Homeostatic control of Argonaute stability by microRNA availability

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Homeostatic mechanisms regulate the abundance of several components in small-RNA pathways. We used Drosophila and mammalian systems to demonstrate a conserved homeostatic system in which the status of miRNA biogenesis controls Argonaute protein stability. Clonal analyses of multiple mutants of core Drosophila miRNA factors revealed that stability of the miRNA effector AGO2 is dependent on miRNA biogenesis. Reciprocally, ectopic transcription of miRNAs within in vivo clones induced accumulation of AGO1, as did genetic interference with the ubiquitin-proteasome system. In mouse cells, we found that the stability of Ago2 declined in Dicer-knockout cells and was rescued by proteasome blockade or introduction of either Dicer plasmid or Dicer-independent miRNA constructs. Notably, Dicer-dependent miRNA constructs generated pre-miRNAs that bound Ago2 but did not rescue Ago2 stability. We conclude that Argonaute levels are finely tuned by cellular availability of mature miRNAs and the ubiquitin-proteasome system.

Argonaute proteins are the effectors of diverse modes of small RNA–mediated gene silencing and are guided to appropriate targets by their associated small RNAs1. Major Argonaute partners include ~22-nucleotide (nt) microRNAs (miRNAs) and ~21-nt short interfering RNAs (siRNAs) that associate with particular members of the Ago-clade subfamily and ~24-nt to 30-nt Piwi-interacting RNAs (piRNAs) that associate with the Piwi-clade subfamily of Argonautes. Much remains to be understood about piRNA biogenesis, but the generation of miRNAs and siRNAs is relatively well documented2. siRNAs generally derive from cleavage of double-stranded RNAs (dsRNAs) by the Dicer RNase III enzyme into siRNA duplexes. Loading of these duplexes into Argonautes is followed by maturation into single-stranded siRNA–Argonaute complexes. Primary miRNA transcripts contain hairpins that are excised by the nuclear RNase III Drosha and its dsRNA-binding partner DGCR8 (known as Pasha in invertebrates). The resulting pre-miRNA hairpins are then cleaved in the cytoplasm by Dicer into duplexes of miRNA and passenger (star) strands that load into Argonautes and mature into single-stranded form. In addition, a variety of noncanonical pathways generate functional miRNAs independently of Drosha and/or Dicer by exploiting diverse RNases that normally catalyze the maturation of other types of transcripts3. siRNA function is mediated by the cleavage activity of Drosophila AGO2 and vertebrate Ago2, whereas miRNA function requires the Argonaute partner GW182, which associates with Drosophila AGO1 and all four vertebrate Ago-class Argonautes4.

Homeostatic and feedback mechanisms coordinate levels of miRNAs with their effector proteins or harmonize the levels of small-RNA–biogenesis factors that function within complexes5. For example, in vertebrates and flies an elegant feedback loop normalizes levels of Drosha and DGCR8 (ref. 6–8). DGCR8 transcripts are destabilized by 5′ hairpins that are directly cleaved by Drosha, whereas DGCR8 protein is reciprocally unstable in the absence of Drosha. These cross-regulatory interactions maintain the appropriate stoichiometry of the Drosha–DGCR8 complex. Further studies in plan5,10 and animal11–13 systems uncovered requirements for the Hsp90 machinery in Argonaute loading and/or stability. Hsp90–cochaperone complexes also facilitate loading of Piwi proteins14, and loss of piRNA-biogenesis components results in cell-autonomous loss of Piwi protein15.

Despite these accumulating links of small-RNA–biogenesis pathways to the stability of their effector Argonautes, multiple previous studies did not reveal alteration of the Drosophila miRNA effector AGO1 upon depletion of miRNA-biogenesis factors in cultured S2 cells16–19. We re-evaluated this in Drosophila by using null-mutant clones of drosha, pasha and Dcr-1, all of which demonstrate reduction of AGO1. We show that AGO1 is degraded by the ubiquitin-proteasome system in Drosophila, and AGO1 is detectably stabilized by transgenic expression of miRNAs, demonstrating a homeostatic mechanism. We extend these findings to mammalian cells and show that Ago2 has lower stability in Dicer1-knockout cells. We were able to rescue Ago2 stability by inhibiting the proteasome or by introducing Dicer-independent miR-451–type constructs. Notably, noncleavable pre-miR-451 hairpins associated with Ago2 but did not rescue its stability, which provides evidence that the active miRNA-induced silencing complex (miRISC) and not simply RNA-associate Argonaute is protected from degradation. The deep conservation of a strategy by
Figure 1  

Drosophila AGO1 protein level is reduced in the absence of miRNA biogenesis and accumulates upon depletion of GW182. (a) Ovarioles bearing negatively marked mutant clones (lacking GFP in green channel) stained for AGO1 protein (gray-scale channel); scale bar, 20 μm. A pair of egg chambers is shown for each genotype, of which the bottom one contains a germline clone outlined in dashed lines and marked –/–. Control clone with the FRT42D chromosome is shown. (b) Wing imaginal discs bearing positively marked clones (expressing GFP, green channel) stained for AGO1 protein (gray-scale channel); scale bar, 20 μm. Control clone with the FRT82B chromosome is shown. (c) Epistasis analysis of Dcr-1 and GW182. Positively marked wing-disc clones were stained for various markers (gray-scale channel) as labeled; scale bar, 20 μm. Additional data are in Supplementary Figures 1–3.

which Argonaute protein levels are sensitive to miRNA availability reflects its importance for appropriate miRNA-effector function.

RESULTS

In vivo stability of fly AGO1 depends on miRNA biogenesis

Tests from several different laboratories, albeit usually conducted for other reasons, have not revealed substantial alteration of the protein level of miRNA effector AGO1 upon knockdown of any miRNA-biogenesis factors.16–19. Having generated a panel of mutants in core miRNA factors8,30, we evaluated whether AGO1 protein was sensitive to miRNA biogenesis in Drosophila. We generated homozygous mutant ovarian germline clones by using the Flp-FRT system, which negatively marked mutant cells by the absence of GFP. We stained these with AGO1-specific antibodies30, which in ovaries bearing control germline clones detected somatic and germline expression, with higher levels in the oocyte (Fig. 1a). As expected, germline clones of AGO1 mutants showed decreased staining (Fig. 1a). Notably, germline clones of pasha and Dcr-1 similarly showed reduction of AGO1 protein (Fig. 1a), and analysis of three additional Dcr-1 alleles yielded similar results (Supplementary Fig. 1).

We confirmed these findings in somatic tissues and used the mosaic analysis with a repressible cell marker (MARCM) system to positively mark mutant clones with GFP. Whereas control MARCM clones in wing imaginal discs did not show alteration of AGO1 levels (Fig. 1b), clones bearing null alleles of pasha, drosha and Dcr-1 were all strongly deficient for AGO1 protein (Fig. 1b). In addition, clones of two pasha mutants and six Dcr-1 mutants in larval brain lobes all exhibited decreased AGO1 staining (Supplementary Fig. 2). Therefore, accumulation of the miRNA effector AGO1 depends on miRNA biogenesis in diverse tissues and developmental stages.

A previous report did not reveal alteration of AGO1 protein level upon depletion of its cofactor GW182 in S2 cells19. As GW182 (official gene symbol gw) is encoded on chromosome 4, which is not amenable to mitotic recombination, we used a transgene to induce RNA-interference knockdown of GW182 (GW182-RNAi). We validated that expression of this transgene reduces GW182 protein in a cell-autonomous manner (Fig. 1c) and derepresses the endogenous miRNA target Mei-P26 (refs. 8,21) to similar levels as those seen in pasha, drosha and Dcr-1 clones (Supplementary Fig. 3a). With knowledge of these data, we reassessed the effects in Drosophila S2 cells. Although previous studies confirmed efficient depletion of target proteins and/or accumulation of miRNA precursors, the literature indicates the difficulty of fully depleting mature miRNAs by knockdown of miRNA-biogenesis components18,24–26. We used a double-knockdown strategy (4-d dsRNA soaking followed by cell-number normalization and an additional 4-d soaking with fresh dsRNA) for maximal efficacy. This protocol yielded strong loss of compromised growth21, they maintain relatively normal levels of AGO1 (Supplementary Fig. 3b). The opposing effects of loss of core miRNA-biogenesis factors and the loss of GW182 on AGO1 accumulation provided an opportunity for in vivo epistasis analysis. We generated GFP-labeled MARCM clones that were homozygous for a null allele of Dcr-1 and expressed GW182-RNAi. These clones clearly displayed the AGO1 protein loss phenotype (Fig. 1c), demonstrating that accumulation of AGO1 upon GW182 loss is strictly dependent on active miRNA biogenesis.

Post-transcriptional control of AGO1 stability

With knowledge of these in vivo data, we reassessed the effects in Drosophila S2 cells. Although previous studies confirmed efficient depletion of target proteins and/or accumulation of miRNA precursors, the literature indicates the difficulty of fully depleting mature miRNAs by knockdown of miRNA-biogenesis components18,24–26. We used a double-knockdown strategy (4-d dsRNA soaking followed by cell-number normalization and an additional 4-d soaking with fresh dsRNA) for maximal efficacy. This protocol yielded strong loss of
Figure 2 miRNA biogenesis stabilizes AGO1 post-transcriptionally, but siRNA biogenesis does not stabilize AGO2 in Drosophila. (a) Western analysis of AGO1 and α-tubulin loading control following knockdown of miRNA factors in S2 cells. (b) Northern blot analysis of miR-34 under the same conditions as in a. Asterisk denotes altered length distribution that may indicate re-sorting of miR-34 to AGO2. (c) Assays of independent quantitative PCR (qPCR) amplicons for AGO1 upon depletion of miRNA-biogenesis factors in S2 cells. Assays were performed in triplicate for each sample. Error bars, s.e.m. (d) qPCR analysis of miRNA pathway factors in pasha mutant third-instar larvae and yellow white (yw) control larvae. Assays were performed in triplicate for each sample. Error bars, s.e.m. (e) Genotyping of Dcr-2+ (wild type, WT) and Dcr-2(L811fsX) with allele-specific primer pairs to confirm the status of the wild-type (Canton S) and Dcr-2 homozygous mutant backgrounds carrying FH-AGO2 genomic transgenes. (f) Western blot analysis of Flag-AGO2 levels and α-tubulin loading control in the absence of Dcr-2. Additional data are in Supplementary Figure 6.

AGO1 protein upon knockdown of drosha, pasha and Dcr-1 as compared to levels in untreated cells or control cells with GFP knockdown (Fig. 2a). In contrast, knockdown of GW182 reproducibly increased AGO1 levels (Fig. 2a).

These effects were paralleled by the behavior of endogenous miR-34, which strongly decreased in abundance upon knockdown of miRNA-biogenesis factors and mildly increased upon GW182 knockdown (Fig. 2b). The latter result may suggest that AGO1 turnover is altered when the effector complex is disrupted. A population of mature miR-34 was maintained in AGO1-knockdown cells despite near absence of AGO1; these species exhibited an altered length profile (Fig. 2b) that is likely to reflect sorting to AGO2. We tested independent AGO1 ampiclons and observed no reproducible alteration of AGO1 transcripts following knockdown of miRNA-biogenesis factors (Fig. 2c), a result suggesting a post-transcriptional mechanism. This was supported by similar AGO1 transcript levels in homozygous pasha larvae as compared to those of wild type (Fig. 2d).

Overall, our ability to recapitulate phenotypes from mutant clones with S2-cell knockdowns demonstrated that positive regulation of AGO1 stability by miRNA biogenesis is a general phenomenon, and it appears to act post-transcriptionally.

RNAi effector AGO2 is not dependent on siRNA biogenesis

Given these results, we investigated whether the RNAi effector AGO2 was sensitive to siRNA biogenesis. We introduced a hemagglutinin-AGO2 genomic transgene (FH-AGO2) that supports
full RNAi function\textsuperscript{26,27} into the background of the Dcr-2[81fS]/X homozygous mutant, which lacks siRNA-biogenesis capacity and RNAi capacity\textsuperscript{28}, and confirmed genotypes by PCR (Fig. 2e). We extended our previous studies\textsuperscript{27} by showing that AGO2 protein levels were unaltered in Dcr-2–null ovaries and heads (Fig. 2f). Therefore, although both AGO1 and AGO2 are loaded by Hsp90-dependent mechanisms\textsuperscript{11,12}, the miRNA effector AGO1 is uniquely subject to turnover in the unloaded state.

**Increasing miRNA transcription can stabilize AGO1 in vivo**

We reciprocally asked whether overproduction of miRNAs could influence AGO1 levels. Imaginal-disc clones expressing DsRed or different individual upstream activation sequence (UAS)-miRNA transgenes did not reveal substantial effects on AGO1 protein levels (Fig. 3a). However, expression of genomic clusters containing three miRNAs (mir-12, mir-283 and mir-304) or four miRNAs (mir-9c, mir-79, mir-9b and mir-306) resulted in clear cell-autonomous accumulation of AGO1 protein (Fig. 3b). The strongest effects were seen in clones expressing the eight-miRNA cluster containing mir-309, mir-3, mir-286, mir-4, mir-5, mir-6-1, mir-6-2 and mir-6-3 (Fig. 3b). Thus, increasing AGO1 levels correlated with the number of ectopically expressed miRNAs. In contrast, clonal overexpression of an RNAi trigger to generate ectopic siRNAs (shep-RNAi) did not alter AGO1 (Fig. 3c). This was consistent with the bulk sorting of siRNAs into the *Drosophila* RNAi effector AGO2.

We also analyzed the effect of ectopic miRNAs in the ovary. Similarly to the results for imaginal discs, ectopic expression of *mir-124*, but not a nonprocessed mutant *mir-124* construct, elevated AGO1 in ovarian follicle cells (Fig. 3d). Altogether, these data fulfill a homeostatic mechanism in that loss and gain of miRNA biogenesis induce reciprocal effects on AGO1 accumulation.

**The ubiquitin-proteasome system limits AGO1 accumulation**

We hypothesized that the ubiquitin–proteasome system might mediate AGO1 homeostasis. We exploited a transgene encoding a dominant temperature-sensitive subunit of the proteasome β6 subunit Pros26p (UAS-DTSS), which we expressed along the anterior-posterior boundary of the wing imaginal disc by using *ptc-GAL4* (Fig. 3e). We introduced a heat shock–inducible transgene (hs-AGO1) in the genetic background so that we could monitor the outcome of a pulse of AGO1 expression across a time course, comparing wild-type cells with neighboring cells in which proteasome activity was impaired. After a 15-min heat shock, AGO1 protein levels were greatly elevated throughout the disc, with further accumulation within the DTS5-expressing domain (Fig. 3e). By 4 h after induction, AGO1 returned to normal endogenous levels throughout the disc except within the DTS5-expressing domain (Fig. 3e). These data demonstrate that exogenous AGO1 is rapidly turned over by a proteasome-dependent mechanism.

We complemented these tests by assessing whether endogenous AGO1 is regulated by ubiquitination. We were unable to recover imaginal-disc clones expressing an RNAi transgene to the sole ubiquitin-activating enzyme Uba1 (data not shown), probably owing to cell competition or cell lethality. However, we could recover such clones in the ovarian follicle epithelium, and these clones accumulated AGO1 protein (Fig. 3f). Therefore, endogenous AGO1 protein is restricted by ubiquitination.

**Stability of mammalian Ago2 depends on miRNA availability**

We sought to extend these findings to mammalian cells. An excellent model is a stable *Dicer1−/−* mouse embryonic fibroblast (MEF) cell line that we have extensively characterized\textsuperscript{29}. Although western blot analysis detected endogenous mouse Ago2 protein in these cells, time-course analyses following cycloheximide treatment showed that Ago2 was destabilized in *Dicer1−/−* cells (Fig. 4a). We assessed whether Ago2 accumulation was sensitive to the restoration of endogenous miRNA biogenesis. Transfection of *Dicer1−/−* cells with a Flag-Dicer construct rescued processing of endogenous let-7a into mature miRNAs (Fig. 4b). Concomitant with this, completion of miRNA biogenesis substantially increased Ago2 stability (Fig. 4c).

We attempted to assess Ago1 but did not detect its endogenous accumulation in *Dicer1−/−* cells (Supplementary Fig. 4). As an alternative, we transfected myc-tagged human Ago1 into *Dicer1−/−* cells and observed that its decay was slower than that of endogenous Ago2 (Supplementary Fig. 4b). However, myc-tagged human Ago2 did not recapitulate the instability of mouse Ago2. Moreover, transfection of either construct impaired the decay of endogenous Ago2 (Supplementary Fig. 4b). We infer that this may reflect the recruitment of ectopic and endogenous Argonaute proteins into aggregates\textsuperscript{30}, whose loading potential and stability control might be altered. We therefore concentrated our further studies on endogenous Ago2.

Ago2 transcript levels in *Dicer1−/−* cells were unaltered by cycloheximide or Flag-Dicer plasmid (Fig. 4d), a result pointing to regulation of Ago2 protein stability. Analogous to genetic
Supplementary Fig. 5a

Figure 5 Accumulation of mouse and Drosophila Argonautes is dependent on loading with functional small RNAs. (a) Northern blot analysis of mouse Ago2 immunoprecipitates (IP) of Dicer1−/− cells transfected with tRNA–mir-451 or tRNA–mir-155 fusion constructs. (b) Visualization of 5′-labeled RNAs in mouse Ago2-immunoprecipitated RNA samples. Signals in the boxed areas correspond to mature miR-451 and pre-miR-155 hairpin. (c) Western blot of Ago2 in cells expressing wild-type Dicer-independent miR-451 or Dicer-dependent miR-155 or TRNA wildtype construct; α-tubulin loading control is shown. (d) Northern blot analysis of input and immunoprecipitated Ago2 material from Dicer1−/− cells transfected with wild-type or cleavage mutant tRNA–mir-451 CM. (e) Western blot of Ago2 in the presence of tRNA–mir-451CM or tRNA–mir-451 constructs; α-tubulin loading control is shown. (f) Luciferase sensor assays in Drosophila S2 cells. Sensor constructs were empty or contained complementary sites to miR-276a-5p or miR-276a-3p. Assays were performed in biological quadruplicate for each sample. Error bars, s.d. Student’s two-tailed, t-test was performed. (g) Effects of transfection of synthetic miR-276a-miR-276a* duplex or scrambled siRNA duplex onAGO1 accumulation in S2 cells depleted of miRNA-biogenesis factors with the indicated dsRNAs or control GFP dsRNA; α-tubulin loading control is shown. Full-length blots are shown in Supplementary Fig. 6.

Manipulations of the Drosophila proteasome, addition of the proteasome inhibitor MG132 suppressed Ago2 protein decay in Dicer1−/− cells treated with cycloheximide (Fig. 4e). Given the recent report that autophagy regulates Argonaute levels in mammalian cells31, we also tested Dicer1−/− cells with the autophagy inhibitor bafilomycin A1. This did not perturb Ago2 levels during a time course of 2–6 h, even though we confirmed strong inhibition of autophagy by accumulation of LC3-II at all time points (Supplementary Fig. 5a). Moreover, bafilomycin A1 did not rescue the instability of AGO1 in Drosophila S2 cells depleted of various miRNA-biogenesis factors (Supplementary Fig. 5b). Double treatments suggested a mild stabilizing effect of bafilomycin A1 in Dicer1−/− cells beyond the effect of MG132 (Supplementary Fig. 5c). However, we were cautious to interpret this as a parallel pathway, given the possibility of indirect effects in this triple drug treatment. Notably, we could also improve Ago2 stability in Dicer1−/− cells just by transfecting them with synthetic siRNAs (Fig. 4f). Similar findings were reported that synthetic siRNAs stabilize mammalian Argonautes in the presence of Hsp90 inhibitors33. These observations implied that RNA-loaded Ago2 is more resistant to degradation.

Genetic rescue of Ago2 stability in Dicer-knockout cells

We tested cellular strategies to rescue miRNA biogenesis in Dicer1−/− cells. Vertebrate miR-451 matures through a Dicer-independent mechanism that involves direct loading of its short pre-miRNA hairpin into Ago2 and subsequent slicing of the complementary strand and resection of its 3′ end32. We have previously shown that functional miR-451 can be efficiently generated in a Drosha- and Dicer-independent fashion by a fusion construct (tRNA–mir-451) comprising tRNA (official symbol TRNAK3) and miRNA genes33. We exploited this as a genetic strategy to express mature miRNAs from a nuclear context in Dicer1−/− MEFs, and these miRNAs were efficiently immunoprecipitated with Ago2 (Fig. 5a). For comparison, we generated a tRNA–mir-155 fusion that includes the endogenous Dicer substrate pre-miR-155 hairpin. Northern blotting of Ago2 immunoprecipitates from Dicer1−/− MEFs transfected with these constructs showed that they contain pre-miR-155 hairpin but not mature miR-155 (Fig. 5a).

Northern blotting does not directly demonstrate that the detected small RNAs constitute a substantial fraction of total small RNAs in these cells. We therefore performed end-labeling of total RNAs from Ago2 immunoprecipitates. Notably, we observed that cells transfected with tRNA–mir-451 accumulated specific RNAs in the <30 nt range corresponding to mature miR-451 species (Fig. 5b). However, cells transfected with tRNA–mir-155 accumulated an ~60-nt band (Fig. 5b) that matched the expected size of pre-miR-155. Therefore, these tRNA constructs generate sufficient levels of small RNAs to affect the total small-RNA pool in Dicer1−/− cells.

With these validations in hand, we compared the effects of these RNAs on Ago2 levels. Transfection of tRNA–mir-451 substantially rescued instability of Ago2 in Dicer1−/− cells following cycloheximide treatment (Fig. 5c). In contrast, tRNA–mir-155 provided only slight improvement of Ago2 stability as compared to empty tRNA33 vector alone (Fig. 5c). Immunoprecipitation experiments confirmed the reduction of Ago2 abundance under these conditions (Supplementary Fig. 4c). Therefore, although a population of pre-miRNA hairpins has the capacity to associate with Ago2 in the absence of Dicer (Fig. 5a,b), these are not competent to protect Ago2 from turnover.

To provide further evidence for this, we generated a tRNA–mir-451 cleavage-mutant construct (tRNA–mir-451CM) that bears centrally bulged sites, a configuration that is competent for Argonaute loading but
incompetent for Ago2 cleavage. We confirmed that tRNA–miR-451-tm loads into Ago2 in Dicer-1−/− cells but remains arrested as a pre-miR-451 hairpin (Fig. 5d). In contrast, cells expressing wild-type tRNA-miR-451 mildly accumulate the hairpin precursor, but Ago2 complexes strictly contain cleaved and matured forms of miR-451 (Fig. 5d). We observed that tRNA–miR-451-tm was unable to stabilize Ago2 (Fig. 5e), and this provides further evidence that mere RNA association may not suffice for Ago2 stability. We hypothesize that the conformation of pre-miRNA–loaded Argonaute differs from the mature single-stranded complex and that only the latter can achieve full stability in vivo.

**Rescue of Drosophila AGO1 by synthetic miRNAs but not siRNAs**

Finally, we returned to the Drosophila system to ask whether the reintroduction of small RNAs could rescue the instability of AGO1 in cells with impaired miRNA biogenesis. As Dicer-independent miRNAs have not yet been identified in this species, we turned to synthetic small-RNA duplexes. This experimental strategy has the advantage of using the Drosophila-specific feature of Argonaute sorting to potentially distinguish the impact of siRNA loading from that of miRNA loading on AGO1 stability.

We previously used synthetic miR-276a duplexes to dissect the mechanism of AGO1 loading in vitro. Here, we evaluated the capacity of synthetic wild-type miR-276a duplex to repress target sensors when transfected into S2 cells. Both mature and star species of miR-276a are competent for target regulation of cognate luciferase sensors when expressed as primary miRNA transcripts from transfected plasmids (Fig. 5f). However, we obtained much stronger repression of these sensors in S2 cells transfected with the synthetic miR-276a duplex, a result indicating that this duplex efficiently populates functional Argonaute complexes. We used S2 cells that were doubly treated with GFP, drosha, pasha, or Dcr-1 dsRNA and further introduced either synthetic fully paired siRNA duplexes (used in mammalian cell tests in Fig. 4f) or miR-276a duplex. We observed a mild improvement of AGO1 stability in the absence of miRNA biogenesis with the siRNA duplex but observed strong recovery of AGO1 accumulation in the presence of synthetic miR-276a duplex (Fig. 5g). These tests specifically demonstrate that sorting of small RNAs to AGO1 are indeed required for maintenance of AGO1 stability.

**DISCUSSION**

Model figures of biogenesis pathways for small RNAs often give the impression that these processes are constitutive, inflexible and inexorable. However, diverse mechanisms that regulate small-RNA biogenesis and function have been uncovered recently. Notably, many of these mechanisms provide homeostatic control over the levels of small-RNA–biogenesis factors and/or their resultant small RNAs. The revised picture is that small-RNA pathways are subject to constant tuning to maintain appropriate levels of the factors involved and to provide systems that permit reversibility or enhancement of small-RNA functions. In this respect, knowledge of small-RNA pathways is now coming to par with that of other signaling pathways and regulatory networks, which are almost universally subject to multilayer regulation.

In this study, we used both Drosophila and mammalian systems to demonstrate sensitive mechanisms that normalize miRNA biogenesis to the abundance of Argonaute effector proteins. Recent structural studies of full-length mammalian Argonaute provide a framework for rationalizing the turnover of unloaded Argonautes, including the observation that Ago2 adopts a protease-resistant, tight structure upon complexation with a small RNA. This perspective is complemented by studies using Hsp90 inhibitors that demonstrate that conformational changes are required to load Argonautes with small RNAs and lead to a stable state.

A recent study reported that loss of miRNA biogenesis in mouse embryonic stem cells (bearing DGCR8 or Dicer1 mutations) results in post-transcriptional loss of Ago2 protein. Our work agrees with this and broadens this concept across multiple species, in both germ line and somatic cells. In addition, our studies highlight not only that loss of miRNA biogenesis destabilizes Argonaute proteins, but also that Argonaute proteins are normally made in excess and can be stabilized by increasing miRNA transcription. We demonstrated this by clonal analysis of single-copy inducible miRNA transgenes within intact animals and showed that activating the transcription of various miRNA loci was sufficient to elevate AGO1 protein levels.

Such a homeostatic response was not expected, given that elevation of Argonaute proteins can increase the abundance of siRNAs or miRNAs and that quantitative measurements indicated an excess of miRNA species over Argonaute proteins, at least in certain systems. We note that the effects of elevated Argonaute proteins has mostly been studied with respect to cotransfected miRNA constructs and siRNAs; thus, influence of this manipulation on endogenous miRNA pools remains to be understood. In addition, miRNA/Argonaute stoichiometry was examined in cancer cells and remains to be studied in tissues or at least in primary cells. We believe that it makes physiological sense for Argonaute proteins to be initially made in excess relative to miRNAs because if Argonautes were limiting then tissue-specific changes in the transcription of miRNA loci might result in competition for Argonaute occupancy. This would not rule out that the transcription of Argonaute genes might be regulated to help normalize their protein levels with overall tissue-specific changes in bulk miRNA transcription; perhaps both strategies work in concert.

Notably, we find that the Drosophila RNAi effector Ago2 is not sensitive to the status of siRNA biogenesis. Why might homeostatic mechanisms exist specifically for miRNA-effector Argonautes? One possibility is to prevent the misloading of unintended small RNAs into miRISCs because this regulatory complex has the capacity to regulate off-targets bearing minimal sequence complementarity. We showed that the RNAi pathway actively prevents loading of endogenous siRNAs into AGO1 (ref. 27), and a trimming and tailing mechanism further culls siRNAs loaded into miRISCs on the basis of their atypically extensive target pairing. The destabilization of unloaded miRNA effectors may be another mechanism to ensure purity of this Argonaute pool. However, the rarity of endogenous, extensively paired ‘siRNA-type’ targets may mean that misloading of Ago2 is better tolerated. Perhaps a pool of unloaded Ago2 may ensure a timely response to viral infections by allowing large amounts of viral siRNA-dependent RISC to be generated quickly.

The ubiquitin-proteasome system contributes to Argonaute homeostasis, and this extends a theme from other recent studies that demonstrate temporal or cell state-specific ubiquitination and degradation of Argonaute and Piwi proteins. In support of this notion, we used not only chemical inhibitors but also genuine genetic manipulations of the proteasome. We showed that Drosophila AGO1 is increased in cells depleted of Uba1 (the sole Drosophila ubiquitin-activating enzyme) or that express a dominant-negative proteasome subunit (DTSS), indicating that excess miRNA Argonaute effectors are being turned over in vivo. Recently, autophagy was reported to degrade Ago2 in HeLa cells and in DGCR8−/− embryonic stem cells. The latter work is consistent with conclusions regarding instability of Argonautes in the absence of miRNA biogenesis. However, we did not observe substantial effects of autophagy inhibition in either our Drosophila or mouse cell-culture systems, even though we confirmed defective autophagy by accumulation of LC3-II (Supplementary Fig. 5). It is conceivable that both degradation pathways contribute...
to the observed phenomenon, with the relative contributions being determined by cell type and culture conditions.

Finally, our studies used new noncanonical miRNA-expression vectors to distinguish between the pre-miRNA-associated Argonaute state and the mature, functional miRISC state. In particular, our results indicate that Argonaute stability is not only sensitive to the overall availability of miRNAs but also to its state as a mature complex (Fig. 5). Therefore, even though Dicer-1/-cells accumulate pre-miRNAs, and these can associate with Argonaute proteins, RNA association alone does not seem sufficient for full stability. In combination with our data that Drosophila AGO1 can be stabilized by increasing miRNA transcription, we conclude that metazoan cells have sensitive mechanisms that actively harmonize the levels of miRNA-effector Argonautes to the status of miRNA biogenesis.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

P.S., G.A. and J.-L.L. made the initial observations that demonstrated AGO1 homeostasis in vivo. Mechanistic experiments were carried out in flies by P.S., in s2 cells by P.S. and J.-S.Y. and in mammalian cells by J.-S.Y. P.S., J.-S.Y. and E.C.L. wrote the text with input from G.A. and J.-L.L.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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Drosophila stocks, clonal analysis and immunostaining. Mutant alleles and transgenes described in this work includeAGO1(kb8121)\textsuperscript{48}, AGO1(1)\textsuperscript{49}, Drosha[21K11]\textsuperscript{18}, Dcr-2[3R MARCM stock: yw, hsFLP, UAS-CD8GFP, FRT42D, tub-GAL80; tub-GAL4 / TM6B 3R MARCM stock: yw, hsFLP, UAS-CD8GFP; +/tub-GAL4, FRT82B, tub-GAL80 / TM6B 3R MARCM stock: yw, hsFLP, UAS-CD8GFP; +/tub-GAL4, FRT82B, tub-GAL80 / TM6B

Drosophila S2 cell cultures. We depleted small-RNA factors from S2R+ cells by soaking with dsRNA as described\textsuperscript{56}, using previously validated templates\textsuperscript{24}. All soaking experiments are the result of two rounds of RNAi soaking for 4 d each. Four days after the first dsRNA soaking, total cell number was renor malized before the second round of soaking. For S2-cell western blots, we used rabbit anti-AGO1 (1:1,000, Abcam, cat. no. ab53070), mouse anti-α-tubulin (1:1,000, gift from H. Somji), rabbit anti-GW182 (1:500 (this study), rabbit anti-GAL-DBD (1:150, Santa Cruz, cat. no. sc-577).

To genotype the Dcr-2 alleles, the following allele-specific PCR primers were used: Dcr2[1R11X] test F CAGTCACAAAGGCCTGATAAGAGC and Dcr2[1R11] wt ASO R ACAAAAAGAGCTGAGTATCTCCTC for the wild-type Dcr-2 allele; Dcr2[1R11X] ASO ATGGATATTTCCGAGATATCTGGCACGGA-3 and Dcr2[1R11X] test R CACCTTTTGTCAGCACAGG for the Dcr2[1R11X] allele.

Mammalian plasmids and siRNAs. Dicer-expressing plasmid (pCCK-Flag-Dicer) was a gift of V.N. Kim (Seoul National University, Korea). The scrambled siRNA control was purchased from IDT. The miRNA-expressing and control constructs (miRNA\textsuperscript{55}, miRNA-\textit{mir}-451, miRNA-\textit{mir}-451CM and tRNA-\textit{mir}-155) were cloned into pCR2.1-TOPO vector (Life Technologies) as previously described\textsuperscript{35}. Oligo sequences for miRNA cloning were as follows:

| Oligo Name | Sequence |
|------------|----------|
| tRNAlys3\textsubscript{F} | GCCCGGATAGCTCGAGTACGACGTCAGTACGCTTTTAA |
| tRNAlys3\textsubscript{R} | GAGAAGAAAAAGATACCGCCCGAACAGGGAGCTTGA |
| tRNAlys3\textsubscript{F} | GCCCGGATAGCTCGAGTACGACGTCAGTACGCTTTTAA |
| tRNAlys3\textsubscript{R} | GAGAAGAAAAAGATACCGCCCGAACAGGGAGCTTGA |

siRNAs

siRNA of Vdrc ID 37863, hs-AGO2 (5' AGTGGGAATGATTGCATCTG-3') were cloned into the pDHSmiR-276a expression vector. siRNA of AGO1-1195- (5' AGTGGGAATGATTGCATCTG-3') were cloned into the pDHSmiR-276a expression vector. siRNA of AGO1-1195- (5' AGTGGGAATGATTGCATCTG-3') were cloned into the pDHSmiR-276a expression vector.

Mammalian cell studies. Dicer-1\textsuperscript{−/−} mouse embryonic fibroblasts (MEFs) and human HEK293T cells were maintained in DMEM, high-glucose GlutaMAX with 10% heat-inactivated FBS (Sigma) and 1% penicillin-streptomycin (Life Technologies) and incubated in a humidified incubator at 37 °C, 5% CO\textsubscript{2}. Lipofectamine 2000 (Life Technologies) was used to transfect cells according to the manufacturer's manual. To study mouse Ago2 stability, 50 µg/ml cycloheximide (CHX, Calbiochem) was added to Dicer-1\textsuperscript{−/−} MEFs 24 h after transfection. The cells were harvested at 5 h and at 10–12 h after CHX addition. To inhibit proteasome activity, cells were treated with CHX (25 µg/ml) and either MG132 (25 µM) or DMSO for the indicated time. For the indicated time. The cells were harvested at 5 h and at 10–12 h after CHX addition. To inhibit proteasome activity, cells were treated with CHX (25 µg/ml) and either MG132 (25 µM) or DMSO for the indicated time. The cells were harvested at 5 h and at 10–12 h after CHX addition. To inhibit proteasome activity, cells were treated with CHX (25 µg/ml) and either MG132 (25 µM) or DMSO for the indicated time. The cells were harvested at 5 h and at 10–12 h after CHX addition. To inhibit proteasome activity, cells were treated with CHX (25 µg/ml) and either MG132 (25 µM) or DMSO for the indicated time. The cells were harvested at 5 h and at 10–12 h after CHX addition. To inhibit proteasome activity, cells were treated with CHX (25 µg/ml) and either MG132 (25 µM) or DMSO for the indicated time. The cells were harvested at 5 h and at 10–12 h after CHX addition. To inhibit proteasome activity, cells were treated with CHX (25 µg/ml) and either MG132 (25 µM) or DMSO for the indicated time. The cells were harvested at 5 h and at 10–12 h after CHX addition. To inhibit proteasome activity, cells were treated with CHX (25 µg/ml) and either MG132 (25 µM) or DMSO for the indicated time. The cells were harvested at 5 h and at 10–12 h after CHX addition. To inhibit proteasome activity, cells were treated with CHX (25 µg/ml) and either MG132 (25 µM) or DMSO for the indicated time. The cells were harvested at 5 h and at 10–12 h after CHX addition.
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