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Characterization of protein and small RNA components of Argonaute complexes

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Erklärung

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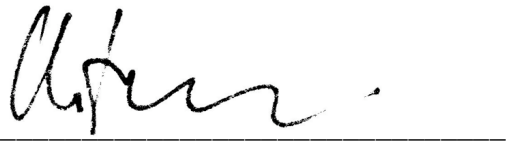

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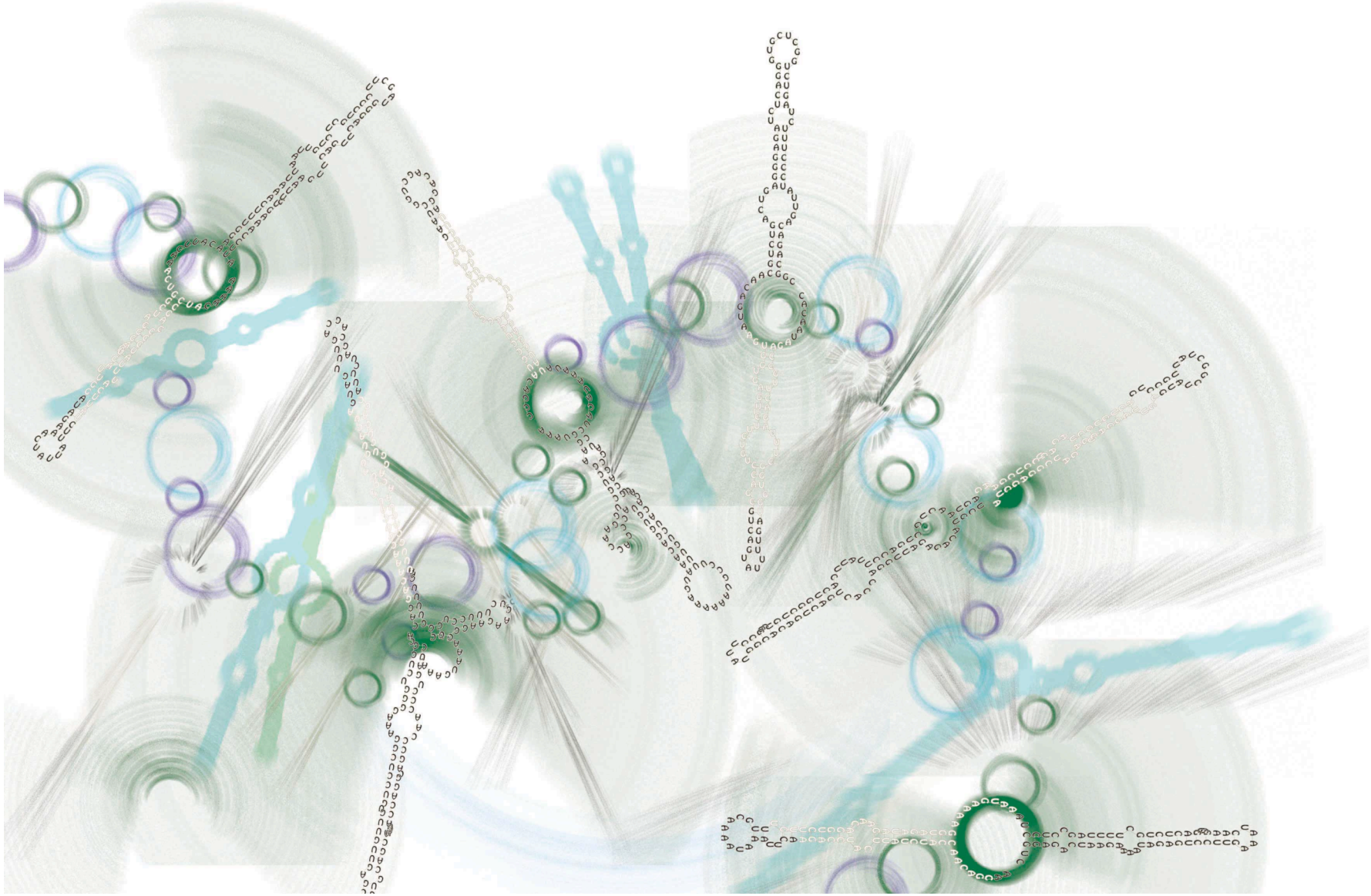


Summary

Members of the Argonaute (Ago) protein family associate with small RNAs and regulate gene expression at the level of transcription, mRNA stability or translation. Although a number of small RNAs and proteins involved in gene silencing have been identified so far, the spectrum of Ago-mediated silencing implies an interplay of various additional factors. We therefore purified Ago complexes from human cells in order to analyze their protein as well as their RNA content. We found that Ago1 and Ago2 reside in three complexes of different sizes, showing distinct Dicer and RNA-induced silencing complex (RISC) activities. The smallest complex is formed by multiple subcomplexes with distinct enzymatic activities. The two larger complexes form large ribonucleoprotein particles (RNPs) carrying a variety of different RNA binding or regulatory proteins, providing new insights into the function of Ago proteins.

The second part of this work focuses on the identification of small RNAs associated with human Ago1 and Ago2. Endogenous Ago proteins were purified, followed by small RNA cloning and large-scale sequencing. The majority of the cloned RNAs belong to the class of miRNAs. Interestingly, individual read numbers suggest preferential Ago binding for at least some miRNAs. Besides miRNAs, we found RNA fragments mapping to mRNAs, transposons and other non-coding RNAs like tRNAs. Moreover, small RNAs with a length of about 20-22 nucleotides originating from snoRNAs have been found. The most prominent sequence read mapping to snoRNA H/ACA45 is capable of guiding Ago2-mediated cleavage of complementary target RNAs. Furthermore, processing of H/ACA45 is Drosha-independent but depending on Dicer. Remarkably, the seed sequence of the H/ACA45 processing product is perfectly complementary in the 3' UTR of several mRNAs. We could demonstrate that H/ACA45-derived small RNAs possess an endogenous mRNA target that is translationally repressed. Together with the finding that many snoRNA-derived reads from various deep sequencing libraries are conserved in mammals, the data suggest that snoRNA processing is a more general mechanism and snoRNA-derived small RNAs can act as miRNAs.

1 Introduction

1.1 RNA silencing, a historical overview

Less than 2% of the human genome is translated into proteins but far more is transcribed into RNA. These non-coding RNAs (ncRNAs) act as important regulators of diverse cellular processes. Transfer RNAs (tRNAs) are decoding genetic information into proteins, ribosomal RNAs (rRNAs) form part of the ribosome and recognize portions of mRNAs and tRNAs, small nuclear RNAs (snRNAs) are involved in splicing and small nucleolar RNAs (snoRNAs) play a role in the biogenesis of other RNAs. Moreover, there are classes of ncRNAs downregulating gene expression, a phenomena which is also termed RNA silencing.

RNA silencing was first described in 1990 by Napoli and Jorgensen (Napoli et al., 1990). They overexpressed a pigment synthesis enzyme responsible for the deep violet coloration in petunia flowers and unexpectedly got white flowers in the end. Because both the transgene and the endogenous gene were repressed, this phenomenon was termed “cosuppression”. Two years later Romano and Macino described a similar phenomenon in *Neurospora crassa* where it was named “quelling” (Romano and Macino, 1992). In animals, RNA silencing was first documented by Guo and Kemphues (Guo and Kemphues, 1995). They found that introduction of both sense and antisense RNA resulted in degradation of par-1 mRNA in *Caenorhabditis elegans*. A major breakthrough was achieved in 1998 by Fire and Mello who reported that not single-stranded (ss) but double-stranded (ds) RNA was the trigger for gene silencing (Fire et al., 1998). They explained that integrated transgenes were responsible for the production of dsRNA in plants and fungi and contamination of sense RNA by dsRNA in worms. During the following years it was shown in plants and *Drosophila* extracts that dsRNA is converted into shorter intermediates of about 22 nt in length (Hamilton and Baulcombe, 1999; Hammond et al., 2000; Zamore et al., 2000). These small RNAs, which could induce cleavage of homologous target mRNAs were termed small interfering RNAs (siRNAs) and the phenomenon itself RNA interference (RNAi). Long dsRNA has not been found to induce RNAi in mammalian cell culture. However, this could be explained by the observation that long dsRNA binds and activates the protein kinase R (PKR) which in turn activates the interferon pathway, leading to a shut down of protein biosynthesis and may result in apoptosis (Kaufman, 1999). The major breakthrough was achieved by Tuschl and coworkers who could show that 21 nt siRNA duplexes specifically induce RNAi in mammalian

cells (Elbashir et al., 2001). This finding and the potential of RNAi to suppress any gene make it particularly attractive for gene therapy approaches (Grimm and Kay, 2007).

During the time of the discovery of RNA silencing, another class of small RNAs was discovered in *C. elegans*. They were found to be important for the developmental timing of worms and therefore called small temporal RNA (stRNA). The first stRNA was described in 1993 by the labs of Ambros and Ruvkun (Lee et al., 1993; Wightman et al., 1993). They proposed that the stRNA lin-4 regulates lin-14 mRNA by imperfect base pairing in the 3' untranslated region (3' UTR). It took another seven years to discover a second stRNA in worms, let-7 (Pasquinelli et al., 2000; Reinhart et al., 2000; Slack et al., 2000). Following the discovery of stRNAs, several labs identified numerous 22 nt small RNAs from different organisms not only implicated in developmental timing (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001; Llave et al., 2002). Therefore, the new class of evolutionary conserved RNAs with stRNAs as founding members was termed microRNAs (miRNAs). Currently, at least a thousand miRNAs are predicted to function in humans and new classes of small RNAs are constantly identified by new sequencing technologies.

Although similar in lengths, no formal connection between siRNAs and miRNAs was made until 2001 where it was shown that long dsRNA are converted into siRNAs and miRNA precursors (pre-miRNAs) into mature miRNAs by the same RNAse III enzyme (Bernstein et al., 2001; Grishok et al., 2001; Hutvágner et al., 2001; Ketting et al., 2001; Knight and Bass, 2001). Today it is known that both miRNAs and siRNAs share a number of similar proteins for their biogenesis and function.

1.2 Classes of small RNAs in gene silencing

Small ncRNAs involved in gene silencing are of about 20-30 nt in length and associated with a member of the Argonaute (Ago) protein family. Although many classes of small RNAs have been discovered, they are divided into three main categories: miRNAs, siRNAs and Piwi-interacting RNAs (piRNAs), all of them present in eukaryotes (Carthew and Sontheimer, 2009).

MiRNAs are involved in the regulation of diverse cellular processes and changes in their expression pattern are linked to diseases, in particular cancer (Alvarez-Garcia, 2005; Esquela-Kerscher and Slack, 2006). They are of about 22 nt in length and are processed from endogenously transcribed precursors. Associated with an effector protein, they can lead to endonucleolytic cleavage, translational inhibition and/or deadenylation of a target mRNA.

Figure 1.1. Overview of small RNAs

Name	Lengh in nt	Organism	Function
miRNA	20-25	Plants, algae, animals, viruses, protists	Translational inhibition, mRNA destabilization
exo-siRNAs	~ 21 (24)	Plants, Animals, fungi, protists	Posttranscriptional silencing, antiviral defense
endo-siRNA	~ 21	Plants, algae, animals, fungi protists	Post-transcriptional and transcriptional silencing of transcripts and transposons
casiRNA, hcRNAs rasiRNA	24	mainly in plants	Chromatin modification
tasiRNA	21	plants	Post-transcriptional regulation
natsiRNA	21-24	plants	Regulation of stress response genes
piRNA	24-31	Metazoans	Transposon silencing
rasiRNA	24-28	Drosophila, zebrafisch	Transposon silencing

Overview of different classes of small RNAs. Many laboratories have identified small RNAs involved in gene silencing in different approaches and different organisms. Therefore, the nomenclature of different subgroups can vary in the literature. Some groups have several names or are overlapping with other groups, as also described in the text.

SiRNAs are about 21 nt in length and generally lead to endonucleolytic cleavage of perfect complementary RNA targets in association with an effector protein. They can be derived from long dsRNA from exogenous sources (exo-siRNAs) or from natural sense-antisense pairs (endo-siRNAs). Depending on their origin and mechanism of action, there are several subgroups of endo-siRNAs (Fig. 1.1). Cis-acting RNAs (casiRNAs) are guiding DNA methylation and histone modification at their loci of origin. (Chan et al., 2004; Ghildiyal and Zamore, 2009; Hamilton et al., 2002; Llave et al., 2002; Mette et al., 2000; Tran et al., 2005; Xie et al., 2004; Zilberman et al., 2003). Some of them are also refered to as heterochromatic small RNAs (hcRNAs) or fall into a class of repeat-associated RNAs (rasiRNAs) (Farazi et al., 2008). Trans-acting siRNAs (tasiRNAs) are generated by the overlap of miRNA and siRNA pathways. (Ghildiyal and Zamore, 2009; Vazquez et al., 2004). Natural antisense transcript-derived siRNAs (natsiRNAs) originate from stress-induced transcription resulting in production of dsRNA and subsequent RNA cleavage (Borsani et al., 2005; Ghildiyal and Zamore, 2009; Katiyar-Agarwal et al., 2006).

PiRNAs, the third main category of small RNAs involved in gene silencing are primarily found in animals. They are restricted to the germ line, where they are important for germ line development (Ghildiyal and Zamore, 2009). Their biogenesis and function are still poorly understood. In contrast to miRNAs and siRNAs, their precursors are single-stranded and in genomic clusters. Moreover, they bind to a different subfamily of effector proteins. A subset of rasiRNAs cloned from Drosophila and zebrafish are thought to belong to the piRNA class as they are associated with the same protein subfamily.

1.3 Biogenesis of miRNAs and siRNAs

Many miRNA loci are in close proximity to each other and transcribed from a polycistronic transcription unit. MiRNAs can derive from coding or non-coding genes and from intronic or exonic regions (Calin et al., 2002; Rodriguez et al., 2004; Saini et al., 2007). They are generally transcribed from RNA polymerase II (Pol II). The transcripts are capped and polyadenylated and can be regulated like other Pol II transcripts (Cai et al., 2004; Lee et al., 2004a).

The stem-loop structured primary miRNA transcripts (pri-miRNAs) are processed in the nucleus by proteins that form the so-called microprocessor complex, containing the RNAse III enzyme Drosha and its cofactor DGCR8 (Figure 1.2). The transcripts are cleaved at the stem of the hairpin structure releasing a 70 nt long miRNA precursor (pre-miRNA) (Lee et al., 2003). Cleavage products have a 5' phosphate and a 2 nt 3' overhang, characteristic for RNAse III cleavage. However, this is not the only way to generate pre-miRNAs. An alternative pathway exists for miRNAs residing in introns that precisely have the size and feature of a pre-miRNA (Figure 1.2). The small RNAs derived from short intronic hairpins are called mirtrons and their precursors are directly liberated by splicing (Berezikov et al., 2007; Okamura et al., 2007; Ruby et al., 2007). Some mirtrons contain 5' or 3' tails and might require exonucleolytic trimming besides splicing.

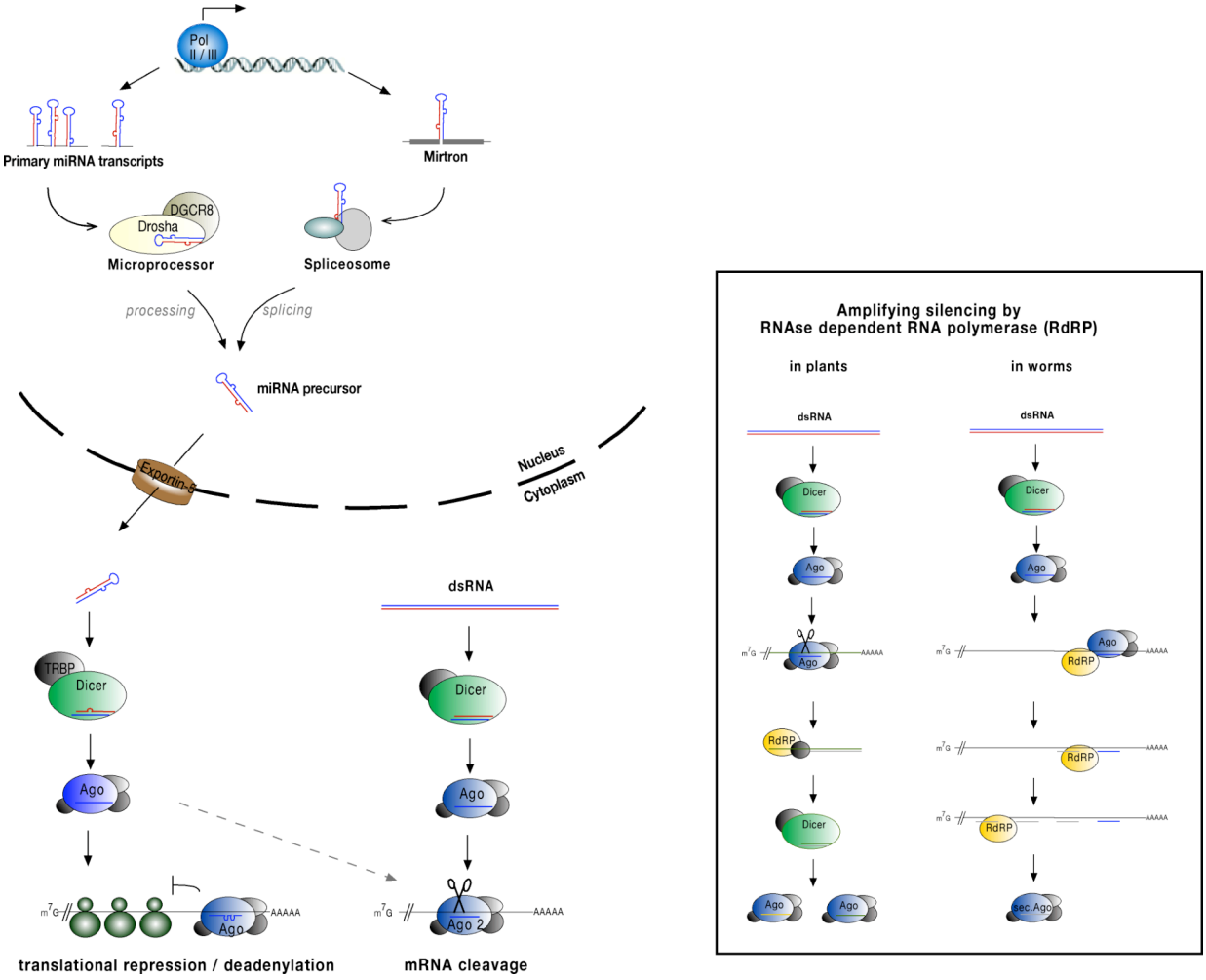
After the first processing step, pre-miRNAs are exported into the cytoplasm by Exportin-5, a member of the nuclear export receptor family first identified as export factor for tRNAs (Bohnsack et al., 2004; Calado et al., 2002; Lund et al., 2004; Yi et al., 2005). Nuclear binding of a pre-miRNA by Exportin-5 requires the GTP-bound form of the cofactor Ran and releases its cargo in the cytoplasm upon hydrolysis of Ran-GTP to Ran-GDP. In the cytoplasm, pre-miRNAs are processed by the RNAse III enzyme Dicer and its partners TRBP and probably PACT into small dsRNA of about 22 nt in length with a 5' phosphate and a 2 nt 3' overhang (Figure 1.2), (Bernstein et al., 2001; Chendrimada et al., 2005; Grishok et al., 2001; Haase et al., 2005;

Hutvágner et al., 2001; Ketting et al., 2001; Knight and Bass, 2001; Lee et al., 2006). The small RNA duplex gives rise to a mature miRNA sequence whereas the opposing strand, the so-called miRNA* sequence, is thought to be degraded. The mature miRNAs is incorporated into protein complexes and guide them to specific mRNAs target sites. Perfect complementarity between the small RNA and its target lead endonucleolytic mRNA cleavage whereas imperfect base-pairing induces translational inhibition and/or deadenylation (Fig. 1.2).

MiRNAs and siRNAs both associate with the same effector proteins and show similarities in their processing pathways. However, in contrast to miRNAs, siRNA are processed independent of Drosha from long dsRNA from exogenous sources or natural sense-antisense pairs, (Fig. 1.2). Depending on the organism, exogenous siRNAs can be derived by cleavage of viral RNAs, by introducing long, perfectly base-paired dsRNA into the cytoplasm or by direct uptake from the environment. (Mello and Conte, 2004). In plants and worms, siRNAs do not need to be double-stranded because of the help of the RNA-dependent RNA polymerase (RdRP). In contrast to typical RNA polymerases they use RNA templates instead of DNA. In plants, RdRPs use mRNA fragments previously cleaved by primary siRNAs as template to synthesize long dsRNA (Fig. 1.2, Box). The newly synthesized dsRNA can then be further processed into secondary siRNAs (Bouché et al., 2006; Deleris et al., 2006; Fusaro et al., 2006; Ghildiyal and Zamore, 2009; Vaistij et al., 2002; Xie et al., 2004). There is slightly different mechanism in worms: primary siRNAs are directly guided to the mRNA target; however, this does not induce mRNA cleavage but leads to the recruitment of RdRP and subsequent synthesis of secondary siRNAs (Fig. 1.2, Box). In contrast to plant secondary siRNAs, they have a 5’ diphosphate or triphosphate, indicating that they are produced by transcription rather than by Dicer cleavage (Aoki et al., 2007; Ghildiyal and Zamore, 2009; Pak and Fire, 2007; Sijen et al., 2001; Sijen et al., 2007; Smardon et al., 2000).

Besides exo-siRNAs there are also endo-siRNAs, first discovered in plants and *C. elegans* (Ambros et al., 2003b; Hamilton et al., 2002; Mello and Conte, 2004; Zilberman et al., 2003). The production of endo-siRNAs often involves RdRP activity. However, endo-siRNAs have recently been found in animals such as flies and mice, where a gene encoding for RdRP is absent. These endo-siRNAs are derived from transposable elements, natural antisense transcripts, long intermolecular paired hairpins and pseudogenes. (Chung et al., 2008; Czech et al., 2008; Ghildiyal et al., 2008; Kawamura et al., 2008; Okamura et al., 2008a; Okamura et al., 2008b; Yang and Kazazian, 2006).

Figure 1.2. Overview of the biogenesis pathway of miRNAs and siRNAs



On the left, an overview of the miRNA and siRNA pathway is shown. The box on the right shows amplified silencing in plants and in worms. A detailed description of these processes is found in the text, chapter 1.3, Biogenesis of miRNA and siRNAs. Sec. Ago means Ago protein with secondary siRNAs.

1.3.1 The RNAse III enzymes Drosha and Dicer

Two RNAse III enzymes are involved in small RNA maturation: Drosha and Dicer. RNAse III enzymes are grouped into three classes. Bacterial and yeast RNAse III proteins belong to class I, having one RNAse III domain (RIIID) and a double-stranded RNA binding domain (dsRBD). Drosha is a prominent member of class II, with two RIIIDs and a dsRBD, whereas Dicer belongs to class III possessing additional domains such as an N-terminal DExD/H-box helicase domain, a domain of unknown function (DUF) and a PAZ domain (MacRae and Doudna, 2007).

Drosha is a protein of about 130-160 kDa, only found in animals. It functions as monomer forming an intramolecular dimer structure with its two RNAse III domains, recognizing the 3' and 5' end of the pri-miRNA stem (Han et al., 2006; Han et al., 2004). Drosha cleavage generates 5' phosphates and 2 nt 3' overhangs which are recognized efficiently by Exportin-5 and Dicer. As its dsRBD is not sufficient for substrate binding Drosha alone can not cleave pri-miRNAs but needs its partner DGCR8, also known as Pasha (partner of Drosha) in *C. elegans* and *D. melanogaster*. DGCR8 interacts with Drosha through its C-terminal domain forming a complex called the microprocessor complex. DGCR8 is about 120 kDa in size, contains two dsRBDs recognizing dsRNA-ssRNA junctions and can position the ribonuclease domain of Drosha eleven nt away from the junction (Han et al., 2006). DGCR8 also interacts with the about 33 bp long stem and the terminal loop of the pri-miRNA. Therefore DGCR8 is responsible for substrate recognition and determines the correct cleavage position for Drosha. Moreover, it enhances the stability of its partner Drosha. In turn, DGCR8 is negatively regulated by the microprocessor complex itself through mRNA cleavage in the 5' untranslated region and the coding sequence. As a consequence, the expression of DGCR8 is reduced when sufficient microprocessor activity is available (Han et al., 2009). Besides the cross-regulation of Drosha and DGCR8, microprocessor-mediated cleavage can also be regulated by miRNA-specific mechanisms. Some miRNAs need the interaction of the two DEAD-box RNA helicases p72 and p68 (DDX5) to interact with the microprocessor complex for efficient processing, pri-miR-18a is bound specifically by hnRNP A1 to facilitate Drosha cleavage and TGF-β signaling enhances Drosha processing by inducing SMAD binding to pri-miR-21 (Davis et al., 2008; Fukuda et al., 2007; Guil and Cáceres, 2007; Winter et al., 2009). A more recent study shows that p53 enhances the maturation of several miRNAs with growth suppressive functions by interacting with the RNA helicase p68 (DDX5) (Suzuki et al., 2009).

Drosha cleavage occurs co-transcriptionally, in both independently transcribed miRNA genes and intron-encoded miRNAs. Moreover, it is suggested that Drosha processing might take place

after recruiting the early spliceosome complex but before splicing (Ballarino et al., 2009; Kim and Kim, 2007; Morlando et al., 2008; Pawlicki and Steitz, 2008).

Following nuclear processing, pre-miRNAs are exported to the cytoplasm and further processed by Dicer. In contrast to Drosha, Dicer does not need a stem with a large terminal loop and flanking sequences but basically cleaves any dsRNA with preferably a 2 nt 3' overhang terminus. With this feature, not surprisingly, Dicer is involved in both miRNA and siRNA maturation. Human Dicer is a protein of about 220 kDa and highly conserved throughout eukaryotes, except *S. cerevisiae*. Mammals and *C. elegans* have only one Dicer whereas other organisms encode for several Dicer homologues with distinct roles. In plants, where Drosha is not expressed, Dicer-like (Dcl) protein 1 is responsible for processing both the long primary miRNA and the precursor miRNA. Dcl-2, 3 and 4 are involved in different siRNA pathways. In *Drosophila*, where two Dicer forms exist, Dicer-1 is required for miRNA biogenesis and Dicer-2 for processing of dsRNA into siRNA (Lee et al., 2004b).

Like Drosha, human Dicer has two RNAse III domains and one C-terminal dsRBD. In addition, there is also a domain of unknown function (DUF283), a PAZ domain and a N-terminal ATPase/DEXD helicase domain. Interestingly, dsRNA processing by Dcr-2 in *Drosophila* and Dcr-1 in *C. elegans* is stimulated by ATP whereas human Dicer appears to cleave dsRNA without requirement of ATP (Bernstein et al., 2001; Ketting et al., 2001; Liu et al., 2003; Nykänen et al., 2001; Zhang et al., 2002). Dicers of *G. intestinalis*, *Dictyostelium discoideum*, and *T. brucei* are even devoid of the helicase/ATPase domain (Macrae et al., 2006; Martens et al., 2002; Shi et al., 2006). In general, Dicers of lower eukaryotes frequently have a less complex domain organization. While bacterial Dicer works as a homodimer, human Dicer works as an intramolecular dimer with its two RNAse III domains. The processing center lies within the active site of the two RNAse III domains. Each of them cleaves one of the two strands, generating ends with a 5' phosphate and a 2 nt 3' overhang (Zhang et al., 2004). The α-helical region between the PAZ and the RNAse III domain serves as a "ruler" measuring the length of the excised RNA fragment. The PAZ domain preferentially recognizes 2 nt 3' protruding ends. However, blocking the ends of dsRNA with RNA tetraloops or DNA–RNA duplexes revealed that free ends are not absolutely required: the terminally blocked dsRNA is cleaved internally, with reduced kinetics. After the initial internal cleavage, normal kinetics is restored as 2-nt 3′-overhang-containing ends become available (Zhang et al., 2004).

Although Dicer on its own can mediate cleavage of dsRNA or pre-miRNAs, it associates with dsRNA binding proteins. Human Dicer associates with TRBP (Tar RNA binding protein) and/or

PACT (protein activator of PKR), which is almost 50% similar to TRBP (Chendrimada et al., 2005; Haase et al., 2005; Lee et al., 2006). Both seem not to be required for Dicer cleavage but contribute to the formation of the small RNA effector complexes. In *Drosophila*, Dicer-1 requires Loquatious (Loqs) for pre-miRNA processing and Dicer-2 R2D2 (Förstemann et al., 2005; Liu et al., 2003; Saito et al., 2005). *Drosophila* Dicer-2 plays a role in both endo- and exo-siRNAs. However, depletion of its partner protein R2D2 has almost no effect on endo-siRNAs. They seem to depend on an isoform of Loqs, usually known to be the partner of Dicer-1 in the miRNA pathway (Hartig et al., 2009; Zhou et al., 2009). In plants, miRNAs, viral siRNAs, transgene siRNAs and tasiRNAs are processed by Dcl-1-4 and their different cofactors, for example different members of the HYL1 family (Margis et al., 2006). These finding suggest that different categories of siRNAs may require different Dicers and different binding partners.

Besides its role in siRNA and miRNA maturation, Dicer is suggested do be involved in mammalian development. Dicer-deficient mice die early in development (E7.5). Dicer deficient embryonic stem cells (ES cells) show significant proliferation and differentiation defects. They are unable to process pre-miRNAs or dsRNAs but are still able to perform siRNA-mediated gene silencing (Bernstein et al., 2003; Kanellopoulou et al., 2005; Murchison et al., 2005). The severe consequences upon Dicer depletions are probably due to the loss of miRNAs. However, DGCR8 depleted cells show a less severe phenotype. DGCR8 knockout embryos arrest early in development and knockout ES cells are defective in differentiation but unlike Dicer knockout cells, they do not fully downregulate pluripotency markers, retain the ability to produce ES cell colonies and even do express some markers in differentiation. This could be explained by the fact that a few DGCR8 independent miRNAs are still functioning in the knockout cells. An analysis of DGCR8 depleted cells revealed that four canonical miRNAs, several mirtrons and other miRNA-like sequences are still present (Babiarz et al., 2008). However, the more severe phenotype in Dicer deficient cells could also indicate that Dicer in addition has miRNA-independent roles in ES cell functions (Wang et al., 2007).

1.3.2 Argonaute proteins

After Dicer cleavage, the miRNA or siRNA is loaded into an effector complex containing an Argonaute protein as the core component. Ago proteins were named after the AGO1 knockout phenotype in *Arabidopsis thaliana*, resembling the tentacles of the octopus *Argonauta argo* (Bohmert et al., 1998). Based on sequence homology, Argonaute proteins can be divided into two subclasses: one resembling *Arabidopsis* AGO1 and the other related to *Drosophila* Piwi. The Ago subclass in humans consisting of hsAgo1-4 is ubiquitously expressed and associates

with miRNAs and siRNAs. The Piwi members are mainly restricted to the germ line, associating with piRNAs. The human genome encodes four Piwi proteins named Piwil1 (Hiwi), Piwil2 (Hili), Piwil3 and Piwil4 (Hiwi2) (Sasaki et al., 2003). Recently, a third subclass has been identified, named group 3. It is worm-specific and mainly contains Ago proteins lacking endonucleolytic activity (Tolia and Joshua-Tor, 2007).

Ago proteins are conserved throughout species and many organisms encode for multiple members, ranging from one in *S. pombe*, five in *Drosophila*, eight in humans, ten in *Arabidopsis* up to 27 in *C. elegans*. They have a molecular weight of about 100 kDa and can also be called PPD proteins because of their two signature domains, the PAZ domain (Piwi/Argonaute/Zwille) and the PIWI domain. Ago proteins are also found in some prokaryotes, but their function in these organisms remains unclear. However, crystallographic studies of archaeal *Pyrococcus furiosus* and eubacterial *Aquifex aeolicus* Ago brought more insight into structure and function of these proteins (Ma et al., 2004; Parker et al., 2004; Rashid et al., 2007; Song et al., 2004; Yuan et al., 2005). It was found that the PIWI domain resembles the structure of bacterial RNAse H which can cleave an RNA strand in a RNA/DNA hybrid (Keck et al., 1998). Later it was discovered that the catalytic activity of miRNA effector complexes, also termed Slicer, resides in the Argonaute protein itself. Interestingly, not all Argonaute proteins show endonucleolytic activity. In humans, only Ago 2 has been shown to cleave the phosphodiester bond of a target RNA opposite of nt 10 and 11 of the siRNA although the conserved sequence motif DDH important for divalent metal ion binding and catalytic activity is present in other human Ago proteins as well (Liu et al., 2004; Meister et al., 2004; Rivas et al., 2005). More recently, the structural studies have been extended to eubacterial *Thermus thermophilus* Argonaute containing a guide strand only or a guide DNA strand and a target RNA duplex (Wang et al., 2008b; Wang et al., 2009; Wang et al., 2008c). The structure is divided into two lobes. One lobe contains the PAZ domain with the N-terminal domain connected through a linker region L1. The second lobe consists of the middle domain (mid domain) and the PIWI domain. The PAZ domain, shared with Dicer enzymes, forms a specific binding pocket for the 3' protruding end of the RNA or DNA in case of *Thermus thermophilus* in a sequence independent manner (Lingel et al., 2003; Ma et al., 2004; Song et al., 2003). The 5' phosphate is positioned in a specific binding pocket in the mid domain (Ma et al., 2005; Parker et al., 2005). The protein-DNA contacts are dominated by sugar-phosphate backbone interactions, as expected for a protein that can accommodate a wide range of guide sequences. The target RNA is base pairing with the guide

DNA at least in the seed sequence, a region especially important for target recognition, but is not touching the protein (Wang et al., 2008b; Wang et al., 2009; Wang et al., 2008c)}.

1.4 MiRNA and siRNA effector complexes

To perform their effector functions, miRNAs and siRNAs are incorporated into Argonaute proteins which are the key components of ribonucleoprotein complexes (miRNPs), often referred to as RNA induced silencing complex (RISC). During RISC assembly, the RNA duplex must be unwound into individual strands. One strand, called guide strand or mature miRNA, remains associated with the Argonaute protein. The opposing strand, called passenger strand or miRNA* is thought to be degraded. Strand selection for siRNAs follows the asymmetry rules, meaning the strand with its 5' terminus at the thermodynamically less stable end becomes part of the active RISC (Tomari et al., 2004b). For miRNAs these rules seem to work only to a certain extent as some duplexes produce mature miRNAs from both strands (Khvorova et al., 2003; Wei et al., 2009). Currently, it is not known how small RNA duplexes are separated into two single strands. It was shown that the passenger strand can be cleaved by *Drosophila* Ago proteins and hAgo2 (Leuschner et al., 2006; Matranga et al., 2005; Miyoshi et al., 2005; Rand et al., 2005). However, this is only possible for perfect complementary duplexes loaded in Ago2. It is not known how miRNA duplexes that are usually not perfectly complementary at the Ago2 cleavage site are unwound and how cleavage-incompetent Ago proteins are loaded. In this case, a helicase might contribute to duplex unwinding but is probably not generally required as RISC loading of miRNAs was shown to be ATP independent (Maniataki and Mourelatos, 2005). In contrast, a recent study shows that Drosophila Ago1 RISC loading is ATP dependent but duplex unwinding is a passive process that does neither need Slicer activity nor ATP (Kawamata et al., 2009).

In humans, RISC assembly is not well understood. The RISC assembly complex consists of Dicer, its dsRBD protein partner TRBP (and/or PACT) and an Argonaute protein (Chendrimada et al., 2005; Haase et al., 2005; Lee et al., 2006). This trimeric complex is able to load a mature miRNA according to the asymmetry rules and cleave a complementary target mRNA (Gregory et al., 2005; Maniataki and Mourelatos, 2005). However, the data that Dicer deficient ES cells are unable to process pre-miRNAs or dsRNAs but are still able to perform siRNA-mediated gene silencing indicate that mammalian Dicer may not play a major role in RISC formation (Kanellopoulou et al., 2005; Murchison et al., 2005). This is consistent with a recent study in *Drosophila* where Dcr-1 seems to be dispensable for Ago1-RISC loading (Kawamata et al., 2009). In contrast, Dcr-2 and R2D2 both seem to be involved in RISC loading and sensing

thermodynamic asymmetry of the siRNA duplex. R2D2 binds the duplex with orientation to the thermodynamic more stable 5' end and is therefore critical for strand selection (Tomari et al., 2004b). Although Dicer alone can process dsRNA into siRNAs, RISC assembly is dependent on both Dicer-2 and R2D2, suggesting that RISC loading is separable from siRNA production (Liu et al., 2003; Liu et al., 2006; Matranga et al., 2005; Tomari et al., 2004b). Indeed, it was shown that both Dcr-2 and R2D2 can act *in vivo* to load Ago2 with miR-277, a miRNA produced by Dcr-1 and Loqs. It could be possible that the RNA duplexes are released after processing by Dicer and then rebound by Dcr-1/Loqs or Dcr-2/R2D2 complexes, the Ago1- or Ago2 loading machinery, respectively (Förstemann et al., 2007; Tomari et al., 2007). This could also explain that miRNA and miRNA* both can be incorporated into mature RISC.

Ever since the first miRNAs and siRNAs were discovered, a lot of investigations on the protein machinery have been done by analyzing where siRNA mediated cleavage activity is residing. Several RISC components have been found that differ in size and protein composition. Besides Argonaute proteins three additional proteins have been identified in *Drosophila* RISC: Vasa intronic gene (VIG) protein, Tudor SN and fragile X related protein (dFXR) (Caudy et al., 2003; Caudy et al., 2002). Moreover dFXR-miRNA complexes including the RNA helicase Dmp68 and the ribosomal proteins L5 and L11 have been found (Ishizuka et al., 2002). In humans, the homolog of dFXR, FMRP (fragile X mental retardation protein) has also been identified. Moreover, miRNAs and Ago2 were found in a 15S complex containing Gemin 3 and Gemin 4 (Mourelatos et al., 2002). A proteomic analysis of human Ago1 and Ago2 complexes revealed the Arginine methyltransferase PRMT5 and the putative DEAxD-box helicase Mov10. MOV10 is a candidate ortholog of the plant protein SDE- 3 and the Drosophila protein Armitage, both of which are involved in RNAi (Dalmay et al., 2001; Tomari et al., 2004a). Genetic screens in *C. elegans*, RNAi screens in *D. melanogaster*, and biochemical purifications of Argonaute complexes in human cells showed interaction of GW182 protein family with Argonaute proteins (Ding et al., 2005; Eulalio et al., 2008b; Jakymiw et al., 2005; Liu et al., 2005; Meister et al., 2005; Rehwinkel et al., 2005). GW182 is termed after its molecular weight and the presence of glycine and tryptophan repeats and was shown to be localized in processing bodies (p-bodies) or GW bodies, cytoplasmic regions enriched with proteins involved in mRNA turnover (Eystathioy et al., 2002; Eystathioy et al., 2003). Insects have one GW182 protein, C. elegans have two (AIN1 and AIN2) and humans three paralogs (TNRC6A/GW182, TNRC6B and TNRC6C) (Eulalio et al., 2009a). In *Drosophila*, depletion of GW182 leads to upregulation of miRNA targets (Behm-Ansmant et al., 2006a; Eulalio et al., 2009a; Eulalio et al., 2008b; Eulalio

et al., 2009b; Eulalio et al., 2009c; Rehwinkel et al., 2005). Moreover, tethering of GW182 to the mRNA represses translation independently of Drosophila Ago1, demonstrating that it is an important effector protein functioning more downstream of Ago functioning in gene silencing (Behm-Ansmant et al., 2006a).

1.5 Regulation of gene expression by miRNAs and siRNAs

MiRNAs and siRNAs guide RNA silencing effector complexes containing an Ago protein to their target mRNA. A key determinant of the regulatory mechanism of RNA silencing is the degree of complementarity between the small RNA and the target RNA. Perfect complementarity promotes Ago2-mediated endonucleolytic cleavage whereas mismatches in the central region of the small RNA lead to repression of mRNA translation (Doench et al., 2003).

1.5.1 Translational repression

The mechanism of translational repression by miRNAs is still an ongoing debate. Studies were carried out in different laboratories with different experimental approaches leading to various results. Several models are under discussion, such as inhibition of translation at initiation steps or at post initiation steps, including co-translational protein degradation or ribosome drop-off.

1.5.1.1 Inhibition of translation at initiation steps

mRNA translation starts with the recognition of the 5'-terminal cap by the eIF4E subunit of the eukaryotic translation initiation factor (eIF) eIF4F, also containing eIF4A and eIF4G. Interaction of eIF4F with eIF3 leads to the recruitment of the 40S ribosomal subunit, which starts scanning the 5' UTR of the mRNA. After joining of the 60S subunit, translation begins at the start codon. Translational efficiency is stimulated by circularization of the mRNA. This interaction is mediated by interaction of eIF4G with the poly(A)-binding protein 1 (PABP1) associated with the poly(A) tail. Some mRNAs can initiate translation cap-independent and without or only a few initiation factors due to the presence of an internal ribosome entry site (IRES) (Filipowicz et al., 2008).
Several laboratories provided support that miRNA-mediated repression occurs at early steps of translation. In *Drosophila* and mouse Krebs2 ascite cells, miRNAs inhibited the association with either the small ribosomal subunit or 80S ribosome (Mathonnet et al., 2007; Thermann and Hentze, 2007). Based on density gradient fractions it was shown that mRNAs repressed by miRNAs are not sedimenting in polysome fractions but shift to the free mRNP pool (Pillai et al., 2005). Furthermore, it was shown that mRNAs containing binding sites for miRNAs can be repressed if they contain a functional m^7G cap but not if they only contain an IRES or a non-

functional cap (Humphreys et al., 2005; Mathonnet et al., 2007; Pillai et al., 2005; Wakiyama et al., 2007). Cap dependency was also shown by experiments with bicistronic constructs. Only the activity of the first cap-dependent cistron was repressed by the endogenous let7 miRNA but not the downstream cistron driven by the initiation factors eIF4E or eIF4G directly tethered at the intercistronic region (Pillai et al., 2005).

Supporting the role of the cap in translational repression is a study showing that providing the cap-binding complex to a cell free system can relieve miRNA-mediated repression (Mathonnet et al., 2007). These observations can be explained by a model that Ago contains a cap-binding motif and can compete with eIF4E for m^7G binding (Kiriakidou et al., 2007). A study in *Drosophila* agrees with the importance of the described Ago motif, however, explains that it is not important for cap binding but rather for interaction with GW182, a protein important for translational repression and deadenylation (Eulalio et al., 2008b).

A second model shows that Ago2 can recruit the eukaryotic initiation factor eIF6, thereby blocking the association of the ribosomal subunits and causing translational repression. EIF6 is involved in 60S ribosomal subunit biogenesis and prevents its premature association with the 40S subunit. It was shown that depletion of eIF6 in human cells and *C. elegans* rescues mRNA targets from inhibition (Chendrimada et al., 2007). Moreover, miRNA-targeted mRNAs in reticulocyte lysate become enriched for 40S but not 60S ribosomal units (Wang et al., 2008a). However, studies in *Drosophila*, where depletion of eIF6 has no or little effect on miRNA silencing are contradicting the involvement of eIF6 in the mechanism of repression (Eulalio et al., 2008a).

Other studies were performed to analyze the poly(A) tail requirement. In reticulocyte lysate and human cells translational repression occurred only when target mRNAs contained both a cap and poly(A)tail (Wakiyama et al., 2007; Wang et al., 2006). In HEK293 lysate mRNAs containing miRNA binding sites underwent deadenylation no matter whether they contained a m^7G cap, a cap analogue or whether translation was IRES mediated (Wakiyama et al., 2007). These data support a model that translation is repressed because the cap and the deadenylated tail cannot interact through proteins, preventing circularization of the mRNA needed for stimulation of translation. Contradicting to this model are studies where mRNA transcripts lacking a poly(A) tail can still undergo miRNA mediated repression (Humphreys et al., 2005; Pillai et al., 2005; Wu et al., 2006).

1.5.1.2 Inhibition of translation at post-initiation steps

In contrast to these studies, data in *C. elegans* suggest a mechanism of miRNA repression after initiation of translation by showing that target mRNAs of lin-4 miRNA remain associated with polysomes (Olsen and Ambros, 1999; Petersen et al., 2006; Seggerson et al., 2002). Incubation of active translating polysomes with translational inhibitors led to ribosome dissociation and shifted miRNAs to lighter gradient fractions, suggesting that miRNPs may physically associate with polyribosomes (Maroney et al., 2006; Nottrott et al., 2006; Petersen et al., 2006). Other studies with bicistronic constructs showed that the second cistron under control of an IRES can be repressed (Lytle et al., 2007; Petersen et al., 2006).

Based on these observations two models have been proposed. One model suggested that miRNAs cause premature ribosome dissociation or ribosome drop-off (Petersen et al., 2006). The other proposes that the nascent polypeptide chain might be degraded co-translationally. However, polypeptide chains could not be detected (Nottrott et al., 2006; Petersen et al., 2006). Moreover, translational repression was not inhibited when reporter proteins were directly targeted to the endoplasmatic reticulum (ER) to exclude the possibility that nascent proteins are degraded in the cytosol (Pillai et al., 2005).

1.5.1.3 Inhibition of translation: initiation versus post-initiation steps

So far, it is difficult to propose a unified model of how miRNAs inhibit translation. The discrepancies in the current data are not easy to explain. Cosedimentation of miRNPs with polysomes does not necessarily indicate post-initiation repression but might simply reflect the association of miRNPs with mRNAs undergoing productive translation. Also, binding of miRNPs to the 3' UTR in Drosophila extracts was shown to induce formation of pseudo-polysomes. These mRNP aggregates similar to polysomes were observed even in the absence of translation, suggesting that in some studies polysomes might have been misinterpreted (Thermann and Hentze, 2007). However, there are no simple technical or experimental explanations to explain all the different data obtained. It is possible that some data could be secondary effects of miRNA-mediated silencing but it is rather likely that miRNAs are able to mediate translational inhibitions in different ways. For a more comperehensive understanding of the underlying mechanisms, further studies will be required.

1.5.1.4 MiRNA-mediated deadenylation and destabilization of target mRNAs

Recently, it has been demonstrated that miRNAs can not only guide translational repression but also destabilization of target mRNAs (Bagga et al., 2005; Behm-Ansmant et al., 2006a;

Chendrimada et al., 2007; Eulalio et al., 2007; Giraldez et al., 2006; Wu et al., 2006). It was shown that mRNA targets for overexpressed miRNAs become less abundant and overexpression of miR-430 in zebrafish correlates with degradation of several maternal mRNAs containing miR-430 binding sites (Giraldez et al., 2006; Lim et al., 2005). Furthermore, downregulation of miRNA pathway components such as Dicer and Ago proteins lead to an increase of predicted miRNA targets (Behm-Ansmant et al., 2006b; Eulalio et al., 2007; Giraldez et al., 2006; Rehwinkel et al., 2006; Schmitter et al., 2006). In animals, RNA decay by miRNAs is mediated by the general mRNA degradation machinery initiated by deadenylation and decapping rather then through endonucleolytic cleavage - unless the miRNA is fully complementary to its target RNA as in the case of miR-196 and its target HoxB8 in mice (Behm-Ansmant et al., 2006b; Eulalio et al., 2007; Giraldez et al., 2006; Wu et al., 2006; Yekta et al., 2004).

The mechanism of miRNA-mediated decay but not translational repression requires proteins of the mRNA degradation machinery such as the CCR4-NOT deadenylase complex, the decapping enzyme DCP2 and several decapping activators including DCP1, Ge-1, EDC3 and RCK/p54. Furthermore, it depends on a member of the Ago and GW182 protein family (Behm-Ansmant et al., 2006a; Eulalio et al., 2007). A recent study in mouse Krebs-2 ascites extractes gives more insight into the mechanism by which GW182 is involved in silencing and how translational repression and mRNA decay contribute to silencing (Fabian et al., 2009). Consistently with previous data, it is observed that miRNA-mediated deadenylation requires a GW182 ortholog and the CCR-NOT deadenylase complex containing CAF1. Since miRNA loaded RISC complex is known to interact with the poly(A)-binding protein PABP and CAF1, it is shown that CAF1 is at least partially responsible for miRNA-mediated deadenylation and PABP-GW182 interaction is required to facilitate the process (Landthaler et al., 2008); (Fabian et al., 2009). PABP-eIF4G binding seems to interfere with miRNA-mediated deadenylation and it is possible that GW182 may compete with eIF4G in PABP binding. Furthermore, Fabian and collegues show that miRNA-mediated deadenylation is detected 1.3h after incubating a reporter construct in Krebs extract whereas inhibition of cap-dependent translation is detected within the first hour. However, repression further increases after longer incubation time and is partially inhibited in CAF-1 depleted extract. This could explain the results obtained in a different study in mammalian cells where it is shown that let-7 reporter constructs are deadenylated preceding measurable translational repression and that blocking deadenylation partially impairs translational repression (Beilharz et al., 2009). Taken together, these data suggest that miRNA-mediated deadenylation

can contribute to translational repression and mRNA decay but might also have additional repressive effects in addition to the initial inhibition of translation.

1.5.2 Other miRNA functions (miRNAs activating gene expression)

Recent data showed that a reporter mRNA containing the tumor necrosis factor α (TNFα) AU-rich element (ARE) has a miRNA binding site. AREs are highly conserved 3'UTR sequences that can stimulate mRNA decay mediated by ARE-binding proteins such as TTP, AUF-1 or BRF1 or regulate mRNA translation with the help of HuR or TIA-1 (Bhattacharyya and Filipowicz, 2007). Binding of a miR-369 to the reporter mRNA containing TNFαARE can stimulate translation in G1 arrested cells whereas no stimulation occurs if the miRNA is absent. However, miR-369 represses translation during other cell cycle phases. Under repressing conditions, Ago2 was not associated to FXR1 but under conditions inducing translational stimulation, both Ago2 and FXR1 were present and essential for upregulation to occur (Vasudevan and Steitz, 2007; Vasudevan et al., 2007). However, they both have been found to mediate translational repression in earlier studies.

A first positive regulatory function for a miRNA has been reported in liver cells, where miR-122 has stimulatory effects on the replication of Hepatitis C Virus by binding directly to a site in the 5'-UTR of the viral RNA. miR-122 increases the association with ribosomes with HCV RNA, leading to translational stimulation whereas miR-122 complementary sequences in the 3' UTR have no effects on translation (Henke et al., 2008). However, it is shown by another group that placing the site into the 3' UTR leads to translational repression. The authors suggested, the location of the miR-122-binding site dictates its effect on gene expression (Jopling et al., 2008). Similarly, more recent data showed that interaction of miR-10a interact with the 5' UTR of certain ribosomal subunits leading to their activated translation, whereas interaction with the 3' UTR leads to repression (Ørom et al., 2008). However, other studies showed that miRNA interacting with the 5' UTR can also repress translation (Lytle et al., 2007).

In a different study it was shown that miRNAs targeting promoter sequences can induce gene expression. The gene promoter of E-cadherin has a putative target site for miR-373. Both miR-373 and its precursor hairpin can induce expression of the candidate in a Dicer dependent way (Place et al., 2008). However, this is not a general mechanism as different data showed that miR-320 targeting the promoter of POLR3D seems to have a repressive function (Kim et al., 2008). Interestingly, promoter associated small RNAs seem not to be rare but their function needs to be investigated (Janowski et al., 2005; Janowski et al., 2006; Kapranov et al., 2007; Schwartz et al., 2008; Taft et al., 2009a).

Taken together, examples for miRNAs activating gene expression are quite rare and more comprehensive analysis needs to be done to observe if this can be a more general mechanism of miRNAs. In theses further studies it is important to consider the whole context of the cell and take into account different cell cycle stages and the influence on different regulatory elements on a single mRNA to study miRNA mechanisms.

1.5.3 Transcriptional gene silencing

Transcriptional gene silencing was first discovered in plants by the discovery that small RNAs derived from transgenes and viral RNAs could guide methylation of homologous DNA sequences (Hamilton and Baulcombe, 1999; Mette et al., 2000; Wassenegger et al., 1994). Further studies showed that DNA methylation of a specific transgene is Dicer and Ago dependent and linked to histone H3 lysine 9 (H3K9) methylation (Henderson et al., 2006; Lippman and Martienssen, 2004; Zilberman et al., 2003). In contrast to plants, fission yeast can only induce histone methylation. In *S. pombe,* deletion of the single Ago, Dicer or RdRP protein result in loss of heterochromatin gene silencing, reduced H3K9 methylation at centromeric repeats and accumulation of ncRNAs transcribed from centromeric repeats and processed into siRNAs (Reinhart and Bartel, 2002; Volpe et al., 2003). The Ago1 containing effector complex in *S. pombe* is termed RNA-induced transcriptional silencing complex (RITS) (Cam et al., 2005; Verdel et al., 2004). RITS associates with nascent transcripts and RNA polymerase. A histone methyltransferase leads to H3K9 methylation, the recruitment of the chromodomain-containing protein Swi6 and to subsequent heterochromatin formation (Bühler et al., 2006; Djupedal et al., 2005; Kato et al., 2005; Lippman and Martienssen, 2004). Furthermore, RITS interacts with the RdRP to reinforce and spread silencing (Sugiyama et al., 2005).

In mammals, siRNAs directed to promoter genes can induce H3K9 and H3K27 methylation (Kim et al., 2006). It was found that besides chromatin modifying complexes, mammalian cells need Ago1 or Ago1&2 for siRNA-mediated transcriptional silencing (Janowski et al., 2006; Kim et al., 2006). Furthermore, two different studies describe a role for promoter-directed human miRNAs in facilitating transcriptional gene silencing (Gonzalez et al., 2008; Kim et al., 2008). Promoter-targeting RNAs have also been involved in activation of transcription (Janowski et al., 2007; Schwartz et al., 2008). These results suggest that small RNA-mediated DNA and histone modifications are also occurring in mammals and the discovery of endo-siRNA increases the chance that there will be more small RNAs involved in chromatin modification.

1.6 MiRNAs and target mRNAs

The annotation of new miRNAs is based on several rules: expression of the new miRNA gene should be verified by experimental evidence and one of the following criteria should be met: (1) the mature miRNA should be included in one arm of a predicted fold-back precursor structure with extensive base-pairing in the miRNA region not containing any large internal loops or bulges, (2) the fold-back structure should be phylogenetically conserved and (3) the precursor should be shown to accumulate in organisms with impaired Dicer function (Ambros et al., 2003a; Mendes et al., 2009). These annotation criteria have inspired most current computational methods, however they differ in approaches to phylogenetic conservation and features to identify good stem-loop candidates (Berezikov et al., 2005; Grad et al., 2003; Lai et al., 2003; Lim et al., 2003). More recent methods include “machine learning”, meaning they try to generalize from a positive set of known miRNA and a negative set of stem-loops (Mendes et al., 2009).

An up to date collection of published miRNAs sequences is provided by miRBase (http://microrna.sanger.ac.uk/). The growing number of miRNAs identified in the most recent years is mainly due to the discovery of deep-sequencing technologies, which has opened doors to detecting and profiling known and novel, low abundant miRNAs. However, the identification of the large pool of sequenced transcripts from a deep sequencing run remains a major challenge. Mapping of the small transcripts is not trivial and miRNAs need to be separated from the pool of other sequenced small RNAs or degradation products. A number of bioinformatical algorithms help to analyze the huge datasets, among them miRDeep. The idea of miRDeep is to detect miRNAs by analyzing how sequenced RNAs are compatible with how miRNA precursors are processed in the cell (Friedländer et al., 2008).

The understanding of the function and the biological impact of miRNAs are defined by the target mRNAs and the effect on their expression. Most miRNA binding sites are found in the 3’ UTR, however recent experiments showed that there are also miRNA binding sites in the 5’ UTR or the coding region mRNAs (Duursma et al., 2008; Lal et al., 2008; Ørom et al., 2008; Rigoutsos, 2009; Tay et al., 2008). Number and position of binding sites can play important roles. More miRNA binding sites are more effective and two sites close together lead to cooperative action, meaning they are even more potent than two single sites (Saetrom et al., 2007). Moreover, the position within the 3’ UTR can be critical too. RNAs with binding sites in a AU-rich neighborhood, or not too far away from the stop codon and away from the center of long 3’ UTRs are more easily regulated (Grimson et al., 2007; Nielsen et al., 2007). Furthermore, a different study

shows that target accessibility might play an important role for the extent of repression (Kertesz et al., 2007).

In plants, it is common that miRNAs match with near-perfect complementary to their mRNA targets whereas animal miRNAs usually only show complementarity between the miRNA-mRNA duplex from nucleotides 2 to 8 of the miRNA, the so-called seed sequence. Bulges and mismatches are present in the central region and therefore endonucleolytic target cleavage is excluded. In the 3' half of the miRNA some bulges are allowed but there should be some complementarity particularly in nt 13 to 16, especially if matching in the seed region is suboptimal (Brennecke et al., 2005; Lewis et al., 2005). However, many miRNA-target site interactions violate the seed rule (Ha et al., 1996; Ørom et al., 2008; Reinhart et al., 2000; Stern-Ginossar et al., 2007). Moreover, some sites are recognized by miRNAs whereas other regions showing similar or higher complementarity are not. This might be due to the interplay of different target sites and due to various proteins binding to the target mRNA. For example, HUR/ELAV1, an AU-rich element (ARE) binding protein associates with the 3' UTR of the CAT1 mRNA after stress, relieving it from miR-122-mediated repression. By this CAT1 is recruited from p-bodies to the cytoplasm where it can associate with polysomes and become translationally active. (Bhattacharyya et al., 2006). A similar phenomenon was first discovered in Zebrafish. By binding U-rich mRNA regions of miR-430 targets, the RNA-binding protein Dead end (Dnd1) can block miRNA accessibility and therefore inhibit silencing (Kedde et al., 2007). MiRNA activity at synapses appears to be regulated in a similar manner. By adding a specific neurotrophic factor to neurons, miR-134-mediated repression of its mRNA target can be relieved (Schratt et al., 2006). In contrast to factors relieving miRNA function there are proteins enhancing the activity. In *C. elegans*, the Trim-NHL protein NHL-2 is required for optimal let-7 and lys-6 miRNA activity (Hammell et al., 2009). In mammals, Trim32 plays a role in the enhancement of let-7a- mediated differentiation of neural progenitor cells by interacting with Ago1 (Loedige and Filipowicz, 2009; Schwamborn et al., 2009). As a conclusion, proteins that are not part of the miRNA complex can tune miRNA silencing. This makes miRNA target identification more challenging and it should be noted that it is important not to analyze single miRNA binding sites but to look at the whole context of the 3' UTR.

Since animal miRNA-mRNA duplexes contain mismatches, gaps and G:U wobbles, they are difficult to predict. Most approaches involve conserved 3' UTR sites with favorable thermodynamic hybridization energies and use the detection of seed matches as a major condition. The current prediction programs as for example Target Scan, PicTar and EMBL

require strict seed base pairing and therefore have a high degree of overlap. However, there are still differences due to the different algorithms. Another problem is that different prediction programs use different UTR databases, miRNA sequences and different ranking of the results. Other approaches to predict miRNA targets include rules of target site recognition from confirmed targets (Mendes et al., 2009). Many of these methods successfully predict real miRNA targets, but the number of false positives remains high and ways to experimentally verify target mRNAs get more and more important.

Besides bioinformatical target identification, several biochemical identification approaches have been done. Two labs performed proteome analysis after miRNA overexpression or deletion (Baek et al., 2008; Selbach et al., 2008). Other labs co-immunoprecipitated targets with Ago proteins (Beitzinger et al., 2007; Easow et al., 2007; Hammell et al., 2008; Hendrickson et al., 2008; Karginov et al., 2007; Landthaler et al., 2008; Zhang et al., 2007). Similarly, Ago proteins were purified in a method named Hits-CLIP, where Ago-RNA complexes are cross-linked before purification to allow mapping of Ago-miRNA and Ago-mRNA binding sites (Chi et al., 2009). A third approach uses biotinylated RNAs to isolate miRNA targets (Orom and Lund, 2007). However, the false-positive rate of these target analysis remains high and experimental target approaches have to be further optimized.

1.7 PiRNAs

PiRNAs are single stranded RNAs of about 24-33nt in length and are not conserved between species. In contrast to animal miRNAs, they carry a 2'O-methyl modification at their 3' end which is added by a RNA methyltransferase. PiRNAs are restricted to the germ line and bind to the PIWI subclass of Argonaute proteins (Aravin et al., 2006); (Girard et al., 2006). The Piwi clade consist of Piwi, Aubergine (AUB) and AGO3 in flies, Mili (Piwil1), Miwi (Piwil2) and Miwi2 (Piwil4) in mice and Hili (Piwil2) Hiwi1 (Piwil1), Hiwi2 (Piwil4) and Hiwi3 (Piwil3) in humans.

1.7.1 PiRNA function and transposon silencing

Mutations in Drosophila Piwi proteins lead to defects in oogenesis and depletion of germline cells whereas Aub mutations disrupt gametogenesis (Cox et al., 1998; Cox et al., 2000; Harris and Macdonald, 2001; Klattenhoff et al., 2007). In mouse, all Piwi proteins are important for spermatogenesis (Deng and Lin, 2002); (Kuramochi-Miyagawa et al., 2004); (Carmell et al., 2007). The discovery of their small RNA binding partners helped to further elucidate the function of these proteins. The first population of RNAs binding to Piwi proteins was performed in Drosophila, where small RNAs mapping to repetitive elements were identified, leading to the

term repeat-associated small interfering RNAs (rasiRNAs) (Aravin et al., 2003), (Brennecke et al., 2007); (Saito et al., 2007); (Gunawardane et al., 2007). Similar RNAs have also been identified in mammalian testes and zebrafish where they were called piRNAs (Aravin et al., 2006); (Girard et al., 2006) (Houwing et al., 2007). Sharing some common features, rasiRNAs are often refered to as a subgroup of piRNAs. In mammals, piRNA expression changes during stages of sperm development. Therefore, mammalian piRNAs can be divided into pre-pachytene and pachytene piRNAs according to the stage of meiosis at which they are expressed in developing spermatocytes. Pre-pachytene piRNAs predominantly correspond to repetitive and transposon rich sequences and interact with MILI and MIWI2 whereas pachytene piRNAs associate with MILI and MIWI (Aravin et al., 2006; Aravin et al., 2007; Aravin et al., 2008; Girard et al., 2006; Kuramochi-Miyagawa et al., 2008). Plants lack Piwi proteins but have a different RNAi-based strategy for transposon control. In *Arabidopsis*, Ago4 is loaded with transposon-derived small RNAs and involved in silencing of transposable elements (Slotkin and Martienssen, 2007).

Besides piRNAs there are also transposon-derived endo-siRNAs. Transcripts from some mobile elements are derepressed in ago2 mutant heads and ovaries, dicer-2 mutant heads and dicer-2 and ago2 knockdown S2 cells (Chung et al., 2008; Czech et al., 2008; Ghildiyal et al., 2008; Kawamura et al., 2008; Rehwinkel et al., 2006). These studies show that the endo-siRNA pathway contributes to transposon repression. It is possible that in the germline the piRNA pathway and the endo-siRNA pathway cooperate with each other. In the male germline, piRNA pathway mutations have a severe effect on fertility whereas it appears to be dispensable in the female germline of mammals, which contain a rich endo-siRNA population (Tam et al., 2008; Watanabe et al., 2008). With the exception of the esi-2-endo-siRNA, the role of endo-siRNA processed from structured loci and convergent transcripts needs to be further investigated (Czech et al., 2008; Okamura et al., 2008b).

1.7.2 PiRNA biogenesis

Although there are thousands of piRNAs identified they derive from discrete genomic clusters (Aravin et al., 2006; Girard et al., 2006). PiRNA biogenesis was best studied in *Drosophila*. Piwi and Aubergine bind piRNAs antisense to transposon RNAs whereas Ago3 typically binds the sense strands. AUB- and PIWI associated piRNAs show preference for a Uracil at their 5' end, Ago3-associated piRNAs mostly have an adenine at nt 10. With the discovery that the first 10 nt of antisense piRNAs are often complementary to the sense piRNAs, the "ping pong" model has been proposed (Brennecke et al., 2007; Gunawardane et al., 2007). AUB or Piwi associated with

antisense piRNAs cleave sense retrotransposon transcripts thereby creating the 5' end of sense piRNAs that then associate with Ago3. Ago3 then subsequently cleaves antisense retrotransposon transcripts generating the 5' end of antisense piRNA that subsequently bind to Piwi. It is not known how the 3' end of the piRNAs is generated. By this continuous cycle, piRNAs are amplified and retrotransposon silencing can be maintained. How piRNA biogenesis is initiated it not really understood. At least AUB and PIWI are maternally inherited to embryos. It is possible that piRNAs associated to those proteins act as primary piRNAs and initiate the ping-pong cycle (Brennecke et al., 2007; Nishida et al., 2007).

The ping-pong model found in *Drosophila* might also be applicable for mouse piRNAs. MILI cleaves the precursors thereby generating the 5' end of the piRNA subsequently accepted by MIWI2. MIWI2 then cleaves the opposite strand precursor thereby generating the 5' end of the piRNA subsequently binding to MILI. As in *Drosophila* it is not known how the 3' end of the piRNA is generated. The ping-pong model is not applicable to pachytene piRNAs associated to MILI and MIWI and moreover it is unclear how primary piRNAs are processed (Aravin et al., 2008).

1.8 Aim of the thesis

Members of the Argonaute protein family are the major cellular protein interactors of small RNA-mediated gene silencing. However, many protein as well as RNA interactors of individual human Ago protein complexes are unknown so far. Therefore, the aim of this work was to biochemically characterize Ago1- and Ago2-containing protein complexes in human cells and analyze their protein content and small RNA components.

Previous biochemical purifications of Ago proteins have already identified several protein interacting partners, many of them being directly involved in the miRNA biogenesis pathway. However, it is not known how many additional proteins are required for gene silencing. New interaction partners of Ago proteins might help to get more insight into the mechanistic detail of gene silencing, which is so far still not fully understood. In addition, it would be interesting to find out if proteins bound to target mRNAs can interact with Ago and thereby effect silencing. Furthermore, there might be factors promoting or inhibiting Ago binding to their targets. One aim of the thesis was therefore to contribute to a more comprehensive analysis of different Ago complexes and their protein network. For this purpose, Ago1 and Ago2 complexes were purified and separated by gradient centrifugation. Protein components were identified by mass spectrometry and Ago interacting proteins were validated by different experimental approaches.

The second aim of the thesis focused on small RNA binding partners of Ago proteins, known to bind to a variety of small RNAs with different biogenesis and function, such as miRNAs, siRNAs and piRNAs. In humans, four different Ago proteins seem to associate with the same miRNA species regardless of their sequence, as shown in previous results (Meister et al., 2004). However the full spectrum of Ago1- and Ago2-associated small RNAs, their individual biogenesis pathway and potential targets in human somatic cells is still not known. The aim of the thesis was to analyze the small RNA components bound to Ago proteins in order to identify new small RNA species by small RNA cloning and deep sequencing. The processing and function of the new identified small RNAs were analyzed and characterized by using different biochemical and molecular biological approaches.

2 Results

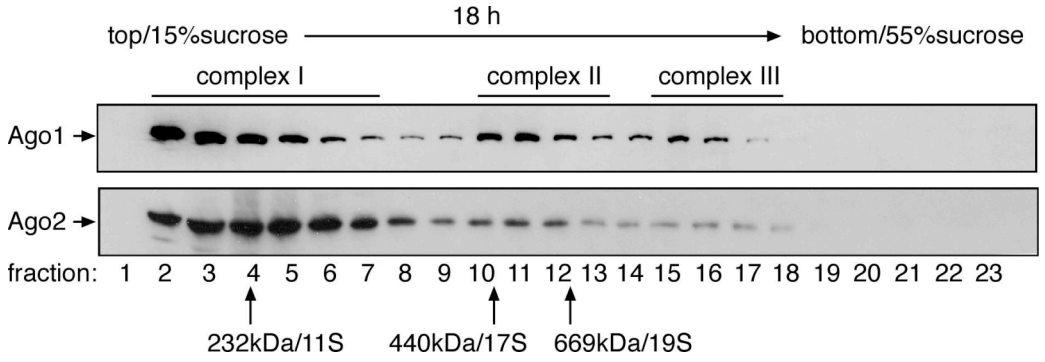
2.1 Analysis of Argonaute containing mRNA protein complexes in human cells

Members of the Argonaute protein family are key components of RNA silencing effector complexes. They are guided to their targets by small RNAs and can induce RNA cleavage, translational repression and/or deadenylation, depending on the complementarity of the small RNA and its respective target. The identification of different Ago protein complexes will lead to a better understanding of miRNAs and their function and help to elucidate the mechanisms of gene silencing.

2.1.1 Ago1 and Ago2 are incorporated into three distinct protein complexes

For a detailed characterization of Argonaute proteins Ago1 and Ago2 containing complexes were biochemically purified and analyzed. Lysate from HEK 293 cells was separated by 15%-55% sucrose density gradient centrifugation. 23 fractions were collected and analyzed by western blotting using antibodies against Ago1 and Ago2 (Höck et al., 2007) (Fig. 2.1).

Figure 2.1. Ago1 and Ago2 complexes reside in three different complexes

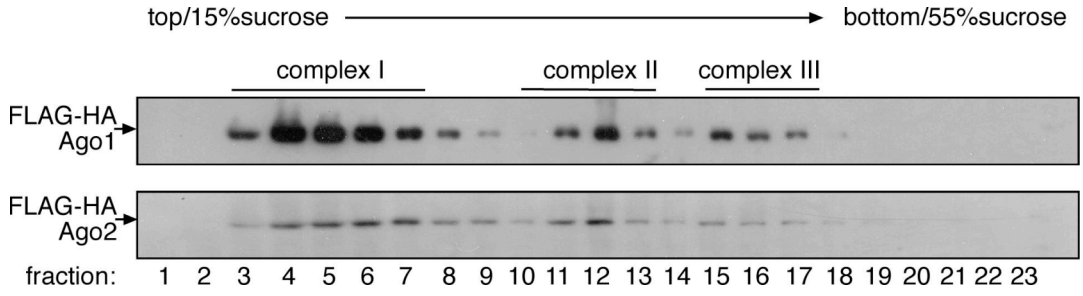


Lysates from wild-type HEK 293 cells were separated by sucrose density centrifugation under conditions that allow the separation of mRNPs. Endogenous Ago1 and Ago2 of individual fractions were analyzed using anti-Ago1 (monoclonal) and anti-Ago2 (polyclonal) (Höck et al., 2007).

Both Ago1 and Ago2 were sedimenting in three different complexes referred to as complex I-III (Fig. 2.1). Complex I contains the largest Ago fraction (40-60%) and has a molecular weight of about 250-350 kDa. Complex II has a molecular weight of about 600-700 kDa or 19S and complex III sediments at a molecular weight of more than 900 kDa or 25-30S. Complex II and III are RNAse A sensitive (Höck et al., 2007). Moreover, a luciferase reporter construct carrying the 3'UTR of KRAS mRNA, known to be regulated by let-7a, sediments with complex III when transfected to 293 cells and analyzed by gradient centrifugation as described above (Höck et al., 2007; Johnson et al., 2005). This suggests that complex III contains mRNPs with miRNA targets. Since Ago proteins are key components in RNA silencing and act as guides for small RNAs, the miRNA association of the three complexes was analyzed by semi-quantitative PCR. Two different miRNAs, namely miR-16 and let-7a are found to associate with all three Ago complexes as described by Höck et al. (Höck et al., 2007).

For further characterization, immunoprecipitates of the three complexes were required. As the Ago antibodies used for western blotting were not efficient enough for immunoprecipitation, the experiments were recapitulated with tagged Ago1 and Ago2 proteins. Lysate from HEK 293 cells transiently transfected with Flag-HA-Ago1 and Flag-HA-Ago2 expressing plasmids was separated by 15%-55% sucrose density gradient centrifugation. The fractions were collected and analyzed by western blotting using anti-HA antibodies (Fig. 2.2). Both Flag-HA-Ago1 and Flag-HA-Ago2 showed the same sedimentation pattern as described for endogenous Ago1 and Ago2 (Fig. 2.1). Therefore the tagged constructs were used for further analysis.

Figure 2.2. Flag-HA tagged Ago1 and Ago2 complexes associate with native protein complexes



HEK293cell extracts containing Flag-HA–Ago1 (upper panel) or Flag-HA–Ago2 (lower panel) were separated by gradient centrifugation. The presence of Flag-HA–Ago1 and Flag-HA–Ago2 was analyzed by western blotting using HA antibodies.

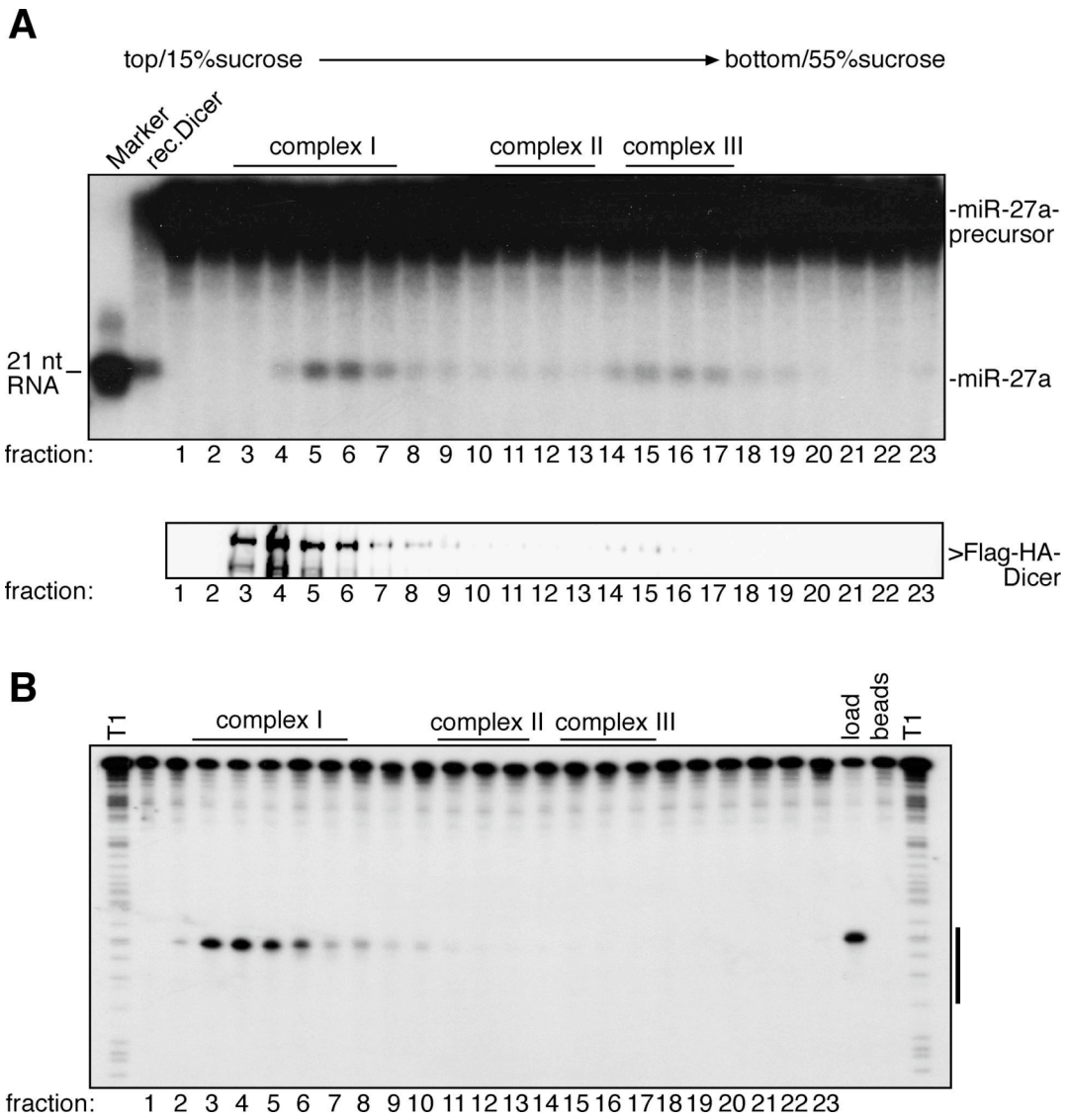
To identify cofactors of Ago1 and Ago2 residing in the three different complexes, lysates transfected with plasmids expressing Flag-HA-Ago1 and Flag-HA-Ago2 were loaded onto a 15-55% sucrose gradient. Subsequent to fractionation by density centrifugation fractions 3-8, 10-13 and 15-18 representing complex I, II and III respectively, were pooled and immunoprecipitated using anti-FLAG or control antibodies. The co-immunoprecipitated proteins were separated by SDS-PAGE and further analyzed by mass spectrometry. The proteins specifically purified with anti-FLAG antibody are summarized in (Table 2.1). A detailed analysis of the identified proteins is described in Höck et al. (Höck et al., 2007).

2.1.2 RISC and Dicer activity associate with distinct Ago complexes

A prominent interaction partner of Ago proteins is Dicer. A region in the PIWI domain of Ago proteins binds directly to the RNAse III domain of Dicer (Tahbaz et al., 2004). The two proteins form a stable complex that can generate small RNAs from dsRNA precursor (Gregory et al., 2005). In the mass spectrometry results (Table 2.1) Dicer was found to reside in two different complexes, complexes I and III. To further verify this, lysates expressing Flag-HA-Ago1 and Flag-HA-Ago2 were analyzed by density gradient centrifugation as described above. Individual fractions were immunoprecipitated using an anti-HA antibody and incubated with in-vitro transcribed and P32 labeled pre-miR 27 transcripts. Cleavage products were analyzed by denaturing RNA-PAGE (Fig. 2.3A). Consistent with the mass spectrometry results, a cleavage product of about 22nt in length is observed in complexes I and III whereas no cleavage product can be detected in complex II. The same results are obtained with lysates transfected with Flag-HA-Dicer expressing plasmids.

In humans, Ago 2 has been shown to cleave the target mRNA opposite of nt 10 and 11 of the miRNA (Liu et al., 2004; Meister et al., 2004; Rivas et al., 2005). To investigate in which of the three complexes resides RISC activity, lysate from HEK 293 cells transiently transfected with Flag-HA-Ago2 expressing plasmids was separated by sucrose density gradient centrifugation as described above. The individual fractions were immunoprecipitated using anti-HA antibody, incubated with a ^{32}P-cap labeled artificial target mRNA bearing a region fully complementary to endogenous miR-19b. Cleavage products were observed by denaturing RNA-PAGE (Fig. 2.3B). Cleavage activity is immunoprecipitated with individual fractions of complex I whereas no cleavage signal is observed in any other higher molecular weight fractions. This indicates that Ago2 complex I represents human RISC.

Figure 2.3. Dicer and RISC activity reside in distinct complexes



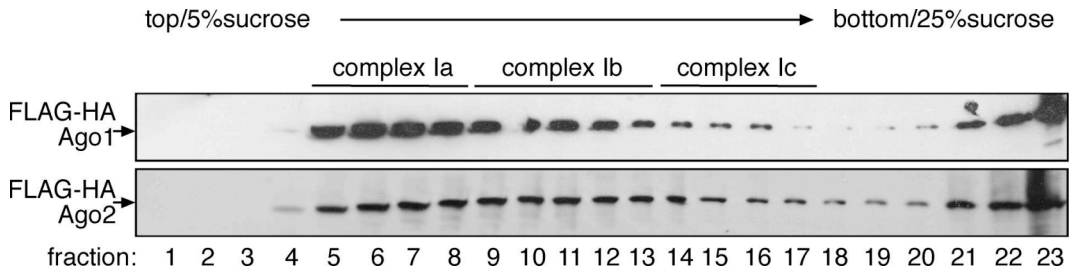
(A) Lysates from Flag-HA–Ago2-transfected HEK293 cells were separated and immunoprecipitated as described in Figure 2.1. The immunoprecipitates or recombinant Dicer were incubated with an internally labeled pre-miR-27a substrate. A21-nucleotide marker is shown to the left. (A) Flag-HA–AGO1-containing HEK293 lysate was separated and immunoprecipitated as described in Figure 2. Immunoprecipitates were incubated with a ^{32}P-cap-labelled RNA, which contained a perfect complementary sequence to the endogenous miR-19b. Lanes indicated with T1 show RNaseT1 digestions of the RNA substrates. The RNA sequence complementary to miR-19b is indicated by a black bar on the right.

2.1.3 Ago complex I consists of distinct subcomplexes

There is a huge number of proteins found in Ago complex I, which would form a much bigger complex than the actual molecular weight of about 250-350 kDa. For further separation of potential smaller complexes, lysates transfected with plasmids expressing Flag-HA-Ago1 and Flag-HA-Ago2 were loaded onto a 5-25% sucrose gradient. After density centrifugation individual fractions were analyzed by western blotting using anti-HA antibodies. Ago complex II and III migrate at the bottom of the gradient whereas complex I is present in almost all fractions (Fig. 2.4).

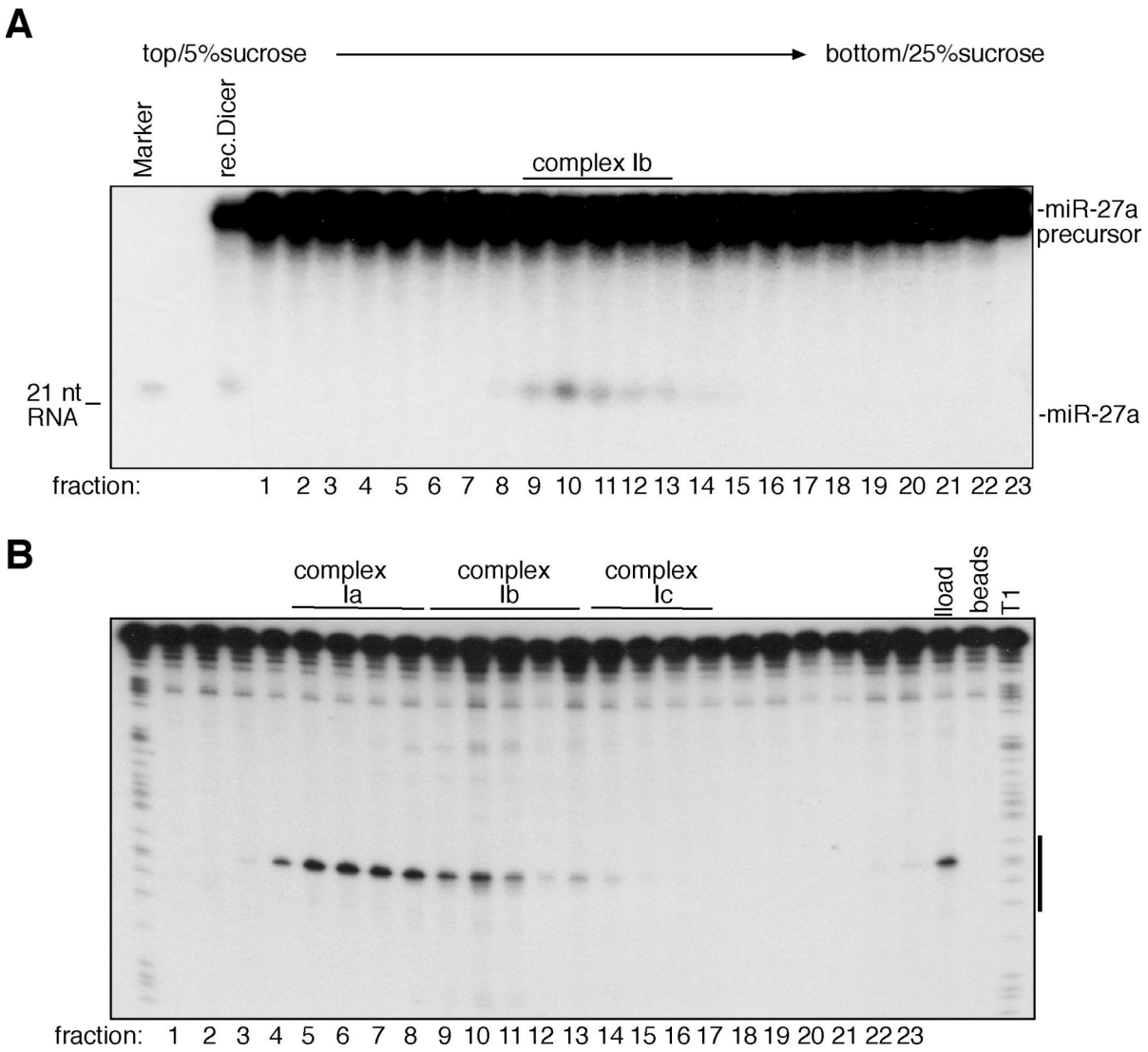
To be able to differentiate between different subcomplexes, fractions were analyzed for Dicer activity. Flag-HA-Ago2 was immunoprecipitated from individual fractions of a 5-25% gradient using anti-Flag antibodies. The precipitated complexes were incubated with in-vitro transcribed and ^{32}P labeled pre-miR 27 transcript and cleavage products were analyzed by denaturing RNA-PAGE (Fig. 2.5A). Dicer activity can be observed in fractions 9-13 with a peak in fraction 10. To get more insight into the different subcomplexes fractions were analyzed for Slicer activity. Flag-HA- Ago2 was immunoprecipitated as described above and incubated with a P32-cap labeled substrate RNA complementary to the endogenous miR-19b. RISC activity can be observed in fractions 4-14 (Fig. 2.5B). Slicer and Dicer activity assays suggest that Ago2 complex I consists indeed of multiple subcomplexes here referred to as complex I a-c.

Figure 2.4. Ago complex I consists of distinct subcomplexes



HEK293 lysates containing FLAG-HA-Ago1 or FLAG-HA-Ago2 were separated by sucrose gradient centrifugation ranging from 5% to 25%. Individual fractions were analyzed by western blotting using anti-HA-antibodies

Figure 2.5. Ago subcomplex I shows different Dicer and RISC activities



(A) Flag-HA–Ago2 was immunoprecipitated from the gradient fractions using anti-FLAG antibodies and incubated with an internally labeled pre-miR-27a substrate. Recombinant Dicer was used incubated with the same substrate as a positive control. A 21-nucleotide marker is shown to the left. (B) Immunoprecipitates were incubated with a ^{32}P-cap-labelled RNA, which contained a perfect complementary sequence to the endogenous miR-19b. Lanes indicated with T1 show RNaseT1 digestions of the RNA substrates. The RNA sequence complementary to miR-19b is indicated by a black bar on the right.

Complex Ia might represent Ago on its own. Complex 1b associates with Dicer as well as RISC and could therefore be identical with a previously described trimeric complex consisting of Ago, Dicer and TRBP (Gregory et al., 2005). Complex Ic might interact with other proteins identified by mass spectrometry.

In summary, it was shown that Ago1 and Ago2 complexes can be separated into three distinct complexes using density gradient centrifugation. All complexes can bind miRNAs. Previously validated miRNA targets sediment in complex III, suggesting that this complex forms large mRNPs with miRNA target mRNAs. Dicer activity associates with complex I, which is the smallest complex, and complex III, the largest complex. RISC activity is only observed in complex I. Furthermore, complex I consist of at least three different subcomplexes with minimal RISC as complex Ia and the trimeric complex Ago, Dicer and TRBP as complex Ib. Moreover, mass spectrometry results of the three main Ago complexes give more detailed information of the Ago protein interaction network.

Table 2.1 Proteins associated with human Ago1 and Ago2

Name	Domains/motif	Ago1-complex	Ago2-complex	Acc. No.
Proteins involved in gene silencing				
Dicer	DEAD box, RNase III, PAZ, dsRBD, DUF	I, III	I, III*	gi|21665773/ gi|5019620
TNRC6B	RRM	-	I	gi|14133235
MOV10	DExH box	III	III	gi|14424568
TRBP	dsRBD	I*	I*	gi|107904
Gemin3	DEAD box		II*	gi|14209614
Gemin4	Leucin Zipper	II*, III	II, III	gi|7657122
DEAD/DEAH box containing proteins				
RNA helicase A (RHA)/ DHX9	DEAH box, helicase domain, dsRBD, DUF1605	II, III	II, III	gi|1806048/ gi|1082769
DHX30	DEAH box, helicase domain, dsRBD, DUF1605	II, III	II, III	gi|20336294
RENT1/Upf1	DEAD box, exoV	III	-	gi|1575536
DHX36	DEAH box, helicase domain, DUF1605	II*, III*	II*	gi|7959237/ gi|23243423
DDX21/RNA helicase GuA	DEAD box, helicase domain, GUCT	II, III	II	gi|2135315
DDX50/RNA helicase GuB	DEAD box, helicase domain, GUCT, RESIII	III	-	gi|55664207
DDX46	DEAH box, helicase domain, DUF1605	II*	II*	gi|2696613
DDX48	DEAD box, helicase domain	II*, III	-	gi|496902
DDX18	DEAD box, helicase domain	III	-	gi|1498229
DDX5/p68	DEAD box, helicase domain	-	II*	
DDX39/BAT1	DEAD box, helicase domain	III*	II*	gi|1905998
DDX47	DAED box, helicase domain, Apolipoprotein L	III	-	gi|20149629
hnRNPs				
hnRNP-U	SAP, SPRY, SCOP	II, III	II, III	gi|32358
hnRNP-U-like	SAP, SPRY, SCOP	I*	-	gi|3319956
hnRNP-H2/H'	RRM, RNPHF zinc finger	II*	-	gi|6065880
hnRNP-F	RRM, RNPHF zinc finger	II*	I*	gi|16876910
hnRNP-C	RRM	II, III	II, III	gi|13937888/ gi|14250048
hnRNP-E2	KH1, KH2	III*	-	
NSAP1	Phox-like, PX-associated motif, RRM	II, III	-	gi|5031512
hnRNP-L	Enoyl-CoA hydratase/isomerase, RRM	III*	-	gi|11527777
mRNA binding proteins				
Poly-A binding proteins	RRM	II, III	II, III	gi|46367787/ gi|693937
Nuclear cap binding protein 80kDa	MIF4G	III	-	gi|3153873
YB-1	Cold shock domain	II	II, III	gi|181486/ gi|55451
FMRp	Agenet, KH1	III*	-	gi|182673
FXR1	Agenet, KH1	-	III	gi|1730139
FXR2	Agenet, KH1	III	-	gi|4758410
ZBP-1	RRM, KH1	II, III	III	gi|7141072/ gi|56237027
ZBP-3	RRM, KH1	III	-	gi|30795212
HuR	RRM	III*	-	gi|1022961
RBM4	RRM, Zn-finger	-	III*	gi|4506445
Proteins involved in RNA metabolism				
NF90/ILF3/ NFAR-1	dsRBD, DZF	II, III	II	gi|1082856/ gi|5006602
NF45/ILF2	DZF	II, III	II, III	gi|532313

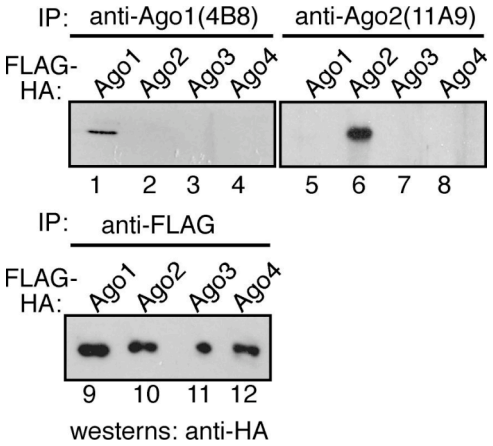
SART3	Lsm interaction motif, RRM	I, II, III	-	gi|7661952
RBM10	D111/G-patch, RRM, zn finger, Ran binding	-	I*, II*	gi|12644371
Fibrillarin	Fibrillarin motif	-	II*, III*	gi|182592
NOP56	Pre-mRNA processing RNP, NOP5NT, NOSIC	III	-	gi|2230878
Nucleolin	RRM	III	-	gi|128841
eIF2bδ	Initiation factor 2B	I*	-	gi|6563202
eIF4b	RRM	-	I*	gi|288100
FLJ20758	Pentatricopeptide repeat	II	II	gi|38683855
Other proteins				
Myb binding protein 1a	DNA polymerase V	III	III*	gi|7657351
Matrin 3	RRM, Zn finger	III*	III*	gi|6563246
Motor protein	-	II, III		gi|516764
ZNF326	AKAP95	II, III	-	gi|31807861/ gi|47125447
Ku70	Ku70/80 motif, DNA binding SAP	-	II*	gi|57165052
DDB1	CPSF A subunit	I	I*	gi|418316
RuvB-like II	AAA ATPase, Tip49b	I	I, II	gi|5730023/ gi|12653319
Coatomer protein	WD-40, COPB2	III	II	gi|1002369

*identified by a single peptide

2.2 Argonaute proteins and their small RNA binding partners

Besides the biochemical isolation and identification of human Ago1 and human Ago2 protein complexes the aim of this thesis was to analyze the small RNA components of Ago proteins. Members of the Ago protein family are key components of RISC complexes. They are guided to their targets by small RNAs. Depending on the complementarity of the small RNA and its target they can lead to target RNA cleavage, translational repression and/or deadenylation (Peters and Meister, 2007). For a detailed characterization of Argonaute proteins, monoclonal antibodies specific for Ago1 and 2 were generated. For their characterization, plasmids expressing FLAG-HA-tagged Ago1 and Flag-HA-Ago2 were transfected into HEK293 cells. After immunoprecipitation with anti-Ago1 and anti-Ago2 antibodies, proteins were analyzed by western blotting using anti-HA antibodies (Fig. 2.6). All antibodies immunoprecipitated only one specific Ago protein. Therefore, they can be used as powerful tools to purify endogenous Ago complexes and analyze protein-components, associated mRNAs and associated small RNAs.
For more detailed antibody characterization see also Beitzinger et al. and Rüdel et al. (Beitzinger et al., 2007; Rüdel et al., 2008).

Figure 2.6 Characterization of monoclonal anti-Ago1 and anti-Ago2 antibodies

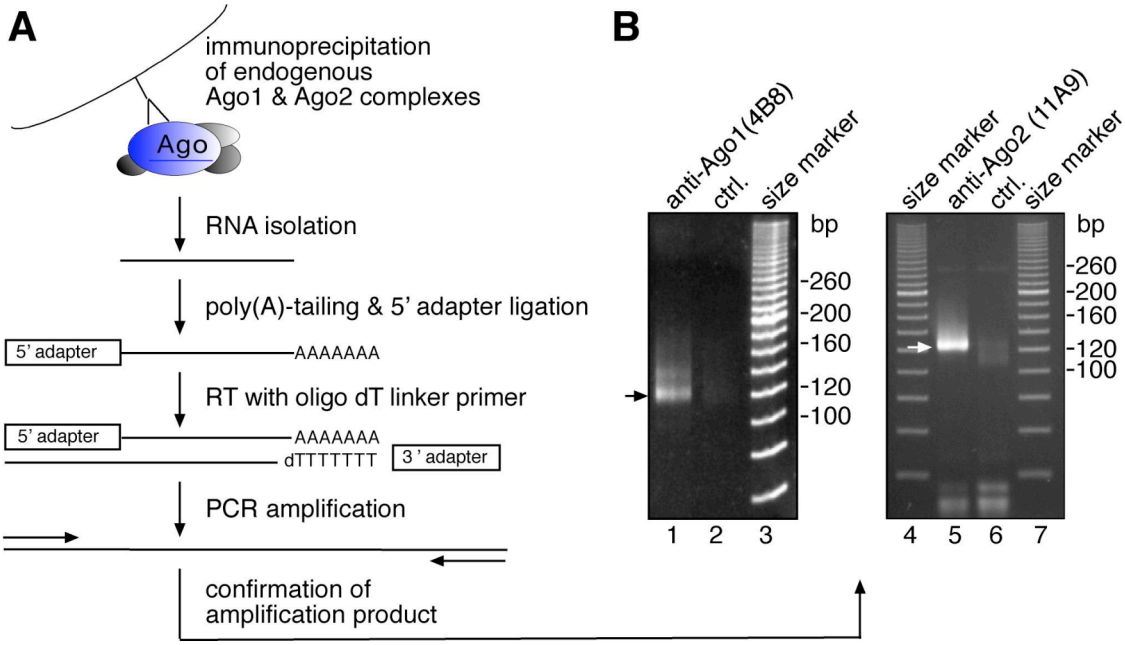


FLAG-HA-tagged Ago1-4 were subjected to immunoprecipitations using anti-Ago1(4B8) (lanes 1–4), anti-Ago2(11A9) (lanes 5–8), and anti-FLAG (lanes 9–12) antibodies. Immunoprecipitated FLAG-HA-Ago proteins were analyzed by western blotting using anti-HA antibodies.

2.2.1 Small RNAs associated with human Ago1 and Ago2

Since there are several Ago paralogs in an organism it is intriguing to find whether every small miRNA species can be incorporated into all of them or whether certain miRNAs are selectively incorporated into a specific Ago protein. Ago sorting is best studied in *Drosophila*, where the structure of the precursor is the major determinant of sorting small RNAs into Ago proteins. Duplexes with central mismatches are preferentially sorted into Ago1 whereas perfectly complementary duplexes are sorted into Ago2 (Förstemann et al., 2007; Tomari et al., 2007).

Figure 2.7. Cloning and library generation of small RNAs for 454 sequencing



(A) Endogenous Ago1 and Ago2 complexes were immunoprecipitated using the specific monoclonal antibodies described in Figure 2.6. An anti-FLAG or an anti-GST antibody served as controls. Without any size fractionation, extracted Ago-associated RNAs were poly(A)-tailed and an adaptor was ligated to the 5' phosphate of the miRNAs. First-strand cDNA synthesis was performed using an oligo(dT)- linker primer and reverse The resulting cDNAs were PCR amplified in 22 cycles. (B). The 120–135 bp amplification products were loaded onto an agarose gel and visualized by ethidium bromide staining. Massively parallel sequencing was performed by 454 Life Sciences (Branford, USA) and MWG Biotech (Germany) using the Genome Sequencer 20 system.

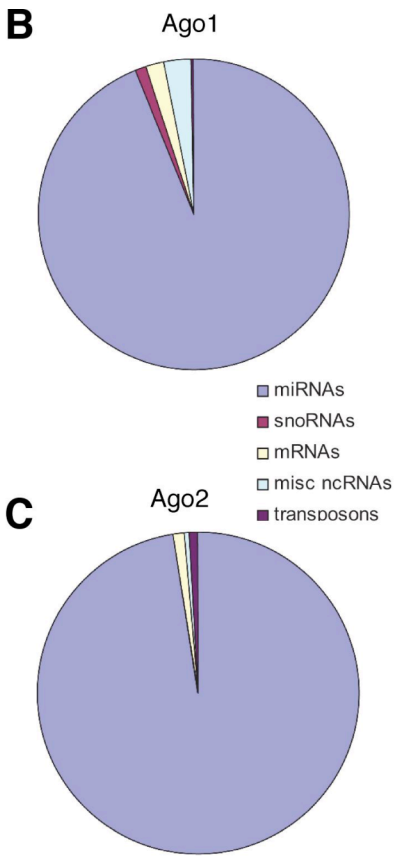
Similarly, in *C. elegans* small RNAs from mismatched or perfect precursors are incorporated into the Ago proteins ALG-1 or RDE-1 respectively (Steiner et al., 2007). In humans, four different Ago proteins seem to associate with the same miRNA species regardless of their sequence (Azuma-Mukai et al., 2008; Meister et al., 2004). However the spectrum of Ago-associated small RNAs in human somatic cells is presently not known. Therefore, monoclonal antibodies specific to human Ago1 (4B8) and Ago2 (11A9) were used for Ago isolation from total HEK 293 cell lysates. Co-immunoprecipitated RNAs were extracted and cloned without size fractionation as described in Figure 2.7. Moreover, the new deep sequencing technologies bring major advantages, as there is no need for cloning into bacterial vectors and for amplification and purification of individual templates. Briefly, the DNA library is mixed with capture beads (carrying complementary sequences to the library adapters), PCR reagents and oil, creating an emulsion. The oil droplets function as tiny microreactors where the DNA is amplified, still immobilized on the beads. After the PCR reaction is complete, the DNA containing beads are isolated and placed into a PicoTiterPlate for sequencing. Each Plate contains 1.6 million wells and only a single capture bead fits into each well. After loading of tiny enzyme beads, the plate is placed into the 454 Sequencing System instrument. Bases are flown sequentially and always in the same order across the plate. A nucleotide complementary to the template is incorporated and releases pyrophosphate. Addition of adenosine 5' phosphosulfate (APS) and luciferin allows ATP sulfurylase to convert pyrophosphate and APS to ATP. Luciferin is oxidated by luciferase in the presence of ATP and emits light, which is recorded by a CCD camera. Finally, a software generates a flowgram for each well, which can be further analyzed (454 technology). 20448 sequenced reads were found to be associated with the Ago1 and 42604 reads with the Ago2 library. All reads with known annotation are depicted in a pie chart (Fig. 2.8). More than 80% could be annotated to miRNAs; however there were also some reads mapping to snoRNAs, mRNAs, other non-coding RNAs and transposons (Fig. 2.8A-C). Using a Dicer substrate identification algorithm (miRDeep), the presence of 158 known miRNAs in the combined Ago1 and Ago2 libraries was confirmed (Friedländer et al., 2008). All miRNAs that are present in the libraries bind to Ago1 as well as Ago2 (see Appendix for individual numbers of miRNAs). However, plotting individual reads of Ago1 bound miRNAs against Ago2 bound miRNAs shows that some show a higher relative abundance in one or the other library, suggesting a preferential binding to either Ago1 or Ago2 (Fig. 2.9). This is consistent with the published data on Ago2 and Ago3 miRNA association (Azuma-Mukai et al., 2008). Using miRDeep, 8 new miRNA candidates could be identified, as shown in Fig 2.10. Candidate 7 is particularly interesting as it is localized

in an intron, directly flanked by exons. During its further analysis, this sequence was confirmed to be a miRNA that is Drosha independent but is directly liberated by splicing (Berezikov et al., 2007). Moreover, all new miRNA candidates identified in this study were published by other labs during manuscript preparation of Ender et al. (Ender et al., 2008).

Figure 2.8. Small RNAs associated with Ago1 and Ago2

A

	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
# rRNA	**313**	**52**
# tRNA	**159**	**71**
# scRNA	**2**	**3**
# snRNA	**10**	**4**
# Transposable elements (LINE, SINE, LTR)	**157**	**92**
# Other	**6**	**38**



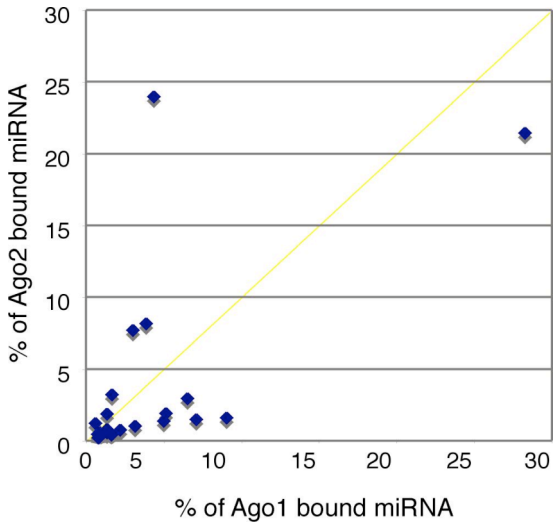
(A) Summary of the sequencing data obtained from deep sequencing of human Ago1 and Ago2 associated small RNAs. (B and C) Schematic representation of individual small RNA classes that are associated with human Ago1 or Ago2 complexes. Only reads with known annotation are represented.

However, although candidate 4 (Fig. 2.10) was annotated as a miRNA it was found during this study that it maps to a sequence annotated as a snoRNA. More intensive analysis revealed that there are in all Ago-associated RNA libraries small RNAs with a length of about 20-22 nt mapping to snoRNAs. Interestingly, the sequenced reads derive only from one half of the snoRNA, either the hairpin formed by the 3' half or the 5' half. Both arms of the individual stems are present in the libraries and the sequence with the lower abundance is indicated as "star" sequences.

2.2.2 SnoRNAs: structure, assembly and function

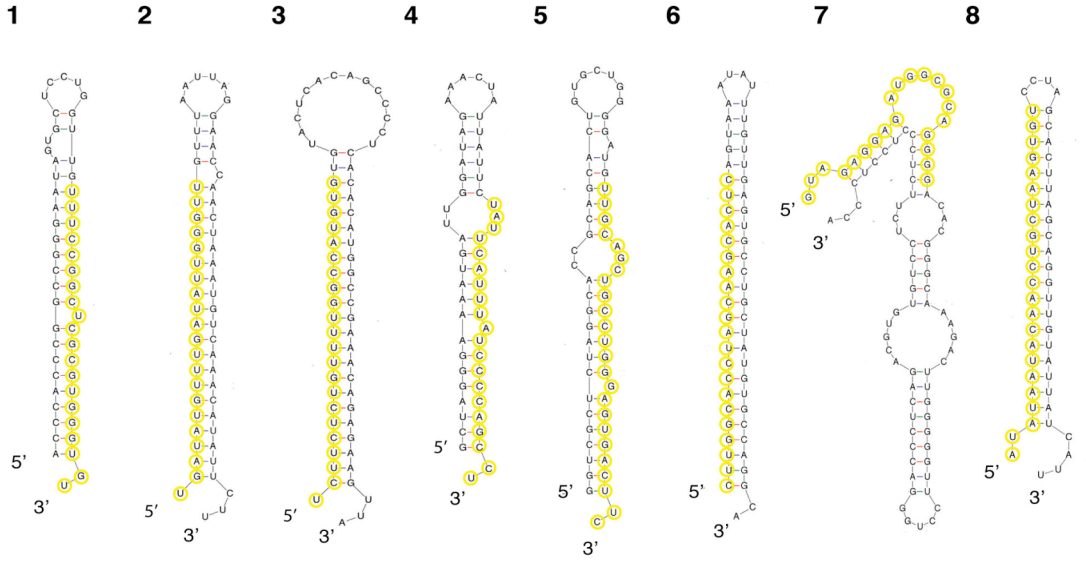
SnoRNAs are small nucleolar RNAs of 60-300 nt in length existing as snoRNA:protein complexes (snoRNPs). They are well conserved and present in archaea as well as in eukaryotes, indicating that they arose over 2-3 billion years ago. SnoRNAs can be divided into two distinct classes: the box C/D and the box H/ACA snoRNAs (Fig 11). Box C/D RNAs are characterized by two sequence motifs, box C (UGAUGA) and box D (CUGA) which direct

Figure 2.9. Analysis of preferential Ago binding of miRNAs



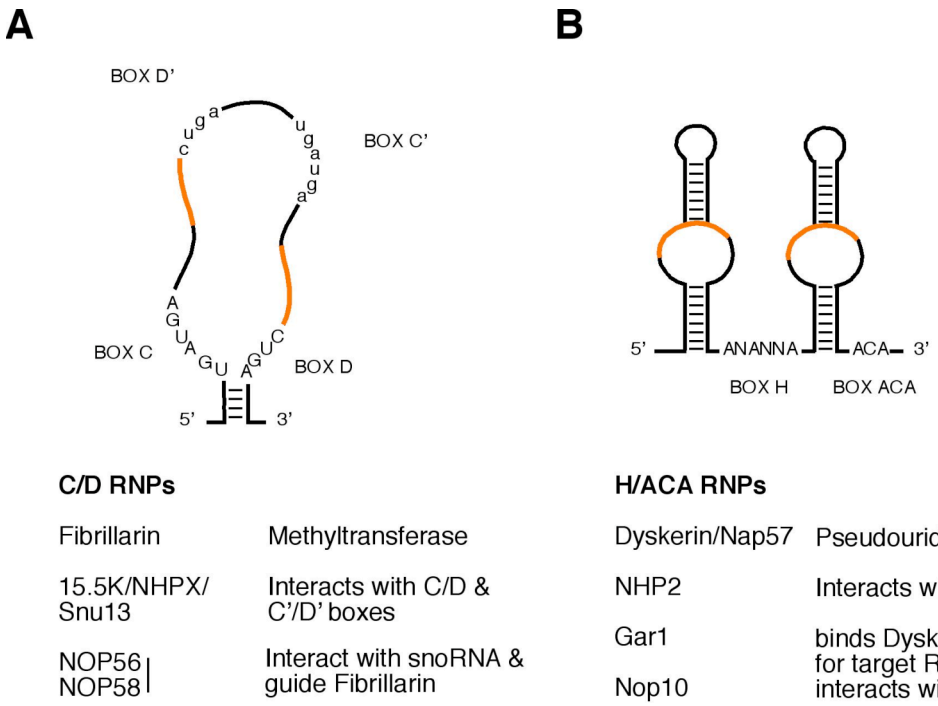
Plotting the 30 most abundant miRNAs immunoprecipitated with Ago1 and Ago2 complexes. All reads annotated as miRNA for Ago1 and for Ago2 libraries respectively, equals 100%. Individual numbers of all miRNA reads are shown in the appendix.

Figure 2.10. New miRNA candidates identified with miRDeep



(1) This candidate precursor is located in an intron in the sense orientation. It has a total frequency of 28 reads mappings. It is now annotated as hsa-miR-1180 (Subramanian et al., 2008).
(2) This candidate precursor is well conserved and is predicted to have a conserved secondary structure. The mature sequence is very similar to the human hsa-miR-190 sequence. It has a read frequency of two reads. It is been annotated as hsa-miR-190b (Landgraf et al., 2007).
(3) The precursor of candidate 3 is located in an intron in the sense orientation. It has a total frequency of 43 reads mapping. It has been annotated as hsa-miR-942 (Lui et al., 2007).
(4) This candidate precursor is well conserved and is located in an intron in the sense orientation. It has a frequency of five reads and has now been annotated as hsa-miR-942 (Lui et al., 2007). Interestingly, it maps to a sequence annotated as snoACA36B (see also figure 2.22).
(5) Candidate 5 is well conserved and is located in an intron in the sense orientation. It has a read frequency of four reads. It was predicted by Berezikov and has been annotated as hsa-miR-1301 (Berezikov et al., 2006; Morin et al., 2008; Zhu et al., 2009).
(6) This candidate precursor is well conserved and is located in an intron in the sense orientation. It is predicted to have a conserved secondary structure. The candidate mature sequence shares the nucleus sequence with the miR-96 family, but is not conserved beyond the nucleus. It has a read frequency of 14. It has been annotated as hsa-miR 1271 (Morin et al., 2008; Nygaard et al., 2009).
(7) The mature and star sequences are conserved. It is located in an intron, flanked directly by exons. The read frequency is 18, and both mature and star sequences are represented. It has been annotated as hsa-miR 877 and later identified as a mammalian mirtron (Berezikov et al., 2007; Landgraf et al., 2007).
(8) This candidate precursor is well conserved and located in an intron in the sense orientation. It is predicted to have a conserved secondary structure. The mature sequence is very similar to members of the miR-374 family. It forms a cluster with the known hsa-miR-421. It has a read frequency of 42 reads and has been annotated as hsa-miR-374b (Landgraf et al., 2007).

Figure 2.11. Classes of snoRNAs and their protein partners



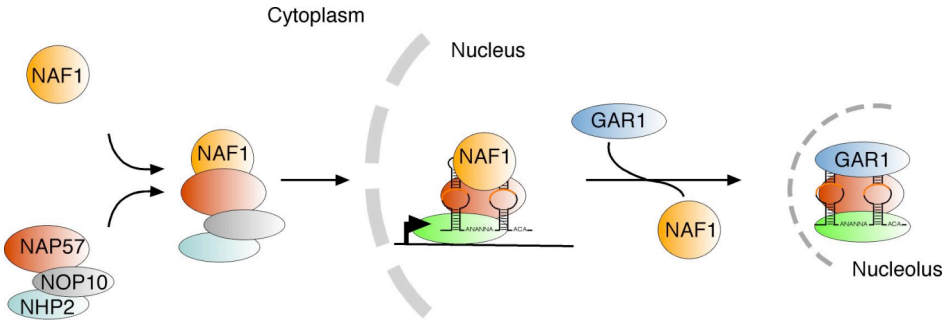
C/D RNPs	
Fibrillarin	Methyltransferase
15.5K/NHPX/ Snu13	Interacts with C/D & C'/D' boxes
NOP56 NOP58	Interact with snoRNA & guide Fibrillarin

H/ACA RNPs	
Dyskerin/Nap57	Pseudouridine synthase
NHP2	Interacts with snoRNA
Gar1	binds Dyskerin & is important for target RNA interaction
Nop10	interacts with Dyskerin

Schematic representation of snoRNA structures. SnoRNAs can be divided into two major classes. On top the structure of the two classes are shown and below the protein partners are listed. (A) Box C/D snoRNA share the sequence motif PuUGAUGA (box C) and CUGA (box D) located near the 5`and or 3' end respectively. In addition, most snoRNAs contain additional C- and D- like motifs termed boxes C' and D'. Sequences depicted in orange are usually complementary to conserved sequences of rRNAs. (B) Box H/ACA snoRNAs are characterized by the sequence motif ANANNA (box H) and ACA (box ACA). Box ACA is usually positioned three nt upstream from the 3' end. H/ACA snoRNAs show a hairpin-hinge-hairpin-tail secondary structure where each hairpin forms two duplexes with the rRNA (pseudouridinylation pocket, indicated in orange). It is possible that only one of the two antisense regions is functional, however the two modules do not function independently as it is observed for C/D box snoRNAs. Figure 2.11 is adapted from Dragon et al. (Dragon, 2006).

binding of fibrillarin, Nop56p, Nop58p and 15.5K/NHPX/Snu13 snoRNP proteins. Box H/ACA carry the sequence motif box H (ANANNA – with N = any nucleotide) and ACA and are associated with Dyskerin/Nap57, NHP2, Gar1 and Nop10. Three of the four proteins are already associated with the H/ACA RNA during transcription. In addition, the nuclear assembly factor-1 (NAF1), which already associates with Nap57 in the cytoplasm, binds to the transcript. This pre-RNP is inactive until NAF1 is exchanged for GAR1, which only binds to functionally engaged snoRNPs (Fig. 2.12) (Darzacq et al., 2006; Matera et al., 2007).

Figure 2.12. Human H/ACA RNP assembly



NAF1 associates with NAP57 and probably also already with NOP10 and NHP2 in the cytoplasm. The complex is recruited to the nucleus to the site of H/ACA RNA transcription where NAF1 is replaced by GAR1, leading the mature RNPs into the nucleolus

SnoRNAs are involved in the processing or modification of ribosomal RNAs (rRNAs). The small and large subunit rRNAs are transcribed as part of a large precursor. After post-transcriptional modifications the precursor is subsequently cleaved and trimmed to release mature 18S, 5.8S and 25-28S rRNAs. The modified regions are key regions of rRNAs and are essential for ribosome function. Most snoRNAs are rather involved in modification than in processing of pre-rRNAs. C/D RNAs direct 2'O-ribose methylation. H/ACA RNAs guide pseudouridinylation (conversion of uridine to pseudouridine). Forming direct base-pairing interactions with the substrate RNA, a feature that is also known for miRNAs, specifies the sites of modification. Both 2'-O-methylation and pseudouridinylation change the chemical nature of RNA and therefore its structure. Besides rRNA maturation, snoRNAs are also involved in snRNA modifications in eukaryotes, transfer RNAs in Archaea, spliced leader RNAs in trypanosomes and for telomere synthesis (Darzacq et al., 2002); (Dennis et al., 2001); (Uliel et al., 2004); (Collins, 2006). Moreover, there is still a high number of orphan snoRNAs that do not target established substrates indicating that snoRNAs also function on targets and in processes that remain to be identified.

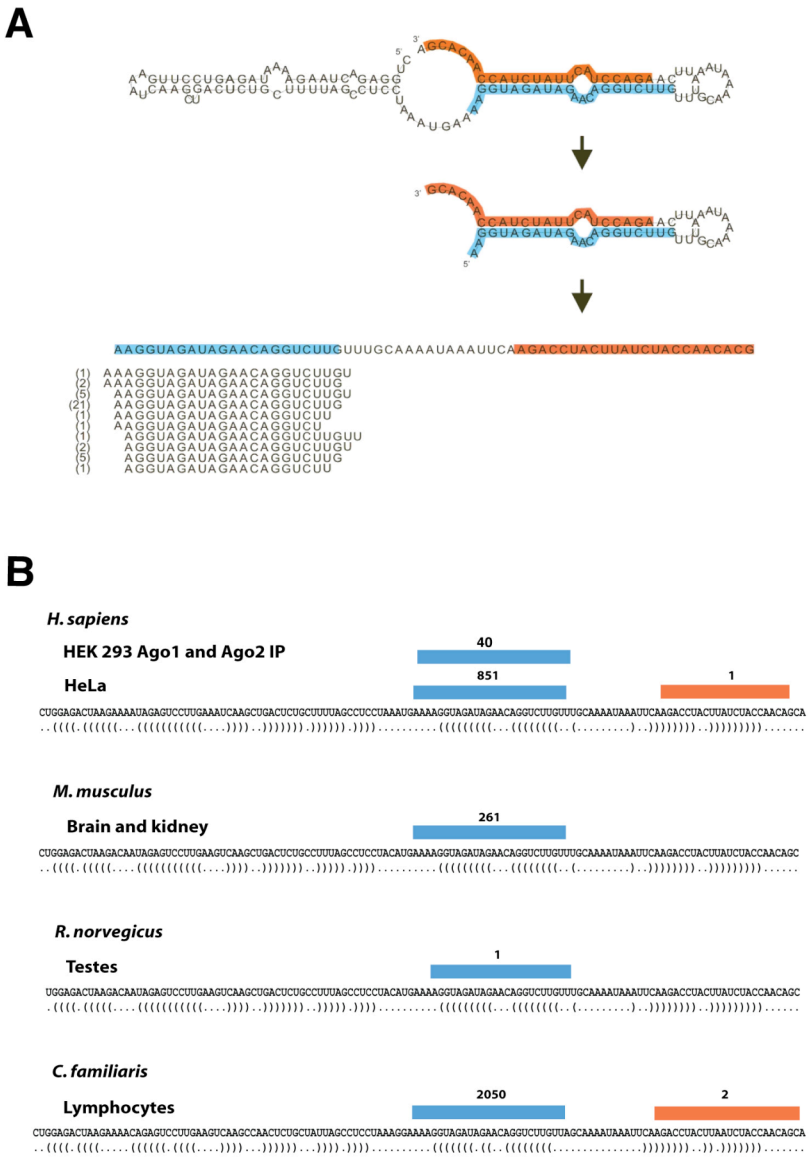
In contrast to yeast, most vertebrate snoRNAs are encoded in introns. Many derive from polycistronic transcripts containing several RNAs and only a few mammalian snoRNAs are known to be located in individual genes with their own promoters, typically transcribed by polymerase II. Genes containing intronic snoRNAs are often encoding proteins involved in ribosome assembly or nucleolar processes. However, the spliced RNA of some snoRNA host

genes do not have protein encoding potential and the functional product of these genes is contained in introns only (Dragon, 2006). Individual snoRNAs are liberated by splicing. After splicing, the intron lariat is degraded by exonucleases from both ends but trimming stops at the end of the snoRNA border, probably due to proteins bound to the RNA, together building the snoRNP. Boxes C/D, H and ACA may serve as binding sites for the snoRNP proteins. However, a few snoRNAs are not liberated by splicing but rather excised from the intron by endonucleases and subsequently trimmed by exonucleases. As a result of trimming, mature snoRNAs carry a 5' phosphate and a 3' OH.

2.2.3 ACA45 processing products are conserved across species

In the Ago-associated RNA libraries there were reads of about 22nt in length originating from snoRNAs, the most abundant one matching to snoRNA ACA45 (Fig 2.13A). This particular snoRNA was therefore chosen for further and more detailed studies. The sequenced reads of ACA45 derive only from the 3' half of the snoRNA. To look for sequence conservation, the reads were mapped to different Solexa libraries made from total RNA. 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 (submitted to Gene Expression Omnibus database, GEO accession number GSE10829) (Friedlander et al., 2008). The mouse data sets were produced by deep sequencing of small RNAs from mouse brain and kidney tissues (unpublished data) and rat data was produced by deep sequencing of column-purified small RNAs from testes extracts, all of them using the 454 technology (GEO accession number GSE5026) (Lau et al., 2006). The last data set was obtained by sequencing small RNAs from dog lymphocytes using Solexa technology (GEO accession number GSE10825) (Friedlander et al., 2008). Mapping the snoACA45 reads to these different Solexa libraries revealed that they are conserved in mammals, suggesting that they are indeed specific processing products (Fig. 2.13B).

Figure 2.13. Conserved reads mapping to snoRNA associate with Ago1 and Ago2 libraries



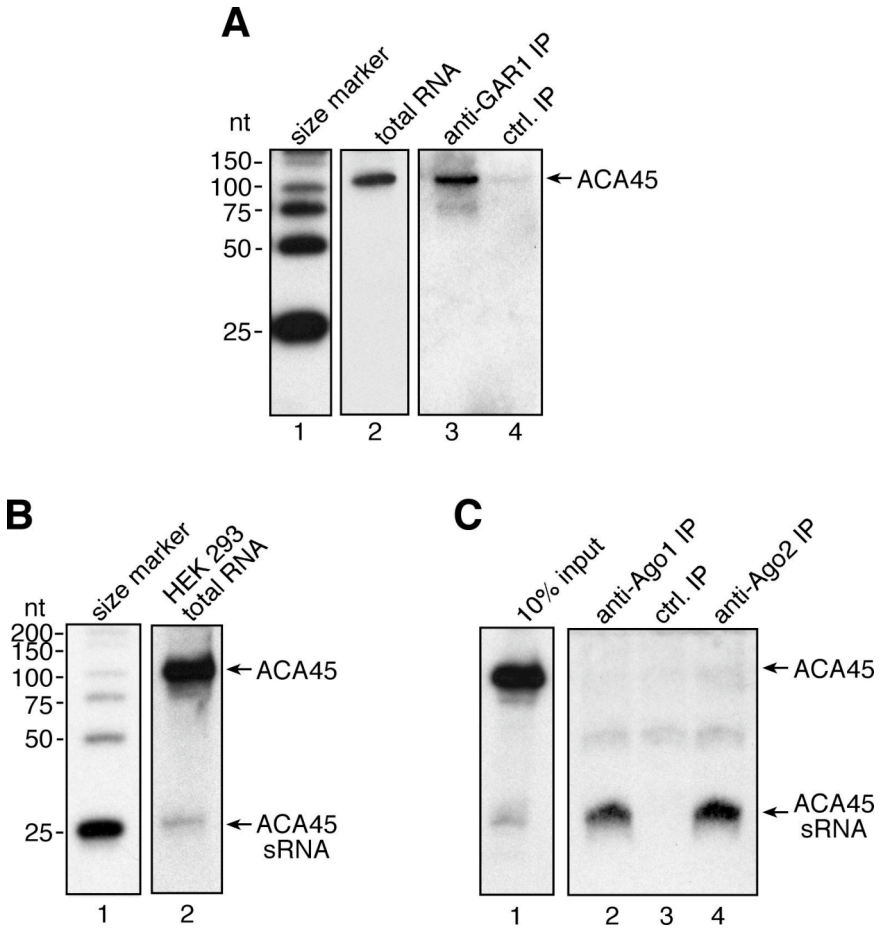
(A) Sequence reads found in Ago1 and/or Ago2 libraries are highlighted in orange and blue. Numbers of individual sequences are shown in parentheses. (B) Specific ACA45-processing products are conserved across species. The ACA45 sequences for *homo sapiens*, *rattus norvegicus*, *mus musculus*, and *canis familiaris* are shown by sequence and structure. Independent deep sequencing data from each species have been obtained and mapped against the corresponding homolog. The bars indicate the positions of mapped reads. 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.

2.2.4 SnoRNA ACA45 is processed to small RNAs

Homo sapiens ACA45 is predicted to guide pseudouridinylation of residue U37 for the spliceosomal U2 snRNA (Shibata et al., 1975). As a modification guide of RNA pol II-transcribed spliceosomal snRNA it presumably resides in Cajal bodies and is also known as scaRNA15. Cajal bodies are subcellular regions specialized for aspects of RNA biogenesis and function. ACA45 is 127nt in length and encoded within the large intron II (18497 bp) of the fibronectin type III and SPRY domain-containing protein 2. Although ACA45 was identified in a screen for functional snoRNAs, it is possible that it represents a miRNA gene that has been mis-annotated as a snoRNA (Kiss et al., 2004). In order to prove that ACA45 is indeed a functional snoRNA, GAR1 binding of ACA45 was analyzed (Fig. 2.14A). Endogenous GAR1 was immunoprecipitated from HEK293 total lysate using anti-GAR1 antibodies. Associated RNAs were extracted and further analyzed by northern blotting using a probe specific to ACA45. Indeed, full-length ACA45 was readily detectable in the anti-GAR1 but not in control immunoprecipitates. These data confirm that ACA45 represents a functional snoRNA.

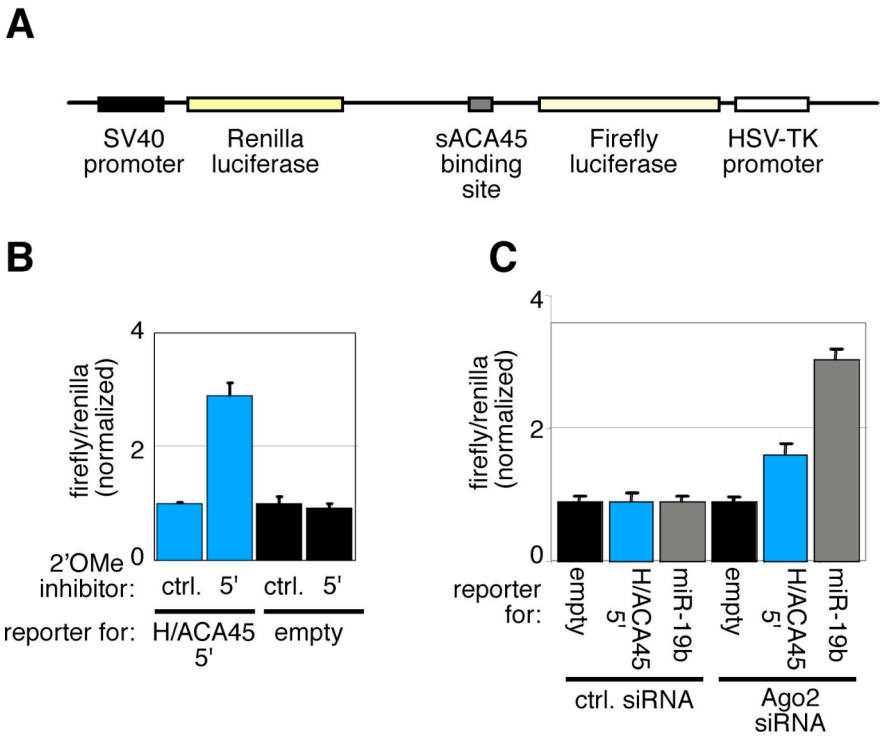
Fragments of about 22 nt in length mapping to the 3' half of ACA45 were identified in the libraries of immunoprecipitated Ago1 and Ago2 complexes. The reads are conserved in different libraries sequenced from mammals, indicating that they are specific processing products. The processing of ACA45 to small RNAs could be validated by northern blotting (Fig. 2.14B). A probe complementary to the prominent sequence in the 5' arm (Fig. 2.13A, blue) detected the full-length ACA45 snoRNA as well as a band of approx. 22-23 nt in total RNA indicating that part of the cellular ACA45 pool is indeed processed to small RNAs. Since only a minor portion of ACA45 is processed to small RNAs it was furthermore investigated if ACA45 processing products are specifically enriched in Ago protein complexes. Endogenous Ago1 or Ago2 were immunoprecipitated from HEK293 total lysate using anti-Ago1 and anti-Ago2 antibodies and associated RNAs were analyzed by northern blotting against ACA45 processing products (Fig. 2.14C). Consistently with the cloning data, the small RNA derived from ACA45 was indeed enriched in Ago1 as well as Ago2 immunoprecipitates, indicating that ACA45 processing products specifically associate with Ago proteins. This functional small RNA was therefore termed ACA45 small RNA (ACA45 sRNA).

Figure 2.14. SnoRNA ACA45 Is processed to small RNAs and associates with Ago1 and Ago2



(A) 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 small RNA. Lane 2 shows total RNA extracted from 10% of the input lysate, and lane 1 shows a size marker. (B) 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. (C) Endogenous Ago1 (lane 2) or Ago2 (lane 4) were immunoprecipitated from HEK293 lysates, and the coimmunoprecipitated RNAs were extracted and analyzed by northern blotting. Lane 1 shows 10% of the extracts used for the immunoprecipitations, and lane 3 shows a control immunoprecipitation.

Figure 2.15. ACA45 sRNA can mediate RNA cleavage



(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 pre- transfected with control siRNAs and siRNAs against Ago2. Firefly luciferase activity was normalized to renilla activity. Error bars are derived from four individual experiments.

2.2.5 ACA45 small RNAs can function like miRNAs

Since ACA45 processing products are similar to miRNA precursors, it was investigated if ACA45 sRNAs are also functionally similar to miRNAs. To analyze this, a luciferase reporter construct containing a Renilla luciferase and a complementary binding site for the abundant 5' arm of the snoRNA precursor downstream of a Firefly luciferase was generated (Fig. 2.15A). The luciferase plasmid was cotransfected together with 2' O-methylated (2' OMe) antisense inhibitors against ACA45 sRNA into HEK293 cells. Luciferase activity was strongly increased when endogenous ACA45-derived small RNAs were inhibited compared to cotransfection with a control 2' OMe

oligo (Fig. 2.15B). Therefore, it can be concluded that the reporter construct cannot be cleaved upon inhibition of ACA45 sRNA. To analyze if the reporter cleavage is Ago2 dependent, the luciferase reporter was cotransfected with siRNAs against Ago2 and was compared to cotransfection with a control siRNAs. Indeed, luciferase activity was also increased, indicating that sACA45 is not cleavage competent upon Ago2 depletion (Fig. 2.15C). In summary, these data demonstrate that ACA45 small RNA can function like a miRNA.

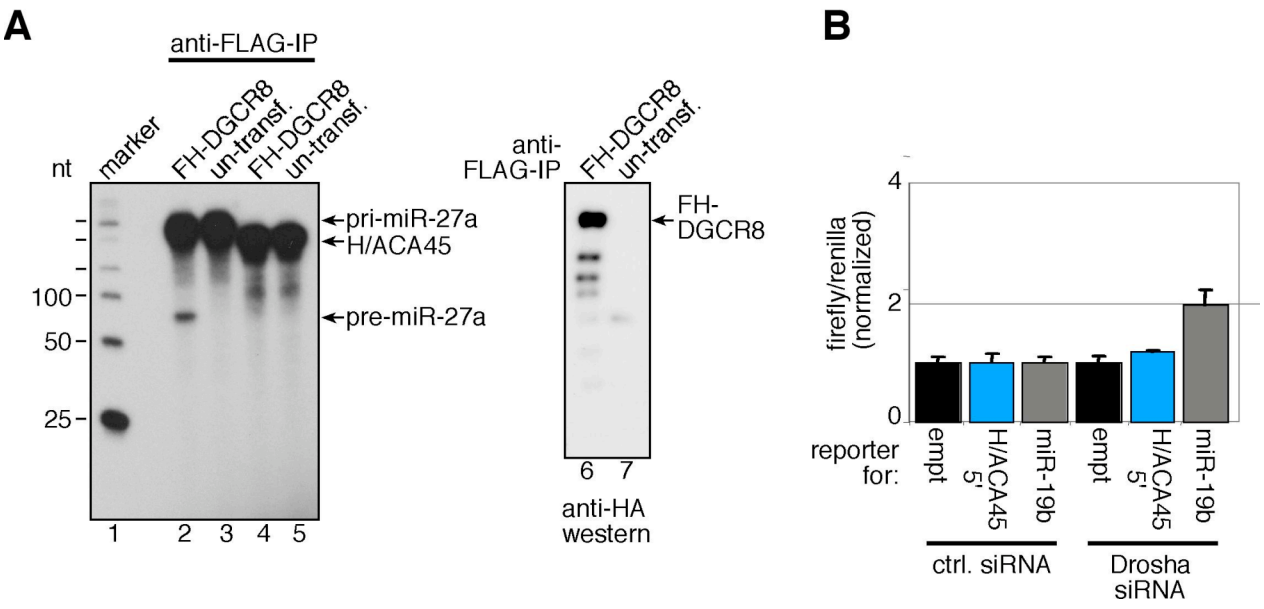
2.2.6 ACA45 processing is independent of the Drosha/DGCR8 complex

Similar to miRNAs, ACA45 sRNA can cleave reporter constructs containing a perfectly complementary binding site in an Ago2 dependent manner. It was therefore intriguing to investigate whether also the processing of ACA45 is linked to the miRNA pathway. Primary miRNAs are usually cleaved by the microprocessor complex, containing the RNAse III enzyme Drosha and its binding partner DGCR8, into smaller precursors. The cleavage signature of the stem-loop-structured processing intermediate is different from the typical 2 nt 3' overhangs generated by Drosha. However, Drosha and DGCR8 complex requirements for ACA45 processing were tested by using *in vitro* as well as *in vivo* approaches.

Plasmids expressing FLAG-HA-DGCR8 were transfected into HEK293 cells. After immunoprecipitation using anti-Flag antibodies purified proteins were incubated with either a ^{32}P-labeled primary miR-27a transcript or ACA45 and cleavage products were analyzed by denaturing RNA-PAGE (Fig. 2.16A). A specific cleavage product representing pre-miR-27a was observed in the anti-FLAG-HA-DGCR8 immunoprecipitates whereas no signal was observed when ACA45 was used as substrate.

Drosha requirement was further investigated using the luciferase reporter construct described above (Fig. 2.16B). The reporter construct was cotransfected with siRNAs against Drosha and control siRNAs. Consistently with the *in vitro* results there was no elevated luciferase activity upon Drosha depletion, whereas luciferase activity of a miR-19b-responsive reporter was significantly increased. Taken together, the described results suggest that ACA45-processing is independent of the Drosha/DGCR8 complex.

Figure 2.16. ACA45 processing is independent of the microprocessor complex



(A) 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. (B) The luciferase reporter described in 2.15A, the empty vector, and a luciferase reporter containing a complementary binding site to miR-19b were transfected into HEK293 cells that have been pre-transfected with control siRNAs and siRNAs against Drosha. Firefly luciferase activity was normalized to renilla activity. Error bars are derived from four individual experiments.

2.2.7 ACA45 processing requires Dicer

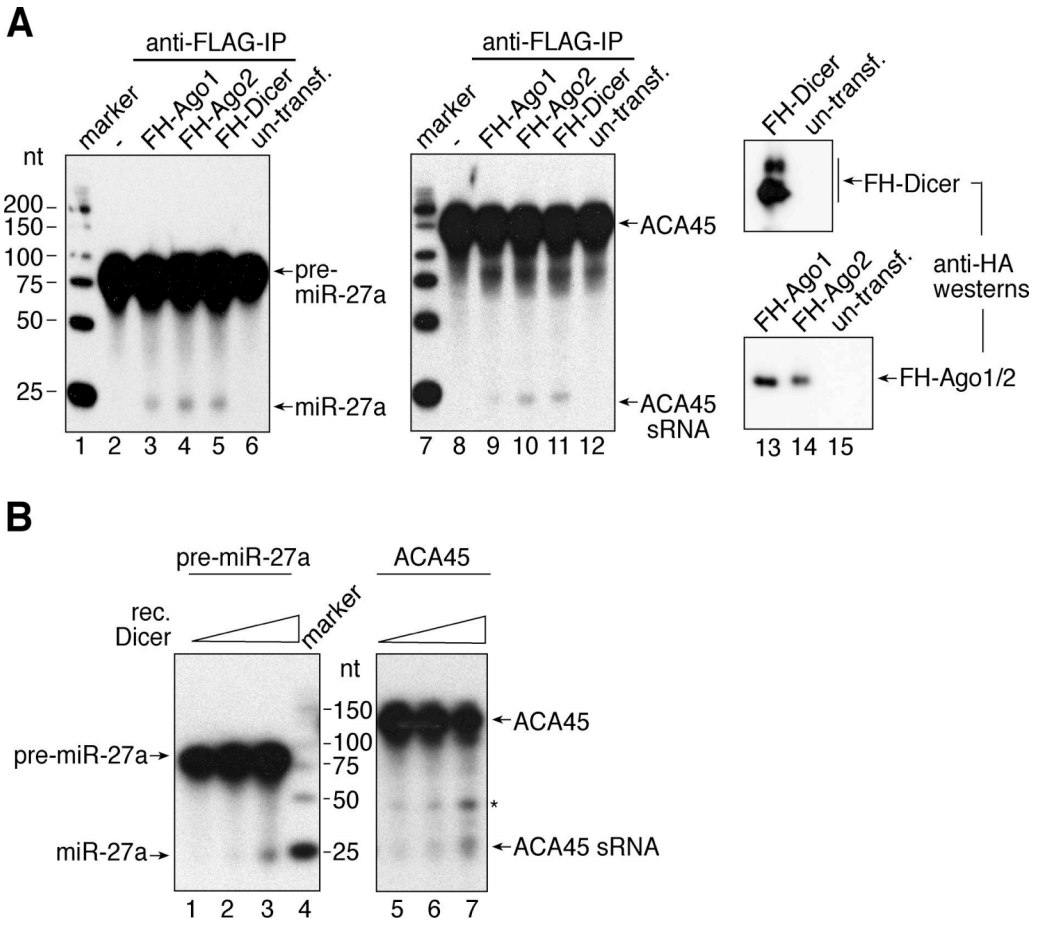
After processing by the microprocessor complex and export to the cytoplasm pre-miRNAs are further processed by Dicer into small RNAs duplexes. The PAZ domain of Dicer preferentially recognizes 2 nt 3' protruding ends. However, it was shown that with RNA tetraloops terminally blocked dsRNA can be cleaved internally (Zhang et al., 2004). Since ACA45 processing is independent of Drosha and DGCR8 it was investigated whether Dicer is required and might even be sufficient to cleave ACA45 into small RNAs.

As it was shown in the first part of this work, Dicer activity can be coimmunoprecipitated with Ago proteins. Therefore, plasmids expressing Flag-HA-Ago1 (FH-Ago1), Flag-HA-Ago2 (FH-

Ago2) or Flag-HA-Dicer (FH-Dicer) were transfected into HEK293 cells and immunoprecipitated using anti-HA antibodies. The complexes were incubated with a ^{32}P labeled pre-miR-27a or full-length ACA45 and analyzed on a denaturing RNA-PAGE (Fig. 2.17A). As expected, both Flag-HA-Ago2 and Flag-HA-Dicer immunoprecipitates efficiently processed the pre-miR-27a. Furthermore, Flag-HA-Ago1, Flag-HA-Ago2 and Flag-HA-Dicer immunoprecipitates processed the ^{32}P labeled full-length ACA45 as well, suggesting that Dicer is required for generation of ACA45 small RNAs.

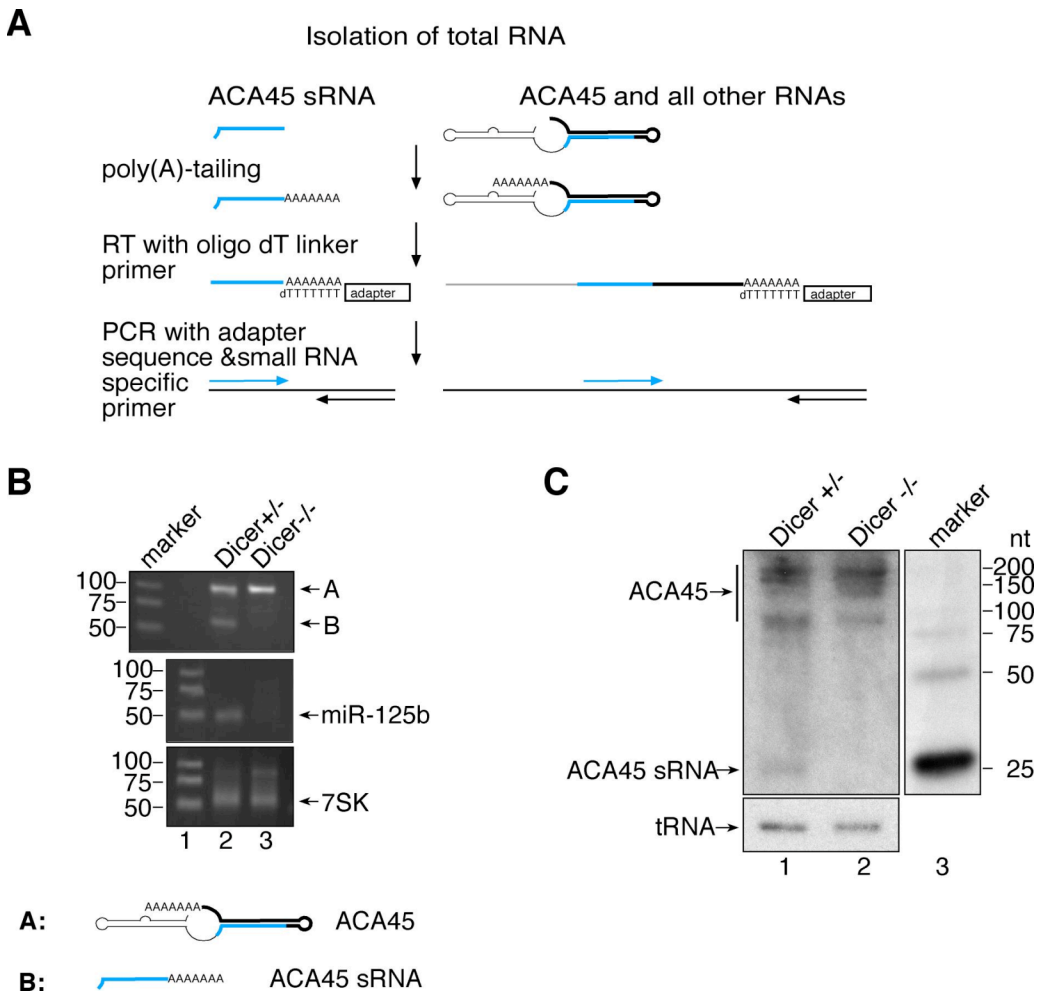
Notably, Dicer generates longer RNAs as well, which might represent processing intermediates (see asterisk in Figure 2.17A). To further investigate Dicer's function in ACA45 processing, it was analyzed if Dicer alone is sufficient for ACA45 processing *in vitro*. ^{32}P-labeled ACA45 was incubated with increasing amounts of recombinant Dicer and cleavage products analyzed by RNA-PAGE (Fig. 2.17B). 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. Finally, the role of Dicer in ACA45 processing was analyzed *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 semi-quantitative real time PCR (qRT-PCR) (Fig. 2.18A). Strikingly, no PCR product was detectable in the Dicer -/- cells, whereas a PCR product originating form the ACA45 small RNA was readily detectable in Dicer +/- cells (Fig. 2.18B). 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 (Fig. 2.18C). In summary, these data indicate that Dicer processes ACA45 to small RNAs independently of the Drosha-containing microprocessor complex.

Figure 2.17. Dicer can process ACA45 into small RNAs



(A) Flag-HA-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. (B) ^{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.

Figure 2.18. Processing of ACA45 requires Dicer

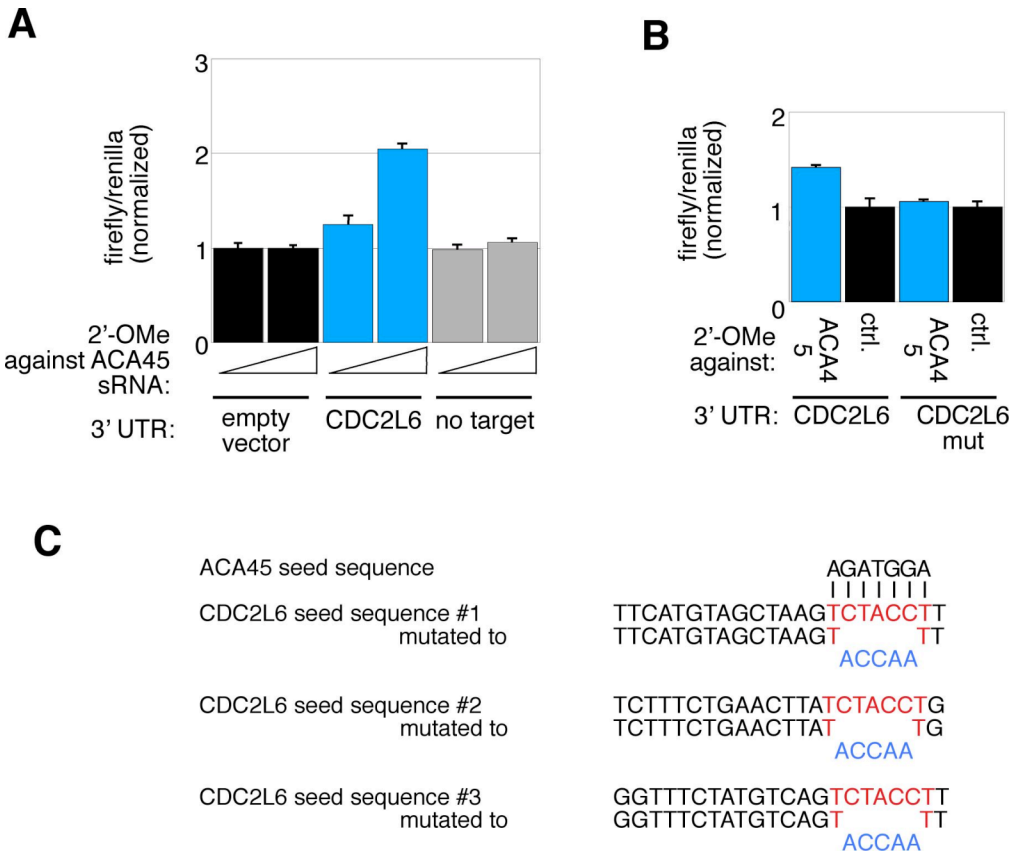


(A) RT PCR of small RNAs. After poly(A)-tailing of the RNAs, an oligo dT linker primer with a unique universal sequence (adapter) is annealed, followed by reverse transcription. The obtained cDNA can then be amplified using specific primers for the small RNA and the universal sequence. (B) Total RNA 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. (C) 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.

2.2.8 Validation of an endogenous ACA45-derived small RNA target

It is thought that complementary Watson-Crick base pairing of the seed sequence (nucleotides 2-8 counted from the 5' end) is a key feature of miRNA:mRNA target recognition. It is also known that highly conserved 7mers 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, several target mRNAs could be predicted for the ACA45-derived small RNA (Krek et al., 2005). For experimental validation, a number of 3' UTRs selected from the predicted target mRNAs were fused to the luciferase reporter gene described above. Luciferase reporter constructs were cotransfected with 2'-OMe oligonucleotides antisense to the ACA45 small RNA. Many of the tested 3' UTRs did not respond to the 2'-OMe inhibitors suggesting that the small RNA does not target these mRNAs or that the small RNA – target mRNA interactions are not relevant in the tested cell line. Strikingly, the activity of the luciferase reporter fused to the CDC2L6 (CDK11) 3' UTR is increased when the endogenous ACA45 small RNA is inhibited (Fig. 2.19A). The CDC2L6 gene product is a component of the mediator complex and therefore important for transcription (Conaway et al., 2005a; Conaway et al., 2005b). For further validation of ACA45 sRNA effects on CDC2L6 expression, all predicted ACA45 sRNA binding sites were mutated in the CDC2L6 3' UTR (Fig. 2.19C). Indeed, a luciferase reporter containing the mutated CDC2L6 3' UTR was not upregulated when endogenous ACA45 sRNA was inhibited (Fig. 2.19B), indicating that ACA45 sRNA seed sequence matches are important for CDC2L6 expression. In summary, these data demonstrate that ACA45 is processed to a small RNAs that can function like a miRNA on the endogenous target CDC2L6, identifying the ACA45 sRNA as potential transcriptional regulator in human cells.

Figure 2.19. ACA45-derived small RNAs regulated CDC2L6 expression

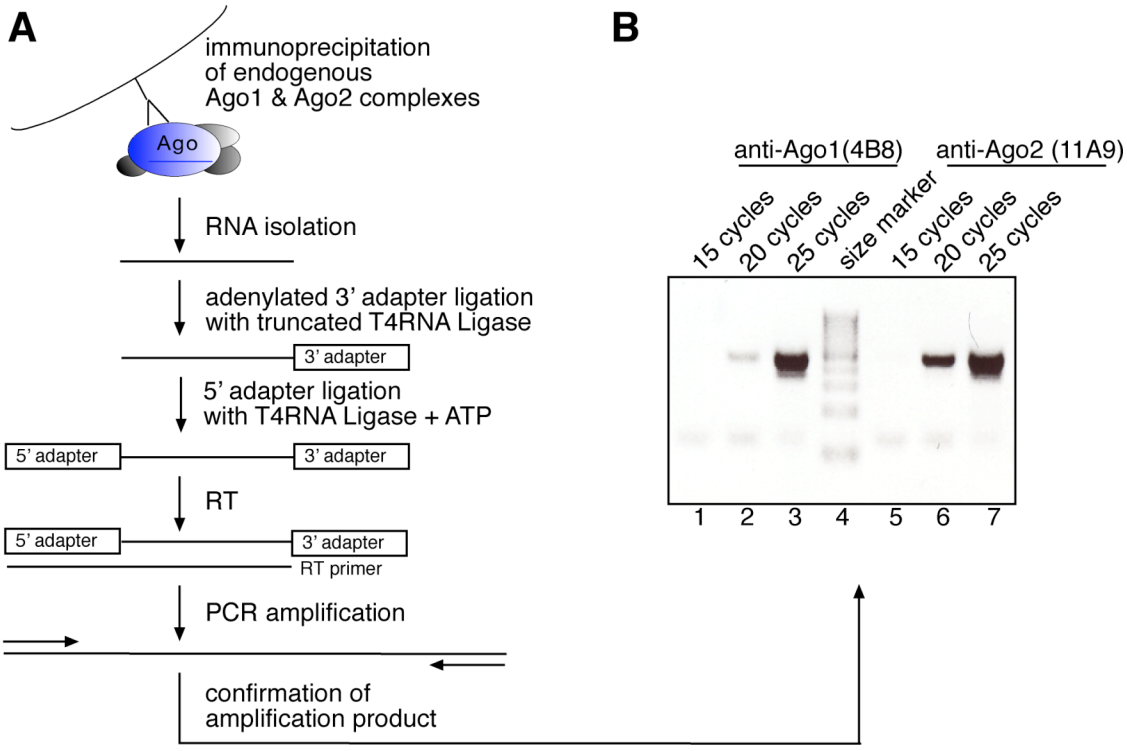


(A) Luciferase reporter constructs containing the 3' UTR of CDC2L6 or BAP-1 (no target) or the empty vector were cotransfected with increasing concentrations of 2' -OMe inhibitors against the ACA45 sRNA. Firefly luciferase activity was normalized to Renilla activity. Error bars are derived from four 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. (C) Mutated ACA45 sRNA-Binding Sites of the 3′UTR reporter construct. The seed sequence of the ACA45 sRNA is shown in the upper panels and the binding sites on the CDC2L6 3'UTR are displayed in the middle panels. Mutated binding sites are shown in the lower panels. Seed sequence binding sites and mutated nucleotides on the CDC2L6 3'UTR are highlighted in red and blue, respectively.

2.2.9 Small RNA processing signatures are not restricted to snoACA45

The findings that ACA45 is processed into a small RNA repressing CDC2L6 led to the question whether this could be a more general fate for snoRNAs. The numbers of orphan RNAs that do not apparently target established substrates indicates that snoRNAs might indeed be involved in more cellular pathways. For a more comprehensive study anti-Ago1 and anti-Ago2 antibodies were used for Ago isolation from total HEK 293 cell lysates. After isolation, co-immunoprecipitated RNAs were fractioned into RNA fragments of 19 – 24 nt in length and cloned as described in Fig. 2.20.

Figure 2.20. Cloning and library generation of small RNAs for Solexa Sequencing



(A) Endogenous Ago1 and Ago2 complexes were immunoprecipitated using the specific monoclonal antibodies described in Figure 2.6. RNA was size fractionated between 19 and 24 nt. The preadenylated 3' adapter was ligated to the 3' hydroxyl group of the small RNA using a truncated T4 RNA ligase which is impaired in adenylate transfer from ATP to the 5' phosphate of RNA. The 3' adapter is blocked at the 3' end with a C6 amino linker. 5' adapter ligation is ligated with the classical T4 RNA ligase. After reverse transcription, the cDNA is amplified and analyzed on an Agarose gel after 15, 20 and 25 PCR cycles (B).

The generated libraries were subsequently sequenced by Illumina, which allows a higher quantity of sequence reads in parallel than 454 sequencing. Briefly, the DNA library is immobilized on a flow cell surface. Solid-phase amplification creates up to 1000 identical copies of each template. Because this process does not involve photolithography, mechanical spotting or positioning of beads into wells, very high densities of single-molecule clusters are achieved. Four modified fluorescently labeled nucleotides, primers and DNA polymerase are added. After laser excitation, the emitted fluorescence from each cluster is captured, the first base is identified and the next cycle can start. Finally, the sequencing results can be analyzed with the use of a specific software (Illumina Sequencing technology).

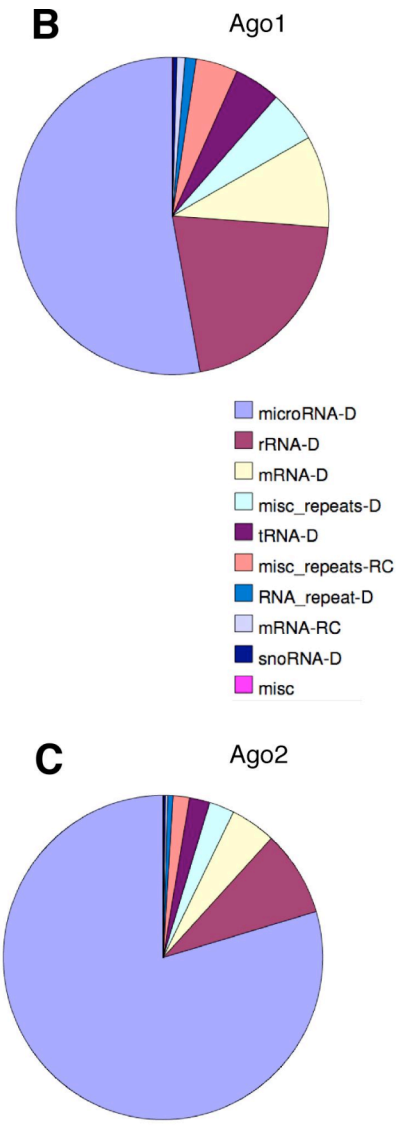
About 5 million reads were found to be associated with the Ago1 and 4.5 million reads with the Ago2 library. All reads which could be annotated are depicted in the pie chart in (Fig. 2.21B/C) and a more detailed overview is given in Figure 2.21A. As for the 454 sequencing results, the majority of the reads could be annotated as miRNAs (Fig 2.21) but there were also reads mapping to snoRNAs, mRNAs, other non-coding RNAs and repetitive elements.

To check if processing of snoRNAs into specific small RNAs is not restricted to ACA45, snoRNA reads from the Solexa library were studied in more detail. From 1909205 annotated reads associated to Ago1 and 2753916 associated to Ago libraries, 7218 or 4951 reads respectively could be mapped to snoRNAs. H/ACA Box RNAs and scaRNAs secondary structure consist of two hairpins. Figure 2.22 shows scaRNAs & H/ACA Box RNAs where at least 80% of all reads mapping to them come from the two strands of a stem-loop structure. Reads originating from stem-loop structures within the snoRNAs ACA45, ACA47, ACA36b, U92, HBI-100, ACA56, ACA3, ACA50 and U3 were identified. All the examples shown have reads in at least two data sets between Ago1-4 (Ago3 and 4 datasets are not discussed in this work) and most often in all four. Not all scaRNAs and H/ACA RNAs seem to have clearly the same processing signature. Most of them have a 3' protruding end. Cleavage seems to occur at the hinge between the two hairpins of the scaRNA or H/ACA RNA. Interestingly, a similar signature, however without 3' protruding end is present in the reads of the CD Box snoRNAs, U3. It has a different secondary structure, but does contain one hairpin. In summary, the 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.

Figure 2.21. Small RNAs associated with Ago1 and Ago2

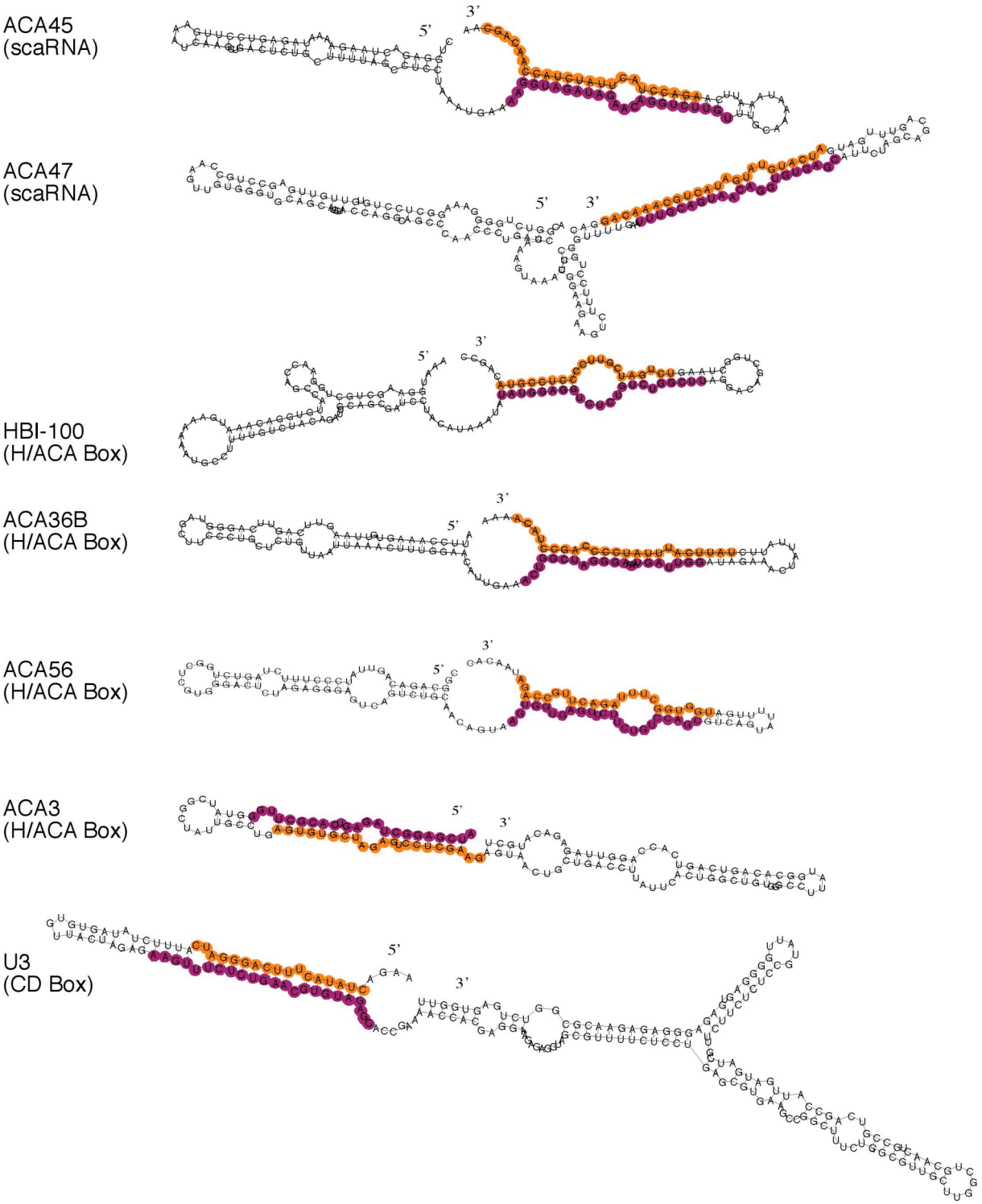
A

	Ago1	Ago2
Total # of reads	**5014986**	**4520829**
Mapped reads	**2163447**	**2891425**
Have known annotation	1909205	2753916
# miRNAs	**1007494**	**2188988**
# sno/scaRNAs	**7218**	**4951**
- CDBox	5454	2546
- HAcaBox	574	513
- scaRNA	1190	1892
# mRNAs	**175982**	**128005**
- 3' UTR	58948	45980
- 5' UTR	20052	13289
- Coding exon	96982	68736
# mRNA RC	**15292**	**5905**
# rRNA	**403406**	**239702**
# tRNA	**92669**	**70751**
# scRNA	**8237**	**4567**
# snRNA	**12840**	**10154**
# repeats	**102554**	**56706**
- LINE	40503	17369
- SINE	32533	23545
- LTR	15788	8194
- others	13730	7598
- LINE RC	28588	18590
- SINE RC	24601	11142
- LTR RC	18110	7974
- others RC	11214	5800



(A) Summary of the sequencing data obtained from deep sequencing of human Ago1 and Ago2 associated small RNAs. (B and C) Schematic representation of individual small RNA classes that are associated with human Ago1 or Ago2 complexes. Only reads with known annotation are represented.

Figure 2.22. Several human snoRNAs show miRNA-like processing signatures



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

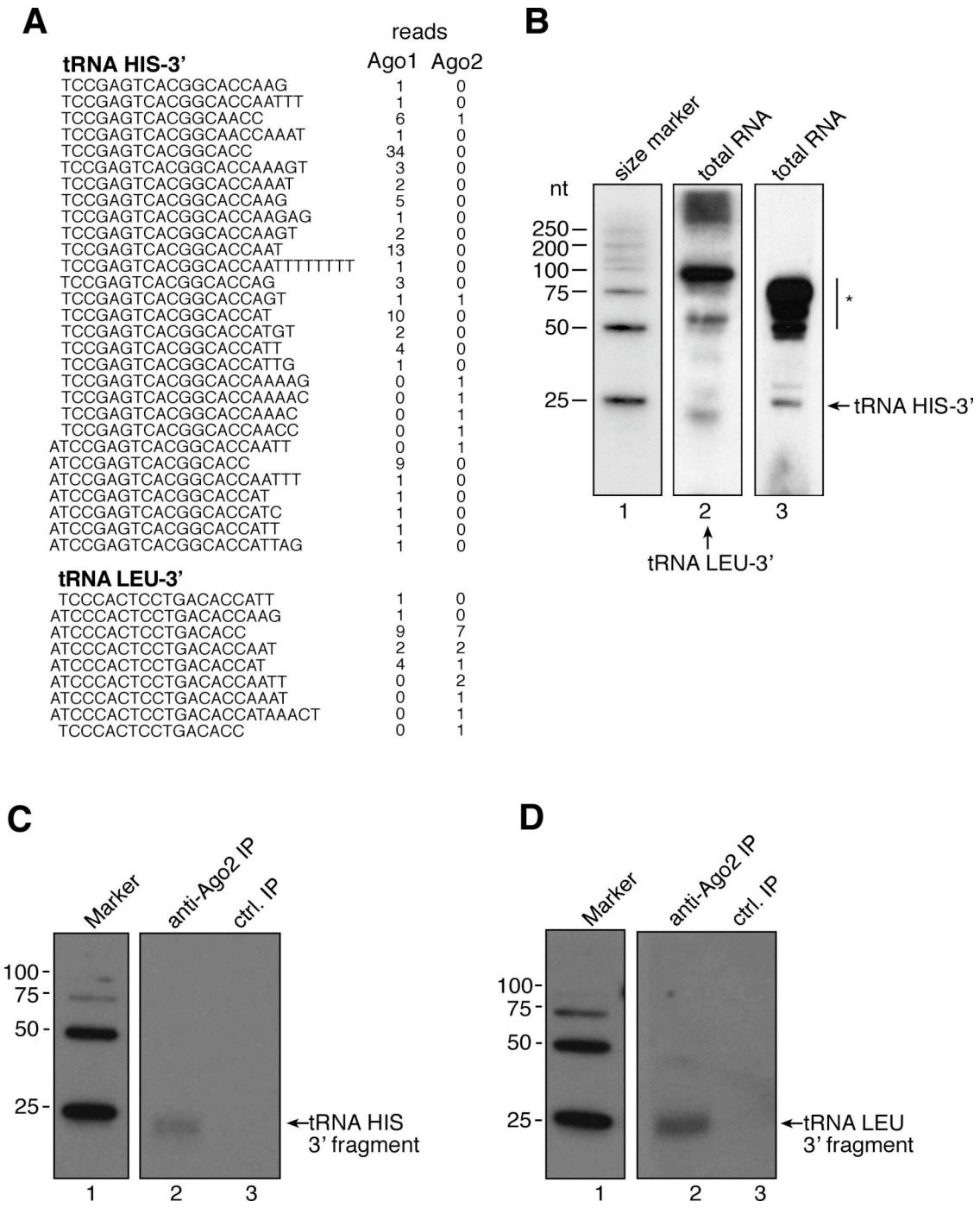
2.2.10 Small RNA reads mapping to tRNAs immunoprecipitate with Ago

Besides reads mapping to snoRNAs a small portion of the Ago-associated small RNAs of 17-22 nt in length were mapping to tRNAs (Fig. 2.8). The expression of two tRNAs referred to as tRNA LEU-3' and tRNA HIS-3' were validated by northern blotting (Figure 2.23B). They both derive from the 3' end of a full length tRNA. Probes complementary tRNA LEU-3' and tRNA HIS-3' detected small RNAs of about 23-25 nt in length indicating that the identified small RNAs are indeed expressed in HEK 293 cells. Much stronger bands with sizes between 50 and 80 nt were observed for both RNAs, which may correspond to processing intermediates and full length tRNAs. It was furthermore investigated if the small tRNA fragments are specifically enriched in Ago protein complexes (Fig. 2.23C/D). Endogenous Ago2 was immunoprecipitated from HEK293 total lysate using anti-Ago2-11A9 antibody and associated RNAs were analyzed by northern blotting against tRNA processing products. Consistently with the cloning data, the small RNAs derived from tRNAs were indeed enriched in Ago2 immunoprecipitates, indicating that tRNA processing products specifically associate with Ago proteins.

2.2.11 tRNA fragments mediate RNA cleavage *in vitro*

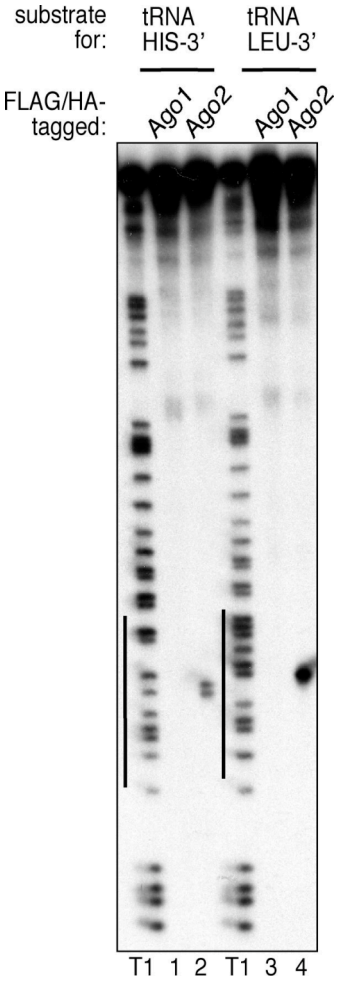
Since tRNA fragments similar in length as miRNAs associate with Ago proteins it was investigated whether they can induce RNA cleavage. As described before, Ago2 has been shown to cleave the target mRNA opposite of nts 10 and 11 of the miRNA (Liu et al., 2004; Meister et al., 2004; Rivas et al., 2005). Therefore, Flag-HA-Ago1 and Flag-HA-Ago2 complexes were immunoprecipitated using anti-Flag antibodies and incubated with a ^{32}P-cap labeled artificial target mRNA bearing a region fully complementary to endogenous tRNA LEU-3' and tRNA HIS-3' fragments. Cleavage products were observed by denaturing RNA-PAGE (Fig. 2.23). Indeed, both endogenous tRNA fragments directed Ago2-mediated cleavage (lanes 2 and 4). Ago1 that is catalytically inactive, does not co-purify with any cleavage activity although both tRNA fragments are found in the Ago1 library. These results suggest that small RNAs are generated from tRNAs that may function in an RNAi-like pathway.

Figure 2.23. tRNA fragments associate with Ago proteins



(A) Sequence reads found in Ago1 and/or Ago2 454 libraries. Numbers of individual sequences are shown. (B) Total RNA from HEK293 cells (5 μg) was blotted, and the membrane was incubated with a probe complementary to tRNA HIS-3' (lane 3) and tRNA LEU-3' (lane 2). A size marker is shown in lane 1. (C; D) Endogenous Ago2 (lane 2) was immunoprecipitated from HEK293 lysates, and the coimmunoprecipitated RNAs were extracted and analyzed by northern blotting using the probe described above. Lane 3 shows a control immunoprecipitation.

Figure 2.24. tRNA fragments can mediate Ago2-dependent cleavage *in vitro*

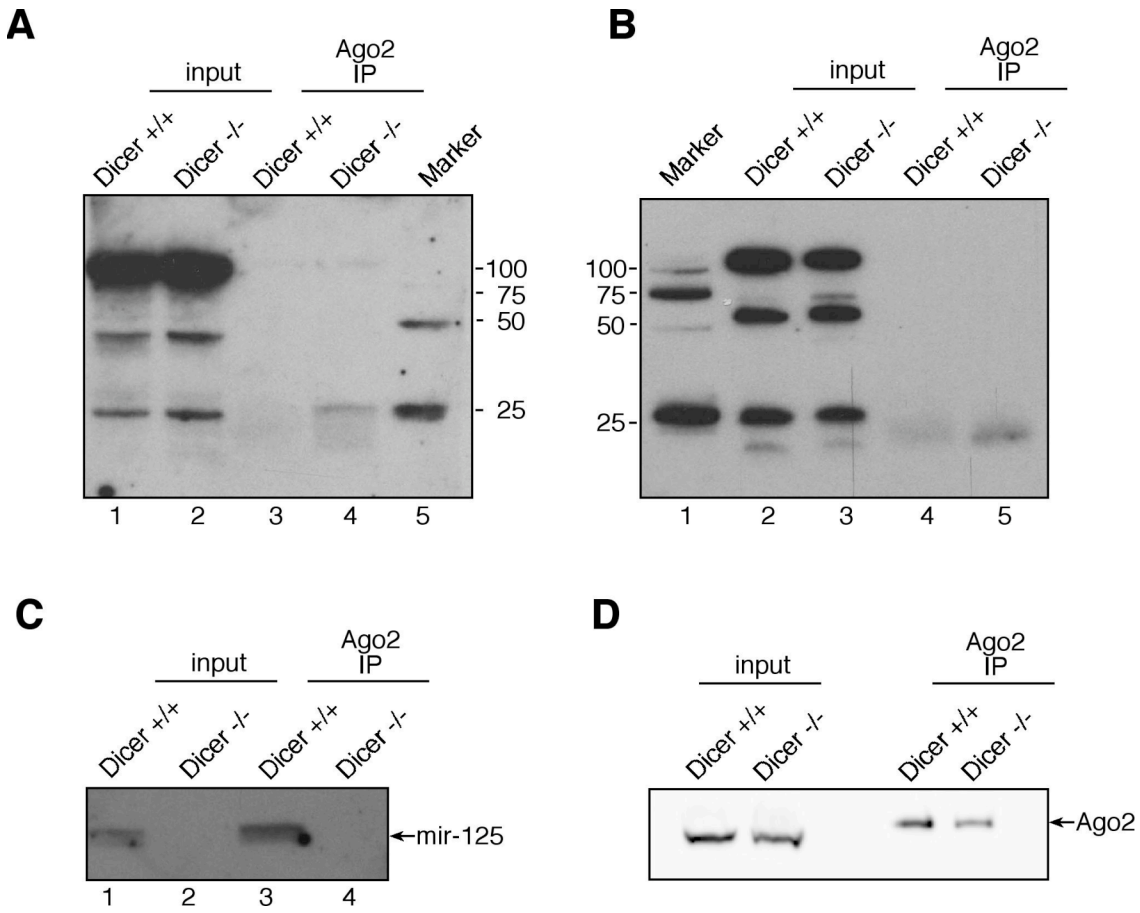


Immunoprecipitated Flag-HA-Ago1 and Flag-HA-Ago2 were incubated with a ^{32}P-cap-labeled RNA, which contained a perfect complementary sequence to the endogenous tRNA HIS-3' and tRNA LEU-3'. Lanes indicated with T1 show RNaseT1 digestions of the RNA substrates. The RNA sequence complementary to tRNA HIS-3' and tRNA LEU-3' is indicated by a black bar on the left.

2.2.12 tRNA processing is Dicer independent

Finally, the role of Dicer in tRNA processing of the two candidates was analyzed *in vivo*. Total RNA from mouse embryonic stem cells carrying homozygous Dicer deletions was analyzed for the presence of tRNA LEU-3' and tRNA HIS-3' fragments by northern blotting. Probes complementary to both tRNA fragments detected small RNAs of about 23-25 nt in length in both wild type and Dicer depleted ES cells, indicating that the processing of tRNAs is Dicer independent. A northern blot using a probe complementary to miR-125 was used as control. As expected, miR-125 can be detected in wild type ES cells but not in Dicer depleted cells. Interestingly, northern blot analysis with immunoprecipitated RNA showed a much stronger signal for tRNA fragments in Dicer knockout cells compared to wild type cells. It is possible that due to the lack of miRNA, Ago has a higher capacity to bind Dicer independent tRNA fragments.

Figure 2.25. tRNA fragments are present in Dicer knockout cells



(A, B) Total RNA and RNA from Ago2 immunoprecipitated complexes from Dicer knockout cells (lane A2 and B3) or ES wild type cells (lane A1 or B2) were analyzed by northern blotting using probes specific for tRNA LEU-3' and tRNA HIS-3' small RNAs. Lane A5 and B1 show a size marker. (C) Northern blot as described above using probes against miR-125. (D) Protein levels of endogenous Ago2 detected by western blotting using anti-Ago2 (6F4).

3 Discussion

3.1 Characterization of Ago-containing protein complexes

Ago protein complexes are core components of RNA silencing effector complexes such as RISC or miRNPs and directly bind to small RNAs. To obtain further insight into the function of human Ago proteins, proteomic and biochemical approaches were performed to characterize so far unkown protein components of human Ago1- and Ago2- containing complexes.

The results in this work indicate that both Ago1 and Ago2 reside in three distinct protein complexes ranging in size from 11S to more than 30S. The smallest complex, termed complex I, contains the largest Ago fraction (40-60%) and has a molecular weight of about 250-350 kDa. Complex II has a molecular weight of about 600-700 kDa or 19S and complex III sediments at a molecular weight of more than 900 kDa or 25-30S. Ago2 complex I shows RISC activity, whereas Ago complexes I and III are associated with Dicer. Interestingly, Ago complex II does not contain RISC and shows little detectable Dicer activity. Consistently with the observations by Dicer assays, Dicer was found in complexes I and III by mass spectrometry. However, low amounts of TRBP are identified only in Ago complex I. It could be possible that active Dicer complexes lacking TRBP exist. It was shown that TRBP knock down cells are still able to process pre-let-7a to mature miRNAs, however to a lesser extend compared to wild type cells. TRBP may facilitate pre-miRNA cleavage by Dicer but is primarily responsible for assembly and functioning of RISC (Haase et al., 2005). However, it is also very likely that TRBP was beyond detection level in the mass spectrometry results of this work.

The Dicer containing complex I shows a huge number of proteins identified by mass spectrometry, which would form a much bigger complex than the actual molecular weight of 250-350 kDa. Indeed, gradient centrifugation shows that complex I consists of three distinct subcomplexes referred to as complex I a-c with different Dicer and RISC activities. Complex Ia might represent Ago2 on its own. Complex 1b associates with Dicer as well as RISC and could therefore be identical with the described trimeric complex Ago, Dicer and TRBP (Gregory et al., 2005). Complex Ic might interact with other proteins identified by mass spectrometry.

Besides Dicer, TRBP and a few already known interactors of Ago, many DEAD/H box-containing proteins have been identified. Among them are Gemin3 which has been found to sediment in a 15S complex with Ago, and RHA (DEAH box polypeptide 9) which has been shown to be important for effective RNA silencing and probably RISC loading (Mourelatos et al., 2002; Robb and Rana, 2007). Moreover, DDX5, an ortholog of Drosophila p68, which has been shown to associate with Drosophila Ago2, and DDX18, a putative helicase that has been implicated in Drosha function (Gregory et al., 2004; Ishizuka et al., 2002) have been found. It is tempting to speculate that one of the DEAD/H proteins identified could be the helicase that promotes small RNA duplex unwinding. However, DExD/H proteins can also be implicated in remodeling or assembly of RNA complexes (Linder, 2006).

Besides DEAH/D box helicases, isoforms of poly-A-binding proteins and many mRNA binding proteins involved in translation were present in the analysis, such as YB-1, ZBP1/3, HuR, RBM4, FMRp and its homologues FXR1 and FXR2. FXR has been identified in Drosophila RISC and FMRp has also been identified in humans (Caudy et al., 2003; Caudy et al., 2002). RBM4 was found to be important for cleavage of reporter constructs with a perfect complementary site to a miRNA or for repression of target RNAs (Höck et al., 2007). Surprisingly, RMB4 interacts with Ago in a RNA-dependent manner. It is possible that it helps recruiting Ago-miRNA complexes to their mRNA targets by further stabilizing the interaction of the miRNA with its target site.

The identification of all these different proteins identified in complex II and III suggest that they both form large RNPs. Indeed, both complexes are RNAse sensitive. Moreover, the let-7 target KRAS co-migrates with Ago complex III in the Sucrose gradient, suggesting that complex III forms large mRNPs with Ago bound miRNAs targeting mRNAs. This does not allow for binding and cleaving of a substrate RNA and explains why complex III does not show RISC activity.

In summary, the results presented here and the more comprehensive study in Höck et al. suggest that Ago complexes are recruited to miRNA target mRNAs carrying a variety of different RNA binding or regulatory proteins (Höck et al., 2007). miRNPs might then function in concert with this mRNA regulatory network unique to each individual mRNA and fine-tune translational regulation. It is therefore important to analyze miRNA function in the light of the whole protein network. Moreover, it suggests a way of identifying possible miRNA targets by immunopurification of Ago complexes and analysis of the precipitated mRNAs as it has recently been done by many laboratories (Beitzinger et al., 2007; Easow et al., 2007; Hammell et al., 2008; Hendrickson et al., 2008; Karginov et al., 2007; Landthaler et al., 2008; Zhang et al., 2007).

3.2 Small RNA binding partners of Argonaute proteins

Besides the biochemical isolation and identification of human Ago1 and human Ago2 protein complexes a second aim of this thesis was to analyze the small RNA components of Ago protein complexes. It has been proposed before, that in humans, four different Ago proteins seem to associate with the same miRNA species regardless of their sequence (Azuma-Mukai et al., 2008; Meister et al., 2004). However, a detailed analysis of Ago1- and Ago2-associated RNA species has not been done. Therefore, monoclonal antibodies specific to human Ago1 and Ago2 were used for Ago isolation from total HEK 293 cell lysates. Co-immunoprecipitated RNAs were extracted, cloned and sequenced. Ago proteins are the cellular binding partners of small RNAs and it is not surprising that a high amount of miRNAs could be identified in the libraries. Interestingly, there were also some reads mapping to snoRNAs, mRNAs, other non-coding RNAs and transposons. Most published cloning approaches used total RNA for cloning of small RNAs and therefore unspecific degradation products of larger RNAs are present in the libraries and it is difficult to find classes of functional RNAs. However, in this work immunoprecipitated endogenous Ago complexes were analyzed it is very likely that small RNAs that associate with Ago proteins are functional RNA molecules rather than just degradation products.

3.2.1 A human snoRNA acting as a miRNA

SnoRNAs form a highly abundant class of non-coding RNAs in many different organisms. They localize to the nucleolus and guide specific modifications of rRNAs or snRNAs (Matera et al., 2007). In the immunoprecipitated RNA of endogenous Ago complexes there are reads of about 22 nt in length originating from snoRNAs, the most abundant one matching to snoRNA ACA45 (Fig. 13A). The sequenced reads derive only from the 3' half of the snoRNA and are conserved in mammals (Fig. 13B). These findings further suggest that the identified reads termed ACA45 sRNAs are specific processing products rather than snoRNA degradation product. Consistent with the cloning data, the small RNAs could be validated by northern blotting and are enriched in Ago immunoprecipitations. Moreover, ACA45 sRNA can silence a luciferase reporter with perfect complementary binding sites in an Ago2 dependent manner, demonstrating that it can function like a miRNA.

3.2.2 Processing of ACA45

The cleavage signature of the stem-loop-structured processing intermediate is different from the typical 2 nt 3' overhangs generated by Drosha. Consistently, ACA45 sRNA can mediate luciferase reporter cleavage upon depletion of Drosha, suggesting that processing of ACA45 is microprocessor independent. Moreover, immunoprecipitated DGCR8 was not able to cleave a ^{32}P-labeled in vitro transcribed ACA45. However, a specific cleavage product was observed in the anti-DGCR8 immunoprecipitates using primary miR-27a as transcript (Fig. 16A). These results are supported by the analysis of the libraries from DGCR8 knockout ES cells where ACA45 sRNA is present (Babiarz et al., 2008).

It was shown by northern blotting and semi-quantitative PCR that ACA45 processing requires Dicer (Fig. 2.18). Moreover, Dicer can process the full length ACA45 *in vitro*. *In vivo*, only a minor portion is processed to a small miRNA-like RNA, as shown by the analysis of total RNA in northern blots, where the strongest signal originates from the full-length ACA45. 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).

In the model described in figure 3.1A, ACA45 is transcribed and functions presumably in cajal bodies of human cells. 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. The finding that recombinant Dicer as well as Dicer-containing Ago protein complexes are capable of generating full-length ACA45 small RNAs in vitro supports this hypothesis.

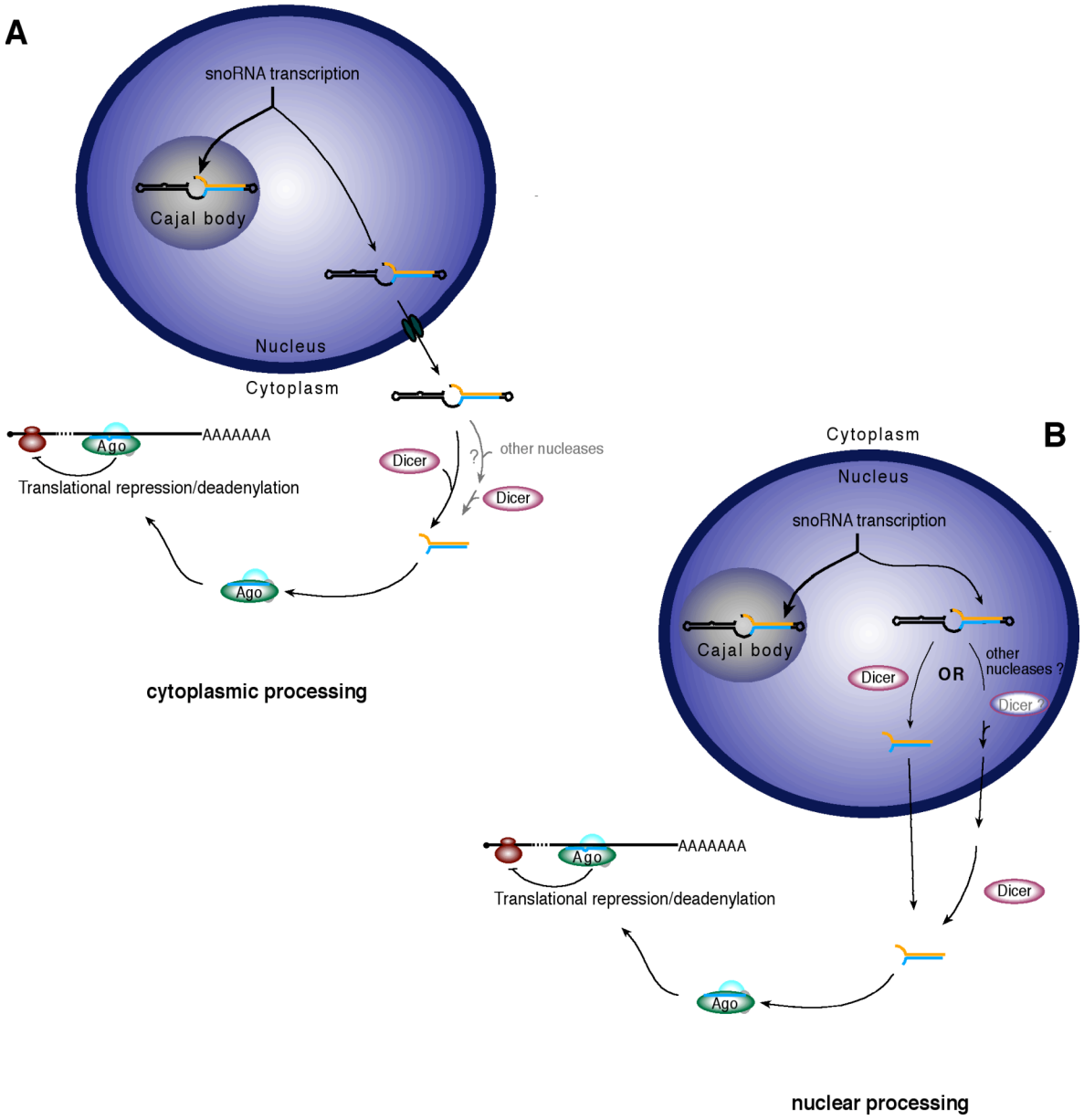
Most snoRNAs have not been reported to be transported to the cytoplasm. However U3, U8 and U13 snoRNA have been shown to be associated with the phosphorylated export adaptor PHAX and U3 additionally to the cap-binding complex, Ran, the exportin CRM1, all involved in snRNA maturation, and the nucleo-cytoplasmic shuttle protein Nopp140 (Boulon et al., 2004; Leary et al., 2004; Watkins et al., 2007; Watkins et al., 2004). This could indicate that the capped mammalian pre-snoRNAs are exported to the cytoplasm. Indeed, all four precursors and the mature U8 were found in nucleoplasmic extracts, whereas the cytoplasmic extract contained two precursor forms. For U3 and U13 both the precursors and the mature snoRNAs were present in the cytoplasm. However, these snoRNAs are transcribed from independent transcription units. The primary transcripts of these genes possess an m7G cap structure. During biogenesis, the cap is hypermethylated to an $m^{2,2,7}G$ cap (m_3G cap) and can be recognized by export factors mentioned above. A similar hint for export of other snoRNAs has not been observed yet. It would

be intriguing to find out if H/ACA snoRNAs, in particular ACA45 is associated with Ran or other export factors. Moreover, it should be analyzed if mature snoRNAs or their precursor can be found in the cytoplasm.

Alternatively to cytoplasmic processing of ACA45, ACA45 is cleaved in the nucleus already and one half is recognized as miRNA precursor by the pre-miRNA export pathway (Fig. 3.1B). However, to avoid cleavage of the majority of the ACA45 pool, which is needed for classical snoRNA functions, a nuclear cleavage activity would have to be very inefficient or specially regulated, as it is for example known for microprocessor-mediated cleavage of some specific miRNAs (Davis et al., 2008); (Fukuda et al., 2007); (Guil and Cáceres, 2007); (Winter et al., 2009). In the case of nuclear cleavage, only a part of ACA45 would be exported. This could explain the observation that only reads from the 3' half of ACA45 were found in the libraries, although the other half folds like a typical miRNA precursor as well.

Dicer mainly localizes to the cytoplasm but nuclear localization has been shown as well (Daniels et al., 2009). Therefore, it could also be possible that Dicer cleaves ACA45 in the nucleus (Fig. 3.1B). In both cases, nuclear or cytoplasmic cleavage, it is not clear whether Dicer is the only nuclease or if a second nuclease cleaves ACA45 before Dicer processing as it does not structurally represent a classical Dicer substrate with 2 nt 3' protruding ends. However, the involvement of another nuclease seems rather unlikely as it was observed that Dicer alone can cleave ACA45 *in vitro* (Fig. 2.17B). Indeed, it was shown that free ends are not absolutely required for Dicer cleavage: terminally blocked dsRNA is cleaved internally, with reduced kinetics. After the initial internal cleavage, normal kinetics is restored as 2-nt 3′-overhang-containing ends become available (Zhang et al., 2004). This two-step cleavage could explain the processing intermediate shown in Fig. 2.17B (indicated with asterisks). After all, Dicer seems to be a very likely candidate to process ACA45 on its own. It has been shown that Drosha cleaves miRNAs co-transcriptionally, after recruiting the early spliceosome complex but before splicing (Ballarino et al., 2009; Kim and Kim, 2007; Morlando et al., 2008; Pawlicki and Steitz, 2008). Keeping in mind that Dicer might function in the nucleus, it would be interesting to see if Dicer is found associated to the chromatin with transcribed snoRNA. If so, this would also suggest that snoRNA processing does not need the mature snoRNA. However, to answer this question it is also possible to knock down snoRNA assembly factors such as Naf1 and analyze if ACA45 sRNA can still be observed.

Figure 3.1. ACA45 processing and sACA45 function in human cells



Two simplified models of snoRNA processing are shown. (A) ACA45 is transported to the cytoplasm and processed by Dicer (in addition possibly by another nuclease creating a processing intermediate) to a miRNA-like small RNA functioning in gene silencing. (B) ACA45 is processed by nuclear Dicer to ACA45 small RNA, transportetd to the cytoplasm and acting like a miRNA. Nuclear processing could also be performed by an unknown nuclease or Dicer creating a processing intermediate. The intermediate can then be exported to the cytoplasm, followed by a second processing step by Dicer

3.2.3 SnoRNA-derived miRNAs are a general mechanism of small RNA generation

By analyzing larger data sets, several small RNAs with miRNA-like processing signatures originating from snoRNAs were found, namely ACA47, ACA36b, U92, HBI-100, ACA56, ACA3, and ACA50 (Figure 2.22). Only snoRNAs were counted with reads in at least two data sets between Ago1-4 where both arms of the individual stems are present. Many more candidates can be identified where the star sequences is not present. Consistently with the work described here, more miRNA-like snoRNAs have been identified in independent studies. A report investigating the small RNAs present in human cells describes small RNA fragments mapping to four box H/ACA snoRNAs (Kawaji et al., 2008) By analyzing several small RNA libraries, Taft et al found short RNA sequences derived from snoRNAs in human, mouse, chicken, fruit fly, *Arabidopsis* and fission yeast (Taft et al., 2009b). Analysis of the size distribution in humans showed that H/ACA box derived reads were about 22 nt in length whereas C/D box derived sRNAs were predominantly 17-19 nt and 30 nt. Moreover, they found that reads from H/ACA box snoRNAs are predominantly from the 3' end whereas C/D box derived sRNAs are rather from the 5' end. These findings support the hypothesis that a considerable number of snoRNAs are natural precursors for functional small RNAs. In a third study, it is summarized that 14 known box H/ACA snoRNAs encode fragments of the size of miRNAs that have been experimentally detected, 7 of them in this work here, three of them have already been annotated as miRNAs previously (Scott et al., 2009). Moreover, they get to a similar result from another point of view. They find 20 genomic regions encoding miRNAs that have a highly significant similarity to snoRNAs, both on the level of their surrounding genomic context as well a their predicted folded structure.

SnoRNAs acting as miRNAs have also been found in *Gardia lamblia*, a unicellular and binucleated protozoan parasite (Saraiya and Wang, 2008). It is one of the earliest branching eukaryotes with many primitive features. No RNA interference has been observed in Gardia but analysis of the genome showed a Dicer and Ago homolog. The structure of Dicer has been resolved and it has been shown to cleave dsRNA *in vitro* and support RNAi in *S. pombe* Dicer deletion mutants. Interestingly, the genome of Giardia shows no Drosha or Exportin-5 homolog.

Saraiya and colleagues isolated, cloned and sequenced small RNAs from Giardia and identified snoRNA sequences among them. One of them, miR-2, was identified as a Dicer-digested product from GlsR17, previously identified as a box C/D snoRNA in Giardia. Putative target sites for miR-2 were identified at the 3'-UTRs of many variant surface protein (VSP) mRNAs. Expression of a reporter mRNA carrying these putative target sites was specifically inhibited by

miR-2 without affecting the mRNA level. Subsequent analysis also indicated the dependence of this inhibition on the presence of Argonaute, thus verifying the ability of a snoRNA derived miRNA to function in miRNA-mediated translational repression in Giardia.

The finding of miRNA-mediated repression using snoRNA precursors in such an ancient organism is interesting as it is possible that snoRNAs, besides their canonical functions, may have become involved in gene regulation, meaning some miRNAs have evolved from snoRNAs.

3.2.4 Orphan snoRNAs

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 there are snoRNAs which do not contain sequences complementary to rRNA or any other RNA species. It is unknown whether or not these candidates represent functional snoRNAs. Because of lacking complementary sequences in the genome, these snoRNAs are called 'orphan' snoRNAs. Some of them might regulate alternatively spliced transcripts. It has been reported that the snoRNA HBII-52 regulates alternative splicing of the serotonin receptor 2C via a conserved region complementary to the snoRNA (Kishore and Stamm, 2006). HBII-52 is a brain-specific snoRNA and its locus is maternally imprinted. Maternally imprinted genes are generally expressed only when inherited from the father. Loss of paternally expressed genes lead to the development of the Prader-Willi syndrome, characterized by neonatal hypertonia, failure to thrive during infancy and later obesity and hypogonadism. Kishore at al showed that HBII-52 is likely to bind a silencer element in exon Vb, increasing its inclusion in the mRNA of the serotonin receptor 2C. MRNAs lacking exon Vb are not functional. Interestingly, the sites of HBII-52 complementary to the serotonin receptor 2C are 18 nt in length. It would be interesting to analyze whether base pairing is done by a processed form of the snoRNA rather than by full length HBII-52.

It is tempting to speculate that orphan snoRNAs are specifically processed to small RNAs functioning in gene silencing. However, it would also be intriguing to analyze if a snoRNAs can act both as a canonical snoRNA and as sRNA precursor. Many snoRNAs being a precursor for miRNAs, including ACA45, have a predicted target pseudouridinylation site. It will be interesting to see whether they can act indeed on both their canonical snoRNA target and their miRNA target. However, snoRNAs identified in Scott et al have a AGA sequence instead of a ACA box, indicating that they are no longer functional (Scott et al., 2009). Further analysis has to be done to clarify the relationship between miRNAs and snoRNAs.

3.2.5 tRNA processing

Working as an adapter molecule, tRNAs decode genetic information that is translated into proteins by the ribosome. TRNAs are 75 – 95 nt in length and have four domains, the D-arm, the anticodon arm, the TψC and the acceptor stem. They are transcribed by polymerase III and the internal promoters constitute two discrete regions corresponding to nt 8–19 (box A) and 52–62 (box B) of a tRNA. Nucleotides 8, 14, 18 and 19 in box A, and nt 53–56, 58 and 61 in box B are highly conserved among all tRNAs because of the tRNA tertiary structure (Goodenbour and Pan, 2006). TRNA precursors have a 5' and 3' trailer. The 5' trailer is cleaved by RNAse P and the 3' trailer by RNAse Z, yielding a tRNA primed for addition of the CCA motif by the CCA-adding enzyme. The CCA nucleotide sequence is essential for amino acid attachment and interaction with the ribosome. Moreover, tRNAs get modified to stabilize their structure (modifications in the core regions) and modifications are important for codon pairing and accurate recognition by the cognate aminoacyl-tRNA synthetase (modifications in the anticodon loop) (Nakanishi and Nureki, 2005).

In humans, there are 49 families of tRNAs (encoding for 20 amino acids plus selenocystein) grouped according to their isoacceptor (Lander et al., 2001). Codon degeneracy requires up to 5 tRNAs with distinct anticodons (tRNA isoacceptors) to read codons for each amino acid. Moreover, there is a huge number of tRNA pseudogenes and tRNA genes having the same anticodon sequence but differences elsewhere in the tRNA body. The very high number of tRNA genes makes it difficult to annotate short reads derived from small RNA cloning to a precise tRNA locus. Data generated in this work indicate that tRNAs are processed into small RNAs of about 18-22 nt in length. At least some of them seem to derive from mature tRNAs as their 3' end carries a CCA tail. They coimmunoprecipitate with Ago proteins, indicating that they are functional small RNAs rather than just random degradation products. Indeed, immunoprecipitated Ago complexes can cleave target RNAs with a perfect complementary site to specific tRNA fragments *in vitro*. The small tRNA fragments analyzed in this work are present in Dicer knock out cells and therefore it remains a mystery so far how these tRNA fragments are processed. However, several observations by other laboratories indicate that tRNAs can be endonucleolytically cleaved as stress response. In *Escherichia coli* (*E. coli*), tRNAs can be cleaved in response to a bacteriophage infection (Levitz et al., 1990). TRNA cleavage occurs during amino acid starvation in Tetrahymena and during oxidative stress in human, plant and *S. cerevisiae*. (Lee and Collins, 2005; Thompson et al., 2008; Yamasaki et al., 2009). So far, preliminary results (data not shown) do not show any connection between small tRNA fragments

associated to Ago proteins and oxidative stress. Moreover, stress induced tRNA cleavage occurs mainly in the anticodon loop, creating longer fragments than those observed in this work. Therefore, further analysis of tRNA cleavage should be concentrated on RNAse Z or RNAse P, which are responsible for tRNA maturation. Moreover, it should be investigated if tRNA can induce target cleavage *in vivo* and possible target sites should be identified. A general conclusion can not be made at this point and a more comprehensive analysis about small tRNAs will be performed.

4 Material & Methods

4.1 Material

4.1.1 Chemicals and enzymes

Unless stated otherwise, all chemicals were purchased from *Amersham Bioscience* (Buckinghamshire,UK), *Biorad* (Hercules, USA), *Merck* (Darmstadt, Germany), *Qiagen* (Hilden, Germany), *Roche* (Penzberg, Germany), *Roth* (Karlsruhe, Germany) and *Sigma-Aldrich* (Munich, Germany). Radioactive chemicals were provided by *Perkin Elmer* (Waltham, USA). Enzymes were delivered from *New England Biolabs* (Ipswich, USA) and *Fermentas* (Burlington, Canada).

4.1.2 Plasmids

pMIR-RNL	encodes for the *Phototinus pylaris* luciferase (in this work termed firefly) under the control of a CMV promoter and a *Renilla reniformis* luciferase (in this work termed renilla) under the control of a SV40 promoter. The firefly coding sequence is flanked by a multiple cloning site (MCS) at its 3' end, allowing for the introduction of regulatory sequences into the 3'UTR. The plasmid carries an ampicillin resistance (Beitzinger et al., 2007).
pBluescript KS(+)	was used for in vitro transcription (Fermentas). The sequence includes a polylinker sequence (MCS), antibiotic resistance sequence to ampicillin and an *E. coli* and f1 helper phage origin of replication. The MCS is flanked by a T7 and a T3 promoter.

4.1.3 Antibodies

α-human Ago1 (4B8)	monoclonal, rat hybridoma supernatant (Beitzinger et al., 2007)
α-human Ago2 (11A9)	monoclonal, rat hybridoma supernatant (Rüdel et al., 2008)

α-human Ago3 (5A3)	monoclonal, rat hybridoma supernatant (Weinmann et al., 2009)
α-human Ago4 (6C10)	monoclonal, rat hybridoma supernatant (Weinmann et al., 2009)
α-human RmC (16D2)	monoclonal, rat hybridoma supernatant
α-human Gar1	kindly provided by Witold Filipowicz
α-HA	Covance
α-mouse Ago2 (6F4)	monoclonal, rat hybridoma supernatant
α-mouse IgG	peroxidase conjugated, Sigma
α-rat IgG	peroxidase conjugated, The Jackson Laboratory

4.1.4 Bacterial strains and cell lines

Bacterial strains:	*E. coli* XL1 blue
Cell lines:	HeLa
	HEK 293T
	Dicer +/- ES cells (Murchison et al., 2005)
	Dicer -/- ES cells (Murchison et al., 2005)

4.1.5 Cell culture media

For cultivation of the cell lines the following media were used:

Normal medium:	500 ml	DMEM (PAA, Pasching, Austria)
	10%	fetal bovine serum (Biochrom, Berlin, Germany)
	1%	Penicillin/Streptomycin (PAA, Pasching, Austria)

4.1.6 Buffers and solutions

Phosphate buffered saline	130 mM	NaCl
(PBS)	774 mM	Na_2HPO_4
	226 mM	NaH_2PO_4

1x TBE buffer	89 mM	Tris pH 8.3
	89 mM	boric acid
	2.5 mM	EDTA
SDS running buffer	200 mM	Glycin
	25 mM	Tris pH 7.5
	25 mM	SDS
Towbin-blotting buffer 10x	192 mM	Glycin
	0.025 mM	Tris
	20%	Methanol
Western blotting wash buffer	300 mM	Tris pH 7.5
	150 mM	NaCl
	0.25%	Tween-20
Chemiluminescence	100 mM	Tris pH 8.5
detection	1.2 mM	Luminol in 10 ml
	0.68%	p-cumaric acid in 150 µl
		H_2O_2 (30%) 11 µl
		mix all components before use
HEPES 2x for Calcium	274 mM	NaCl
phosphate transfection	54.6 mM	HEPES
	1.5 mM	Na_2HPO_4
MOPS buffer 50x	1M	MOPS (pH 7)
EDC cross-link solution	0.13 M	1-Methylimidazole (pH 8)
	0.16 M	EDC

LB (lysogeny broth) media	1% (w/v)	Trypton
	1% (w/v)	NaCl
	0.5% (w/v)	Yeast extract
20x SSC	3 M	NaCl
	0.3 M	Sodium citrate
		adjust pH to 7.1
RNA loading dye 1x	90%	Formamide
	0.025%	Xylene cyanol
	0.025%	Bromophenol blue
		in 1x TBE
Protein sample buffer 5x	400 mM	Tris/HCl pH 6.8
	5 mM	EDTA
	0.01%	Bromphenol blue
	50%	Glycerin
	1%	SDS
DNA loading dye 5x	15 g	Saccharose
	50 ml	H_2O
	0.025%	Xylene cyanol
Polyacrylamide gels for SDS-PAGE		
5% stacking gel	5%	Acrylamide 37,5:1
	75 mM	Tris pH 6.8
	0.1 %	SDS
	0.1 %	APS
	4 μM	TEMED

10% separation gel	10%	Acrylamide 37.5:1
	400 mM	Tris pH 8.0
	0.1%	SDS
	0.1%	APS
	4 μM	TEMED
PAGE elution buffer	300 mM	NaCl
	2 mM	EDTA
IP wash buffer	300 mM	NaCl
	50 mM	Tris pH 7.5
	5 mM	$MgCl_2$
	1 mM	NaF
	0.01% (v/v)	NP-40
Cell lysis buffer I	150 mM	KCl
	25 mM	Tris pH 7.5
	2 mM	EDTA
	1 mM	NaF
	0.5%	NP-40
Cell lysis buffer II	300 mM	NaCl
	2.5mM	MgCl
	0.5%	NP-40
	20 mM	Tris pH 7.5
RIPA buffer	50 mM	Tris
	500 mM	NaCl
	1%	NP-40
	0.5%	Sodium deoxycholate
	0.1%	SDS

Gradient buffer	150 mM	KCl
	25 mM	Tris (pH 7.4)
	2 mM	EDTA
Protease K buffer, 2x	300 mM	NaCl
	200 mM	Tris pH 7.5
	25 mM	EDTA
	2%	SDS
50x Denhardt's solution	1%	Albumin fraction V
	1%	Polyvinylpyrrolidon K30
	1%	Ficoll 400
Hybridizitation solution	7.5 ml	20x SSC
	21.0 ml	10% SDS
	0.6 ml	1 M Na_2HPO_4 pH 7.2
	0.6 ml	50x Denhardt's solution
Renilla buffer	2.2 mM	EDTA
	220 mM	K_2PO_4 pH 7.2
	0.44 mg/ml	BSA
	1.1 M	NaCl
	1.3 mM	NaN_3
	1.43 μM	Coelenterazine (P.J.K., Kleinblitterdorf, Germany)

Firefly buffer	470 μM	D-luciferin (P.J.K., Kleinblitterdorf, Germany)
	530 μM	ATP (P.J.K., Kleinblitterdorf, Germany)
	270 μM	Coenzyme A (P.J.K., Kleinblitterdorf, Germany)
	20 mM	Tricine
	5.34 mM	$MgSO_4$ pH 7.2
	0.1 mM	EDTA
	33.3 mM	DTT

Denaturing polyacrylamide gels for sample purification
using the SequaGel®-Kit from National Diagnostics (Atlanta, USA).

12.5%	29 ml	concentrate
	25 ml	diluent
	6 ml	buffer
	500 μl	Ammonium persulfate (APS)
	100 μl	TEMED

Denaturing polyacrylamide gels for northern blotting

12%	25.2 g	Urea
	18 ml	Acrylamide (40%; 19:1)
	1.2 ml	MOPS-NaOH (pH 7) 1M
	360 μl	APS
	21 μl	TEMED

4.2 Methods

4.2.1 Molecular biological methods

4.2.1.1 General methods

Any less detailed descriptions of molecular biological standard methods (DNA/RNA gel-electrophoresis, -extraction, -precipitation and the determination of concentrations of nucleic acids, PCR, etc.) were performed as described in Sambrook et al. (Sambrook, 1989) or according to the manufacturers' instructions, respectively. There, one can also find the composition of not listed buffers and solutions.

The isolation of plasmid-DNA from *E. coli* was carried out by using the ‚Plasmid MiniKit I®' (Omega BioTek, Darmstadt, Germany) or the ‚NucleoBond® XtraMidi'-Kit (Macherey Nagel, Düren, Germany), respectively. For the elution of DNA fragments from agarose gels, the NucleoSpin®-Kit (Macherey Nagel, Düren, Germany) was used.

4.2.1.2 Cloning of 3'UTRs from genomic DNA into pMIR-RNL

pMIR-RNL miRNA reporter plasmids which express the firefly luciferase ORF fused to a cleavage site complementary to a small RNA or a regulatory 3'UTR sequence and renilla luciferase as a transfection control were generated from pMIR-REPORT (Ambion) as described in Höck et al. (Höck et al., 2007). The following DNA oligonucleotides containing a sequence perfectly complementary to miR-19b and snoACA45 5' were annealed, digested with SacI and NaeI and inserted into the *SacI* and *NaeI* restriction sites of the reporter plasmid: miR-19b 5'-
CGCTGAGCTCATCGCCACCTTGTTTAAGCCTCAGTTTTGCATGGATTTGCACAATTAGACCT
ACGCACTCCAGGCCGGCTCGC -3' and
GCGAGCCGGCCTGGAGTGCGTAGGTCTAATTGTGCAAATCCATGCAAAACTGAGGCTTAAA
CAAGGTGGCGATGAGCTCAGCG -3'; snoACA45: 5'-
CGCTGAGCTCATCGCCACCTTGTTTAAGCCCAAGACCTGTTCTATCTACCTTATTAGACCTA
CGCACTCCAGGCCGGCTCGC -3' and 5'-
GCGAGCCGGCCTGGAGTGCGTAGGTCTAATAAGGTAGATAGAACAGGTCTTGGGCTTAAAC
AAGGTGGCGATGAGCTCAGCG -3'.

3'UTRs were amplified by polymerase chain reaction (PCR). As template, genomic DNA of HEK293 cells was used. PCR amplification was performed by the Phusion™ polymerase (Finnzymes, Espoo, Finland). PCR products were cloned into the reporter plasmid pMIR-RNL.

4.2.1.3 RNA Extraction from cultured cells

a) Total RNA extraction (mRNA and miRNAs)

Extraction of RNA was performed following the TriFast® protocol (Peqlab, Erlangen, Germany). Briefly, cells were homogenized by pipetting up and down several times in an appropriate amount of TriFast. Samples were incubated for a few minutes at room temperature to let nucleoprotein complexes dissociate. 200 µl chloroform per 1 ml TriFast were added, the samples mixed for 20 seconds and centrifuged for 20 minutes at full speed in a desk top centrifuge resulting in the dissociation of organic and aqueous phase. The upper phase (approx. 500 µl) was pipetted into a new sterile reaction tube containing 1 ml isopropanol. RNA was precipitated at -20°C. After pelleting the RNA for 15 minutes by centrifugation at full speed, isopropanol was removed thoroughly and the pellet washed with 80% Ethanol. After pelleting and removing the Ethanol the RNA was air-dried for 5 minutes and was dissolved at 70°C in an appropriate amount of ddH_2O.

b) RNA exraction of immunopurified complexes and input samples

Cells were usually lysed in 1ml lysis buffer per 15 cm plate. For input samples, usually 100 µl lysate (generally 1/10 of lysate used for IP) was digested in 200 µl 2x proteinase K buffer containing 40 mg Proteinase K. RNA from immunopurified samples was directly isolated from antibody coupled beads by adding 200 µl 2x proteinase K buffer containing 40 mg Proteinase K. Proteinase K digestion was performed for 20 minutes at 65°C, followed by two times phenol/chloroform/isoamylalcohol extractions. For subsequent RNA precipitation, the aqueous phase was mixed with three volumes (miRNAs) or two volumes (mRNAs) of absolute ethanol and incubated at -20°C. After pelleting the RNA for 30 minutes by centrifugation at 17'000g, isopropanol was removed thoroughly and the pellet was air-dried for 5 -10 minutes. RNA was dissolved at 70°C in an appropriate amount of ddH_2O.

4.2.1.4 Ago complex purification

HEK293 cells were lysed in cell lysis buffer II 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 PBS and incubated with 10 µl anti-Ago1 (4B8), anti-Ago2 (11A9), anti-FLAG (3H3), or anti-GST at 4°C with gentle agitation overnight. After several wash steps with PBS, beads were incubated with HEK293 cell lysate of 6 x 15 cm plates for 3 h. Anti-Ago1-coated beads were extensively washed with lysis buffer II containing 300 mM NaCl followed by a

wash with PBS. Anti-Ago2-coated beads were washed five times using RIPA buffer. RNA was isolated with 40 mg Proteinase K in 200 µl Proteinase K buffer 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 h at 4°C with rotation. Beads were extensively washed and coimmunoprecipitated RNA was extracted as described above.

4.2.1.5 RNA polyacrylamide gel electrophoresis

For the detection of RNA species (miRNAs, mRNAs) denaturing RNA polyacrylamide gel-electrophoresis was performed using the SequaGel® System Kit (National Diagnostics, Atlanta, USA) or MOPS based urea gels according to Hamilton et al. (Pall and Hamilton, 2008). The acrylamide concentration was between 8 and 15%. Before loading, the samples were provided with 2x dye and denatured for 5 min at 95°C. The gel was pre-run for 10-15 min at 400 V. 0.5-1x TBE or 0.5x MOPS (for MOPS containing gels) was used as running buffer. Samples were loaded after rinsing each pocket with buffer and the gel was run 500 V.

4.2.1.6 Northern blotting

After adding formamide loading dye, the RNA was separated by electrophoresis usually on a 12% denaturing polyacrylamide gel and transferred to Hybond-N membrane (Amersham Bioscience, Buckinghamshire, UK) by semidry blotting for 30 min with 20 V. H_2O was used to prewet three sheets of Whatman, the membrane and another three sheets of Whatman on top of the gel. After blotting was completed, the damp membrane was removed and placed with RNA side face up on top of a Whatman paper saturated in freshly prepared crosslinking EDC solution. Wrapped in Saran the membrane was incubated for 1 h at 50 °C. After crosslinking the membrane was rinsed in excess RNase-free distilled water and dried. Prehybridization was performed in 5x SSC; 20 mM Na_2HPO_4, pH 7.2; 7% SDS and 1x Denhardt's solution for 1 h at 50°C, before a 5'-^{32}P-labeled probe (see below) was added for hybridization for 4 h up to overnight at 50°C. Subsequently, the membrane was washed in 10-min intervals twice with 5x SSC and 1% SDS and once with 1x SSC and 1% SDS. Exposure to Kodak BioMax MS films was performed with an intensifying screen (Kodak, Stuttgart, Germany) at –80°C.

For northern probe preparation, 10 pmol of synthetic DNA-oligonucleotides (Metabion, Martinsried, Germany) or 2'OMe-oligonucleotides reverse complementary to the appropriate miRNA were radiolabeled in a T4-Polynucleotide kinase (Fermentas, Burlington, Canada) reaction in the presence of [γ-^{32}P]-ATP (GE Healthcare, Munich, Germany) according to

standard protocols. Subsequently, the probe was purified by gel filtration using MicroSpin G-25 columns (Amersham Bioscience, Buckinghamshire, UK).

The following probes for northern blotting were used: 5'- AAGACCTGTTCTATCTACCT -3' for sACA45, 5'- TGATGCTCTACCGACTGAGCTATCCGGGC -3' for Lys tRNA, 5'- TGGTGCCGTGACTCGGA -3' for tRNA HIS 3', 5'- ATGGTGTCAGGAGTGGGA -3' for tRNA LEU 3'.

4.2.1.7 Dicer & DGCR8 cleavage assay

In vitro transcribed pri-27a substrate used in this study was described previously (Landthaler et al., 2004; Meister et al., 2005). The template for pre-27a transcription was created by annealing the following primers: 5'-

TTAATACGACTCACTATAGCTGAGGAGCAGGGCTTAGCTGCTTGTGAGCAGGGTCCACACC AAGTCGTGTTCACAGTGGCTAAGTTCCGCCCCCCAGC -3' and 5'-

GCTGGGGGGCGGAACTTAGCCACTGTGAACACGACTTGGTGTGGACCCTGCTCACAAGCA GCTAAGCCCTGCTCCTCAGCTATAGTGAGTCGTATTAA. 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 transcribed *in vitro*. Briefly, 8 µl 5x NTP mix (A/C/G/U = 5/5/8/0.1 mM), 8 µl 5x T7 buffer, 0.2 µl DTT (1M), 1 µl T7 RNA polymerase (Fermentas), 5 µl (α-^{32}P)-UTP (3000Ci/mmol) and 1 µl PCR product or 3 µl linearized plasmid in a 40 µl reaction was incubated for 2 h at 37°C. After adding RNA sample buffer, RNA was purified by 8% denaturing RNA-PAGE, detected by autoradiography, gel eluted over night and recovered by Ethanol precipitation. The construction of human FLAG-HA-Ago1, FLAG-HA-Ago2, and FLAG-HA-Dicer has been reported earlier (Meister et al., 2005). FLAG-HA-DGCR8 was purchased from Addgene. Immunoprecipitations were performed as described earlier. 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 RNA (2Bq/cm^2) at 37°C. The reaction was stopped by adding 200 µl proteinase K buffer containing proteinase K (0.2 mg/ml). RNA was isolated by Phenol/Chloroform extraction, precipitated with Ethanol and analyzed by 8% or 12% denaturing RNA-PAGE. Signals were detected by autoradiography.

4.2.1.8 RISC cleavage assay

For mapping RNA cleavage, a transcript was generated using the following primers: 5'-TAATACGACTCACTATAGAACAATTGCTTTTACAG -3' as T7 primer, 5'-ATTTAGGTGACACTATAGGCATAAAGAATTGAAGA -3' as SP6 primer, 5'-GAACAATTGCTTTTACAGATGCACATATCGAGGTGAACATCACGTACGTGGTGCCGTGACTCGGATCGGTTGGCAGAAGCTAT 3' for tRNA HIS and 5'-GAACAATTGCTTTTACAGATGCACATATCGAGGTGAACATCACGTACGATGGTGTCAGGAGTGGGATCGGTTGGCAGAAGCTAT 3' for tRNA LEU and 5'-GGCATAAAGAATTGAAGAGAGTTTTCACTGCATACGACGATTCTGTGATTTGTATTCAGCCCATATCGTTTCATAGCTTCTGCCAACCGA for the sequence comlementary to the oligonucelotide with the specific cleavage sequence. Regions complementary to tRNA fragments are underlined. The following PCR reaction was performed: 37 µl H_2O, 10 µl 5x buffer (Finnzymes), 1 µl dNTPs (10 mM), 0.3 µl SP6 primer (100 µM), 0.3 µl T7 primer (µM), 1 µl primer with specific cleavage sequence, 1 µl primer comlementary to the specific cleavage sequence and 1 µl Phusion polymerase (Finnzyme).

PCR products were *in vitro* tanscribed using the following reacion: 20 µl 5x NTP mix (A/C/G/U = 5/5/8/2 mM), 20 µl 5x T7 buffer, 0.5 µl DTT (1M), 2 µl T7 RNA polymerase (Fermentas) and 5 µl PCR product in a 100 µl reaction were incubated for at least 2 h at 37°C. After adding RNA sample buffer, RNA was purified by 8% denaturing RNA-PAGE, detected by UV shadowing, gel eluted over night and recovered by Ethanol precipitation.

For RISC activity assays, substrates were ^{32}P-cap labelled as follows: 1.5 µl (α-^{32}P)-GTP (3000Ci/mmol), 2 µl 10x buffer (0.4 M Tris pH 8.0, 60 mM MgCl2, 100 mM DTT, 20 mM spermidine), 0.25 µl RNasin (Promega), 1 µl S-adenosyl-Met (500 µM), 1 µl DTT (1M), 1 µl Guanyltransferase and ½ RNA from the 20 µl *in vitro* transcription reaction was incubated for 3 h at 37°C. After adding RNA sample buffer, RNA was purified by 8% denaturing RNA-PAGE, detected by autoradiography, gel eluted over night and recovered by Ethanol precipitation.

10 µl of Ago complex-containing anti-FLAG beads were incubated in a 25 µl reaction containing 5 nM target RNA, 1 mM ATP, 0.2 mM GTP, 10 U/mL RNasin (Promega), 100 mM KCl, 1.5 mM $MgCl_2$ and 0.5 mM DTT for 1.5 h at 30°C. RNA was isolated as described above and analyzed by 8% denaturing RNA-PAGE. Signals were detected by autoradiography using BioMax MS film (Kodak) and an intensifying screen (Kodak).

4.2.1.9 Reverse transcription - cDNA synthesis

RNA was isolated as described in "RNA Extraction from cultured cells", b) and dissolved in 12 µl H_2O. RNA was first treated with DNaseI to remove DNA contaminations using the following reaction mix: 12 µl RNA, 1.5 µl DNaseI buffer (Fermentas), 1 µl DNaseI (Fermentas) and 0.5 *µ*l RiboLock (Fermentas). DNaseI digest was performed for 30 minutes at 37°C. Afterwards the enzyme was inactivated for 10 minutes at 70°C and reaction was cooled on ice. Then, cDNA synthesis was performed in a final 30 µl reaction using "First Strand cDNA Synthesis-Kit"" from Fermentas (Burlington, Canada), according to the manufacturer's protocol. For quantitative real-time PCR (qRT PCR), cDNA from immunopurified samples were adjusted to a final volume of 40 µl, total RNA to a final volume of 60 µl. 1 µl cDNA in 10.5 µl H_2O was used per well for a 25 µl PCR reaction.

4.2.1.10 Poly(A) tailing of miRNAs for qRT-PCR

In order to detect miRNAs in a qRT-PCR reaction the mature miRNAs have to be modified as described in Hurteau et al. (Hurteau et al., 2006). RNA was extracted using Trifast followed by DNaseI digestion as described above. MiRNAs were subsequently poly(A) tailed using the "Poly(A) Tailing-Kit" from Ambion with the follwing reaction: 5 µl DNaseI treated RNA, 5 µl 5x *E. coli* poly(A) polymerase I (E-PAP) buffer, 2.5 µl 25mM $MnCl_2$, 2.5 µl 10mM ATP, 1 µl E-PAP and 9 µl nuclease free H_2O.

Reverse transcription was performed with 10 µl of the E-PAP treated total RNA using the "First Strand cDNA Synthesis-Kit" from Fermentas. Instead of the random-primer mix provided, 1 µl l of a 100 mM universal reverse transcription primer (URT primer; 5'-AACGAGACGACGACAGACTTTTTTTTTTTTTTT 3') was used. This primer comprises a poly(T) and a unique URT primer sequence and anneals to poly(A) tails. cDNA was amplified by qRT-PCR using Mesa Green qPCR MasterMix Plus (Eurogentec), specific forward primers for the small RNA (5'- AAGGUAGAUAGAACAGGUCUUG for ACA45, 5'-TCCCTGAGACCCTAACTTGTGA for miR-125b, and 5'- ACACATCCAAATGAGGCG for 7SK) and the URT sequence as the universal reverse PCR primer. The PCR products were analyzed by 4% agarose gel electrophoresis.

4.2.1.11 qRT-PCR

Quantitative PCR analysis was performed using a BioRad (Hercules,USA) real-time detection system and the Mesa Green qPCR MasterMix Plus (Eurogentec, Cologne, Germany).
The qRT-PCR reactions were set up as 25 µl reactions: 12.5 µl MESA GREEN qPCR kits for SYBR® Assay, 0.5 µl, forward RT-primer (10 µM), 0.5 µl reverse RT-primer (10 µM), 11.5 µl cDNA (diluted, see chapter 5.1.9)

4.2.1.12 Generation of small RNA libraries for 454 sequencing

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'-GCCTCCCTCGCGCCATCAGCTNNNNGACCTTGGCTGTCACTCA -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'-GCCTTGCCAGCCCGCTCAGACGAGACATCGCCCCGC[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 analysis. Both cDNA pools were mixed in equal amounts and subjected to gel fractionation. The 120–135 bp fraction was electroeluted from 6% polyacrylamide gels. After isolation with Nucleospin Extract II (Macherey and Nagel), cDNA pools were dissolved in 5 mM Tris (pH 8.5) with a concentration of 10 ng/ml 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.)

4.2.1.13 Generation of small RNA libraries for Solexa sequencing

The protocol was adapted from Pfeffer et al. (Pfeffer, 2007).

Adenylation of 3'Adapter

For the adenylation of the 3' adapter, 5 µl phosphorylated adapter (500 µM; 5'-TCGTATGCCGTCTTCTGCTTG-3') and 75 µl of the antisense adapter (100 µM; 5'-CTGGAATTCGCGGTTAAATATAGTGCAGT) were mixed with 0.25 µl Tris pH 7.5 (2 M), 5 µl NaCl (0.3 M), 10 µl EDTA (1 mM) and 19.75 µl H_2O. The two oligonucleotides were annealed by

heating at 95°C for 3 min and cooling on ice for 5 min. The solution was then brought to 215 µl total volume containing 40 µl MOPS (0.2 M), 2 µl $MgCl_2$ (1 M), 20 µl DTT (0.1 M), 20 µl ATP (100 mM) and 18 µl concentrated T4 DNA Ligase (30U/µl, Fermentas). The total mixture was incubated for 24h at 25°C. Samples were then loaded on a 18% denaturing acrylamide/bisacrylamide gel, and the band corresponding to the adenylated oligonucleotide was excised and eluted in elution buffer after visualization by ultraviolet (UV)-shadowing. After RNA preciptiation, pellet was dissolved in water and adjusted to a concentration of 50 µM.

Labelling of the 19nt and 24nt markers

The 19nt and 24nt containing PmeI restriction sites (5'- CGUACGCGGGUUUAAACGA -3' and 5'- CGUACGCGGAAUAGUUUAAACUGU -3') were prepared as follows:

1 µl RNA marker (20 µM), 2 µl polynucleotide kinase (PNK) buffer (10x, Fermentas), 14.5 µl H_2O, 2 µl [γ-^{32}P]-ATP and 0.5 µl PNK (Fermentas) were incubated for 20 min at 37°C, loaded on a 18% denaturing acrylamide gel, excised and eluted in elution buffer over night after visualization by autoradiography. After Ethanol precipitation, the pellet was dissolved in 20 µl of water.

Isolation of small RNA fraction and adapter ligation

The RNA extracted from Ago immunoprecipitations was spiked with radiolabelled oligonucleotides of 19 and 24 nt in size (1 µl of a freshly made 20 nM stock). After denaturation for 30 sec, RNA was loaded onto a 15% denaturing RNA-PAGE and exposed to a phosphoimaging screen. Gel pieces of 19-24 nt in size were sliced out, gel eluted and precipitated.

For ligation of the 3' adapter, the RNA pellet was dissolved in 9 µl H_2O and mixed with 2 µl 10x ligation buffer (0.1 M $MgCl_2$, 0.1 M 2-Mercaptoethanol, 0.5 M Tris pH 7.6 and 1 mg/ml acetylated bovine serum albumin), 6 µl DMSO (50%) and 1 µl 3' Adapter (50 µM) and denatured for 30 sec at 90°C. After cooling on ice, 2 µl truncated T4 RNA ligase 2 (Rnl2, New England Biolabs) were added, and the mixture was incubated at 37°C for 1 h. The reaction was stopped by adding RNA sample buffer and samples were separated on a denaturing 15% acrylamide gel. The ligation product was visualized after phosphorimaging of the gel for 45 min and was excised. After gel elution in 300 µl 0.3 M NaCl in siliconized tubes, the RNA was subsequently precipitated.

For ligation of the 5' adapter, the RNA-pellet was dissolved in 8.6 µl H_2O, 2 µl 10x ligation buffer (0.1 M $MgCl_2$, 0.1 M 2-Mercaptoethanol, 0.5 M Tris pH 7.6 and 1 mg/ml acetylated bovine serum

albumin), 0.4 µl ATP (100 mM), 6 µl DMSO (50%) and 1 µl 5' adapter RNA oligo (100 µM; 5' GUUCAGAGUUCUACAGUCCGACGAUC 3'). After denaturation of the RNA for 30 s at 90°C and cooling on ice, 2 µl of T4 RNA ligase (New England Biolabs) were added and the sample was incubated at 37°C for 1 h. The reaction was stopped by addition of 1 volume of formamide loading solution, and samples were separated on a denaturing 15% acrylamide/bisacrylamide gel. The ligation product was collected as in the previous step after phosphorimaging of the gel and eluted overnight in 300 µl 0.3 M NaCl in siliconized tubes supplemented with 1 µl of a 100 mM solution of the reverse transcription primer (5'-CAAGCAGAAGACGGCATACGA-3'). The RNA was subsequently precipitated.

Reverse Transcription, PCR amplification and *PmeI* digestion

The precipitated RNA was dissolved in 5.6 µl H_2O and denatured by heating for 30 sec at 90°C. Then, 1.5 µl of 0.1 M DTT, 3 µl of 5x first-strand buffer and 4.2 µl of 2 mM dNTPs were added, the reaction was incubated for 3 min at 50°C and 0.75 µl of reverse transcriptase (200 U/µl Superscript III, RNase H [-] M-MLV reverse transcriptase, Invitrogen) were added. The reaction was carried out at 50°C for 45 min. The remaining RNA was hydrolyzed by adding 40 µl of 150 mM KOH/20 mM Tris and incubating 10 min at 90°C and the solution was neutralized by addition of 40 µl of 150 mM HCl to obtain a pH between 7.0 and 9.5.

To amplify the cDNA from previous step, 10 µl of 2 mM dNTPs, 10 µl of 10x PCR buffer, 1 µl of 100 mM 3' primer (same as RT primer), 1 µl of 5' primer (5'-AATGATACGGCGACCACCGACAGGTTCAGAGTTCTACAGTCCGA 3'), 67 µl of water, and 1 µl of Taq DNA polymerase (5U/µl) were added to 10 µl of cDNA, and cycling was performed with the following parameters: 45 s at 94°C , 85 s at 50°C , and 60 s at 72°C for 25 cycles. An aliquot of 5 µl of PCR was taken after 15, 20, and 25 cycles. The PCR aliquots were visualized after loading on a 3% NuSieve agarose gel (Cambrex) using a 25 bp DNA ladder (Invitrogen) as a size marker to determine the number of PCR cycles corresponding to the linear range of amplification. A new PCR using the same conditions and the correct number of cycles was then performed. After amplification, NaCl was added to a final concentration of 0.3 M, the DNA was extracted with phenol and phenol/chloroform and precipitated with ethanol.

To get rid of the cloned size markers, the pellet was dissolved in 10 µl 10x NEB buffer 4.88 µl water, and 2 µl *PmeI* and incubated at 37°C for 2 h. The digested product was extracted once with phenol/chloroform after addition of NaCl to a final concentration of 0.3 M and once with chloroform and precipitated with ethanol. The pellet was dissolved in 30 µl 1x PCR buffer and

loaded on a 3% NuSieve agarose gel. After migration, the band was visualized on a UV table, and cut out. The gel slice was weighted, at least 1 volume of 0.4 M NaCl was added and placed at 65°C for 5 min. The DNA was then extracted once with phenol pH 8 previously heated to 65°C, once with phenol/chloroform, and once with chloroform. After addition ethanol, the PCR product was precipitated and the pellet was dissolved in 50 µl H_20, ready for sequencing.

4.2.2 Cell biological methods

4.2.2.1 Culturing of mammalian cells

Mammalian cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM, PAA, Pasching, Austria) at 37°C and 5% CO_2 in culture plates of different sizes. The medium was supplied with 10 % (v/v) fetal bovine serum (FBS, Biochrome, Berlin, Germany) as well as 1% (v/v) penicillin/streptomycin (PAA, Pasching, Austria). To split the cells, medium was aspirated off, cells were washed once with PBS (pH 7.5) and detached by Trypsin-EDTA (PAA, Pasching, Austria) treatment.

4.2.2.2 Calcium Phosphate transfections

If not co-transfected with 2'OMe-miRNA-inhibitor, plasmids were transfected using Calcium Phosphate transfection methods. Approximately 5h prior to transfection cells were plated at 30% confluency. Per 15 cm dish 5-20 µg plasmid DNA was diluted in 1075 µl H_2O and 153µl 2M $CaCl_2$. 1250 µl 2× HEPES-buffered saline (274 mM NaCl, 1.5 mM Na_2HPO_4, 54.6 mM HEPES-KOH pH 7.1) was added drop-wise under gentle agitation. The transfection solution was then sprinkled onto the cells. Medium was changed 1 d post-transfection and cells were harvested 2d post-transfecion.

4.2.2.3 siRNA transfections

For knockdown experiments siRNAs were transfected with Lipofectamine RNAiMax (Invitrogen) according to the manufacturer's protocol 5d prior to harvest of the cells or 4d prior to cotransfection of pMIR-RL 3'UTR and 2'-OMe-miRNA-inhibitor. The following siRNAs were used: 5'- AAGTCACGAAGCCTACTCGTT -3' and 5'- CGAGTAGGCTTCGTGACTTTT -3' for Drosha, 5'- CTTGCTGAATACTTGGTCCTT -3' and 5'- GGACCAAGTTCAGCAAGTT -3' for Dicer and 5'- GCACGGAAGTCCATCTGAATT -3' and 5'- TTGTCTTGCATTCGACTAATT -3' for Ago2. Knockdowns were validated by quantitative real-time PCR.

4.2.2.4 2’ OMe transfections

If not used for Luciferase assys and not cotransfected with plasmids, 2’ OMe-miRNA-inhibitors were transfected with Lipofectamine RNAiMax (Invitrogen) according to the manufacturers protocol 2 d prior to harvest of the cells. The following 2’ OMe-miRNA-inhibitors were used: 5’-AAGACCTGTTCTATCTACCTT 3’ for sACA45 and 5’- GCAAGGGCGAATGCAGAAAATAT 3’ for BART2. BART2 is a miRNA derived from the Epxtein-Barr virus and used as a control for unspecific effects caused by the 2’ OMe-oligonucleotides.

4.2.2.5 Cotransfection of 2’ OMe and luciferase reporter constructs

The day before transfection, cells were plated subconfluently on a 48-well plate. Co-transfection for luciferase assays were performed with 50 ng pMIR-RL 3'UTR and 20 pmol 2'OMe-miRNA-inhibitor per 2x104 cells in a 48-well plate using Lipofectamine 2000 (Invitrogen, Paisley, UK).

4.2.2.6 Luciferase assays

Luciferase activity was measured 24 h post transfection using a dual luciferase reporter system as described by the manufacturer (Promega). Firefly luciferase expression was normalized to Renilla luciferase expression. Error bars are derived from four individual experiments.

4.2.2.7 Western blotting

For western blotting, protein sample buffer was added to lysates or the immunoprecipitates and the precipitated proteins were analyzed by SDS-PAGE followed by semidry western blotting using 1x Towbin blotting buffer.

4.2.2.8 Sucrose density analysis

For complex purification and co-immunoprecipitations, HEK 293 cells were lysed in cell lysis buffer I containing 0.5 mM dithiothreitol and protease inhibitors (Roche, Penzberg, Germany) and centrifuged at 10,000g for 10 min at 4°C. For fractionations, gradients from 15% (w/v) to 55% (w/v) sucrose in 150 mM KCl, 25 mM Tris (pH 7.4) and 2 mM EDTA were used. Lysates were separated by centrifugation at 30,000 r.p.m. for 18 h in an SW41 rotor at 4°C. To determine indicated S values, catalase (11S), apoferritin (17S) and thyroglobin (19S) were used. For the analysis of RNA-dependent interactions, extracts were preincubated with 100 mg/ml RNase A (Qiagen, Hilden, Germany) at 4°C for 1 h.

4.2.2.9 Computational methods for 454 data analysis

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 50 end or the last three nt at the 30 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 us- ing the tables from UCSC database (Karolchik et al., 2003). A read was anno- tated 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 us- ing NCBI megablast with the following options: 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 (Friedla¨ nder 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 posi- tions and frequencies of the reads mapped to the individual hairpin, using Bayes- ian 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).

For conservation analysis of human ACA45, the 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 origin of the different data sets is indicated in the text.

To map the total of 17362367 sequence reads obtained by sequencing Ago1–4 IP using Solexa technology to the genome, we used the locally devel- oped suffix array-based tool (to be published elsewhere). Candidate snoRNAs with miRNA-like processing were selected 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.

Abbreviations

3’ UTR	3’ untranslated region
Ago	Argonaute
CasiRNA	cis-acting small interfering RNA
Dcl	Dicer-like
Dcr	Dicer
ds	double-stranded
dsRBD	double stranded RNA binding domain
DUF	domain of unknown function
eiF	eukaryotic initation factor
endo-siRNA	endogenous small interfering RNA
ER	endoplasmatic reticulum
ES cells	embryonic stem cells
exo-siRNA	exogenous small interfering RNA
FH	Flag-HA
hcRNA	heterochromatic RNAs
IRES	internal ribosomal entry site
Loqs	Loquatious
miRNA	microRNA
miRNP	micro ribonucleoprotein
mRNP	messenger Ribonucleoprotein
NatsiRNA	natural antisense-transcript small interfering RNA
ncRNA	non-coding RNA
nt	nucleotides
p-bodies	processing bodies
Pol	polymerase
pre-miRNA	precursor microRNA
pri-miRNA	primary microRNA
rasiRNA	repeat-assocaited small interfering RNA
RdRP	RNA dependent RNA polymerase
RIIID	RNAse III domain
RISC	RNA induced silencing complex

RITS	RNA induced transcriptional gene silencing
RNAi	RNA Interference
RNP	ribonucleoprotein
rRNA	ribosomal RNA
siRNA	small interfering RNA
snoRNA	small nucleolar RNA
snRNA	small nuclear RNA
ss	single-stranded
stRNA	small temporal RNA
tasi RNA	trans-acting small interfering RNA
tRNA	transfer RNA

Figure Index

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Appendix

MiRNAs associated with human Ago1 and Ago2 (454 Sequencing)

	read numbers		% of total annotated reads		% of total mirna reads	
miRNA	Ago1	Ago2	Ago1	Ago2	Ago1	Ago2
hsa-miR-17	4702	6698	26.41	20.95	28.28	21.46
hsa-miR-106a	1508	500	8.47	1.56	9.07	1.60
hsa-miR-25	1183	471	6.64	1.47	7.11	1.51
hsa-miR-106b	1084	920	6.09	2.88	6.52	2.95
hsa-miR-15b	857	593	4.81	1.85	5.15	1.90
hsa-miR-151-5p	832	427	4.67	1.34	5.00	1.37
hsa-miR-20a	729	7486	4.09	23.41	4.38	23.98
hsa-miR-92a	646	2547	3.63	7.97	3.89	8.16
hsa-miR-103	523	329	2.94	1.03	3.15	1.05
hsa-miR-16	502	2402	2.82	7.51	3.02	7.70
hsa-miR-221	364	244	2.04	0.76	2.19	0.78
hsa-miR-93	278	1004	1.56	3.14	1.67	3.22
hsa-miR-23b	273	129	1.53	0.40	1.64	0.41
hsa-miR-15a	226	581	1.27	1.82	1.36	1.86
hsa-miR-26b	223	179	1.25	0.56	1.34	0.57
hsa-miR-27b	222	256	1.25	0.80	1.34	0.82
hsa-miR-222	159	137	0.89	0.43	0.96	0.44
hsa-miR-128	147	145	0.83	0.45	0.88	0.46
hsa-miR-191	140	177	0.79	0.55	0.84	0.57
hsa-miR-23a	138	77	0.78	0.24	0.83	0.25
hsa-miR-652	128	76	0.72	0.24	0.77	0.24
hsa-miR-196a	122	146	0.69	0.46	0.73	0.47
hsa-miR-320	100	379	0.56	1.19	0.60	1.21
hsa-miR-378	87	72	0.49	0.23	0.52	0.23
hsa-miR-424	78	272	0.44	0.85	0.47	0.87
hsa-miR-218	74	322	0.42	1.01	0.45	1.03
hsa-miR-615-3p	62	26	0.35	0.08	0.37	0.08
hsa-miR-27a	60	133	0.34	0.42	0.36	0.43
hsa-miR-30estar	58	19	0.33	0.06	0.35	0.06
hsa-miR-361-5p	57	95	0.32	0.30	0.34	0.30
hsa-miR-152	49	9	0.28	0.03	0.29	0.03
hsa-miR-17star	43	25	0.24	0.08	0.26	0.08
hsa-miR-532-3p	34	35	0.19	0.11	0.20	0.11
hsa-miR-532-5p	30	22	0.17	0.07	0.18	0.07
hsa-miR-19b	30	360	0.17	1.13	0.18	1.15
hsa-miR-151-3p	28	22	0.16	0.07	0.17	0.07
hsa-miR-455-3p	28	12	0.16	0.04	0.17	0.04
hsa-miR-185	27	61	0.15	0.19	0.16	0.20
hsa-miR-26a	26	119	0.15	0.37	0.16	0.38

hsa-miR-374b	23	60	0.13	0.19	0.14	0.19
hsa-miR-183	23	33	0.13	0.10	0.14	0.11
hsa-miR-182	22	60	0.12	0.19	0.13	0.19
hsa-miR-30c	22	126	0.12	0.39	0.13	0.40
hsa-miR-766	21	18	0.12	0.06	0.13	0.06
hsa-miR-942	21	30	0.12	0.09	0.13	0.10
hsa-miR-30b	19	106	0.11	0.33	0.11	0.34
hsa-miR-30astar	19	9	0.11	0.03	0.11	0.03
hsa-miR-195	18	26	0.10	0.08	0.11	0.08
hsa-miR-18a	18	799	0.10	2.50	0.11	2.56
hsa-miR-877	16	4	0.09	0.01	0.10	0.01
hsa-miR-1226	16	12	0.09	0.04	0.10	0.04
hsa-miR-345	14	13	0.08	0.04	0.08	0.04
hsa-miR-425	13	96	0.07	0.30	0.08	0.31
hsa-miR-423-5p	13	19	0.07	0.06	0.08	0.06
hsa-miR-24	12	32	0.07	0.10	0.07	0.10
hsa-miR-148a	12	30	0.07	0.09	0.07	0.10
hsa-miR-34a	12	27	0.07	0.08	0.07	0.09
hsa-miR-196b	11	41	0.06	0.13	0.07	0.13
hsa-miR-125b	10	7	0.06	0.02	0.06	0.02
hsa-miR-22star	10	7	0.06	0.02	0.06	0.02
hsa-miR-30d	10	59	0.06	0.18	0.06	0.19
hsa-miR-7-1star	10	19	0.06	0.06	0.06	0.06
hsa-miR-194	10	14	0.06	0.04	0.06	0.04
hsa-miR-132	10	32	0.06	0.10	0.06	0.10
hsa-miR-92b	9	23	0.05	0.07	0.05	0.07
hsa-miR-99b	9	51	0.05	0.16	0.05	0.16
hsa-miR-197	9	18	0.05	0.06	0.05	0.06
hsa-miR-199b-3p	9	2	0.05	0.01	0.05	0.01
hsa-miR-423-3p	9	74	0.05	0.23	0.05	0.24
hsa-miR-221star	9	5	0.05	0.02	0.05	0.02
hsa-miR-744	9	10	0.05	0.03	0.05	0.03
hsa-miR-130b	8	18	0.04	0.06	0.05	0.06
hsa-miR-503	8	37	0.04	0.12	0.05	0.12
hsa-miR-378star	8	21	0.04	0.07	0.05	0.07
hsa-miR-187	7	10	0.04	0.03	0.04	0.03
hsa-miR-454	7	26	0.04	0.08	0.04	0.08
hsa-miR-9	7	26	0.04	0.08	0.04	0.08
hsa-miR-186	7	65	0.04	0.20	0.04	0.21
hsa-miR-125a-5p	7	20	0.04	0.06	0.04	0.06
hsa-miR-324-3p	7	51	0.04	0.16	0.04	0.16
hsa-miR-497	7	3	0.04	0.01	0.04	0.01
hsa-miR-590-3p	7	126	0.04	0.39	0.04	0.40
hsa-miR-22	6	34	0.03	0.11	0.04	0.11
hsa-miR-210	6	0	0.03	0.00	0.04	0.00
hsa-miR-598	6	20	0.03	0.06	0.04	0.06

hsa-miR-107	6	1	0.03	0.00	0.04	0.00
hsa-miR-501-3p	6	4	0.03	0.01	0.04	0.01
hsa-miR-18astar	6	10	0.03	0.03	0.04	0.03
hsa-miR-30e	6	53	0.03	0.17	0.04	0.17
hsa-miR-7	5	46	0.03	0.14	0.03	0.15
hsa-miR-126	5	20	0.03	0.06	0.03	0.06
hsa-miR-181b	5	8	0.03	0.03	0.03	0.03
hsa-miR-365	5	27	0.03	0.08	0.03	0.09
hsa-miR-10a	5	65	0.03	0.20	0.03	0.21
hsa-miR-212	5	10	0.03	0.03	0.03	0.03
hsa-miR-96	5	8	0.03	0.03	0.03	0.03
hsa-miR-28-3p	5	13	0.03	0.04	0.03	0.04
hsa-miR-502-3p	4	2	0.02	0.01	0.02	0.01
hsa-miR-340star	4	13	0.02	0.04	0.02	0.04
hsa-miR-21	4	112	0.02	0.35	0.02	0.36
hsa-miR-421	4	39	0.02	0.12	0.02	0.12
hsa-miR-101	4	32	0.02	0.10	0.02	0.10
hsa-miR-148b	4	12	0.02	0.04	0.02	0.04
hsa-miR-484	4	74	0.02	0.23	0.02	0.24
hsa-miR-301a	4	10	0.02	0.03	0.02	0.03
hsa-miR-760	4	0	0.02	0.00	0.02	0.00
hsa-miR-342-3p	3	49	0.02	0.15	0.02	0.16
hsa-miR-122	3	0	0.02	0.00	0.02	0.00
hsa-miR-19a	3	72	0.02	0.23	0.02	0.23
hsa-miR-200c	3	8	0.02	0.03	0.02	0.03
hsa-miR-877star	3	0	0.02	0.00	0.02	0.00
hsa-miR-140-3p	3	31	0.02	0.10	0.02	0.10
hsa-miR-330-3p	3	1	0.02	0.00	0.02	0.00
hsa-miR-16-2star	3	19	0.02	0.06	0.02	0.06
hsa-miR-624star	3	10	0.02	0.03	0.02	0.03
hsa-miR-215	3	1	0.02	0.00	0.02	0.00
hsa-miR-10b	2	26	0.01	0.08	0.01	0.08
hsa-miR-486-5p	2	1	0.01	0.00	0.01	0.00
hsa-miR-582-5p	2	4	0.01	0.01	0.01	0.01
hsa-miR-29a	2	42	0.01	0.13	0.01	0.13
hsa-miR-192	2	4	0.01	0.01	0.01	0.01
hsa-miR-500star	2	2	0.01	0.01	0.01	0.01
hsa-miR-9star	2	25	0.01	0.08	0.01	0.08
hsa-miR-331-3p	2	3	0.01	0.01	0.01	0.01
hsa-miR-374a	2	54	0.01	0.17	0.01	0.17
hsa-miR-629	2	2	0.01	0.01	0.01	0.01
hsa-miR-30dstar	2	0	0.01	0.00	0.01	0.00
hsa-miR-425star	2	4	0.01	0.01	0.01	0.01
hsa-miR-93star	2	2	0.01	0.01	0.01	0.01
hsa-miR-362-5p	2	8	0.01	0.03	0.01	0.03
hsa-miR-31	2	12	0.01	0.04	0.01	0.04

hsa-miR-629star	2	1	0.01	0.00	0.01	0.00
hsa-miR-328	2	1	0.01	0.00	0.01	0.00
hsa-miR-139-5p	2	2	0.01	0.01	0.01	0.01
hsa-miR-486-3p	2	3	0.01	0.01	0.01	0.01
hsa-miR-28-5p	2	20	0.01	0.06	0.01	0.06
hsa-miR-190	2	8	0.01	0.03	0.01	0.03
hsa-miR-505	2	5	0.01	0.02	0.01	0.02
hsa-miR-548c-5p	2	7	0.01	0.02	0.01	0.02
hsa-miR-140-5p	1	3	0.01	0.01	0.01	0.01
hsa-miR-146a	1	0	0.01	0.00	0.01	0.00
hsa-miR-200b	1	1	0.01	0.00	0.01	0.00
hsa-miR-25star	1	0	0.01	0.00	0.01	0.00
hsa-miR-455-5p	1	2	0.01	0.01	0.01	0.01
hsa-miR-483-5p	1	0	0.01	0.00	0.01	0.00
hsa-miR-32	1	5	0.01	0.02	0.01	0.02
hsa-miR-642	1	0	0.01	0.00	0.01	0.00
hsa-miR-657	1	0	0.01	0.00	0.01	0.00
hsa-miR-339-5p	1	4	0.01	0.01	0.01	0.01
hsa-miR-339-3p	1	6	0.01	0.02	0.01	0.02
hsa-miR-542-3p	1	0	0.01	0.00	0.01	0.00
hsa-miR-181a-2star	1	3	0.01	0.01	0.01	0.01
hsa-miR-625	1	1	0.01	0.00	0.01	0.00
hsa-miR-324-5p	1	7	0.01	0.02	0.01	0.02
hsa-miR-550star	1	1	0.01	0.00	0.01	0.00
hsa-miR-181a	1	7	0.01	0.02	0.01	0.02
hsa-miR-410	1	0	0.01	0.00	0.01	0.00
hsa-miR-204	1	1	0.01	0.00	0.01	0.00
hsa-miR-106bstar	1	2	0.01	0.01	0.01	0.01
hsa-miR-504	1	0	0.01	0.00	0.01	0.00
hsa-miR-708	1	4	0.01	0.01	0.01	0.01
hsa-miR-590-5p	1	13	0.01	0.04	0.01	0.04
hsa-miR-30a	1	18	0.01	0.06	0.01	0.06
hsa-miR-222star	1	1	0.01	0.00	0.01	0.00
hsa-miR-193b	1	2	0.01	0.01	0.01	0.01
hsa-miR-548b-5p	1	1	0.01	0.00	0.01	0.00
hsa-miR-374astar	1	4	0.01	0.01	0.01	0.01
hsa-miR-338-3p	1	0	0.01	0.00	0.01	0.00
hsa-miR-874	1	0	0.01	0.00	0.01	0.00
hsa-miR-362-3p	1	18	0.01	0.06	0.01	0.06
hsa-miR-340	1	12	0.01	0.04	0.01	0.04
hsa-miR-15bstar	1	4	0.01	0.01	0.01	0.01
hsa-miR-10astar	1	6	0.01	0.02	0.01	0.02
hsa-miR-369-3p	1	0	0.01	0.00	0.01	0.00
hsa-miR-296-3p	1	1	0.01	0.00	0.01	0.00
hsa-miR-330-5p	1	0	0.01	0.00	0.01	0.00
hsa-miR-450a	1	17	0.01	0.05	0.01	0.05

hsa-miR-628-3p	1	1	0.01	0.00	0.01	0.00
hsa-miR-346	1	0	0.01	0.00	0.01	0.00
hsa-miR-98	1	0	0.01	0.00	0.01	0.00
hsa-miR-361-3p	1	6	0.01	0.02	0.01	0.02
hsa-miR-126star	1	11	0.01	0.03	0.01	0.04
hsa-miR-199b-5p	1	1	0.01	0.00	0.01	0.00
hsa-miR-589	1	0	0.01	0.00	0.01	0.00
hsa-miR-34c-3p	1	0	0.01	0.00	0.01	0.00
hsa-miR-95	1	1	0.01	0.00	0.01	0.00
hsa-miR-190b	1	0	0.01	0.00	0.01	0.00
hsa-miR-29b	1	8	0.01	0.03	0.01	0.03

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Curriculum Vitae

PERSONAL DETAILS

Name	Christine Ender
Date of Birth	10.07.1980
City of Birth	Neuchâtel, Switzerland
Citizenship	Swiss

EDUCATION

10/2005	Diploma in Molecular Biology, University of Basel, Switzerland
12/1999	Matura/Abitur, Gymnasium Münchenstein, Switzerland

SCIENTIFIC WORK EXPERIENCE

11/2005 – present	Ph.D. student with Dr. Gunter Meister, Max-Planck-Institute of Biochemistry, Martinsried near Munich, Germany
03/2004 – 06/2005	Diploma student with Dr. Witold Filipowicz, Friedrich Miescher Institute for Biomedical Research, Basel, Switzerland Part of Novartis Research Foundation

RESEARCH ARTICLES (PEER-REVIEWED)

2009	**Ender C**., Meister G. Argonaute proteins at a glance *Journal of Cell Science, submitted*
2008	**Ender C**., Krek A., Friedländer M.R., Beitzinger M., Weinmann L., Chen W., Pfeffer S., Rajewsky N. & Meister G. (2008) A human snoRNA with microRNA-like functions. *Molecular Cell*, 32, 519-528. *Featured article; front cover of this issue created by C. Ender*
2007	Höck J., Weinmann L., **Ender C**., Rüdel S., Kremmer E., Raabe M., Urlaub H. & Meister G. (2007) Proteomic and functional analysis of Argonaute-containing mRNA-protein complexes in human cells. *EMBO Reports*, 8, 11, 1052-1060.

SPEAKER AT SEMINARS AND CONFERENCES

2009	Meeting “RNA Forschergruppe”, EMBL Heidelberg, GERMANY
	Special Seminar Series, Victor Chang Institute for Cardiac Research, Sydney, AUSTRALIA
2008	Meeting on Translational Control, Cold Spring Harbour, NY, USA
	Meeting “RNA Forschergruppe”, Halle, GERMANY
	“Microsymposium on small RNAs”, Vienna, AUSTRIA
	Interdepartmental Seminar, Max-Planck-Institute of Biochemistry, Martinsried, GERMANY

RESEARCH STUDENT TRAINING EXPERIENCE

11/2008 – 06/2009	Bachelor student, later Master student at King's College, London
08/2007 – 08/2008	Diploma student, later Ph.D. student at Gene Center, LMU, Munich

EXTRA CURRICULAR ACTIVITIES

2009 – present	Co-organizer of the interdepartmental Research in Progress Seminar Series, Max-Planck-Institute of Biochemistry, Martinsried, Germany
2009	Graphic Design for Poster & Flyer of EMBL Conference 2010 on mRNAs
2008	Design of front cover of Molecular cell, Vol 32, No 4, 2008
2005	Member of the executive committee of the "career guidance conference in life sciences 2005" in Basel, http://www.fmi.ch/cgc2005/index.html
2000 – 2005	Board member of the association of students "Verein Studierender der Biologie" (VSB) at Biozentrum Basel
	Event organization for the students association, student representative in the Biozentrum conference and in the curriculum committee, cooperation in two appointment committees for the Biozentrum
2003 – 2005	President of the association of students (VSB) at the Biozentrum

NON-SCIENTIFIC WORK EXPERIENCE

2005	MCH: Swiss Exhibition Group, Switzerland Hostess "City Info" during international shows
02/2002 – 08/2003	Town hall, Aesch, Switzerland Human resources department (part-time)
10/2001 – 08/2002	Sports shop "Ochsner Sport", Pratteln, Switzerland (part-time)
02/2000 – 08/2000	Insurance company "Schweizerische National-Versicherung", Basel, Switzerland Human resources department (full-time)

LANGUAGE SKILLS

Fluent in German and English, practical knowledge of French