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ATP-dependent human RISC assembly pathways

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Abstract

The assembly of RNA-induced silencing complex (RISC) is a key process in small RNA–mediated gene silencing. In humans, small interfering RNAs (siRNAs) and microRNAs (miRNAs) are incorporated into RISCs containing the Argonaute (AGO) subfamily proteins Ago1–4. Previous studies have proposed that, unlike *Drosophila melanogaster* RISC assembly pathways, human RISC assembly is coupled with dicing and is independent of ATP. Here we show by careful reexamination that, in humans, RISC assembly and dicing are uncoupled, and ATP greatly facilitates RISC loading of small-RNA duplexes. Moreover, all four human AGO proteins show remarkably similar structural preferences for small-RNA duplexes: central mismatches promote RISC loading, and seed or 3′-mid (guide position 12–15) mismatches facilitate unwinding. All these features of human AGO proteins are highly reminiscent of fly Ago1 but not fly Ago2.

Small RNAs, including microRNAs (miRNAs) and small interfering RNAs (siRNAs), regulate various biological functions by downregulating the expression of the target genes. Small RNAs do not work alone, but rather function as the specificity determinants of the effector ribonucleoprotein complexes, called RNA-induced silencing complexes (RISCs)[1–4]. Although a number of proteins have been identified as candidates for RISC components[5–8], exactly which proteins compose RISC is not well defined. One established exception is a member of the Argonaute (Ago) family of proteins, the very core component of RISC. Ago family proteins can be divided into ubiquitously expressed AGO subfamily proteins and gonadally expressed *P*-element–induced wimpy testis (PIWI) subfamily proteins[1–4]. *Drosophila* has two AGO proteins, Ago1 and Ago2, whereas humans have four, Ago1–4.

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AUTHOR CONTRIBUTIONS

M.Y. performed *in vivo* experiments; M.Y., T.K. and S.I. performed biochemical experiments; Z.P., X.Y. and Q.L. expressed and purified recombinant Ago2 and performed initial experiments using it; Y.T. supervised the study; M.Y. and Y.T. wrote the manuscript; all authors discussed the results and approved the manuscript.

Both miRNAs and siRNAs are processed by Dicer enzymes during their biogenesis to yield double-stranded intermediates—miRNA-miRNA* duplexes and siRNA duplexes—that are initially loaded into AGO proteins as double strands[9–13]. Such AGO protein complexes containing small-RNA duplexes are called pre-RISCs. In *Drosophila*, miRNA-miRNA* duplexes and siRNA duplexes are actively sorted into Ago1-RISC and Ago2-RISC, respectively14, according to their intrinsic structures but not their Dicer-dependent biogenesis15,16; central mismatches direct small-RNA duplexes to fly Ago1 (ref. 13) while preventing them from entering Ago2 (ref. 16). Similarly, miRNAs enter Argonaute-like gene 1 and 2 (ALG-1 and ALG-2) proteins and siRNAs enter RNAi defective 1 (RDE-1) proteins in *Caenorhabditis elegans*, according to the structures of small-RNA duplexes[17,18]. In *Arabidopsis thaliana*, the 5′-terminal nucleotide serves in small-RNA sorting[19–21]. However, it remains unclear whether small RNAs are actively sorted in humans. Although early reports have shown that both miRNAs and siRNAs enter all four AGO proteins irrespective of their sequences[22,23], some recent reports suggest that a small-RNA sorting system, albeit much less stringent than those in other animals or plants, may exist in humans[24,25].

Once pre-RISC is formed, the two strands of the small-RNA duplex are subsequently separated or unwound within AGO proteins, with only one strand (the guide strand) retained and the other (the passenger strand) discarded. Which strand serves as the guide depends on the thermodynamic stabilities of the base pairs at the 5′ ends of the two strands[26,27]. AGO protein complexes containing single-stranded small RNAs are called mature RISCs, holo-RISCs or simply RISCs. *Drosophila* Ago2 and human Ago2, both of which possess efficient target cleavage (‘slicer’) activity, unwind perfectly complementary siRNA duplexes by cleaving the passenger strand, as if it were a target RNA, to facilitate its rapid release and decay[9–12]. In contrast, we have recently shown that *Drosophila* Ago1, whose slicer activity is very limited, unwinds small-RNA duplexes without cleaving the passenger strand[13]. Such slicer-independent unwinding by fly Ago1 does not require ATP but does require mismatches in the seed (guide positions 2–8) and/or 3′-mid (guide positions 12–15) regions, which reflects the unique geometry of RNAs in AGO proteins[13].

It is well established that, in flies, both Ago1 and Ago2 require ATP for RISC loading13,28–30. ATP is presumably consumed to trigger the dynamic conformational opening of AGO proteins so that they can accept small-RNA duplexes (discussed in ref. 13). However, previous studies using immunopurified31,32 or recombinantly reconstituted33 human Ago2 complex (therein referred to as human RISC-loading complex (RLC)) containing Ago2, Dicer and TAR RNA-binding protein (TRBP) show that human RISC is assembled in a manner independent of ATP hydrolysis, arguing for a potential difference between fly and human systems.

The coupling of dicing and RISC assembly is another subject of debate. It was previously proposed that, after dicing, small-RNA duplexes need to dissociate from Dicer and then rebind to RISC-loading machinery that senses the thermodynamic asymmetry of the duplex, because the guide strand of an siRNA duplex diced from long double-stranded RNA can be either in the sense or antisense orientation, and a pre-miRNA can harbor the mature miRNA strand either in the 5′ or 3′ arm[34,35]. Indeed, the strict small RNA–sorting system in flies, reflecting the structure but not the biogenesis of the small-RNA duplexes, strongly supports the possibility that dicing and RISC assembly are uncoupled[16]. In contrast, it was reported that immunopurified or reconstituted human Ago2 complex could use pre-miRNAs but not pre-diced miRNA-miRNA* duplexes or siRNA duplexes for target cleavage, leading to a conclusion that dicing of pre-miRNAs and RISC assembly are functionally coupled in humans[31,32]. Such apparent discrepancies between flies and humans complicate our

understanding of the RISC assembly pathway, a key process in small RNA–mediated gene silencing.

Here we carefully reexamined the human RISC-assembly pathways using the classical target-cleavage assay and a new agarose native gel system that we recently established to study fly Ago1-RISC assembly[13]. We show that, just as in flies, human RISC assembly is uncoupled from dicing, and ATP greatly facilitates RISC loading of small-RNA duplexes. Moreover, all four human AGO proteins show remarkably similar structural preferences for small-RNA duplexes: central mismatches promote RISC loading and seed or 3′-mid mismatches facilitate unwinding. All these features of human AGO proteins are highly reminiscent of fly Ago1 but not of fly Ago2, the best-characterized AGO protein.

RESULTS

Human RISC assembly is uncoupled from dicing

To clarify the paradoxical situation between flies and humans, we first reexamined the question of whether dicing and RISC assembly are coupled in humans. Based on a perfectly complementary firefly luciferase siRNA (Fig. 1**a**, luc siRNA duplex), we designed a functionally asymmetric small-RNA duplex (Fig. 1**a**, miR-luc–miR-luc* duplex) that structurally resembles typical miRNA-miRNA* duplexes containing mismatches and wobbles in the central and seed regions (ref. [13] and see below). We also constructed an artificial pre-miRNA–like hairpin RNA, pre–miR-luc, by connecting miR-luc–miR-luc* duplex and the terminal short stem-loop sequence of human miR-22 (Fig. 1**a**). Control experiments showed that dicing of pre–miR-luc precisely produced miR-luc–miR-luc* duplex (Supplementary Fig. 1a).

We transfected these three trigger RNAs, a *Renilla reniformis* luciferase (RL) reporter harboring a perfectly complementary target site in HeLa cells and a firefly luciferase control. Both luc siRNA duplex and miR-luc–miR-luc* duplex induced strong repression of the target, whereas pre–miR-luc induced much milder repression (Fig. 1**b**). This suggests that coupling with dicing does not promote RISC assembly *in vivo*. Similarly, an *in vitro* cleavage assay using HeLa cell lysate showed that miR-luc–miR-luc* duplex and luc siRNA duplex cleaved a target mRNA more rapidly than pre–miR-luc, supporting the proposition that dicing is the limiting step and is uncoupled from RISC assembly (Fig. 1**c**, left).

We then immunopurified Ago2 complex from cells expressing Flag-tagged Ago2 using antibody to Flag (anti-Flag). This Ago2 complex retained detectable dicing activity (Supplementary Fig. 1a) and could use miR-luc–miR-luc* duplex better than pre–miR-luc to cleave the target (Fig. 1**c**, middle), apparently consistent with our data *in vivo* and in HeLa cell lysate. Strangely, however, the luc siRNA duplex, which was highly active *in vivo* and in HeLa cell lysate, could barely cleave the target (Fig. 1**c**, middle). To simplify the assay system, we purified recombinant Ago2 using a baculovirus expression system to apparent homogeneity (Supplementary Fig. 1b). Because the recombinant Ago2 was devoid of any dicing activity (Supplementary Fig. 1a), pre–miR-luc in theory should not form any RISC. However, to our surprise, the recombinant Ago2 alone could use pre–miR-luc, as well as miR-luc–miR-luc*, to cleave the target (Fig. 1**c**, right). In contrast, perfectly complementary luc siRNA duplex was barely active (Fig. 1**c**, right). Our observation is consistent with a previous report showing that purified recombinant Ago2 from *Escherichia coli* can incorporate single-stranded RNAs but not double-stranded siRNAs[36]. A plausible but unexpected explanation is that undiced pre–miR-luc in fact entered Ago2 as a long, single-stranded RNA, and its 5′-arm region served as the guide. Supporting this idea, 21-nt single-stranded miR-luc and 58-nt single-stranded RNA containing the miR-luc sequence at the 5′ end, which cannot form the pre-miRNA–like hairpin structure, were efficiently incorporated

into recombinant Ago2 and cleaved the target (Fig. 1**d**). The high activities of miR-luc–miR-luc* and pre–miR-luc can be explaind by their loose structures with mismatches and wobbles. Therefore, the cleavage activities previously observed with immunopurified[31,32] or recombinantly reconstituted[33] human Ago2 complex could reflect such bypass incorporation of single-stranded forms of small-RNA duplexes and hairpin RNAs.

Human RISC assembly is fueled by ATP

We next asked whether ATP is truly dispensable for human RISC assembly. We performed a target cleavage assay using HeLa cell lysate, immunopurified Ago2 complex and purified recombinant Ago2 in the presence (~1 mM) or absence (<0.2 nM, measured by an ATP assay) of ATP. Because ATP chelates Mg^{2+} much more strongly than ADP[37], simple omission of ATP or hexokinase-mediated depletion of ATP frees up Mg^{2+} in the buffer. The resultant excess concentration of free Mg^{2+} can inhibit target cleavage (unpublished data), which may produce the spurious appearance that ATP has enhanced RISC activity. Therefore, we substituted ATP with EDTA for the –ATP condition to normalize the free Mg^{2+} concentration. In HeLa cell lysate, ATP enhanced the target cleavage activity of miR-luc–miR-luc* duplex by more than threefold (Fig. 1**e**, left). Luc siRNA duplex and pre–miR-luc also required ATP for efficient target cleavage in HeLa cell lysate (Supplementary Fig. 1c). Thus, although it is not essential, ATP greatly facilitates human RISC assembly. In contrast, target cleavage of miR-luc–miR-luc* duplex, pre–miR-luc and single-stranded RNA by immunopurified or recombinant Ago2 was unaffected by ATP (Fig. 1**e**, middle and right, and Supplementary Fig. 1d). Stably double-stranded luc siRNA was inactive with recombinant Ago2 regardless of the presence of ATP (data not shown). Taking these data together, we concluded that ATP fuels the RISC assembly of small-RNA duplexes in humans, just as in *Drosophila*. Conversely, our results suggest that the immunopurified or reconstituted Ago2 complex used in previous studies[31–33] does not reflect the canonical human RISC-assembly pathway, but rather represent an ATP-independent ‘bypass’ pathway whereby Ago2 itself incorporates single-stranded RNAs.

Identification of complexes in human Ago2-RISC assembly

To identify which step(s) in the human RISC assembly pathway depends on ATP, we sought to distinguish the RISC-loading step, in which small-RNA duplexes are incorporated into AGO proteins, and the RISC maturation step, in which small-RNA duplexes are unwound. We used an agarose native gel system that we recently established to study the *Drosophila* Ago1-RISC assembly[13]. We prepared three small-RNA duplexes, duplex A, B and C, all of which contained an identical guide-strand sequence (Fig. 2**a**). Duplex B corresponded to a functionally asymmetric siRNA duplex, in which the guide and passenger strands were fully paired except at guide position 1. Duplex A contained two additional mismatches, one at guide position 5 (in the seed region) and another at 10 (in the central region), whereas duplex C contained only one additional mismatch at guide position 10.

We incubated HeLa cell lysate with 5′ ^{32}P-radiolabeled small-RNA duplexes and ~1,500 nt nonradiolabeled target mRNA containing complementary sites to the guide strand except for a central mismatch. We detected six distinct complexes on an agarose native gel, which we tentatively named complexes I–VI (Fig. 2**b**). These complexes were not formed in the absence of HeLa cell lysate (data not shown). Duplex A mainly formed complex II, duplex B formed both complexes I and II, and duplex C predominantly formed complex I. Complexes I and II disappeared when >95% of endogenous Ago2 was depleted by RNAi, suggesting that they contain Ago2 (Fig. 2**b**). This is consistent with a previous report that, among the four human AGO proteins, Ago2 is most prominently expressed in HeLa cells[22]. We then used a cross-linking technique to identify the protein(s) directly associated with small-RNA duplexes in each complex. After incubating the three duplexes at 25 °C for 30

min, the reaction mixtures were photo-cross-linked with 254-nm UV light. Complexes were separated by the agarose native gel and cut out from the gel, and then the protein(s) covalently cross-linked to the radiolabeled duplexes were analyzed by SDS-PAGE (Fig. 2**c**). A ~100-kDa band, which matches the size of Ago2, was detected in complexes I and II (Fig. 2**c**). This ~100-kDa band was undetectable in lysate depleted of Ago2 by RNAi (Supplementary Fig. 2a). We concluded that small RNAs directly contact Ago2 in complexes I and II.

We next determined whether the complexes contain single-stranded or double-stranded small RNAs. We incubated duplexes A–C and 2′-*O*-methyl antisense oligonucleotide (ASO), perfectly complementary to the guide strand, as a target in HeLa cell lysate. The complexes were separated and cut out from the gel, and RNA was extracted from each gel slice by deproteination and separated on 15% polyacrylamide native gel. Complex I mostly contained double-stranded small RNA, whereas complex II primarily contained single-stranded guide annealed with the target ASO (Fig. 2**d**), suggesting that complex I is pre-Ago2-RISC and complex II is mature-Ago2-RISC.

Finally we monitored the kinetics of the complex formation by a ‘chase’ experiment. HeLa cell lysate was incubated with radiolabeled duplex A and ~1,500 nt target mRNA for 0.5 min to assemble complexes III–VI. We then added a 20-fold excess of nonradiolabeled duplex A to prevent further incorporation of the radiolabeled duplex A into complexes III–VI and continued to monitor complex formation. Complexes III–VI were not chased into complexes I or II, indicating that complexes III–VI are not involved in Ago2-RISC formation (Supplementary Fig. 2b). Similarly, we formed complex I by a 5-min incubation at 15 °C (where unwinding is blocked), added a 20-fold excess of nonradiolabeled duplex, and immediately shifted the temperature from 15 °C to 25 °C (to initiate unwinding). Complex I was efficiently chased into complex II (Fig. 2**e**), confirming that complex I is a precursor of complex II. Taking these data together, we concluded that complexes I and II are pre-Ago2-RISC and mature Ago2-RISC, respectively.

ATP facilitates RISC loading but is dispensable for unwinding

We then asked whether ATP is required at the RISC-loading step or the unwinding step. ATP was depleted from HeLa cell lysate with glucose and hexokinase, and radiolabeled duplex C and ~1,500 nt target mRNA were added. Pre–Ago2-RISC formation was markedly impaired in the absence of ATP, indicating that the RISC-loading step requires ATP (Fig. 3**a**). We next formed pre–Ago2-RISC with duplex A by a 5-min incubation at 15 °C, depleted ATP, added a 20-fold excess of nonradiolabeled duplex A as in the chase experiment and immediately shifted the temperature to 25 °C. The conversion of pre–Ago2-RISC into mature Ago2-RISC was not affected by ATP (Fig. 3**b**). Thus, ATP facilitates RISC loading but is dispensable for unwinding.

Structural preferences for Ago2-RISC loading and unwinding

In *Drosophila*, perfectly complementary siRNA duplexes are actively loaded into Ago2-RISC, whereas typical miRNA–miRNA* duplexes containing central mismatches are sorted into Ago1-RISC[13,16]. In addition to central mismatches, *Drosophila* Ago1 requires mismatches in the seed or the 3′-mid regions for efficient unwinding[13]. However, the structural requirements for RISC loading and unwinding by the four human AGO proteins remain unknown. To investigate this, we prepared a series of 17 small-RNA duplexes: a perfectly complementary duplex (duplex mm1) and its 16 derivatives bearing one additional mismatch at every guide position (duplexes mm2–mm 17)[13]. All the duplexes contained a mismatch at position 1 to make them strongly asymmetric. At 15 °C, a temperature at which unwinding is blocked, duplexes mm8–mm11 formed pre–Ago2-RISC more efficiently than

duplexes mm3–mm7 and mm12–mm17, indicating that central mismatches facilitate human Ago2-RISC loading (Fig. 4**a**). We next prepared another series of 16 small-RNA duplexes: a duplex with a central mismatch at guide position 10 (duplex MM10-mm1) and its 15 derivatives bearing one additional mismatch at every guide position except for position 10 (duplexes MM10-mm2 to MM10-mm17)[13]. At 25 °C, pre-Ago2-RISC containing MM10-mm2 to MM10-mm7 and MM10-mm12 to MM10-mm15, but not MM10-mm8 to MM10-mm11, were efficiently converted to mature Ago2-RISC (Fig. 4**b**). Therefore, mismatches in the seed or 3′-mid regions are required for unwinding. Notably, these structural preferences of human Ago2 have a remarkable similarity to those of fly Ago1, but not fly Ago2, for both RISC loading and unwinding.

All four human AGO proteins have similar structural preferences

To examine the RISC assembly pathways of the other AGO proteins, we transiently expressed Flag-tagged Ago1, Ago2, Ago3 or Ago4 in human embryonic kidney (HEK) 293T cells; HEK 293T cell lysate by itself formed much smaller amounts of AGO complexes on agarose native gels than did HeLa cell lysate (data not shown), allowing sensitive detection of the complexes formed by overexpressed Flag-tagged AGO proteins (Supplementary Fig. 3a). Upon overexpression of each AGO protein, we detected two complexes (Supplementary Fig. 3a). The migrations of the two complexes varied slightly among AGO proteins, probably reflecting the size and the charge of each AGO protein. Examination of RNA species in the complexes revealed that the upper complex (complex I) contained small-RNA duplexes, and the lower complex (complex II) contained single-stranded small RNAs annealed with target ASO, indicating that they represent pre-RISC and mature RISC, respectively (Supplementary Fig. 3b). Using the mm series of duplexes to monitor RISC loading, we found that central mismatches facilitate RISC loading of all four AGO proteins (Fig. 5**a**). Similarly, native gel analysis using the MM10-mm series of duplexes revealed that the conversion of pre-RISC to mature RISC was promoted by seed or 3′-mid mismatches for all four AGO proteins (Fig. 5**b**). These results, consistent with the results of endogenous Ago2 in HeLa cell lysate (Fig. 4), suggest that all four human AGO proteins have markedly similar structural preferences for both RISC loading and unwinding.

miRNA-like small-RNA duplexes can be used by all four AGO proteins

Among the four human AGO proteins, only Ago2 possesses the slicer activity. As expected, only Ago2 among the four could efficiently unwind perfectly complementary duplex B (Fig. 6**a**). This unwinding is mediated by passenger-strand cleavage, because a nonslicer mutant form of Ago2, in which an aspartate residue in the catalytic core was substituted with alanine (D597A), failed to unwind duplex B (Fig. 6**b**). In contrast, all Ago1–4 and the noncatalytic mutant of Ago2 could rapidly unwind duplex A, which contains a central mismatch and a seed mismatch (Fig. 6**a**,**b**). Duplex C, which has only a central mismatch, could not be efficiently unwound by any of the four AGOs or the mutant Ago2 (Fig. 6**a**,**b**). Consistent with the results of the native gel data, duplex C was barely active in the cleavage assay that measures Ago2 activity (Supplementary Fig. 3c). These observations suggest that conventional perfectly complementary siRNA duplexes can be effectively used only by Ago2, but miRNA-like small-RNA duplexes with central and seed (and/or 3′-mid) mismatches can be rapidly used by all four AGO proteins. Notably, miRNA-like duplex A was slightly but consistently more active than siRNA-like duplex B in the native gel analysis (Supplementary Fig. 3c) and in the cleavage assay (Figs. 1**c** and 6**a**).

DISCUSSION

Here we show that the human RISC assembly is uncoupled from dicing and is fueled by ATP. This is in contrast to previous reports using immunopurified or reconstituted Ago2

complex[31–33], which appears to represent a pathway that can use only single-stranded RNAs, bypassing the canonical RISC loading of small-RNA duplexes (Fig. 1). Our data show that ATP is used to effectively load small-RNA duplexes into AGO proteins but is dispensable for subsequent unwinding (Fig. 3). Moreover, the four human AGO proteins show remarkably similar structural preferences for small-RNA duplexes (Figs. 4 and 5); central mismatches promote RISC loading, and seed or 3′-mid mismatches facilitate unwinding. All these features of human AGO proteins are highly reminiscent of fly Ago1 (ref. 13) but not fly Ago2 (ref. 16). The fly Ago2 pathway shows an inverse structural preference of small-RNA duplexes for RISC loading; fly Ago2 RLC, containing Dicer-2 and its partner protein R2D2, acts as a gatekeeper to exclude small-RNA duplexes with central mismatches16. Therefore, although fly Ago2 pathway is the best RISC-assembly pathway characterized to date9,16,[28,29,38,39], its properties should be generalized only with due caution. Indeed, comparison of residue sequences (Supplementary Fig. 4; for a more complete dataset, see ref. 1) supports the idea that human Ago1–4 and fly Ago1 are highly homologous, whereas fly Ago2 is quite distinctive. The mechanism by which fly Ago2 represses translation also seems to be unique: fly Ago2-RISC competes with eukaryotic translation initiation factor (eIF) 4G for eIF4E and blocks the function of the cap structure without the aid of a P-body protein, GW182 (ref. 40), whereas fly Ago1 and all human AGO proteins act via GW182 to induce deadenylation and translational repression40–42.

It is well established that Dicer-2 and R2D2 are essential for fly Ago2-RISC loading[16,28,29,38], and this is why a complex containing the Dicer-2–R2D2 heterodimer is called “RISC-loading complex.” However, our recent study shows that neither Dicer-1 nor Dicer-2 is required for fly Ago1-RISC loading[13]. Similarly, immunodepletion of human Dicer did not compromise RISC activity *in vitro*[43], and Dicer-null mouse embryonic stem cells fully supported siRNA-directed RNAi[44,45]. These observations, together with our data in this study, suggest that the previously designated “human RLC” composed of Ago2, Dicer, and TRBP does not correspond to a canonical RISC-loading complex, therefore warranting further studies on how small-RNA duplexes are loaded into human AGO proteins and fly Ago1.

In *Drosophila*[13] and *C. elegans*[17,18], small-RNA duplexes are actively and faithfully sorted into distinct AGO proteins according to their structural features. In contrast, our data show that there is no obvious difference in structural preferences among AGO proteins in human RISC-assembly pathways (Fig. 5). The sole difference between RISC-assembly pathways of human AGO proteins is that only Ago2 can effectively unwind highly complementary small-RNA duplexes via passenger-strand cleavage (Fig. 6). This exclusive feature of Ago2 may explain the recent observation of weak small-RNA sorting in humans[24,25].

Methods

Methods and any associated references are available in the online version of the paper at http://www.nature.com/nsmb/.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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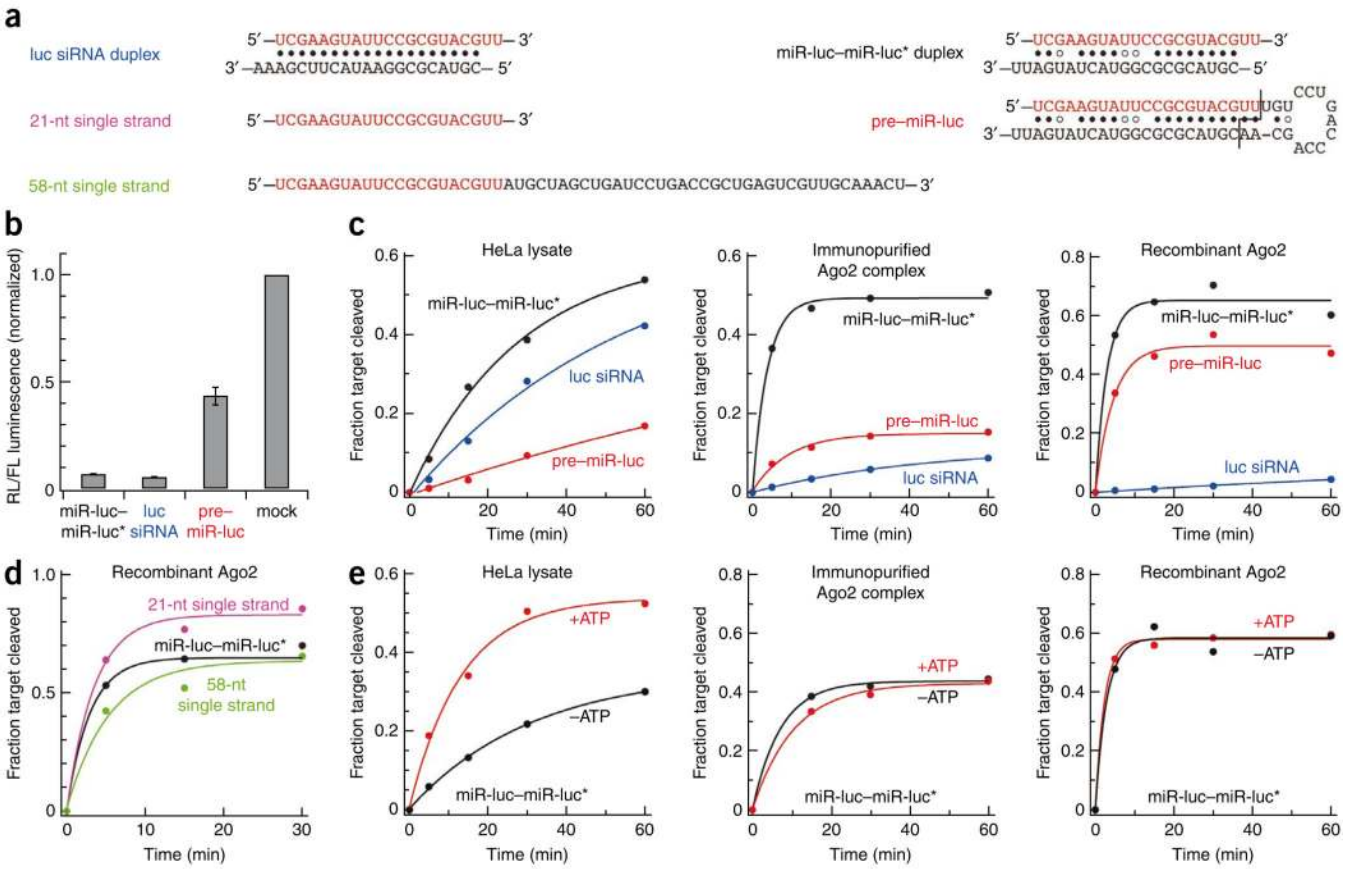

Figure 1.

Human RISC assembly is uncoupled from dicing and dependent on ATP. (**a**) Small-RNA duplexes, a pre-miRNA–like hairpin RNA, and single-stranded RNAs used in **b**–**e**. miR-luc (guide strand) is shown in red. The dicing site of pre–miR-luc is indicated by a black line. (**b**) RISC assembly is uncoupled from dicing in HeLa cells. The dual luciferase reporter assay was performed 24 h after transfection. miR-luc–miR-luc* duplex and luc siRNA duplex repressed the perfectly complementary reporter, much better than pre–miR-luc. Mock refers to no siRNA treatment. (**c**) RISC assembly is uncoupled from dicing in HeLa cell lysate, but immunopurified Ago2 complex and purified recombinant Ago2 represent a bypass pathway that can use only single-stranded RNAs. Target cleavage by miR-luc–miR-luc* duplex, luc siRNA duplex and pre–miR-luc were monitored. (**d**) Purified recombinant Ago2 can use single-stranded RNAs that cannot form hairpin structure. Target cleavage by miR-luc–miR-luc* duplex and 21-nt and 58-nt single-stranded RNAs were monitored. (**e**) ATP facilitates RISC assembly in HeLa cell lysate, but not in immunopurified Ago2 complex or purified recombinant Ago2. Target cleavage by miR-luc–miR-luc* duplex was monitored in the presence or absence of ATP. The energy regenerating system was omitted, and ATP was substituted with EDTA for the –ATP condition.

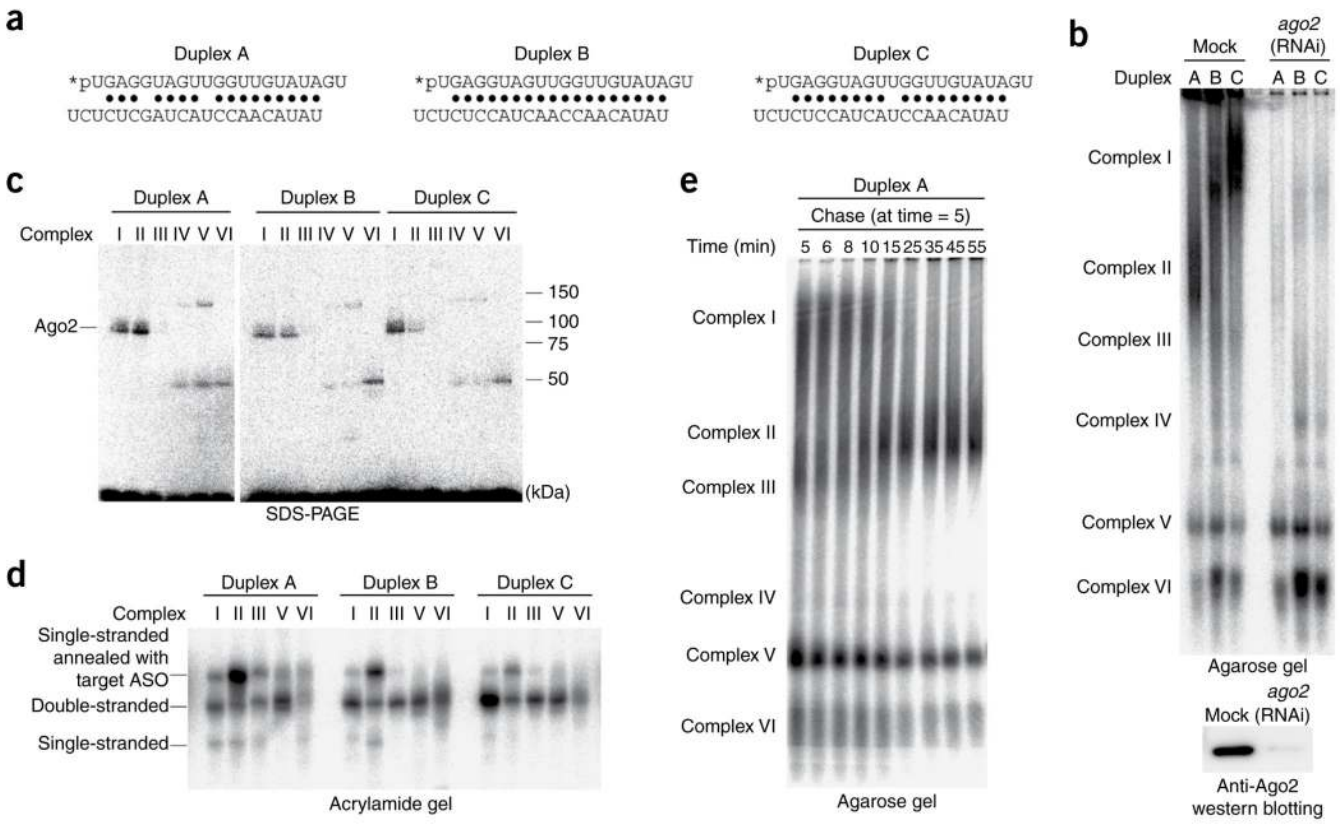

Figure 2.

Complex I and complex II are pre–Ago2 RISC and mature–Ago2 RISC, respectively. (**a**) Small-RNA duplexes used in this study. Each duplex contained an identical guide strand sequence and a 5′ ^{32}P radiolabel (indicated by an asterisk). (**b**) Agarose native gel analysis of wild-type and *ago2*(RNAi) HeLa cell lysate. Six distinct complexes were detected, and complexes I and II disappeared in *ago2*(RNAi) lysate. Anti-Ago2 western blotting showed that >95% of endogenous Ago2 was depleted by RNAi. (**c**) Complexes I and II contain Ago2. The reaction mixtures were photo-cross-linked and separated by an agarose native gel electrophoresis. The complexes were cut out from the gel and then analyzed by SDS-PAGE. The ~100-kDa bands detected in complexes I and II corresponded to Ago2. (**d**) Complex I contained double-stranded small RNA, whereas complex II contained single-stranded guide annealed with the target ASO. The complexes were cut out from the gel, and RNA was extracted and analyzed by 15% native PAGE. The identity of the bands were assigned by their comigration with the corresponding synthetic markers. (**e**) Complex I is a precursor of complex II. Complex I was formed with radiolabeled duplex A at 15 °C, a 20-fold excess nonradiolabeled duplex was added and complex formation was further monitored at 25 °C. Complex I was efficiently ‘chased’ into complex II.

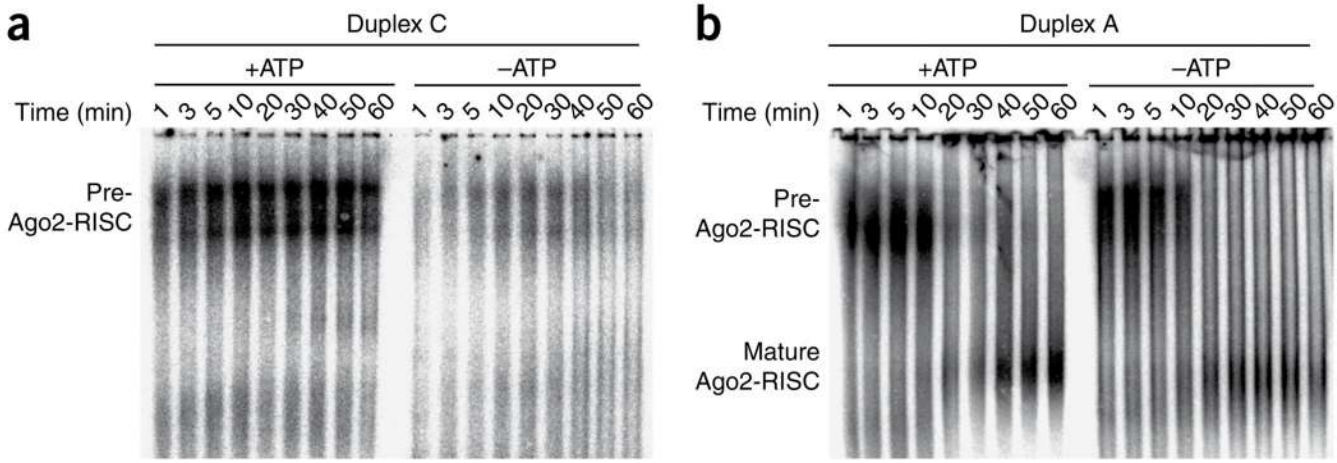

Figure 3.

ATP facilitates RISC loading but not unwinding. (**a**) Formation of pre–Ago2-RISC is promoted by ATP. HeLa cell lysate was incubated with radiolabeled duplex C at 15 °C in the presence or absence of ATP. Pre–Ago2-RISC formation was markedly impaired in the absence of ATP. (**b**) Conversion of pre–Ago2-RISC into mature–Ago2-RISC does not require ATP. Pre–Ago2-RISC was formed by incubating radiolabeled duplex A in HeLa cell lysate in presence of ATP. ATP was then depleted by hexokinase and glucose, a 20-fold excess nonradiolabeld duplex was added, and complex formation was monitored. Pre–Ago2-RISC was chased into mature Ago2-RISC regardless of the presence of ATP.

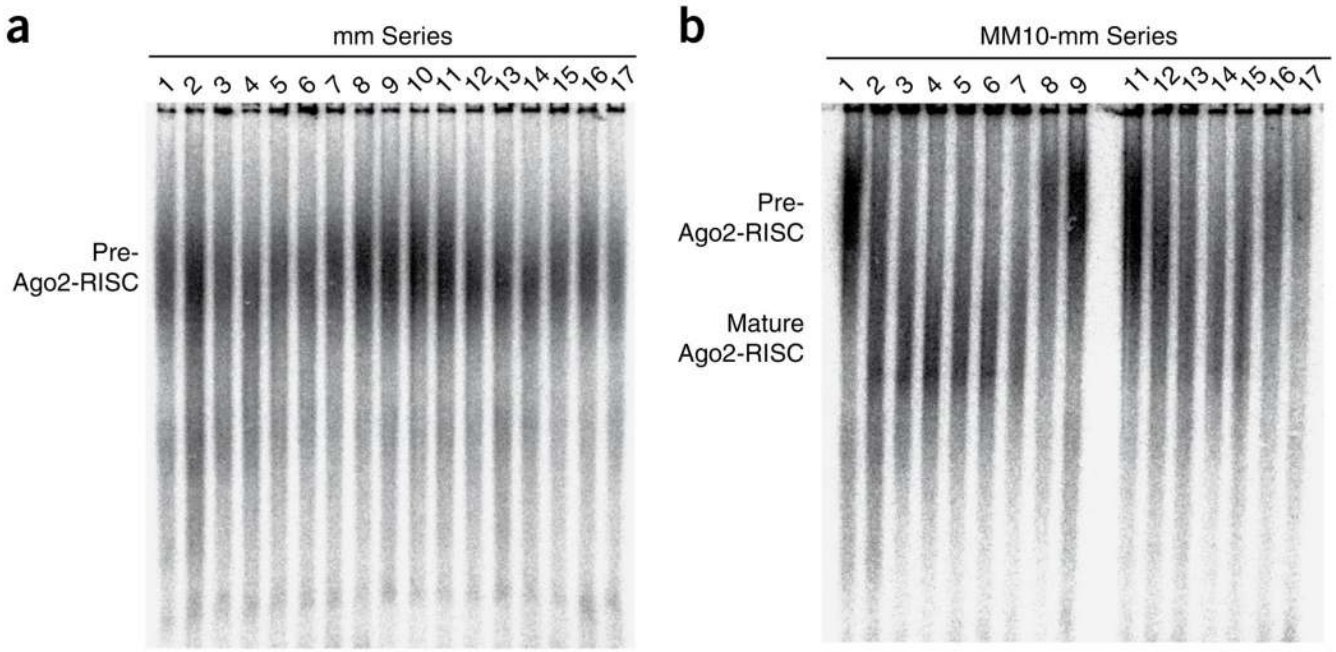

Figure 4.

Central mismatches promote Ago2-RISC loading, and seed and 3′-mid mismatches facilitate unwinding. (**a**) HeLa cell lysate was incubated with mm series of 17 small-RNA duplexes (mm1–mm17) at 15 °C. Pre–Ago2-RISC formation was enhanced with duplexes containing central mismatches. (**b**) HeLa cell lysate was incubated with MM10-mm series of small-RNA duplexes (MM10-mm1 to MM10-mm17) at 25 °C. Conversion of pre–Ago2-RISC into mature–Ago2-RISC was promoted by seed or 3′-mid mismatches.

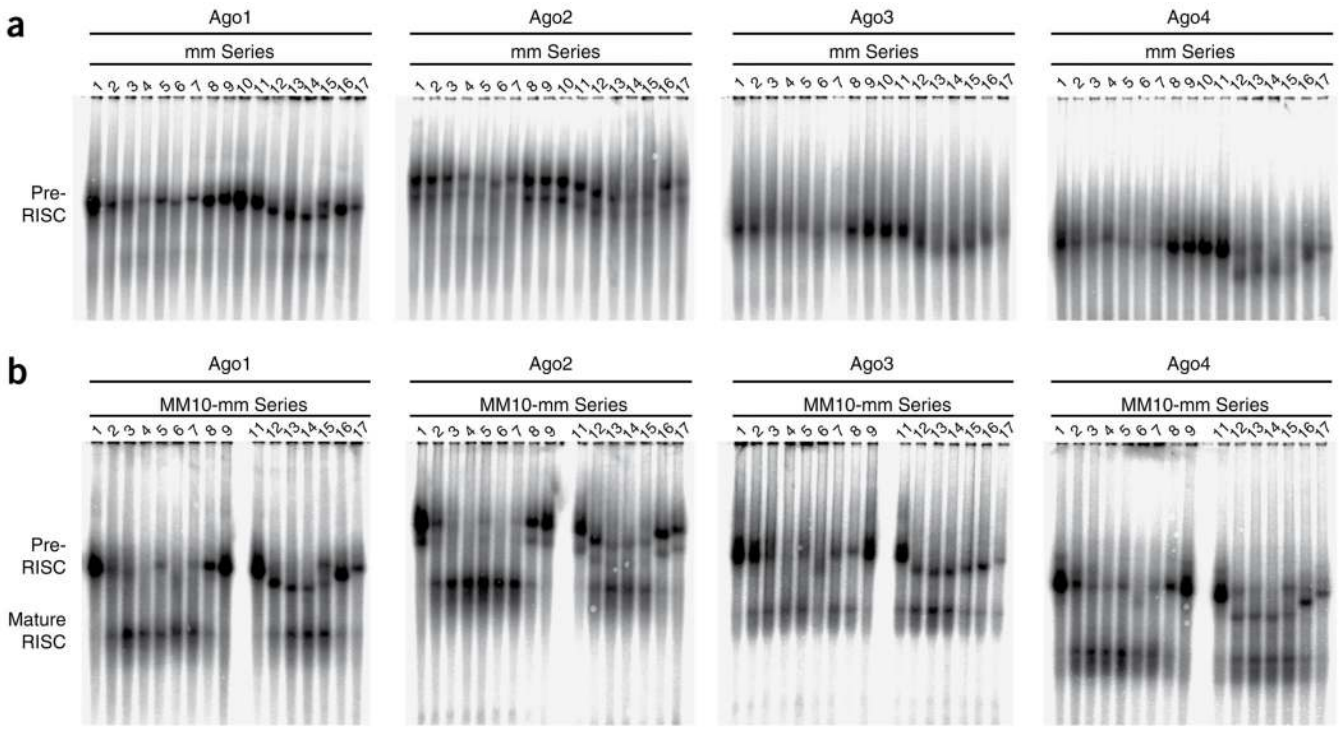

Figure 5.

Four human AGO proteins have similar structural preferences for small-RNA duplexes for RISC loading and unwinding. (**a**) RISC loading. The mm series of duplexes (mm1–mm17) were incubated at 15 °C in lysate from HEK 293T cells expressing Flag-tagged Ago1, Ago2, Ago3 or Ago4. (**b**) Unwinding. The MM10-mm series of duplexes (MM10-mm1 to MM10-mm17) were similarly incubated at 25 °C.

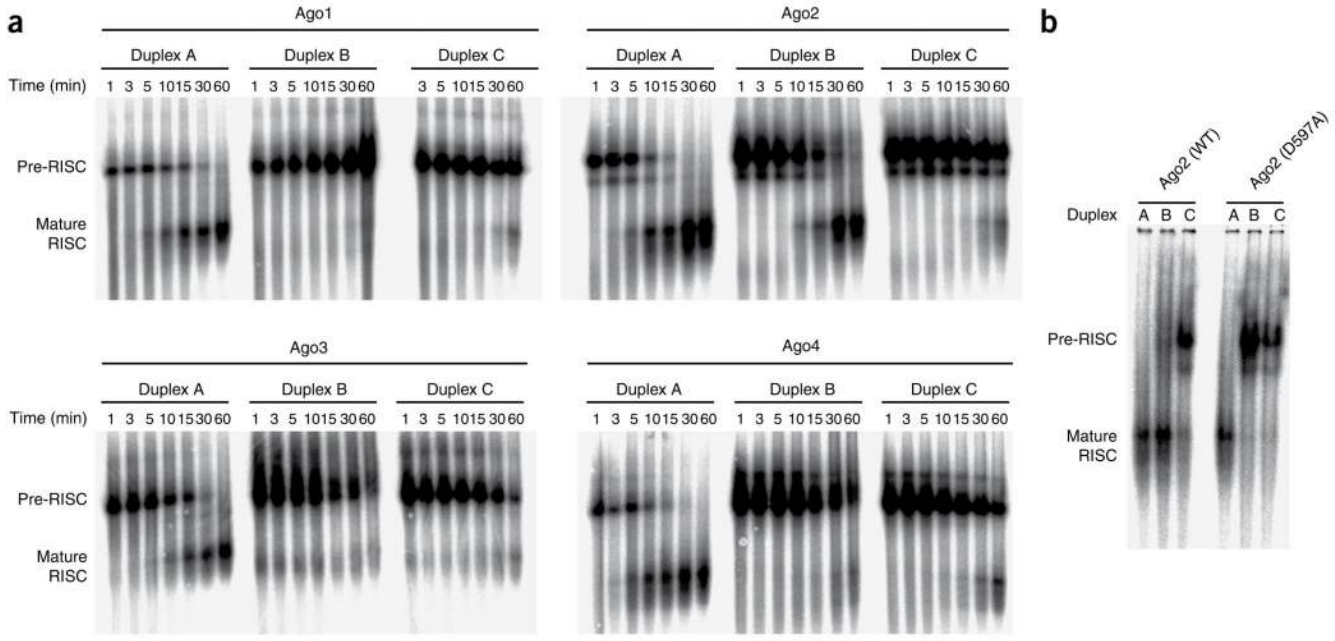

Figure 6.

Ago1–4 can use miRNA-like duplexes, whereas only Ago2 can efficiently unwind siRNA duplexes. (**a**) Lysates from HEK 293T cells expressing Flag-tagged Ago1, Ago2, Ago3 or Ago4 were incubated with radiolabeled duplexes A–C and complex formation was monitored. Ago1–4 could unwind miRNA-like duplex A but not central-bulged duplex C. Only Ago2 could unwind perfectly complementary duplex B via passenger-strand cleavage. (**b**) A noncatalytic mutant of Ago2 (D597A) cannot unwind perfectly complementary duplex B, indicating that this process is mediated by passenger-strand cleavage.