MOLECULAR AND CELLULAR BIOLOGY, Apr. 2006, p. 2965–2975 Vol. 26, No. 8
0270-7306/06/$08.00+0 doi:10.1128/MCB.26.8.2965–2975.2006
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Genome-Wide Analysis of mRNAs Regulated by Drosha and Argonaute Proteins in *Drosophila melanogaster*†

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Received 13 October 2005/Returned for modification 22 November 2005/Accepted 3 February 2006

RNA silencing pathways are conserved gene regulation mechanisms that elicit decay and/or translational repression of mRNAs complementary to short interfering RNAs and microRNAs (miRNAs). The fraction of the transcriptome regulated by these pathways is not known, but it is thought that each miRNA may have hundreds of targets. To identify transcripts regulated by silencing pathways at the genomic level, we examined mRNA expression profiles in *Drosophila melanogaster* cells depleted of four Argonaute paralogs (i.e., AGO1, AGO2, PIWI, or Aubergine) that play essential roles in RNA silencing. We also profiled cells depleted of the miRNA-processing enzyme Drosha. The results reveal that transcripts differentially expressed in Drosha-depleted cells have highly correlated expression in the AGO1 knockdown and are significantly enriched in predicted and validated miRNA targets. The levels of a subset of miRNA targets are also regulated by AGO2. Moreover, AGO1 and AGO2 silence the expression of a common set of mobile genetic elements. Together, these results indicate that the functional overlap between AGO1 and AGO2 in *Drosophila* is more important than previously thought.

RNA silencing pathways are conserved mechanisms that regulate gene expression at both the transcriptional and post-transcriptional levels in a sequence-specific manner (1, 28, 33). These pathways are triggered by the presence of double-stranded RNAs (dsRNAs) of diverse origins. Long dsRNA molecules may originate from viral replication; transcription of endogenous microRNA (miRNA) genes, pseudogenes, and repetitive sequence elements; or during transposition of mobile genetic elements (1, 28, 33). dsRNAs can also be introduced into the cell artificially. To enter silencing pathways, long dsRNA molecules and miRNA primary transcripts are first processed by the RNase III-like enzymes Drosha and/or Dicer (1, 28, 33). In *Drosophila melanogaster*, Dicer-2 converts long dsRNAs into 21- to 22-nucleotide (nt) small interfering RNAs (siRNAs) (1, 25, 28, 33). Processing of primary miRNA hairpins encoded in the genome into ca.-22-nt-long miRNAs requires the consecutive action of Drosha and Dicer-1 (10, 15, 24, 25).

The siRNAs and miRNAs are incorporated into multimeric RNA-protein complexes referred to as RNA-induced silencing complexes (RISCs), which elicit decay or translational repression of complementary mRNA targets (1, 28, 33). siRNAs are fully complementary to their targets and elicit mRNA degradation via a pathway known as RNA interference (1, 28, 33). Similarly, plant miRNAs are often fully complementary to their targets and elicit mRNA decay. In contrast, animal miRNAs are only partially complementary to their targets and either elicit mRNA decay or repress translation without affecting transcript levels (1, 4, 26, 28, 33).

Members of the Argonaute (AGO) protein family are essential components of the RISC (17). The *Drosophila* genome encodes 5 AGO paralogs, but there are 8 in human and more than 20 in *Caenorhabditis elegans* (7, 8, 17, 33). This family of highly basic proteins is characterized by a central PAZ domain and a C-terminal Piwi domain (7, 8, 17, 33). The PAZ domain is involved in the specific recognition of the 2-nt 3′ overhangs of siRNAs and miRNAs (27, 46, 53). The Piwi domain adopts an RNase H-like fold (32, 38, 47). The Piwi domains of human and *Drosophila* AGO2 and of *Drosophila* AGO1 are catalytically active and can cleave mRNAs fully complementary to siRNAs or miRNAs (29, 34, 35, 40).

Current evidence suggests that, despite their similar domain organizations, Argonaute paralogs are not redundant in *Drosophila* (9, 11, 35, 36, 37, 40, 51). Indeed, *Drosophila* PIWI and Aubergine (AUB) have been implicated in heterochromatin formation and are required for the establishment and maintenance of the germ line (9, 28, 37). *Drosophila* AGO2 mediates siRNA-guided endonucleolytic cleavage of mRNAs, whereas *Drosophila* AGO1 plays a role in translational repression or mRNA decay triggered by miRNAs (4, 35, 36, 40). This lack of redundancy is further supported by the observation that mutations or knockouts of Argonaute paralogs in *Drosophila* have different phenotypes (11, 36, 51). An additional Argonaute paralog, AGO3, is encoded by the *Drosophila* genome but has not yet been characterized (51).

To identify mRNAs regulated by Argonaute proteins at the genomic level, we examined expression profiles in *Drosophila* cells individually depleted of AGO1, AGO2, PIWI, and AUB. We also profiled cells depleted of the miRNA-processing enzyme Drosha. Our analyses reveal a core set of transcripts

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whose levels are regulated by the miRNA pathway in *Drosophila* cells and demonstrate a partial functional overlap between AGO1 and AGO2.

MATERIALS AND METHODS

RNA interference. RNA interference was performed essentially as described before (41). dsRNAs used in this study correspond to fragments encompassing about 700 nt of the coding sequences.

Western blotting and immunoprecipitations. Western blot analyses were performed as described before (41). Antibodies to *Drosophila* AGO2 and AUB (PAZ domains) were raised in rabbits and rats, respectively, immunized with glutathione *S*-transferase fusions of the proteins expressed in *Escherichia coli*. For Western blot analyses, the polyclonal antibodies were diluted 1:1,000. Antibodies to *Drosophila* AGO1 and Drosha were kindly provided by H. Siomi and G. Hannon, respectively. Rabbit polyclonal anti-REF1 antibodies (41) were diluted 1:10,000. Hemagglutinin (HA)-tagged proteins were detected using polyclonal anti-HA antibody (Sigma). Bound primary antibodies were detected with alkaline phosphatase-coupled secondary antibodies (Western-Star kit from Tropix).

For immunoprecipitations, S2 cells were transfected with plasmids expressing HA-tagged versions of AGO1, AGO2, or maltose binding protein (MBP). Three days after transfection, 1.5×10^7 cells were lysed in 1.2 ml of NET buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% NP-40, and 1 mM EDTA) supplemented with protease inhibitors (Roche Complete protease inhibitor). After a 15-min incubation on ice, the lysate was spun for 15 min at 14,000 rpm at 4°C. The supernatant was incubated with monoclonal anti-HA antibodies (15 μl antibody; Covance) for 1 h on a rotating wheel at 4°C. Next, protein G-Sepharose beads were added (150 μl of a 1:1 suspension in NET buffer) and incubated for an additional hour on a rotating wheel at 4°C. Beads were washed three times in NET buffer and once in phosphate-buffered saline. An aliquot of beads was directly resuspended in protein sample buffer for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. The remaining beads were treated with proteinase K (1.5 mg/ml in 50 mM Tris, pH 7.4, 300 mM NaCl, 5 mM EDTA, 1.5% SDS) for 2.5 h at 50°C. RNA was extracted by two consecutive phenol extractions, followed by precipitation in the presence of 1 μg glycogen. RNA samples were analyzed in 15% denaturing polyacrylamide gels and transferred to Hybond-N+ membranes by semidry transfer. Membranes were blocked for 1 h at 50°C in hybridization buffer (5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 20 mM Na phosphate, pH 7.0, 7% SDS, 1× Denhardt's, and 0.1 mg/ml salmon sperm DNA). miRNAs were detected by hybridization at 50°C with 5′-end-labeled deoxyoligonucleotides complementary to the sequence of the mature miRNA. Filters were washed at 50°C (twice in 3× SSC and 5% SDS and once in 1× SSC and 1% SDS).

RNA isolation and genome-wide expression analysis. Total RNA was isolated using TRIzol reagent (Life Technologies). To reduce potential variations in the preparation of the RNA, two RNA preparations were isolated from a single knockdown experiment. These preparations were pooled with the equivalent preparations isolated from an independent knockdown to minimize differences in knockdown efficiencies. These pools of four RNA preparations from two independent knockdowns are referred to as RNA samples.

Northern blotting and reverse transcription-PCR (RT-PCR) were performed as described before (41). High-density oligonucleotide microarrays (*Drosophila* array 2; Affymetrix) covering more than 18,500 transcripts from *Drosophila* were used.

Biotinylated targets were prepared from 5 μg of total RNA by following standard Affymetrix procedures. Standard Affymetrix protocols were used for hybridization, washing, and data acquisition (Fluidics station 400, GeneArray 2500 scanner, Microarray Suite version 5.1; Affymetrix). Control parameters were within recommended limits. Data were imported into GeneSpring 6 (Silicon Genetics) (mock-treated cells, control channel; knockdown experiment, signal channel). All experiments were normalized using an intensity-dependent normalization scheme (Lowess). When two independent total RNA samples were compared, all spots had an average ratio of 1.07 ± 0.50 after intensity-dependent normalization. We therefore applied a 1.5-fold cutoff to identify regulated transcripts. This cutoff value is somewhat lax and may lead to the identification of false positives. However, for the list of core transcripts, the number of false positives is strongly reduced by applying this filtering criterion to two independent profiles obtained for Drosha and to five of six profiles obtained for AGO1.

GO analysis. Gene ontology (GO) terms (3) associated with regulated genes were identified using a gene ontology mining tool (www.affymetrix.com) and exported to Microsoft Excel. The enrichment of GO terms among regulated genes was assessed by the probability (*P*) that an equally high or higher enrichment could be obtained by chance given the frequency of the GO terms among detectable genes. We calculated the *P* value as the hypergeometric sum, which corresponds to randomly drawing an equal number of genes and obtaining the same number of genes associated with specific GO terms. The enrichment of miRNA targets among the up-regulated genes was assessed correspondingly by considering the frequency of all predictions for a given miRNA among all detectable mRNAs (following Stark et al. [49]).

Luciferase reporters. Wild-type 3′ untranslated regions (UTRs) of predicted miRNA targets were amplified by PCR from a *Drosophila* S2 cell cDNA library and cloned downstream of the firefly luciferase coding region between the XbaI and XhoI sites of plasmid pJ-Luc, as described before (42). All 3′ UTRs contained 3′ polyadenylation signals. *Renilla* luciferase was cloned between the EcoRI and XhoI sites of vector pAc5.1 (Invitrogen); this plasmid served as a transfection control. For expression of miRNAs, a genomic fragment of ca. 200 nt encompassing the miRNA gene was amplified from genomic DNA and cloned in vector pAc5.1A downstream of the actin 5C promoter. Plasmid pAc5.1-miR-13 expresses both miR-13a and miR-13b. Transfections were performed in 6-well plates by using Effectene transfection reagent (QIAGEN). The transfection mixtures contained 50 ng of firefly luciferase reporter plasmid, 0.3 μg of the transfection control, and 1 μg of plasmids expressing miRNA primary transcripts. Cells were collected 4 days after transfection. Luciferase activity was measured using a dual-luciferase reporter assay system (Promega).

Microarray accession number. The microarray data have been submitted to the ArrayExpress database at EBI under accession number E-MEXP-295.

RESULTS

Genome-wide identification of transcripts regulated by RNA silencing pathways. To identify transcripts regulated by the Argonaute proteins, we analyzed expression profiles of *Drosophila* Schneider cells (S2 cells) individually depleted of AGO1, AGO2, PIWI, or AUB by using whole-genome oligonucleotide microarrays. To distinguish clearly transcripts whose levels are regulated by the miRNA pathway, we also profiled RNA expression levels in cells depleted of Drosha (10, 15, 24). We assessed the efficacy of the depletions by Western blotting. Four days after addition of dsRNA, the steady-state expression levels of Drosha, AGO1, AGO2, and AUB had declined to about 10% of the levels detected in untreated cells (Fig. 1A, lanes 4 versus lanes 1). On day 9, the residual levels of the proteins were less than 10% of those observed in control cells (Fig. 1A, lanes 5). Depletion of Drosha, AGO1, or AGO2 also inhibited cell proliferation, confirming the effectiveness of the dsRNAs (see Fig. S1A in the supplemental material). In the absence of specific antibodies against PIWI, we determined the extent of its depletion by RT-PCR (Fig. 1B). Importantly, AGO2 depletion had no effect on AGO1 or Drosha expression levels (data not shown).

For each depleted protein, we obtained two (Drosha, AUB, and PIWI), three (AGO2), or six (AGO1) independent expression profiles from RNA samples isolated on day 9 (see Materials and Methods for a description of the RNA samples). For AGO1, whose depletion leads to more-widespread changes in RNA levels, we also performed a time course and analyzed expression profiles from RNA samples collected on days 3, 5, and 9 of the same knockdown (Fig. 1C). Total RNA was isolated from mock-treated cells as a reference (control) sample. To identify mRNAs regulated nonspecifically in response to the dsRNA treatment, we examined mRNA profiles in cells treated with green fluorescent protein (GFP) dsRNA (data not shown).

We assigned detectable transcripts to three classes according to their relative expression levels. These were transcripts at

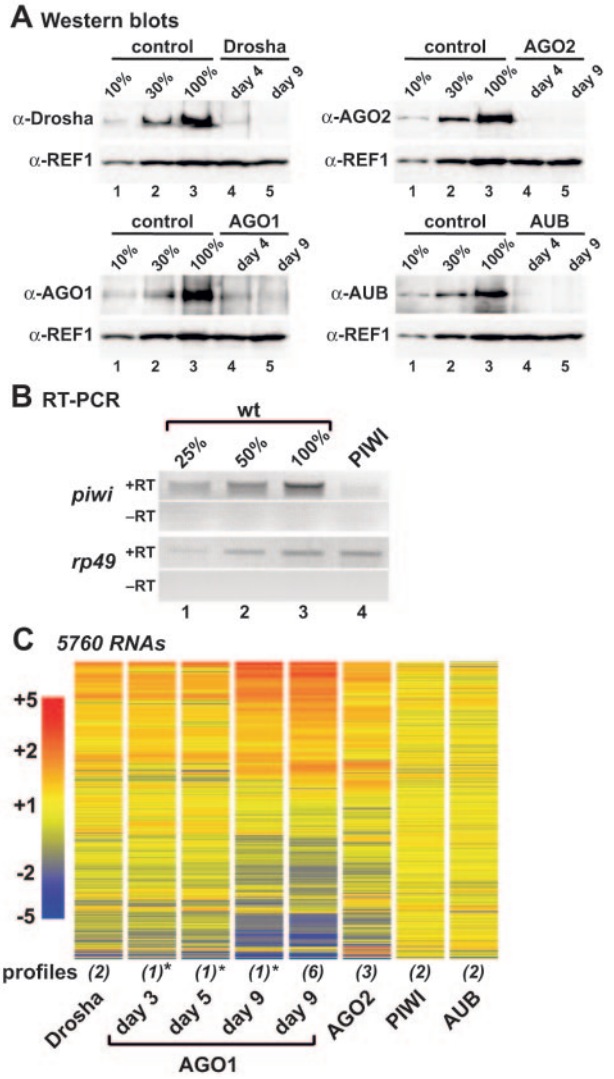

FIG. 1. Expression profiles of *Drosophila* S2 cells depleted of Drosha or Argonaute proteins. (A) S2 cells were treated with the dsRNAs indicated above the lanes. The effectiveness of the depletions was analyzed by Western blotting with the antibodies indicated on the left. In lanes 1 to 3, dilutions of the sample isolated on day 0 were loaded to assess the efficacy of the depletion. Antibodies against the nuclear antigen REF1 (α-REF1) were used as a loading control. (B) The effectiveness of PIWI depletion was analyzed by RT-PCR. The positive or negative signs on the left of the panels indicated that the reverse transcriptase was included (+RT) or omitted (−RT). The rp49 mRNA served as an internal control. wt, wild type. (C) Comparison of the average expression levels of detectable transcripts (5,760 RNAs) in all profiles. Numbers to the left indicate change (*n*-fold) in expression level. Blue, transcripts at least 1.5-fold underrepresented compared to the reference sample; yellow, transcripts not significantly changed (less than 1.5-fold different from the reference); red, transcripts at least 1.5-fold overrepresented. The number of independent expression profiles obtained per protein is indicated in parentheses below the columns. Asterisks indicate profiles derived from a single knockdown.

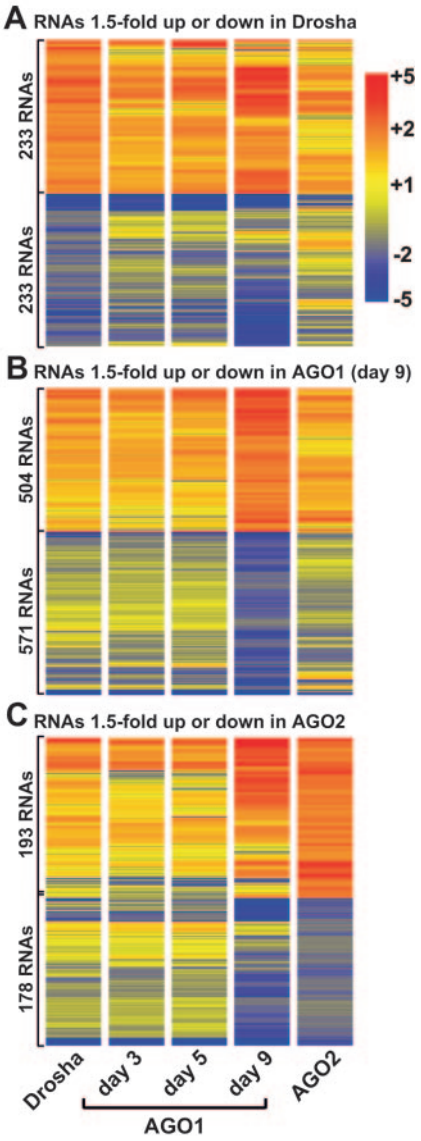

FIG. 2. RNAs regulated by Drosha, AGO1, or AGO2. (A) Expression profiles of RNAs at least 1.5-fold over- or underrepresented in the two independent profiles obtained for Drosha (see Table S2 in the supplemental material). Numbers to the right indicate change (*n*-fold) in expression level. (B) Expression profiles of RNAs at least 1.5-fold over- or underrepresented in at least five of six profiles obtained for AGO1 on day 9 (see Table S3 in the supplemental material). (C) Expression profiles of RNAs at least 1.5-fold over- or underrepresented in the three independent profiles obtained for AGO2 (see Table S4 in the supplemental material). In all panels, transcripts detectable in Drosha-, AGO1-, and AGO2-depleted cells (5,868 RNAs) are considered.

least 1.5-fold underrepresented compared to the reference sample, not significantly changed (less than 1.5-fold different from the reference), and at least 1.5-fold overrepresented (Fig. 1C). We considered a transcript only if it could be assigned to the same class in the two (Drosha, AUB, and PIWI), the three (AGO2), or five of the six (AGO1) independent profiles obtained on day 9 for these proteins. We validated changes in RNA levels for selected mRNAs by Northern blotting (see below; also data not shown).

Fewer than 2% of transcripts showed altered expression in cells depleted of PIWI or AUB (Fig. 1C; also see Table S1 in the supplemental material). Most of these transcripts have low levels of expression in wild-type cells, and we did not investigate them further. In cells depleted of Drosha, AGO1, or

AGO2, between 6% and 18% of transcripts were differentially expressed (Fig. 1C).

The expression profiles in cells depleted of Drosha or AGO1 (day 9) were significantly correlated (rank correlation coefficient [r] = 0.7) (Fig. 1C), indicating that depletion of these proteins affects the expression of a common set of RNAs. Some of the RNAs in this set changed levels concordantly in AGO2-depleted cells (Fig. 1C). In agreement with this, for AGO2 and AGO1 (day 9) profiles, $r = 0.7$, and for Drosha and AGO2 profiles, $r = 0.4$, indicating that Drosha, AGO1, and AGO2 regulate the expression levels of common targets.

Depletion of Drosha and depletion of AGO1 lead to similar expression profiles. To investigate further the similarity of cellular response to the depletion of Drosha, AGO1, or AGO2, we selected mRNAs belonging to specific classes in the Drosha knockdown (at least 1.5-fold over- or underrepresented, respectively) and analyzed their levels in the AGO1 or AGO2 knockdowns. We observed that of the 233 transcripts at least 1.5-fold overrepresented in Drosha-depleted cells, 58% and 16% were at least 1.5-fold up-regulated in the AGO1 (day 9) and AGO2 knockdowns, respectively (Fig. 2A; also see Table S2 in the supplemental material). Similarly, of the 233 down-regulated RNAs in Drosha-depleted cells, 61% and 16% exhibited the same regulation in AGO1-depleted (day 9) and AGO2-depleted cells, respectively (Fig. 2A; also see Table S2 in the supplemental material).

Likewise, RNAs showing differential expression in AGO1-depleted cells (day 9) had expression profiles similar to those in the Drosha knockdown, although the relative changes in expression levels were more pronounced in AGO1-depleted cells on day 9 (Fig. 2B; also see Table S3 in the supplemental material). These results indicate that Drosha and AGO1 regulate common targets, in agreement with the role of these proteins in the miRNA pathway (10, 15, 24, 36). As mentioned above, a subset of transcripts regulated by AGO2 showed similar expression levels in cells depleted of Drosha or AGO1 (Fig. 2C; also see Table S4 in the supplemental material), suggesting functional overlap between the three proteins.

Predicted miRNA targets are significantly enriched among up-regulated transcripts. Given the role of Drosha and AGO1 in the miRNA pathway (10, 15, 24, 36), changes in mRNA levels observed after their depletion are most likely to be caused by the inactivation of this pathway. We therefore investigated whether the transcripts up-regulated in Drosha- or AGO1-depleted cells were among predicted miRNA targets. The overlap between both sets can be used to distinguish between transcripts whose levels are directly or indirectly affected by miRNAs. The *Drosophila* genome encodes ca. 100 miRNAs (2, 16, 21, 22), of which 53 have been cloned (2, 21, 22) and 39 have unique (nonredundant) seed sequences (i.e., eight most-5′ nucleotides). We tested the enrichment for targets of nonredundant cloned miRNAs predicted by Stark et al. (49) by using an algorithm based on experimentally derived rules for miRNA target recognition (6) and found a significant enrichment for predicted miRNA targets among transcripts up-regulated in the Drosha knockdown ($P = 5.8 \times 10^{-24}$) and AGO1 knockdown ($P = 3.0 \times 10^{-34}$) (see Table S5 in the supplemental material). Interestingly, transcripts up-regulated in the AGO2 knockdown were also significantly enriched in miRNA predicted targets ($P = 1.8 \times 10^{-9}$) (see Table S5 in the supplemental material), suggesting that some miRNAs may not discriminate between AGO1- or AGO2-containing RISCs. Targets predicted in other studies were also represented in the list of up-regulated genes (13, 43, 48). No significant enrichment for predicted targets was found among down-regulated transcripts (P values of order unity), suggesting that these transcripts represent secondary targets of the miRNA pathway.

Identification of a core set of transcripts regulated by the miRNA pathway. To identify potential miRNA targets, we generated a list of transcripts up-regulated at least 1.5-fold in the two profiles obtained for Drosha and in at least five of six profiles obtained for AGO1 (day 9). We found 136 mRNAs in this class, representing 2.3% of detectable RNAs (Fig. 3A; also see Table S6 in the supplemental material). Although the cutoff ratio of 1.5 is low relative to the standard deviation of all detectable spots in the array (see Materials and Methods), the stringent filtering criterion (i.e., regulation in at least seven of eight independent profiles) reduces the likelihood of selecting false positives. Consistent with this, only four of these transcripts changed levels more than 1.5-fold in cells treated with AUB, PIWI, or GFP dsRNA (see Table S6 in the supplemental material). We define these RNAs as core transcripts, whose levels are regulated by the miRNA pathway.

The list of core transcripts includes hid and reaper mRNAs, which are validated miRNA targets (5, 48). Indeed, we found that both hid and reaper mRNAs were at least twofold up-regulated in cells depleted of Drosha or AGO1 (day 9) (see Table S6 in the supplemental material). Unexpectedly, both hid and reaper were at least 1.7-fold up-regulated in AGO2-depleted cells (see Table S6 in the supplemental material). Furthermore, among the 136 core transcripts, 31 were at least 1.5-fold up-regulated in the three independent profiles obtained for AGO2 (Fig. 3A; also see Table S6 in the supplemental material). This lends additional support to the hypothesis that some miRNAs may not discriminate between AGO1- or AGO2-containing RISCs.

The miRNAs with the most significant target gene enrichment among the core transcripts were the K-Box miRNAs (i.e., miR-2, miR-13, miR-6, and miR-11 [P of $\sim 10^{-12}$ to 10^{-6}]) (Table 1). Targets of miR-308, miR-8, and miR-314 were also significantly enriched (P of $\sim 10^{-9}$ to 10^{-6}). The enrichment levels for miR-14 ($P = 6 \times 10^{-4}$) and miR-9a and miR-9b (miR-9a/b) ($P = 1 \times 10^{-2}$) targets (Table 1) were also significant, although these miRNAs have not been shown to be expressed in S2 cells. Our results suggest that miR-9 and miR-14 might be expressed in S2 cells under our experimental conditions. Indeed, these miRNAs are detectable in S2 cells (Fig. 3B).

Analysis of the biological function of the proteins encoded by core transcripts done using gene ontology terms (3) revealed that some functional groups are overrepresented in the list of core transcripts in comparison to the detectable transcripts (Fig. 3C; also see Table S6 in the supplemental material). In particular, we observed a significant enrichment of genes involved in developmental processes ($P = 5 \times 10^{-3}$), axonogenesis ($P = 4 \times 10^{-3}$), organogenesis ($P = 7 \times 10^{-3}$), cell adhesion ($P = 1 \times 10^{-2}$), and signal transduction ($P = 1 \times 10^{-2}$).

Compared to the distribution of abundance of detectable transcripts, core transcripts show a bias towards low abundance in wild-type cells but an almost normal distribution in AGO1-

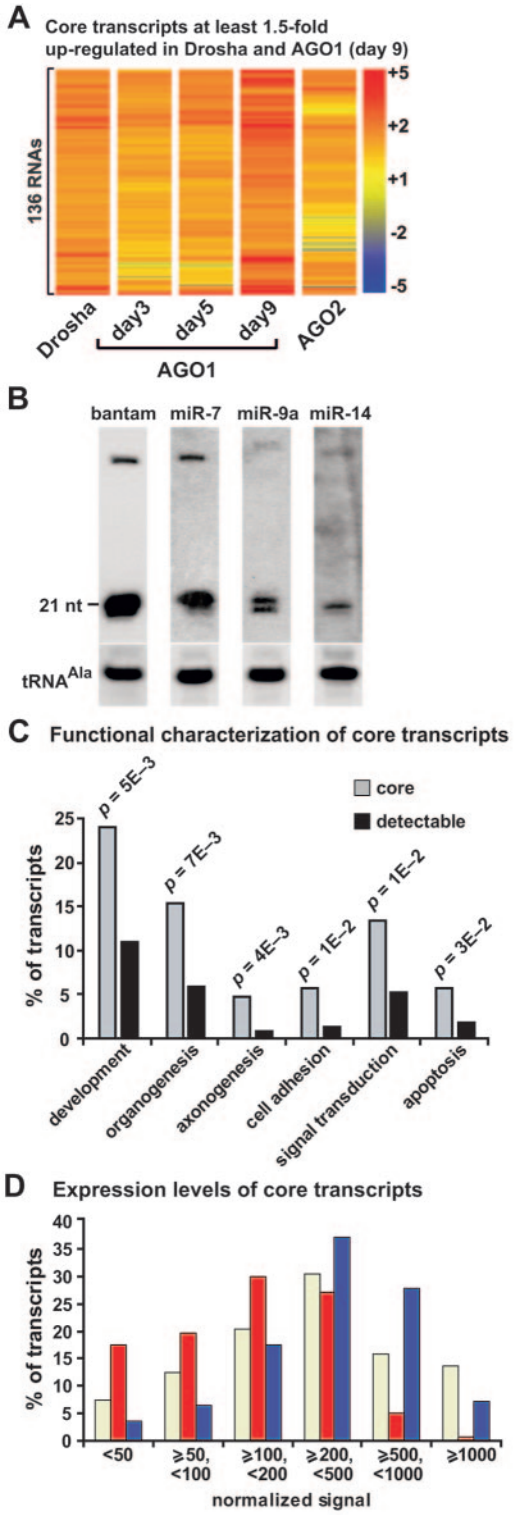

FIG. 3. Core transcripts regulated by the miRNA pathway. (A) Expression profiles of RNAs at least 1.5-fold overrepresented in Drosha- and AGO1-depleted (day 9) cells (core transcripts) (see Table S6 in the supplemental material). Numbers to the right indicate change (*n*-fold) in expression level. (B) Northern blot analysis of total RNA samples isolated from S2 cells. Probes specific to the miRNAs indicated above the lanes were used. $tRNA^{Ala}$ served as a loading control. (C) GO terms significantly enriched within the lists of core transcripts (gray bars). Black bars indicate the percentage of detectable transcripts associated with a specific GO term. (D) The histogram shows the distribution of signal intensities for all transcripts detected in samples isolated from control cells (yellow bars; average signal intensity of 569) and for the list of core transcripts in control cells (red bars; average signal intensity of 188) or in AGO1-depleted cells (day 9) (blue bars; average signal intensity of 465).

TABLE 1. Enrichment of predicted miRNA targets among core transcripts

P value	miRNA category or designation	No. of core targets/ no. of detectable targets
1.2E-22	Cloned, redundant miRNAs	90/1,674
2.6E-22	Cloned, nonredundant miRNAs	89/1,645
1.8E-12	miR-6	23/148
8.2E-12	miR-13b	22/144
8.2E-12	miR-2a	22/144
8.2E-12	miR-2b	22/144
1.1E-11	miR-13a	22/146
2.3E-09	miR-314	15/82
1.5E-08	miR-308	18/142
2.7E-06	miR-8	16/162
4.3E-06	miR-11	11/78
2.4E-04	miR-184	5/22
4.9E-04	miR-92b	12/155
6.0E-04	miR-14	8/75
6.5E-04	miR-317	7/58
3.3E-03	miR-92a	9/121
3.7E-03	miR-34	6/59
4.0E-03	miR-279	6/60
4.0E-03	miR-286	6/60
7.3E-03	miR-263b	6/68
8.6E-03	miR-9b	8/116

depleted cells (Fig. 3D), suggesting that these transcripts are not intrinsically of low abundance but rather are down-regulated by the miRNA pathway in wild-type cells.

Core transcripts represent authentic miRNA targets. To investigate whether predicted miRNA targets in the list of core transcripts represent authentic targets, 3′ UTRs derived from eight core transcripts were cloned into a firefly luciferase sensor reporter (42). We selected transcripts that were also regulated by AGO2. Four of them were predicted miR-9a/b targets. A previously validated miR-9b target, Nerfin (49), served as the positive control. When cotransfected with at least one of the predicted cognate miRNAs, six of eight of the 3′ UTRs led to a reduction of luciferase activity (relative to the activity observed in the absence of the miRNA) (Fig. 4A).

We found that predicted miR-9 targets were often regulated exclusively by either miR-9a or miR-9b (Fig. 4A) (e.g., CG10011 and Nerfin), indicating that these miRNAs are not redundant, despite their sequence similarity. Also, for some reporters (e.g., CG4851, Sema-1b, and CG12505) coexpression of an miRNA led to an increase in luciferase protein expression (Fig. 4A). One possible explanation for these results is that these miRNAs silence the expression of a negative regulator.

The results described above raised the question of whether predicted miRNA targets not included in the list of core transcripts also represent authentic targets. We therefore tested two 3′ UTRs derived from transcripts (CG30337 and CG33087) that

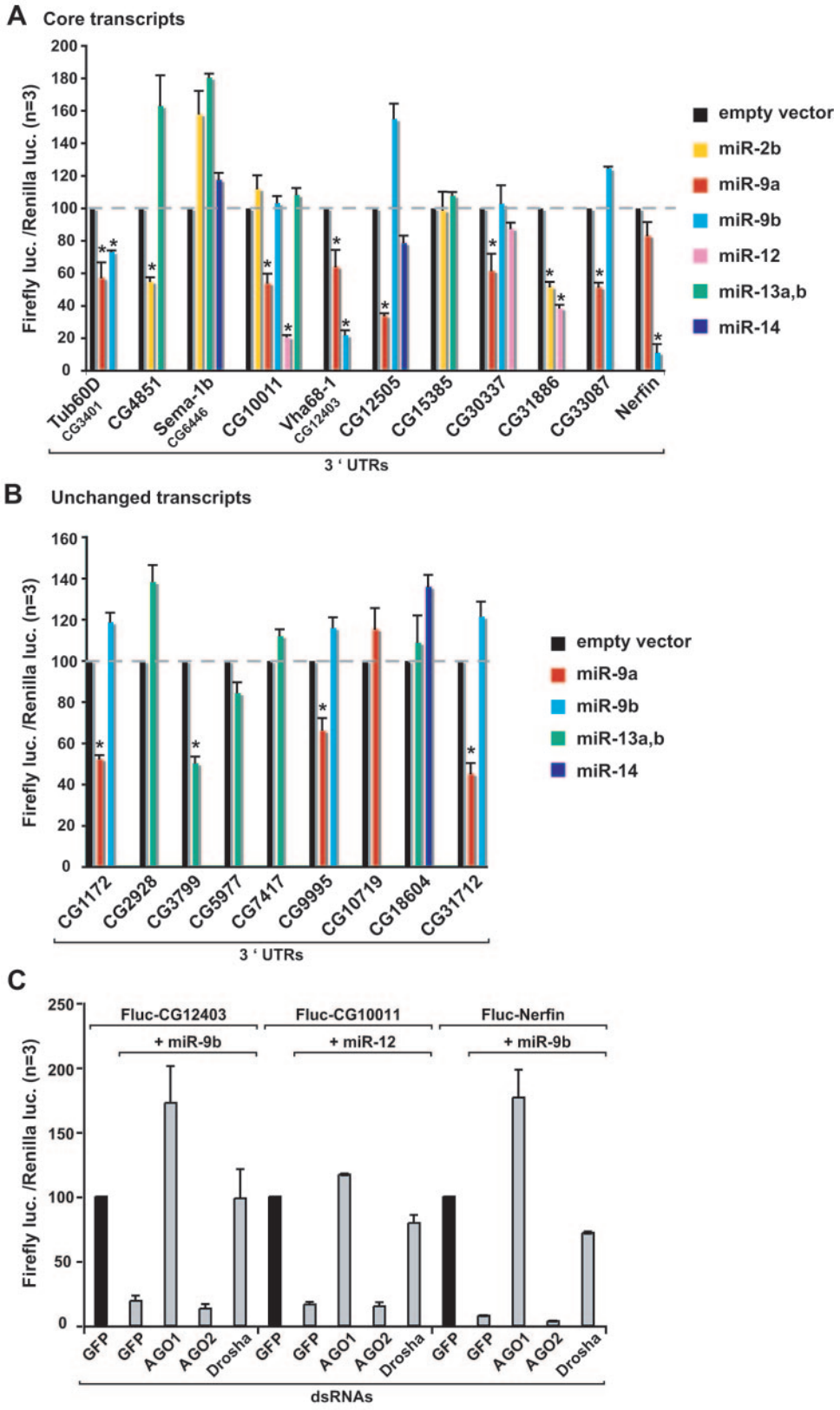

FIG. 4. Core transcripts represent authentic miRNA targets. (A and B) Reporter plasmids constitutively expressing firefly luciferase (luc.) flanked by the 3′ UTRs of predicted miRNA targets and plasmids expressing miRNA primary transcripts were cotransfected in S2 cells as indicated. *Renilla* luciferase was included as a transfection control. Firefly luciferase activity was normalized to the level of the *Renilla* luciferase activity in three independent experiments ($n = 3$). Normalized firefly luciferase activities in the absence of miRNAs were set to 100% (horizontal

were regulated in Drosha- and AGO1-depleted cells but were not in the list of core transcripts because they were not detectable in two experiments. These reporters were also down-regulated by at least one of the miRNAs predicted to recognize these 3′ UTRs (Fig. 4A). This observation confirms the assumption that the filtering criterion to select core transcripts (regulation in seven of eight independent profiles and detectable in all profiles) is stringent and that some genuine targets are excluded.

We also selected nine 3′ UTRs from predicted targets of miR-9a/b, miR-13a/b, and miR-14 whose expression levels remained unchanged in depleted cells and were comparable to those of the core transcripts in wild-type cells. Four out of nine 3′ UTRs tested repressed luciferase expression in the presence of the cognate miRNA (Fig. 4B). Note that for these 3′ UTRs we have not tested all miRNAs predicted to have binding sites, so the fraction of these transcripts representing authentic miRNA targets is likely to be underestimated.

We conclude that although the majority of predicted miRNA targets in the list of core transcripts are genuine targets of the miRNA pathway, this list is not comprehensive and additional targets may be identified when less stringent criteria are applied. Furthermore, not all miRNA targets are subject to down-regulation of mRNA levels, and some miRNA targets might not be regulated at all in S2 cells.

AGO2 associates with miRNAs. In a previous study, we showed that expression of firefly luciferase from the reporters harboring Vha68-1 or CG10011 3′ UTRs in the presence of miR-9b or miR-12 could be restored in cells depleted of AGO1 but not of AGO2 (42), despite Vha68-1 and CG10011 mRNA levels being regulated in AGO2-depleted cells. We obtained similar results for the reporter containing the Nerfin 3′ UTR (Fig. 4C). Depletion of Drosha also led to a partial restoration of firefly luciferase expression from these reporters, providing further evidence for a regulation of these reporters via the miRNA pathway (Fig. 4C). The lack of restoration in AGO2-depleted cells is not caused by an inefficient depletion, because silencing of firefly luciferase expression by cotransfecting a fully complementary siRNA (Luc-siRNA) is impaired in these cells (see Fig. S1B in the supplemental material) (42). Thus, depletion of AGO2 inhibits siRNA-guided but not miRNA-guided gene silencing, as reported by Okamura et al. (36).

These results contrast with the observation that AGO1 and AGO2 regulate the expression levels of a common set of miRNA targets. We therefore reasoned that regulation by AGO2 may not be observed with the reporter assays described above, as in this case both the reporter and the miRNAs are overexpressed. To investigate whether AGO2 associates with endogenous miRNAs, we performed immunoprecipitations from total lysates of S2 cells expressing a HA-tagged version of AGO1, AGO2, or MBP as a control. The presence of miRNAs associated with the precipitated proteins was analyzed by Northern blotting. Although the expression levels of these proteins were comparable, HA-tagged AGO1 immunoprecipitated very inefficiently (Fig. 5A). Nonetheless, miR-13b and bantam coimmunoprecipitated with HA-tagged AGO1 (Fig. 5B). HA-tagged AGO2 also immunoprecipitated these miRNAs above background levels, indicating that a small fraction of endogenous miRNAs can be found in association with AGO2 (Fig. 5B). These results provide an explanation for the observation that a subset of miRNA targets is regulated by AGO2.

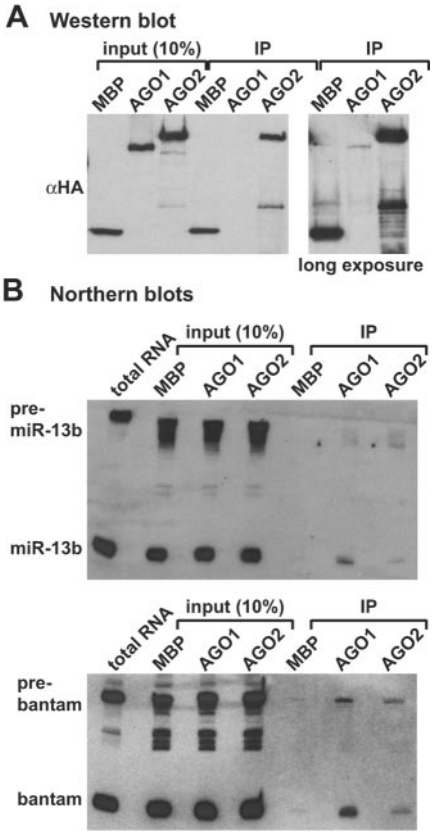

FIG. 5. AGO2 associates with miRNAs. (A) Immunoprecipitation of HA-tagged AGO1, AGO2, or MBP from total cell lysates. The right panel shows a longer exposure of the immunoprecipitated samples to visualize the presence of AGO1. α-HA, anti-HA. (B) The presence of miR-13b or bantam in the immunoprecipitates (IP) shown in panel A was analyzed by Northern blotting.

A few transcripts are regulated exclusively in the individual knockdowns. To determine whether Drosha, AGO1, and AGO2

dashed line). Asterisks indicate a significant reduction of firefly luciferase activity. In panel A, all miRNAs predicted to have binding sites in a given 3′ UTR were tested, while in panel B only a subset of miRNAs having potential binding sites were tested per reporter. (C) S2 cells were treated with the indicated dsRNAs on days 0 and 4. On day 6, cells were transfected with a mixture of plasmids: plasmids expressing firefly luciferase (Fluc) flanked by the indicated 3′ UTRs, plasmids expressing miRNA primary transcripts (gray bars) or the corresponding empty vector (black bars), and a plasmid expressing *Renilla* luciferase. Firefly and *Renilla* luciferase activities were measured 4 days after transfection. Firefly luciferase activity was normalized to the level of the *Renilla* luciferase activity and set to 100% for cells transfected with the empty vector and treated with GFP dsRNA (black bars). For all panels, mean values are shown and error bars represent standard deviations from three independent experiments.

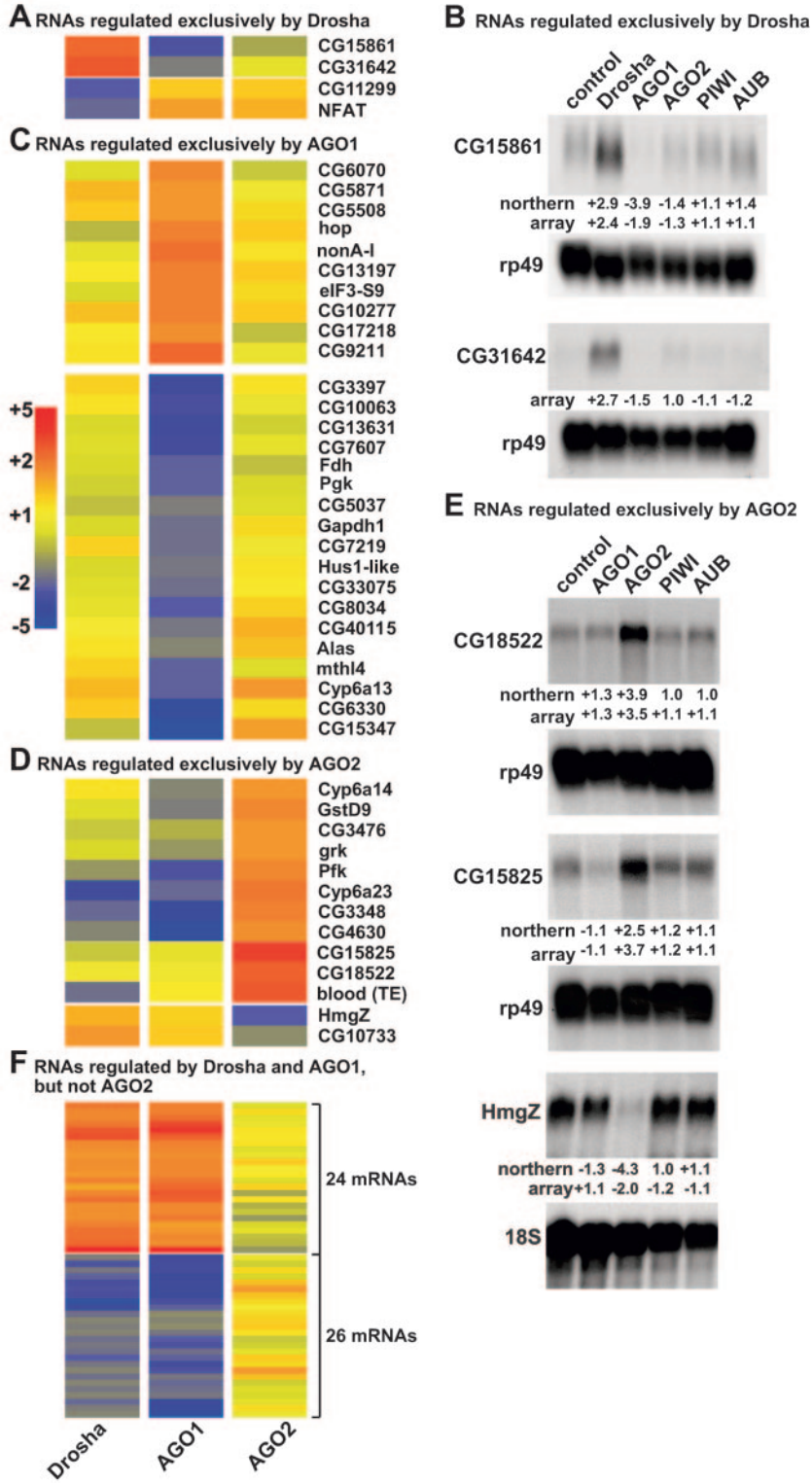

FIG. 6. RNAs regulated exclusively in the individual knockdowns. RNAs regulated exclusively by Drosha (A and B), AGO1 (C), or AGO2 (D and E) showing noncorrelated expression in the other knockdowns. (F) RNAs regulated exclusively in the four profiles obtained for Drosha and AGO1 (day 9). Numbers to the left indicate change (*n*-fold) in expression level. In panels B and E, the signals from the Northern blot analyses were normalized to rp49 mRNA or 18S rRNA. These values were compared with the values measured by microarray (averages of independent profiles). Values given between the blots are relative to the values obtained with mock-treated (control) cells (positive values, overrepresented; negative values, underrepresented).

have evolved specialized functions, we searched for transcripts regulated exclusively in one of the knockdowns but clearly unaffected (less than 1.3-fold) or showing inverse correlation in the other knockdowns. Only four transcripts were found to be regulated exclusively in Drosha-depleted cells (Fig. 6A and B). It would be of interest to determine whether Drosha regulates the expression of these transcripts by a mechanism not involving miRNAs.

We were also able to identify transcripts regulated by AGO1 but not by Drosha or AGO2 and transcripts regulated by AGO2 but not by Drosha or AGO1 (Fig. 6C, D, and E). The latter would be explicable if AGO2 regulates the expression of these transcripts by a mechanism involving, for example, siRNAs that are not processed by Drosha.

We also detected transcripts regulated by Drosha and AGO1 but unaffected in AGO2-depleted cells (Fig. 6F) or transcripts regulated by AGO1 (showing correlated expression in AGO2-depleted cells) but unaffected by Drosha depletion (not shown). Finally, we noticed that Dicer-1 and Dicer-2 mRNAs were at least 1.5-fold up-regulated in AGO1-depleted cells (in four of six profiles). *Drosophila* Dicer-1 mRNA has one target site, for miR-314, and Dicer-2 mRNA has sites for miR-280 and miR-315 (note that these sites are not conserved in *D. pseudoobscura*). This suggests that a feedback mechanism regulates the expression of genes involved in RNA silencing. Similarly, expression of Dicer-like 1 (DCL1) is regulated by miR-162 in *Arabidopsis thaliana* (52) and expression of AGO1 is regulated by miR-168 (50).

Among the transcripts regulated exclusively by AGO2 depletion, we found the transposable element (TE) blood (Fig. 6D). This prompted us to investigate whether additional transposon-derived transcripts are regulated in depleted cells. There are 96 families of transposable element in *Drosophila*, which represent 22% of the genome (19, 20). TEs are represented by 85 probe sets on the array, most of which correspond to long terminal repeat and non-long terminal repeat retrotransposon families. We found that 21% and 41% of detectable TEs were at least 1.5-fold up-regulated in cells depleted of AGO1 or AGO2, respectively (see Fig. S1C and D and Tables S3 and S4 in the supplemental material). With two exceptions, transposons up-regulated in cells depleted of AGO1 were also up-regulated in the AGO2 knockdown, providing further evidence for functional cross talk between these proteins.

DISCUSSION

Using microarray analysis of *Drosophila* cells depleted of Drosha and Argonaute proteins, we show that transcripts whose levels are likely to be directly regulated by silencing pathways (up-regulated transcripts) represent less than 20% of the *Drosophila* S2 cell transcriptome. Computational predictions of miRNA targets indicate that more than 30% of the transcriptome is targeted by miRNAs (13, 26, 43, 48, 49). There are several possible explanations for these seemingly contradictory observations. First, we show that not all authentic targets change levels in a detectable manner. This indicates that although microarrays are a valuable tool to identify miRNA targets (see also reference 26), many targets may escape detection using this approach. Second, some miRNAs and targets are expressed in a tissue-specific manner, so it is likely that only a subset of miRNA/target pairs is expressed in S2 cells (13, 14, 21, 23, 49). Finally, current models of miRNA function suggest that miRNAs expressed in a given cell type target transcripts that are already expressed at low levels but avoid housekeeping genes or genes that are expressed in these cells at high levels (14, 26, 49). These targets may escape detection by microarray analysis. Nevertheless, among transcripts regulated by the Argonaute proteins we found several that are expressed at relatively high levels, suggesting that miRNAs not only silence the expression of undesirable, low-abundance transcripts but may also play a role in fine-tuning the expression of abundant mRNAs.

Cross talk between AGO1 and AGO2. AGO1 and AGO2 are thought to have nonoverlapping functions in *Drosophila* (36, 40). In this study, we show that these proteins regulate the expression levels of a common set of miRNA targets. The observation that Drosha also regulates these transcripts strongly supports the idea that regulation is mediated by miRNAs. In agreement with this, we observed that AGO2 can associate with endogenous miRNAs, although less efficiently than does AGO1. In this way, AGO2 may also regulate the expression levels of a subset of miRNA targets. Nonetheless, when we assayed miRNA function by overexpressing miRNAs together with luciferase-based mRNA reporters, we observed that miRNA-mediated translational repression requires AGO1 but not AGO2. It is therefore possible that in this assay the fraction of miRNAs incorporated into AGO2-containing RISC is too small to observe changes in the expression levels of the reporter. Dicer-1 is involved in miRNA biogenesis and is also required for the assembly of RISC complexes (25), so our observations suggest that Dicer-1 may load AGO2-containing RISCs with miRNAs, at least to some extent.

A partial functional overlap between AGO1 and AGO2 is also suggested by the observation that these proteins regulate the expression of a common set of transposable elements. It remains, however, to be established whether this regulation occurs via similar mechanisms and whether it happens at the transcriptional or posttranscriptional level.

Apart from the common regulated transcripts, we have also identified transcripts regulated exclusively by AGO2 but not by Drosha or AGO1, suggesting that AGO2 may regulate the expression of these transcripts by an miRNA-independent mechanism that might involve endogenous siRNAs.

miRNAs affect mRNA expression levels. The levels of hid and reaper mRNAs (two experimentally validated miRNA targets [5, 48]) increase in cells in which the miRNA pathway is impaired. Moreover, by analyzing changes in mRNA levels, we have identified and validated additional miRNA targets in *Drosophila*. The observation that miRNA targets change levels following inhibition of the miRNA pathway lends further support to the idea that miRNAs can reduce the levels of the targeted transcripts and not just the expression of the translated protein (4, 26). Along these lines, it has recently been shown that miRNAs can trigger a strong reduction in target levels in *C. elegans* (4). We observed that among the 136 core transcripts, 21% are between 1.5- and 2-fold up-regulated, 73% exhibited changes in the 2- to 5-fold range, and 6% were at least 5-fold up-regulated in AGO1-depleted cells. Thus, although changes in transcript levels can be used to validate miRNA targets (26), the effects can be modest and, as men-

tioned above, not all targets can be identified using this approach.

In human cells, the Argonaute proteins localize to P-bodies (30, 39, 44, 45). These are specialized cytoplasmic foci in which the enzymes involved in mRNA degradation in the 5′-to-3′ direction colocalize (e.g., the DCP1:DCP2 decapping complex and the 5′-to-3′ exonuclease XRN1 [44, 45]). In addition, mRNA decay intermediates, miRNA targets, and miRNAs have been observed in P-bodies, suggesting a functional link between P-bodies and RNA silencing pathways (44, 45). Consistent with this, we and others have recently shown that P-body components play a crucial role in silencing pathways (12, 18, 31, 42). In particular, the RNA-binding protein GW182 (a P-body component in metazoa) and the DCP1:DCP2 decapping complex are required for miRNA-mediated gene silencing in *Drosophila* cells (42). Likewise, human GW182 plays a role in silencing mediated by miRNAs and siRNAs (18, 31). Finally, the *C. elegans* protein AIN-1, which is related to GW182, is also required for regulation of a subset of miRNA targets (12). Together with the observation that miRNAs inhibit cap-dependent but not cap-independent translation initiation (39), these observations suggest a model in which miRNA targets are stored in P-bodies after translation inhibition, where they are maintained in a silenced state by associating with proteins that prevent translation or possibly by removal of the cap structure (12, 30, 39, 42, 44, 45). Decapping or simply the storage of miRNA targets in P-bodies may make these mRNAs susceptible to degradation, providing a possible explanation for the reduction in mRNA levels (30, 42, 44, 45). In agreement with this, depletion of a 5′-to-3′ exonuclease in *C. elegans* partially restores the levels of miRNA targets (4).

Nevertheless, not all authentic miRNA targets change expression levels. Thus, it is possible that the extent of the degradation depends on the number of miRNA binding sites and/or the stability of the miRNA:mRNA duplexes. It is also possible that the rate of mRNA decay triggered by miRNAs for some targets does not exceed the rate of transcription and that thus the steady-state levels of these targets remain unchanged. It would therefore be of interest to determine whether miRNAs generally cause a reduction in the half-life of targeted transcripts.

ACKNOWLEDGMENTS

We are grateful to members of the Gene Core Facility at EMBL for performing the array hybridizations, G. Hannon and H. Siomi for providing antibodies, and D. J. Thomas for comments on the manuscript.

This study was supported by the European Molecular Biology Organization (EMBO) and the Human Frontier Science Program Organization (HFSPO).

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