Molecular Cell
Article

A Human snoRNA with MicroRNA-Like Functions

**Christine Ender,[1,6] Azra Krek,[2,3,6] Marc R. Friedländer,[2] Michaela Beitzinger,[1] Lasse Weinmann,[1] Wei Chen,[4]
Sébastien Pfeffer,[5] Nikolaus Rajewsky,[2,*] and Gunter Meister[1,*]**
[1]Center for Integrated Protein Science Munich (CIPSM), Laboratory of RNA Biology, Max Planck Institute of Biochemistry,
Am Klopferspitz 18, 82152 Martinsried, Germany
[2]Max Delbrück Center for Molecular Medicine, Robert-Rössle-Strasse 10, 13125 Berlin, Germany
[3]Department of Physics, New York University, 4 Washington Place, New York, NY 10003, USA
[4]Department of Human Molecular Genetics, Max Planck Institute of Molecular Genetics, Ihnestrasse 73, 14195 Berlin, Germany
[5]IBMP-CNRS, 12 Rue du General Zimmer, 67084 Strasbourg Cedex, France
[6]These authors contributed equally to this work
*Correspondence: rajewsky@mdc-berlin.de (N.R.), meister@biochem.mpg.de (G.M.)
DOI 10.1016/j.molcel.2008.10.017

SUMMARY

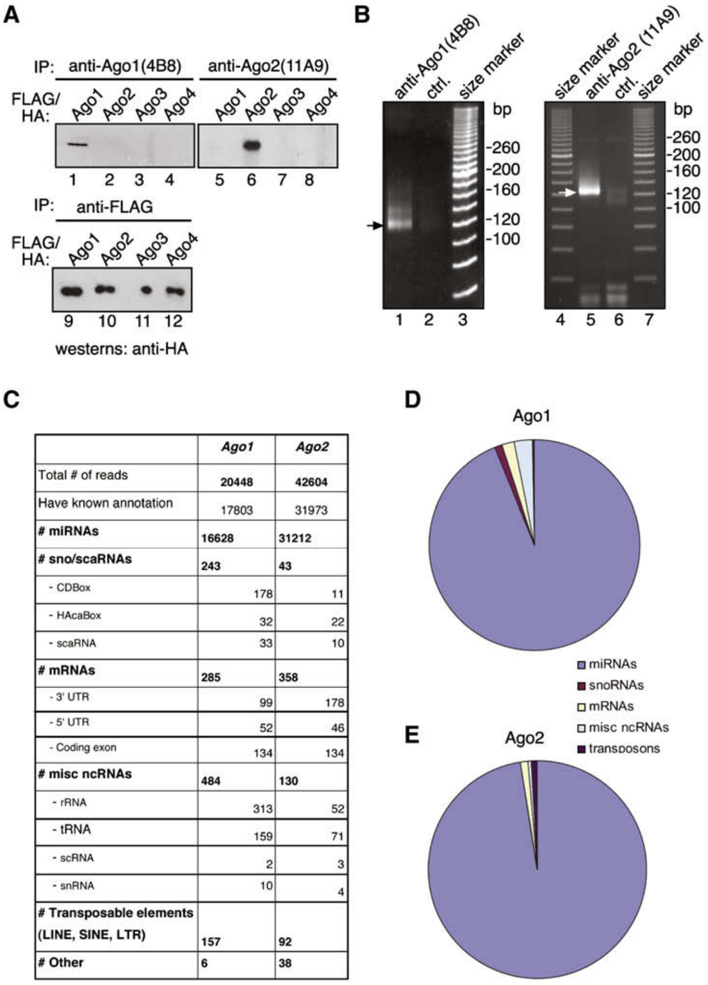
Small noncoding RNAs function in concert with Argonaute (Ago) proteins to regulate gene expression at the level of transcription, mRNA stability, or translation. Ago proteins bind small RNAs and form the core of silencing complexes. Here, we report the analysis of small RNAs associated with human Ago1 and Ago2 revealed by immunoprecipitation and deep sequencing. Among the reads, we find small RNAs originating from the small nucleolar RNA (snoRNA) ACA45. Moreover, processing of ACA45 requires Dicer activity but is independent of Drosha/DGCR8. Using bioinformatic prediction algorithms and luciferase reporter assays, we uncover the mediator subunit CDC2L6 as one potential mRNA target of ACA45 small RNAs, suggesting a role for ACA45-processing products in posttranscriptional gene silencing. We further identify a number of human snoRNAs with microRNA (miRNA)-like processing signatures. We have, therefore, identified a class of small RNAs in human cells that originate from snoRNAs and can function like miRNAs.

INTRODUCTION

Small noncoding RNAs, including microRNAs (miRNAs), short interfering RNAs (siRNAs), and Piwi-interacting RNAs (piRNAs), are important regulators of gene expression (Filipowicz et al., 2005; Meister and Tuschl, 2004; Seto et al., 2007). miRNAs and siRNAs guide sequence-specific cleavage, deadenylation, or translational repression of target mRNAs (Chen and Rajewsky, 2007; Pillai et al., 2007). piRNAs are specifically expressed in testes (Seto et al., 2007) and control retrotransposition in the mammalian germ line (Aravin et al., 2007).

In many gene-silencing pathways, small RNAs are generated from double-stranded RNA (dsRNA) molecules by distinct processing steps (Tomari and Zamore, 2005). miRNA genes are transcribed by RNA polymerases II or III as primary miRNAs that are further processed to hairpin-structured miRNA precursors (pre-miRNAs) by the nuclear microprocessor complex containing the RNase III enzyme Drosha and its cofactor DGCR8 (Borchert et al., 2006; Denli et al., 2004; Gregory et al., 2004; Landthaler et al., 2004; Lee et al., 2003, 2004). Pre-miRNAs are transported to the cytoplasm, where the RNase III enzyme Dicer cleaves off the loop of the miRNA hairpin, thereby generating a short dsRNA of about 20–25 nucleotides (nt) in length (Bohnsack et al., 2004; Grishok et al., 2001; Hutvágner et al., 2001; Lund et al., 2004). Such dsRNA intermediates are subsequently unwound, and the single-stranded mature miRNA is incorporated into effector complexes often referred to as miRNPs (Mourelatos et al., 2002). In the siRNA pathway or RNA interference (RNAi), long dsRNA is processed by Dicer as well (Bernstein et al., 2001). The mature siRNA is incorporated into the RNA-induced silencing complex (RISC). The biogenesis of piRNAs is only poorly understood and probably does not require the function of Drosha or Dicer.

Argonaute (Ago) proteins are the cellular binding partners of small RNAs and form the core of gene silencing effector complexes (Parker and Barford, 2006; Peters and Meister, 2007). In humans, eight different Argonaute genes exist, which can be phylogenetically divided into four Ago and four Piwi subfamily members (Peters and Meister, 2007; Tolia and Joshua-Tor, 2007). Whereas Piwi proteins interact with piRNAs in the germ line (Seto et al., 2007), Ago subfamily members associate with miRNAs in somatic cells. Argonaute proteins are generally characterized by Piwi-Argonaute-Zwille (PAZ) and PIWI domains (Parker and Barford, 2006; Peters and Meister, 2007). A third domain, termed MID domain, anchors the 5′ end of the small RNA (Ma et al., 2005; Parker et al., 2005). The PAZ domain binds the 3′ end of the small RNA, and the PIWI domain, which is structurally similar to RNase H, cleaves the complementary target RNA (Parker and Barford, 2006; Patel et al., 2006; Tolia and Joshua-Tor, 2007). However, not all Argonaute proteins are endonucleases, although critical residues within the PIWI domain are conserved. In mammals, only Ago2 has been shown to act as endonuclease in RNAi (Liu et al., 2004; Meister et al., 2004). Argonaute proteins with endonuclease activity are often referred to as Slicers. Although Ago subfamily members have been extensively studied in the past, only little is known about their individual small RNA-binding specificities. It has been reported that all Ago proteins bind miRNAs or siRNAs indiscriminately of their



	Ago1	*Ago2*
Total # of reads	**20448**	**42604**
Have known annotation	17803	31973
# miRNAs	**16628**	**31212**
# sno/scaRNAs	**243**	**43**
- CDBox	178	11
- HAcaBox	32	22
- scaRNA	33	10
# mRNAs	**285**	**358**
- 3' UTR	99	178
- 5' UTR	52	46
- Coding exon	134	134
# misc ncRNAs	**484**	**130**
- rRNA	313	52
- tRNA	159	71
- scRNA	2	3
- snRNA	10	4
# Transposable elements (LINE, SINE, LTR)	**157**	**92**
# Other	**6**	**38**

Figure 1. Small RNAs Associated with Ago1 and Ago2 Complexes

(A) Characterization of monoclonal anti-Ago1 and anti-Ago2 antibodies. FLAG/HA-tagged Ago1 through 4 were subjected to immunoprecipitations using anti-Ago1(4B8) (lanes 1–4), anti-Ago2(11A9) (lanes 5–8), and anti-FLAG (lanes 9–12). Immunoprecipitated FLAG/HA-Ago proteins were analyzed by western blotting using anti-HA antibodies.

(B) Endogenous Ago1 (lane 1) and Ago2 (lane 5) complexes were immunoprecipitated using the specific monoclonal antibodies described in (A). An anti-FLAG (lane 2) or an anti-GST antibody (lane 6) served as controls. Coimmunoprecipitated RNAs were extracted, cloned, and sequenced. Cloned PCR products containing 5′ adaptors, poly(A) tails, and 3′ adaptor sequences were loaded onto an agarose gel and visualized by ethidium bromide staining.

(C) Summary of the sequencing data obtained from deep sequencing of human Ago1 and Ago2 associated small RNAs.

(D and E) Schematic representation of individual small RNA classes that are associated with human Ago1 or Ago2 complexes.

sequence (Liu et al., 2004; Meister et al., 2004). However, a recent study analyzed small RNAs that are associated with human Ago2 and Ago3 and suggested that Ago proteins might have preferences for individual miRNA species, although all miRNAs that have been investigated bind to both Ago2 and Ago3 (Azuma-Mukai et al., 2008).

Here, we report the characterization of small RNAs associated with human Ago1 and Ago2 by immunoprecipitation and deep sequencing. We find that Ago1 and Ago2 bind to similar sets of miRNAs, although some miRNAs are more prominent in Ago2 libraries and vice versa. More importantly, we find small RNAs that originate from small nucleolar RNAs (snoRNAs). snoRNAs are nucleolar noncoding RNAs, which have important functions in the maturation of other noncoding RNAs such as ribosomal RNAs (rRNAs) or small nuclear RNAs (snRNAs) (Matera et al., 2007). We demonstrate that the bona fide snoRNA ACA45 is processed to small 20- to 25-nt-long RNAs that stably associate with Ago proteins. Processing is independent of the Drosha/DGCR8 complex but requires Dicer. Finally, we identify a cellular target mRNA that is regulated by the ACA45-derived small RNA, indicating that snoRNA-derived small RNAs can function like miRNAs.

RESULTS

Small RNAs Associated with Human Ago1 and Ago2

Different Ago proteins associate with the same miRNA species regardless of their sequence (Azuma-Mukai et al., 2008; Meister et al., 2004). However, the spectrum of Ago-associated small RNAs in human somatic cells is presently not known. Therefore, we used monoclonal antibodies specific to human Ago1 (Ago1 [4B8]) (Beitzinger et al., 2007) and Ago2 (Ago2 [11A9]) (Rudel et al., 2008) for Ago isolation from total HEK293 cell lysates (Figure 1A). Coimmunoprecipitated RNAs were extracted and cloned without size fractionation (Figure 1B). Using 454 deep sequencing, we obtained 20448 reads from the Ago1-associated and 42604 reads from the Ago2-associated small RNA libraries (Figures 1C–1E). Using a Dicer substrate identification algorithm (Friedländer et al., 2008), the presence of 166 known miRNAs in the combined Ago1 and Ago2 libraries was confirmed. We next investigated whether miRNAs are differentially bound to Ago1 or Ago2 in HEK293 cells (Table S1 available online). All miRNAs that are present in the libraries bind to Ago1 as well as Ago2. Similarly to the published data on Ago2 and Ago3 miRNA association (Azuma-Mukai et al., 2008), some miRNAs are more highly represented in one or the other library, suggesting a preferential Ago binding.

Processing of Functional Small RNAs from the Bona Fide snoRNA ACA45

In the Ago-associated RNA libraries, we have identified small RNAs with a length of about 20–22 nt that originate from snoRNAs particularly from ACA45 (Figure 2A). Notably, the sequenced reads derive only from the hairpin formed by the 3′ half of ACA45. The found reads are conserved in mammals (Figure 2B), suggesting that they are, indeed, specific processing products.

Although ACA45 was identified in a screen for functional snoRNAs (Kiss et al., 2004), it is conceivable that it represents a miRNA gene that has been misannotated as snoRNA. Due to

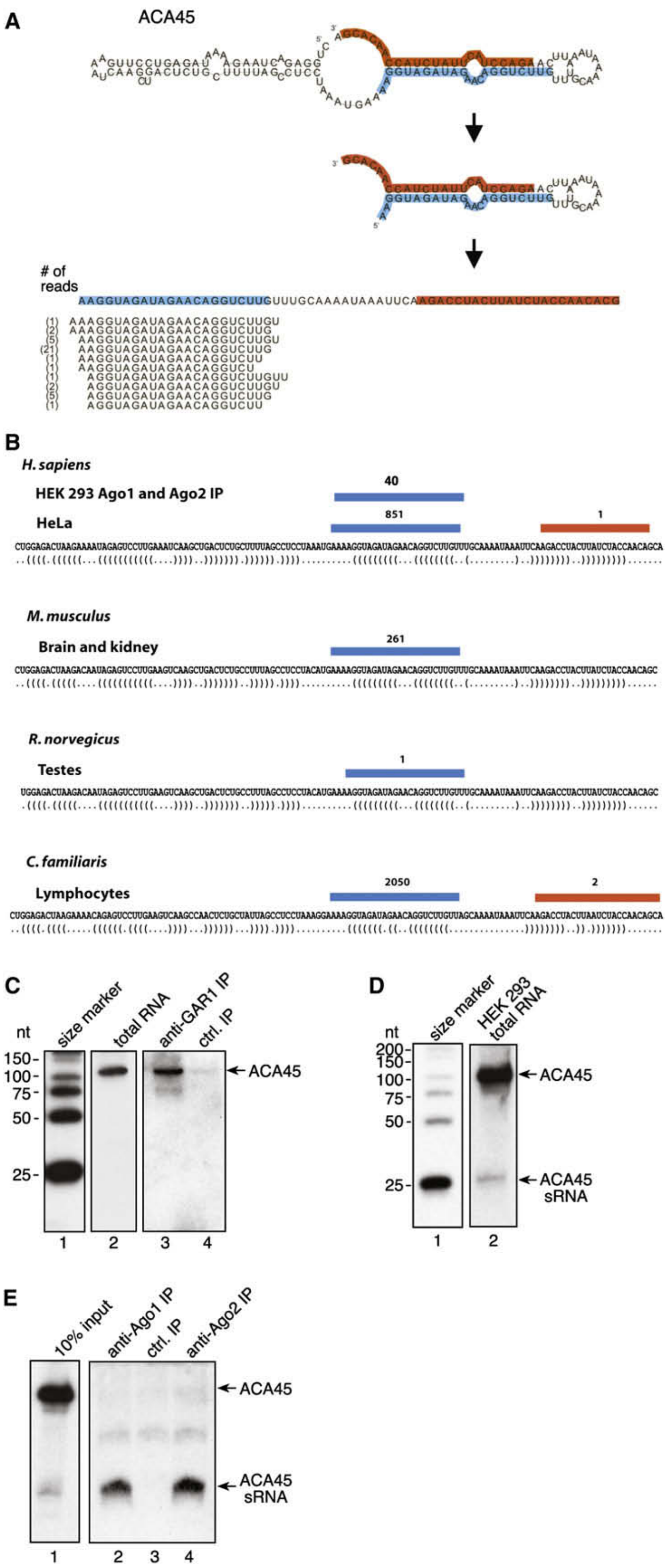


Figure 2. snoRNA ACA45 Is Processed to Small RNAs

(A) Sequence reads found in Ago1 and/or Ago2 libraries are highlighted in orange and blue. Numbers of individual sequences are shown in parentheses.

(B) Specific ACA45-processing products are conserved across species. The ACA45 sequences for *Homo sapiens*, *Rattus norvegicus*, *Mus musculus*, and *Canis familiaris* are shown by sequence and structure. Independent deep sequencing data from each species have been obtained and mapped against the corresponding homolog (data sets referenced in Experimental Procedures). The bars indicate the positions of mapped reads; consensus positions are shown where small variations exist. The blue bars represent putative "mature" Dicer products, while the red bars represent putative "star" products. The numbers above the bars indicate the number of reads.

(C) ACA45 associates with the snoRNP component GAR-1. HEK293 lysates were immunoprecipitated using anti-GAR-1 (lane 3) or control antibodies (lane 4). Coimmunoprecipitated RNA was extracted and analyzed by northern blotting using a probe specific to ACA45. Lane 2 shows total RNA extracted from 10% of the input lysate, and lane 1 shows a size marker.

(D) Total RNA from HEK293 cells (30 μg) was blotted, and the membrane was incubated with a probe complementary to nt 65–85 (lane 2) of ACA45. A size marker is shown in lane 1.

(E) Endogenous Ago1 (lane 2) or Ago2 (lane 4) were immunoprecipitated from HEK293 lysates, and the coimmunoprecipitated RNAs were extracted and analyzed by northern blotting using the probe described in (D). Lane 1 shows 10% of the extracts used for the immunoprecipitations, and lane 3 shows a control immunoprecipitation.

their specific structures and functions, snoRNAs can be grouped in H/ACA and Box C/D class snoRNAs. snoRNAs associate with specific protein components such as GAR-1 (H/ACA) or fibrillarin (Box C/D) to form functional snoRNPs (Matera et al., 2007). In order to prove that ACA45 is, indeed, a functional snoRNA, we analyzed GAR-1 binding to ACA45 (Figure 2C). Endogenous GAR-1 was immunoprecipitated using anti-GAR-1 antibodies. Associated RNAs were extracted and further analyzed by northern blotting using the probe specific to ACA45. Indeed, full-length ACA45 was readily detectable in the anti-GAR-1 (lane 2), but not in control immunoprecipitates (lane 3). Our data, therefore, confirm that ACA45 represents a functional snoRNA.

We next validated the processing of ACA45 to small RNAs by northern blotting (Figure 2D). A probe complementary to the 5′ arm (Figure 2A, indicated in blue) detected the full-length ACA45 snoRNA as well as a band of ~22–23 nt in total RNA, indicating that a portion of the cellular ACA45 pool is, indeed, processed to small RNAs. Using quantitative northern blotting, we analyzed ACA45 sRNA molecule numbers per cell (data not shown). We find that less than 1000 molecules are present per cell, which is similar to a low abundant miRNA (Lim et al., 2003). Since only a minor portion of ACA45 is processed to small RNAs, we next investigated whether ACA45 processing products are specifically enriched in Ago protein complexes (Figure 2E). Endogenous Ago1 (lane 2) or Ago2 (lane 4) were immunoprecipitated, and associated RNAs were analyzed by northern blotting against ACA45-processing products. Consistent with the cloning data, the small RNA derived from ACA45 was enriched in Ago1 as well as Ago2 immunoprecipitates, indicating that ACA45-processing products specifically associate with Ago proteins. Therefore, we refer to this functional small RNA as ACA45 small RNA (ACA45 sRNA).

ACA45 Small RNAs Can Function Like miRNAs

The striking similarity of ACA45-processing products to miRNA precursors prompted us to investigate whether ACA45 sRNAs are functionally similar to miRNAs. We generated a luciferase reporter construct containing a complementary binding site for the abundant 5′ arm of the snoRNA precursor (Figure 3A). Luciferase activity was strongly increased when the endogenous ACA45-derived small RNAs were inhibited using 2′-O-methylated (2′-OMe) antisense inhibitors (Figure 3A). Moreover, luciferase activity was also increased when the RNAi endonuclease Ago2 was depleted (Figure 3B), indicating that small RNAs that are processed from ACA45 can function like miRNAs.

ACA45 Processing Is Independent of the Drosha/DGCR8 Complex but Requires Dicer

The cleavage signature of the stem-loop-structured processing intermediate is different than the typical 2 nt 3′ overhangs generated by Drosha. Therefore, we analyzed whether ACA45 processing requires activity of the Drosha/DGCR8 complex using in vitro as well as in vivo approaches (Figures 3B and 3C). FLAG/HA(FH)-tagged DGCR8 was immunoprecipitated, and the immunoprecipitate was incubated with either a ^{32}P-labeled primary miR-27a transcript or ACA45. A specific cleavage product representing pre-miR-27a was observed in the anti-DGCR8 immunoprecipitates, whereas no signal was observed when ACA45 was used as substrate. We further investigated Drosha requirements using the luciferase reporter construct described above (Figure 3B). Indeed, we did not observe elevated luciferase activity upon Drosha depletion (siRNAs have been validated in Landthaler et al. [2004]), whereas luciferase activity of a miR-19b-responsive reporter was significantly increased. Taken together, our results suggest that ACA45 processing is independent of the Drosha/DGCR8 complex.

Next, we investigated Dicer requirements for ACA45 processing. It has been demonstrated that Ago proteins form a stable complex with Dicer, and Dicer activity can be coimmunoprecipitated with antibodies against Ago proteins (Gregory et al., 2005; Maniataki and Mourelatos, 2005; Meister et al., 2005). Therefore, FH-tagged Ago proteins, as well as FH-Dicer, was immunoprecipitated from HEK293 lysates and incubated with ^{32}P-labeled pre-miR-27a or full-length ACA45 (Figure 3D). As expected, both FH-Ago2 and FH-Dicer immunoprecipitates efficiently processed the miR-27a precursor (Figure 3D, left panel). Furthermore, FH-Ago1, FH-Ago2, and FH-Dicer immunoprecipitates processed the ^{32}P-labeled full-length ACA45 as well (Figure 3D, right panel), suggesting that Dicer is required for the generation of ACA45 small RNAs. To further investigate Dicer's function in ACA45 processing, we analyzed whether Dicer alone is sufficient for ACA45 processing in vitro. ^{32}P-labeled ACA45 was incubated with increasing amounts of recombinant Dicer, and cleavage products were analyzed by RNA-PAGE (Figure 3E). Indeed, recombinant Dicer produced small RNAs from the full-length ACA45 in a concentration-dependent manner, suggesting that Dicer alone is sufficient for ACA45 processing. Notably, Dicer generates longer RNAs as well, which might represent processing intermediates (see asterisk in Figure 3D). Finally, we analyzed the role of Dicer in ACA45 processing in vivo. Total RNA from mouse embryonic stem (ES) cells carrying homozygous or heterozygous Dicer deletions (Murchison et al., 2005) was analyzed for the presence of ACA45 small RNAs by semiquantitative real-time PCR (qRT-PCR) (Figure 3F). Strikingly, no PCR product was detectable in the Dicer$^{-/-}$ cells, whereas a PCR product originating from the ACA45 small RNA was readily detectable in Dicer$^{+/-}$ cells. Notably, the full-length ACA45 was present in both Dicer$^{-/-}$ and Dicer$^{+/-}$ cells. Similar results were obtained when total RNA from Dicer$^{-/-}$ and Dicer$^{+/-}$ cells was analyzed by northern blotting using a probe complementary to the ACA45 small RNA (Figure 3G). In summary, our data indicate that Dicer processes ACA45 to small RNAs independently of the Drosha-containing microprocessor complex.

Validation of an Endogenous ACA45-Derived Small RNA Target

It is thought that complementary Watson-Crick base pairing of the seed (nucleotides 2–8 counted from the 5′ end) is a key feature of miRNA:mRNA target recognition. It is also known that highly conserved 7-mers in 3′UTRs are often complementary to seed sequences of known miRNAs (Chen and Rajewsky, 2007). Remarkably, the seed of ACA45 22-nt-long processing product is perfectly complementary to a significantly conserved 3′UTR motif (top 3% of all possible seed sites). Using the miRNA target prediction algorithm PicTar (Krek et al., 2005), we have predicted target mRNAs for the ACA45-derived small RNA

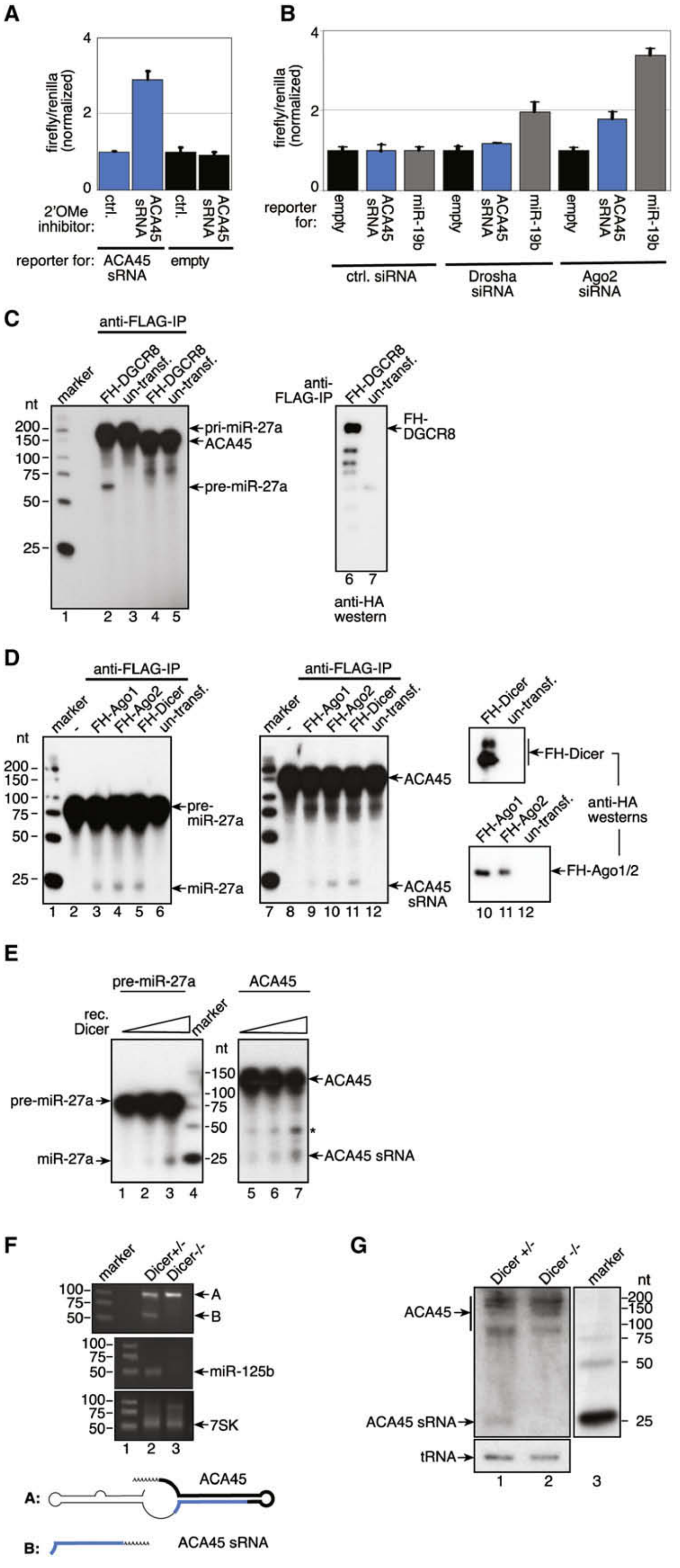


Figure 3. ACA45 Processing Requires Dicer but Is Independent of Drosha

(A) A luciferase reporter construct containing a perfectly complementary binding site for the ACA45 sRNA or the empty vector was cotransfected with 2′-O-methylated antisense inhibitors directed against the ACA45 sRNA.

(B) The luciferase reporter described in (A), the empty vector, and a luciferase reporter containing a complementary binding site to miR-19b were transfected into HEK293 cells that have been pretransfected with control siRNAs, siRNAs directed against Drosha, and siRNAs against Ago2. Firefly luciferase activity was normalized to Renilla activity. Error bars are derived from four individual experiments.

(C) FH-DGCR8 or untreated cells were immunoprecipitated using anti-FLAG antibodies. Immunoprecipitates were incubated with ^{32}P-labeled pri-miRNA-27a (lanes 2 and 3) or ACA45 (lanes 4 and 5). Lane 1 represents a size marker, and lanes 6 and 7 represent the protein input.

(D) FH-Ago2 (lanes 4 and 10), FH-Ago1 (lanes 3 and 9), and FH-Dicer (lanes 5 and 11) were incubated with ^{32}P-labeled pre-miR-27a (lanes 2–6) or ACA45 (lanes 8–12) and analyzed by RNA PAGE. In lanes 6 and 12, lysate from untransfected HEK293 cells was used. Lanes 13–15 show anti-HA western blots of the protein input. Lanes 1 and 7 show size markers.

(E) ^{32}P-labeled pre-miR-27a (lanes 1–3) or ACA45 (lanes 5–7) were incubated with increasing amounts of recombinant Dicer. Cleavage products were analyzed by RNA PAGE. Lane 4 shows a size marker. A putative processing intermediate is indicated by an asterisk.

(F) Total RNA from Dicer$^{-/+}$ (lane 2) or Dicer$^{-/-}$ cells was analyzed by semi-qRT-PCR using primers specific for the ACA45 sRNA (upper panel), miR-125b (middle panel), and 7SK RNA (lower panel). The origin of the PCR products indicated as A and B are highlighted in bold below the figure.

(G) Total RNA from Dicer$^{+/-}$ (lane 1) or Dicer$^{-/-}$ (lane 2) cells was analyzed by northern blotting using probes specific for the ACA45 small RNA described above. Lane 3 shows a size marker.

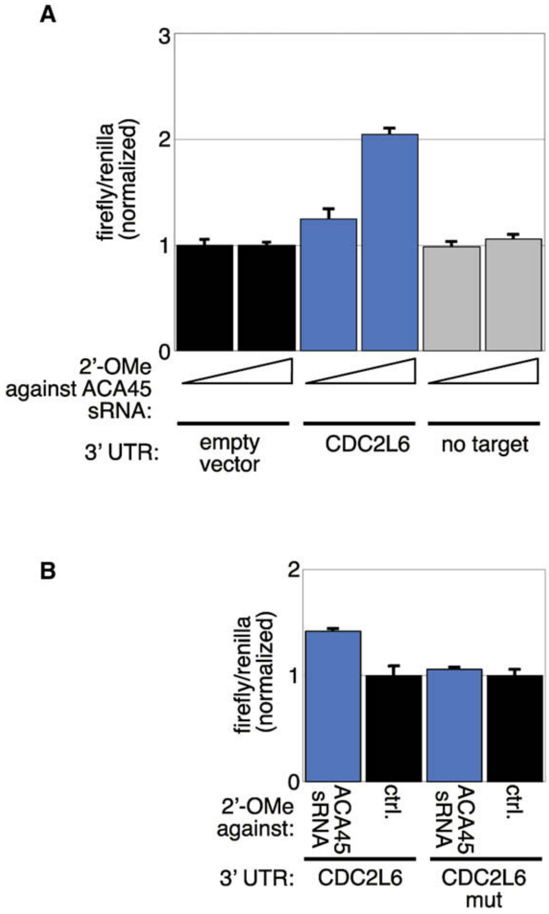


Figure 4. ACA45-Derived Small RNAs Regulated CDC2L6 Expression

(A) Luciferase reporter constructs containing the 3′UTR of CDC2L6 or BAP-1 (no target) or the empty vector were cotransfected with increasing concentrations of 2′-OMe inhibitors against the ACA45 sRNA. Firefly luciferase activity was normalized to Renilla activity. Error bars are derived from four individual experiments.

(B) Luciferase reporter constructs containing the 3′UTR of CDC2L6 or the CDC2L6 3′UTR with mutated ACA45 sRNA-binding sites were cotransfected with 2′-OMe inhibitors against the ACA45 sRNA. Firefly luciferase activity was normalized to Renilla activity. Error bars are derived from four individual experiments.

(data not shown). For experimental validation, we fused a number of 3′UTRs that we selected from the predicted target mRNAs to a luciferase reporter gene. Luciferase reporter constructs were cotransfected with 2′-OMe oligonucleotides antisense to the ACA45 small RNA. Many of the tested 3′UTRs, however, did not respond to the 2′-OMe inhibitors, suggesting that the small RNA does not target these mRNAs or that small RNA-target mRNA interactions are not relevant in the cell line that has been used (Figure 4A and data not shown). Strikingly, we found that activity of the luciferase reporter fused to the CDC2L6 (CDK11) 3′UTR is increased when the endogenous ACA45 small RNA is inhibited. The CDC2L6 gene product is a component of the mediator complex and, therefore, important for transcription (Conaway et al., 2005). For further validation of ACA45 sRNA effects on CDC2L6 expression, we mutated all predicted ACA45 sRNA-binding sites in the CDC2L6 3′UTR (Figures 4B and S1). Indeed, a luciferase reporter containing the mutated CDC2L6 3′UTR was not upregulated when endogenous ACA45 sRNA was inhibited (Figure 4B), indicating that ACA45 sRNA seed sequence matches are important for CDC2L6 expression.

In summary, our data demonstrate that ACA45 is processed to a small RNA that can function like a miRNA on the endogenous target CDC2L6, identifying the ACA45 sRNA as a potential transcriptional regulator in human cells.

Cellular snoRNAs with miRNA Processing Signatures

The intriguing finding that ACA45 can function like a miRNA prompted us to analyze processing of other snoRNAs. We generated small RNA libraries from human Ago1–4 complexes and mapped the sequence reads to snoRNAs (the detailed composition of the Ago1–4 libraries are currently analyzed and will be published elsewhere). We find reads originating from stem-loop structures within the snoRNAs ACA47, ACA36b, U92, HBI-100, ACA56, ACA3, and ACA50 (Figure 6). Both arms of the individual stems are present in the libraries, and the sequence with the lower abundance is indicated as “star” sequences in Table S1 (see also Tables S2 and S3 for individual snoRNA-derived sequence reads and read lengths). Our data obtained from larger sequencing data sets suggest that processing of snoRNAs to functional small RNAs is not unique to ACA45 and can be observed for other snoRNAs as well.

DISCUSSION

snoRNAs form a highly abundant class of noncoding RNAs in many different organisms. snoRNAs localize to the nucleolus and guide specific modifications of rRNAs or snRNAs (Matera et al., 2007). Moreover, snoRNAs have also been implicated in alternative splicing events (Kishore and Stamm, 2006). Here, we show that the snoRNA ACA45 is processed to a small RNA that can function like a miRNA. ACA45 processing is independent of the Drosha-containing microprocessor complex but requires Dicer. At least in vitro, Dicer can process the full-length ACA45, although it does not structurally represent a classical Dicer substrate. In northern blots, however, the strongest signal originates from the full-length ACA45, and only a minor portion is processed to a small miRNA-like RNA. This observation is consistent with the finding that ACA45 exists as a functional snoRNA that forms snoRNPs with the protein factor GAR-1 (Matera et al., 2007). Therefore, we propose a model in which ACA45 is transcribed and functions in the nucleolus of human cells (Figure 5). However, a minor portion is transported to the cytoplasm by a so far unknown export receptor. In the cytoplasm, Dicer immediately processes the full-length snoRNA to a miRNA-like small RNA that functions in gene silencing. This hypothesis is supported by our finding that recombinant Dicer, as well as Dicer-containing Ago protein complexes, are capable of generating ACA45 small RNAs in vitro. However, it cannot be excluded that other nucleases contribute to ACA45 processing in the cytoplasm. Alternatively, ACA45 is cleaved in the nucleus already, and one half is recognized as miRNA precursor by the miRNA pathway. However, such a scenario might be unlikely because a nuclear

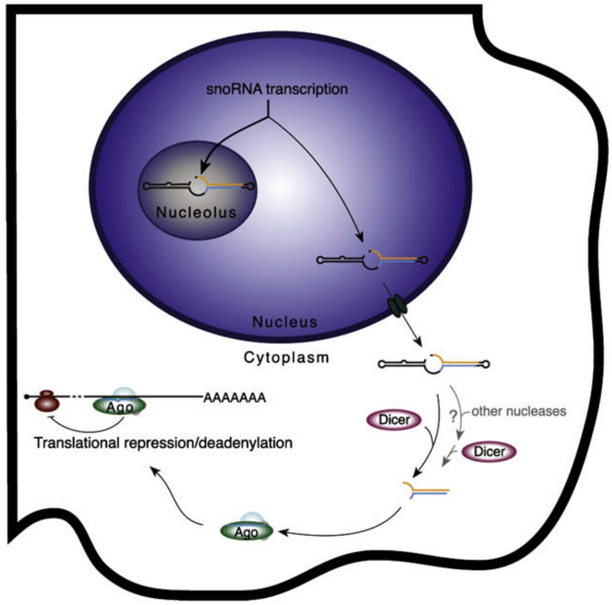


Figure 5. Model for ACA45 Processing and ACA45 sRNA Function in Human Cells
ACA45 snoRNA is transcribed in the nucleus, and the majority localizes to the nucleolus where it fulfills its specific functions by modifying other noncoding RNAs. However, a minor portion of ACA45 is exported to the cytoplasm, where Dicer, probably with the help of other nucleases, processes it to small RNAs that are specifically loaded into Ago protein-containing complexes. The ACA45-derived small RNA guides Ago protein complexes to partially complementary binding sites in the 3′UTR of target genes and represses its expression. AAAA, poly(A) tail.

cleavage activity might cleave the majority of the ACA45 pool, which is needed for classical snoRNA functions. Moreover, only one half of ACA45 would be exported by this model, although the other half folds like a typical miRNA precursor as well. Alternatively, a potential nuclear snoRNA cleavage activity could be physically separated from the snoRNAs as well. Further experiments aiming at the identification of specific snoRNA export pathways will help to elucidate the biogenesis pathways of small RNAs derived form snoRNAs.

Many small RNA cloning and sequencing projects have been carried out, but small RNAs derived from snoRNAs or other noncoding RNAs have not been reported. Here, we have immunoprecipitated endogenous Ago complexes, and it is very likely that small RNAs that associate with Ago proteins are functional RNA molecules rather than just degradation products. Most published cloning approaches size fractioned total RNA before cloning and, therefore, all unspecific degradation products are present in the libraries and it is difficult to find classes of functional small RNAs. Therefore, we suggest that cloning projects aiming at the identification of new classes of Ago-associated small noncoding RNAs of about 18–35 nt in length should be carried out from anti-Ago immunoprecipitations.

Using cloning and sequencing approaches, a variety of different snoRNA genes have been identified in the past (Bachellerie et al., 2002). However, many of these snoRNA candidates have not been characterized in detail, and it is unknown whether or not these candidates represent functional snoRNAs. Therefore, it is tempting to speculate that more snoRNAs are specifically processed to functional small RNAs. Indeed, by analyzing larger data sets, we find several small RNAs with miRNA-like processing signatures that originate from snoRNAs (Figure 6). These candidate sRNAs are derived from a subset of snoRNAs comprised of H/ACA snoRNAs and small Cajal body RNAs (scaRNAs), whose secondary structure is characterized by two hairpins linked by a hinge similar to ACA45 (Figure 2A). These findings support our hypothesis that a considerable number of snoRNAs are natural precursors for functional small RNAs. Moreover, we add another so far unrecognized function in post-transcriptional gene silencing to the list of snoRNA functions. A detailed functional characterization of all mammalian snoRNAs will help to elucidate the impact of snoRNA processing in RNA-guided gene silencing.

EXPERIMENTAL PROCEDURES

Ago Complex Purification

HEK293 cells were lysed in buffer containing 20 mM Tris HCl (pH 7.5), 150 mM NaCl, 0.25% NP-40, and 1.5 mM $MgCl_2$ and centrifuged at 10,000 × g for 10 min at 4°C.

For immunoprecipitation of endogenous Ago complexes, 100 μl protein G Sepharose (GE Healthcare) was washed with phosphate-buffered saline (PBS) and incubated with 10 ml anti-Ago1-4B8, anti-Ago2-11A9, anti-FLAG-3H3, or anti-GST at 4°C with gentle agitation overnight. After washes with PBS, beads were incubated with HEK293 cell lysate of 6 × 15 cm plates for 3 hr. Anti-Ago1-coated beads were extensively washed with 300 mM NaCl, 2.5 mM $MgCl_2$, 0.5% NP40, and 20 mM Tris-HCl (pH 7.5) followed by a wash with PBS. Anti-Ago2-coated beads were washed five times using RIPA buffer (50 mM Tris-HCl, 500 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS). RNA was isolated with 40 μg Proteinase K in 200 μl Proteinase K buffer (300 mM NaCl, 25 mM EDTA, 2% SDS, 200 mM Tris HCl [pH 7.5]) followed by Phenol/Chloroform extraction and Ethanol precipitation.

For immunoprecipitation of FLAG/HA-tagged Ago complexes, cell lysate from two 15 cm dishes were incubated with 20 μl FLAG M2 agarose beads (Sigma) for 2 hr at 4°C with rotation. Beads were extensively washed, and coimmunoprecipitated RNA was extracted as described above.

Small RNA Cloning

Small RNA cloning was carried out by Vertis Biotechnology (Weihenstephan, Germany) and has been described earlier (Tarasov et al., 2007). Without any size fractionation, extracted Ago-associated RNAs were poly(A)-tailed using poly(A) polymerase, and an adaptor was ligated to the 5′ phosphate of the miRNAs: (5′ end adaptor [43 nucleotides]: 5′-GCCTCCCTCGCGCCATCAG CTNNNNGACCTTGGCTGTCACTCA-3′). NNNN represents a “barcode” sequence. Next, first-strand cDNA synthesis was performed using an oligo(dT)-linker primer and M-MLV-RNase H reverse transcriptase (3′ end oligo [dT] linker primer [61 bases]: 5′-GCCTTGCCAGCCCGCTCAGACGAGACATCGCCCCG C$[T]_{25}$-3′). The resulting cDNAs were PCR amplified in 22 cycles using the high-fidelity Phusion polymerase (Finnzymes). The 120–135 bp amplification products were confirmed by polyacrylamide gel electrophoresis (PAGE) analysis. Both cDNAs pools were mixed in equal amounts and subjected to gel fractionation. The 120–135 bp fraction was electroeluted from 6% PAA-gels. After isolation with Nucleospin Extract II (Macherey and Nagel), cDNA pools were dissolved in 5 mM Tris/HCl (pH 8.5) with a concentration of 10 ng/μl and used in single-molecule sequencing. Massively parallel sequencing was performed by 454 Life Sciences (Branford, USA) using the Genome Sequencer 20 system as well as MWG Biotech (Germany). The complete sequencing data is available at the Gene Expression Omnibus (GEO, Accession number: GSE13370.)

A

type	ID	tot. # reads	“mature” seq. [# of reads]	“star” seq. [# of reads]	3' over -hang
scaRNA	ACA45	3516	aagguagauagaacaggucuugu [3405]	agaccuacuuaucuaccaacagc [55]	Y
scaRNA	ACA47	136	auuugcaguaacaggugugagc [107]	aucauguaugauacugcaaacag [6]	Y
scaRNA	U92	9	uaacggacagauacggggcagaca [5]	acugccuuuugaugaccgggacg [4]	Y
H/ACA Box RNA	HBI-100	591	uauggaggucucugucuggcuu [544]	ucugaucguuccccuccaua* [3]	Y
H/ACA Box RNA	ACA36B**	269	acuggcuagggaaaaugauugg [207]	uauucauuuauccccagccuaca [20]	N
H/ACA Box RNA	ACA56	102	agguggugaguucuucuguccagc [95]	ugguggcuuuagacuugccaga [2]	N
H/ACA Box RNA	ACA3	98	aucgaggcuagagucacgcuugg [87]	agugugcuagaguccucgaag [1]	Y
H/ACA Box RNA	ACA50	11	aagcacugccuuugaaccugaugu [8]	acgggccaagcaacagugcuaga [3]	Y (5nt)

B

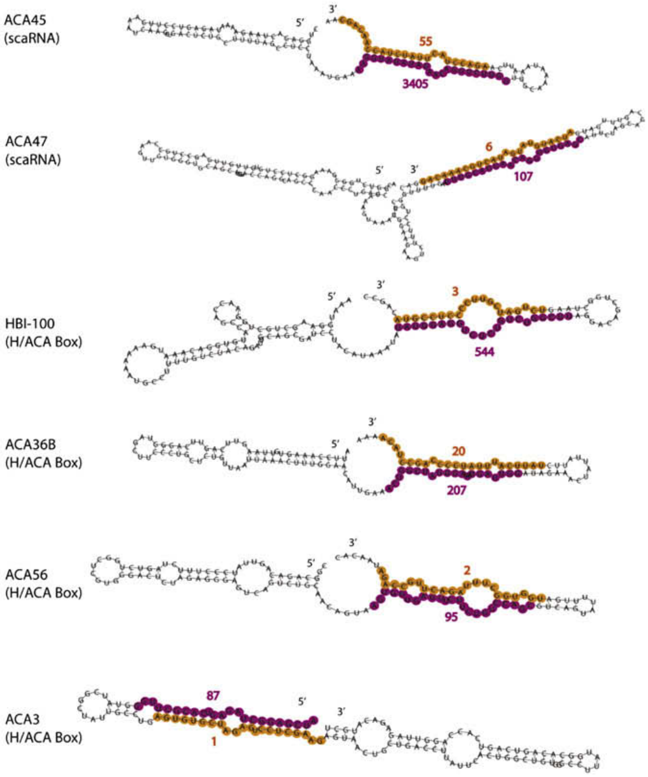


Figure 6. Several Human snoRNAs Show miRNA-Like Processing Signatures

(A) Small RNA reads originating from human snoRNAs that have been found in large sequencing data sets from Ago immunoprecipitates. The more abundant read is indicated as “mature,” and the complementary strand is indicated as “star” read. All reads that have been found for individual snoRNAs are indicated as “total reads.”

*The official genomic sequence is tctgatcgttcccctcc gta, but all of the reads mapping to this position have a mismatch, and all have “a” at position 18 and there is an annotated SNP at this position.

**The ACA36b sRNA candidate is identical to the annotated miRNA miR-664.

(B) Schematic representation of the secondary structure of full-length snoRNAs. Ago-associated reads are highlighted in purple and yellow.

RNA Cleavage Experiments

In vitro transcribed pri-27a substrate used in this study was described previously in Landthaler et al. (2004) and Meister et al. (2005). The template for pre-27a transcription was created by annealing the following primers: 5′-TTAATACGACTCACTATAGCTGAGGAGCAGGGCTTAGCTGCTTGTGAGCAGGGTCCACACCAAGTCGTGTTCACAGTGGCTAAGTTCCGCCCCCCAGC and 5′-GCTGGGGGGCGGAACTTAGCCACTGTGAACACGACTTGGTGTGGACCCTGCTCACAAGCAGCTAAGCCCTGCTCCTCAGCTATAGTGAGTCGTATTAA. ACA45 was cloned from genomic DNA using the primers 5′-ACGAGCTCCTGGAGACTAAGAAAATAGAGTCCTTGA and 5′-ACGGTACCTGCTGTTGGTAGATAAGTAGGTCTTGAA, digested with SacI and KpnI, and inserted into the SacI and KpnI restriction sites of the pBluescript. Plasmid was linearized using the KpnI restriction site and in vitro transcribed as described in Landthaler et al. (2004). The construction of human FLAG/HA-Ago1, FLAG/HA-Ago2, and FLAG/HA-Dicer was reported earlier (Meister et al., 2005). FLAG/HA-DGCR8 was purchased from Addgene.

Immunoprecipitations were performed as described above. For cleavage activity assays, 10 μl of Ago or Dicer complex-containing anti-FLAG beads were incubated in 20 μl PBS containing 5 mM ATP, 7.5 mM $MgCl_2$, 10 U/ml RNasin (Promega), and about 100 counts (~50 fmol) of internally labeled RNA for 1 hr at 37°C. The reaction was stopped by adding 200 μl proteinase K buffer (300 mM NaCl, 25 mM EDTA, 2% SDS, 200 mM Tris HCl [pH 7.5]) containing proteinase K (0.2 mg/ml). RNA was isolated with Phenol/Chloroform and analyzed by 8% or 12% denaturing RNA PAGE. Signals were detected by autoradiography.

Northern Blotting and Semiquantitative RT-PCR

Immunoprecipitated RNA and 30 μg total RNA isolated from HEK293 cells using Trifast (Peqlab) was separated by 12% denaturing RNA PAGE and transferred to a nylon membrane (GE Healthcare) by semidry electroblotting. Membranes were crosslinked by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) chemical crosslink incubating for 1 hr at 50°C, prehybridized for 1 hr, and hybridized overnight at 50°C with probes complementary to snoRNA ACA45 or tRNA. The following probes have been used: 5′-AAGACCTGTTCTATCTACCT complementary to snoRNA ACA45 and 5′-CTGATGCTCTACCGACTGAGCTATCCGGGC complementary to lysine tRNA. After hybridization, membranes were washed twice 10 min with 5 × SSC and once 10 min with 1 × SSC. Radioactive signals were detected by exposure of BioMax MS film (Kodak) using an intensifying screen (GE Healthcare).

For semiquantitative RT-PCR, extracted RNA was modified by addition of poly(A) tail using poly(A) tailing kit from Ambion. Reverse transcription was performed using the cDNA synthesis kit (Fermentas) with the universal RT primer 5′ AACGAGACGACGACAGACTTTTTTTTTTTTTTTT (described in Hurteau et al. [2006]). DNA was amplified using Mesa Green qPCR MasterMix Plus (Eurogentec), a universal reverse primer identical to the 18 bp tag added during the RT step and the following specific primers: 5′-AAGGUAGAUAGAACAGGUCUUG for ACA45, 5′-TCCCTGAGACCCTAACTTGTGA for miR-125b, and 5′-ACACATCCAAATGAGGCG for 7SK. The PCR products were analyzed by 4% agarose gel electrophoresis.

Conserved Processing of ACA45

The human ACA45 sequence was obtained at the snoRNABase (http://www-snorna.biotoul.fr/). The ACA45 mouse, rat, and dog homologs were identified by mapping the human sequence against each genome, retaining only unambiguous matches. Subsequently, a number of deep sequencing data sets were mapped to the ACA45 homologs. Each data set was mapped to the homolog of the species from which the data set originated, and only perfect matches were retained. The human data consisted of the data sets produced for this study using the 454 Life Sciences technology, as well as a data set produced by deep sequencing the small RNA fraction of HeLa cells using the Solexa/Illumina technology (GEO accession number GSE10829) (Friedländer et al., 2008). The mouse data sets were produced by deep sequencing small RNAs from mouse brain and kidney tissues using the 454 technology (unpublished data). The rat data set was produced by deep sequencing column-purified small RNAs from testes extracts using the 454 technology (GEO accession number GSE5026) (Lau et al., 2006). The dog data set was produced by sequencing small RNAs from dog lymphocytes using the Solexa technology (GEO accession number GSE10825) (Friedlander et al., 2008).

Computational Methods

A total of 64733 reads was obtained by deep sequencing the RNA that immunoprecipitated with Ago1 and Ago2. Of this, 20834 belonged to the Ago1 set and 43899 to the Ago2 set. Upon removal of adapters, the sequences shorter than 17 nt were discarded, resulting in 20448 and 42604 reads in Ago1 and Ago2 sets, respectively. These reads were mapped to human genome (hg 18, UCSC database [Karolchik et al., 2003]) using NCBI blastn (Altschul et al., 1990) with the minimum word length set to 7. The mapping with the best E value was associated with each read. The only mismatches allowed were the first nt at the 5′ end or the last three nt at the 3′ end of the read. In case a read mapped with the same E value to several locations, they were all taken into consideration. The genomic loci of best matches were annotated using the tables from UCSC database (Karolchik et al., 2003). A read was annotated as a DNA repeat (including LINE, SINE, LTR) only if the genomic locus it mapped to had no other annotation.

For purposes of identification of known and novel miRNAs, reads from the Ago1 and Ago2 libraries were combined and mapped to the human genome using NCBI megablast with the following options: −W 12 −p 100. Only perfect mappings (full length, 100% identity) were retained. These were used as input to miRDeep, an algorithm designed for the discovery of Dicer substrates such as miRNAs from deep sequencing data (Friedländer et al., 2008). The algorithm intersects the mappings with local genomic sequence to identify potential Dicer hairpin substrates. These are then scored according to the distribution of positions and frequencies of the reads mapped to the individual hairpin, using Bayesian statistics. The energetics and stability of the hairpins and the cross-species conservation of the seed sequence also contribute to the score. Human snoRNA sequences were downloaded from snoRNABase (Lestrade and Weber, 2006).

To map the total of 17362367 sequence reads obtained by sequencing Ago1–4 IP using Solexa technology to the genome, we used the locally developed suffix array-based tool (to be published elsewhere). Candidate snoRNAs with miRNA-like processing were selected (Table S1) if the combined Ago1–4 data set contained reads mapping to both strands of a hairpin and if these reads represented more than 85% of all reads mapping to a given snoRNA.

SUPPLEMENTAL DATA

The Supplemental Data include Supplemental Experimental Procedures, one figure, and three tables and can be found with this article at http://www.molecule.org/supplemental/S1097-2765(08)00733-8.

ACKNOWLEDGMENTS

We are grateful to Sabine Rottmüller and Bernd Haas for technical support, Vertis Biotechnologie AG (Weihenstephan, Germany) for small RNA cloning, and MWG biotech (Munich, Germany) for deep sequencing. We thank Sihem Cheloufi and Greg Hannon for providing RNA from Dicer$^{-/-}$ as well as Dicer$^{-/+}$ cells and Witold Filipowicz for the anti-GAR-1 antibody. Our research is supported by the Max Planck Society. This work was supported, in part, by EU grant LSHG-CT-2006-037900 (SIROCCO; G.M.) and the Deutsche Forschungsgemeinschaft (Me 2064/2-1 to G.M.).

Received: August 18, 2008
Revised: October 16, 2008
Accepted: October 27, 2008
Published: November 20, 2008

REFERENCES

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. (1990). Basic local alignment search tool. J. Mol. Biol. *215*, 403–410.

Aravin, A.A., Sachidanandam, R., Girard, A., Fejes-Toth, K., and Hannon, G.J. (2007). Developmentally regulated piRNA clusters implicate MILI in transposon control. Science *316*, 744–747.

Azuma-Mukai, A., Oguri, H., Mituyama, T., Qian, Z.R., Asai, K., Siomi, H., and Siomi, M.C. (2008). Characterization of endogenous human Argonautes and their miRNA partners in RNA silencing. Proc. Natl. Acad. Sci. USA *105*, 7964–7969.

Bachellerie, J.P., Cavaille, J., and Huttenhofer, A. (2002). The expanding snoRNA world. Biochimie *84*, 775–790.

Beitzinger, M., Peters, L., Zhu, J.Y., Kremmer, E., and Meister, G. (2007). Identification of human microRNA targets from isolated argonaute protein complexes. RNA Biol. *4*, 76–84.

Bernstein, E., Caudy, A.A., Hammond, S.M., and Hannon, G.J. (2001). Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature *409*, 363–366.

Bohnsack, M.T., Czaplinski, K., and Gorlich, D. (2004). Exportin 5 is a RanGTP-dependent dsRNA-binding protein that mediates nuclear export of pre-miRNAs. RNA *10*, 185–191.

Borchert, G.M., Lanier, W., and Davidson, B.L. (2006). RNA polymerase III transcribes human microRNAs. Nat. Struct. Mol. Biol. *13*, 1097–1101.

Chen, K., and Rajewsky, N. (2007). The evolution of gene regulation by transcription factors and microRNAs. Nat. Rev. Genet. *8*, 93–103.

Conaway, R.C., Sato, S., Tomomori-Sato, C., Yao, T., and Conaway, J.W. (2005). The mammalian Mediator complex and its role in transcriptional regulation. Trends Biochem. Sci. *30*, 250–255.

Denli, A.M., Tops, B.B., Plasterk, R.H., Ketting, R.F., and Hannon, G.J. (2004). Processing of primary microRNAs by the Microprocessor complex. Nature *432*, 231–235.

Filipowicz, W., Jaskiewicz, L., Kolb, F.A., and Pillai, R.S. (2005). Post-transcriptional gene silencing by siRNAs and miRNAs. Curr. Opin. Struct. Biol. *15*, 331–341.

Friedländer, M.R., Chen, W., Adamidi, C., Maaskola, J., Einspanier, R., Knespel, S., and Rajewsky, N. (2008). Discovering microRNAs from deep sequencing data using miRDeep. Nat. Biotechnol. *26*, 407–415.

Gregory, R.I., Yan, K.P., Amuthan, G., Chendrimada, T., Doratotaj, B., Cooch, N., and Shiekhattar, R. (2004). The Microprocessor complex mediates the genesis of microRNAs. Nature *432*, 235–240.

Gregory, R.I., Chendrimada, T.P., Cooch, N., and Shiekhattar, R. (2005). Human RISC couples microRNA biogenesis and posttranscriptional gene silencing. Cell *123*, 631–640.

Grishok, A., Pasquinelli, A.E., Conte, D., Li, N., Parrish, S., Ha, I., Baillie, D.L., Fire, A., Ruvkun, G., and Mello, C.C. (2001). Genes and mechanisms related to RNA interference regulate expression of the small temporal RNAs that control *C. elegans* developmental timing. Cell *106*, 23–34.

Hurteau, G.J., Spivack, S.D., and Brock, G.J. (2006). Potential mRNA degradation targets of hsa-miR-200c, identified using informatics and qRT-PCR. Cell Cycle *5*, 1951–1956.

Hutvágner, G., McLachlan, J., Pasquinelli, A.E., Bálint, E., Tuschl, T., and Zamore, P.D. (2001). A cellular function for the RNA interference enzyme Dicer in small temporal RNA maturation. Science *293*, 834–838.

Karolchik, D., Baertsch, R., Diekhans, M., Furey, T.S., Hinrichs, A., Lu, Y.T., Roskin, K.M., Schwartz, M., Sugnet, C.W., Thomas, D.J., et al. (2003). The UCSC Genome Browser Database. Nucleic Acids Res. *31*, 51–54.

Kishore, S., and Stamm, S. (2006). The snoRNA HBII-52 regulates alternative splicing of the serotonin receptor 2C. Science *311*, 230–232.

Kiss, A.M., Jady, B.E., Bertrand, E., and Kiss, T. (2004). Human box H/ACA pseudouridylation guide RNA machinery. Mol. Cell. Biol. *24*, 5797–5807.

Krek, A., Grun, D., Poy, M.N., Wolf, R., Rosenberg, L., Epstein, E.J., MacMenamin, P., da Piedade, I., Gunsalus, K.C., Stoffel, M., and Rajewsky, N. (2005). Combinatorial microRNA target predictions. Nat. Genet. *37*, 495–500.

Landthaler, M., Yalcin, A., and Tuschl, T. (2004). The human DiGeorge syndrome critical region gene 8 and its D. melanogaster homolog are required for miRNA biogenesis. Curr. Biol. *14*, 2162–2167.

Lau, N.C., Seto, A.G., Kim, J., Kuramochi-Miyagawa, S., Nakano, T., Bartel, D.P., and Kingston, R.E. (2006). Characterization of the piRNA complex from rat testes. Science *313*, 363–367.

Lee, Y., Ahn, C., Han, J., Choi, H., Kim, J., Yim, J., Lee, J., Provost, P., Radmark, O., Kim, S., and Kim, V.N. (2003). The nuclear RNase III Drosha initiates microRNA processing. Nature *425*, 415–419.

Lee, Y., Kim, M., Han, J., Yeom, K.H., Lee, S., Baek, S.H., and Kim, V.N. (2004). MicroRNA genes are transcribed by RNA polymerase II. EMBO J. *23*, 4051–4060.

Lestrade, L., and Weber, M.J. (2006). snoRNA-LBME-db, a comprehensive database of human H/ACA and C/D box snoRNAs. Nucleic Acids Res. *34*, D158–D162.

Lim, L.P., Lau, N.C., Weinstein, E.G., Abdelhakim, A., Yekta, S., Rhoades, M.W., Burge, C.B., and Bartel, D.P. (2003). The microRNAs of *Caenorhabditis elegans*. Genes Dev. *17*, 991–1008.

Liu, J., Carmell, M.A., Rivas, F.V., Marsden, C.G., Thomson, J.M., Song, J.J., Hammond, S.M., Joshua-Tor, L., and Hannon, G.J. (2004). Argonaute2 is the catalytic engine of mammalian RNAi. Science *305*, 1437–1441.

Lund, E., Guttinger, S., Calado, A., Dahlberg, J.E., and Kutay, U. (2004). Nuclear export of microRNA precursors. Science *303*, 95–98.

Ma, J.B., Yuan, Y.R., Meister, G., Pei, Y., Tuschl, T., and Patel, D.J. (2005). Structural basis for 5′-end-specific recognition of guide RNA by the A. fulgidus Piwi protein. Nature *434*, 666–670.

Maniataki, E., and Mourelatos, Z. (2005). A human, ATP-independent, RISC assembly machine fueled by pre-miRNA. Genes Dev. *19*, 2979–2990.

Matera, A.G., Terns, R.M., and Terns, M.P. (2007). Non-coding RNAs: lessons from the small nuclear and small nucleolar RNAs. Nat. Rev. Mol. Cell Biol. *8*, 209–220.

Meister, G., and Tuschl, T. (2004). Mechanisms of gene silencing by double-stranded RNA. Nature *431*, 343–349.

Meister, G., Landthaler, M., Patkaniowska, A., Dorsett, Y., Teng, G., and Tuschl, T. (2004). Human Argonaute2 mediates RNA cleavage targeted by miRNAs and siRNAs. Mol. Cell *15*, 185–197.

Meister, G., Landthaler, M., Peters, L., Chen, P.Y., Urlaub, H., Luhrmann, R., and Tuschl, T. (2005). Identification of novel argonaute-associated proteins. Curr. Biol. *15*, 2149–2155.

Mourelatos, Z., Dostie, J., Paushkin, S., Sharma, A., Charroux, B., Abel, L., Rappsilber, J., Mann, M., and Dreyfuss, G. (2002). miRNPs: a novel class of ribonucleoproteins containing numerous microRNAs. Genes Dev. *16*, 720–728.

Murchison, E.P., Partridge, J.F., Tam, O.H., Cheloufi, S., and Hannon, G.J. (2005). Characterization of Dicer-deficient murine embryonic stem cells. Proc. Natl. Acad. Sci. USA *102*, 12135–12140.

Parker, J.S., and Barford, D. (2006). Argonaute: a scaffold for the function of short regulatory RNAs. Trends Biochem. Sci. *31*, 622–630.

Parker, J.S., Roe, S.M., and Barford, D. (2005). Structural insights into mRNA recognition from a PIWI domain-siRNA guide complex. Nature *434*, 663–666.

Patel, D.J., Ma, J.B., Yuan, Y.R., Ye, K., Pei, Y., Kuryavyi, V., Malinina, L., Meister, G., and Tuschl, T. (2006). Structural biology of RNA silencing and its functional implications. Cold Spring Harb. Symp. Quant. Biol. *71*, 81–93.

Peters, L., and Meister, G. (2007). Argonaute proteins: mediators of RNA silencing. Mol. Cell *26*, 611–623.

Pillai, R.S., Bhattacharyya, S.N., and Filipowicz, W. (2007). Repression of protein synthesis by miRNAs: how many mechanisms? Trends Cell Biol. *17*, 118–126.

Rudel, S., Flatley, A., Weinmann, L., Kremmer, E., and Meister, G. (2008). A multifunctional human Argonaute2-specific monoclonal antibody. RNA *14*, 1244–1253.

Seto, A.G., Kingston, R.E., and Lau, N.C. (2007). The coming of age for Piwi proteins. Mol. Cell *26*, 603–609.

Tarasov, V., Jung, P., Verdoodt, B., Lodygin, D., Epanchintsev, A., Menssen, A., Meister, G., and Hermeking, H. (2007). Differential regulation of microRNAs by p53 revealed by massively parallel sequencing: miR-34a is a p53 target that induces apoptosis and G1-arrest. Cell Cycle *6*, 1586–1593.

Tolia, N.H., and Joshua-Tor, L. (2007). Slicer and the argonautes. Nat. Chem. Biol. *3*, 36–43.

Tomari, Y., and Zamore, P.D. (2005). Perspective: machines for RNAi. Genes Dev. *19*, 517–529.