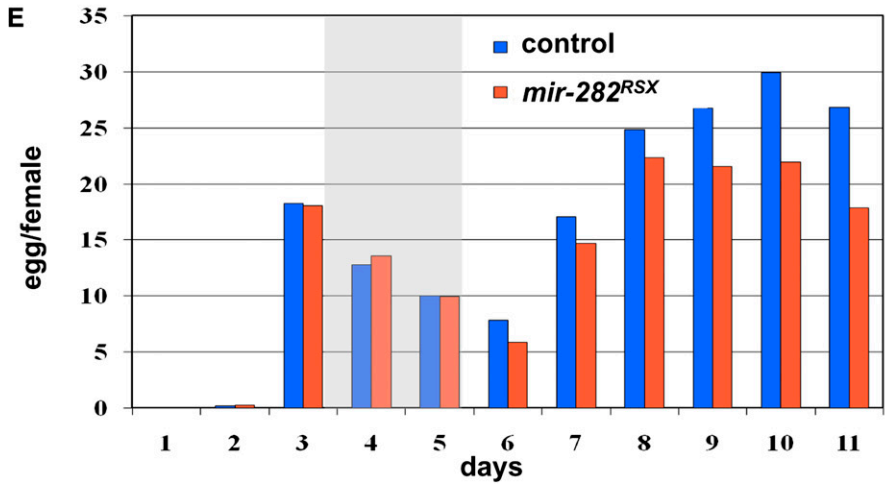


Figure 4 Starvation is not the cause of the elevated apoptotic activity in *mir-282* mutant ovaries. (A–D) The ovaries of 10-day-old control (A) and *mir-282* mutant (B–D) females. Green color indicates ApoBrDU-FITC signal; DNA stained with propidium iodide is shown in red. Mutant ovaries have reduced numbers of ovarioles with apparent accumulation of apoptotic debris in the proximal end of the oviduct. Apoptosis in egg chambers after stage 8 is indicated by arrows. (E) Starvation test reveals that *mir-282* mutants show normal response to temporary starvation. Gray highlight indicates the period of food deprivation.

not required for oogenesis but rather for the very first steps of embryonic development.

Finding targets and thus functions for microRNA genes is a difficult task in microRNA research. Mutants often exhibit mild or no defects; there are hundreds of predicted targets, and the lists of target genes available in the different databases usually do not overlap. Using bioinformatic and molecular methods, we have identified the *rutabaga* gene, which is a nervous system-specific adenylate cyclase, as a target of miR-282. Since the *rut* mRNA has predicted regulating sites for four different microRNAs and miR-282 has many potential targets (miRBase), it is hard to tell at present whether the regulation of *rut* is the only or the main function of *mir-282*. However, based on our data, it is conceivable that one of the main functions of *mir-282* in *D. melanogaster* is the regulation of adenylate cyclase activity in the nervous system during

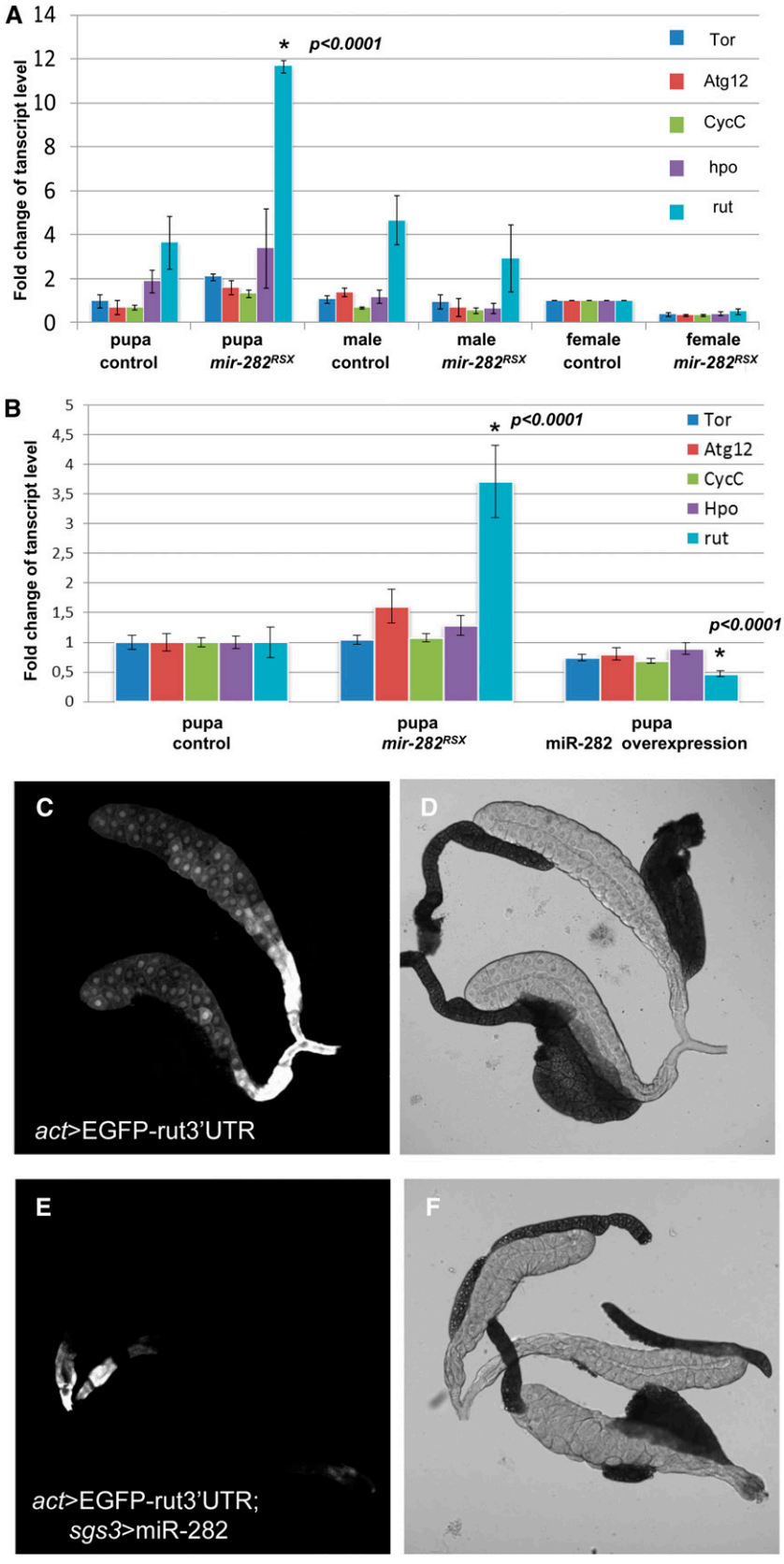


Figure 5 The *Rutabaga* gene is regulated by miR-282. (A and B) Quantitative RT-PCR using primers specific for the transcript of five potential target genes (*Tor*, *Atg12*, *CycC*, *Hpo*, and *rut*) of miR-282. Assays were performed on total RNA from pupae and adult males and females of both the control (RS5453e) and the null mutant (*mir-282*^{RSX}) genotypes as well as from pupae overexpressing miR-282. Fluorescence values were standardized to the *rp49* transcript and normalized to the female control sample in A and the control pupae in B. Fold increase of transcript levels is shown on the *y*-axis; significant changes are marked by an asterisk. (C and D) The EGFP transcript coupled with the 3'-UTR of *rut* is expressed in larval salivary gland cells as indicated by the EGFP signal (white color). (E and F) Transcript level is downregulated when miR-282 is simultaneously overexpressed, as shown by the lack of GFP signal.

metamorphosis. Considering this, the semilethality at the pupal stage in *mir-282* mutants is likely to be the consequence of either a general somatic weakness or developmental problems occurring during metamorphosis. The latter notion is further supported by our findings that the *mir-282* gene is active at the pupal stage and it is conserved in holometabolic insects, which undergo complete metamorphosis. Moreover, in genetic interaction screens, *mir-282* overexpression was found to have effect on pupal wing development and nervous system function (Bejarano *et al.* 2008; Cao *et al.* 2008).

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