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Microreview

RNA interference in protozoan parasites

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Summary

RNA interference or RNAi is defined as the mechanism through which gene-specific, double-stranded RNA (dsRNA) triggers degradation of homologous transcripts. Besides providing an invaluable tool to downregulate gene expression in a variety of organisms, it is now evident that RNAi extends its tentacles into both the nucleus and the cytoplasm and is involved in a variety of gene silencing phenomena. Here we review the current status of RNAi in protozoan parasites that cause diseases of considerable medical and veterinary importance throughout Africa, Asia and the Americas. RNAi was first discovered in *Trypanosoma brucei,* a species of the family *Trypanosomatidae*, and it rapidly became the method of choice to downregulate gene expression in these organisms. At the same time, mechanistic studies exposed a role for RNAi in the control of retroposon transcript abundance. Whereas RNAi is also present in *T. congolense*, other members of the same family of organisms, namely *T. cruzi* and *Leishmania major*, are RNAi-negative. In apicomplexan parasites, there is experimental evidence for RNAi in *Plasmodium*, but this is not supported by their genetic make up. In contrast, the genome of *Toxoplasma gondii* harbours gene candidates with convincing similarity to 'classical' RNAi genes. Thus, as previously shown in fungi, protozoan parasites are genetically heterogeneous as far as the RNAi pathway is concerned. Finally, database mining predicts that *Entamoeba histolytica* and *Giardia intestinalis* have an RNAi pathway and the presence of RNAi genes in *Giardia* supports the view that gene silencing by dsRNA appeared very early during evolution of the eukaryotic lineage.

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Overview of RNA interference

Ever since the first report in 1998 (Fire *et al*., 1998), RNA interference (RNAi) has swept through all fields of eukaryotic biology and has provided a tool of immense value to analyse gene function in a variety of organisms, especially those that are not amenable to classical genetics. Beside its value as a genetic tool to downregulate gene expression by double-stranded RNA (dsRNA), the RNAi pathway is implicated in gene silencing phenomena as diverse as heterochromatin assembly (Volpe *et al*., 2002) and maintenance (Hall *et al*., 2002), DNA and histone methylation (Zilberman *et al*., 2003), DNA elimination (Mochizuki *et al*., 2002), promoter silencing (Mette *et al*., 2000) and developmental control (Lau *et al*., 2001; Lee and Ambros, 2001). All these processes are guided by small RNAs in the size range of 20–26 nt which, depending on the process they are involved in, have different characteristics and names. So far, three major classes of small RNAs have been described: (i) small interfering RNAs or siRNAs (Elbashir *et al*., 2001) are double-stranded and are derived from cleavage of long endogenous or exogenous dsRNAs. siRNAs guide degradation of target mRNAs via base pairing with complementary sequences and are the hallmark of the 'classical' RNAi pathway; (ii) microRNAs or miRNAs (Pasquinelli, 2002) constitute a large class of regulatory RNAs that are single-stranded and are processed from ~70 nt-long hairpins, containing a few bulged nucleotides. So far miRNAs have only been characterized in higher eukaryotes. In the few cases where their function has been studied, miRNAs appear to inhibit translation by binding to the 3′ UTR of target mRNAs; (iii) small heterochromatic RNAs are thought to be similar in structure to siRNAs (Reinhart and Bartel, 2002) and have been proposed to be derived from dsRNA transcripts representing heterochromatic chromosomal regions, such as the centromeric repeats in *Schizosaccharomyces pombe* (Volpe *et al*., 2002). Although it is not completely understood how

each class of small RNAs functions, it is evident that small regulatory RNAs have become pervasive in eukaryotic biology and as a consequence our view of gene regulation has been profoundly altered.

In this review we will primarily concentrate on the 'classical' experimental definition of RNAi, namely the mechanism through which dsRNA triggers specific degradation of homologous cellular transcripts. In this pathway long dsRNA, derived from endogenous, transgenic or viral transcripts, is first cleaved to 20–26 nt-long siRNAs by an RNAse III-related enzyme called Dicer, which is a multidomain protein comprising helicase, PAZ, RNAse III and dsRNA-binding domains (Bernstein *et al*., 2001; Hannon, 2002). A *Drosophila* protein with two dsRNA binding domains, dubbed R2D2, is found in a complex with Dicer (Liu *et al*., 2003) and is involved in handing over siRNAs to a multiprotein complex termed RISC, or RNAi-induced silencing complex (Hammond *et al*., 2000). Next, in an ATP-dependent step siRNAs are unwound, RISC is activated (Nykanen *et al*., 2001) and becomes competent to carry out the last step in RNAi, namely recognition and cleavage of target mRNAs. Among the proteins that have been identified in the *Drosophila* RISC are AGO2 (Hammond *et al*., 2001), an argonaute (AGO) family member, VIG (vasa intronic gene), FXR (fragile-X related protein, the *Drosophila* homologue of the human fragile–X mental retardation syndrome protein or FMRP), a helicase p68 (Caudy *et al*., 2002; Ishizuka *et al*., 2002) and Tudor-SN, a protein containing five staphylococcal/micrococcal nuclease-like domains and a Tudor domain (Caudy *et al*., 2003). Tudor binds to modified amino acids, such as dimethyl arginine present in certain RNA binding proteins (Sprangers *et al*., 2003). How recognition and cleavage of target mRNA is accomplished by RISC is not yet understood, except that cleavage requires extensive, if not complete, sequence complementarity between the target mRNA and an antisense siRNA (Elbashir *et al*., 2001; Hannon, 2002; Schwarz *et al*., 2003).

At the genetic level there are two proteins that constitute the universal hallmark of RNAi and related phenomena: the aforementioned Dicer and members of the AGO protein family (Hannon, 2002). The prototypical AGO1 gene was discovered in *Arabidopsis thaliana* and mutations in this gene stunt plant growth resulting in abnormal leaf development (Bohmert *et al*., 1998). Later on the link between RNAi and AGO proteins was established by genetic analysis in *C. elegans* with the discovery that Rde-1, an AGO-like protein, is essential for RNAi (Tabara *et al*., 1999). All organisms that are known to exploit dsRNA as a gene silencing trigger possess one or more members of the AGO gene family, as well as one or more Dicer-like genes. At the biochemical level, the *D. melanogaster* AGO2 protein is an essential component of RISC (Hammond *et al*., 2001). In addition, AGO proteins are known to interact with Dicer (Hammond *et al*., 2001; Tabara *et al*., 2002) and are found in ribonucleoprotein complexes containing miRNAs (Mourelatos *et al*., 2002).

Members of the AGO protein family have conserved structural features: they are basic proteins of approximately 100 kDa and they are identified by the presence of two domains, called PAZ and Piwi (Fig. 2 and Carmell *et al*., 2002). The PAZ domain, which is also found in Dicer, precedes the Piwi domain, is approximately 150 amino acids long and contains a fold that binds RNA (Lingel *et al*., 2003; Song *et al*., 2003; Yan *et al*., 2003). The Piwi domain is about 330 amino acids in length, is located at the C-terminus of AGO and its sequence is quite well conserved, making it a useful tool for database mining. One of the functions of the Piwi domain is to interact with the RNAse III domain of Dicer (Tahbaz *et al*., 2004). However, it is likely that the Piwi domain is endowed with additional functions, as Piwi-containing proteins exist also in organisms that are RNAi negative (see below).

In certain organisms, like *Neurospora crassa* (Cogoni and Macino, 1999), *C. elegans* (Smardon *et al*., 2000), *S. pombe* (Volpe *et al*., 2002) and plants (Baulcombe, 1999), an RNA-dependent RNA polymerase (RdRP) is also essential for dsRNA-triggered gene silencing. One hypothesis is that RdRP amplifies the RNAi response by using siRNAs as primers for conversion of target RNAs into dsRNA, thus generating more substrate for Dicer cleavage and a secondary 'wave' of siRNAs (Sijen *et al*., 2001). However, RdRP genes are not present in other organisms, like *Drosophila* and mammals, where robust RNAi responses have been demonstrated. Thus, it appears that RdRp is not a universal component of the RNAi pathway and that its role is organism specific.

Another fascinating and important aspect of the RNAi response concerns the ability of the silencing signal to spread throughout an organism (Baulcombe, 2002). Systemic silencing occurs in plants and *C. elegans,* but not in unicellular eukaryotes, *Drosophila* or mammals. Recent evidence suggests that a membrane protein, termed SID, is involved in systemic silencing in *C. elegans*, possibly by providing a route of entry for long dsRNA (Winston *et al*., 2002; Feinberg and Hunter, 2003).

For further information on the mechanism and components of the RNAi pathway the reader is referred to recent reviews (Hannon, 2002; Dykxhoorn *et al*., 2003).

RNAi in *Trypanosoma brucei*

Applications of RNAi

RNAi was serendipitously discovered in *T. brucei* in our laboratory and its first report dates back to an abstract presented at the 1997 Molecular Parasitology Meeting in

Woods Hole (MA). At the time the implications of our findings were not fully understood, but a few months later it became clear that trypanosomes (Ngo *et al*., 1998), like *C. elegans* (Fire *et al*., 1998), have the machinery to specifically degrade mRNA upon exposure to homologous dsRNA. Since then, a number of laboratories, including ours, have generated vectors for heritable and inducible RNAi (Bastin *et al*., 2000; LaCount *et al*., 2000; Shi *et al*., 2000; Wang *et al*., 2000). RNAi is now recognized as the method of choice to downregulate gene expression in *T. brucei*. There are two basic strategies to express dsRNA in *T. brucei*. The first one employs cloning a fragment of the gene of interest in-between opposing T7 RNA polymerase promoters and terminators. The second approach is to express a hairpin structure of the gene of interest. The technical aspects of using RNAi in *T. brucei* have recently been covered in a review and will therefore not be discussed further (Tschudi *et al*., 2003).

In the last few years RNAi has found genome-wide applications in *T. brucei* as a tool for forward genetic approaches through the establishment of an inducible RNAi library (Wang and Englund, 2001), as well as for global analysis of gene function, a project funded by the Wellcome Trust in England. Even though RNAi has proved invaluable to further our understanding of the biology of *T. brucei*, one should keep in mind that RNAi only downregulates, but does not abolish gene function. In particular, the extent of downregulation of a specific gene by RNAi very much depends on the corresponding protein abundance and half-life, as well on the amount of dsRNA that enters the RNAi pathway. One curious aspect that emerged from RNAi experiments in *T. brucei* is that downregulation of essential genes by RNAi can sometimes lead to the selection of cell populations that escape killing by the specific dsRNA trigger. One possibility to explain these results is that the 'resistant' cells have lost the dsRNA cassette by recombination of the inverted repeats that flank the fragment of the gene of interest in both types of dsRNA-producing vectors mentioned above. Alternatively, there might be selection for RNAi-negative cells, namely cells that are no longer capable of responding to dsRNA challenge. Indeed, we have observed that it is relatively easy to select for RNAi-negative trypanosomes by consecutive cycles of electroporation with α-tubulin dsRNA, a treatment that downregulates tubulin synthesis, blocks cytokinesis and eventually kills the cells (H. Shi, C. Tschudi and E. Ullu, in preparation). Nevertheless, RNAi has already provided novel insights into the biology of *T. brucei* and undoubtedly will continue to do so in the future.

The RNAi mechanism in T. brucei

Figure 1 illustrates what is presently known about the mechanism of RNAi in *T. brucei*. Synthetic dsRNA electroporated into cells (Ngo *et al*., 1998; LaCount *et al*., 2000), dsRNA expressed from transgenes transiently introduced into cells (Ngo *et al*., 1998; LaCount *et al*., 2000) or stably integrated into the genome (Bastin *et al*., 2000; Shi *et al*., 2000; Wang *et al*., 2000), or endogenous dsRNA derived from retroposon transcripts (Djikeng *et al*., 2001) is processed to siRNAs by a Dicer-like enzyme. Although siRNAs have been cloned and characterized in *T. brucei* (A. Djikeng, C. Tschudi and E. Ullu, 2001) and a Dicer activity can be detected in cell-free extracts (A. Djikeng, C. Tschudi and E. Ullu, unpubl. obs.), database mining has so far failed to identify a putative Dicer homologue in the *T. brucei* genome. Besides the obvious possibility that the genome sequence is not complete, one should also consider that the *T. brucei* 'Dicer' might be so divergent that it cannot be recognized using the *Dicer* consensus sequence.

What is clearly apparent in the *T. brucei* genome is a member of the AGO protein family, named TbAGO1. The structure of TbAGO1 is schematically shown in Fig. 2 (Shi *et al*., 2004). A characteristic feature of this protein is the presence at the amino terminus of a repeated sequence rich in RGG residues, in which the arginine residues have the potential to be methylated. RGG residues are found in other AGO family members, but not to the extent present in the trypanosome protein. TbAGO1 is essential for RNAi in procyclic trypanosomes, but it appears not to be an essential gene, as viable AGO1KO cells can be propagated in culture (Durand-Dubief and Bastin, 2003; Shi *et al*., 2004). AGO1KO cells have been established in two different genetic backgrounds, namely our laboratory procyclic strain *T. b. rhodesiense* YTat1.1 (Shi *et al*., 2004) and a derivative of the *T. brucei* 427 procyclic strain (Durand-Dubief and Bastin, 2003). Both AGO1KO mutant strains display a slow growth phenotype, but the magnitude of this defect is greater in the 427 than in the YTat1.1 genetic background (H. Shi, N. Chamond, C. Tschudi and E. Ullu, in preparation). At present the reason(s) for this difference is not understood.

Genetic and biochemical studies are consistent with AGO1 being a component of RISC (Shi *et al*., 2004). AGO1, a predominantly cytoplasmic protein, is in a complex with siRNAs and we suspect that the protein is binding directly to siRNAs. This is based on affinity purification of AGO1, which depletes extracts of siRNAs, but does not reveal the presence of any other protein present in stochiometric amounts with AGO1 (A. Djikeng, C. Tschudi and E. Ullu, unpubl. obs.). Thus, TbAGO1 could be the siRNA binding subunit of RISC.

Interestingly, a proportion of the siRNA–AGO1 complexes are found associated with translating ribosomes (Djikeng *et al*., 2003) and this interaction is mediated by the N-terminal RGG domain of AGO1 (see below; H. Shi, C. Tschudi and E. Ullu, in preparation). The identity

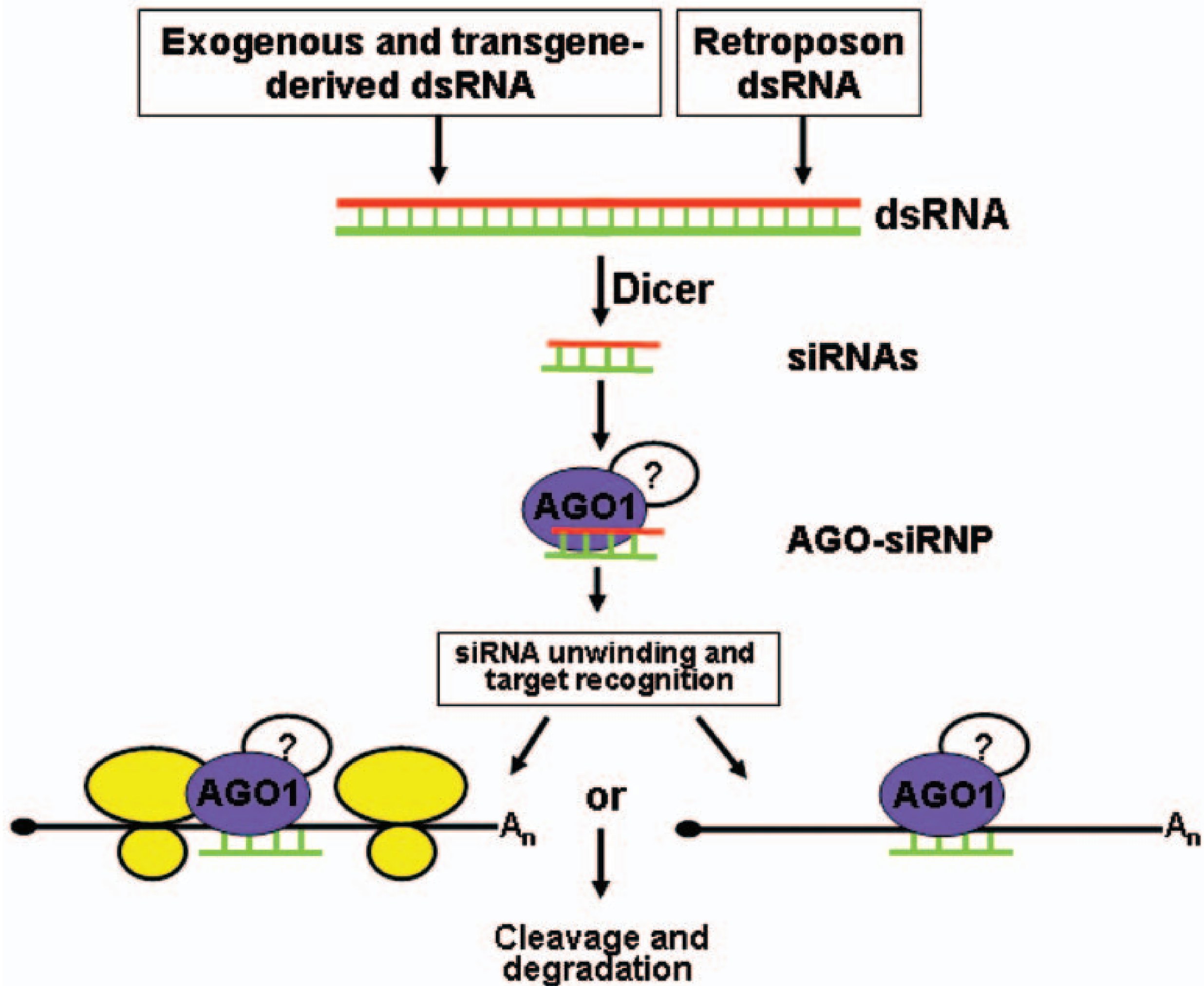

Fig. 1. Schematic representation of the RNAi pathway in *T. brucei*. The identity of the Dicer enzyme is at present unknown. siRNAs are shown in a complex with AGO1. By analogy to what is known in other organisms, it is likely that additional proteins are part of this complex, but their existence in *T. brucei* has not yet been established. The AGO1-siRNP is found in part associated with translating ribosomes. Cleavage and degradation of target mRNA is hypothesized to occur either when the mRNA is being translated or in a ribosome-free state.

of the ribosome component(s) interacting with the AGO1-containing ribonucleoprotein particle remains to be determined. We have proposed that the association between AGO1–siRNA complexes and polyribosomes facilitates recognition of target mRNA by RISC, while the mRNA is being translated, or perhaps marks the translated mRNA for subsequent degradation in the cytoplasm (Djikeng *et al*., 2003). Our findings are in agreement with the original observation that RISC activity in *Drosophila* is enriched in a high-speed pellet containing ribosomes (Hammond *et al*., 2001). Moreover, a link between the translation and RNAi machineries is supported by the following findings. First, recruitment of mRNA to polysomes is necessary for the RNAi response during development of *Drosophila* oocytes (Kennerdell *et al*., 2002). Second, the *Drosophila* homologue of human FMRP, a protein known to be associated with polyribosomes, is a component of RISC (Caudy *et al*., 2002; Ishizuka *et al*., 2002).

In an alternative pathway, depicted in Fig. 1, the AGO1–siRNA complex might directly associate with ribosome-free mRNA and cleavage of mRNA might occur without a direct interaction between the translation and RNAi machineries.

AGO1 is at present the only characterized component of the RNAi machinery in *T. brucei*. As mentioned above, a Dicer homologue is not apparent in the current database and RdRp, VIG or FMRP gene candidates cannot be recognized in the *T. brucei* genome. Although a Tudor-SN homologue (Caudy *et al*., 2003) is present in the *T. brucei* genome (E.Ullu, unpubl. obs.), this gene is also present in *Leishmania major* and *T. cruzi,* which are RNAi-negative (see below). Thus, its role in RNAi will have to be carefully evaluated.

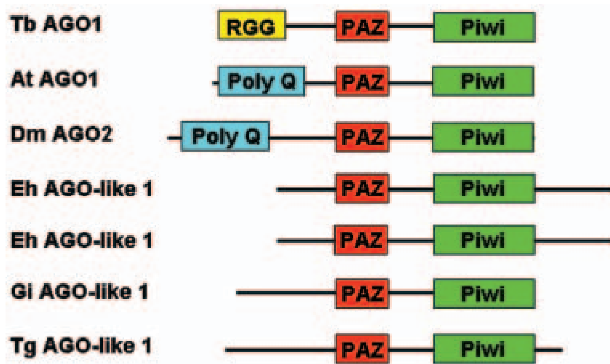

Fig. 2. Domain structure of AGO-like proteins in parasitic protozoa and selected other organisms. The drawing is not to scale. Abbreviations: Tb AGO1, *T. brucei* Argonaute 1 (Sanger Centre accession # TRYP10.0.000587–99 and Shi *et al*., 2004); At AGO1, *A. thaliana* Argonaute 1 (accession # U91995); Dm AGO2, *D. melanogaster* Argonaute 2 (accession # AE003530); Eh AGO-like1 and Eh Ago-like 2, *E. hystolytica* Argonaute-like protein 1 (accession # EH01440 and EH1685); Gi AGO-like 1 (*Giardia intestinalis* Argonaute protein 1, accession # EAA43025); Tg AGO-like 1 (*T. gondii* Argonaute-like protein; accession # Tg9x_994281_gs_44). The location of the PAZ and Piwi domains is only approximate. RGG indicates the domain of TbAGO1 containing arginine-glycine-glycine repeats. Poly Q, polyglutamine-containing domain. Solid lines indicate sequences with no recognizable domains.

The biological significance of RNAi in T. brucei

The available evidence suggests that RNAi in *T. brucei* is a constitutive mechanism of genome defence to silence retroposon transcripts. This view is supported by the finding that siRNAs cloned from the polyribosomal fraction of *T. brucei* procyclics contain a relatively high proportion of sequences derived from the retroposons Ingi and SLACS (Djikeng *et al*., 2001). Furthermore, ablation of the TbAGO1 gene leads to almost complete disappearance of the retroposon-derived siRNAs, with a concomitant three- to fivefold increase in the steady-state level of Ingi and SLACS transcripts (Shi *et al*., 2004). This is brought about by a higher stability of the transcripts, as well as by an elevated transcription rate. How these two phenomena are coordinated and whether transcriptional activation is directly dependent on AGO1 function remains to be established. Nevertheless, considering that retroposons are usually found in heterochromatic portions of the genome, it is tempting to speculate that the RNAi machinery has a role in the nucleus of *T. brucei,* and perhaps contributes to chromatin remodelling, as it is the case in *S. pombe* (Volpe *et al*., 2002) and in plants (Zilberman *et al*., 2003). In this scenario, the increased transcription rate of retroelements might result from a failure in heterochromatin assembly. As mentioned above, 427-AGO1KO procyclics display a prominent reduction in the growth rate (Durand-Dubief and Bastin, 2003) with accumulation of zoids (anucleated cytoplasmic fragments containing a kinetoplast or mitochondrial genome), which are diagnostic of aberrant cell division. Furthermore, a proportion of 427-AGO1KO cells showed abnormal mitotic spindles with unequal DNA distribution between the daughter nuclei, suggesting that chromosome segregation is defective in the absence of AGO1 function (Durand-Dubief and Bastin, 2003). This is reminiscent of the phenotype of *S. pombe* RNAi mutants that have defects in mitotic, as well as meiotic chromosome segregation (Hall *et al*., 2003). In this organism, a functional RNAi pathway is required for centromeric heterochromatin assembly (Volpe *et al*., 2002; 2003), and thus for the assembly of a functional centromere. So far, the structure of *T. brucei* centromeres has been elusive. Thus, at present it is not feasible to ascertain whether centromeric heterochromatin structure is altered in AGO1KO cells. Moreover, it remains to be demonstrated whether the effects seen on mitotic division in one genetic background, namely *T. brucei* procyclic 427 cells, are directly or indirectly related to the RNAi pathway. Because in AGO1KO cells retroposon transcripts abundance is considerably increased (Shi *et al*., 2004), there is the distinct, although as yet unproven, possibility that retroposition is activated in AGO1KO cells. Given the long culture times required for establishing KO cell lines, retroposon hopping could have deleterious consequences on cell viability, including defects in mitotic division. The establishment of a conditional AGO1KO cell line should allow us to distinguish direct from indirect effects due to an ablation of the RNAi pathway.

The existence of an RNAi-like mechanism in the *T. brucei* nucleus is also suggested by the finding that dsRNA homologous to certain small nucleolar RNAs (snoRNAs) can induce specific silencing of the corresponding snoRNAs (Liang *et al*., 2003). It remains to be ascertained whether the snoRNA silencing phenomenon is dependent on the activity of RNAi genes. An alternative possibility is that antisense snoRNA transcripts anneal to the corresponding RNAs in the nucleolus and the resulting dsRNA is subject to degradation by an RNAse III enzyme unrelated to Dicer (Liang *et al*., 2003).

Besides retroposon silencing and a potential role in chromatin remodelling, are there any other functions for RNAi in trypanosomes? For instance, are there regulatory RNAs of the miRNA class, which could provide new and powerful means for controlling gene expression at the post-transcriptional level? Possible avenues to address these issues are the characterization of large libraries of siRNAs and the identification of transcripts that are upregulated in cells where the RNAi pathway has been ablated by different means.

Is the RNAi pathway present in other protozoan parasites?

The demonstration of RNAi in *T. brucei* prompted a num-

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ber of groups to investigate whether RNAi was functioning in other protozoan parasites, such as the trypanosomatids *T. congolense*, *T. cruzi* and *L. major* and the apicomplexan *Plasmodium falciparum* and *Toxoplasma gondii*. Although quite sophisticated reverse genetics tools are available for most of these organisms, it is evident that RNAi could greatly facilitate a genome-wide functional analysis and help in the validation of drug targets.

The case of T. congolense*,* T. cruzi *and* L. major

The good news is that *T. congolense,* a parasite causing a severe cattle disease in Africa, is RNAi positive (Inoue *et al*., 2002). This was convincingly demonstrated by establishing a tetracycline-regulated system for expression of dsRNA in these organisms. In addition, database mining of the unfinished *T. congolense* genome reveals the existence of partial open reading frames with homology to TbAGO1 (E. Ullu, unpubl. obs.). However, it came as a surprise that *L. major* and *T. cruzi*, members of the same family of organisms, appeared to be RNAi negative. Using experimental strategies similar to those that revealed RNAi in *T. brucei,* strong and convincing evidence led to the conclusion that neither *L. major* nor *T. cruzi* are able to utilize dsRNA to trigger degradation of target mRNA (Zhang and Matlashewski, 2000; Robinson and Beverley, 2003; DaRocha *et al*., 2004). Furthermore, the current *T. cruzi* and *L. major* genome databases lack an AGO1 homologue or any other gene involved in RNAi, consistent with the absence of a functional RNAi pathway in these organisms.

Nevertheless, what became apparent from these database searches was the presence in *T. brucei*, *L. major, T. vivax* and *T. cruzi* of a predicted protein of approximately 1000 amino acids with a solo Piwi domain, but without a recognizable PAZ domain (Fig. 3). This protein is annotated as *T. brucei* Piwi-like protein 1 at NCBI (accession #AAR10811) and there is no evidence that this gene functions in the RNAi pathway (E. Ullu, unpubl. obs. and Durand-Dubief and Bastin, 2003). Thus, we propose that trypanosomatids identify a new class of Piwi-containing proteins and we shall refer to them as Piwi ‘solo’ (PiwiS), in order to distinguish them from the PAZ-Piwi-containing proteins. The predicted trypanosomatid PiwiS proteins show a considerable degree of amino acid identity over the entire length of the sequence, suggesting that they perform similar functions (*Supplementary material,* Fig. S1). As neither *L. major* nor *T. cruzi* appear to have a functional RNAi pathway, the presence of PiwiS genes does not correlate with the presence of the RNAi pathway. PiwiS genes are also found in certain prokaryotes, both archaea and bacteria (Cerutti *et al*., 2000), but their function is not known. Except for the Piwi domain, their sequence is not related to the trypanosomatid PiwiS proteins (Fig. 3). In addition, searching the genomes of *Drosophila* and mammals we found predicted PiwiS proteins, but whether their structures are accurate remains to be established. The function of PiwiS proteins is at present unknown.

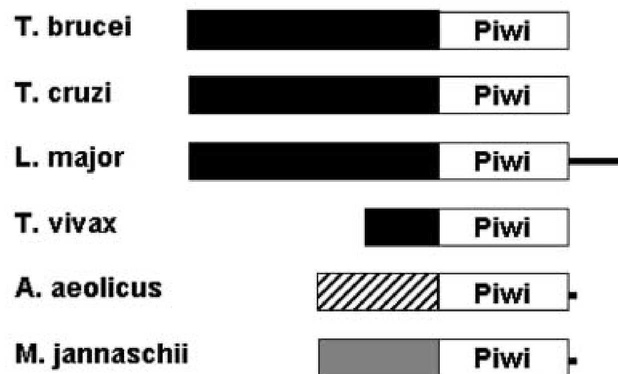

Fig. 3. Schematic structure of trypanosomatid and selected archaeal and bacterial proteins with a solo Piwi domain (PiwiS proteins). *T. brucei* (accession numbers Tb10.70.5530 and Tb10.70.5520, the two putative ORFs are separated by a termination codon that is likely to represent a sequencing error); *T. cruzi* (accession # 1103103, conceptual translation of nt 1689–4796); *L. major* (accession # Q9GRQ5); *T. vivax* (accession # Tviv428b08.p1k_0.pro, note that the *T. vivax* sequence is incomplete); *A. aeolicus* (*Aquifex aeolicus*, accession # NP_213999): *M. jannaschii* (*Methanococcus jannaschii*, accession # NP_248321). The drawing is not to scale. Homologous sequences in the trypanosomatid PiwiS proteins are indicated by solid boxes. The homology between the *A. aeolicus* and *M. jannaschii* PiwiS proteins is restricted to the Piwi domain. Non-homologous sequences are indicated by differently shaded boxes. Solid lines represent non-homologous sequences that are present after the Piwi domain at the C-terminus of some of the PiwiS proteins.

The apparent absence of the RNAi pathway in *T. cruzi* and *L. major* came as a surprise considering that these organisms all belong to the trypanosomatid lineage, with *T. brucei* representing the earliest branch. Thus, the most plausible explanation is that the RNAi pathway has been lost in *T. cruzi* and in *L. major*. This is not unprecedented, as it is known that the genome of the yeast *Saccharomyces cerevisiae* is devoid of RNAi genes, whereas the RNAi pathway is operational in several other fungi, namely *S. pombe*, *N. crassa* (Catalanotto *et al*., 2000) and *Cryptococcus neoformans* (Liu *et al*., 2002), among others. What evolutionary forces shaped the genomes of eukaryotic organisms to either retain or lose the RNAi pathway is puzzling, especially in light of the many functions that RNAi genes are implicated in. One possibility is that in some organisms the RNAi pathway has become redundant and RNAi genes were lost. Interestingly, certain plant single-stranded RNA viruses encode RNAi repressors that appear to function by sequestering siRNAs (Silhavy *et al*., 2002). If at some point during evolution, *L. major* and/or *T. cruzi* acquired an RNAi repressor as a result of a viral infection, then the RNAi pathway, or a portion of it, might have been disabled in these organism. Recently, it

was also proposed that RNAi loss in these lineages might be a reflection of a successful viral attack on these organisms (Beverley, 2003). Nevertheless, the apparent absence of dsRNA-mediated mRNA degradation does not exclude the possibility that small non-coding RNAs play a role in the regulation of gene expression in *L. major* and *T. cruzi*. It will be interesting to analyse whether *T. cruzi* and/or *L. major* accumulate gene-specific 20–26 nt-long small RNAs.

Considering that the *T. cruzi* genome is rich in retroposon-like elements (Wickstead *et al*., 2003), one intriguing aspect is how *T. cruzi* copes with the potential damage of retroposon mobilization? Perhaps, given the large number of organisms that are produced during an infection, the survival of *T. cruzi* at the population level is not affected by the appearance of a few mutants at each generation. Another possibility is that the maintenance of genome integrity in *T. cruzi* is achieved by a surveillance mechanism different from RNAi. For instance, in mammalian cell nuclei long dsRNAs, which might include retroposon transcripts, are subjected to extensive adenosine deamination. This generates inosine-containing molecules that bind to a specific set of proteins and are sequestered in the nucleus away from the cytoplasmic RNAi enzymes (Kumar and Carmichael, 1998; Carmichael, 2003). In fact, mammalian cells do not produce retroposon-derived siRNAs (Dykxhoorn *et al*., 2003).

At the other end of the spectrum is the case of *L. major,* whose genome is devoid of mobile elements and is RNAi-negative (Beverley, 2003). Whether retroelements were ever present in the ancestral *Leishmania* genome and were subsequently lost is not known.

The case of apicomplexan parasites

Whether RNAi is functioning in *Plasmodium* species is still a debatable issue, in spite of the publication of a few papers on this topic. First, using dsRNA against dihydroorotate dehydrogenase (DHODH), an enzyme essential for the pyrimidine biosynthesis of *P. falciparum,* it was shown that the growth of the parasite was impaired (McRobert and McConkey, 2002). Although, by RT-PCR the level of DHODH messenger RNA was reduced in dsRNA-treated parasites, the data was not supported by Northern analysis, and unspecific effects of dsRNA cannot be ruled out. Soon afterwards, evidence was presented for downregulation of the *P. falciparum* cysteine proteases, falcipain-1 and falcipain-2, by homologous dsRNA (Malhotra *et al*., 2002). Cells treated with dsRNA against falcipain had enlarged food vacuoles consistent with the inhibition of falcipain activity. The authors went on to show accumulation of siRNA-like molecules and of a 70 nt-long intermediate of unknown origin. Unfortunately, there is no evidence that the 25 nt small RNA species are true siRNAs, nor that they are produced by the parasites and not by potentially contaminating white cells. Next, a report appeared where injection of *P. berghei* infected mice with siRNAs specific to the *P. berghei* cysteine protease berghepain resulted in downregulation of berghepain (Mohmmed *et al*., 2003). Also in this case the siRNA-treated parasites had enlarged food vacuoles. RNA isolated from parasites grown in siRNA-injected mice showed diminution of berghepain-1 and berghepain-2 mRNA and the presence of small RNAs of multiple sizes specific to berghepain-2 RNA. However, the origin of the berghepain small RNAs was not clear. We and others (Aravind *et al*., 2003) asked whether the genomes of *Plasmodium* species contain genes that are known to be essential for the RNAi pathway. By database mining with the consensus domain sequences for Dicer, Piwi, PAZ or RdRp we failed to identify RNAi gene candidates in any of the *Plasmodium* databases that are currently available. Thus, the existence of a ‘classical’ RNAi pathway in these organisms is not supported by their genetic makeup. Although one should keep in mind that no genome sequence is ever complete, it is possible that what has been reported so far in *Plasmodium* is the result of an antisense RNA rather than a dsRNA effect.

At the present time there is only one report that RNAi might be operational in *Toxoplasma gondii* (Al-Anouti and Ananvoranich, 2002). Expression of dsRNA homologous to uracil phosphoribosyltransferase (UPRT) mRNA, but not of antisense RNA, was shown to downregulate the function of the corresponding enzyme, as assayed by scoring the resistance of the parasites to 5-fluoro-2′-deoxyuridine (FDUR). Although Northern blot analysis demonstrated specific reduction of the UPRT mRNA level, this study did not ascertain whether there was production of siRNAs, the hallmark of the RNAi pathway. Therefore, the connection of these results with the RNAi pathway was not firmly established. Nevertheless, database mining of the *T. gondii* predicted coding regions revealed the existence of putative ORFs with convincing homology to the classical RNAi genes, namely potential homologues of AGO, Dicer and RdRp. The *T. gondii* AGO-like protein (Fig. 2) contains both a PAZ and a Piwi domain, as can be appreciated from the alignment shown in Fig. S2 in the *Supplementary material*. Furthermore, the expression of the *T. gondii* AGO-like protein is supported by a number of tachyzoite cDNAs. Thus, this protozoan is most likely endowed with a functional RNAi pathway. The fact that several laboratories have tried and failed to establish RNAi for downregulation of gene expression in *Toxoplasma* might be due to several reasons. Our experience has taught us that to elicit a strong RNAi response in *T. brucei*, it is necessary to express large amounts of dsRNA under the control of a strong promoter. This requirement for high levels of expression might be due to the possibility that

only a small proportion of dsRNA can enter the RNAi pathway, because the cellular machinery is saturated by endogenous siRNAs. Indeed, when we sequenced *T. brucei* siRNAs from cells expressing an actin dsRNA transgene we found that only about 1–2% of the sequences were derived from actin dsRNA (Djikeng *et al*., 2001). Alternatively, in *T. gondii* the RNAi genes might function in the production of other regulatory RNAs, like miRNAs.

The case of Entamoeba *and* Giardia

At the time of writing this review there was no published report of a functional RNAi pathway in *Giardia or Entamoeba*. However, it is evident from database mining that the genomes of both protozoa possess the genes that are the hallmark of the RNAi pathway (Fig. 2 for the AGO-like genes and the sequence alignment for *Giardia* AGO-like protein in Fig. S3 of the *Supplementary material*). Thus, we anticipate that reports on RNAi in *Giardia* and/or *Entamoeba* will be forthcoming. Interestingly, recent evidence has shown that transcriptional gene silencing can occur in *Entamoeba* by expressing amoebapore sense transgenes with flanking regulatory elements and that the 5′ flanking regions was sufficient for the silencing phenomenon (Bracha *et al*., 2003). As no siRNAs corresponding to the amoebapore gene sequences were detected, it remains to be established whether transcriptional gene silencing depends on RNAi genes. In the case of *Giardia,* Hugo Lujan and colleagues have provided evidence that the RNAi pathway in *Giardia* controls expression of the variant-specific surface proteins (vsps) and that the function of RdRp is necessary to restrict expression of the vsp gene repertoire to a single gene at any one time (H. Lujan, pers. comm.). Moreover, we have recently cloned 20–30 nt-long RNAs from *G. intestinalis* total RNA and found sense and antisense sequences derived from the most abundant retroposon elements present in the *Giardia* genome (Arkhipova and Morrison, 2001; E. Ullu, H. Lujan, A. G. McArthur and C. Tschudi, in preparation). This suggests that, like in *T. brucei,* RNAi in *Giardia* functions as a genome defence mechanism to silence retroposon transcripts. As *Giardia* represents the earliest divergent eukaryote characterized to date, this would suggest that the RNAi pathway appeared very early in the eukaryotic lineage and that retroposon silencing is the ancestral function of the RNAi pathway. Interestingly, it is known that certain strains of *Giardia* can be infected by a dsRNA virus, called giardiavirus (Wang *et al*., 1993), and that there are abundant sense and antisense transcripts originating from transcription of the *Giardia* genome (Elmendorf *et al*., 2001). One wonders how the RNAi pathway can co-exist with the potential for dsRNA formation and the fact that giardiavirus can complete a productive life cycle at least in certain *Giardia* strains. As giardiavirus most likely replicates in the cytoplasm, this points to the possibility that the RNAi machinery is either compartimentalized in the nucleus or that giardiavirus is capable of escaping the RNAi response through some unknown mechanism, perhaps by producing an RNAi repressor. On the other hand, in the case of the sense and antisense transcripts it is not known whether there is formation of dsRNA *in vivo* and whether these putative dsRNAs ever enter the RNAi pathway. Clearly, further experiments need to be carried out to elucidate the role of sense/antisense transcripts in the biology of *Giardia*.

Conclusion

The last few years have provided the scientific community with a new field of biology, namely the discovery that many eukaryotic organisms use small regulatory RNAs as guides for gene silencing mechanisms that take place both in the nucleus and in the cytoplasm. In parasitic protozoa we have just begun to unravel the mechanism and biology of RNAi in *T. brucei*, but it is becoming evident from direct experimentation and database mining that the RNAi pathway is likely to be functional in *T. gondii*, *E. histolytica* and *G. intestinalis*. What remains to be achieved in these organisms is to harness the power of RNAi for gene downregulation. This might be facilitated by understanding the biological role of RNAi and by engineering powerful expression systems for dsRNA. It is also evident that RNAi is not a constant feature in different species of the same family, as *L. major* and *T. cruzi,* are RNAi-negative. As unfortunate as this might be, once the genes involved in RNAi in *T. brucei* are identified it might be possible to use these genes to try to reconstruct the RNAi pathway in those organisms that have lost them.

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Supplementary material

The following material is available from http://www.blackwellpublishing.com/products/journals/suppmat/cmi/cmi399/cmi399sm.htm

Fig. S1. Clustal alignment of trypanosomatid PiwiS putative coding regions (see legend to Fig. 3 for details).The Piwi domain is boxed in green.

Fig. S2. Clustal alignment of *T. gondii* putative AGO-like protein1 (TgAGO-L1) with the eIF-2C consensus sequence (cons), annotated as conserved domain KOG1041 and At AGO1 (*Arabidopsis thaliana* AGO1). See legend to Fig. 2 for accession numbers. The PAZ and Piwi domains are boxed in red and green respectively.

Fig. S3. Clustal alignment of *G. intestinalis* AGO-like protein1 (GiAGO-L1, for the accession number see legend to Fig. 2) with the eIF-2C consensus sequence (cons), annotated as conserved domain KOG1041, and *Homo sapiens* piwi (Hspiwi, accession # AAK69348). The PAZ and Piwi domains are boxed in red and green respectively.

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