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Identification of Novel Argonaute-Associated Proteins

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Summary

RNA silencing processes are guided by small RNAs known as siRNAs and microRNAs (miRNAs) [1–4]. They reside in ribonucleoprotein complexes, which guide the cleavage of complementary mRNAs [3, 4] or affect stability and translation of partial complementary mRNAs [1, 2, 5]. Argonaute (Ago) proteins are at the heart of silencing effector complexes and bind the single-stranded siRNA and miRNA [4, 6]. Our biochemical analysis revealed that Ago2 is present in a pre-miRNA processing complex that is able to transfer the miRNA into a target-mRNA cleaving complex. To gain insight into the function and composition of RNA silencing complexes, we purified Ago1- and Ago2-containing complexes from human cells. Several known Ago1- and/or Ago2-associated proteins including Dicer were identified, but also two novel factors, the putative RNA helicase MOV10, and the RNA recognition motif (RRM)-containing protein TNRC6B/KIAA1093. The new proteins localize, similar to Ago proteins, to mRNA-degrading cytoplasmic P bodies, and they are functionally required to mediate miRNA-guided mRNA cleavage.

Results and Discussion

Proteomic Analysis of Human Ago1 and Ago2 Complexes

We generated HeLa-cell lines stably expressing FLAG/HA-tagged Ago1 or Ago2 [7] and biochemically copurified Ago-associated proteins from HeLa-cell cytoplasmic extracts by double-affinity purification. Cytoplasmic extract was first passed over anti-FLAG-antibody-coated beads. Bound proteins were eluted under native conditions with FLAG peptides, and the eluate was subsequently incubated with anti-HA-antibody-coated beads. The bound proteins were recovered and analyzed by SDS-PAGE and silver staining (Figure 1A, lanes 2 and 3). The visible bands were subjected to ESI tandem MS (LC-MS/MS) analysis. The most prominent proteins with molecular weights of about 100 kDa were identified as FLAG/HA-tagged Ago2 (lane 2) and Ago1 (lane 3). Additionally, Gemin3 and Gemin4, two proteins previously reported to be associated with human Ago2 [8], were also copurified with FLAG/HA-Ago2. The RNase III enzyme Dicer was also found to be stably associated with Ago1 and Ago2, consistent with very recent reports [9].

Among the silver-stained bands in both purifications, we identified four proteins that have not been previously linked to small-RNA-regulated gene silencing in human systems. The 175 kDa band was identified as TNRC6B isoform 1, also known as KIAA1093, a poorly characterized protein. The gene is annotated as trinucleotide repeat containing 6B, which encodes glycine-tryptophan (GW) repeats. The amino acid sequence of TNRC6B also contains an RNA recognition motif (RRM) at the C terminus, suggesting TNRC6B may function as a single-stranded-RNA binding protein. The 130 kDa band was identified as MOV10, a putative DExD-box helicase. MOV10 is a candidate ortholog of the plant protein SDE-3 and the *Drosophila melanogaster* protein Armitage [10–12], both of which are involved in RNAi. A third protein migrating at about 70 kDa was identified as the arginine methyltransferase PRMT5. The protein migrating at 50 kDa was found to be the translation factor eEF1α.

The specific interactions of the affinity-purified components of the Ago complexes with Ago1 and Ago2 proteins were further examined by cotransfection and co-immunoprecipitation (co-IP) experiments. N-terminally tagged FLAG/HA-Ago1, FLAG/HA-Ago2, or FLAG/HA-GFP was cotransfected with N-terminally tagged myc-TNRC6B into HEK 293 cells (Figure 1B). Myc-TNRC6B coimmunoprecipitated with FLAG/HA-Ago1 and FLAG/HA-Ago2 (lanes 5 and 6), and myc-TNRC6B was absent in the FLAG/HA-GFP co-IP (lane 7). FLAG/HA-Ago1, FLAG/HA-Ago2, and FLAG/HA-GFP IPs were tested for association with PRMT5 via a specific polyclonal anti-PRMT5 antibody (Figure 1B, right panel). PRMT5 was detected only in the FLAG/HA-Ago1 and FLAG/HA-Ago2 IPs (lanes 9 and 10) and not in the FLAG/HA-GFP control IP (lane 11). To verify a specific interaction of MOV10 with the Ago proteins, N-terminally FLAG/HA-tagged MOV10 was cotransfected with N-terminally tagged myc-Ago1 or myc-Ago2 (Figure 1C). The FLAG/HA-tagged proteins were immunoprecipitated with anti-FLAG beads, and the bound proteins were analyzed by western blotting against the myc-tag (middle and right panel) and the FLAG/HA-tag (left panel). FLAG/HA-MOV10 coprecipitated with myc-Ago1 and myc-Ago2 (lanes 4 and 6), whereas no Ago proteins were detected in the FLAG/HA-GFP control IP (lanes 3 and 5). We were unable to confirm an association of eEF1α with Ago1 or Ago2 via coimmunoprecipitation

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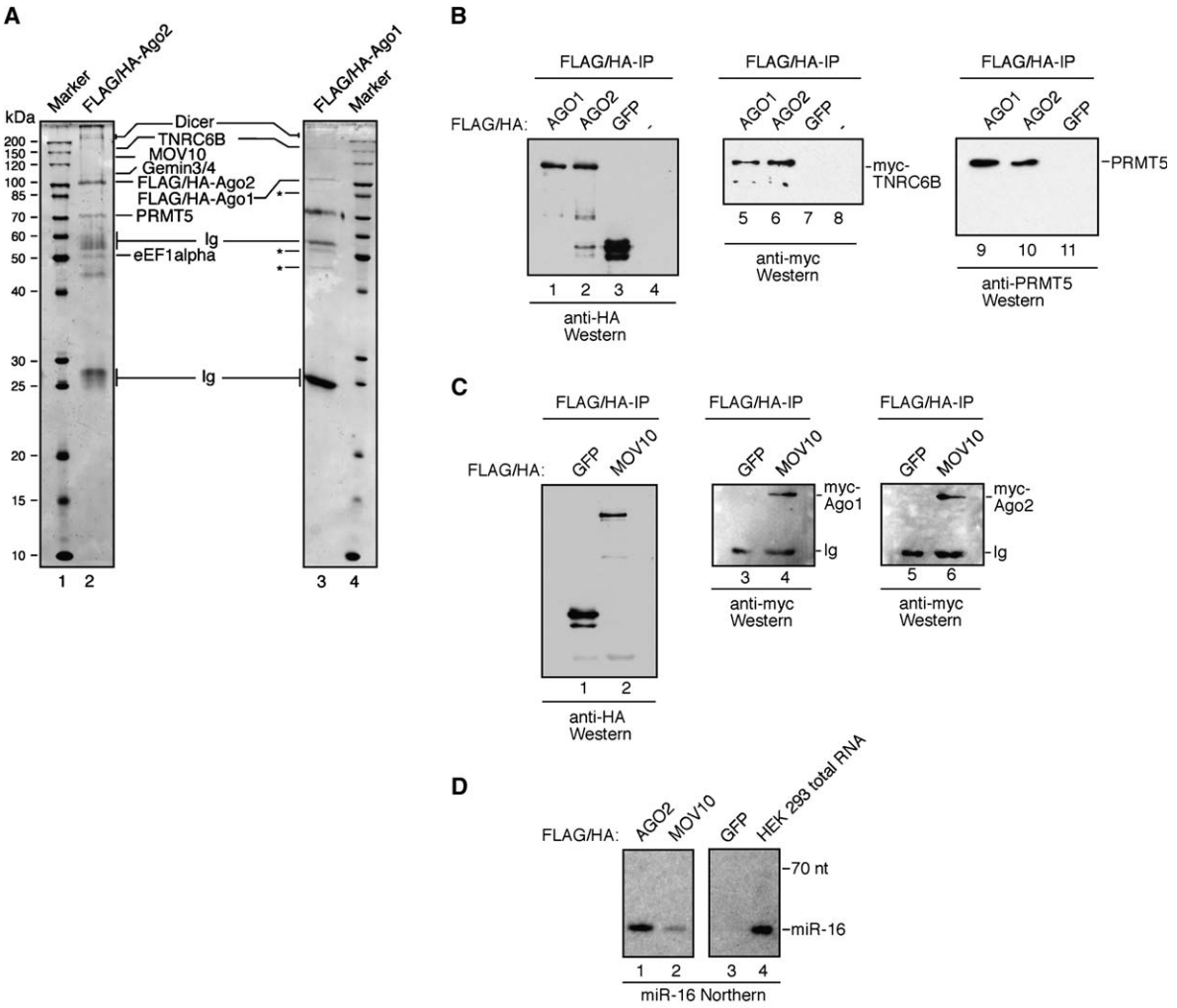

Figure 1. Biochemical Purification and Identification of Novel Ago-Associated Protein Factors

(A) Ago protein complexes were double-affinity-purified from HeLa cells stably transfected with FLAG/HA-tagged Ago1 (lane 3) and Ago2 (lane 2) plasmids. The proteins were separated by SDS-PAGE followed by silver staining and identification of the bands by ESI tandem MS (LC-MS/MS). The asterisks indicate so-far-unidentified proteins. Lanes 1 and 4 show molecular-weight markers.

(B) FLAG/HA-tagged Ago1 (lanes 1 and 5), FLAG/HA-tagged Ago2 (lanes 2 and 6), and FLAG/HA-tagged GFP (lanes 3 and 7) were cotransfected with myc-TNRC6B. In lanes 4 and 8, no FLAG/HA plasmid was cotransfected. Anti-FLAG immunoprecipitates were analyzed by western blotting with anti-HA (lanes 1–4) or anti-myc antibodies (lanes 5–8). FLAG/HA-tagged Ago1 (lane 9), FLAG/HA-tagged Ago2 (lane 10), and FLAG/HA-tagged GFP (lane 11) were transfected into 293 HEK cells. Anti-FLAG immunoprecipitates were analyzed by western blotting with specific polyclonal anti-PRMT5 antibodies.

(C) FLAG/HA-tagged MOV10 (lanes 2, 4, 6) and FLAG/HA-tagged GFP (lanes 1, 3, 5) were cotransfected with myc-tagged Ago1 (lanes 3 and 4) or with myc-tagged Ago2 (lanes 5 and 6). Anti-FLAG immunoprecipitates were analyzed by western blotting with anti-HA (lanes 1 and 2) or anti-myc antibodies (lanes 3–6).

(D) FLAG/HA-tagged Ago2 (lane 1), FLAG/HA-tagged MOV10 (lane 2), and FLAG/HA-tagged GFP were transfected into HEK 293 cells. RNA was extracted from anti-FLAG immunoprecipitates and analyzed by northern blotting with a probe specific to miR-16. Total RNA extracted from HEK 293 cells is shown in lane 4.

approaches. Therefore, it is unclear whether the binding of eEF1α to the Ago2 complex is specific.

Ago proteins specifically associate with mature miRNAs [7]. We therefore investigated whether MOV10 interacts with miRNA-loaded Ago protein complexes. We transiently transfected FLAG/HA-tagged Ago2, FLAG/HA-tagged MOV10, and FLAG/HA-tagged GFP into HEK 293 cells (Figure 1D). FLAG/HA-tagged proteins were immunoprecipitated with anti-FLAG antibodies. Coprecipitated RNAs were extracted from the beads and analyzed by northern blotting with a probe specific to miR-16, a highly abundant miRNA in HEK 293 cells. miR-16 was specifically detected in the FLAG/HA-Ago2 as well as the FLAG/HA-MOV10 immunoprecipitate (lanes 1 and 2), and this detection is indicative of an interaction of MOV10 with mature-miRNA-containing Ago protein complexes. No signal was observed in the control-IP (lane 3).

Taken together, our results indicate that MOV10, TNRC6B, and PRMT5 specifically associate with Ago proteins. We were unable to detect MOV10 and PRMT5 in the FLAG/HA-Ago1 purification, presumably

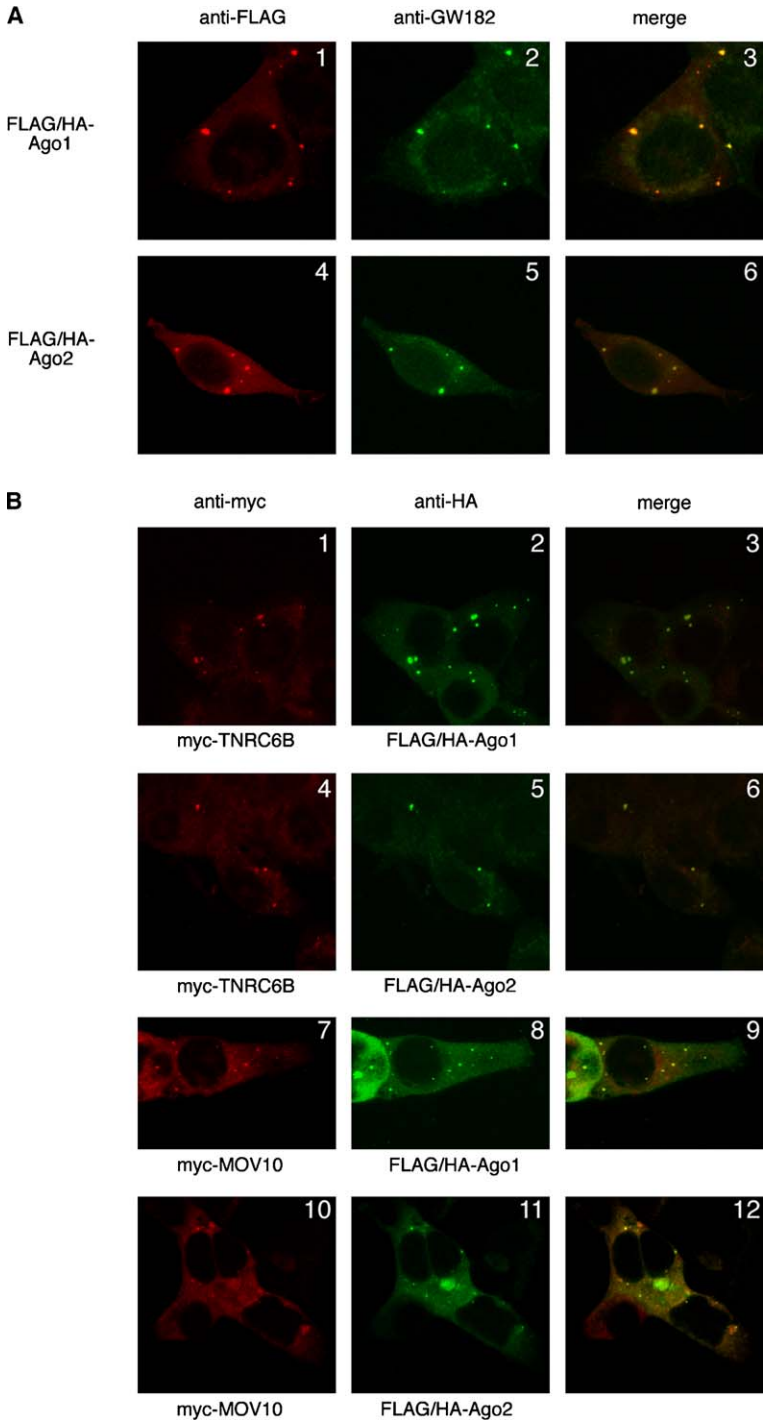

Figure 2. Human Ago1 and Ago2 Colocalize with TNRC6B and MOV10 in Cytoplasmic P Bodies

(A) FLAG/HA-Ago1 was transiently expressed in HEK 293 cells, and the fixed cells were probed with rabbit anti-FLAG and monoclonal mouse anti-GW182 antibodies. The cells were stained with secondary fluorescein-conjugated anti-mouse and Texas Red-conjugated anti-rabbit antibodies. The cells were analyzed with a TCS SP2 confocal laser microscope (Leica). (A_1) shows FLAG/HA-Ago1, (A_4) shows FLAG/HA-Ago2, and (A_2) and (A_5) show GW182 localization. (A_3) and (A_6) show the merged images.

(B) FLAG/HA-Ago1 ($B_{1–3}$ and $B_{7–9}$), FLAG/HA-Ago2 ($B_{4–6}$ and $B_{10–12}$), and myc-TNRC6B ($B_{1–6}$) as well as myc-MOV10 ($B_{7–12}$) were coexpressed in HEK 293 cells. The fixed cells were probed with mouse anti-HA and rabbit anti-myc antibodies. Protein localization was analyzed as described in (A).

because the amounts available for purified FLAG/HA-Ago1 complexes were less compared to FLAG/HA-Ago2 complexes (Figure 1A, compare lane 2 and 3).

MOV10 and TNRC6B Colocalize with Ago Proteins in Cytoplasmic P Bodies

Recently, it has been demonstrated that Ago proteins as well as miRNAs localize to specific cytoplasmic loci termed processing bodies, or P bodies [13–15]. Furthermore, TNRC6B is a protein homologous to the P body component GW182 [16], which is also known as TNRC6A. Therefore, we tested whether the novel biochemically identified Ago-complex components localize to P bodies as well. We transiently expressed FLAG/HA-tagged Ago1 and Ago2 in HEK 293 cells. After 48 hr, the cells were fixed and immunofluorescence studies were performed with anti-FLAG antibodies. Both Ago1 and Ago2 were concentrated in cytoplasmic P bodies (Figure 2A, left panel) as shown by costaining with antibodies directed against the P body marker protein GW182 (Figure 2A, middle and right panel). For colocalization studies of Ago1 and Ago2 with the biochemically

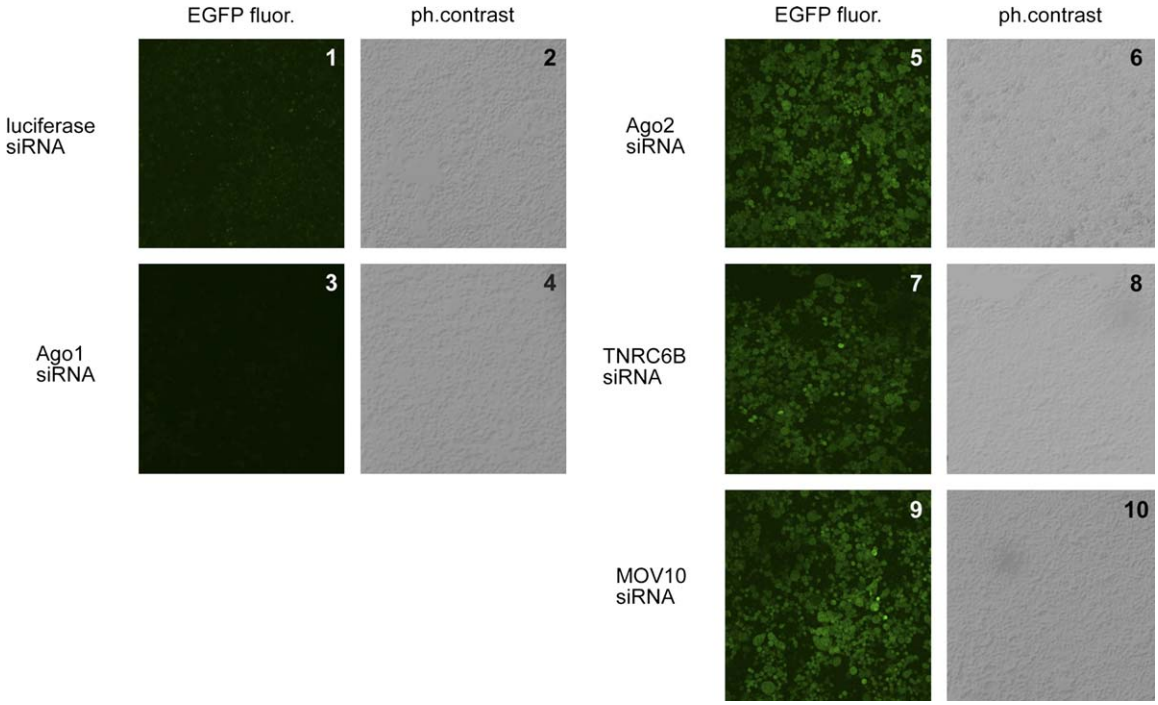

Figure 3. MOV10 and TNRC6B Are Required for miR-21-Guided Cleavage Activity in HeLa-Cell Culture

A stable HeLa-cell line that expresses EGFP carrying a sequence with perfect complementarity to miR-21 in its 3′ UTR was transfected with control siRNAs complementary to the luciferase mRNA (1 and 2) or Ago1 (3 and 4), Ago2 (5 and 6), TNRC6B (7 and 8) and MOV10-specific siRNA duplexes (9 and 10). Fluorescence and phase-contrast images were recorded 5 days after transfection with a TCS SP2 confocal laser microscope (Leica Microsystems, Germany).

identified complex components TNRC6B and MOV10, we transiently expressed FLAG/HA-Ago1 and FLAG/HA-Ago2 together with myc-TNRC6B or myc-MOV10 in HEK 293 cells (Figure 2B). Immunostaining with anti-myc antibodies demonstrated that both TNRC6B and MOV10 localized to cytoplasmic granules (Figures $2B_1$, $2B_4$, $2B_7$, and $2B_{10}$). Moreover, these granules also contained FLAG/HA-Ago1 or FLAG/HA-Ago2 as shown by probing the same cells with anti-HA antibodies (Figures $2B_2$, $2B_5$, $2B_8$, and $2B_{11}$). An overlay of the respective images shows that both Ago1 and Ago2 colocalize with TNRC6B and MOV10 in cytoplasmic P bodies (Figures $2B_3$, $2B_6$, $2B_9$, and $2B_{12}$). No specific signal has been observed when the primary anti-FLAG, anti-HA, or anti-myc antibodies were omitted from the staining procedure (Figure S1 in the Supplemental Data available with this article online).

MOV10 and TNRC6B Are Required for miRNA-Guided mRNA Cleavage In Vivo

To learn about the function of Ago2 and its complex components TNRC6B and MOV10 in cultured cells, we used a previously developed GFP-based positive-readout reporter cell line [7, 17]. In this cell line, the expression of GFP is repressed by miR-21. Transfection of 2′-O-methyl oligonucleotides antisense to miR-21 blocked endogenous miR-21 and derepressed GFP expression [7, 17]. In this reporter system, we depleted Ago1, Ago2, TNRC6B, and MOV10 mRNAs by using siRNAs (Figure 3). The knockdown of the specific mRNA was monitored by quantitative RT-PCR (qRT-PCR, Figure S2). Similar to previous findings, depletion of Ago1 (Figure 3_3), as well as transfection of control siRNAs, (Figure 3_1) has no influence on GFP expression, whereas knockdown of Ago2 resulted in strong upregulation of GFP (Figure 3_5) [7]. Knockdown of MOV10 (Figure 3_9) as well as TNRC6B mRNA (Figure 3_7) resulted in GFP-signal upregulation comparable to targeting of Ago2, indicating that MOV10 and TNRC6B were required for miR-21-guided mRNA cleavage in cultured cells.

Very recently, it has been shown that also *Caenorhabditis elegans* and *D. melanogaster* homologs of the TNRC6 family are required for miRNA-guided gene silencing and are present in P bodies [18, 19]. Because members of the human Ago subfamily as well as miRNAs and their target mRNAs were reported to localize to cytoplasmic P bodies [13–15], it is tempting to speculate that some of the newly identified factors, including TNRC6B, mediate targeting of Ago complexes to P bodies, while at the same time they might act in assembly of the silencing complexes and/or in guiding target recognition.

In Vitro Reconstitution of Target-RNA Cleaving RISC/miRNPs Supplying dsRNA Precursors

Because Dicer copurified with FLAG/HA-Ago1 and FLAG/HA-Ago2, we tested whether Dicer also associated with other Ago proteins and whether Dicer-containing Ago complexes showed biochemical Dicer activity. We transiently expressed FLAG/HA-tagged Ago1 through Ago4 and MOV10 in HEK 293 cells. After 48 hr, cells were harvested and the FLAG/HA-tagged proteins were immunoprecipitated. The IP was incubated with internally

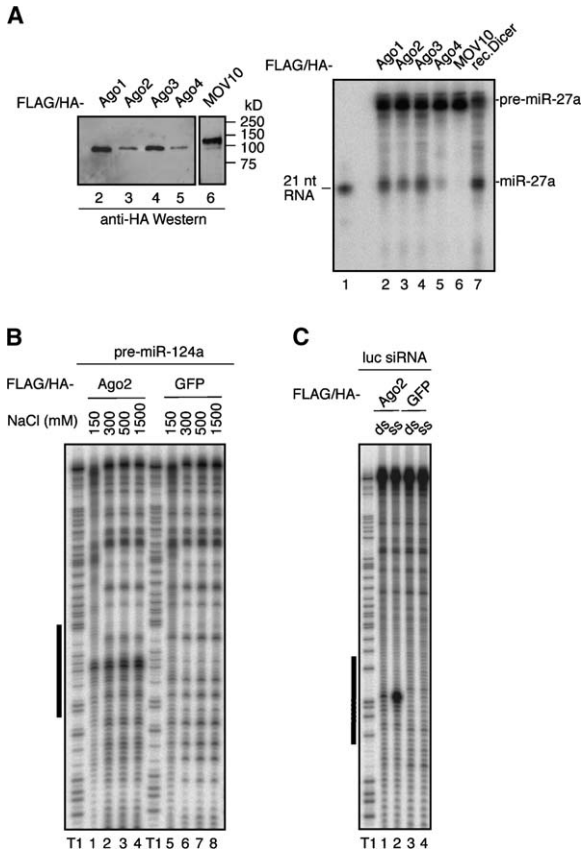

Figure 4. Human Ago2 Is Associated with Dicer, Allowing Cleavage of miRNA Precursors, Incorporation of the miRNA into Ago2 Complexes, and Cleavage of Complementary Target RNA

(A) HEK 293 cells were transiently transfected with FLAG/HA-tagged Ago1, 2, 3, 4, and FLAG/HA-tagged MOV10 (lanes 2–6). Immunoprecipitates were incubated with a Dicer substrate RNA derived from the miR-27a precursor that has been internally ^{32}P labeled. The processed RNA products were analyzed by 15% denaturing RNA PAGE followed by phosphoimaging. In lane 7, recombinant Dicer was used, and lane 1 shows a 21-nt-long RNA as size marker. An anti-HA western blot indicating the amounts of FLAG/HA-tagged Ago proteins used for the Dicer reaction is shown to the left.

(B) Immunoprecipitations of FLAG/HA-tagged Ago2 (lanes 1–4) or FLAG/HA-tagged GFP (lanes 5–8) were washed with 150 mM, 300 mM, 500 mM, or 1500 mM NaCl and subsequently preincubated with an in vitro-transcribed miR-124a-1 precursor followed by incubation with a ^{32}P-cap-labeled target RNA that carried a sequence element perfectly complementary to the mature miR-124.

The reactions shown in (C) were preincubated with single-stranded or double-stranded siRNAs directed against a luciferase target RNA. Cleaved RNA products shown in (B) and (C) were separated by 8% denaturing RNA PAGE and visualized by phosphoimaging. Partial nuclease T1 digestions of the miR-124 and the luciferase target RNAs are indicated as T1. The target-RNA sequence element that is covered by miR-124 and the siRNA against the luciferase mRNA is shown as black lines to the left of the gels.

radioactively labeled pre-miR-27a, and the processing products were subsequently analyzed by denaturing PAGE (Figure 4, right panel). As a positive control, we incubated pre-miR-27a with commercially available recombinant human Dicer (Stratagene). All of the FLAG/HA-Ago IPs (lanes 2–5) as well as the positive control (lane 7) supported pre-miRNA processing to 21-nt RNA products, indicating co-IP of Dicer cleavage activity. The FLAG/HA-MOV10 IP, however, did not contain Dicer activity, suggesting that MOV10 functions downstream of the Dicer cleavage step (lane 6) or that it is only transiently associated with the RISC loading or assembly complex.

The coexistence of Dicer and Ago proteins in Ago-purified complexes and the presence of Dicer activity prompted us to examine whether RISC can be reconstituted in vitro by using pre-miRNAs as input. Previously, it was shown that single-stranded 21-nt RNAs but not duplex siRNAs reconstitute cleavage activity by using partly purified or recombinant Ago2 [20, 21]. To avoid contamination of endogenously expressed miRNAs from HeLa or HEK 293 cells in such an assay, we chose the neuron-specific pre-miR-124a-1. Pre-miR-124a-1 was transcribed in vitro and mimicked the Drosha processing products carrying a 2 nt 3′ overhang at the base of the stem loop. FLAG/HA-Ago2 or FLAG/HA-GFP IPs from HEK 293 cells were preincubated with pre-miR-124a-1 and followed by the addition of a ^{32}P-cap-labeled substrate RNA perfectly complementary to miR-124a. The cleaved RNA products were analyzed by denaturing PAGE (Figure 4B). Only the incubation with FLAG/HA-Ago2 IP (lane 1) but not the FLAG/HA-GFP control IP (lane 5) resulted in a specific miR-124a-guided cleavage product, which indicated that Dicer, RISC-loading, and RISC activity coprecipitated with FLAG/HA-Ago2. The activity of the complexes is increased after a high-salt wash up to 1.5 M NaCl (lanes 1–4), possibly suggesting that endogenously associated dsRNA may have been removed and the proteins were free to incorporate the exogenously added pre-miR-124a. We also examined whether RISC can be reconstituted by using duplex siRNA (Figure 4C). FLAG/HA-Ago2 or FLAG/HA-GFP IPs were preincubated with the duplex siRNA directed against firefly luciferase mRNA. A ^{32}P-labeled substrate RNA was subsequently added, and the cleaved RNA products were analyzed as described above. The FLAG/HA-Ago2 IP, but not the FLAG/HA-GFP IP, supported target-RNA cleavage. The cleavage activity reconstituted on FLAG/HA-Ago2 beads when input duplex siRNA was used was about 10–20-fold weaker than when single-stranded siRNA was used at the same concentrations (lane 2), presumably as a result of very low amounts of coprecipitated protein complex(es) that are required for unwinding and/or RISC loading. In summary, we demonstrated that human Ago2 coprecipitated with components required for miRNA processing, unwinding of the small RNA duplex, RISC loading, and cleavage of a substrate RNA.

Integration of the Factors in an Interaction Network

Crystallographic studies of Ago proteins revealed unique structural and enzymatic features required for binding small RNAs processed by RNase III enzymes and for guiding target-mRNA recognition as well as cleavage [21–28]. To define the protein network of individual members of the human Ago subfamily, we applied biochemical methods to identify such interactors. Among the identified proteins, we found Dicer stably associated with Ago1- and Ago2-containing complexes; Dicer was previously shown to bind to the PIWI-box of Ago proteins through one of its RNase III domains [29]. Human Dicer was also shown to be present in a complex together with the dsRNA binding domain (dsRBD) protein TRBP,

and that this complex interacted with Ago proteins [9, 30]. However, it is only here where functional biochemical studies support the existence of such a small-RNA processing and targeting "holo-RISC" complex.

MOV10 is a human homolog of the *D. melanogaster* DExD-box protein Armitage. In *D. melanogaster* lysates, Armitage is required for early RISC assembly as demonstrated in gel-shift experiments [12]. Armitage is also essential for miRNA-guided translational regulation in *D. melanogaster* and suggests a general role for Armitage in all RNA silencing processes [10, 12]. MOV10 might have a similar function in assembling miRNA-containing silencing complexes and/or acts in subsequent steps.

The arginine-methyltransferase PRMT5 specifically generates symmetric dimethyl-arginines in its target proteins [31]. Among other RNA binding proteins, such targets are "like-Sm" proteins (LSm proteins), which are involved in pre-mRNA splicing and mRNA turnover [32]. Interestingly, LSm-proteins also localize to cytoplasmic P bodies [33], and it will be very interesting to examine whether PRMT5 and/or LSm-proteins are required for the localization of Ago proteins to cytoplasmic P bodies. Though we assume that PRMT5 is part of human "holo-RISC" and mediates methylation of arginine residues on some yet-to-be-identified "holo-RISC" component(s), we did not identify classic target motifs composed of arginines usually flanked by two glycines in known components of silencing complexes. Further biochemically studies are needed to address the role of these new enzymatic activities present in RNA-silencing-mediating complexes.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures and two Supplemental Figures and are available with this article online at: http://www.current-biology.com/cgi/content/full/15/23/2149/DC1/.

Acknowledgments

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Note Added in Proof

This text differs slightly from the version previously published online, in which MOV10 was incorrectly identified as a DEAD-box helicase; MOV10 actually belongs to the DExD superfamily. In addition, the authors wish to cite the following paper as evidence that Gemin3 and Gemin4 functionally interact with Ago2: Hutvagner, G., and Zamore, P.D. (2002). A microRNA in a multiple-turnover RNAi enzyme complex. Science *297*, 2056–2060.