Chapter 8
The RNA Interference Pathway in *Trypanosoma brucei*

Elisabetta Ullu, Nikolay G. Kolev, Rebecca L. Barnes, and Christian Tschudi

Abstract In most eukaryotic cells, expression or delivery of long double-stranded RNA (dsRNA) signals the presence of foreign and/or potentially dangerous nucleic acids, such as viruses or transcripts derived from retroposons and transposons. As a consequence cells go on red alert and activate specific defense mechanisms to eliminate the invaders. Among such mechanisms is the RNA interference (RNAi) pathway, which is triggered by long dsRNAs, destroys target transcripts in a sequence-specific manner, and is widespread throughout eukaryotic evolution. Here we summarize our current understanding of the RNAi mechanism in *Trypanosoma brucei*, a protozoan parasite and early divergent eukaryote, and highlight similarities and differences with the RNAi machinery and its function in higher eukaryotes.

Keywords Argonaute • Dicer • RNAi genes • siRNA

E. Ullu (✉)
Department of Internal Medicine, Yale University School of Medicine, 295 Congress Avenue, New Haven, CT 06536-0812, USA

Department of Cell Biology, Yale University School of Medicine, 295 Congress Avenue, New Haven, CT 06536-0812, USA
e-mail: elisabetta.ullu@yale.edu

N.G. Kolev • C. Tschudi
Division of Epidemiology of Microbial Diseases, Yale University School of Public Health, 295 Congress Avenue, New Haven, CT 06536-0812, USA
e-mail: nikolay.kolev@yale.edu; christian.tschudi@yale.edu

R.L. Barnes
Department of Internal Medicine, Yale University School of Medicine, 295 Congress Avenue, New Haven, CT 06536-0812, USA
e-mail: rebecca.l.barnes@yale.edu

A. Bindereif (ed.), *RNA Metabolism in Trypanosomes*, Nucleic Acids and Molecular Biology 28, DOI 10.1007/978-3-642-28687-2_8,

8.1 Overview of RNA Interference

Genetic interference triggered by double-stranded RNA (dsRNA), commonly referred to as RNA interference or RNAi, was first described in 1998 by the laboratories of Andrew Fire and Craig Mello (Fire et al. 1998). The subsequent discovery of a plethora of small 19–30 nucleotide (nt) noncoding RNAs that function in diverse pathways, ranging from mRNA degradation, translational repression, heterochromatin formation, and DNA elimination, has radically changed our understanding of how gene expression is regulated in eukaryotes. In addition, RNAi provided the scientific community with a tool of immense value for the analysis of gene function, especially in organisms not easily amenable to classical genetic approaches.

As surmised by studies in several model organisms, the "natural" or endogenous RNAi pathway functions as a defense mechanism to maintain genome integrity by destroying transcripts derived from molecular parasites, namely viruses, transposons, retroposon, and transgenes, thus limiting viral infection and the catastrophic consequences of transposon/retroposon mobilization (Siomi and Siomi 2009). RNAi is widespread throughout the eukaryotic lineage, from protists to man and based on phylogenetic considerations, it is likely that the ancestral eukaryote was endowed with a functional RNAi pathway (Cerutti and Casas-Mollano 2006). In this review, we focus on the parasitic protozoa *Trypanosoma brucei*, one of the few pathogenic single-cell eukaryotes where the mechanism and functional significance of RNAi has been studied in detail. For more details on RNAi, readers are referred to recent reviews (Siomi and Siomi 2009; Liu and Paroo 2010).

8.2 RNAi in Model Organisms

The minimal set of RNAi components consists of Dicer, an RNase III-family enzyme presiding over the processing of dsRNAs into small interfering RNAs (siRNAs); a "slicer" Argonaute (AGO), an endonuclease with an RNase H-like domain required for target RNA cleavage; and a dsRNA-binding protein (dsRBP), which facilitates Dicer cleavage and assists in transferring siRNAs into AGO (Liu and Paroo 2010). In certain organisms, including *Arabidopsis thaliana*, *Caenorhabditis elegans*, and *Schizosaccharomyces pombe*, a fourth RNAi factor, an RNA-dependent-RNA polymerase (RdRP), amplifies the initial RNAi response, resulting in the production of secondary siRNAs (Cerutti and Casas-Mollano 2006).

In the current model, the RNAi pathway is subdivided into two phases, namely the initiation and the effector steps. In the first phase Dicer, in a complex with the dsRBP and AGO, processes dsRNA into duplex siRNAs with characteristic two nucleotide 3′ extensions, which are then transferred to AGO slicer. AGO cleaves the "passenger" siRNA strand, the strand that is destined to be ejected, while retaining the "guide" siRNA strand. Recent evidence indicates that the process of

siRNA loading into AGO is facilitated by the Hsp90/70 chaperone system, which probably maintains AGO in a conformation suitable for accepting siRNAs (Iwasaki et al. 2010; Miyoshi et al. 2010). The assembly of the AGO-guide siRNA complex leads to the formation of active RISC (RNA-induced silencing complex), of which AGO is the catalytic engine. In the effector step the AGO ribonucleoprotein seeks out and then cleaves homologous target RNA, approximately in the middle of the region of complementarity with the siRNA. The cleaved RNA fragments are then released and degraded by cellular ribonucleases, while RISC is recycled for another round of slicing. It should be noted that the above pathway is based in large parts on studies in higher eukaryotes and considerably less is known in single-cell eukaryotes.

8.3 RNAi in *Trypanosoma brucei*

Ever since the discovery of RNAi in *Trypanosoma brucei* in 1998 (Ngo et al. 1998), a new era began in the study of the biology of this important human pathogen. It is not an exaggeration to say that since the beginning of the year 2000 the way experiments are carried out in *T. brucei* has undergone a major revolution, and gene silencing by RNAi has found wide applications, including forward genetic screens and global analysis of gene function (see Sect. 8.3.10).

To date functional studies have uncovered three components that are essential for RNAi in *T. brucei*: *Tb*AGO1, a member of the Argonaute family of proteins (Durand-Dubief and Bastin 2003; Shi et al. 2004b) and two distinct Dicer-like enzymes, namely *Tb*DCL1 and *Tb*DCL2 (Shi et al. 2006b; Patrick et al. 2009). However, comparative genomics indicates that there are at least two more genes that are candidates for being RNAi factors (see note added in proof), as they are present in RNAi-proficient trypanosomatids, namely the African trypanosomes, *Leishmania Viannia braziliensis* and *Crithidia fasciculata*, but absent in *T. cruzi*, *L. major*, and *L. donovani* (Lye et al. 2010), which are RNAi-negative (Robinson and Beverley 2003; DaRocha et al. 2004). Inspection of the *T. cruzi* and old-world *Leishmania* genomes shows no remnants of the DCL1 and DCL2 genes, whereas in the case of AGO1 a pseudogene is present in *Leishmania* but absent in *T. cruzi*. The loss of the RNAi genes appeared to have occurred twice in the trypanosomatid lineage (Lye et al. 2010), once at some undefined point in the lineage leading to *T. cruzi* and a second time after the divergence of the *Viannia* subgenus from other *Leishmania* species.

A schematic representation of our current view of the RNAi pathway in *T. brucei* is illustrated in Fig. 8.1. *Tb*AGO1, in a complex with single-stranded siRNAs, is found predominantly in the cytoplasm and is essential for mRNA degradation. However, functional evidence supports the model that *Tb*AGO1 is active in the nucleus as well (see Sect. 8.3.3). Notably, the two Dicers are localized in different cellular environments, with *Tb*DCL1 and *Tb*DCL2 predominantly in the cytoplasm

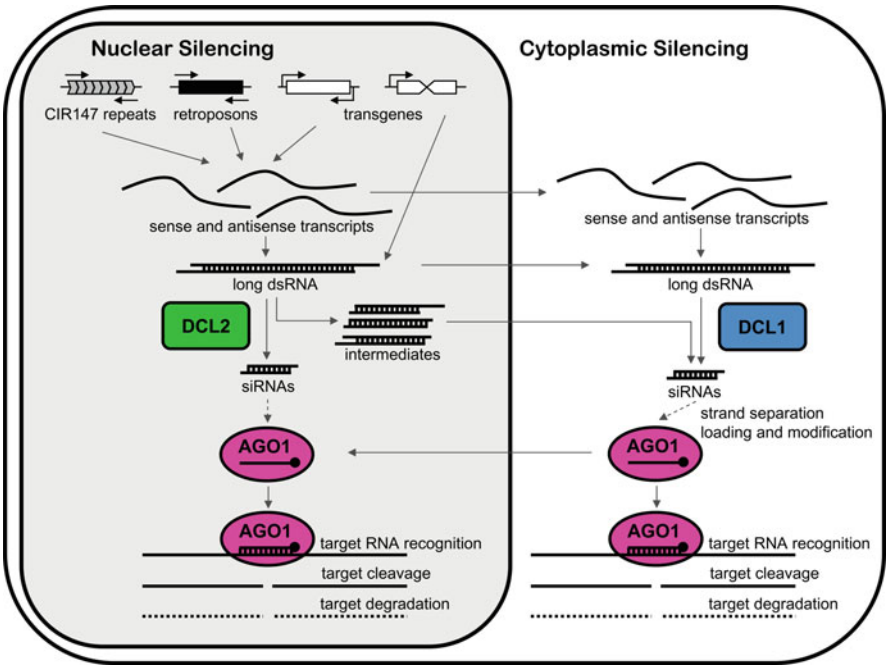

Fig. 8.1 Current working model of RNAi in *T. brucei*. Depicted are the two branches (nuclear and cytoplasmic) of silencing. Sense and antisense (or inverted repeat) RNAs transcribed in the nucleus form long double-stranded RNA (dsRNA) in both cellular compartments. DCL2 and DCL1 process the long RNA duplexes into siRNA duplexes in the nucleus and the cytoplasm, respectively. DCL2 also generates intermediate size dsRNAs, which are converted to siRNAs by DCL1. Argonaute (AGO1) is programmed with single-stranded "guide" siRNA as a result of duplex siRNA strand separation, loading, and modification of the 3′ end of the siRNA. AGO1 finds transcripts complementary to the bound siRNA in both compartments of the cell, and base pairing between the siRNA and the target leads to endonucleolytic cleavage and subsequent degradation of the target RNA

and the nucleus, respectively. In the following sections, we will review our current understanding of the functional significance for the compartmentalization of the two Dicers.

8.3.1 The Cytoplasmic Dicer-Like Protein TbDCL1

A number of early observations predicted the existence of Dicer or Dicer-like protein(s) in *T. brucei*, including the identification of siRNAs (Djikeng et al. 2001), the in vivo RNAi response to transfected long dsRNAs (Ngo et al. 1998), and the detection of an enzymatic activity in trypanosome extracts generating siRNA-size molecules (Best et al. 2005). Dicer and Dicer-like proteins are large multidomain proteins with the canonical signature of two neighboring RNase III

domains, termed RNase IIIa and RNase IIIb (Jaskiewicz and Filipowicz 2008). However, initial homology searches of the *T. brucei* genome with the *Aquifex aeolicus* RNase III domain only returned predicted ORFs with a single RNase III domain (Shi et al. 2006b). Nevertheless, what caught our attention was that two ORFs of 1,648 and 948 amino acids were present in *T. brucei*, the only RNAi-positive trypanosomatid protozoa known at that time, but absent in the RNAi-negative organisms *T. cruzi* and *L. major* (Ullu et al. 2004). Furthermore, as judged by the cellular distribution of GFP fusion proteins, the 1,648 and 948 amino acid-long proteins localized predominantly to the cytoplasm and nucleus, respectively (Shi et al. 2006b). Subsequent refined bioinformatics analysis and the availability of additional genomes of RNAi-positive African trypanosomes allowed the identification of a second RNase III domain in both candidates. Thus, the ORFs of 1,648 and 948 amino acids were named Dicer-like protein 1 or *Tb*DCL1 (Tb927.8.2370) and Dicer-like protein 2 or *Tb*DCL2 (Tb927.3.1230), respectively. *Tb*DCL2 will be discussed in Sect. 8.3.2.

Typically, RNase IIIa and RNase IIIb domains in metazoan and fungal Dicers are adjacent to each other (Jaskiewicz and Filipowicz 2008). However, in *Tb*DCL1 the RNase IIIa domain is close to the amino terminus, and the RNase IIIb domain is located approximately in the center of the molecule. No domains commonly associated with Dicer or Dicer-like proteins, like helicase, PAZ, DUF283, or dsRNA-binding domains, were recognizable along the *Tb*DCL1 polypeptide chain (Shi et al. 2006b). Although the apparent lack of additional domains was surprising at first, the large dataset available to date clearly revealed that Dicer and Dicer-like proteins have diversified substantially throughout eukaryotic evolution, making the two RNase III domains the only universal feature of this class of proteins. It is likely that the diverse domain structure observed in protozoan, fungal, plant, and metazoan proteins reflects organism-specific adaptations.

The RNase IIIa and IIIb domains in *Tb*DCL1 have the potential to form an intramolecular pseudodimer and to build the typical Dicer dsRNA processing center (Jaskiewicz and Filipowicz 2008) composed of two active sites (from the two RNase III domains), each able to perform scission of one of the two strands in the RNA duplex. Indeed, partially purified *Tb*DCL1 catalyzes Mg^{2+}-dependent processing of dsRNA into 24–26 nucleotide-long siRNAs, a size range typical for endogenous siRNAs in *T. brucei* (Shi et al. 2006b). In vivo, the activity of *Tb*DCL1 is responsible, but only in part, for the production of siRNAs from retroposon transcripts, which possibly escape the nucleus, for processing of transfected long dsRNA, and for the cleavage of 35–65 nucleotide dsRNA intermediates generated by *Tb*DCL2 (see Sect. 8.3.2). However, how *Tb*DCL1 recognizes a dsRNA substrate is at present unknown, since PAZ and dsRNA-binding motifs, which are essential for positioning the dsRNA at the catalytic center (MacRae et al. 2007), have so far not been identified.

Although Dicer enzymes have a primary role at the initiation step of RNAi, namely the processing of the dsRNA trigger to siRNAs, there is also evidence for a role in the effector step, namely the cleavage of the target transcript by AGO slicer (Jaskiewicz and Filipowicz 2008). This appears to be the case for *Tb*DCL1 as well,

since a point mutation of a conserved residue outside of the RNase III domains (D966G) does not detectably affect the production of siRNAs, but has a significant dominant negative effect on the cleavage of target mRNAs by *Tb*AGO1 (Shi et al. 2006b). Thus, one prediction would be that *Tb*DCL1 and *Tb*AGO1 interact, yet evidence for such a complex has been elusive so far.

8.3.2 The Nuclear TbDCL2 Initiates the Endogenous RNAi Response

In addition to the bioinformatic analysis described above, experimental evidence provided clues that *T. brucei* contained a second Dicer or Dicer-like enzyme. Firstly, genetic ablation of *Tb*DCL1 did not result in the upregulation of ingi or SLACS retroposon transcripts or transcripts derived from a family of 147-nucleotide-long chromosomal internal repeats, termed CIR147 repeats (Patrick et al. 2009), which are located at putative centromeric regions (Obado et al. 2005). This phenomenon was first observed in *Tb*AGO1 null cells and constitutes the hallmark of RNAi deficiency (Shi et al. 2004b). Secondly, the accumulation of CIR147 siRNAs was independent of *Tb*DCL1, and the siRNAs from ingi and SLACS were only partially depleted in *Tb*DCL1 null cells (Patrick et al. 2009). Thus, we reasoned that a second Dicer must be at work in the trypanosome RNAi pathway and *Tb*DCL2 was the most likely candidate, since sequence homology analysis and three-dimensional structure modeling revealed two RNase III domains and the remnants of a dsRNA-binding domain at the very C terminus (Patrick et al. 2009). Similar to *Tb*DCL1, the two RNase III domains in *Tb*DCL2 are not adjacent, but are found in close proximity to the two ends of the polypeptide, with RNase IIIa at the N terminus and RNase IIIb at the C terminus. One would assume that the two RNase III domains form an intramolecular pseudodimer, since partially purified wild-type *Tb*DCL2 is capable of processing long dsRNA into siRNA-size products in a Mg^{2+}-dependent fashion and single-point mutations in the active site of the RNase IIIb module (K879A and E886A) severely compromised this activity (Patrick et al. 2009). *Tb*DCL1 and *Tb*DCL2 appear to generate duplex siRNA products of slightly different size, as revealed by in vitro dicing assays with *Tb*DCL1 null and *Tb*DCL2 null whole cell extracts. The size range of *Tb*DCL2-generated siRNAs is one nucleotide shorter than the *Tb*DCL1-generated siRNAs; however, the significance of this difference is currently not clear. In addition, *Tb*DCL2 is responsible for the production of intermediate-size RNA molecules (35–65 nt long), which accumulate in the absence of *Tb*DCL1 (Patrick et al. 2009). These molecules are possibly double stranded, since in the case of CIR147 intermediates, both sense and antisense sequences are detected with similar frequency and their distribution is evenly spaced across the entire CIR147 repeat. The intermediate-size RNAs contain a 5′-monophosphate, as indicated by their sensitivity to treatment with Terminator exonuclease (Patrick et al. 2009), which is

consistent with their biogenesis depending on *Tb*DCL2. Although the relevance of the intermediates in the RNAi pathway remains to be investigated, one possibility is that they are side products of *Tb*DCL2 processing of long dsRNA. If *Tb*DCL2 requires substrates with a double-stranded region longer than ~65 bp for binding and processing, then RNA molecules with duplexes shorter than 35–65 bp and single-stranded extensions (5′ or 3′) would not be recognized. These "noncanonical" substrates appear to find their way to *Tb*DCL1, and this Dicer readily accepts them. In vivo, the intermediate RNAs gradually disappear upon increasing the levels of *Tb*DCL1 in the cell (Patrick et al. 2009) with concomitant increase in the amount of corresponding siRNAs, which indicates that the intermediates contain double-stranded regions of at least ~25 bp and serve as a source for siRNA production by *Tb*DCL1.

A *Tb*DCL2-GFP fusion protein localized predominantly to the nucleus, suggesting that the RNAi machinery is present in this compartment. Three lines of evidence support this hypothesis. Firstly, CIR147 transcripts in *Tb*AGO1 null cells are found exclusively in the nucleus, close to or at the nucleolus (Patrick et al. 2009). Secondly, in the absence of *Tb*DCL2, CIR147 siRNAs are below the limit of detection, indicating that their biogenesis is mostly dependent on *Tb*DCL2 (Patrick et al. 2009). Thirdly, work by the group of Michaeli has shown that small nucleolar RNAs (snoRNAs), which as the name indicates are only found within the confines of the nucleus, could be targeted for destruction by expressing homologous dsRNA, a phenomenon termed snoRNAi (Liang et al. 2003). In addition, our two laboratories have recently provided genetic evidence that snoRNAi requires *Tb*DCL2 and *Tb*AGO1, but not cytoplasmic *Tb*DCL1 (Gupta et al. 2010). Taken together, these observations strongly support the existence of a nuclear phase of the RNAi pathway. Since in the absence of *Tb*DCL2 retroposon and CIR147 transcripts accumulate, a phenotype observed in *Tb*AGO1 null but not in *Tb*DCL1 null cells, we proposed that nuclear *Tb*DCL2 initiates the endogenous RNAi response (Patrick et al. 2009). Indeed, *Tb*DCL2 appears to be the sole enzymatic activity producing siRNAs from dsRNAs retained in the nucleus, and its presence is critical for successful RNAi-mediated knockdown approaches targeting nuclear RNAs (Gupta et al. 2010). Notably, knockout of *Tb*DCL2 results in "hypersensitivity" to exogenous dsRNA and synthetic siRNAs, a phenotype that likely reflects decreased competition in *Tb*DCL2 null cells for the formation of active RISC between siRNAs from endogenous and exogenous dsRNA triggers (Patrick et al. 2009). This observation further highlights the crucial role played by *Tb*DCL2 in initiating and sustaining the endogenous RNAi pathway.

Why do trypanosomes have two Dicers? Whereas some of the roles of *Tb*DCL1 and *Tb*DCL2 overlap, namely the generation of siRNAs from retroposons and ensuring *Tb*AGO1 programming against transcripts from these mobile genetic elements to safeguard the trypanosome genome, the two Dicers also have specific functions in the RNAi pathway. *Tb*DCL2 is predominantly nuclear and is the enzyme producing siRNAs from dsRNAs formed by transcripts that do not leave the cell nucleus, e.g., the CIR147 and retroposon RNAs. *Tb*DCL2 appears to have more strict requirements for its substrates, since it leaves behind the intermediate-size

RNAs. *Tb*DCL1 is the second line of defense against dsRNA producing transcripts and accepts a broader arsenal of substrates, including the intermediates. Any RNA that escapes the confines of the nucleus and contains or forms (on its own or with an antisense partner) a double-stranded segment longer than ~25 bp is converted to siRNAs in the cytoplasm by *Tb*DCL1. An additional role for *Tb*DCL1 is in the effector step of the cytoplasmic RNAi branch, since it appears to be required for RISC-mediated cleavage of target transcripts. However, it remains to be determined whether *Tb*DCL2 has a similar function in the nucleus. In summary, trypanosomes have found an evolutionary advantage of having two Dicers with similar properties, but segregated into different cellular compartments, to tackle dsRNA challengers in a "two lines of defense" mechanism and initiate their destruction.

8.3.3 TbAGO1 Is the Argonaute Slicer

The *T. brucei* genome encodes two members of the Argonaute family of proteins, namely *Tb*AGO1, which is essential for the RNAi response (Durand-Dubief and Bastin 2003; Shi et al. 2004b) and *Tb*Piwi-like1 (Durand-Dubief and Bastin 2003), which is present in all trypanosomatid protozoa, irrespective of whether they are RNAi proficient (Ullu et al. 2004). *Tb*Piwi-like 1 is dispensable for the RNAi response (Durand-Dubief and Bastin 2003), but its function is yet to be discovered.

*Tb*AGO1 is required in experimental RNAi for cleavage of mRNA targets in the cytoplasm (Shi et al. 2004b) and snoRNAs in the nucleus (Gupta et al. 2010), and in the endogenous RNAi pathway for cleavage of retroposon- and repeat-derived transcripts, both in the nucleus and in the cytoplasm (Shi et al. 2004b; Patrick et al. 2009). Single-stranded siRNAs form a ribonucleoprotein complex with *Tb*AGO1 (Djikeng et al. 2003), which is highly abundant at 60,000 copies/cell (unpublished observations), as compared to 150–200,000 ribosomes. However, significantly larger *Tb*AGO1-containing complexes are detected in cells ablated of *Tb*DCL1 or *Tb*DCL2 (unpublished observations), possibly representing stalled intermediates in the formation of RISC. Cell fractionation and the microscopic localization of GFP- (Durand-Dubief and Bastin 2003) or epitope-tagged *Tb*AGO1 (Shi et al. 2004b) consistently place the majority of the protein in the cytoplasm. Nevertheless, the requirement of *Tb*AGO1 for downregulation of retroposon- and repeat-derived transcripts and for snoRNAi is consistent with *Tb*AGO1 also functioning in the nucleus, where it presumably travels to pick up siRNAs produced by *Tb*DCL2 and then slices single-stranded and potentially harmful transcripts. How *Tb*AGO1 gets access to the nucleus remains to be addressed experimentally. Lastly, a proportion of *Tb*AGO1 is found associated with polyribosomes (Djikeng et al. 2003; Shi et al. 2009). We have shown that this association is specific for translating ribosomes and appears to be mediated by ionic interactions, as *Tb*AGO1 is released from polyribosomes under high-salt conditions. We proposed that *Tb*AGO1 on polyribosomes functions as a sentinel ready for action, if potentially dangerous retroposon transcripts should become associated with polyribosomes (Shi et al. 2009).

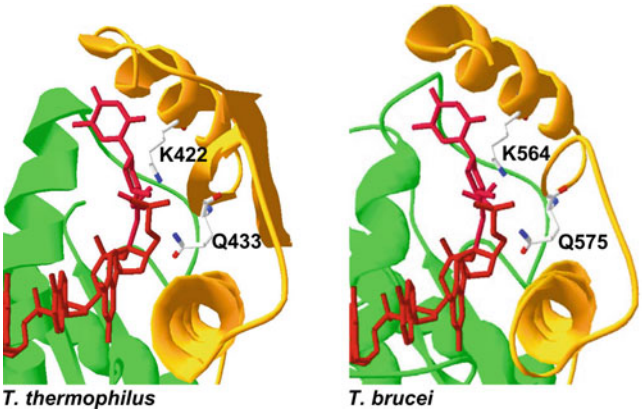

Fig. 8.2 Ribbon (AGO) and stick (DNA) diagrams of the *T. thermophilus* AGO MID domain bound to the 5′-end of a 21-base DNA guide strand (PDB ID 3DLH; *left panel*; (Wang et al. 2008)) and of the predicted model of *Tb*AGO1 with a superimposed DNA guide strand (*right panel*). The positions of two conserved residues in the binding pocket are indicated

8.3.4 Structural and Functional Motifs in Trypanosomatid AGO1

AGO-family proteins are about 100 kDa, highly basic and distinguished by four domains: the N-terminal, PAZ, MID, and PIWI domains, with the PIWI domain showing the highest degree of sequence conservation (Tolia and Joshua-Tor 2007). *Tb*AGO1, as well as its homologues in RNAi-proficient trypanosomatids, conform to the consensus structure of AGO family members, although their primary sequence is highly divergent from that of higher eukaryotic AGOs. The various domains have distinct roles, which have emerged from functional studies and the crystal structures of prokaryotic AGO paralogues (Tolia and Joshua-Tor 2007; Faehnle and Joshua-Tor 2010). The 5′-phosphate and the 3′-end of the guide small RNA are anchored by the MID and PAZ domains binding pockets, respectively, whereas the PIWI domain adopts a fold similar to that of RNase H-type enzymes and confers endonuclease activity to AGO "slicers." Using the crystal structure of *Thermus thermophilus* AGO as a template (Wang et al. 2008), a considerable part of the *Tb*AGO1 C terminus was amenable to three-dimensional modeling, despite low conservation at the primary sequence level (unpublished observation). In particular, the PIWI and part of the MID domain revealed structural similarities to *T. thermophilus* AGO, including the 5′-phosphate-binding pocket made up of the MID domain (Fig. 8.2). In vivo studies further indicated that predicted key amino acid residues in the *Tb*AGO1 5′-phosphate-binding pocket are critical for target RNA cleavage activity (unpublished observation).

The catalytic center of AGO slicers includes the amino acid triad DDH, which is found in all AGO proteins for which slicer activity has been documented (Fig. 8.3; Tolia and Joshua-Tor 2007). However, the presence of these active site residues

Fig. 8.3 Alignment of the motifs containing the catalytic triad DDH in selected Argonaute proteins from humans, fruit fly, and RNAi-proficient trypanosomes. The DDH residues are highlighted in *red* and residues conserved in all of the shown sequences in *blue*

does not guarantee that the AGO protein is endowed with endonuclease activity (Tolia and Joshua-Tor 2007). Of note, the trypanosomatid AGO1 proteins from *T. brucei*, *T. congolense*, *T. vivax*, *L. (V.) braziliensis*, *L. (V.) panamensis*, and *C. fasciculata* are not endowed with the DDH catalytic triad (Fig. 8.3). Whereas the first aspartate residue is conserved in AGO1 of African trypanosomes, it is absent in the *L. Viannia* and *Crithidia fasciculata* counterparts and replaced by valine. Another notable difference between trypanosomatid AGO1s is the presence of a conserved tyrosine at the histidine-equivalent position in the *C. fasciculata* and *L. Viannia* proteins, whereas this position is occupied by alanine in *Tb*AGO1 and by serine in the *T. congolense* and *T. vivax* proteins. Although intriguing, the functional significance of these amino acids substitutions needs to be verified experimentally.

Sequence alignments of the myriad of AGO proteins revealed additional invariant residues, including an arginine residue at position 735 of *Tb*AGO1, just one amino acid upstream from the second aspartate residue of the catalytic triad (Fig. 8.3). Mutation of arginine 735 to alanine in *Tb*AGO1 leads to severe impairment of mRNA cleavage, suggesting that this protein acts as a slicer (Shi et al. 2004c). In support of this model are two additional pieces of evidence. First, upon transfection of α-tubulin dsRNA we observe accumulation of discrete fragments with the size expected for endonucleolytic cleavage of the target mRNA (Shi et al. 2009) and, secondly, we showed that human AGO2 slicer can partially complement the cytoplasmic RNAi response when *Tb*AGO1 is ablated (Shi et al. 2006a). However, the absence in AGO1 of the "classic" catalytic triad of RNase H-type enzymes is puzzling, and this issue can only be resolved by in vitro experiments, which unfortunately have been unsuccessful so far. A possible reason behind our failure to reconstitute *Tb*AGO1 slicing activity in vitro is suggested by the observation that when the pool of newly synthesized *Tb*AGO1 is depleted, the RNAi response declines severely (Shi et al. 2007). We proposed that newly synthesized *Tb*AGO1 is preferentially loaded with newly processed siRNAs, possibly because newly synthesized *Tb*AGO1 is in a conformation that readily accepts siRNAs. We further speculated that the Hsp90 chaperone system may be

involved in facilitating the process of siRNA loading. This prediction has recently gained support by in vitro studies in *Drosophila* and tobacco extracts (Iwasaki et al. 2010; Miyoshi et al. 2010). Interestingly, reconstitution of RISC activity in tobacco could only be achieved by coupling translation of AGO with loading of synthetic siRNAs in a homologous in vitro extract.

8.3.5 Function of the RGG-Rich N Terminus of Tb*AGO1*

One of the striking features of the *T. brucei* AGO1 N-terminal domain is the high representation of arginine-glycine-glycine (RGG) motifs, which make up almost 50% of the first 59 amino acids (Shi et al. 2009). In particular, between positions 9 and 59 there are 10 RGG motifs, which are part of an 11-amino acid repeating unit with the consensus sequence G(Y/R)RGGRGGG(E/F)G. An N-terminal RGG domain is present in all trypanosomatid AGO1 homologues (Shi et al. 2009), indicating a conserved and important function. In addition, certain AGO-family members from higher eukaryotes are also endowed with RGG motifs. Interestingly, the N-termini of certain murine Piwi family proteins have methylated arginines, which guide interactions with Tudor proteins and affect protein stability and localization (Kirino et al. 2009; Vagin et al. 2009).

Through mutagenesis studies we have shown that the RGG domain of *Tb*AGO1, and specifically the arginine residues, are required for efficient cleavage of target mRNA in the cytoplasm and downregulation of retroposon transcripts in the nucleus. A second phenotype observed in cells expressing mutant *Tb*AGO1 carrying partial or complete deletions of the RGG domain or bearing substitutions of all arginine residues by lysine or alanine is the inhibition of the association of a proportion of *Tb*AGO1 with polyribosomes (Shi et al. 2009). A similar phenotype was reported for the *Toxoplasma gondii* AGO protein, which like *Tb*AGO1 contains an RGG-rich N terminus (Braun et al. 2010). Mass spectroscopy analysis, as well as reactivity with antibody Y12, which recognizes symmetric dimethylated arginine (sDMA), support the modification of at least some arginine residues in the *Tb*AGO1 RGG domain to sDMA (Shi et al. 2009). At present we do not know the identity of the specific protein arginine methyltransferases (PRMTs) responsible for modification of *Tb*AGO1. Based on the current model that sDMA is recognized by Tudor domain-containing proteins, we (unpublished observations) and others (Alsford et al. 2010) tested the possibility that *T. brucei* TudorSN, a protein with a recognizable Tudor domain, was required for RNAi, but the results have been negative. Thus, several questions concerning the function of the RGG domain remain to be answered. Which PRMT methylates arginine residues in the RGG domain? Are methylated arginines recognized by a specific, yet to be identified, factor? Does the RGG domain function as a protein and/or an RNA recognition module? Is this domain involved in guide siRNA-target RNA recognition or perhaps in determining the accessibility of the target RNA to the catalytic center?

8.3.6 Endogenous Small Interfering RNAs

siRNAs in a complex with AGO slicer guide recognition of target RNAs. The realization that siRNAs are essential mediators in RNAi was heralded by the seminal discovery in *Arabidopsis* that short 20–25 nt RNAs accumulated in transgenic plants undergoing "cosuppression," namely downregulation of cellular gene expression mediated by ectopic expression of homologous *trans*-genes (Hamilton and Baulcombe 1999). Within a short period of time siRNAs were reported in several different organisms, including *T. brucei* (Djikeng et al. 2001). A decade ago we generated and sequenced the first comprehensive library of endogenous siRNAs (esiRNAs) and discovered that the majority of molecules were derived from the retroposons ingi and SLACS, consistent with the known role of RNAi as a defense mechanism to maintain genome integrity (Djikeng et al. 2001). Once the *T. brucei* genome was completed, our collection of esiRNAs expanded to include the CIR147 siRNAs (Patrick et al. 2009). Recently, deep sequencing of *Tb*AGO1-associated siRNAs has confirmed that retroposons and CIR147 repeats are the major esiRNA-producing loci in *T. brucei*. However, the RNAi machinery processes many different transcripts that have the potential to anneal and form dsRNA (unpublished observations), similarly to what has been described for the esiRNAs in higher eukaryotes (Joshua-Tor and Hannon 2011). Thus, it appears that the nature of esiRNA-producing loci has been conserved throughout eukaryotic evolution. So far, experimental evidence for micro RNAs (miRNAs) is lacking in *T. brucei*.

T. brucei esiRNAs, as well as siRNAs produced from transgenic hairpins, are 24–26 nt in length (Djikeng et al. 2001) making them slightly longer than those described in model organisms, which tend to be around 21–22 nt. As expected from Dicer-mediated cleavage of dsRNA, *T. brucei* siRNAs carry a monophosphate group at the 5′ end (Patrick et al. 2009). The 3′ ends are insensitive to β-elimination, suggesting the presence of a modification on the ribose. Most likely the 2′-OH of the terminal ribose is methylated, similarly to what has been originally described for plant small regulatory RNAs (Li et al. 2005). Addition of the methyl group is carried out by the HEN1 family of methyltransferases and, not surprisingly, the *T. brucei* genome codes for a candidate paralogue of higher eukaryotic HEN1. The current evidence indicates that HEN1 modification protects the small RNA 3′ end from tailing and/or exonucleolytic degradation (Li et al. 2005; Kurth and Mochizuki 2009; Ameres et al. 2010) and is consistent with a model in which the 3′ end of siRNAs is dislodged from the PAZ domain when there is extensive complementarity between the 3′ half of the guide strand and the target. 3′ end methylation would be required to stabilize siRNAs depending on the specific AGO protein, the requirement for pairing between the siRNA and the target, and the rearrangement of the siRNA 3′ end upon binding to the target. Considering that extensive complementarity between siRNAs and their target is required to elicit the RNAi response in *T. brucei* (Aphasizheva et al. 2009) and that siRNAs are modified (Patrick et al. 2009), it is tempting to speculate that the 3′ end of the siRNA is extruded from *Tb*AGO1 after annealing to the target and that 3′ end modification is required to impede siRNA destabilization.

8.3.7 Diversification of the RNAi Mechanism Among Trypanosomatids

A number of observations provocatively suggest that the RNAi mechanism in trypanosomatid protozoa is more diverse than originally anticipated. For instance, the sequence comparison in Fig. 8.3 points to the possibility that the *L. Viannia* and *Crithidia* AGO1s are functionally different from the African trypanosome counterparts. In support of this hypothesis, we found that *L. V. braziliensis* AGO1 was not able to complement RNAi deficiency in *Tb*AGO1 null cells (unpublished observation). Further highlighting a possible mechanistic difference is the observation that in contrast to *T. brucei*, siRNA 3′ ends are not modified in *L. V. braziliensis* and a HEN1 candidate gene is absent in the genome (unpublished observation). Comparative genomic analysis also informed us that whereas DCL1 and DCL2 are found at syntenic loci in African trypanosomes and *L. V. braziliensis*, the AGO1 genes are not. In addition, inspection of the old-world *Leishmania* genomes revealed remnants of an AGO1 gene in a locus syntenic with *L. V. braziliensis* AGO1. Taken together, these as yet unconnected observations might suggest that the ancestor to the trypanosomatid lineage was endowed with two different AGO1 genes and that the African trypanosome and *L. Viannia* lineages did not retain the same gene.

8.3.8 RISC Activation

At the core of RISC activation is the formation of the AGO-guide siRNA complex (Liu and Paroo 2010). In humans, *Drosophila* and *Neurospora crassa* duplex siRNAs are loaded into AGO slicer and the passenger strand is first cleaved by the AGO endonuclease activity and then the resulting fragments are released. This latter step can occur either by destabilization of the passenger strand fragments, which is expected after cleavage, or with the assistance of accessory factors, such as *Nc*QIP (qde2/AGO-interacting protein1), a protein with a 3′–5′ exonuclease domain related to the epsilon exonuclease subunit of prokaryotic DNA Pol III (Maiti et al. 2007) or the heterodimeric endonuclease C3PO (Liu et al. 2009), which has been shown to stimulate RISC activity. Alternatively, AGO itself could unwind the duplex as supported by the observation that human AGO2 can melt a perfect duplex in vitro (Yoda et al. 2010). How does this process take place in *T. brucei?* The current evidence suggests that formation of active RISC in these organisms follows different rules, as *Tb*AGO1 mutants that are severely impaired in target mRNA cleavage are invariably found in a complex with single-stranded siRNAs (Shi et al. 2009). In contrast, active site mutations in *Drosophila*, human or *Neurospora* AGO slicers result in the accumulation of the corresponding AGO loaded with duplex siRNAs (Liu and Paroo 2010). There are at least two scenarios that can reconcile these observations. First, it is possible that in *Tb*AGO1 the passenger strand and

mRNA endonuclease activities are mechanistically distinct, as it has been suggested for human AGO1 and AGO2 from in vitro studies (Yoda et al. 2010). Alternatively, a specific factor may be required to cause double- to single-strand siRNA transition and loading of the guide strand into trypanosome AGO1. A clarification in the mode of RISC activation in *T. brucei* might arise with the aid of comparative genomics, which indicates that there are at least two more candidate RNAi genes, due to their presence in RNAi-proficient, but absence in RNAi-negative trypanosomatids (Lye et al. 2010). In particular, one candidate has been predicted to have a C-terminal domain resembling the DnaQ superfamily of 3′–5′ exonucleases (Zhang et al. 2008) and thus, similarly to *Neurospora* QIP, could play a role in the transition of double- to single-stranded siRNAs (see note added in proof).

8.3.9 Consequences of Genetic Ablation of the RNAi Pathway

As soon as *Tb*AGO1 was identified, our laboratory (Shi et al. 2004b) as well as Philippe Bastin's group (Durand-Dubief and Bastin 2003) examined the consequences of deleting the RNAi pathway by knocking out the AGO1 gene. RNAi deficiency resulted in a severe growth defect in Lister 427 trypanosomes (Durand-Dubief and Bastin 2003) and in a milder lengthening of the cell cycle in our laboratory procyclic strain derived from human infective *T. b. rhodesiense* (Shi et al. 2004a). Consistent with these observations, we showed that RNAi-deficient trypanosomes, which were selected through resistance to multiple rounds of transfection with α-tubulin dsRNA, displayed a growth defect that increased in severity with the degree of inhibition of the RNAi response and correlated with inhibition in the late stages of cytokinesis (Shi et al. 2004a). On the other hand, Lister 427 AGO1 null trypanosomes had pronounced chromosome segregation alterations (Durand-Dubief and Bastin 2003; Durand-Dubief et al. 2007), a phenotype that could account for their severe growth phenotype. Perhaps the RNAi pathway is involved in establishing pericentric heterochromatin domains, as it has been described in *S. pombe* (Martienssen et al. 2005). However, a direct link between *Tb*AGO1, heterochromatin formation and centromere function has not yet been established. Nevertheless, it is an attractive hypothesis that *Tb*AGO1 function in the nucleus may affect chromosome segregation. Intriguingly, *Tb*AGO1 ablation in two different blood-stream form trypanosome strains adapted to in vitro culture, one a derivative of Lister 427 and the other the pleomorphic strain STIB247, showed no apparent defects in growth, silencing of the variable surface glycoprotein expression sites or life-cycle progression (Janzen et al. 2006). At the present time it is difficult to reconcile these various and somewhat discordant observations into a coherent model. Perhaps, in vitro cultured trypanosome strains have acquired specific adaptations, which may account for the different observed phenotypes.

At the RNA level, *Tb*AGO1 ablation manifests three major phenotypes (1) the siRNAs dramatically decline in abundance (Shi et al. 2004b); (2) there is

upregulation of steady-state transcripts derived from potential mobile elements or their decayed progeny, namely the retroposons ingi and SLACS (Shi et al. 2004b); and (3) transcription of ingi and SLACS retroposons in permeabilized trypanosomes substantially increases (Shi et al. 2004b). Upregulation of retroposon steady-state and newly synthesized transcripts is also observed in in vitro selected RNAi-deficient trypanosomes (Shi et al. 2004a), as well as in *Tb*DCL2 null cells (Patrick et al. 2009), and similar observations apply to the CIR147-derived transcripts (Patrick et al. 2009; unpublished observation). From the above observations we surmise that *Tb*AGO1 and *Tb*DCL2 preside to downregulation of potentially harmful transcripts, which if left intact could promote deleterious hopping of mobile elements. In support of this hypothesis, long-term culturing of *Tb*AGO1 null trypanosomes is accompanied by rearrangements of the SLACS elements (Patrick et al. 2008). The increased transcription of SLACS, ingi, and CIR147 repeats indicate that directly or indirectly the RNAi pathway may regulate the accessibility of these elements to RNA polymerase II transcription, possibly by playing a role in heterochromatin formation, which is a well-established mechanism to silence expression of mobile elements and repeats alike.

8.3.10 RNAi Technology

Soon after its first description in *T. brucei* (Ngo et al. 1998), the power of RNAi has been harnessed for functional genomics studies by generating vectors for heritable and inducible RNAi. This advancement would not have been possible without the efforts of the laboratories of Christine Clayton (Wirtz and Clayton 1995) and George A.M. Cross (Wirtz et al. 1999), who in the meantime had built tetracycline-inducible systems for expression of toxic gene products. The availability of conditional dsRNA expression allowed investigators to apply the RNAi tool to explore the functions of hundreds of genes, both for individual research purposes and for genome-wide screens. Importantly, the rapid validation of the large number of potential drug targets, which are currently under consideration in various centers, would not have been possible without the RNAi tool.

Although the RNAi technology has proven to be invaluable in the analysis of gene function in *T. brucei*, its potential for forward genetics screens has so far not been fully explored on a genome-wide scale. Genomic DNA insert library screens (Morris et al. 2002; Schumann Burkard et al. 2010) rely on the generation of a sufficient number of trypanosome clones (much larger than the actual number of genes) in order to provide several-fold genome coverage. Systematic RNAi screens have been performed only for chromosome I in *T. brucei* (Subramaniam et al. 2006) and were based on knowledge of predicted open reading frames. With the recently obtained genome-wide map of mRNA boundaries through next-generation RNA sequencing (Kolev et al. 2010; Nilsson et al. 2010; Siegel et al. 2010), the stage is set for the systematic design of a genome-wide library producing dsRNA against all identified trypanosome transcripts to significantly reduce the number

of transformant cell lines needed (to ~10,000). Ideally, the genome-wide inducible RNAi library would produce hairpin dsRNA (for its superiority in eliciting an RNAi response) and the vector carrying the inverted repeat would be integrated at a single, defined locus in the genome (Alsford and Horn 2008) to minimize epigenetic influence of surrounding chromatin on dsRNA expression.

8.4 Conclusion

In the last 5 years there has been a tremendous acceleration in all fields of trypanosome biology due to the completion of the genome sequence and the wide application of RNAi. In addition, studies of the RNAi pathway have so far identified three RNAi factors that cooperate to bring about the RNAi response not only in the cytoplasm, but surprisingly also in the nucleus. Nuclear RNAi is fundamental to downregulate expression of transcripts derived from retroposons and putative centromeric repeats and we suspect it may have additional functions to clear away dsRNA originating from other regions of the genome, including the numerous sites of convergent transcription. However, the precise scope of the nuclear RNAi pathway needs to be further investigated in order to understand its impact on trypanosome biology.

Note added in proof Our laboratory recently reported the characterization of two novel and essential RNAi factors, namely *Tb*RIF4, a 3′–5′ exonuclease of the DnaQ superfamily with a critical role in the conversion of duplex siRNAs to the single-stranded form, and *Tb*RIF5, a possible *Tb*DCL1 cofactor (Barnes et al. 2012).

Acknowledgments Research in the author's laboratory was supported by Public Health Service grants AI28798 and AI56333 to E.U and AI43594 to C.T.

References

Alsford S, Horn D (2008) Single-locus targeting constructs for reliable regulated RNAi and transgene expression in *Trypanosoma brucei*. Mol Biochem Parasitol 161:76–79

Alsford S, Kemp LE, Kawahara T, Horn D (2010) RNA interference, growth and differentiation appear normal in African trypanosomes lacking Tudor staphylococcal nuclease. Mol Biochem Parasitol 174:70–73

Ameres SL, Horwich MD, Hung JH, Xu J, Ghildiyal M, Weng Z, Zamore PD (2010) Target RNA-directed trimming and tailing of small silencing RNAs. Science 328:1534–1539

Aphasizheva I, Ringpis GE, Weng J, Gershon PD, Lathrop RH, Aphasizhev R (2009) Novel TUTase associates with an editosome-like complex in mitochondria of *Trypanosoma brucei*. RNA 15:1322–1337

Barnes RL, Shi H, Kolev NG, Tschudi C, Ullu E (2012) Comparative genomics reveals two novel RNAi factors in *Trypanosoma brucei* and provides insight into the core machinery. PLoS Pathog (in press)

Best A, Handoko L, Schluter E, Goringer HU (2005) In vitro synthesized small interfering RNAs elicit RNA interference in African trypanosomes: an *in vitro* and *in vivo* analysis. J Biol Chem 280:20573–20579

Braun L, Cannella D, Ortet P, Barakat M, Sautel CF, Kieffer S, Garin J, Bastien O, Voinnet O, Hakimi MA (2010) A complex small RNA repertoire is generated by a plant/fungal-like machinery and effected by a metazoan-like Argonaute in the single-cell human parasite *Toxoplasma gondii*. PLoS Pathog 66(5):e1000920. doi:10.1371/journal.ppat.1000920

Cerutti H, Casas-Mollano JA (2006) On the origin and functions of RNA-mediated silencing: from protists to man. Curr Genet 50:81–99

DaRocha WD, Otsu K, Teixeira SM, Donelson JE (2004) Tests of cytoplasmic RNA interference (RNAi) and construction of a tetracycline-inducible T7 promoter system in *Trypanosoma cruzi*. Mol Biochem Parasitol 133:175–186

Djikeng A, Shi H, Tschudi C, Ullu E (2001) RNA interference in *Trypanosoma brucei*: cloning of small interfering RNAs provides evidence for retroposon-derived 24-26-nucleotide RNAs. RNA 7:1522–1530

Djikeng A, Shi H, Tschudi C, Shen S, Ullu E (2003) An siRNA ribonucleoprotein is found associated with polyribosomes in *Trypanosoma brucei*. RNA 9:802–808

Durand-Dubief M, Bastin P (2003) TbAGO1, an Argonaute protein required for RNA interference is involved in mitosis and chromosome segregation in *Trypanosoma brucei*. BMC Biol 1:2

Durand-Dubief M, Absalon S, Menzer L, Ngwabyt S, Ersfeld K, Bastin P (2007) The Argonaute protein TbAGO1 contributes to large and mini-chromosome segregation and is required for control of RIME retroposons and RHS pseudogene-associated transcripts. Mol Biochem Parasitol 156:144–153

Faehnle CR, Joshua-Tor L (2010) Argonaute MID domain takes centre stage. EMBO Rep 11:564–565

Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature 391:806–811

Gupta SK, Hury A, Ziporen Y, Shi H, Ullu E, Michaeli S (2010) Small nucleolar RNA interference in *Trypanosoma brucei*: mechanism and utilization for elucidating the function of snoRNAs. Nucleic Acids Res 38:7236–7247

Hamilton AJ, Baulcombe DC (1999) A species of small antisense RNA in posttranscriptional gene silencing in plants. Science 286:950–952

Iwasaki S, Kobayashi M, Yoda M, Sakaguchi Y, Katsuma S, Suzuki T, Tomari Y (2010) Hsc70/Hsp90 chaperone machinery mediates ATP-dependent RISC loading of small RNA duplexes. Mol Cell 39:292–299

Janzen CJ, van Deursen F, Shi H, Cross GA, Matthews KR, Ullu E (2006) Expression site silencing and life-cycle progression appear normal in Argonaute1-deficient *Trypanosoma brucei*. Mol Biochem Parasitol 149:102–107

Jaskiewicz L, Filipowicz W (2008) Role of Dicer in posttranscriptional RNA silencing. Curr Top Microbiol Immunol 320:77–97

Joshua-Tor L, Hannon GJ (2011) Ancestral roles of small RNAs: an ago-centric perspective. Cold Spring Harb Perspect Biol 3(10):a003772. doi:10.1101/cshperspect.a003772

Kirino Y, Kim N, de Planell-Saguer M, Khandros E, Chiorean S, Klein PS, Rigoutsos I, Jongens TA, Mourelatos Z (2009) Arginine methylation of Piwi proteins catalysed by dPRMT5 is required for Ago3 and Aub stability. Nat Cell Biol 11:652–658

Kolev NG, Franklin JB, Carmi S, Shi H, Michaeli S, Tschudi C (2010) The transcriptome of the human pathogen *Trypanosoma brucei* at single-nucleotide resolution. PLoS Pathog 6(9): e1001090. doi:10.1371/journal.ppat.1001090

Kurth HM, Mochizuki K (2009) 2′-O-methylation stabilizes Piwi-associated small RNAs and ensures DNA elimination in *Tetrahymena*. RNA 15:675–685

Li J, Yang Z, Yu B, Liu J, Chen X (2005) Methylation protects miRNAs and siRNAs from a 3′-end uridylation activity in Arabidopsis. Curr Biol 15:1501–1507

Liang XH, Liu Q, Michaeli S (2003) Small nucleolar RNA interference induced by antisense or double-stranded RNA in trypanosomatids. Proc Natl Acad Sci USA 100:7521–7526

Liu Q, Paroo Z (2010) Biochemical principles of small RNA pathways. Annu Rev Biochem 79:295–319

Liu Y, Ye X, Jiang F, Liang C, Chen D, Peng J, Kinch LN, Grishin NV, Liu Q (2009) C3PO, an endoribonuclease that promotes RNAi by facilitating RISC activation. Science 325:750–753

Lye LF, Owens K, Shi H, Murta SM, Vieira AC, Turco SJ, Tschudi C, Ullu E, Beverley SM (2010) Retention and loss of RNA interference pathways in trypanosomatid protozoans. PLoS Pathog 6(10):e1001161. doi:10.1371/journal.ppat.1001161

MacRae IJ, Zhou K, Doudna JA (2007) Structural determinants of RNA recognition and cleavage by Dicer. Nat Struct Mol Biol 14:934–940

Maiti M, Lee HC, Liu Y (2007) QIP, a putative exonuclease, interacts with the *Neurospora* Argonaute protein and facilitates conversion of duplex siRNA into single strands. Genes Dev 21:590–600

Martienssen RA, Zaratiegui M, Goto DB (2005) RNA interference and heterochromatin in the fission yeast *Schizosaccharomyces pombe*. Trends Genet 21:450–456

Miyoshi T, Takeuchi A, Siomi H, Siomi MC (2010) A direct role for Hsp90 in pre-RISC formation in Drosophila. Nat Struct Mol Biol 17:1024–1026

Morris JC, Wang Z, Drew ME, Englund PT (2002) Glycolysis modulates trypanosome glycoprotein expression as revealed by an RNAi library. EMBO J 21:4429–4438

Ngo H, Tschudi C, Gull K, Ullu E (1998) Double-stranded RNA induces mRNA degradation in *Trypanosoma brucei*. Proc Natl Acad Sci USA 95:14687–14692

Nilsson D, Gunasekera K, Mani J, Osteras M, Farinelli L, Baerlocher L, Roditi I, Ochsenreiter T (2010) Spliced leader trapping reveals widespread alternative splicing patterns in the highly dynamic transcriptome of *Trypanosoma brucei*. PLoS Pathog 6(8):e1001037. doi:10.1371/journal.ppat.1001037

Obado SO, Taylor MC, Wilkinson SR, Bromley EV, Kelly JM (2005) Functional mapping of a trypanosome centromere by chromosome fragmentation identifies a 16-kb GC-rich transcriptional "strand-switch" domain as a major feature. Genome Res 15:36–43

Patrick KL, Luz PM, Ruan JP, Shi H, Ullu E, Tschudi C (2008) Genomic rearrangements and transcriptional analysis of the spliced leader-associated retrotransposon in RNA interference-deficient *Trypanosoma brucei*. Mol Microbiol 67:435–447

Patrick KL, Shi H, Kolev NG, Ersfeld K, Tschudi C, Ullu E (2009) Distinct and overlapping roles for two Dicer-like proteins in the RNA interference pathways of the ancient eukaryote *Trypanosoma brucei*. Proc Natl Acad Sci USA 106:17933–17938

Robinson KA, Beverley SM (2003) Improvements in transfection efficiency and tests of RNA interference (RNAi) approaches in the protozoan parasite *Leishmania*. Mol Biochem Parasitol 128:217–228

Schumann Burkard G, Jutzi P, Roditi I (2010) Genome-wide RNAi screens in bloodstream form trypanosomes identify drug transporters. Mol Biochem Parasitol 175:91–94

Shi H, Chamond N, Tschudi C, Ullu E (2004a) Selection and characterization of RNA interference-deficient trypanosomes impaired in target mRNA degradation. Eukaryot Cell 3:1445–1453

Shi H, Djikeng A, Tschudi C, Ullu E (2004b) Argonaute protein in the early divergent eukaryote *Trypanosoma brucei*: control of small interfering RNA accumulation and retroposon transcript abundance. Mol Cell Biol 24:420–427

Shi H, Ullu E, Tschudi C (2004c) Function of the trypanosome Argonaute 1 protein in RNA interference requires the N-terminal RGG domain and arginine 735 in the Piwi domain. J Biol Chem 279:49889–49893

Shi H, Tschudi C, Ullu E (2006a) Functional replacement of *Trypanosoma brucei* Argonaute by the human slicer Argonaute2. RNA 12:943–947

Shi H, Tschudi C, Ullu E (2006b) An unusual Dicer-like1 protein fuels the RNA interference pathway in *Trypanosoma brucei*. RNA 12:2063–2072

Shi H, Tschudi C, Ullu E (2007) Depletion of newly synthesized Argonaute1 impairs the RNAi response in *Trypanosoma brucei*. RNA 13:1132–1139

Shi H, Chamond N, Djikeng A, Tschudi C, Ullu E (2009) RNA interference in *Trypanosoma brucei*: the role of the amino-terminal RGG domain and the polyribosome association of Argonaute1. J Biol Chem 284:36511–36520

Siegel TN, Hekstra DR, Wang X, Dewell S, Cross GA (2010) Genome-wide analysis of mRNA abundance in two life-cycle stages of *Trypanosoma brucei* and identification of splicing and polyadenylation sites. Nucleic Acids Res 38:4946–4957

Siomi H, Siomi MC (2009) On the road to reading the RNA-interference code. Nature 457:396–404

Subramaniam C, Veazey P, Redmond S, Hayes-Sinclair J, Chambers E, Carrington M, Gull K, Matthews K, Horn D, Field MC (2006) Chromosome-wide analysis of gene function by RNA interference in the African trypanosome. Eukaryot Cell 5:1539–1549

Tolia NH, Joshua-Tor L (2007) Slicer and the Argonautes. Nat Chem Biol 3:36–43

Ullu E, Tschudi C, Chakraborty T (2004) RNA interference in protozoan parasites. Cell Microbiol 6:509–519

Vagin VV, Wohlschlegel J, Qu J, Jonsson Z, Huang X, Chuma S, Girard A, Sachidanandam R, Hannon GJ, Aravin AA (2009) Proteomic analysis of murine Piwi proteins reveals a role for arginine methylation in specifying interaction with Tudor family members. Genes Dev 23:1749–1762

Wang Y, Sheng G, Juranek S, Tuschl T, Patel DJ (2008) Structure of the guide-strand-containing argonaute silencing complex. Nature 456:209–213

Wirtz E, Clayton C (1995) Inducible gene expression in trypanosomes mediated by a prokaryotic repressor. Science 268:1179–1183

Wirtz E, Leal S, Ochatt C, Cross GA (1999) A tightly regulated inducible expression system for conditional gene knock-outs and dominant-negative genetics in *Trypanosoma brucei*. Mol Biochem Parasitol 99:89–101

Yoda M, Kawamata T, Paroo Z, Ye X, Iwasaki S, Liu Q, Tomari Y (2010) ATP-dependent human RISC assembly pathways. Nat Struct Mol Biol 17:17–23

Zhang D, Xiong H, Shan J, Xia X, Trudeau VL (2008) Functional insight into Maelstrom in the germline piRNA pathway: a unique domain homologous to the DnaQ-H 3′-5′ exonuclease, its lineage-specific expansion/loss and evolutionarily active site switch. Biol Direct 3:48