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Conserved association of Argonaute 1 and 2 proteins with miRNA and siRNA pathways throughout insect evolution, from cockroaches to flies

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ABSTRACT

Background: Argonaute proteins are key in RNA silencing. In *Drosophila melanogaster*, the five proteins of the Argonaute family participate in the pathways and mechanisms mediated by three types of small RNAs: piRNAs, miRNAs, and siRNAs. Two Argonaute proteins, Argonaute 1 (Ago1) and Argonaute 2 (Ago2), are associated with miRNA and siRNA mechanisms, which are the most thoroughly studied. The available data points to a sorting specialization of Ago1 for miRNAs and Ago2 for siRNAs. However, this has been demonstrated only in *D. melanogaster*, one of the most modified insects, which emerged some 100 million years ago. Thus, an important question is whether this association of Ago1 with miRNAs and Ago2 with siRNAs occurs generally in insects, or was a specific innovation in higher flies.

Methods: We addressed this question by using RNAi approaches and studying Ago1 and Ago2 functions in the German cockroach, *Blattella germanica*, a much less modified insect that emerged some 320 million years ago.

Results: The results showed that *B. germanica* does preferentially use Ago1 in the miRNA pathway, but can also use Ago2 in some cases. Conversely, Ago2 operates in the RNAi, in siRNA sorting, whereas Ago1 seems to have no relevant role in this process.

Conclusions and general significance: These basic associations are equivalent to those observed in *D. melanogaster*, implying that they have been evolutionary conserved from at least cockroach to flies, and possibly stem from the last common ancestor of extant insects.

1. Introduction

RNA interference (RNAi) pathways mediated by small RNAs have been reported throughout eukaryotic organisms, suggesting that they were present in the last common ancestor of all extant eukaryotes. RNAi mechanisms possibly originated to counteract viral invasion and were later coopted for other functions, like post-transcriptional regulation of gene expression [36]. Although the basic structure of RNAi pathways is similar in all eukaryotes studied, gene duplication and gene loss has occurred in multiple lineages, and work on model organisms has shown that the duplication and loss of core RNAi pathway genes have occurred multiple times [13]. A notable example is provided by the Argonaute proteins, which are at the core of all RNAi pathways [20,29] and notably vary in number in different groups, from 26 in the nematode *Caenorhabditis elegans* to 5 in the fly *Drosophila melanogaster* [24,40].

In *D. melanogaster*, the five proteins of the Argonaute family participate in the three pathways and mechanisms respectively mediated by three types of small RNAs: Piwi-interacting RNAs (piRNAs), microRNAs (miRNAs), and small interfering RNAs (siRNAs). Three *D. melanogaster* Argonaute proteins that belong to the Piwi subfamily (Piwi, Argonaute 3 and aubergine) are associated with piRNA mechanisms [18,38]. The main function of piRNAs is to counteract the action of mobile elements in the genome through binding to Piwi subfamily proteins [38]. The other two Argonaute proteins of *D. melanogaster*, Argonaute 1 (Ago1) and Argonaute 2 (Ago2), are associated with miRNA and siRNA mechanisms, which are the most thoroughly studied [8].

miRNAs are processed from the corresponding hairpin miRNA precursor by an endonuclease called Dicer, which was discovered by Hannon's group [7] in *D. melanogaster*. Processing yields two partially complementary single strand miRNAs: the “mature” miRNA and the “passenger” strand. In insects, this duplex is incorporated into the RNA-induced silencing complex (RISC) combined with the protein Argonaute, discovered by Tuschl's group [28], where the “mature” miRNA is selected. The miRNA then guides the Ago-RISC to the target mRNA, the miRNA binds to its target site in the mRNA by imperfect complementarity and blocks transcript translation [2](see also [6] for a comprehensive history of the discovery of the mechanisms regulating miRNA production and action). siRNAs are generated from long double-

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stranded RNAs (dsRNAs) that can be endogenous or exogenous in origin. The dsRNA is cleaved into siRNAs by a Dicer enzyme, then the siRNAs unwind, the guide strand couples to an Ago-containing RISC, which conveys it to the target mRNA. Finally, the RISC couples to the target mRNA, blocking and degrading it [37] (see also [3] for a comprehensive history of the discovery of the mechanisms regulating RNAi in insects).

The above data point to a sorting specialization of different Dicer enzymes and Ago proteins for miRNAs and siRNAs in insects. In mammals, a single Dicer assorts siRNAs and miRNAs among four Ago proteins, apparently without much discrimination [9]. In *D. melanogaster* there are two Dicer enzymes, Dicer-1 and Dicer-2, which, in general, are involved in the miRNA and siRNA pathways, respectively [23], whereas a parallel sorting specialization of Ago1 for miRNAs and Ago2 for siRNAs has been reported in the same fly [9,10]. This specialization would look as a general rule for insects, but in fact, it has been demonstrated only in *D. melanogaster*, one of the most derived and modified insects on Earth, making it rather unrepresentative of the insect class. Indeed, drosophilids are relative newcomers in the evolutionary history of insects, as they emerged in mid-Cretaceous, some 100 million years ago [17]. An important question is, therefore, whether this parallel association of Ago1 with miRNAs and Ago2 with siRNAs is general in insects, or was a specific innovation of higher flies.

We have addressed this question by using in the German cockroach *Blattella germanica* as the experimental subject. Cockroaches are an ancient group that emerged in the mid-Carboniferous, some 320 million years ago. They show many features that can be presumed to be similar to those of the last common ancestor of winged insects, such as short germ-band embryogenesis and hemimetabolan metamorphosis. This contrasts with *D. melanogaster*, a long germ-band, holometabolan species [48]. Moreover, *B. germanica* is highly sensitive to RNAi [3], making it particularly suitable for experimental gene mRNA depletion using this approach. In previous papers, we studied the role of Dicer-1 in the miRNA pathway [16] and Dicer-2 in the siRNA pathway [26]. In the present work, we wondered whether Ago1 and Ago2 are also respectively specialized in miRNA and siRNA pathways in *B. germanica*.

2. Materials and methods

2.1. Insect sampling and RNA extraction

Freshly ecdysed fifth (penultimate) instar nymphs (N5) of the cockroach *B. germanica* used in the experiments were obtained from a colony reared in the dark at 30 ± 1 °C and 60–70% relative humidity. The entire animal except the head (to avoid interferences with the eye pigments) and the digestive tube (to avoid contamination with parasites) was used for RNA extractions. All dissections and tissue sampling were carried out on carbon dioxide-anaesthetized insects. RNA isolation was carried out with miRNeasy® Mini Kit (QUIAGEN), which increases the yield of small RNAs. The reverse transcription was carried out with the NCode™ miRNA First-Strand cDNA Synthesis and qRT-PCR Kits (Invitrogen), following the manufacturer's protocols, which allows the quantification of the mRNA and miRNAs by quantitative real-time PCR (qRT-PCR). Only in the experiment examining the effect of a dsRNA on Ago2 expression, RNA was extracted with the GenElute Mammalian Total RNA kit (Sigma), and cDNA was prepared with the Transcriptor First Strand cDNA Synthesis kit (Roche).

2.2. Phylogenetic analysis

We obtained the insect sequences labeled as Ago1 and Ago2 from GenBank, and from i5k project (https://i5k.nal.usda.gov/webapp/blast/) [34] by BLAST search using the protein sequences from *B. germanica* and *D. melanogaster* as queries. Finally, the species and protein sequences of Ago1 and Ago2 included in the analysis were those indicated in Supplementary Table S1. As external group, we used the Ago3, Piwi and aubergine sequences of *D. melanogaster* and *B. germanica* (Supplementary Table S1). Alignments were carried out with ClustalX [22] and phylogenetic reconstruction with RAxML [39], based on the maximum-likelihood principle, a JTT matrix, a gamma model of heterogeneity rate, and using empirical base frequencies and estimating proportions. The data was bootstrapped for 100 replicates.

2.3. Characterization of the miRNAs studied

We examined the expression of the following mature miRNAs: bantam (bantam-3-p), let-7 (let-7-5p), miR-1 (miR-1-3p), miR-10 (miR-10-5p), miR-34 (miR-34-5p), miR-184 (miR-184-3p) and miR-276 (miR-276-3p). In the case of miR-10 and miR-276, we also examined the "passenger" strand: miR-10* (miR-10-3p) and miR-276* (miR-276-5p). The discrimination between the mature and passenger strand is based on the level of expression (higher in the mature than in the passenger), as detailed in the Supplementary Data S1 of Ylla et al. [47].

2.4. Quantification by qRT-PCR

For mRNA expression studies by qRT-PCR, 400 ng of total RNA were reverse transcribed. Amplification reactions were carried out using iQ™ SYBR Green Supermix (BioRad) and the following protocol: 95 °C for 2 min, and 40 cycles of 95 °C for 15 s and 60 °C for 30 s, in a MyIQ Real-Time PCR Detection System (BioRad). After the amplification phase, a dissociation curve was obtained to ensure that there was only one product amplified. All reactions were run in triplicate and for, at least, 3 biological replicates. Statistical analysis of relative expression results was carried out with the REST software tool [33]. RNA expression was calculated in relation to the expression of U6 in experiments intended to measure mRNAs and miRNAs, or to Actin 5c in experiments intended to measure only mRNAs. Primer sequences used are indicated in Supplementary Table S2.

2.5. RNAi and treatments with dsRNA in vivo

Basic procedures for RNAi experiments were as described by Rubio et al. [35]. Details on the dsRNAs targeting Ago1 (GenBank accession number HF912424), Ago2 (HF912425), Atrophin (HF912426) and PolyH, a sequence from *Autographa californica* nucleopolyhedrovirus, K01149, used as control dsRNA (dsPolyH), are also detailed in Rubio et al. [35]. The dsRNAs were injected at the chosen doses in 1 μL volume in *B. germanica* females freshly emerged to the fifth (penultimate, N5) nymphal stage (N5D0). Effects on the targeted transcript were examined by qRT-PCR in 4-day-old N5 (N5D4) or in freshly emerged sixth (last) nymphal instar (N6D0), depending on the experiment. The primers used to prepare the dsRNAs are indicated in the Supplementary Table S2. To study the effects of an alien dsRNA, freshly emerged N5 were injected with 3 μg of dsPolyH in 1 μL volume of aqueous solution or with 1 μL of water, and Ago1 and Ago2 expression was measured 6 h later.

2.6. Experiments of RNAi in vitro

B. germanica embryonic cells UM-BGE-1 [21] were grown in suspension at 25 °C in L15 medium (Sigma), modified as recommended by Munderloh and Kurtti [30]. Briefly, the pH was adjusted to 6.5 with sodium hydroxide and the medium was complemented with 5% fetal bovine serum (GIBCO), 1% lipoprotein-cholesterol concentrate (Sigma) and 50 U/mL penicillin + 50 μg/mL streptomycin (GIBCO). The cells were collected by centrifugation, washed twice in PBS and re-suspended in fresh cell culture medium at a density of 10^6 cells/mL. Two hours after transferring the cells to the plate, the chosen dsRNA (dsPolyH, dsAgo1 or dsAgo2 at a final concentration of 30 nM) was added along with FuGENE Transfection Reagent (Promega), at a proportion 3/1 dsRNA/FuGENE, in medium without serum. Six hours later,

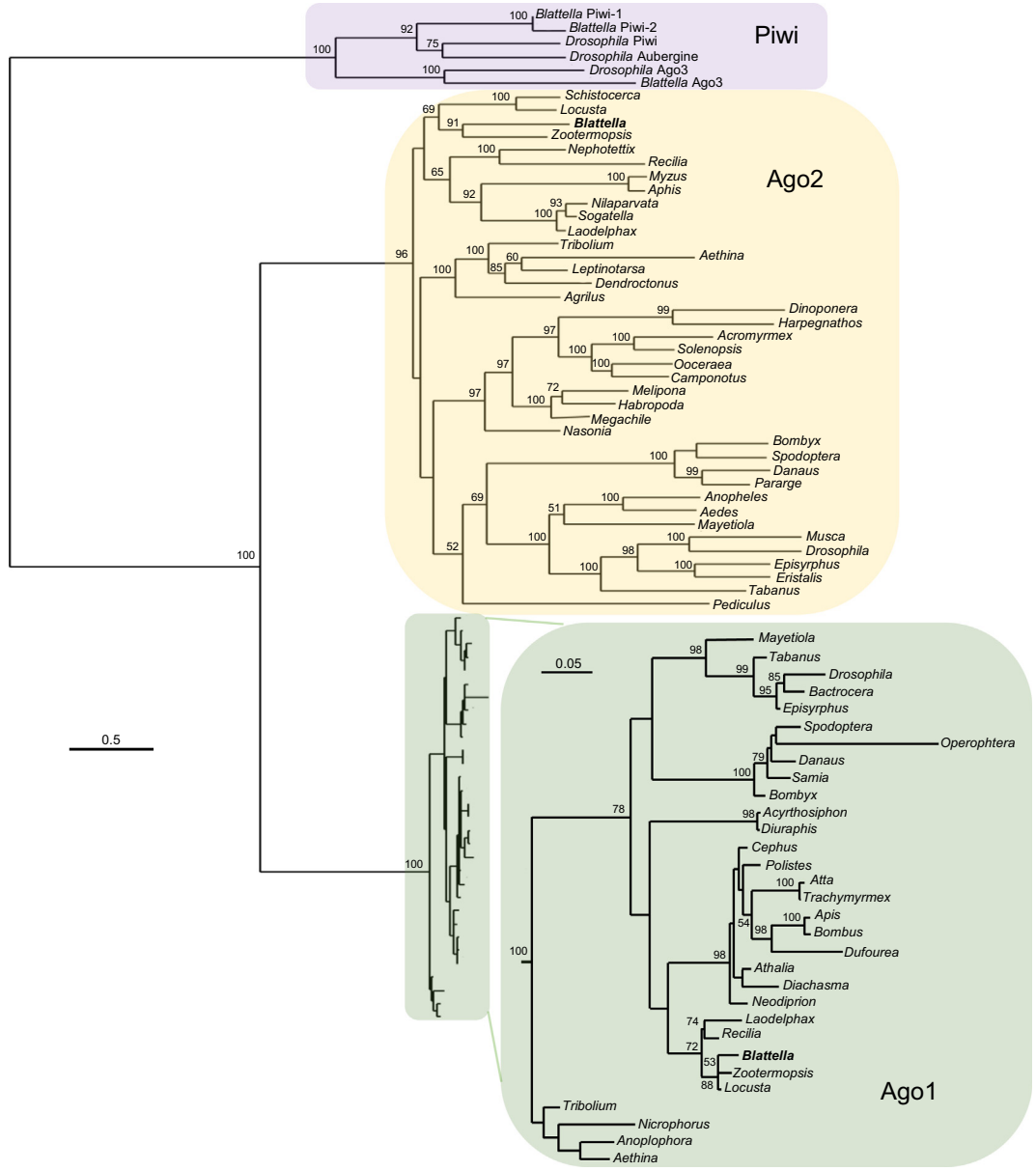

Fig. 1. Phylogenetic analysis of Ago1 and Ago2 in insects, using Ago3, Piwi and aubergine sequences of *Drosophila melanogaster* and *B. germanica* as the external group. The species and accession numbers are indicated in the supplementary Table S1. The protein sequences were aligned using ClustalX and phylogenetic reconstruction with RAxML, based on the maximum-likelihood principle, a JTT matrix, a gamma model of heterogeneity rate, and using empirical base frequencies and estimating proportions. Bootstrap values > 50 are indicated at the corresponding node. Scale bar indicates the number of substitutions per site.

complete medium was added to allow cells grow normally. Then, dsAtro was added 24 h later at the same concentration and conditions. The effect of the treatments on transcript levels was examined 24 h after the dsAtro treatment.

3. Results

3.1. B. germanica Ago1 and Ago2 in a phylogenetic context

Before proceeding with our functional studies, we wanted to confirm that the available Ago1 and Ago2 sequences of *B. germanica* (GenBank HF912424 and HF912425, respectively) were the right orthologues of these proteins in this species. For this purpose, we obtained a representative set of Argonaute 1 and 2 sequences from insects (see the Supplementary Table S1), as well as the sequences of Argonaute 3, Piwi and aubergine from *D. melanogaster* and *B. germanica*, which were used as external groups. A phylogenetic analysis revealed that Ago1 and Ago2 fall into two respective nodes that cluster into the Argonaute subfamily group. On the other hand, Ago3 + Piwi-aubergine cluster in another node, which is the sister group of Ago1 + Ago2 (Fig. 1). Our results confirm that *B. germanica* Ago1 and Ago2 robustly cluster into the respective nodes of the Ago1 and Ago2 subfamilies. The analysis also allowed the identification of one Ago3 and two Piwi-aubergine orthologues in *B. germanica*. Moreover, the structure of Ago1, Ago2 and Piwi proteins of *B. germanica* show the canonical organization in domains of each one, homologous to that found in reference species like *D. melanogaster* (Supplementary Fig. S1).

3.2. General effects of Ago1 and Ago2 depletion

Doses of 3.75 or 1.5 µg of dsAgo1 administered to freshly emerged fifth (penultimate) instar nymphs (N5D0) provoked 100% mortality. Controls (treated with 3 µg of dsPolyH) (n = 10) molted to normal N6, 6 days after the treatment, and then to normal adults. The dsAgo1-

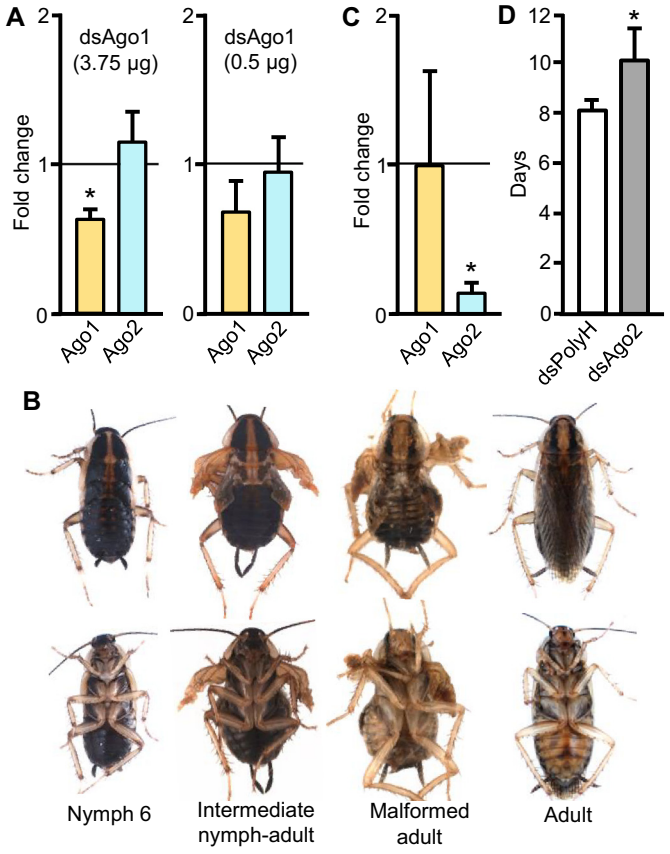

Fig. 2. Effect of RNAi of Ago1 and Ago2 in *Blattella germanica*. (A) Effects of dsAgo1 treatment at the doses indicated on Ago1 and Ago2 transcript levels. (B) Representative phenotypes resulting from the treatment with dsAgo2 at a dose of 0.5 µg. (C) Effects of dsAgo2 treatment at a dose of 2.5 µg on Ago1 and Ago2 transcript levels. (D) Effects of dsAgo2 treatment at a dose of 2.5 µg on the length of the last nymphal instar (N6). dsAgo1 or dsAgo2 was administered on female nymphs freshly ecdysed to the penultimate instar (N5D0) and transcript levels were measured 4 days later (N5D4). In A and C, each point represents at least three biological replicates and is normalized against the controls (reference value corresponding to dsPolyH treatment, which is 5.35 ± 2.63 for Ago1and 18.22 ± 7.57 for Ago2, copies per 1000 copies of U6, n = 3 in both cases); the asterisk indicates statistically significant differences with respect to controls ($p < 0.05$) according to the REST software tool [33]. In D, each point represents 10 biological replicates and the asterisk indicates statistically significant differences with respect to controls ($p < 0.05$) according to the student's *t*-test.

treated insects (n = 10) died either during the molting to N6 or just after molting (Supplementary Fig. S2). At the highest dose, Ago1 mRNA levels were significantly depleted (38% as average), whereas those of Ago2 were practically unaffected (Fig. 2A). Subsequently, we used a dose of 0.5 µg of dsAgo1 administered to freshly emerged N5. In this experiment, Ago1 mRNA levels were also reduced (33% as average, although the differences with respect to the controls were not statistically significant), whereas Ago2 mRNA levels were practically unaffected (Fig. 2A). In this experiment, controls (n = 40) again molted normally to N6 and then to adults. Conversely, the treatment with 0.5 µg of dsAgo1 in N5 (n = 80) gave the following phenotypes: 54 treated insects (68%) died in the process of molting from N5 to N6; 13 insects (16%) molted to an apparently normal N6, remained in this stage for 40–50 days, and then died; 5 insects (6%) molted to normal N6 6 days after the treatment, and then molted again to an intermediate nymph-adult 8 days later; and 8 insects (10%) molted to malformed adults 8 days after treatment (Fig. 2B and Supplementary Fig. S2)·The intermediate nymph-adult phenotype had a blackish coloration and a general nymphal morphology, but the wings were rudimentary and completely wrinkled. The malformed adult phenotype showed the yellowish coloration and general features typical of the adult, but the wings not properly extended and the abdomen was flattened dorsoventrally (Fig. 2B).

For Ago2, we carried out equivalent RNAi experiments, using a dose of 2.5 µg of dsAgo2 administered to freshly emerged N5. Transcript measurements showed that Ago2 mRNA levels were significantly depleted (86% as average), whereas those of Ago1 showed average values similar to controls, although with high deviations, which suggest that Ago1 might have been targeted by dsAgo2 in some cases (Fig. 2C). Both groups, Ago2-depleted (n = 10) and control (n = 10), molted to normal N6, and then to normal adults. The only difference was the length of the N6 stage, which was 25% longer in Ago2-depleted (Fig. 2D).

3.3. Effects on the miRNA pathway

First, we studied the influence of Ago1 on miRNA levels, using the following mature miRNAs as case studies: bantam (bantam-3-p), let-7 (let-7-5p), miR-1 (miR-1-3p), miR-10 (miR-10-5p), miR-34 (miR-34-5p), miR-184 (miR-184-3p) and miR-276 (miR-276-3p). Moreover, we also studied the "passenger" strand of two of them: miR-10* (miR-10-3p) and miR-276* (miR-276-5p). We treated freshly emerged N5 with 0.5 µg of dsAgo1, and we measured the levels of these miRNAs in freshly emerged N6 (N6D0). Bantam, let-7, miR-1, miR-184, and miR-276 were significantly down-regulated, miR-10 tended to be down-regulated, miR-34 was practically unaffected, whereas miR-10* and miR-276* were significantly up-regulated (Fig. 3A).

Using an equivalent approach, we studied the effect of Ago2-depletion. Freshly emerged N5 were treated with 2.5 µg of dsAgo2, and the miRNAs were measured in freshly emerged N6. Results showed that only let-7 and miR-1 were significantly down-regulated, whereas all the others were not significantly affected, although miR-10*, miR-184 and miR-276 tended to be down-regulated (Fig. 3B).

3.4. Effects on the siRNA pathway

Next, we studied the influence of Ago1 and Ago2 on the siRNA pathway. Our approach consisted of assessing whether Ago1 or Ago2 depletion impaired the RNAi efficiency when targeting a given transcript. As a case-study transcript we used Atrophin, which is efficiently depleted by RNAi in *B. germanica* [35]. In the first set of experiments we treated freshly emerged N5 with either dsAgo1 (0.5 µg) or dsAgo2 (2.5 µg), and, subsequently, with dsAtro (3 µg) just after the molt to N6. Controls were treated in a similar way with dsPolyH (3 µg in N5 and 3 µg in N6). All control insects (n = 20) molted to normal N6 and then to normal adults. The treatment with dsPolyH in N5 and dsAtro in N6 (n = 20) triggered a significant decrease of Atrophin mRNA levels (55% as average) (Fig. 4A). The insects molted from N6 to adults with partially extended and wrinkled wings (Fig. 4B), as a consequence of slower and imperfect ecdysis. As expected, these effects are coincident with those reported by Rubio et al. [35].

All insects treated with dsAgo1 in N5 and dsAtro in N6 (n = 15) died the day after the second treatment. Those treated with dsAgo2 in N5 and dsAtro in N6 (n = 20) molted from N6 to normal adults or had, at most, the forewings (tegmina) slightly separated from one another (Fig. 4B). On day 4 of N6 the levels of Ago2 mRNA were still significantly lower (50% as average) than in the controls, whereas Atrophin levels were similar to the controls (Fig. 4A).

The lethal effects of Ago1 depletion in these in vivo experiments prevented us demonstrating whether Ago1 operates in the siRNA pathway and the RNAi process. For this reason, we decided to follow an approach in vitro using the *B. germanica* embryonic cells UM-BGE-1. The cells were first treated with dsPolyH, dsAgo1, or dsAgo2, and then 24 h later with dsPolyH or dsAtro. Thereafter, 24 h after the last treatment, the levels of the corresponding mRNAs were measured. The

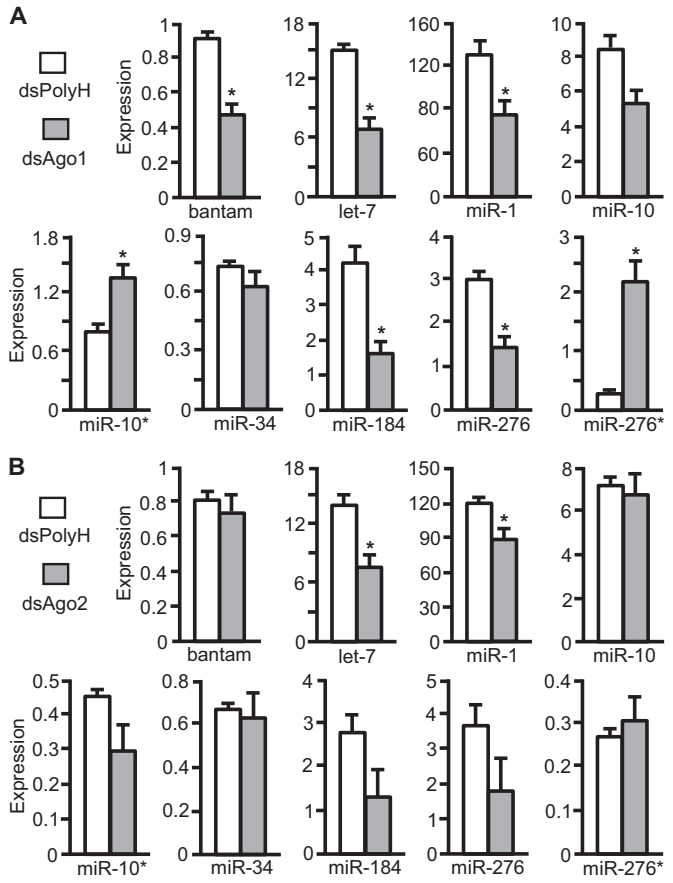

Fig. 3. Effect of Ago1 or Ago2 depletion on the expression of miRNAs in *Blattella germanica*. dsAgo1 (0.5 μg) or dsAgo2 (2.5 μg) were administered on in freshly ecdysed fifth instar female nymphs (N5D0), and miRNAs were measured just after molting to the next instar(N6D0). Data are expressed as miRNA copies per 10 copies of U6. Each point represents three biological replicates; the asterisk indicates statistically significant differences with respect to controls ($p < 0.05$) according to the REST software tool [33].

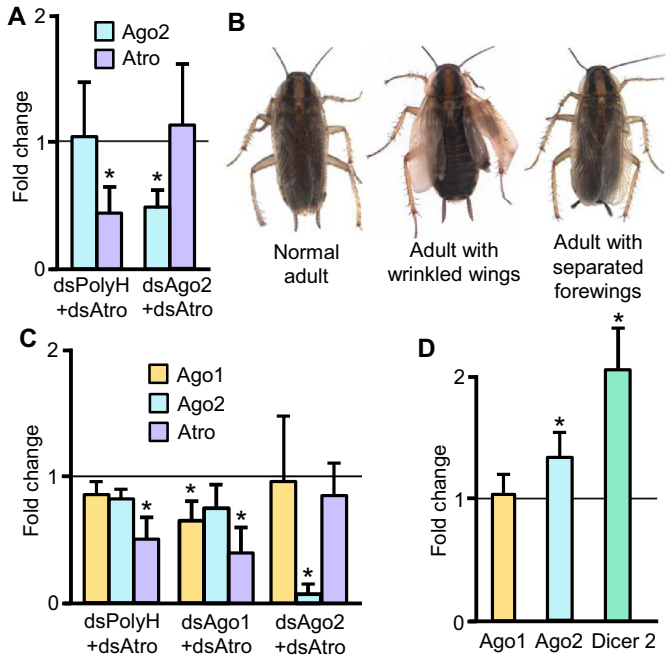

Fig. 4. Effect of Ago1 or Ago2 depletion on RNAi of Atrophin in *Blattella germanica*, and effect of dsPolyH on Ago1 and Ago2 expression. (A) Effects in vivo of a first treatment of dsPolyH (3 μg) and a second treatment of dsAtro (3 μg), or a first treatment of dsAgo2 (2.5 μg) and a second treatment of dsAtro (3 μg) on Atrophin (Atro) and Ago2 mRNA levels; the first treatment was performed in freshly emerged female nymphs in fifth instar (N5) and the second just after the molt to the next instar (N6). (B) Phenotypes resulting from the treatment described in panel "a". (C) Effects in UM-BGE-1 cells cultured in vitro of a first treatment with dsPolyH, dsAgo1 or dsAgo2, and a second treatment with dsAtro; the second treatment was performed 24 h after the first one, and mRNAs were measured 24 h after the second treatment; dsRNAs were used at a final concentration of 30 nM. (D) Effect of dsPolyH treatment on Ago1 Ago2 and Dicer-2 expression; dsPolyH was administered to female nymphs freshly ecdysed to the penultimate instar (N5D0) and transcript levels were measured 6 h later. Data on transcript levels are expressed as mRNA copies per 1000 copies of U6 (A and C) or Actin 5c (D); each point represent three biological replicates and is normalized against the controls (reference value corresponding to dsPolyH treatment, which in A is 66.26 ± 18.97 for Ago2 and 11.58 ± 5.38 for Atro, copies per 1000 copies of U6, n = 3 in both cases; in C is 14.35 ± 4.31 for Ago1, 129.67 ± 8.34 for Ago2 and 46.31 ± 7.10 for Atro, copies per 1000 copies of U6, n = 3 in all cases; and in D is 7.28 ± 1.20 for Ago1, 26.50 ± 3.27 for Ago2 and 7.07 ± 0.65 for Dicer-2, copies per 1000 copies of Actin 5c, n = 3 in all cases); the asterisk indicates statistically significant differences with respect to controls ($p < 0.05$) according to the REST software tool [33].

results showed that Ago1 depletion practically did not affect the RNAi effects of dsAtro. Conversely, depletion of Ago2 precluded RNAi effects, as shown by the high levels of Atrophin mRNA in these experiments, despite the treatment with dsAtro (Fig. 4C).

3.5. Effects of an alien dsRNA on Ago1 and Ago2 expression

Finally, we wondered whether a dsRNA treatment would stimulate the expression of Ago2, as occurs with Dicer-2, as reported by Lozano et al. [26]. Freshly emerged N5 were injected with 3 μg of dsPolyH in 1 μL volume of aqueous solution or with 1 μL of water, and expression was measured 6 h later. Results showed that Ago2 was significantly up-regulated (34% as average) by the dsPolyH treatment, whereas Ago1 was unaffected. As a positive control, we measured the expression of Dicer-2, which was also up-regulated (127% as average) (Fig. 4D), as expected according to Lozano et al. [26].

4. Discussion and conclusions

4.1. AGO and Piwi family genes in B. germanica

Sequence comparisons and analyses led to the unequivocal identification of the orthologues of Ago1 and Ago2 in *B. germanica*. The topology obtained in our phylogenetic tree is similar to that found by Wang et al. [43]. Thus, Ago1 and Ago2 belong to the Ago subfamily, while Ago3 and Piwi-aubergine pertain to the Piwi subfamily [1]. The shorter branches of the Ago1 node with respect to those of Ago2 indicate that Ago1 sequences are much more conserved each other than those of Ago2. This is perhaps related to the high evolvability of the Ago2 gene, which is frequently duplicated and has a highly variable copy number in arthropods [32]. Our study led to the characterization of the orthologues of Ago3 and Piwi-aubergine. The topology of this node suggests that there were independent duplications of Piwi-like genes in the respective *D. melanogaster* and *B. germanica* branches, thus precluding the establishment of direct Piwi and aubergine orthologues between the two species. For this reason, we named the two *B. germanica* sequences Piwi-1 and Piwi-2. Finally, our discovery of an Ago3 orthologue in the cockroach *B. germanica* contradicts the hypothesis that this gene was lost in Dictyoptera [13]. The organization of the respective protein families also shows the close similarity between the Ago1 and Ago2 families, and the divergence of the Piwi family.

4.2. Ago1 is a vital gene in B. germanica

Depletion of Ago1 in N5 using 3.75 or 1.5 μg doses of dsRNA provoked 100% mortality, suggesting that Ago1 is involved in vital processes in *B. germanica*, at least in late nymphal instars. The 0.5 μg dose still provoked 68% mortality, whereas the survivors that molted to N6 showed three different phenotypes: a permanent N6 stage, a subsequent molt to an intermediate nymph-adult, and a subsequent molt to a malformed adult. These results indicate that Ago1 is crucial in molting and metamorphosis. In the beetle *Tribolium castaneum*, RNAi depletion of Ago1 in larvae produced developmental defects and impaired pupation [41]. Further studies showed that Ago1 depletion up-regulated the expression of Methoprene-tolerant (Met) and Krüppel homolog-1 (Kr-h1), crucial transducers of the antimetamorphic signal of juvenile hormone [5,19], as well as resulting in defects in ecdysis and the pupa, and concomitant mortality in late larval or pupal stages, effects that are mediated by miRNAs [45]. In *B. germanica*, the phenotypes observed are reminiscent of those obtained when depleting Dicer-1, which consequently affects miRNA levels and impairs adult morphogenesis [16]. Other work revealed that miR-2, which targets Kr-h1, is predominantly responsible for the phenotype observed [4,27]. Therefore, we presume that the severe phenotypes observed after depleting Ago1 derive from miRNA deficiencies (see next section). Conversely, the efficient depletion of Ago2 in N5 using a 2.5 μg dose of dsRNA produced no phenotypic effects but merely resulted in a slight delay of the imaginal molt. This suggests that Ago2 is not needed for housekeeping homeostasis in *B. germanica* nymphs.

4.3. B. germanica preferentially use Ago1 in the miRNA pathway, but can also use Ago2

To test the participation of Ago1 and Ago2 in the production of miRNAs, we analyzed the change in expression of different miRNAs after depleting Ago1 or Ago2. The levels of most of the miRNAs measured in Ago1-depleted specimens were down-regulated or had a tendency to down-regulation, with the exception of miR-10* and miR-276*, which were significantly up-regulated. This suggests that in *B. germanica* miRNAs generally follow the canonical miRNA pathway, through which the duplex miR/miR* is sorted by Ago1 that then selects the mature strand [10]. Nevertheless, this is not always the case. The sorting of miR/miR* duplexes between Ago1 and Ago2 depends on the duplex structure and sequence information. In *D. melanogaster*, mismatches at central positions direct duplex loading towards Ago1, whereas central pairing increases Ago2 loading. In addition, Ago1-loaded miRNAs have a very strong preference for U at 5′, whereas Ago2-loaded miRNAs show a moderate preference for C [10,15]. Furthermore, and particularly in the case of miR/miR* duplexes with high complementarity, the strand with the thermodynamically less stable 5′ end is more often retained [10,31]. These peculiarities produce that, whereas most of the small RNAs that come from miR/miR* duplexes are mature miRNAs loaded into Ago1, Ago2 is significantly depleted of mature miRNAs and enriched for miRNAs* [10,15]. This may explain the up-regulation of miR-10* and miR-276* in our Ago1-depleted specimens. In control animals, mature miR-10 and miR-276 levels are much higher than their corresponding miR-10* and miR-276* [48]. However, our present results indicate that Ago1 depletion reduces the mature and increases the star strands of these miRNAs, pointing to a loading of the duplexes into the Ago2 complex and an increase in miR-10* and miR-276* selection. Consistent with these notions, Ago1 depletion in *D. melanogaster* S2 cells triggers a reduction of mature miR-276a and an increase of miR-276a* [10], suggesting that miR-276a* is preferentially loaded into the Ago2 complex [31]. Finally, as Ago2 depletion triggers a decrease of miR-1 and let-7 levels, we propose that these two miRNAs are preferentially selected by the Ago2 complex. As indicated above, Ago1 depletion also produces a decrease in miR-1 and let-7 levels, pointing to Ago1 sorting for these duplexes, this being the more common pathway for most miRNAs [9]. However, the duplexes miR-1/miR1* and let-7/let-7* show high complementarity in the middle of the structure [48], which could alternatively direct them, to some extent, to Ago2. miR-10* also tends to be reduced in Ago2-depleted specimens, suggesting that some of the processing of miR-10/miR-10* is carried out by Ago2. On the other hand, miR-276* levels do not change with this treatment, suggesting that Ago2 is not the main pathway for processing miR-276/miR-276*.

4.4. Ago2 operates in the siRNA pathway in B. germanica

Our experiments combining treatments with dsAgo1, dsAgo2 and dsAtro, in vivo and in vitro, indicated that in *B. germanica* Ago2 is required for the RNAi mechanism, whereas Ago1 is dispensable. These results suggest that in *B. germanica* RNAi mechanisms and siRNA sorting depend on Ago2, whereas Ago1 does not appear to play relevant roles in this process. This is quite general in insects, as first demonstrated in *D. melanogaster* [10,14] and then in other species, like the hemipteran *Nilaparvata lugens* [46], the lepidopteran *Bombyx mori* [25] and the coleopteran *Diabrotica virgifera* [42]. However, a few studies report that Ago1 is involved in the RNAi process. For example, Williams and Rubin [44] described that mutations in the Ago1 gene in *D. melanogaster* suppress RNAi in embryos. However, the effect results from a reduced ability to degrade mRNA in response to dsRNA in vitro, suggesting that Ago1 functions downstream of siRNA production. More recently, Yoon et al. [49] reported that depletion of Ago1 and aubergine/Piwi in the Colorado potato beetle *Leptinotarsa decemlineata*, reduces the RNAi effect against IAP (Inhibitor of apoptosis). Moreover, dsAgo1 reduced siRNAs formation from the GFP dsRNA, suggesting that Ago1 plays a role in the production of siRNAs. The authors propose that the action of Ago1 is at the level of intracellular transport of dsRNA and/or recruitment of Dicer enzymes, thus, upstream of siRNA production [49].

4.5. Allien dsRNA stimulates the expression of Ago2 but not Ago1

Interestingly, an injection of dsPolyH stimulated the expression of Ago2, in comparison with an injection of water, which parallels the results obtained in a previous work reporting that Dicer-2 is also stimulated by dsPolyH [26]. Conversely, dsPolyH did not affect the expression of Ago1. In RNA-based antiviral immunity in insects, dsRNAs are recognized as molecules associated with pathogens and, as a defensive mechanism, they are processed into siRNAs by host Dicer-2 [12]. Moreover, evidence for a role of Dicer-2 as a sensor of viral infection and as a key antiviral defense element beyond the RNAi pathway has been demonstrated in *D. melanogaster*, where Dicer-2 mediates the induction of the antiviral gene Vago [11]. Indeed, sensing alien RNAs could have been a specialized function of Dicer-2 after the duplication of the ancestral Dicer gene [26]. The parallel stimulation of Ago2 by an alien dsRNA suggests that it could also play a sensor role associated to Dicer-2, and again supports that Ago2 specializes in the siRNA sorting, whereas Ago1 does not seem to participate in that pathway.

4.6. Conclusions

In *D. melanogaster*, Ago1 associates with miRNAs, and Ago2 with siRNAs [10]. However, there are exceptions to the rule, and inverse relationships have been described, Ago2 sorting miRNAs and Ago1 influencing the RNAi process, although probably upstream or downstream of siRNA sorting. This study focused on the parallel Argonaute sorting of small RNAs, and our results show that *B. germanica* preferentially uses Ago1 in the miRNA pathway, but can also use Ago2. Conversely, Ago2 would operate in the RNAi, in siRNA sorting, whereas Ago1 does not appear to be relevant in this process. These basic associations are equivalent to those observed in *D. melanogaster*, implying that they have been evolutionary conserved from at least cockroach to

flies, and possibly stem from the last common ancestor of extant insects.

Transparency document

The http://dx.doi.org/10.1016/j.bbagrm.2018.04.001 associated this article can be found, in online version.

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Appendix A. Supplementary data

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