Overlapping Functions of Argonaute Proteins in Patterning and Morphogenesis of Drosophila Embryos

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Introduction

RNA silencing represents an important regulatory mechanism of gene expression, in which short RNAs regulate mRNA stability and translation or control transcription via chromatin modification [1]. Such short RNAs include small interfering RNAs (siRNAs) and microRNAs (miRNA). Small RNAs are produced from larger precursors by RNase III–type endonucleases, called Dicer [2,3]. Processed small RNAs are incorporated into the RNA-induced silencing complex (RISC), also called miRNP in the case of miRNAs [4,5]. Active RISC either catalyzes the cleavage of the cognate target mRNA or interferes with its translation. RNA silencing directed by siRNAs has been implicated in heterochromatic silencing in various organisms from yeast to humans [6]. miRNAs are involved in a variety of biological processes such as cell proliferation, cell death, developmental timing, and embryonic patterning [7].

Biochemical and genetic analyses have revealed that the molecular mechanisms underlying RNA silencing crucially require proteins from the Argonaute family [8]. Argonaute proteins are essential components of RISC and represent the catalytic activity of RISC in both the miRNA and the siRNA pathway [9–14]. Argonaute proteins are highly conserved and share at least two functional domains, a PAZ and a PIWI domain [8]. The PAZ domain forms a nucleic acid–binding pocket and binds small RNAs [15,16]. The PIWI domain shares structural similarities with ribonucleases and contains an activity that degrades cognate RNAs [13,14,17–19].

The Drosophila genome encodes five Argonaute family members: Aubergine (Aub), Piwi, Ago1, Ago2, and Ago3 [8,20,21]. Ago2 differs from the other Drosophila Argonautes in that it has a unique amino-terminal extension rich in glutamines (37% of all residues are glutamine). Almost two-thirds of this domain is made up of tandem copies of two types of glutamine-rich repeats (GRRs). In other proteins, glutamine-rich domains can promote protein aggregation such as the PolyQ domains in the mutant forms of Huntingtin linked to neurodegeneration or the PrD domains of many yeast prions [22,23]. For Ago2, however, the functional significance of this domain is unknown.

In Drosophila, siRNA-induced and miRNA-induced silencing vary in their requirement for different Argonaute and Dicer genes. Ago1 is required for miRNA-induced RNA silencing but is dispensable for siRNA-triggered RNA cleavage in vitro.
Synopsis

Cells employ diverse mechanisms to control the activity of their genes, and over the last ten years, a new strategy for gene regulation called RNA silencing has been discovered. Central components responsible for RNA silencing are Argonaute proteins. While many of the molecular properties of Argonaute proteins have been uncovered, little is known about their function in living organisms. In *Drosophila*, it has been puzzling that mutations in individual Argonaute proteins lead to surprisingly mild defects in development, although RNA silencing had been suggested to play major roles in gene regulation. Meyer and coworkers describe that Ago1 and Ago2, two Argonaute family members in *Drosophila*, function in a redundant fashion. Previously, these two proteins were demonstrated to mediate distinct pathways of RNA silencing. The authors show that in early embryos Ago1 and Ago2 work together in two fundamental processes: the generation of polarity within cells, by controlling the unequal distributions of proteins and cell organelles, and the polarity of tissues, by modulating an important cell-cell signaling pathway. These results connect the activity of Argonaute proteins and by extension the mechanisms of RNA silencing with central problems of cell and developmental biology, namely the regulation of polarity in cells and tissues.

[11]. A combination of biochemical and genetic approaches has demonstrated an essential function of Ago2 for RNAi [9,11], but depletion of Ago2 does not impair miRNA-directed RNA cleavage in vitro. A similar distinction has been detected at the level of Dicer: *Dicer-1 (Der-1)* mutants are defective in processing pre-miRNAs, while *Dicer-2 (Der-2)* mutants are defective in processing siRNA precursors [24]. The fact that null mutations in *Der-2* are homozygous viable and fertile suggests that siRNA-triggered RNAi is not essential for normal development. In contrast, homozygous *Der-1* null mutants are lethal supporting the model that regulation through miRNAs is a crucial mechanism during embryogenesis.

We have characterized the maternal-effect mutation *drop out (dop)*, which causes specific developmental defects at the midblastula transition. The mutant embryos show a transient block in membrane growth and fail to undergo a developmental switch in the microtubule-based polarized transport of lipid droplets. Surprisingly, we find that *dop* mutations represent special alleles of *ago2*. Two independently generated *dop* alleles reduce the copy number of the GRRs, providing the first evidence of a functional role of this domain. These mutations render Ago2 only partially defective in siRNA responses. However, these alleles interact genetically with Ago1, suggesting the possibility of crosstalk between Ago1- and Ago2-mediated pathways. This conclusion is further supported by double-mutant analysis using loss-of-function alleles of *ago2* and *ago1*; we demonstrate that the two gene products function redundantly in embryonic patterning. Our results reveal novel functions of Argonaute proteins in early embryogenesis and suggest a regulatory role for the GRR domain of Ago2.

Results

The *dop* Mutation Affects Membrane Growth during Cellularization

To dissect the mechanisms that establish epithelial cell polarity, we reexamined the previously described female-sterile mutation *dop* [25]. Flies homozygous or hemizygous for *dop* are viable, but embryos from *dop* homozygous mothers do not hatch and display defects in larval cuticle formation, reminiscent of bazooka or crumbs alleles, mutations that disturb the formation of the embryonic epidermis (see below). Using videomicroscopy of living embryos, we found that these embryos display severe morphological defects about 3 h after fertilization, at the time of the midblastula transition (MBT).

The MBT is marked by the onset of substantial zygotic transcription and is accompanied by dramatic morphological changes. One of the most striking MBT-specific processes in *Drosophila* is cell formation, and it is this process that is abnormal in *dop* mutants. In the wild-type, the first 13 cleavage divisions of the zygote take place without cytokinesis, and during cycle 14, the time of the MBT, polarized growth of the plasma membrane transforms the syncytial embryo into the polarized blastoderm epithelium [26]. In the *dop* mutant embryos, we detected no significant differences to the wild-type up to cycle 13 (not shown). But the initiation of membrane growth in cycle 14 was significantly delayed (Figure 1A–1C). The defect in membrane growth was also evident by immunolabeling for an endogenous marker for cleavage membranes, Neurotactin (Nrt) (Figure 1D and 1E).

*dop* represents the first example of a maternal-effect mutation that specifically affects morphogenetic events at the MBT.

Cell formation is initiated by the generation of two membrane subdomains: the furrow canal and the basal junction [27]. In *dop* embryos, Slam, a marker for the furrow canal [28], did not accumulate in between the individual nuclei but remained associated with the egg cortex in an irregular pattern throughout the initial stages of cell formation (Figure 1F–II). Similarly, Arm, a marker for basal junctions, failed to accumulate in a typical honeycomb-like pattern but remained diffusely distributed over the egg cortical cytoplasm (Figure 1J–1M). We conclude that *dop* affects early morphological processes during the MBT, namely, initiation of the furrow canal, formation of basal junctions, and membrane growth.

The morphogenetic events during the MBT in *Drosophila* are controlled by a limited number of zygotic genes [29]. We therefore tested whether the *dop* phenotype results from defective expression of Slam, a known zygotic regulator of cellularization. Zygotic mutants for *slam* display a delay in cellularization very similar to the one of *dop* embryos [28,30]. Yet levels of both *slam* transcript and of Slam protein were normal in *dop* embryos, as were their spatial expression patterns (Figure 1F–II and unpublished data). Thus, the phenotype of *dop* embryos does not reflect a defect in the regulation of *slam* expression.

Membrane growth during cellularization requires an intact microtubule cytoskeleton [31,32]. Thus, the cellularization defects in *dop* might indicate problems with microtubule-based transport. Since the mechanisms of microtubule transport during cellularization are not well understood, we instead examined a well-defined microtubule-based transport process in the early embryo, the motion of lipid droplets.

*dop* Embryos Display Aberrant Microtubule-Based Transport of Lipid Droplets

When examined by videomicroscopy, *dop* embryos were...
Function of argonaute Genes in Drosophila
Figure 1. dop1 \(^1\) Affects Cell Formation in the Early Embryo

(A–C) Membrane extension is strongly reduced in dop1 \(^1\) embryos. (A, A’) Frames taken from a video-sequence of a wild-type embryo. In the wild-type, membranes grow slowly for the first 30 min of cycle 14 interphase [slow phase (A)], and then growth speeds up considerably [fast phase (A’)]. (B, B’) dop1 \(^1\) mutant embryo from a video-sequence at corresponding time points. The shape of the nuclei (circles) and the extent of furrow progression are indicated (bars). (C) The progression of the cleavage furrow was plotted against the time after the beginning of mitotic cycle 14. Note that compared to the wild-type furrow progression is significantly slower in embryos derived from homozygous (dop/dop) or hemizygous (dop/\(\text{Df}[3L]XG9\)) dop1 females, within the first 30 min of cellularization. In dop1 homozygotes, membranes advanced 0.07 \(\mu\)m/min during slow phase, compared to 0.25 \(\mu\)m/min in the wild-type. In hemizygous dop1 embryos, membrane formation was even more severely impaired; it was slower during both slow and fast phase.

(D, E) Immunostaining of the endogenous membrane protein Neurotactin (Nrt; green) and DNA (blue) in wild-type (D) and dop1 \(^1\) (E) mutant embryos during progression of cell formation (from left to right panel). While Nrt associates with the egg cortex in both cases, Nrt positive cleavage membranes in dop1 mutant embryos are absent until fast phase.

(F–I) Immunostaining with Slam antibodies (red) indicates the presence of furrow canals in wild-type (F, H) and dop1 \(^1\) (G, H) mutant embryos (F, G: optical cross section; H, I: tangential optical section) at progressively older stages of cell formation.

(J–M) Immunostaining with Arm antibodies (red) indicates the positioning of the basal junctions; two timepoints are shown in each panel: beginning of slow phase to the left and beginning of fast phase to the right.

(F–L) Immunostaining with Slam antibodies (red) indicates the formation of the furrow canal as a regular array of loop-like structures beginning with nuclear elongation. In dop1 mutant embryos, Slam localization does not resolve into this regular array and forms an unevenly distributed network apical to the nuclei. (J–M) Immunostaining with Arm antibodies (red) indicates the positioning of the basal junctions; two timepoints are shown in each panel: beginning of slow phase to the left and beginning of fast phase to the right.

(K, M) In dop1 \(^1\) mutant embryos, Arm remains apical and does not accumulate in basal junctions. Fixed embryos were staged by the extent of nuclear elongation, as described by Lecuit et al. [30].

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abnormally transparent from gastrulation onward (Figure 2A and 2B). Such altered transparency can be a signature of mislocalized lipid droplets, as cytoplasm filled with lipid droplets is opaque [33,34]. In the wild-type, lipid droplets are present throughout the embryo periphery after gastrulation. In contrast, in dop1 \(^1\) embryos, they were highly enriched basally, around the yolk sac (Figure 2C and 2D).

In the wild-type, droplet distribution along the apical-basal axis is temporally coordinated with cellularization: droplets are located throughout the periphery in syncytial stages and are transported basally early during cycle 14 and back apically during gastrulation [33]. As a result, the periphery first becomes transparent (cytoplasmic clearing) and then turns opaque again. Because wild-type and dop1 \(^1\) embryos displayed very similar transparency up until the end of cycle 14, the initial basal droplet transport appeared normal. To

Figure 2. dop1 \(^1\) Compromises Polarized Microtubule-Based Transport

(A, B) During germband extension, the periphery of dop1 \(^1\) embryos (B) is much more transparent than that of wild-type embryos (A).

(C, D) Lipid droplets (green) were stained with the droplet-specific fluorescent dye Nile Red, and their distribution was recorded by epifluorescence microscopy. In the wild-type, lipid droplets are found throughout the periphery. In the mutant, lipid droplets are accumulated around the central yolk. (E–H) Males homozygously deleted for \(\text{halo}\) were crossed to dop1 heterozygous (E, G) or homozygous (F, H) females to generate embryos with reduced \(\text{halo}\) expression. In these embryos, droplet distribution was assessed by overall transparency (E, F) or staining for the regulator Klar (green) (G, H). In late cycle 14, embryos from dop1 homozygous mothers have a more transparent periphery and tighter basal accumulation of Klar puncta.

(I, K) Overall expression and distribution of Klar (green) is very similar in wild-type (I) and ago2\(^{89C}\) embryos (K). (L) In centrifuged early embryos, lipid droplets accumulate in a distinct layer, just above nuclei (blue). In centrifuged dop1 embryos, Klar (green) is highly enriched in the droplet layer, just as in the wild-type [36], indicating that it is physically associated with the droplets. Scale bars represent 200 \(\mu\)m in (A) and 80 \(\mu\)m in (L).

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determine when the difference in droplet transport between these genotypes arises, we employed embryos in which expression of the directionality determinant Halo was reduced. Under these conditions, basal transport is slower and less complete [35]. Embryos that expressed only a single copy of halo appeared similar early in cycle 14 whether they were derived from dop\(^{1}\) homozygous or heterozygous mothers (not shown), but late in cycle 14, lipid droplets in embryos from dop\(^{1}\) homozygotes accumulated more basally (Figure 2E–2H), as judged by both higher embryo transparency and tighter basal accumulation of Klar, a droplet-associated regulator [36]. Thus, droplet distribution in dop\(^{1}\) becomes abnormal late in cycle 14, and droplets fail to switch from basal to apical transport.

The abnormal droplet distribution in dop\(^{1}\) embryos is reminiscent of the droplet-transport defect in embryos mutant for the regulator Klar [33]. However, Klar expression and distribution were very similar between wild-type and dop\(^{1}\) embryos (Figure 2I and 2K), and Klar was physically associated with lipid droplets in the mutant, as it is in the wild-type (Figure 2L). To test whether Klar function was affected in dop\(^{1}\) embryos, we took advantage of the observation that in embryos that completely lack Halo the direction of net transport in early cycle 14 depends on Klar: it is apical in the presence of Klar but basal in the absence of Klar (unpublished data). When we abolished Halo expression pharmacologically, droplet motion was net apical in wild-type and dop\(^{1}\) embryos but net basal in klar embryos (Figure S1). Thus, Klar is not only expressed but also functional in dop\(^{1}\) embryos. Expression and function of Halo also appeared to be unaffected in dop\(^{1}\) embryos (Figure S2).

Lipid droplets move bidirectionally along microtubules, and their apical-basal distribution results from the relative contribution of plus- and minus-end motion [33]. Altered droplet distribution in dop\(^{1}\) embryos was not due to lack of droplet motion per se since droplets moved bidirectionally before, during, and after cellularization (unpublished data). This observation indicates that the motors driving droplet transport as well as the microtubule tracks are grossly intact. We conclude that the basic machinery responsible for droplet motion is intact and that dop\(^{1}\) alters a specific aspect of transport resulting in faulty regulation of net transport direction.

In summary, the dop\(^{1}\) mutation reveals the existence of a maternally established control mechanism to regulate specific events at the MBT: formation and growth of membrane at the start and during cellularization, and a developmentally regulated switch in organelle transport at the end of cellularization. Importantly, dop\(^{1}\) does not pleiotropically impair embryogenesis per se; we did not observe developmental defects prior to the MBT. It remains to be established whether the defects in the dop\(^{1}\) mutants reflect a common problem with microtubule-based transport or independent molecular mechanisms. Uncovering the underlying molecular mechanisms should provide new insights into the regulation of the morphogenetic events associated with the MBT.

dop Is Allelic to ago2

As a first step toward characterizing this new mechanism controlling the MBT, we sought to identify the gene affected by the dop\(^{1}\) mutation. Using chromosomal deletions, we mapped dop\(^{1}\) to a 45-kb genomic region that contains six predicted genes (Figure 3). P-element insertions within this interval were employed to produce small deletions by male recombination [37]. The majority of the recombinant chromosomes that failed to complement dop\(^{1}\) were obtained with two insertions that flank the genes for CG7739 or ago2 (Figure 3B and unpublished data). It seemed therefore likely that dop is represented by one of these two genes. We generated rescue constructs in which the maternal expression of the directionality determinant Halo was restored (Figure 3B and unpublished data). It seemed therefore likely that dop is represented by one of these two genes. We generated rescue constructs in which the maternal expression of the directionality determinant Halo was restored (Figure 3B and unpublished data). It seemed therefore likely that dop is represented by one of these two genes. We generated rescue constructs in which the maternal expression of the directionality determinant Halo was restored (Figure 3B and unpublished data). It seemed therefore likely that dop is represented by one of these two genes. We generated rescue constructs in which the maternal expression of the directionality determinant Halo was restored (Figure 3B and unpublished data).

The conclusion that dop\(^{1}\) is an allele of ago2 is supported by an independent allele, dop\(^{46}\), that we created by transposase-induced reversion of EP(3L)3417, a P-element inserted within the 5' UTR of ago2. Females homozygous or hemizygous for dop\(^{46}\) are sterile, and their embryos can be rescued to viability by expression of the mat::tub-ago2 transgene. Embryos derived from dop\(^{46}\) mothers to viability (Table 1).

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that zygotic expression of Ago2 was sufficient to rescue
ago2
dexpression. To test this notion, we used the
DIAP1RNAi
sequence, the eyes are significantly smaller (Figure 4A and 4B).
are reduced, causing cells to die by apoptosis; as a conse-
quence, the eyes are significantly smaller (Figure 4A and 4B).
are due to compromising a zygotic function and are not
simply the consequences of earlier defects in oogenesis.

from dop
4 homzygous or hemizygous females showed
defects in early development very similar to embryos from
dop
4 mothers. dop
4 failed to complement both the lethality and the
cellularization defects of dop
4. We conclude that dop
4 mutations represent mutant alleles of ago2. In the following,
these alleles will be called ago2
dop
1 and ago2
dop
46, respectively. Embryos derived from homozygous or hemzygous mutant
mothers will be referred to as ago2
dop
 embryos.
The phenotype of ago2
dop
1 strongly suggests that this
mutation specifically disturbs processes at the MBT, as it did not impair syncytial development of the embryo. To test this notion, we used the Gal4/UAS system to drive expression of ago2 cDNA at the onset of zygotic transcription. We found that zygotic expression of Ago2 was sufficient to rescue ago2
dop
1 mutant embryos to viability (Table 2). We therefore conclude that the major defects in ago2
dop
 embryos are due to compromising a zygotic function and are not simply the consequences of earlier defects in oogenesis.

Table 1. Rescue of dop
1 Mutants by Maternally Expressed Full-
Length ago2 cDNA

| Genotype                     | Larvae Hatched (n) | %     |
|------------------------------|-------------------|-------|
| Osr × Osr (control)         | 50 (50)           | 100   |
| ago2
agomttub
× Osr                  | 0 (112)           | 0     |
| ago2
agomttub
ago2
dop
1/ago2
dop
1 × Osr | 162 (533)         | 31.4  |
| ago2
agomttub
ago2
dop
1/ago2
dop
1 × Osr | 143 (564)         | 27.7  |

Eggs were collected from crosses indicated (genotype of mothers [left] and fathers [right]) and the number of hatched larva was examined (Osr = Oregon R, n = total number of eggs analyzed. Rescue by the mat::tub-ago2 transgene is highly efficient as dop
1 homzygotes carrying the transgene can be maintained as a stock.

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Table 2. Genetic Interaction of ago2
dop
1 with ago1: Distribution of Larval Cuticle Phenotypes

| Genotype                      | Normal | Class I | Class II | Class III | Class IV | n   |
|-------------------------------|--------|---------|----------|-----------|----------|-----|
| ago2
41/ago41                   | 0      | 4       | 15       | 29        | 52       | 202 |
| ago2
dop
1/ago2dop
1             | 0      | 31      | 12       | 10        | 47       | 213 |
| ago2
agomttub
ago2
agomttub
ago2
dop
1/ago2
dop
1 mat::Gal4 | 0      | 7       | 10       | 13        | 70       | 509 |
| UAS-Ago2+/+; ago2
agomttub
ago2
dop
1 mat::Gal4 | 2      | 15      | 22       | 22        | 39       | 222 |
| UAS-Ago2+/+; ago2
agomttub
ago2
dop
1 mat::Gal4 | 1      | 32      | 18       | 10        | 39       | 731 |

Numbers represent percentages of observed phenotypes; for classification of cuticle phenotypes see Figure 5. Severity of defects increases from left to right: “normal” represents hatching first instar larvae. Note that in the last two experiments only 50% of the embryos contain both the UAS and the Gal4 transgenes, which are together required for rescue.

Embryos are derived from mothers of the indicated genotypes crossed to fathers of the same genotype.

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41/ago41                   | 0      | 4       | 15       | 29        | 52       | 202 |
| ago2
dop
1/ago2dop
1             | 0      | 31      | 12       | 10        | 47       | 213 |
| ago2
agomttub
ago2
agomttub
ago2
dop
1/ago2
dop
1 mat::Gal4 | 0      | 7       | 10       | 13        | 70       | 509 |
| UAS-Ago2+/+; ago2
agomttub
ago2
dop
1 mat::Gal4 | 2      | 15      | 22       | 22        | 39       | 222 |
| UAS-Ago2+/+; ago2
agomttub
ago2
dop
1 mat::Gal4 | 1      | 32      | 18       | 10        | 39       | 731 |

In this experimental system, the efficiency of RNAi induced by long dsRNA can be assessed by the size of the eye: efficient RNAi results in severely reduced eyes; complete disruption of RNAi leads to normal-sized eyes. In flies with only a single copy of the Dcr-2 gene, this phenotype was partially suppressed (Figure 4C), confirming that the activity of DIAP1RNAi depends on siRNA production through Dcr-2. We also tested a reported null allele of ago2 in this assay: ago2
21B has a deletion of exons 1 and 2 and completely abolishes siRNA-directed RNAi in embryo extracts [11]. In flies homozygous for ago2
21B, the DIAP1RNAi phenotype was completely suppressed (Figure 4D). Together these results are consistent with the biochemical requirements of Dcr-2 and Ago2 for RNAi [11,24].

The ago2
21B mutation impaired RNAi only partially. Animals heterozygous for ago2
21B did not exhibit a significant modification of the DIAP1RNAi phenotype (Figure 4E). We observed mild suppression in ago2
21B homozygotes and stronger suppression in ago2
21B hemizygotes, but even in the most extreme cases suppression was less pronounced than for ago2
21B (Figure 4F and 4G). Thus, the ago2
21B mutation compromises, but does not abolish, Ago2’s function to mediate RNAi induced by long dsRNA.

When we examined ago2
21B mutant embryos, in which RNAi is completely abolished, the embryos appeared largely normal morphologically, consistent with previous reports on the effect of another ago2 null allele, ago2
41 [11,39]. As described recently, a minor fraction of the embryos derived from mothers homozygous for ago2
21B (44% of embryos, n = 786) or ago2
21B (33% of embryos, n = 200) exhibit defects during syncytial cleavages [40]. Embryos displaying syncytial defects were grossly abnormal and did not develop beyond that stage. All other embryos obtained from ago2
21B or ago2
21B homozygous mothers developed normally, and cellularization of these embryos was indistinguishable from the wild-type (Figure S3). We also analyzed the distribution of lipid droplets in ago2
21B homozygous embryos and found no defects in net transport of droplets (Figure S4). Thus, it seems unlikely that the defects in membrane growth and microtubule transport in ago2
21B embryos are due to a failure in the siRNA pathway.

This conclusion is further supported by the lack of morphogenesis defects in Dcr-2 mutants. Lee et al. [24] reported that mutations in Dcr-2 are viable, and we found that embryos derived from Dcr-2 homozygotes exhibit normal cellularization and net lipid-droplet transport (unpublished data). We therefore conclude that ago2
21B mutations alter the activity of the ago2 gene and that the forms of Ago2 encoded
by the ago2<sup>dop1</sup> alleles might interfere with a pathway distinct from siRNA-triggered RNAi.

**ago2 Interacts Genetically with ago1**

One possibility to explain the early embryonic phenotype of ago2<sup>dop1</sup> mutants is that mutations in one Argonaute family member might affect the function of another. A good candidate for this other Argonaute gene is *ago1*. While biochemical studies have concluded that Ago1 is dispensable for siRNA-triggered RNAi [11], it has also been reported that mutations in *ago1* mildly suppress siRNA-triggered RNAi in the embryo [21]. Consistent with the latter report, we found, using the DAPI<sup>RNAi</sup> reporter construct, that reduction of *ago1* gene dosage mildly suppresses the RNAi response in the compound eye (Figure 4H). This suppression can even be enhanced by ago<sup>2dup1</sup>; flies that are heterozygous for *ago1* and homozygous for ago<sup>2dup1</sup> exhibit a stronger suppression of the DAPI<sup>RNAi</sup> phenotype as either mutation alone (Figure 4I and 4J). We conclude that *ago1*, at least in the eye, is required for siRNA-mediated RNAi and thus Ago1 and Ago2 might have partially overlapping functions.

Given the interaction of *ago1* and *ago2* in siRNA-mediated silencing, it is conceivable that these two genes also have overlapping roles at the MBT. Indeed, reduction of *ago1* function enhanced the cellularization phenotype of ago<sup>2dup1</sup> homozygotes: membrane growth was strongly compromised, and only very little membrane extension was observed (Figure 5A). This enhanced defect in cell formation was also evident from a more severe disruption of larval cuticle formation (Figure 5B-5F and Table 2). Remarkably, zygotic expression of UAS::ago1 was sufficient to ameliorate the cuticle defects of ago<sup>2dup1</sup> mutant embryos and to allow some of them to develop into adult flies (Figure 5E and Table 2). Thus, the severity of the ago<sup>2dup1</sup> phenotypes is sensitive to the levels of expression of *ago1*, suggesting that in ago<sup>2dup1</sup> mutants Ago1 function has become limiting.

Since the overlapping functions of Ago1 and Ago2 were detected using our ago<sup>2dup1</sup> alleles, the question arises of whether this genetic interaction is an unusual feature of these particular alleles or whether the two genes cooperate more generally. We were able to distinguish between these possibilities when we discovered a novel phenotype of *ago1* and ago2 in larval cuticle formation (Figure 6). Embryos homozygous for both ago<sup>1k08121</sup> and ago<sup>2dup1</sup> displayed cuticles with characteristic patterning defects, similar to those described for mutations in the *wingless* (wg) or *arm* genes (Figure 6B and 6C). This strong segment-polarity defect was also observed in embryos double mutant for the two loss-of-function alleles ago<sup>1k08121</sup> and ago<sup>21B</sup> (Figure 6D and 6E). Because such defects are never observed in single mutants for *ago1* or *ago2* (20; W. J. Meyer and H. A. J. Müller, unpublished data), these data indicate that *ago1* and *ago2* act in a partially redundant fashion. In addition, these observations reveal a previously unrecognized essential role of Argonaute genes in segmental patterning.

To identify the molecular basis for this patterning defect, we examined the expression of Wg, Engrailed (En), and Arm proteins in the double mutants. In the wild-type, Wg and En are initially expressed in 14 nonoverlapping segmental stripes (Figure 6G and 6H). In response to Wg signaling, levels of cytoplasmic Arm increase and En expression is maintained in the receiving cells (Figure 6I; [41]). In embryos zygotically
Figure 5. Genetic Interaction of ago2<sup>dop1</sup> with ago1
Reduction of the ago1 gene doses enhances the ago2<sup>dop1</sup> cellularization phenotype.
(A) Kinetics of membrane extension during cellularization in embryos derived from ago1<sup>K08121</sup>/CyO; ago2<sup>dop1</sup>/ago2<sup>dop1</sup> females. Embryos from Kr<sup>+/CyO</sup>; ago2<sup>dop1</sup>/ago2<sup>dop1</sup> display the characteristic dop delay in membrane growth during cellularization (purple line; n = 6) relative to the wild-type (Oregon R; black line; n = 6). Embryos from ago1<sup>K08121</sup>/CyO; ago2<sup>dop1</sup>/ago2<sup>dop1</sup> mutants (blue line; n = 9) show a strongly reduced furrow progression even during fast phase.

(B–E) Embryos derived from mothers homozygous or hemizygous for ago2<sup>dop1</sup> do not hatch and produce abnormal larval cuticles, which can be grouped into four classes. (B) Class I (continuous cuticle): such embryos form a continuous cuticle with more or less severely affected denticle belts. (C) Class II (shield): such embryos produce a shield of continuous cuticle, reminiscent of neurogenic mutants. (D) Class III (crumbs-like): such embryos produce only small globular remnants of cuticle, reminiscent of mutations in genes required for epithelial polarity, such as crumbs. (E) Class IV (no cuticle): embryos in this class did not produce any cuticle at all.

(F) Graphic presentation of the distribution of cuticle phenotypes of the different classes; color codes are indicated on (B–E).
Original data are presented in Table 2.
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homzygous for both ago1<sup>K08121</sup> and ago2<sup>51B</sup>. Wg protein was not detected in extended germ-band stages (Figure 6H). The lack of expression of Wg protein in these embryos is sufficient to result in the observed patterning defects. As a consequence of the lack of Wg protein expression, cytoplasmic Arm stripes are not present and En expression is not maintained in these embryos (Figure 6K and 6N). This genetic interaction between ago1 and ago2 alleles indicates a partially redundant function of the two Argonaute family members in the positive regulation of Wg protein expression in the early embryo.

Since Ago1 is an essential component of the miRNA-triggered RNA silencing, we reasoned that the observed partial redundancy with Ago2 might reflect a function of Ago2 in the miRNA pathway. If this assumption were correct, ago1 might exhibit a similar genetic interaction with mutations in other crucial components of the miRNA pathway. The best candidate to test for this interaction is Dcr-1, because Dcr-1 is essential for the processing of pre-miRNAs [24]. Indeed, we found that double mutants of the loss-of-function alleles ago1<sup>K08121</sup> and Der-1<sup>Q1147X</sup> exhibit the same segment-polarity cuticle phenotype and lack of Wg expression as ago1, ago2 double mutants (Figure 6F, 6I, and 6L). In ago1, Der-1 double mutants, Wg protein was not detected even in gastrula stages (Figure 6O and 6P). Embryos singly mutant for Der-1<sup>Q1147X</sup> or double mutant for Der-2<sup>51B</sup> or Der-1<sup>Q1147X</sup> do not show such defects (Figure 6A and unpublished data). Because a reduction in the zygotic expression of two major components of the miRNA pathway can result in segment polarity defects, these data suggest an as-of-yet unrecognized requirement of miRNA function for segmentation and the regulation of Wg expression.

In summary, our genetic analysis has uncovered several instances in which Ago1 and Ago2 appear to act in a partially redundant and partially overlapping manner: in siRNA-mediated RNA interference, in the regulation of Wg expression in the early embryo, and for morphological changes associated with the MBT.

Biochemical Interaction of Ago1 and Ago2
What is the molecular basis for the observed genetic interaction between ago1 and ago2? It is conceivable that depletion of Ago2 leads to a reduction in the levels of Ago1 or shared components of the RISC assembly machinery. To investigate this possibility, we analyzed Ago1 protein levels in ago2 mutants by Western blotting. We find that Ago1 protein levels are wild-type in embryos derived from ago2<sup>dop1</sup> homozygotes, as well as from ago2<sup>51B</sup> or ago2<sup>514</sup> homozygotes (Figure 7A). We also tested the levels of Dcr-1 and Loquacious (Loqs), a dsRNA binding protein that can be part of a protein complex with Ago1 and Dcr-1 and is important for the function of Dcr-1 to process pre-miRNAs [42,43]. Like in the case of Ago1, Loqs and Dcr-1 protein levels were largely unimpaired in ago2<sup>dop1</sup>, ago2<sup>51B</sup>, or ago2<sup>514</sup> homozygotes (Figure 7B and 7C). We therefore conclude that mutations in ago2 do not result in global changes of the protein levels of Ago1, or shared components involved in RISC assembly.

Our genetic data demonstrate that Ago1 and Ago2 act in a partially redundant fashion in siRNA-triggered RNAi as well as during morphogenesis and pattern formation in the
embryo. One possible explanation for this interaction would be that Ago1 and Ago2 have overlapping functions as part of a common protein complex. To examine this possibility, we used Ago2 antibodies to immunoprecipitate Ago2 protein complexes from embryo lysates and assayed for the presence of Ago1. We found that anti-Ago2 antibodies coprecipitate Ago1 protein from these extracts (Figure 7D). These results indicate that subpopulations of Ago1 and Ago2 proteins are present in a common complex, consistent with the idea that Ago1 and Ago2 have overlapping functions.

ago2<sup>dop</sup> Mutations Affect Glutamine-Rich Repeats in the Amino-Terminus of Ago2

Our data on the ago2<sup>dop</sup> alleles indicated that these mutations compromise Ago2’s function in the siRNA pathway and might also disturb the normal function of Ago2 such that it negatively interferes with Ago1 function. This suggests that a molecular characterization of the ago2<sup>dop</sup> mutations should give insight into the mechanism by which Ago2 and Ago1 interact. Our molecular analysis indicates an important role for the previously uncharacterized amino-terminal GRR domain (Figure 8). This domain is rich in glutamines and is largely composed of four imperfect copies of the 6–amino acid (aa) repeat GRR1 and 11 imperfect copies of the 23-aa repeat GRR2 (Figures 8B and S5).

When we compared ago2 expression by RT-PCR or Northern blotting, we found that ago2 mRNA was expressed in ago2<sup>dop1</sup> but that its size was slightly shorter than in the wild-type (Figure 8A). Importantly, despite the size difference, the total levels of ago2 transcript are the same in the wild-type and ago2<sup>dop1</sup> mutants (Figure 8A). We also assessed whether the ago2<sup>dop1</sup> mutation affects the total protein levels of Ago2 and found that Ago2 protein levels are unimpaired in the mutant (Figure 8A). Thus, the ago2<sup>dop</sup> mutation does not affect the normal accumulation of Ago2 mRNA or protein in the embryo.

We found that the genomic sequence of ago2<sup>dop1</sup> exhibits a 69-nucleotide in-frame deletion in exon 3 that leads to the loss of exactly one copy of GRR1 (Figure 8A). Importantly, despite the size difference, the total levels of ago2 transcript are the same in the wild-type and ago2<sup>dop1</sup> mutants (Figure 8A). We also assessed whether the ago2<sup>dop1</sup> mutation affects the total protein levels of Ago2 and found that Ago2 protein levels are unimpaired in the mutant (Figure 8A). Thus, the ago2<sup>dop1</sup> mutation does not affect the normal accumulation of Ago2 mRNA or protein in the embryo.

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when compared to the wild-type (ago2Δ) reduction in the number of either type of GRRs has severe activity in siRNA-triggered RNAi. Strikingly, even a slight protein and that mutations in the GRRs reduce Ago2 domain of Ago2 is essential for the normal function of the data provide the first evidence that the amino-terminal

Ago2Cterm antibodies or with an antibody against GFP as a control; (input). Immunoprecipitations (IP) were performed using anti Ago1 and protein in extracts from wild-type embryos is shown in the left lane (D) Coimmunoprecipitation experiments of Ago1 with Ago2. Ago1 protein levels of Loqs (B) and Dcr-1 (C) are largely unimpaired in extracts from ago2ΔDop1, ago2ΔDop, and ago2ΔDop1 mutants; α-tubulin was used as a loading control. Likewise, the protein levels of Loqs (B) and Dcr-1 (C) are largely unimpaired in extracts from ago2ΔDop1, ago2ΔDop, and ago2ΔDop1 mutants when compared to the wild-type (w1118). In (C) ovary extracts were used instead of embryo extracts.

Discussion

In Drosophila, two major molecular pathways of RNA silencing have recently been defined: miRNA-induced silencing and siRNA-induced RNAi [1]. At the level of Argonaute family members, Ago1 has been implicated in miRNA function while Ago2 was shown to be essential for siRNA function. Our analysis provides genetic and biochemical evidence that Ago1 and Ago2 have overlapping functions both in siRNA-triggered RNAi and during early embryogenesis.

We found that, in addition to the PAZ and PIWI domains conserved in all family members, insect orthologs of Ago2 contain an amino-terminal GRR domain. The ago2ΔDop alleles allow us to probe the function of this domain. Even the subtle alterations in these alleles have striking organismal phenotypes, but the absence of Ago2 (in the reported null alleles) does not. While the mutant Ago2 proteins still support siRNA

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function to some extent, they also interfere with Ago1-dependent processes.

A Function for the Amino-Terminal Glutamine-Rich Repeats of Ago2

In other proteins, glutamine-rich domains have been implicated in protein aggregation, such as in certain neurodegenerative diseases that involve the formation of long-lived protein aggregates (e.g., the PolyQ domain of mutant Huntingtin). Extension of the glutamine-rich region promotes aggregation, and the length of the polyglutamine extension correlates with the severity of the disease [22]. Glutamine-rich domains are also involved in the mechanism by which yeast prions switch between soluble and aggregated states [49, 50]. For the translation factor Sup35, e.g., increases in the copy number of GRRs in the prion domain favor the aggregated, inactive state; decreases in the copy number favor the soluble, active state [47, 51]. Our genetic and molecular analyses of the ago2<sup>dop1</sup> alleles thus raise the tantalizing possibility that the GRRs regulate Ago2 by modulating its aggregation state. Unlike in the polyglutamine diseases, however, it is the reduction, rather than the expansion, of the GRR region that leads to an aberrant Ago2 protein. *Drosophila* Ago2 may therefore provide a unique inroad for dissecting the normal organismal function of glutamine-rich or PolyQ domains.

Since Ago2 is an essential component of protein complexes, such as the RISC, control of its aggregation state is conceivably important for its function. Mammalian Argonaute proteins are localized to GW bodies, cytoplasmic compartments analogous to yeast P-bodies, which are centers of mRNA degradation [52, 53]. Central components of GW bodies, like GW182 and decapping enzymes DCP1:DCP2, have recently been shown to also be involved in miRNA-mediated gene silencing in *Drosophila* cultured cells [54]. The presence of both Ago1 and Ago2 in GW bodies is consistent with our biochemical studies. An important next step for unraveling the molecular function of the Ago2 GRR domain will be to determine whether the ago2<sup>dop1</sup> alleles alter the recruitment of Ago2 to particular cytoplasmic mRNA degradation complexes. Such recruitment via glutamine-rich domains need not necessarily inactivate the protein; in the translation factor CPEB from *Aplysia*, a glutamine-rich prion-like amino-terminal domain promotes protein aggregation, and it is the aggregated form that has the greatest capacity to stimulate translation [55].

Specificities of Ago1 and Ago2 Functions

Previous analyses had suggested a simple model of division of labor between Argonaute proteins in *Drosophila*, with Ago1 specific for miRNA-directed silencing and Ago2 involved in siRNA-triggered RNAi. However, our genetic data add to emerging evidence that these proteins play much broader roles. Ago2, for example, appears to have functions beyond siRNA-induced RNAi. It has been proposed that in larval neurons Ago2 is recruited via the dFMR1 protein to certain RNP complexes, including those containing the PPK1 mRNA. This recruitment is functionally important since in the ago2<sup>11B</sup> allele PPK1 mRNA levels are not properly downregulated; thus, Ago2 may play a role in the turnover of specific transcripts [39].

For Ago1, on the other hand, it is well established that it has a function in miRNA-directed RNA silencing. But while in biochemical assays Ago1 is not essential for siRNA function, ago1 mutations impair the response of siRNA-triggered RNAi in vivo ([11, 21]; the present work). Our data provide further evidence for overlapping functions of Ago2 and Ago1 in siRNA-directed RNAi. It is possible that although Ago2 is in principle sufficient to promote siRNA-directed RNA decay, in vivo the two proteins act in concert to make this process more efficient.

It is unlikely that the morphogenesis phenotypes of ago2<sup>dop1</sup> mutant embryos are simply caused by disturbing the function of Ago2 in RNAi. Unlike ago2<sup>dop1</sup> mutants, ago2 alleles that...
completely abolish experimental siRNA-induced responses do not cause these gross morphological defects and exhibit problems with nuclear migration only during syncytial stages; these phenotypes occur with a moderate penetrance such that animals homozygous for these alleles can be kept as a stock [11,24,39,40]. Rather, our genetic data suggest that ago2dop mutations compromise the function of both Ago2 and Ago1 in controlling specific aspects of the MBT. A genome-wide analysis of mRNA targets regulated by Argonaute proteins has recently shown that Ago1 and Ago2 are required for the regulation of a common set of miRNA targets, despite the fact that only Ago1 is essential for miRNA function in vitro [56]. In S2 cells, both Ago1 and Ago2 coprecipitate with specific miRNAs, suggesting that not only Ago1, but also Ago2, is able to bind miRNAs [56]. Based on our results, it is conceivable that the interaction of miRNAs with Ago2 is indirect, namely that Ago2 coprecipitates those miRNAs that are bound to Ago1. While the exact mechanisms need to be resolved, the available data provide ample support for our conclusion that Ago1 and Ago2 act in a partially redundant fashion during early embryogenesis.

It is conceivable that the ago2dop mutations not only interfere with Ago1 and Ago2 function but might affect a common factor that is essential for both Ago1 and Ago2 or for Argonaute protein function in general. Preliminary observations suggest that mutations in other Argonaute family members, piwi or aubergine, might also interact genetically with ago2dop alleles (unpublished data). We currently favor the model that disrupting both Ago1 and Ago2 function is sufficient to cause the observed defects at the MBT because ago2dop mutations can be rescued by zygotic expression of either ago1 or ago2. A test of this notion will be to determine the phenotypic consequences for embryos when both the maternal and zygotic expression of ago1 and ago2 has been eliminated. In addition, the interactions of ago2dop alleles with other components of RNA silencing pathways should be examined to further understand the genetic and molecular basis for the altered activity of Ago2dop proteins during the MBT.

Function of Argonaute Proteins in Segment Polarity

Mutations in ago1 were originally discovered in a genetic screen for modifiers of the Wg pathway [20]. Overexpression of ago1 rescued a defect in Wg signaling induced by depletion of cytoplasmic Arm in the wing imaginal disc. However, because embryos homozygous for a loss-of-function mutation in ago1 did not exhibit defects in segment polarity [20], the relevance of Ago1 for normal Wg signaling remained unclear. The data presented in this paper now provide an explanation for this result. By combining loss-of-function mutations in both ago1 and ago2, we demonstrate that the two Argonaute genes have partially overlapping functions and together are required for establishing segment polarity.

The function of Ago1 and Ago2 for the initial expression of Wg protein is striking. No other genes have been identified that are similarly essential for the general expression of Wg. We propose two possible explanations for this result. Ago1 and Ago2 might act to eliminate a general repressor of wg transcription or translation. In this case, it is conceivable that specific miRNAs exist that modulate wg expression by negatively regulating a repressive mechanism. Alternatively, Ago1 and Ago2 might be part of RNPs that contain wg mRNA, and the reduction in Argonaute function might interfere with the microtubule motor-driven localization of the transcripts. It is well established that compromising the apical localization of wg mRNA strongly affects the intracellular distribution and the signaling activity of the protein [57,58]. A detailed analysis of the expression and the localization of wg transcripts will be required to discriminate between these possibilities.

Although we have not been able to find direct evidence that any of the ago2 alleles interfere with miRNA function in vivo or in vitro, it is interesting to note that ago1, Der-1 double mutants exhibit the same segment polarity phenotypes as ago1, ago2 double mutants. This result further strengthens the notion that in the embryo Ago1 and Ago2 might both be important for miRNA function (see above). When we employed an eye reporter assay to test if ago2dop alleles interfere with the function of the bantam miRNA, we failed to detect interactions (Figure S6). This result might be due to the observed redundancy of Ago2 with Ago1 function; such a redundancy was recently described for S2 cells ([56]; Figure S6). Future studies to identify the miRNAs involved and their targets might yield novel insight into the regulation of Wg expression.

An alternative explanation is that our analysis has

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Table 3. Amino-Terminal Glutamine-Rich Domains in Predicted Ago2 Orthologs of Insect Species

| Species           | Number of Glutamines | % Glutamine | Repeats |
|-------------------|----------------------|-------------|---------|
| D. melanogaster   | 121/316              | 38%         | 4 × 6 aa + 11 × 23 aa |
| D. simulans       | 83/233               | 35%         | 2 × 81 aa + 2 × 25 aa |
| D. yakuba         | 125/307              | 41%         | 16 × 16 aa |
| D. ananassae      | 62/160               | 39%         | 2 × 12 aa + 3 × 11 aa + 2 × × 10 aa |
| D. pseudoobscura  | 27/77                | 35%         | 4 × 11 aa |
| D. virilis        | 102/318              | 32%         | 8 × 22 aa + 5 × 13 aa |
| D. mojavensis     | 40/170               | 28%         | 4 × 31 aa |
| Anopheles gambiae | 117/173              | 68%         | No clear repeats |
| Aedes aegypti     | 82/122               | 67%         | No clear repeats |
| Apis mellifera    | 143/337              | 42%         | 10 × 29 aa |

In D. melanogaster, exon 3 of ago2 encodes the bulk of the amino-terminal glutamine-rich domain plus an amino acid stretch widely conserved among Argonaute proteins in many species (indicated in purple in Figure S5). Genomic DNA sequences of the indicated species were examined for homologues of ago2 from D. melanogaster, and the regions corresponding to the conserved stretch of exon 3 were identified. In each case, the genomic sequences immediately 5’ have the capacity to encode glutamine-rich domains. The amino-terminal extent of these domains was estimated using exon prediction software or cDNA evidence (see Figure S5 for details). Shown are the glutamine contents of these regions and the copy number of distinct GRRs identified (highlighted in yellow, blue, and green in Figure S5).

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Function of argonaute Genes in Drosophila
uncovered a novel function of Argonaute protein family members. Intriguingly, the study of Kataoka et al. [20] showed that ectopically expressed Ago1 constructs could suppress Wg pathway defects even if they lacked a functional PIWI domain. This result may suggest that Ago1 function in Wg signaling does not involve its PIWI domain, hinting at an uncharacterized biochemical property of Ago1. Although too little is known at this point to speculate what such a new function might entail, it is interesting to note that there are intriguing connections between microtubules and the RNA silencing machinery: Armitage, a putative helicase required to assemble Ago2-containing RISC [59,60], is associated with microtubules in developing oocytes; the dop alleles of Ago2 interfere with microtubule-based processes at the MBT; and it is conceivable that Ago1 and Ago2 control the microtubule-dependent localization of wg mRNA. Whether or not these phenomena are explained by a shared molecular mechanism remains to be established.

In summary, the genetic interactions described in this paper are not easily reconciled with the model that different pathways in gene silencing are strictly separated. Rather, our data suggest that in the living organism these pathways, or at least crucial components of these pathways, might act in concert. Our observation that ago1 and ago2 cooperate in Wg signaling provides a powerful new tool to resolve some of these issues since now the function of these Argonaute proteins can be assessed using a clearly defined phenotype of a well-characterized signaling pathway.

Function of Ago1 and Ago2 for the Midblastula Transition

Freshly laid Drosophila embryos contain large amounts of maternally supplied mRNAs that encode proteins essential for the earliest stages of embryogenesis [61]. As development proceeds, these maternally supplied transcripts need to be replaced by transcripts synthesized by the zygote. This process is a hallmark of the MBT. Maternal transcripts are degraded via two pathways: a maternal pathway switched on at egg activation, and a zygotic pathway activated at the MBT [62]. Our genetic analysis has shown that although ago2Δ/dop alleles represent maternal-effect mutations, they specifically perturb processes shortly after the onset of zygotic transcription at the MBT. We therefore propose that Ago1 and Ago2 are key mediators of the zygotic pathway of maternal transcript degradation. Precedence for such a scenario has recently been provided by the identification of the miR-143 miRNA family in zebrafish. miR-143 expression is strongly upregulated at the MBT and is required to specifically downregulate a set of maternal mRNAs [63]. Conversely, embryos deficient for Dicer activity display defects shortly after the MBT [63]. It remains to be determined whether miRNAs are also required for maternal transcript degradation in Drosophila.

The known functions and structural features of Argonaute proteins suggest a model for the underlying molecular mechanisms. It is well established that Argonaute proteins can act as ribonucleases and provide slicer activity in RISC [14,17,19,64]. During early development, Ago2 and Ago1 might act as ribonucleases that cleave maternal transcripts at the MBT. Abnormal persistence of maternal mRNAs could then interfere with the morphogenetic events usually triggered by zygotic transcription, such as membrane growth during cellularization and correct directionality of lipid-droplet transport. Alternatively, Argonaute proteins might regulate the translation of such maternal or zygotic transcripts. As we have not detected significant changes in the expression pattern of known regulators of membrane growth and droplet transport (Halo, Slam, Klar), the relevant targets are likely novel components of these regulatory pathways. Identifying them should not only give insight into the regulation of these fundamental cell-biological processes but will also shed light on the mechanisms by which the Argonaute proteins Ago1 and Ago2 work together to control developmental events.

Materials and Methods

Drosophila genetics. Flies were kept on standard medium. The following stocks were used: w[11]; dop+/+; red ETM6 [25]; Df(3L)XG7/TM3; Df(3L)Y.hercules; P[vas.W][31]174/TM3; P[EP](AGO2)5147; P[PZ]Cre83576; Kr, P(Delta2-3)H01.21; CyO; F{P{GawB}}3.109d; ago2P26[11]; y w P[tsFLP]; P[neoFLP]82B; Dcr-1(1)1147/TM3; Dcr-2(2)1471/CyO; F{P[tsFLP]}33; klr6[35]; klr4[36]; ago251B [20]; MGR-Cell4; UAS:DIAP1RNAi; ago220P26[38]; and MGR-Cell4; UAS:AGO2[19]. ago2Δ/dop1 was produced by imprecise excision of EP(3)3417. Male recombinant experiments were done according to [65]. Egg collection and cuticle preparations were according to standard methods.

In vivo observations and immunohistochemistry. Flies in vivo observations and embryos were collected, staged on apple juice plates, and mounted in halocarbon oil 27 (Sigma-Aldrich, St. Louis, Missouri, United States) on microscope slides. Videomicroscopy was performed on a Carl Zeiss (Jena) microscope equipped with Nomarski optics, and time-lapse videos were taken on conventional videotape. For immunohistochemistry, embryos were either heat-fixed or fixed using modified Stefanini's fixative and stained with antibodies essentially as described elsewhere [66]. In situ hybridization with digoxigenin-labeled antisense RNA was performed as described before [67]. Nile Red staining was performed after Welte et al. [53]. The following antibodies were used: rabbit anti-Slam [28]; mouse anti-Neurotactin (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, Iowa, United States), mouse anti-Arm [41], rabbit anti-Armcentral (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, Iowa, United States), mouse anti-Arm [41], rabbit anti-Arm-center (Keller and Müller, unpublished data), and mouse anti-Klar [36]. All secondary fluorochrome-conjugated antibodies were from Jackson ImmunoResearch (West Grove, Pennsylvania, United States) or Molecular Probes (Eugene, Oregon, United State). Imaging was performed on a Leica TCS Confocal microscope, and image processing was performed with Adobe Photoshop (Adobe Systems, San Jose, California, United States).

Molecular biology. Molecular cloning was performed following standard protocols. RT-PCR was performed using OneStep RT PCR kit (Qiagen, Valencia, California, United States) with embryonic poly-A+ RNA as template. Primer sequences are available upon request. Products from RT-PCR were directly sequenced (Seqlab, Göttingen, Germany). Northern blotting was performed using polyA-polymerase (Promega, Madison, Wisconsin, United States) and cloned using the TOPO-cloning kit (Invitrogen, Carlsbad, California, United States). DNA sequencing was performed by Seqlab, and sequences were examined using Lasergene (DNASTAR) software (Madison, Wisconsin, United States).

Immunoblotting and immunoprecipitation. For preparation of protein extracts, 0- to 12-h-old embryos were dechorionated and homogenized in CHAPS lysis buffer (20 mM Tris [pH 8], 150 mM NaCl, 10% glycerol, 2 mM EDTA, 10 mM CHAPS) containing protease inhibitors (pepsatin, aprotinin, leupentin, pefabloc, and antitryptic). The solution was kept on ice for 10 min and then centrifuged for 15 min at 4 °C at 13,000 rpm. The supernatant was transferred into a new reaction tube, and the protein concentration was measured using the Bradford assay (Bio-Rad, Hercules, California, United States). For SDS-PAGE, electrophoresis and Western blotting, 30 μg of protein was boiled in SDS sample buffer and loaded onto a discontinuous, horizontal SDS-polyacrylamide gel. Ovaries were dissected in PBS and directly boiled in SDS-sample buffer. The separation of the proteins was performed in electrophoresis buffer.
Figure S3. Cellularization in ago251B and ago2414 Mutant Embryos Is Unimpaired

Embryos were obtained from Oregon R (wild-type) (A–C), or ago251B (D–F), ago251B (G–I), and ago2414 (J–L) homozygous mothers, fixed, and immunolabeled for Arm (red), Nrt (green), and DNA (blue). Consecutive time points during cellularization are shown from left to right panels for each genotype. Note that cellularization occurs normal in ago251B and ago2414 mutant embryos. The kinetics of membrane formation in ago251B and ago2414 mutant embryos is very similar to that in the wild-type (unpublished data).

Found at DOI: 10.1371/journal.pgen.0020134.sg003 (6.3 MB TIF).

Figure S4. Distribution of Lipid Droplets in ago251B and ago2414 Mutant Embryos Was Analyzed by Nile Red Staining

Embryos at the extended germ band stage were fixed and stained with the lipid droplet specific dye Nile Red.

(A) In the wild-type, Nile Red staining is uniformly distributed.

(B) In ago251B mutant embryos, the outer cell layers are devoid of staining indicative of failure of lipid droplets to move apically (compare to Figure 2).

(C) Embryos from klar mutant mothers displays a similar failure of apical transport.

(D) ago2414 mutant embryo displays a wild-type distribution of lipid droplets, indicated by uniform Nile Red staining.

Found at DOI: 10.1371/journal.pgen.0020134.sg004 (6.1 MB TIF).

Figure S5. Amino-Terminal GRPs in Ag0 Proteins from Different Insects

The protein sequence encoded in ago2 exon 3 of D. melanogaster is listed at the top. The corresponding region of Ag02 predicted from genomic sequences is shown for six additional Drosophila species, the malaria mosquito A. gambiae, the yellow fever mosquito A. aegypti, and the honeybee A. mellifera.

For each species, a bipartite structure is apparent: an amino-terminal glutamine-rich region (glutamines indicated in red and bold) of variable sequence followed by a conserved stretch at the 5′ end of exon 3 (purple). In many instances, the glutamine-rich regions contain multiple imperfect copies of distinct repeats (yellow, blue, or green). The 5′ extent of the region to be included was based on EST evidence (A. gambiae), an existing prediction by NCBI using GNOMON (A. mellifera), or splice-site predictions using Genscan (Drosophila species except D. mojavensis).

Supporting Information

Figure S1. Klar Is Still Functional in ago251B Embryos

Embryos from wild-type (A), klarB (B), and ago251B (C) mothers were injected with the transcription inhibitor alpha-amanitin to prevent expression of Halo. In early cycle 14, this global inhibition of transcription causes a droplet transport defect very similar to deletion of holo [35]. In klarB embryos, Klar function is absent, and the peripheral cytoplasm becomes transparent because lipid droplets accumulate basally. In both wild-type and ago251B embryos, droplets accumulate apically resulting in an opaque periphery.

Found at DOI: 10.1371/journal.pgen.0020134.sg001 (2.0 MB TIF).

Figure S2. Expression of holo Transcripts in Wild-Type and ago251B Embryos

Full-length holo digoxigenin-labeled antisense in situ probe was used for in situ hybridization of control (w+) embryos (A, C, E, G) and embryos from ago251B homozygous mothers (B, D, F, H). (A, B) Syncitial blastoderm (C, D) early cycle 14; (E, F) mid-cellularization stages; and (G, H) late cellularization (fast phase). Note that holo exhibits strictly zygotic expression, which is downregulated at the end of cellularization [35]. This expression pattern is largely unimpaired in ago251B mutants.

Found at DOI: 10.1371/journal.pgen.0020134.sg002 (1.8 MB TIF).

Figure S6. The ago251B Mutation Does Not Interfere with the Activity of the mirRNA ban (ban)

To test for miRNA activity, we employed an eye-based reporter assay for the function of ban. ban negatively regulates the expression of the proapoptotic regulator Hid [69]. Expression of ban in the eye using GMR:Gal4 does not grossly affect eye development (A). Expression of GMR:hid induces cell death in the retina and thus results in a strongly reduced eye size (B). This phenoype is only slightly suppressed by the EP insertion banEP3266 (banEP alone) (C) and strongly suppressed by overexpression of banEP3266 using GMR:Gal4 (D). To test interference of ago251B with ban activity, we performed the same experiment in ago250B heterozygous (E), ago250B homzygous (F) or ago251B hemizygous (G) genetic backgrounds. In neither case did we detect a suppression of ban activity, which should result in a reversion to the GMR:hid phenotype and produce a strong reduction of the size of the eye. The increased activity of ban in ago250B homzygous or hemizygous flies is explained by two copies of the banEP3266 insertion present in these animals. We conclude that ago250B does not inhibit the activity of ban in regulating Hid expression in this assay. Interestingly, in an ago251B homozygous background (H), activity of ban seems to be slightly reduced; in the presence of two copies of banEP3266, the size of the eye is considerably smaller as compared to ago250B heterozygotes (I), which contain only one copy of banEP3266.

This result suggests that Ago2 might be involved in the activation of ban to downregulate Hid. The genotypes are indicated above each panel, respectively. TM6 and MKRS correspond to balancer chromosomes.

Found at DOI: 10.1371/journal.pgen.0020134.sg006 (2.0 MB TIF).

Figure S7. Densitometric Analysis of Independent Western Analyses Measuring Ago1, Dcr-1, and Loq Levels in Protein Extracts of Mutant and Wild-Type Cells

The quantification of the Western blots was performed from scanned images in a linear range using ImageJ from NIH Image. The columns...
represent the integrated density levels for independent Western blots detecting Agol (n = 3), Dcr-1 (n = 2), and Loqs (n = 3) in wild-type and age2 mutants (error bars show standard deviation). All values have been normalized against the α-tubulin loading control.

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Accession Numbers
GenBank (http://www.ncbi.nlm.nih.gov/Genbank) accession numbers are whole genome shotgun sequence AAGE01113413 (59568258), BM597722 (18895825), and protein XP_3950482 (66517254).

The National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov) accession numbers are Argonaute 2 (390883), Argonaute 1 (36544), Dicer-1 (42903), Dicer-2 (36993), Loquacious (34751), Klarsicht (38067), Halo (33334), Wingless (34009), Engrailed (36240), Slam (33890), Armadillo (31151), and DIAP1 (39753).

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