The Smaug RNA-Binding Protein Is Essential for microRNA Synthesis During the Drosophila Maternal-to-Zygotic Transition

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ABSTRACT Metazoan embryos undergo a maternal-to-zygotic transition (MZT) during which maternal gene products are eliminated and the zygotic genome becomes transcriptionally active. During this process, RNA-binding proteins (RBPs) and the microRNA-induced silencing complex (miRISC) target maternal mRNAs for degradation. In Drosophila, the Smaug (SMG), Brain tumor (BRAT), and Pumilio (PUM) RBPs bind to and direct the degradation of largely distinct subsets of maternal mRNAs. SMG has also been shown to be required for zygotic synthesis of mRNAs and several members of the miR-309 family of microRNAs (miRNAs) during the MZT. Here, we have carried out global analysis of small RNAs both in wild-type and in smg mutants. Our results show that 85% of all miRNA species encoded by the genome are present during the MZT. Whereas loss of SMG has no detectable effect on Piwi-interacting RNAs (piRNAs) or small interfering RNAs (siRNAs), zygotic production of more than 70 species of miRNAs fails or is delayed in smg mutants. SMG is also required for the synthesis and stability of a key miRISC component, Argonaute 1 (AGO1), but plays no role in accumulation of the Argonaute family proteins associated with piRNAs or siRNAs. In smg mutants, maternal mRNAs that are predicted targets of the SMG-dependent zygotic miRNAs fail to be cleared. BRAT and PUM share target mRNAs with these miRNAs but not with SMG itself. We hypothesize that SMG controls the MZT, not only through direct targeting of a subset of maternal mRNAs for degradation but, indirectly, through production and function of miRNAs and miRISC, which act together with BRAT and/or PUM to control clearance of a distinct subset of maternal mRNAs.

KEYWORDS Smaug miRNA piRNA siRNA RNA-binding protein RNA degradation Argonaute Pumilio Brain tumor

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activation, binds to stem-loop structures in its target mRNAs, and recruits the CCR4/NOT-deadenylylase complex to destabilize these transcripts (Semotok et al. 2005, 2008; Tadros et al. 2007; Benoît et al. 2009; Siddiqui et al. 2012; Chen et al. 2014).

During the MZT, SMG binds to hundreds of maternal mRNA species, serving as the specificity factor that triggers their degradation (Chen et al. 2014). However, in smg mutants, not only these direct targets but hundreds of additional maternal mRNA species fail to undergo clearance (Tadros et al. 2007; Benoît et al. 2009). We have hypothesized that, at least in part, this is because zygotic genome activation fails in the absence of SMG, and, therefore, the transcription-dependent, later waves of maternal mRNA decay are affected indirectly (Benoit et al. 2009). Contributing to one of these later waves is the mirR-309 locus, which encodes a dozen species of miRNAs with the capacity to target several hundred maternal transcripts for degradation (Bushati et al. 2008). We previously showed that several mirR-309 family miRNA species are not produced in embryos derived from smg mutant mothers and that mirR-309-dependent maternal mRNAs remain stable (Benoit et al. 2009).

Here, we have carried out global analyses of small RNAs during the MZT in both wild-type and smg mutants. First, by analyzing activated, unfertilized eggs, which are loaded with maternally encoded products but do not undergo zygotic genome activation, we were able to identify maternally loaded small RNAs. Second, by analyzing two embryonic time-points, one during the early phase of maternal transcript clearance before large-scale zygotic genome activation and the second after completion of the both the early phase of clearance and global zygotic genome activation, and comparing these to unfertilized eggs, we were able to identify changes in small-RNA populations during the MZT. Third, by analyzing these same time-points in embryos from smg mutant females, we were able to define global roles for SMG in small RNA metabolism during the MZT.

Loss of SMG has a profound effect on miRNAs but little effect on piRNAs or siRNAs. Failure to produce zygotic miRNAs in smg mutants correlates with failure to clear maternal miRNAs with seed sequences for these miRNA species. We show that SMG is also required for the accumulation of AGO1 protein during the MZT. Thus, SMG controls the MZT through direct regulation of maternal mRNAs and, indirectly, through mirRISC, which targets additional maternal mRNA species for clearance.

Recently, we identified the direct targets of the BRAT and PUM RBPs in early embryos and showed that these targets are distinct from those of SMG (Laver et al. 2015a). In addition, we showed that BRAT plays an important role in maternal mRNA decay during the MZT (Laver et al. 2015a). Here we show that BRAT and PUM share target mRNAs with those predicted for mirRISC during the MZT, and that the mirR-309 family of miRNAs shares predicted targets with BRAT but not PUM. We hypothesize that SMG’s indirect regulation of maternal mRNA clearance is likely to be implemented by mirRISC acting together with BRAT and/or PUM.

**MATERIALS AND METHODS**

**Experimental methods**

**Fly stocks:** All *Drosophila* cultures were raised at 25°C under standard conditions. The following fly strains were used: (1) w1118, (2) VASA-GFP/CyO; Pr Dv/TM3, Sh, (3) VASA-GFP/CyO; smgP/TM3, Sh, (4) Df(3L)Scf-R6/TM3, Sh (Bloomington Stock #4500), (5) smg7/TM3 (Chen et al. 2014), and (6) w1118, P[FLAG.HA.AGO2]2 (Bloomington Stock #33242). We have shown that the smgP mutation (Dahanukar et al. 1999) results in production of a truncated SMG protein lacking the RNA-binding domain (Benoit et al. 2009), while the smg7 mutation is a deletion that results in a complete lack of SMG protein (Chen et al. 2014). The generation of the SMGW and the point-mutant SMGB transgenic lines has been described previously (Semotok et al. 2008). Embryos from VASA-GFP; Pr Dv/TM3, Sh females were used as the wild-type controls and are referred to throughout the text as “wild-type embryos.” VASA-GFP/CyO; smgP/Df(3L)Scf-R6 females or smg7/hozygous females were used to produce embryos that are referred to throughout the text as “smg mutant embryos” rather than the more cumbersome “embryos produced by smg mutant females.”

**Small RNA library construction and next-generation sequencing:** Samples were collected at the following time-points: 0–2 hr old unfertilized eggs (U0–2h), 0–2 hr old embryos (F0–2h), and 2–4 hr old embryos (F2–4h). Each time-point had three biological replicates. Total RNA was extracted using TRIzol (Invitrogen) following the manufacturer’s protocol. The small-RNA fraction was isolated from the total RNA sample using a published modification of the mirVana (MirVANA kit, Invitrogen) protocol (Gu et al. 2011). 2S RNA depletion followed a published method (Seitz et al. 2008) as did small RNA cloning (Gu et al. 2011). High-throughput sequencing of the small RNA libraries was performed using an Illumina HiSeq2500 platform at the Tufts University Core Facility (http://tucf-genomics.tufts.edu/).

**Western blots:** About 30 μg total protein was resolved by 8% SDS-PAGE. After electrophoresis, proteins were transferred to a PVDF membrane (BioRad, Immob-Blot). The membrane was blocked with 5% milk in TBST (20 mM Tris-Cl, pH 7.5, 150 mM NaCl, and 0.1% Tween20) at room temperature for 1 hr. After blocking, the membrane was incubated overnight at 4°C with primary antibody. After washing three times with TBST, the membrane was incubated for 1 hr at room temperature with secondary antibody. Western blots were imaged and quantified using a BioRad Imaging System (ImageLab).

**Antibodies:** Guinea pig polyclonal anti-SMG antibody (Tadros et al. 2007) was used at 1:10,000, guinea pig polyclonal anti-DP1 antibody (Nelson et al. 2007) at 1:10,000, mouse monoclonal anti-tubulin antibody at 1:10,000 (Sigma); mouse monoclonal antibody for AGO1 (Miyoshi et al. 2005) at 1:1000; and mouse monoclonal anti-FLAG antibody (M2) at 1:1000 (Sigma). Antibodies for Argonautes associated with si- and piRNAs are described in Supplemental Material, File S1.

**RT-qPCR:** Total RNA was isolated from snap-frozen embryo samples using the TRIzol reagent (Life Technologies). The quantity and quality of the extracted RNA were determined by reading the optical densities at 260 and 280 nm (OD 260 / 280) using a NanoDrop spectrophotometer (Thermo Scientific). For mRNA quantification, 1 μg of total RNA was reverse transcribed in a 20 μl reaction using a Vilo Superscript mix (Life Technologies) following the manufacturer’s protocol. The single-stranded cDNA was used to perform quantitative real-time PCR with the SYBR green PCR master mix (Bio-Rad) using a CFX384 Real-Time System (Bio-Rad). Three technical replicates were performed for each RT primer. Relative levels of different transcripts were determined using the ΔΔCT method. RpL32 transcript levels were used for normalization. RT-qPCR primers are listed in Table S1 (Tab 5: RT-qPCR primers).

**Computational and bioinformatics methods**

**Small-RNA extraction and annotation:** Prior to read mapping, small-RNA reads were processed using the FASTX-Toolkit (http://
hannonlab.cshl.edu/fastx_toolkit/) to demultiplex, remove adaptor sequences, filter sequence quality (phred quality $\geq 20$ in 100% of nucleotides), size trim and filter [18–30 nucleotides (nt)], and collapse fastq reads. Filtered reads were mapped to Drosophila Genome Release 5.50 using Bowtie 0.12.8 in the -v alignment mode (Langmead et al. 2009). We considered only reads with perfect matches to the genome in subsequent analyses except where specifically mentioned. For miRNA expression analysis, we remapped these to a custom-made Bowtie index of reference sequences (see below). Reads mapping to multiple loci were distributed uniformly among these loci. For example, if one read mapped to two locations, each location was assigned 0.5 reads.

Samtools (Li et al. 2009) and BEDTools (Quinlan and Hall 2010) were used for data processing and analysis. Representative classes for small RNAs were determined by intersection with the General Feature Format (GFF) file or aligned to a custom-made Bowtie index of reference sequences in the following order: miRNA, small RNA (tRNA, rRNA, snRNA, snoRNA, and ncRNA), cis-NAT-loci, transposable element consensus sequence, exon and intron sequence, and intergenic region.

miRNA: Hairpin.fa, mature.fa, and dme.gff3 (version 19) were downloaded from miRBase (Kozomara and Griffiths-Jones 2011). Noncanonical miRNAs, specifically meaning that they were out of range of annotated mature miRNA sequences, were constructed by extending two nt at the 5’-end and five nt at the 3’-end of annotated mature miRNA species.

Cis-NAT-siRNA: We used lists of previously published cis-NAT siRNA loci in the ovary (Czech et al. 2008; Okamura et al. 2008) to extract cis-NAT-siRNAs (21 nt only).

TE-siRNA and TE-piRNA: The consensus transposable element sequence (Version 9.4.1 from the Berkeley Drosophila Genome Project (http://www.fruitfly.org/p_disrupt/TE.html) was used for transposable element mapping. Previously published genomic piRNA cluster loci (Brennecke et al. 2007) were used to check the distribution of both of TE-siRNAs (21 nt only) and TE-piRNAs (23 nt) in the genome.

Other reference sequences: tRNA, rRNA, snoRNA, snRNA, ncRNA, and exon, intron, and intragenic regions were downloaded from FlyBase (Drosophila Genome Release 5.50).

Data normalization and difference expression: In high-throughput sequencing data, when a small number of highly expressed genes contributes a substantial proportion of the sequenced reads, the remaining genes will falsly appear to be downregulated. For example, the miR-309 cluster accounts for > 60% of miRNA reads in 2–4 hr wild-type embryo samples. To compensate for this, the trimmed mean of M-values (TMM) normalization method was used for small RNA expression normalization. We used reads per million (RPM) for comparison of expression levels between libraries. Small RNA reads RPM > 1 in at least six of 18 libraries was used as a cutoff. We ran a generalized linear model Likelihood Ratio Test (glmLRT, for multifactor experiments) for our datasets. FDR $\leq 5\%$ was considered significant (edgeR, Robinson et al. 2010).

Analysis of miRNAs: To identify nontemplated addition (NTA)-miRNA isoforms, we took the imperfectly matched reads in our small RNA library, trimmed off the last three nucleotides from the 3’-end, and then realigned with the genome reference sequence. A subset of these reads then mapped perfectly to the genome reference; therefore, the mismatch came from the 3’-most three nucleotides. We then realigned these mapped reads to the pre-miRNA sequence without trimming and allowing a maximum of three mismatches. Only the 3’-additional sequences that did not match the pre-miRNA sequence were considered as postcleavage nucleotide modification events. For these three nucleotides, only the following patterns were considered as NTA: match-match-mismatch, match-mismatch-mismatch, and mismatch-mismatch-mismatch.

Combined analysis of the three isoform types: The threshold used was $\Sigma (\text{RPM}_{\text{canonical}} + \text{RPM}_{\text{noncanonical}} + \text{RPM}_{\text{NTA}}) \geq 10$ in one or more of the six sample sets (the six sets comprised the three time-points by two genotypes; each set included three biological replicates). RPM was converted to log2, and then miRNA species were classified by hierarchical clustering (k = 5) in the R software package (Version 3.1.2) (Shen 2013). Table S1: Tab 2, Expression-profiles of miRNA class.

Detailed analysis of a subset of highly expressed noncanonical miRNAs (Figure S2): To be included in the analysis, a noncanonical miRNA species had to meet three criteria: (1) $\Sigma (\text{RPM}_{\text{canonical}} + \text{RPM}_{\text{noncanonical}} + \text{RPM}_{\text{NTA}}) \geq 10$ in one or more of the six sample sets (the six sets comprised the three time-points by two genotypes; each set included three biological replicates); (2) RPM$_{\text{noncanonical}} / \Sigma (\text{RPM}_{\text{canonical}} + \text{RPM}_{\text{noncanonical}} + \text{RPM}_{\text{NTA}}) \geq 0.5$ in one or more of the six sample sets; and (3) $|\text{RPM}_{\text{wild-type eggs}} - \text{RPM}_{\text{wild-type embryos}}| \geq 0.2$. See Table S1: Tab 3, Exp noncanonical and NTA miRNAs.

Comparative analysis of miRNA targets: To define the set of canonical miRNAs downregulated in smg mutants, we chose canonical miRNAs with FDR $\leq 5\%$ at F0–2h or F2–4h (glmLRT) and RPM$_{\text{canonical}} \geq 10$ in one or more of the six sample sets (the six sets comprised the three time-points by two genotypes). To identify miRNA targets of these downregulated miRNAs, we downloaded datasets from TargetScanFly 6.2 (Schnall-Levin et al. 2010) and extracted Drosophila melanogaster conserved target sites in the ORF and 3’UTR. Since TargetScanFly 6.2 does not have the seed sequences for all miRNAs, we only chose canonical miRNAs that were in the TargetScanFly list for comparative analysis as described above. Previously defined lists were used for SMG-dependent-for-decay mRNAs (embryos 2–3 hr, 5% FDR) (Benoit et al. 2009) and SMG-RIP mRNAs (embryos 0–3 hr, 5% FDR) (Chen et al. 2014). Statistical analysis used the one-tailed Fisher’s exact test with the Bioconductor GeneOverlap package (Version 1.6.0) in the R software package (Version 3.1.2) (Shen 2013).

Data availability

The data reported in this study have been deposited in NCBI’s Gene Expression Omnibus and are accessible through GEO series accession number GSE82194.
RESULTS AND DISCUSSION

Identification of small RNAs present during the MZT

To identify small RNA species expressed during the *Drosophila* MZT and to assess the role of SMG in their regulation, we produced and sequenced 18 small RNA libraries: nine libraries from eggs or embryos produced by wild-type females and nine from *smg* mutant females (the genotypes of the females are in the Materials and Methods). The 18 libraries comprised three biological replicates each from the two genotypes and three time-points: (1) 0–2 hr old unfertilized eggs, in which zygotic transcription does not occur and thus only maternally encoded products are present; (2) 0–2 hr old embryos, the stage prior to large-scale zygotic genome activation; and (3) 2–4 hr old embryos, the stage after large-scale zygotic genome activation. After pre-alignment processing (see Materials and Methods), a total of ~144 million high quality small-RNA reads was obtained and 110 million of these perfectly matched the annotated *Drosophila* genome (FlyBase 5.50). The components of our small RNA libraries are shown in Table S1 (Tab 1: Small RNA library components).

We found that loss of SMG had no significant effect on piRNAs and siRNAs, or on the Argonaute proteins associated with those small RNAs: Piwi, Aubergine (AUB), AGO3, and AGO2, respectively. In contrast, loss of SMG resulted in a dramatic, global reduction in miRNA populations during the MZT as well as reduced levels of AGO1, the miRISC-associated Argonaute protein in *Drosophila*. Our analyses of miRNAs and AGO1 are presented in the body of this manuscript (Figure 1, Figure 2, Figure 3, Figure 4, Figure 5, Figure S1, Figure S2, Table 1, Table 2, Table 3, and Table S1) while the data on piRNAs, siRNAs, and the AGO2, AGO3, AUB, and Piwi proteins are exclusively in the supplemental data (Figure S3, Figure S4, Figure S5, Figure S6, File S1, and Table S1).

miRNA isoforms present during the MZT

A pre-miRNA can generate three types of mature miRNA: (1) a canonical miRNA, which has a perfect match to the annotated mature miRNA; (2) a noncanonical miRNA, which shows a perfect match to the annotated mature miRNA but with additional nucleotides at the 5’- or 3’-end that match the adjacent primary miRNA sequence; and (3) a miRNA with nontemplated terminal nucleotide additions (an NTA-miRNA), which has nucleotides at its 3’-end that do not match the primary miRNA sequence.

In our libraries, we identified a total of 364 distinct miRNA species that mapped to miRBase (Version 19), comprising 85% (364/426) of all annotated mature miRNA species in *Drosophila*. Thus, the vast majority of all miRNA species encoded by the *Drosophila* genome are expressed during the MZT. Overall, in the wild type, an average of 75% of all identified miRNAs fell into the canonical category. The remaining miRNAs were either noncanonical (10%) or NTA-miRNAs (15%).

To validate our sequencing results, we compared those mature miRNA species identified in our data that perfectly matched the *Drosophila* genome sequence (i.e., canonical and noncanonical) with a previously published miRNA dataset from 0 to 6 hr old embryos (Ruby et al. 2007). To avoid differences caused by miRBase version, we remapped data sets from that previous study (GSM180330 and GSM180331, 0–6 hr) to miRBase Version 19 and found that 99% of their published miRNA species were on our miRNA list (176/178 mature miRNA species comprising 161 canonical miRNAs and 94 noncanonical miRNAs). There were an additional 181 mature miRNA species in our library that had not been identified as expressed in early embryos in the earlier study.

As a second validation, we compared our list of maternally expressed miRNA species (those present in our 0–2 hr wild-type unfertilized egg samples) with the most recently published list of maternal miRNAs, which had been defined in the same manner (Marco 2015). We found that that 99% of the 86 published maternal miRNA species were on our maternal miRNA list (85/86). We identified an additional 144 maternal miRNA species in our library that had not been observed in that study.

Our identification of a large number of additional miRNA species in unfertilized eggs and early embryos can be attributed to the depth of coverage of the current study. Therefore, our dataset provides the most complete portrait to date of the miRNAs present during the *Drosophila* MZT.

Changes in miRNA populations during the MZT

Next, we analyzed global changes in miRNA species during the MZT in wild-type embryos. We observed a dramatic increase in the proportion of miRNAs relative to other small RNAs, which was due to an increase in absolute miRNA amount rather than a decrease in the amount of other types of small RNAs (Table S1: Tab 1, Small RNA library components). In wild-type 0–2 hr unfertilized eggs, the proportion of our small RNA libraries comprised of canonical and noncanonical miRNAs was 12.8% (mean RPM = 117,068). These represent maternally loaded miRNAs, since unfertilized eggs do not undergo zygotic genome activation. The proportion of small RNAs represented by miRNAs increased dramatically during the MZT, reaching 50.7% in 2–4 hr embryos (mean RPM = 835,638). The other abundant classes of small RNAs underwent either no change or relatively minor changes over the same time-course. We conclude that there is a large amount of zygotic miRNA synthesis during the MZT in wild-type embryos.

For more detailed analysis of the canonical, noncanonical, and NTA isoforms, we focused on 154 miRNA species that possessed an average of ≥10 RPM for all three isoform types in one or more of the six sample sets (the six sample sets comprised three biological replicates each of the two genotypes and three time-points; see Table 1 and Materials and Methods). Here, we focus on changes in wild-type; changes in the *smg* mutant will be presented in the next section. Among all miRNAs, in wild-type the proportion of canonical isoforms increased over the time-course from 69 to 83%, the proportion of noncanonical miRNAs remained constant (from 9 to 10%), and the proportion of the NTA-miRNAs decreased (from 22 to 7%). These results derive from the fact...
that, during the MZT, the vast majority of newly synthesized miRNAs were canonical, undergoing a more than sevenfold increase from 103,105 to 744,043 RPM; that noncanonical miRNAs underwent a comparable, nearly sevenfold, increase from 13,902 to 92,199; whereas NTA-miRNAs underwent a less than twofold increase, from 32,840 to 63,847, thus decreasing in relative proportion.

**Global impact of smg mutations on miRNA populations during the MZT**

Whereas the proportion of the small-RNA population that was comprised of miRNAs increased fourfold over the wild-type time-course, concomitant with increases in overall miRNA abundance, there was no such increase in the *smg* mutant embryos: 21.9% of the small RNAs were miRNAs in 0–2 hr unfertilized *smg* mutant eggs (mean RPM = 203,415) and 20.5% (mean RPM = 196,110) were miRNAs in 2–4 hr embryos (F0–2h). Box plots showing that canonical, noncanonical, and 3′-end nontemplated addition (NTA) miRNAs were significantly downregulated in *smg* mutant embryos at 0–2 hr and 2–4 hr. *P* values are from the Wilcoxon rank sum test: ** ≤ 0.01, and *** ≤ 0.001. FDR, false discovery rate; miRNA, microRNA; wt, wild type.

**Figure 1** miRNA expression is dramatically reduced in *smg* mutant embryos. (A) Scatter-plots showing canonical miRNA expression levels in reads per million (RPM) in wild-type and *smg* mutants in 0–2 hr unfertilized eggs (UF0–2h), 0–2 hr old embryos (F0–2h), and 2–4 hr old embryos (F2–4h). (B) Box plots showing that canonical, noncanonical, and 3′-end nontemplated addition (NTA) miRNAs were significantly downregulated in *smg* mutant embryos at 0–2 hr and 2–4 hr. *P* values are from the Wilcoxon rank sum test: ** ≤ 0.01, and *** ≤ 0.001. FDR, false discovery rate; miRNA, microRNA; wt, wild type.

**Classification of miRNA species into expression classes and the role of SMG**

As described above, during the wild-type MZT, canonical miRNAs comprised the major isomorph that was present (69–83% of miRNAs). Therefore, we next asked whether miRNA species could be categorized into different classes based on their expression profiles during the wild-type MZT. We analyzed 131 canonical miRNA species that had ≥ 10 mean RPM in at least one of the six datasets (see Materials and Methods). Hierarchical clustering of their log2RPM values identified
five distinct categories of canonical miRNA species during the MZT. We then assessed the effects of smg mutations on each of these classes. The data are shown in Figure 2 and Table S1 (Tab 2, Exp-profiles of miR class).

Class A (50 miRNA species): Nonmaternal, low zygotic: Wild type: Maternal deposition of this set of miRNA species was absent or very low with an average RPM of 1.4 in 0–2 hr unfertilized eggs. These species accumulated at low levels upon zygotic genome activation, reaching an average of 72.6 RPM in 2–4 hr embryos. Consistent with our conclusion that this class of miRNAs is zygotically synthesized is the fact that 13 of 21 Class A miRNA species that had been examined in a previous study were shown there to be downregulated when the zygotic transcription factor, Zelda (ZLD), is mutated (Fu et al. 2014). Three of the Class A miRNA species are part of the 12-member miR-309 family, which is expressed zygotically and targets a subset of maternal mRNAs for degradation (Bushati et al. 2008).

smg mutants: Class A miRNAs failed to accumulate in smg mutants with an average RPM of 1.2 in 0–2 hr unfertilized eggs, 1.1 RPM in 0–2 hr embryos, and 2.1 RPM in 2–4 hr embryos. Consistent with these observations, we previously showed that one of the Class A species, a miR-309 family member called mir-3-5p, fails to increase in abundance in smg mutants (Benoit et al. 2009).

Class B (10 miRNA species; low maternal, high zygotic): Wild type: Class B miRNA species were loaded at low levels maternally, averaging 23.7 RPM in 0–2 hr unfertilized eggs, then increasing to an average abundance of 702 RPM in 0–2 hr embryos, and 5557 RPM in 2–4 hr embryos. Nine of 10 Class B miRNA species have been shown to be downregulated in zld mutants (Fu et al. 2014). Half of the members of this class derive from the miR-309 family.

smg mutants: In contrast to the wild type, in smg mutants there was no significant increase in average abundance of Class B miRNA species between 0–2 hr unfertilized eggs (23.9 RPM) and 0–2 hr embryos (38.9 RPM). However, their average abundance increased almost 10-fold by 2–4 hr to 320 RPM. These results suggest that, in smg mutants, synthesis of Class B miRNA species is delayed or reduced rather than absent. This unexpected conclusion was enabled by the global nature of the current analysis relative to our previous study of a small number of miR-309 family miRNAs (Benoit et al. 2009).

Class C (12 miRNA species; high maternal, very high zygotic): Wild type: Class C miRNA species were found to be maternally loaded at high levels as well as synthesized at very high levels zygotically. They were present in 0–2 hr unfertilized eggs at 1301 RPM for degradation (Bushati et al. 2008).

smg mutants: Class C miRNAs were present in smg mutants in 0–2 hr unfertilized eggs at 1255 RPM and 0–2 hr embryos at 1301 RPM. These results suggest that in smg mutants, synthesis of Class C miRNA species is delayed or reduced rather than absent.

Class D (15 miRNA species; low maternal, low zygotic): Wild type: Class D miRNA species were loaded at low levels maternally, averaging 2.5 RPM in 0–2 hr unfertilized eggs, then increasing to an average abundance of 108 RPM in 0–2 hr embryos, and 301 RPM in 2–4 hr embryos. These results suggest that in smg mutants, synthesis of Class D miRNA species is delayed or reduced rather than absent.

smg mutants: Class D miRNAs were present in smg mutants in 0–2 hr unfertilized eggs at 1.5 RPM and 0–2 hr embryos at 2.1 RPM. These results suggest that in smg mutants, synthesis of Class D miRNA species is delayed or reduced rather than absent.

Class E (45 miRNA species; very high maternal, low zygotic): Wild type: Class E miRNA species were loaded at very high levels maternally, averaging 100 RPM in 0–2 hr unfertilized eggs, then increasing to an average abundance of 300 RPM in 0–2 hr embryos, and 500 RPM in 2–4 hr embryos. These results suggest that in smg mutants, synthesis of Class E miRNA species is delayed or reduced rather than absent.

smg mutants: Class E miRNAs were present in smg mutants in 0–2 hr unfertilized eggs at 80 RPM and 0–2 hr embryos at 100 RPM. These results suggest that in smg mutants, synthesis of Class E miRNA species is delayed or reduced rather than absent.

Figure 2 miRNA expression profiles fall into five classes. (A) A heat map showing the expression profiles of each miRNA at the three developmental stages. The miRNA species were classified into five classes (A through E) by hierarchical clustering (k = 5). Each row represents one species of miRNA log2RPM. The gray bar labeled wt.UF.RPM shows the abundance of maternally loaded miRNAs in RPM as indicated. (B) Plots showing the average expression levels of the miRNA species in each class, in wild-type (solid line) and smg mutant (dashed line). UF0-2h, 0–2 hr unfertilized eggs; F0-2h, 0–2 hr embryos; F2-4h, 2–4 hr embryos. miRNA, microRNA; RPM, reads per million; wt, wild type.
and increased to averages of 7821 RPM at 0–2 hr and 24,042 RPM at 2–4 hr. Five of the 12 members of this class derive from the miR-309 family.

**smg mutants:** Class C miRNA species reached a roughly similar abundance in 2–4 hr mutant embryos (5752 RPM average) to that seen at 0–2 hr in the wild type (7820 RPM average), suggesting that, as was the case for Class B miRNAs, zygotic production of Class C miRNA species is delayed or reduced but not absent in the mutant. One of the miR-309 family miRNA species that we previously analyzed by northern blots in *smg* mutants (Benoit et al. 2009), miR-3, belongs to this class.

**Class D (26 miRNA species; low maternal, constant level):** *Wild type:* There was no significant change in the abundance of Class D miRNA species in the embryo time-course relative to unfertilized eggs. They were present in unfertilized eggs at an average of 46.1 RPM that increased to 71.9 by 2–4 hr. Therefore, Class D represents either stable maternal miRNAs with a small amount of zygotic synthesis or a balance in which synthesis of new miRNAs in embryos compensates for turnover of maternal copies.

**smg mutants:** The expression profiles of Class D miRNA species were very similar in *smg* mutants and the wild type. We observed a drop in average levels from 73.9 RPM in unfertilized eggs to 28.1 in 0–2 hr mutant embryos followed by an increase to 47.8 in 2–4 hr embryos. These data are consistent with decay of maternally contributed copies together with delayed (or lower level) synthesis of zygotic copies in *smg* mutants relative to the wild type. The small magnitude of these changes did not, however, permit a firm conclusion.

**Class E (33 miRNA species; high maternal, constant level):** *Wild type:* Wild-type MZT. This is likely to be a consequence of production during the MZT, all 10 NTA isoforms underwent an increase in both proportion and abundance during the MZT in the wild type and (2) there is a failure (or delay) in production of new canonical and noncanonical isoforms in *smg* mutants. Strikingly, six of these increased in relative proportion and abundance during the wild-type MZT but showed little or no increase in either relative proportion or absolute abundance in *smg* mutants (Figure S2 and Table S1: Tab 3, Exp noncanonical and NTA miRNAs). These data are consistent with the hypotheses that, for these miRNA species, (1) there is a higher relative production of the noncanonical than the canonical isoform during the MZT in the wild type and (2) there is a failure (or delay) in production of new canonical and noncanonical isoforms in *smg* mutants.

**noncanonical miRNAs:** Consistent with published observations (Fernandez-Valverde et al. 2010; Berezikov et al. 2011), most of the noncanonical miRNAs in our dataset exhibited the 3′-extended version. The noncanonical isoforms of 10 miRNAs met the above-specified criteria. Strikingly, six of these increased in relative proportion and abundance during the wild-type MZT but showed little or no increase in either relative proportion or absolute abundance in *smg* mutants (Figure S2 and Table S1: Tab 3, Exp noncanonical and NTA miRNAs). These data are consistent with the hypotheses that, for these miRNA species, (1) there is a higher absolute increase in the noncanonical than the canonical isoform during the MZT in the wild type and (2) there is a failure (or delay) in production of new canonical and noncanonical isoforms in *smg* mutants.

**NTA-miRNAs:** Recently, it has been shown that maternally loaded miRNAs are highly adenylated (Lee et al. 2014). Consistent with this, we observed a higher proportion of NTA miRNAs in our wild-type unfertilized egg samples than in our embryo samples (Table 1). Most of the nontemplated terminal modifications detected were mono- or di-nucleotide additions at the 3′-end (Zhou et al. 2012), which are known to influence miRNA stability and target mRNA regulation (Katoh et al. 2009). Adenine has been shown to be the most frequent nontemplated 3′-nucleotide (Burroughs et al. 2010; Fernandez-Valverde et al. 2010; Lee et al. 2014); we found that A-addition occurred for a large percentage of the NTA-miRNAs: A (48–50%), AA (12–20%), U (7–12%), AAA (2–8%), C (3–6%), and G (1–2%) (Figure S1).

The NTA isoforms of 25 miRNAs met the above-mentioned criteria, 10 of which showed particularly striking changes during the MZT in wild-type but not in *smg* mutants (Figure S2 and Table S1: Tab 3, Exp noncanonical and NTA miRNAs). Whereas their canonical versions all underwent increases in both proportion and abundance during the MZT, all 10 NTA isoforms underwent an increase in abundance but a decrease in relative proportion during the wild-type MZT. This is likely to be a consequence of production of canonical and noncanonical isoforms outstripping production of NTA isoforms during the MZT. In contrast, in *smg* mutants, all 10 NTA miRNA isoforms showed either no absolute increase in abundance or a very small increase when compared to the wild type. However, relative to the wild type, in the mutant they showed either a smaller relative decrease in proportion or even an increase in proportion. These results are consistent with replacement of maternally loaded NTA-miRNAs with the zygotically produced canonical or noncanonical counterparts during the MZT. Because of the observed decrease in zygotic synthesis of canonical and noncanonical isoforms in *smg* mutants, the amplitude of the relative decrease was smaller (or there was a relative increase) for the NTA isoform.
Table 2 Overlap of mRNAs that are bound or regulated by Smaug, and those that are targets of miRNAs that are downregulated in smaug mutants

| Dataset                                      | Number | Overlap | P Valuea | Odds Ratio |
|----------------------------------------------|--------|---------|----------|------------|
| Expressed genes for Smaug-dependent decay (embryos 2–3 hr) | 3956   |         |          |            |
| Smaug-dependent decay genes (embryos 2–3 hr, 5% FDR)b | 418    | 151     | 4.9 × 10⁻⁶ | 1.6        |
| Smaug-dependent-miR target genes             | 1507   |         |          |            |
| Expressed genes for Smaug-dependent decay (embryos 2–3 hr) | 3956   |         |          |            |
| Smaug-dependent decay genes (embryos 2–3 hr, 5% FDR)c | 341    | 128     | 3.1 × 10⁻⁶ | 1.7        |
| Smaug-dependent-miR target genes             | 1507   |         |          |            |
| Expressed genes for Smaug-RIP (embryos 0–3 hr)  | 4481   |         |          |            |
| Smaug-RIP genes (embryos 0–3 hr, 5% FDR)      | 339    |         |          |            |
| Smaug-dependent-miR target genes             | 1200   | 101     |          | 0.11       |

FDR, false discovery rate; miR, microRNA.

aOne-tailed Fisher’s exact test.
bSmaug-dependent decay genes list including Smaug-IP-genes.
cSmaug-dependent decay genes list without Smaug-IP-genes.

Indirect clearance of maternal mRNAs by SMG-dependent miRNAs

We previously identified SMG-bound mRNAs and showed that the vast majority of these depend on SMG for clearance during the MZT (Benoit et al. 2009; Chen et al. 2014). However, many maternal mRNAs that depend on SMG for clearance are not bound by SMG, suggesting indirect regulation. With respect to this latter possibility, the current study permitted us to assess on a global scale whether maternal mRNAs that are SMG-bound and/or SMG-dependent for degradation are enriched for predicted target sites for miRNAs that are downregulated in smug mutants. To do so, we extracted from TargetScanFly 6.2 (Schnall-Levin et al. 2010) a list of mRNAs expressed maternally and possessing conserved target sites for those miRNA species with significantly reduced expression in smug mutant embryos (see Materials and Methods and Table S1, Tab 4). We then compared this list to SMG’s direct targets in 0–3 hr embryos (Chen et al. 2014) and the maternal mRNAs dependent on SMG for degradation during the MZT (Benoit et al. 2009).

The results of our analysis are shown in Table 2. There was no significant overlap of the list of predicted mRNA targets of SMG-dependent miRNAs with the list of SMG’s direct targets. However, there was a highly significant overlap of the predicted mRNA targets of SMG-dependent miRNAs with those mRNAs that depend on SMG for degradation. When we removed SMG’s direct targets from the latter list, keeping only indirect targets, the overlap increased in significance.

These data are consistent with a model in which SMG degrades its direct targets without the assistance of miRNAs, whereas a large fraction of the indirectly affected maternal mRNAs in smug mutants fails to be degraded by virtue of being targets of zygotically produced miRNA species that are either absent or present at significantly reduced levels in smug mutants. Thus, SMG is required both for early, maternally encoded decay and for late, zygotically encoded decay. In the former case, SMG is a key specificity component that directly binds to maternal mRNAs; in the latter case, SMG is required for the production of the miRNAs (and AGO1 protein, see below) that are responsible for the clearance of an additional subset of maternal mRNAs (this model is schematized in Figure 5).

SMG is required for AGO1 accumulation during the MZT

In Drosophila, the stability of miRNAs is enhanced by AGO1 and vice versa (Smibert et al. 2013). Since miRNA levels are dramatically reduced in smug mutants, we decided to assess Ago1 mRNA and AGO1 protein levels during the MZT both in the wild type and in smug mutants. In the wild type, AGO1 levels were low in unfertilized eggs and 0–2 hr embryos but then increased substantially in 2–4 hr embryos (Figure 3). These Western blot data are consistent with an earlier, proteomic study that reported a more than threefold increase in AGO1 in embryos between 0–1.5 hr and 3–4.5 hr (Gouw et al. 2009). In contrast to AGO1 protein, we found that Ago1 mRNA levels remained constant during the MZT (Figure 4). Taken together with a previous report that Ago1 mRNA is maternally loaded (Lott et al. 2011), the increase in AGO1 protein levels in the embryo is, therefore, most likely to derive from translation of maternal Ago1 mRNA rather than from newly transcribed Ago1 mRNA.

Next, we analyzed AGO1, AGO2, AGO3, AUB, and Piwi protein levels in eggs and embryos from mothers carrying either of two smug mutant alleles: smug⁴³ and smug⁴⁷ (for details of the molecular lesions see Materials and Methods; Benoit et al. 2009; Chen et al. 2014). The smug mutations had no effect on the expression profiles of AGO2, AGO3, AUB, or Piwi (Figure S5). In contrast, in smug mutant embryos, the amount of AGO1 protein at both 0–2 and 2–4 hr was reduced relative to the wild type, and this defect was rescued in embryos that expressed full-length, wild-type SMG from a transgene driven by endogenous smg regulatory sequences (Chen et al. 2014) (Figure 3, A and B). The reduction of AGO1 protein levels in smug mutants was not a secondary consequence of reduced Ago1 mRNA levels, since Ago1 mRNA levels in both the smug mutant and the rescued-smug mutant embryos were very similar to the wild type (Figure 4).

As mentioned above, a plausible explanation for the decrease in AGO1 levels in smug mutants is the reduced levels of miRNAs, which would then result in less incorporation of newly synthesized AGO1 into functional miRISC and consequent failure to stabilize the AGO1 protein (Smibert et al. 2013). To assess this possibility, we analyzed a time-course in wild-type unfertilized eggs in which zygotic genome activation and, therefore, zygotic miRNA synthesis, does not occur. We found that AGO1 levels were reduced in 2–4 hr wild-type unfertilized eggs compared with wild-type embryos of the same age (Figure 3C). This result is consistent with a requirement for zygotic miRNAs in the stabilization of AGO1 protein.

We next compared wild-type unfertilized egg and smug mutant unfertilized egg time-courses, and found that AGO1 levels were further reduced in the smug mutant relative to the wild type (Figure 3C). This suggests that SMG protein has an additional function in the increase in...
To assess whether this additional function derives from SMG’s role as a posttranscriptional regulator of mRNA, we rescued smg1 mutants either with a wild-type SMG transgene driven by the Gal4-UAS system (SMGWT) or a Gal4-UAS-driven transgene encoding a version of SMG with a single amino acid change that abrogates RNA binding (SMGRBD) and, therefore, is unable to carry out posttranscriptional regulation of maternal mRNAs (Semotok et al. 2008). We found that, whereas AGO1 was detectable in both unfertilized eggs and embryos from SMGWT-rescued mothers, AGO1 was undetectable in unfertilized eggs from SMGRBD-rescued mothers and was barely detectable in embryos from these mothers (Figure 3D). Thus, SMG’s RNA-binding ability is essential for its non-miRNA-mediated role in regulation of AGO1 levels during the MZT.

Since the abundance of SMGWT and SMGRBD proteins is very similar (Semotok et al. 2008), the preceding result excludes the possibility that it is physical interaction between SMG and AGO1 (Pinder and Smibert 2013) that stabilizes the AGO1 protein. We have previously shown that the Ago1 mRNA is not bound by SMG (Chen et al. 2014). Thus, SMG must regulate one or more other mRNAs whose protein products, in turn, affect the synthesis and/or stability of AGO1 protein. It is known that turnover of AGO1 protein requires Ubiquitin-activating enzyme 1 (UBA1) and is carried out by the proteasome (Smibert et al. 2013). We have previously shown that the Uba1 mRNA is degraded during the MZT in a SMG-dependent manner, and that both the stability and translation of mRNAs encoding 19S proteasome regulatory subunits are upregulated in smg mutant embryos (Chen et al. 2014). We speculate that increases in UBA1 and proteasome subunit levels in smg mutants contribute to a higher rate of AGO1 turnover and, thus, lower AGO1 abundance than in the wild type (schematized in Figure 5).

The BRAT and PUM RBPs may cooperate with miRISC in maternal mRNA clearance

Drosophila AGO1 physically associates with BRAT (Neumuller et al. 2008). It is not known whether AGO1 interacts with PUM, but it has been reported that, in mammals and C. elegans, Argonaute family proteins interact with PUM/PUF family proteins (Friend et al. 2012). We recently identified direct target mRNAs of the BRAT and PUM RBPs in early Drosophila embryos and showed, through analysis of brat mutants, that during the MZT, BRAT directs late (i.e., after zygotic genome activation) decay of a subset of maternal mRNAs (Laver et al. 2015a). These data permitted us to ask whether the maternal mRNAs that are predicted to be indirectly regulated by SMG via its role in miRISC production might be coregulated by BRAT and/or PUM.

Figure 3 AGO1 protein levels are dramatically reduced in smg mutant embryos. Western blot analysis of SMG protein expression with either tubulin or DP1 as loading controls. (A) AGO1 levels in smg1/Df(3L)Scf and smg1/Df(3L)Scf embryos are reduced relative to wild-type in 2–4 hr embryos. (B) A full-length smg transgene (FL) rescues AGO1 expression in a smg mutant background. (C) AGO1 levels are also reduced in smg1/Df(3L)Scf 2–4 hr unfertilized eggs relative to wild-type. (D) RNA binding by SMG is necessary for AGO1 expression. The genotype of the females was: UASP[SMGWT or SMGRBD]>, smg1/P[GAL4:VP16-nos.UTR] smg1. The SMGWT transgene rescued expression of AGO1 in both smg1 unfertilized eggs and embryos, whereas the SMGRBD transgene (Semotok et al. 2008) did not rescue smg1. AGO1, Argonaute 1; SMG, Smaug; TUB, tubulin; wt, wild type. UF0-2h, 0-2 hr unfertilized eggs; UF2-4h, 2-4 hr unfertilized eggs; F0-2h, 0-2 hr embryos; F2-4h, 2-4 hr embryos.

AGO1 protein levels that is independent of SMG’s role in zygotic miRNA production (since these are produced in neither wild-type nor smg mutant unfertilized eggs).

To assess whether this additional function derives from SMG’s role as a posttranscriptional regulator of mRNA, we rescued smg1 mutants either with a wild-type SMG transgene driven by the Gal4-UAS system (SMGWT) or a Gal4-UAS-driven transgene encoding a version of SMG with a single amino acid change that abrogates RNA binding (SMGRBD) and, therefore, is unable to carry out posttranscriptional regulation of maternal mRNAs (Semotok et al. 2008). We found that, whereas AGO1 was detectable in both unfertilized eggs and embryos from SMGWT-rescued mothers, AGO1 was undetectable in unfertilized eggs from SMGRBD-rescued mothers and was barely detectable in embryos from these mothers (Figure 3D). Thus, SMG’s RNA-binding ability is essential for its non-miRNA-mediated role in regulation of AGO1 levels during the MZT.

Since the abundance of SMGWT and SMGRBD proteins is very similar (Semotok et al. 2008), the preceding result excludes the possibility that it is physical interaction between SMG and AGO1 (Pinder and Smibert 2013) that stabilizes the AGO1 protein. We have previously shown that the Ago1 mRNA is not bound by SMG (Chen et al. 2014). Thus, SMG must regulate one or more other mRNAs whose protein products, in turn, affect the synthesis and/or stability of AGO1 protein. It is known that turnover of AGO1 protein requires Ubiquitin-activating enzyme 1 (UBA1) and is carried out by the proteasome (Smibert et al. 2013). We have previously shown that the Uba1 mRNA is degraded during the MZT in a SMG-dependent manner, and that both the stability and translation of mRNAs encoding 19S proteasome regulatory subunits are upregulated in smg mutant embryos (Chen et al. 2014). We speculate that increases in UBA1 and proteasome subunit levels in smg mutants contribute to a higher rate of AGO1 turnover and, thus, lower AGO1 abundance than in the wild type (schematized in Figure 5).

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Figure 4 RT-qPCR shows that Ago1 mRNA expression is unaffected in smg mutants whereas miRNA levels are dramatically reduced. (A) RT-qPCR of Ago1 mRNA shows that expression is unaffected in smg mutant eggs and embryos. (B) RT-qPCR shows that a full-length smg transgene restores expression of mir-1-3p, mir-9-5p, and mir-309-3p expression in 2–4 hr smg mutant embryos. AGO1, Argonaute 1; SMG, Smaug; FL, full-length smg transgene; mRNA, messenger RNA; rep, biological replicate; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; wt, wild type.
We found a highly significant overlap between the predicted miRNA-dependent indirect targets of SMG and both BRAT- and PUM-bound mRNAs in early embryos (Table 3). This suggests that BRAT and PUM might function together with miRISC during the MZT to direct decay of maternal mRNAs (schematized in Figure 5).

Given that BRAT and PUM bind to largely nonoverlapping sets of mRNAs during the MZT (Laver et al. 2015a), there are three types of hypothetical BRAT-PUM-miRISC-containing complexes: one with both BRAT and PUM, one with BRAT only, and one with PUM only. To assess this possibility for a specific set of zygotically produced miRNAs, we compared the lists of mRNAs stabilized in 2–3 hr rol de m bry o sf r o m miR-309 deletion mutants (Bushati et al. 2008) to the lists of BRAT and PUM direct-target mRNAs (Laver et al. 2015a). The results are shown in Table 3. There was no significant overlap of PUM-bound mRNAs with those upregulated in miR-309 mutants. However, there was a highly significant overlap of mRNAs upregulated in miR-309-mutant embryos with BRAT-bound mRNAs. These results lead to the hypothesis that BRAT (but not PUM) coregulates clearance of miR-309 family miRNA target maternal mRNAs during the MZT. Experimental tests of this hypothesis are beyond the scope of the present study.

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H.L. with data analysis. The manuscript was written by H.L. and H.D.L., and revised by H.D.L. after feedback from J.M.C. and X.L.

LITERATURE CITED

Benett, B., C. H. He, F. Zhang, S. M. Votruba, W. Tadros et al., 2009 An essential role for the RNA-binding protein Smaug during the Drosophila maternal-to-zygotic transition. Development 136: 923–932.

Berezikov, E., N. Robine, A. Samsonova, J. O. Westholm, A. Naqvi et al., 2011 Deep annotation of Drosophila melanogaster microRNAs yields insights into their processing, modification, and emergence. Genome Res. 21: 203–215.

Blythe, S. A., and E. F. Wieschaus, 2015 Coordinating cell cycle remodeling with transcriptional activation at the Drosophila MBT. Curr. Top. Dev. Biol. 113: 113–148.

Bushati, N., A. Stark, J. Brennecke, and S. M. Cohen, 2008 Temporal reciprocity of miRNAs and their targets during the maternal-to-zygotic transition in Drosophila. Curr. Biol. 18: 501–506.

Chen, L., J. Dumelie, X. Li, M. Cheng, Z. Yang et al., 2014 Global regulation of mRNA translation and stability in the early Drosophila embryo by the Smaug RNA-binding protein. Genome Biol. 15: R4.

Czech, B., C. D. Malone, R. Zhou, A. Stark, C. Schlingeheyde et al., 2008 An endogenous small interfering RNA pathway in Drosophila. Nature 453: 798–802.

Dahanukar, A., J. A. Walker, and R. P. Wharton, 1999 Smaug, a novel RNA-binding protein that operates a translational switch in Drosophila. Mol. Cell 4: 209–218.

Fernandez-Valverde, S. L., R. J. Taft, and J. S. Mattick, 2010 Dynamic isomiR regulation in Drosophila development. RNA 16: 1881–1888.

Friend, K., Z. T. Campbell, A. Cooke, P. Kroll-Conner, M. P. Wickens et al., 2012 A conserved PUF-Ago-eEF1A complex attenuates translation elongation. Nat. Struct. Mol. Biol. 19: 176–183.

Fu, S., C. Y. Nien, H. L. Liang, and C. Rushlow, 2014 Co-activation of microRNAs by Zelda is essential for early Drosophila development. Development 141: 2108–2118.

Gou, J., M. Pinkse, H. Vos, Y. Moshkin, C. Vertijzer et al., 2009 In vivo stable isotope labeling of fruit flies reveals post-transcriptional regulation in the maternal-to-zygotic transition. Mol. Cell. Proteomics 8: 1566–1578.

Gu, W., J. M. Claycomb, F. J. Batista, C. C. Mello, and D. Conte, 2011 Cloning argonaute-associated small RNAs from Caenorhabditis elegans. Methods Mol. Biol. 725: 251–280.

Harrison, M. M., and M. B. Eisen, 2015 Transcriptional activation of the zygotic genome in Drosophila. Curr. Top. Dev. Biol. 113: 85–112.

Katoh, T., Y. Sakaguchi, K. Miyauchi, T. Suzuki, S. Kashiwabara et al., 2009 Selective stabilization of mammalian microRNAs by 3′ adenylation mediated by the cytoplasmic poly(A) polymerase GLD-2. Genes Dev. 23: 433–438.

Kozomara, A., and S. Griffiths-Jones, 2011 miRBase: integrating microRNA annotation and deep-sequencing data. Nucleic Acids Res. 39: D152–D157.

Langmead, B., C. Trapnell, M. Pop, and S. L. Salzberg, 2009 Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biol. 10: R25.

Laver, J. D., X. Li, D. Ray, K. B. Cook, N. A. Hahn et al., 2015a Brain tumor is a sequence-specific RNA-binding protein that directs maternal mRNA clearance during the Drosophila maternal-to-zygotic transition. Genome Biol. 16: 94.

Laver, J. D., A. J. Marsolais, C. A. Smibert, and H. D. Lipshitz, 2015b Regulation and function of maternal gene products during the maternal-to-zygotic transition in Drosophila. Curr. Top. Dev. Biol. 113: 43–84.

Lee, M., Y. Choi, K. Kim, H. Jin, J. Lim et al., 2014 Adenylation of maternally inherited microRNAs by Wispy. Mol. Cell 56: 696–707.

Li, H., B. Handsaker, A. Wysoker, T. Fennell, J. Ruan et al., 2009 The sequence alignment/map format and SAMtools. Bioinformatics 25: 2078–2079.

Lipshitz, H. D. (Editor), 2015 The Maternal-to-Zygotic Transition. Elsevier/Academic Press, London.

Lott, S. E., J. E. Villalta, G. P. Schrotth, S. Luo, L. A. Tonkin et al., 2011 Nonaconical compensation of zygotic X transcription in early Drosophila melanogaster development revealed through single-embryo RNA-seq. PLoS Biol. 9: e1000590.

Marco, A., 2015 Selection against maternal microRNA target sites in maternal transcripts. G3 5: 2199–2207.

Miyoshi, K., H. Tsukumo, T. Nagami, H. Siomi, and M. C. Siomi, 2005 Slicer function of Drosophila Argonautes and its involvement in RISC formation. Genes Dev. 19: 2837–2848.

Nelson, M. R., H. Luo, H. K. Vari, B. J. Cox, A. J. Simmonds et al., 2007 A multiprotein complex that mediates translational enhancement in Drosophila. J. Biol. Chem. 282: 34031–34038.

Neumuller, R. A., J. Betschinger, A. Fischer, N. Bushati, I. Poernbacher et al., 2008 Mei-P26 regulates microRNAs and cell growth in the Drosophila ovarian stem cell lineage. Nature 454: 241–245.

Okamura, K., S. Balla, R. Martin, N. Liu, and E. C. Lai, 2008 Two distinct mechanisms generate endogenous siRNAs from bidirectional transcription in Drosophila melanogaster. Nat. Struct. Mol. Biol. 15: 581–590.

Pinder, B. D., and C. A. Smibert, 2013 microRNA-independent recruitment of Argonaute 1 to nanos mRNA through the Smaug RNA-binding protein. EMBO Rep. 14: 80–86.

Quinlan, A. R., and I. M. Hall, 2010 BEDTools: a flexible suite of utilities for comparing genomic features. Bioinformatics 26: 841–842.

Robinson, M. D., D. J. McCarthy, and K. G. Smyth, 2010 edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics 26: 139–140.

Raby, J. G., A. Stark, W. K. Johnston, M. Kellis, D. P. Bartel et al., 2007 Evolution, biogenesis, expression, and target predictions of a substantially expanded set of Drosophila microRNAs. Genome Res. 17: 1850–1864.

Schnall-Levin, M., Y. Zhao, N. Perrimon, and B. Berger, 2010 Conserved microRNA targeting in Drosophila is as widespread in coding regions as in 3′ UTRs. Proc. Natl. Acad. Sci. USA 107: 15751–15756.

Seitz, H., M. Childiyali, and P. D. Zamore, 2008 Argonaute loading improves the fidelity of both microRNAs and their miRNA* strands in flies. Curr. Biol. 18: 147–151.

Semotok, J. L., R. L. Cooperstock, B. D. Pinder, H. K. Hari, H. D. Lipshitz et al., 2005 Smaug recruits the CCR4/POP2/NOT deadenylase complex to trigger maternal transcript localization in the early Drosophila embryo. Curr. Biol. 15: 284–294.

Semotok, J. L., H. Luo, R. L. Cooperstock, A. Karaiskakis, H. K. Vari et al., 2008 Drosophila maternal Hsp83 mRNA destabilization is directed by multiple SMAUG recognition elements in the open reading frame. Mol. Cell. Biol. 28: 6757–6772.

Shen, L., 2013 GeneOverlap: Test and Visualize Gene Overlaps. R Package. Mt. Sinai College of Medicine, New York.

Siddiqui, N. U., X. Li, H. Luo, A. Karaiskakis, H. Hou et al., 2012 Genome-wide analysis of the maternal-to-zygotic transition in Drosophila primordial germ cells. Genome Biol. 13: R11.

Smibert, P., J. S. Yang, G. Azzam, J. L. Liu, and E. C. Lai, 2013 Homeostatic control of Argonaute stability by microRNA availability. Nat. Struct. Mol. Biol. 20: 789–795.

Tadros, W., and H. D. Lipshitz, 2009 The maternal-to-zygotic transition: a play in two acts. Development 136: 3033–3042.

Tadros, W., A. L. Goldman, T. Babak, F. Menzies, L. Vardy et al., 2007 SMAUG is a major regulator of maternal mRNA destabilization in Drosophila and its translation is activated by the PAN GU kinase. Dev. Cell 12: 143–155.

Walser, C. B., and H. D. Lipshitz, 2011 Transcript clearance during the maternal-to-zygotic transition. Curr. Opin. Genet. Dev. 21: 431–443.

Zhou, H., M. L. Arcila, Z. Li, E. F. Lee, C. Henzler et al., 2012 Deep annotation of mouse iso-miR and iso-moR variation. Nucleic Acids Res. 40: 5864–5875.

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