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Report

Temporal Reciprocity of miRNAs and Their Targets during the Maternal-to-Zygotic Transition in *Drosophila*

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Summary

During oogenesis, female animals load their eggs with messenger RNAs (mRNAs) that will be translated to produce new proteins in the developing embryo. Some of these maternally provided mRNAs are stable and continue to contribute to development long after the onset of transcription of the embryonic (zygotic) genome. However, a subset of maternal mRNAs are degraded during the transition from purely maternal to mixed maternal-zygotic gene expression. In *Drosophila*, two independent RNA degradation pathways are used to promote turnover of maternal transcripts during the maternal-to-zygotic transition [1]. The first is driven by maternally encoded factors, including SMAUG [2], whereas the second is activated about 2 hr after fertilization, coinciding with the onset of zygotic transcription. Here, we report that a cluster of zygotically expressed microRNAs (miRNAs) targets maternal mRNAs for turnover, as part of the zygotic degradation pathway. miRNAs are small noncoding RNAs that silence gene expression by repressing translation of their target mRNAs and by promoting mRNA turnover. Intriguingly, use of miRNAs to promote mRNA turnover during the maternal-to-zygotic transition appears to be a conserved phenomenon because a comparable role was reported for miR-430 in zebrafish [3]. The finding that unrelated miRNAs regulate the maternal to zygotic transition in different animals suggests convergent evolution.

Results and Discussion

A Mutant Deleting the miR-309 MicroRNA Cluster Is Viable and Fertile

The *Drosophila* miR-309 cluster contains eight microRNA (miRNA) genes, which encode six different miRNAs (Figure 1A). Nucleotides 2 to 8 at the miRNA 5′ end comprise the "seed" region, which serves as the primary determinant of target specificity [4, 5]. The cluster encodes miRNAs with five distinct seed sequences, and so has the potential to regulate a broad spectrum of target messenger RNAs (mRNAs) (reviewed in [6]).

By using homologous recombination [7], we generated a mutant in which the 1.1 kb comprising the miR-309 cluster was deleted and replaced with green fluorescent protein (GFP). Northern-blot analysis was used to verify that the first and last miRNAs in the cluster, miR-309 and miR-6, were not produced in the mutant (Figure 1A). Homozygous mutant animals completed embryogenesis with no apparent defects in patterning (data not shown and Figure S1F available online), but approximately 20% died as larvae at different larval stages (Figures 1B and 1C). Some individuals stopped growing at the size of L2 larva and arrested at this developmental stage for a few days before dying. Approximately 80% of mutants survived to adulthood and were viable and fertile. Introduction of a transgene containing a 2.6 kb fragment of genomic DNA spanning the miRNA cluster (Figure 1A) restored survival of the mutants to normal levels (Figure 1B). The mutant animals showed a developmental delay during larval stages. This delay was suppressed in simultaneously collected and staged mutant larvae carrying the rescue transgene (Figure 1C). The phenotypes that result from complete deletion of the three miR-6 miRNA genes (together with the rest of the cluster mRNAs) contrast with the severe embryonic defects that were reported with the use of antisense 2′-O-methyl oligonucleotide injection to deplete miR-6 or miR-286 [8].

RNA samples from precisely staged embryos [9] were used to examine the expression of the miR-309 cluster during early embryogenesis. We compared the levels of mature miR-6 and miR-309 in these samples by quantitative real-time polymerase chain reaction (qPCR). Samples were normalized to two reference miRNAs, miR-310 and miR-184, which we found to be expressed at constant levels when normalized to total RNA. miR-6 and miR-309 were expressed at barely detectable levels in RNA collected from embryos during a 30 min period before the onset of zygotic transcription (Figure 2A, time point T0; [9]). The miRNAs were then strongly induced coincident with the onset of zygotic transcription (Figure 2A, T1). In situ hybridization analysis at this stage, showed expression of the miR-309 cluster primary transcript throughout the embryo, except in pole cells ([10, 11], Figure 2B). This transcript was not detectable in miR-309 cluster mutant embryos.

Although the mature miRNA products persist for some time, the expression of the primary transcript shows a dynamic spatial pattern by in situ hybridization (Figures S1A–S1E; see also [11]). At the midpoint of cellularization, expression of the cluster is turned off at the posterior pole and in a stripe in the anterior region of the embryo. During gastrulation, expression is lost ventrally and laterally, resulting in transient stripes in the dorsal ectoderm. By the onset of germ-band elongation, the primary transcript was essentially undetectable, but in northern blots, the mature miRNAs are detectable until larval stages [8].

The miR-309 cluster is predicted to target many mRNAs, including those of several genes implicated in embryo patterning. However, immunolabelling for the detection of these

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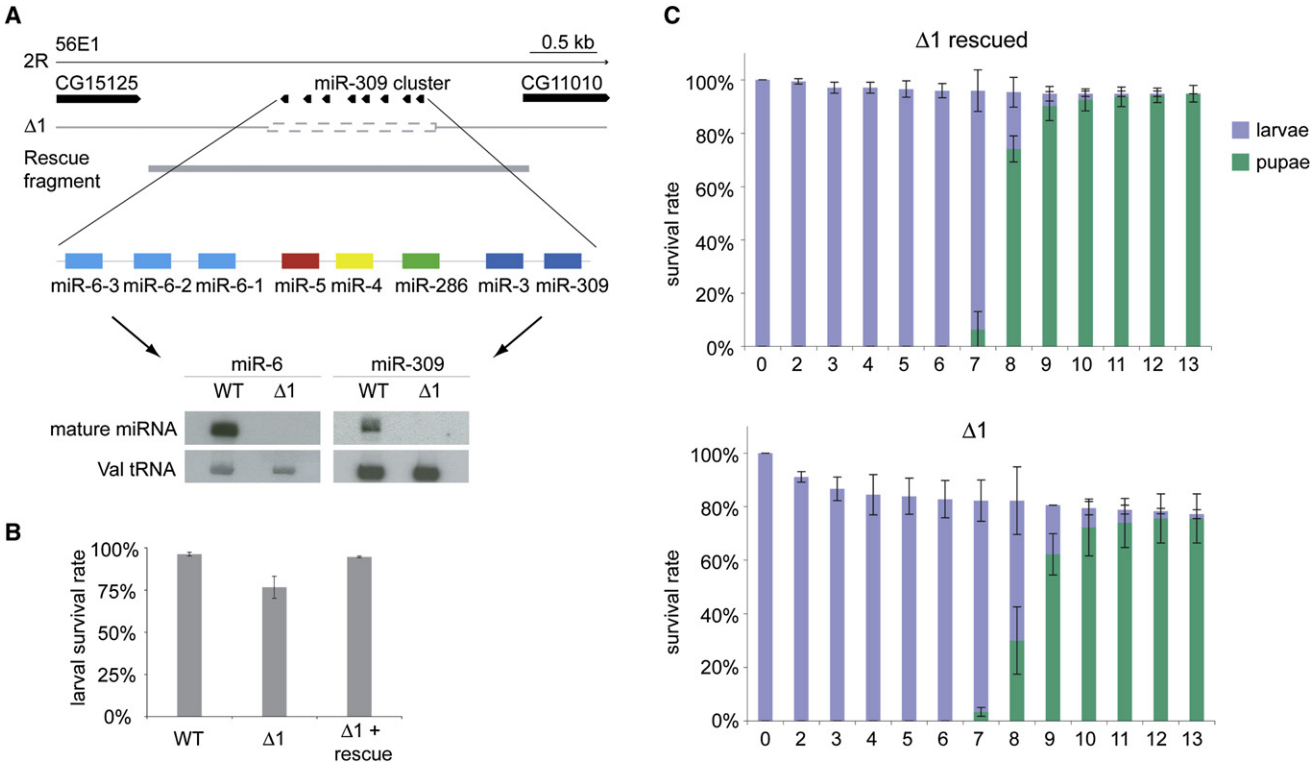

Figure 1. A Mutant Deleting the miR-309 miRNA Cluster

(A) Schematic representation of the genomic organization of the miRNA cluster. The region deleted in *miR-309-6*$^{\Delta 1}$ flies and the genomic rescue construct are indicated. In the middle, colors indicate distinct seed sequences. At the bottom, RNA blots showing miR-309 and miR-6 in control and *miR-309-6*$^{\Delta 1}$ 0-22 hr embryos are shown.

(B and C) Histograms comparing survival of control, *miR-309-6*$^{\Delta 1}$, and rescued larvae. Samples indicate the average of three independent collections. Error bars indicate standard deviation. One hundred first-instar larvae were seeded per vial and surviving pupae counted (B). The reduced survival of homozygous mutant *miR-309-6*$^{\Delta 1}$ larvae was rescued by introduction of an approximately 2.6 kb genomic DNA fragment (see [A]) as a transgene. Fifty first instar-larvae were seeded per vial and surviving larvae and pupae counted on the indicated days (C).

proteins did not reveal alterations in their expression levels or patterns in the miR-309 cluster mutant. For example, Figure S1F compares the expression of the predicted miR-3-miR-309 target Ftz with Even Skipped (which is not a predicted target). There was no striking difference between mutant and control embryos, consistent with the observation that miR-309 cluster mutant embryos did not show discernable embryonic patterning defects. The significance of the dynamics of spatial expression of the cluster miRNAs and the implied potential to regulate genes involved in embryonic patterning remains unclear.

miRNA-Mediated Downregulation of Maternal mRNAs

Given that the early onset of cluster miRNA expression does not appear to play a role in regulating zygotic mRNAs involved in patterning, we turned our attention to their potential to regulate the maternal-to-zygotic-transition. We compared expression of the miR-309 cluster to a high-resolution temporal gene expression profile of early embryonic development [9]. The miRNA expression analysis was performed on the RNA samples used by Pilot et al. ([9]; RNA samples kindly provided by Thomas Lecuit), in order to ensure comparability in the staging of the samples. mRNAs with a temporal expression profile most similar to that of the miR-309 cluster contained significantly fewer 7-mers complementary to miR-309 cluster miRNAs in their 3′ untranslated regions (UTRs) than would be expected by chance (Figure 3A, correlation coefficient bin 0.9 to 1 at far right; Table S1, $p < 0.01$). This suggests that these mRNAs have been under selection to reduce their regulation by the cluster miRNAs with which they are coexpressed. Reciprocally, 7-mer seed matches complementary to cluster miRNAs were enriched in the 3′ UTRs of maternal transcripts that were strongly downregulated as miRNA expression increased (Figure 3A, correlation coefficient bin –1 to –0.9; Table S1). The same trends hold true for 6-mer seed matches to cluster miRNAs. For the 6-mer set, the correlation data are more significant because of overall larger numbers of miRNA targets in each bin (Table S2).

To investigate whether early zygotic miR-309 cluster miRNA expression might contribute to this downregulation, we performed microarray analyses of control and mutant embryos at 0–1 hr and 2–3 hr of embryonic development. During the first hour, miR-309 cluster miRNAs are expressed at barely detectable levels, whereas they are strongly induced during the 2–3 hr interval (Figure 2A). We compared messenger RNA levels in control and miRNA mutant embryos. Messenger RNAs whose expression was upregulated in the absence of the cluster miRNAs were examined with reference to two sets of maternal mRNAs that had previously been classified as being moderately or strongly downregulated during the maternal-to-zygotic transition [12]. Forty-two of the 291 mRNAs (14%) that normally decrease by more than 3-fold between 2 and 3 hr of embryonic development were upregulated by over 1.5-fold in mutant embryos at this stage.

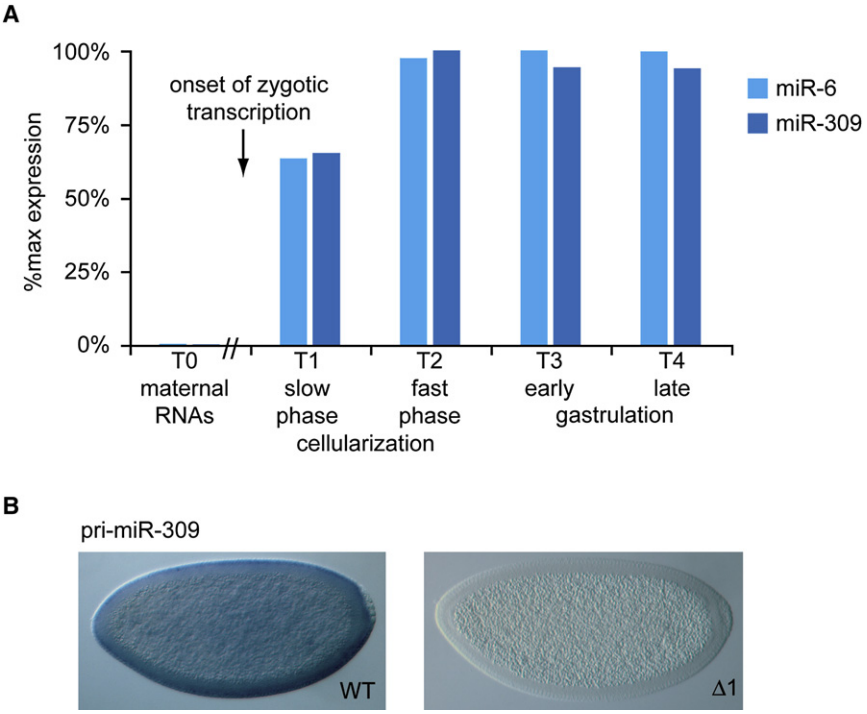

Figure 2. miR-309 Cluster Expression

(A) Quantitative PCR showing levels of mature miR-309 and miR-6, normalized to miR-184. Comparable results were obtained with normalization to miR-310. Time points are defined in [9]. Note that the x axis is not a linear time scale but indicates temporal bins (the larger gap between the T0 and T1 bins is indicated by //).

(B) In situ hybridization to detect the miR-309 cluster primary transcript. Lateral views are shown of control (WT) and *miR-309-6*$^{\Delta 1}$ embryos in the process of cellularization. Anterior is to the left, and dorsal is up.

This represents a 5-fold enrichment among the upregulated mRNAs and is statistically significant ($p < 1 \times 10^{-16}$; Figure 3B, Table S3). The effect of the removal of the miRNAs was stronger in the group of the 32 maternal transcripts annotated to decrease by more than 10-fold at this stage. Thirty-five percent of these were upregulated in the mutant (12/32), a 12.5-fold enrichment ($p < 1 \times 10^{-10}$; Figure 3B, Table S3).

The degree of enrichment of these annotated gene sets among our upregulated transcripts is likely to underestimate the true degree of correlation, because only 30% of the genome was included in the original classification of moderately or strongly downregulated maternal gene sets [12]. To get a more complete picture, we performed a similar analysis on the larger set of maternal mRNAs recently reported by Tadros et al. [2]. One thousand sixty-five mRNAs were classified as unstable maternal transcripts on the basis of expression profiling of RNA from unfertilized wild-type eggs and assessment of the degree of their destabilization over time. One hundred thirty-eight of the 1065 unstable maternal mRNAs were among the 410 mRNAs upregulated in cluster mutant embryos at 2–3 hr (Table S4). This represents more than 4-fold enrichment and is statistically highly significant ($p < 1 \times 10^{-52}$). There was no significant enrichment in 0–1 hr embryos (before the miRNAs are expressed). Much less enrichment was seen in the stable maternal class (1.2-fold; $p = 0.0035$), which contains both stable transcripts and transcripts that are stable in unfertilized eggs but likely degraded by the zygotic pathway in fertilized embryos. For example, some of the stable maternal class mRNAs [2] were classified as 3× down or 10× down by Arbeitman et al. [12]. Sixteen of these mRNAs were upregulated in the miRNA mutant and probably contribute to the 1.2-fold enrichment of mRNAs classified as maternal stable in this set. This analysis indicates that downregulation of maternal transcripts is impaired in the miRNA cluster mutant, suggesting that these miRNAs play a role in the zygotic pathway of maternal mRNA turnover (as hypothesized by Tadros et al.; [2]).

Temporal Reciprocity in miRNA-Target Relationships

The foregoing observations suggest that the miRNA cluster and its targets have largely reciprocal temporal expression patterns, a situation analogous to the spatially reciprocal relationship between many miRNAs and their targets at later stages of embryogenesis [13] and to the temporal relationship between the *C. elegans* heterochronic miRNAs and their targets [14–17]. To assess the significance of these observations, we compared the occurrence of miRNA cluster target sites among the regulated mRNAs with what would be expected to occur by chance. Among the 410 transcripts upregulated in the miRNA cluster mutant, 96 contained 7-mers complementary to the seed of one or more cluster miRNAs (Tables S3 and S4). This represents a statistically significant enrichment of 1.8-fold ($p < 1 \times 10^{-8}$).

Among the mRNAs upregulated in cluster mutant embryos at 2–3 hr, mRNAs from the set of maternal mRNAs examined by Arbeitman et al. (maternal_all), which contained such 7-mer sites, were enriched 3.6-fold [12], (Table S3, $p < 1 \times 10^{-5}$). The enrichment was 6.4-fold in the class of maternal mRNAs 3× downregulated containing such 7-mers ($p < 1 \times 10^{-6}$, Figure 3B lower panel, Table S3) and 48-fold in 10× downregulated set containing miR-309 cluster 7-mer sites ($p < 1 \times 10^{-5}$, Figure 3B lower panel, Table S3). Importantly, no significant enrichment of 7-mers was observed in 0–1 hr embryos, prior to the onset of miRNA cluster expression (Table S3, Figure 3B lower panel). Comparable analysis for the larger set of mRNAs [2] produced similar results (Table S4; Figure 3B lower panel). Maternal mRNAs containing target sites were enriched 2.5-fold ($p < 1 \times 10^{-8}$) and the set of unstable maternal mRNAs carrying target sites 6-fold among the mRNAs upregulated in cluster mutant embryos at 2–3 hr ($p < 1 \times 10^{-18}$). Again, no significant enrichment was seen in the 0–1 hr samples.

These statistical relationships suggest that the regulation of these mRNAs depends on the presence of the miRNA sites. To confirm that such sites are indeed functional, we prepared luciferase reporter constructs containing the 3′ UTRs of 32 of the affected maternal mRNAs from the different functional categories mentioned above and expressed them together with the miR-309 cluster in *Drosophila* S2 cells (Figure 3C). Twenty-nine of the 32 reporters were statistically significantly downregulated upon miR-309 cluster expression, indicating that they carry functional miR-309 cluster target sites.

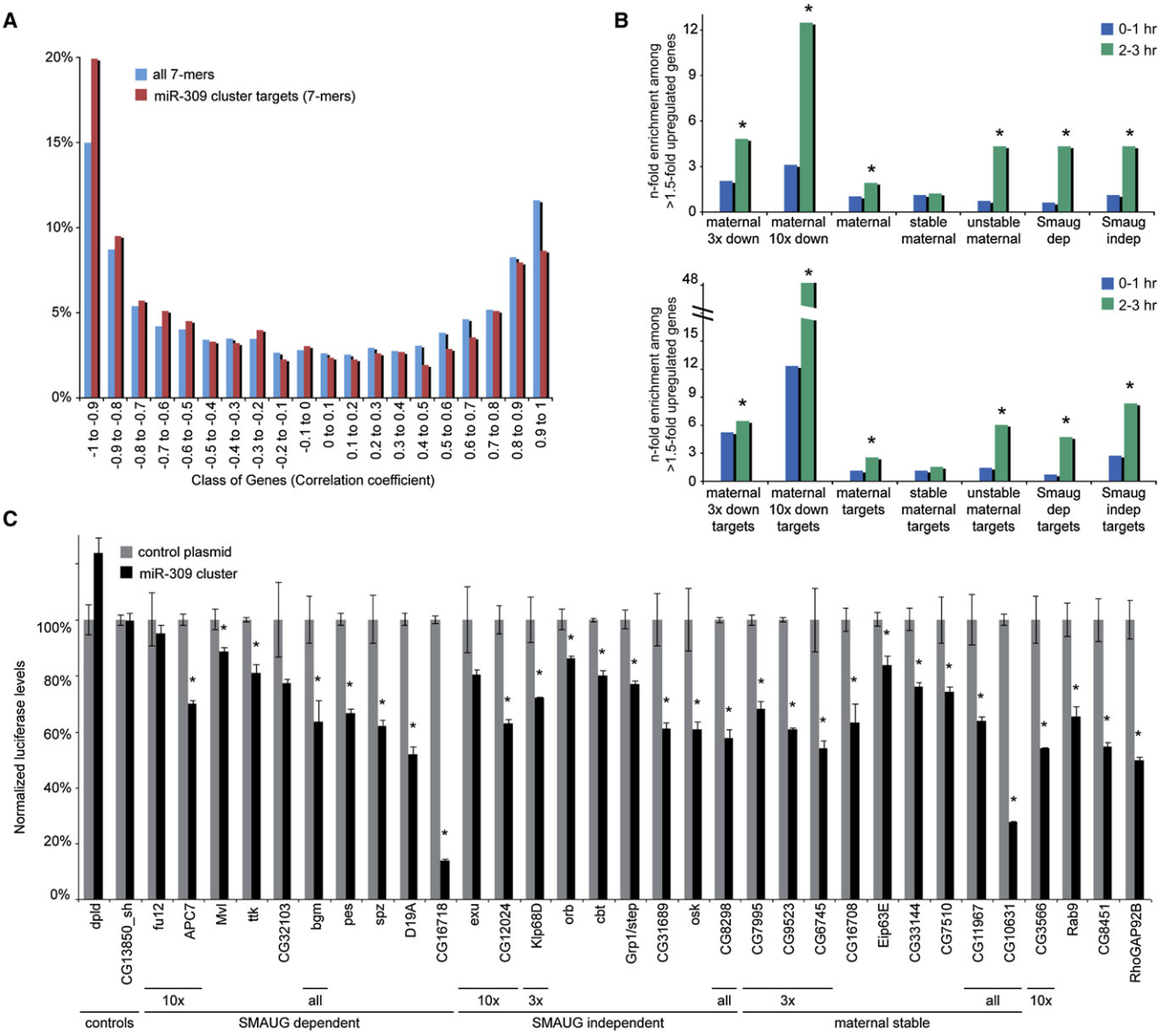

Figure 3. Effects of miR-309 Cluster miRNAs on Target Expression

(A) Histogram showing the proportion of 7-mer seed matches for all miRNAs and for the miR-309 cluster in a set of mRNAs sorted according to the degree of correlation between their temporal expression pattern (determined by expression profiling [9]) and that of the miRNAs in the miR-309 cluster. Note that both expression profiles were done on the very same RNA samples to ensure maximal comparability.

(B) Histograms showing the relative enrichment of maternal mRNAs (upper panel) and maternal mRNAs containing miR-309 cluster 7-mer sites (lower panel) among the transcripts upregulated by more than 1.5-fold ($q < 0.05$) in *miR-309-6*^{d1} 0–1 hr and 2–3 hr embryos. Gene classes maternal 3× down and maternal 10× down are defined in [12]; the remaining gene classes are defined in [2]. * indicates $p < 1 \times 10^{-5}$.

(C) Target validation using 3′ UTR reporters in a luciferase assay. 3′ UTRs of transcripts upregulated in the miR-309 mutant and carrying at least one 7-mer complementary to a miR-309 cluster miRNA in their 3′ UTR were tested. The control 3′ UTRs dpld and CG13850_sh lack miR-309 cluster 7-mers and are not repressed upon miR-309 cluster expression. The classifications by Arbeitman et al. [12] (maternal all, 10× and 3× down indicated as all, 10× and 3×) and by Tadros et al. [2] (maternal stable, maternal unstable, and SMAUG dependent versus independent), are indicated below. Error bars represent the standard deviation. * indicates $p < 0.05$, $n = 3$ (double-sided t test).

The cluster encodes miRNAs with five different seed sequences, reflecting the capacity to regulate different sets of target mRNAs. To assess the contribution of individual miRNAs to the effects of the cluster as a whole, we examined 7-mer seed matches complementary to individual miR-309 cluster miRNAs. Four of the five unique seeds (miR-3 and 309 have the same seed sequence) were significantly enriched among the upregulated mRNAs at 2–3 hr but not at 0–1 hr (Table S5). The magnitude of the enrichment and the statistical significance were stronger for miR-6, suggesting that it may contribute disproportionately to the effects of the cluster. This might be in part because miR-6 is present in three copies and so might be expressed at a higher level than the others. These data suggest that, with the possible exception of miR-286, the five distinct miRNAs encoded in the cluster act in concert to regulate a broad spectrum of mRNAs during the maternal-to-zygotic transition.

Comparison of the Maternal and Zygotic Systems for mRNA Turnover

SMAUG has been identified as a key component of the maternal system for maternal mRNA turnover in the embryo [2], whereas the evidence presented above suggests that the miR-309 cluster acts zygotically to promote turnover of maternal mRNAs. A priori, these systems might be functionally related, acting in concert. Alternatively, they might represent independent systems. To explore these possibilities, we examined the degree to which the sets of targets regulated by these two systems overlap.

Of the 1065 unstable maternal transcripts identified by Tadros et al. [2], 710 were identified as SMAUG targets by expression profiling of RNA from unfertilized eggs laid by *smaug* mutant flies (note: SMAUG is deposited maternally and acts on maternally deposited mRNAs). As mentioned before, 138 of the transcripts upregulated in the miR-309 cluster mutant at 2–3 hr were classified as unstable maternal transcripts, which represents more than 4-fold enrichment (Table S3; Figure 3B upper panel; $p < 1 \times 10^{-52}$). Ninety-two of these transcripts were also targeted by SMAUG, which represents more than 4-fold enrichment (Table S3, Figure 3B upper panel; $p < 1 \times 10^{-33}$). Of these, 20 (21.7%) had 7-mer seed matches complementary to cluster miRNAs in their 3′ UTRs and so might represent a set of mRNAs potentially coregulated by the maternal and zygotic systems. Other mRNAs among the SMAUG targets were not affected in the miRNA cluster mutants—for example, Hsp83, whose downregulation depends strongly on the SMAUG system [18]. Of the 355 unstable transcripts that had been reported to be SMAUG independent [2], 46 were among the 410 mRNAs upregulated in the miR-309 cluster mutant embryos. This represents a more than 4-fold enrichment (Table S3, Figure 3B upper panel; $p < 1 \times 10^{-16}$). Eighteen (39%) of these carry 7-mers complementary to miR-309 cluster miRNAs, an 8-fold enrichment (Table S3, Figure 3B lower panel; $p < 1 \times 10^{-11}$). This set includes mRNAs such as orb, oskar, and exuperantia and may represent the set of mRNAs regulated mainly by the zygotic system. Together, these data suggest that the maternal and zygotic systems regulate distinct but overlapping sets of maternal mRNAs.

The observation that some SMAUG targets also appear to be targets of the zygotic system raised the question of whether there might be a genetic interaction between the two systems. It can be expected that there might be an additive effect of removing two systems that share some common targets (if we assume that the common targets contribute to the mutant phenotype). To address this, we asked whether removing one copy of maternal SMAUG would enhance the severity of the zygotic miR-309 cluster mutant phenotypes. We did not observe a difference in embryonic survival rates between miR-309 cluster mutants and those also lacking one copy of maternal SMAUG. However, there appeared to be a small reduction in survival of miR-309 cluster mutant larvae whose mothers lacked one copy of SMAUG, from 85% ± 5% to 69% ± 12%. This difference was, however, not statistically significant (t test = 0.06). The marginal reduction in survival might reflect an additive effect of perturbing both systems on their common targets. It is possible that a further reduction of SMAUG activity might result in a statistically significant effect. At present, though, it is not possible to conclude that there is an interaction that is more than additive between the two systems.

Conclusions

These findings indicate that the early zygotic onset of miR-309 cluster miRNA expression acts to promote the turnover of many maternally deposited mRNAs. Failure to downregulate maternal mRNAs by this zygotic mechanism has knock-on effects on zygotic gene expression (data not shown) and may result in a late onset phenotype reflected by reduced survival and delayed larval development for many of the surviving animals. Elimination of the early zygotic expression of the miR-430 miRNA gene family also led to substantial misregulation of maternal mRNAs and to a late onset zygotic defect in Zebrafish [3]. Although miRNAs have been shown to act to ensure a proper transition between maternal and zygotic gene expression programs in flies and fish, the miRNAs involved are not conserved. Perhaps the fact that miRNAs act in part by leading to mRNA deadenylation, and subsequent destabilization, provided a means to promote turnover of a selected set of maternally deposited mRNAs. miRNAs may have been co-opted independently during evolution to fulfill a comparable function in different animals. The mechanistic basis for their action and the biological output are both conserved, but the miRNAs themselves and the identity of their targets are not. This may be an example of convergent evolution.

Experimental Procedures

Fly Strains and Genetics

w^{1118} flies were used as the control strain in all experiments. *miR-309-6*$^{\Delta 1}$ mutants were generated with ends-out homologous recombination essentially as described in [7]. For the genomic rescue, a 2.6 kb genomic fragment was amplified from w^{1118} genomic DNA with the primers 5′-GGAGCCCA TAGTGACTTCAATTA-3′ and 5′-GCCACTCGGTTTCCTCTATCCT-3′, cloned into pCasper4 and injected into w^{1118} to create transgenic flies.

miRNA Northern Blotting

Northern blotting was performed as described in [19].

In Situ Hybridization

In situ analysis was performed as described in [13]. Primers used to generate the pri-miR-309 probe were 5′-CAGTCGCCACCTATACAGTTTAAGG-3′ and 5′-TGCCACAACGAACTTCAATGG-3′.

miRNA qPCR

Wild-type total RNA from precisely staged early embryos was generously provided by Thomas Lecuit [9]. Primer sets designed to amplify mature miRNAs (miR-6, miR-309, miR-184, miR-310) were obtained from Applied Biosystems. Products were amplified from 10 ng of total RNA samples with the TaqMan MicroRNA Assay, Quantitative-PCR machine, and software from Applied Biosystems. The n-fold inductions of miR-6 and mir-309 were calculated relative to miR-184. Comparable results were obtained by normalization to miR-310.

Microarray Experiments

One hour egg collections of w^{1118} and *miR-309-6*$^{\Delta 1}$ mutant flies at 25°C were either aged (2–3 hr sample) at 25°C or directly processed (0–1 hr sample). Total RNA was extracted with Trizol reagent (GIBCOBRL) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized from 3 μg of total RNA of six independent samples for each time point according to Affymetrix One-Cycle protocol. Labeling and hybridization on Affymetrix Release 2 microarrays were performed according to Affymetrix protocols. Raw data was normalized with GCRMA [20], and significantly regulated genes were identified by Significance Analysis of Microarrays (SAM) [21, 22] (q value < 0.05 and n-fold change > 1.5).

Larval Survival

We seeded first-instar larvae in fly vials and counted surviving larvae and pupae on the indicated days by floating them out of the food with a 20% sucrose solution and transferring them into fresh vials.

Luciferase Assays

Luciferase assays were performed as described in [13].

Bioinformatics Analyses

To assess whether targets of the cluster miRNAs were preferentially coexpressed or anticorrelated in early wild-type embryos, we calculated the Pearson correlation coefficient for each gene's expression against miRNA expression across all five time points [9]. We binned all genes according to this coefficient (bin size 0.1) and determined the enrichment or depletion of 6-mers or 7-mers complementary to miRNA 5′ ends per bin by a hypergeometric p value. To assess misregulation in miR-309 cluster knockout flies, we selected genes that were 1.5× upregulated compared to the wild-type (q value < 5%). We then determined the enrichment of maternal genes [2, 12], genes containing 7-mers complementary to miRNA 5′ ends, and genes falling into both categories by a hypergeometric p value.

Supplemental Data

One figure and five tables are available at http://www.current-biology.com/cgi/content/full/18/7/501/DC1/.

Acknowledgments

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