Comparative Genomics Reveals Two Novel RNAi Factors in *Trypanosoma brucei* and Provides Insight into the Core Machinery

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**Abstract**

The introduction ten years ago of RNA interference (RNAi) as a tool for molecular exploration in *Trypanosoma brucei* has led to a surge in our understanding of the pathogenesis and biology of this human parasite. In particular, a genome-wide RNAi screen has recently been combined with next-generation Illumina sequencing to expose catalogues of genes associated with loss of fitness in distinct developmental stages. At present, this technology is restricted to RNAi-positive protozoan parasites, which excludes *T. cruzi*, *Leishmania major*, and *Plasmodium falciparum*. Therefore, elucidating the mechanism of RNAi and identifying the essential components of the pathway is fundamental for improving RNAi efficiency in *T. brucei* and for transferring the RNAi tool to RNAi-deficient pathogens. Here we used comparative genomics of RNAi-positive and -negative trypanosomatid protozoans to identify the repertoire of factors in *T. brucei*. In addition to the previously characterized Argonaute 1 (AGO1) protein and the cytoplasmic and nuclear Dicers, *TbDCL1* and *TbDCL2*, respectively, we identified the RNA Interference Factors 4 and 5 (*TbRIF4* and *TbRIF5*). *TbRIF4* is a 3′-5′ exonuclease of the DnaQ superfamily and plays a critical role in the conversion of duplex siRNAs to the single-stranded form, thus generating a *TbAGO1*-siRNA complex required for target-specific cleavage. *TbRIF5* is essential for cytoplasmic RNAi and appears to act as a *TbDCL1* cofactor. The availability of the core RNAi machinery in *T. brucei* provides a platform to gain mechanistic insights in this ancient eukaryote and to identify the minimal set of components required to reconstitute RNAi in RNAi-deficient parasites.

**Introduction**

RNA interference (RNAi) was first described in 1998 and within a short period of time tremendously facilitated the analysis of gene function, especially in organisms where classical genetic approaches are not available. This is particularly evident in the human pathogen *Trypanosoma brucei*, where RNAi has become a primary tool to interrogate its biology aided by the availability of an inducible and heritable system [1,2]. The effectiveness of RNAi in *T. brucei* is documented by over 500 publications in the past ten years and the availability of the genome sequence has opened up the possibility for genome-wide RNAi screens [1]. In particular, the very recently introduced RIT-Seq method (RNA Interference Target Sequencing) took advantage of the power of genome-wide RNAi screens and combined it with the strength and depth of next-generation Illumina sequencing [3]. This strategy provided the scientific community with a catalogue of genes whose knockdown is detrimental to the parasite under a variety of developmental conditions and is likely to find numerous applications in RNAi-positive parasites.

Although RNAi has flourished immensely in *T. brucei*, as highlighted by the above brief synopsis, it was rather disappointing to recognize both experimentally and at the genome sequence level that other protozoan parasites with a major impact on mankind, including *T. cruzi* [4], *Leishmania major*, *L. donovani* [5] and *Plasmodium falciparum* [6], were lacking a functional RNAi pathway. On the other hand, the genome sequence of *L. (Viannia) braziliensis* [7] predicted the existence of the RNAi pathway in this leishmania subgenus, and this prediction was recently experimentally validated [8]. The realization that *T. cruzi* and old world leishmanias are RNAi-negative was quite unfortunate, but it has been argued that once the core genes involved in *T. brucei* RNAi are identified it might be possible to try to reconstruct the pathway in RNAi-negative trypanosomatids [1]. Indeed, the recent success of David Bartel’s group in reconstructing the RNAi pathway in *S. cerevisiae* [9] provides a proof of principle and informs us that it is realistic to try to achieve the same goal in *T. cruzi* or old world *Leishmania* sp.

Comparison of RNAi mechanisms in different model organisms [10] suggests that the common, minimal machinery consisted of firstly, a Dicer endonuclease of the RNase III family that processes long dsRNAs into duplex small interfering RNAs (siRNAs) with characteristic two-nucleotide 3′ overhangs; secondly, an Argonaute (AGO) “slicer” endonuclease that in a complex with a
Author Summary

RNA interference (RNAi), a naturally-occurring pathway whereby the presence of double-stranded RNA in a cell triggers the degradation of homologous mRNA, has been harnessed in many organisms as an invaluable molecular biology tool to interrogate gene function. Although this technology is widely used in the protozoan parasite Trypanosoma brucei, other parasites of considerable public health significance, such as Trypanosoma cruzi, Leishmania major, and Plasmodium falciparum do not perform RNAi. Since RNAi has recently been introduced into budding yeast, this opens up the possibility that RNAi can be reconstituted in these pathogens. The key to this is getting a handle on the essential RNAi factors in T. brucei. By applying comparative genomics we identified five genes that are present in the RNAi-proficient species, but not in RNAi-deficient species: three previously identified RNAi factors, and two novel ones, which are described here. This insight into the core T. brucei RNAi machinery represents a major step towards transferring this pathway to RNAi-deficient parasites.

Results

TbRIF4 and TbRIF5 are two novel T. brucei RNAi factors identified by comparative genomics

In order to identify the core RNAi genes we compared the genomes of RNAi-proficient (T. cruzi and L. major) and RNAi-deficient (T. brucei, T. congolense and L. braziliensis) trypanosomatids and selected ORFs that are exclusively present in all the RNAi-positive organisms, but absent from the RNAi-negative parasites. This analysis only returned five genes. In addition to TbAGO1, TbDCL1 and TbDCL2, the search identified two putative RNAi factors (Tb927.10.8880 and Tb927.10.10730 in T. brucei), which we named T. brucei RNA Interference Factor 4 and 5 (TbRIF4 and TbRIF5, 1509 amino acids long). TbRIF4 contains a C-terminal domain related to 3’-5’ exonucleases of the DnaQ superfamily. In the absence of TbRIF4 protein or function, duplex siRNAs accumulate, and are not associated with TbAGO1. Human AGO2 can replace both TbRIF4 and TbAGO1 functions, suggesting that the slicing function of human AGO2 in RISC maturation is replaced in trypanosomes by the TbRIF4 exonuclease.

Figure 1. Characteristics of TbRIF4. Schematic representation of TbRIF4 (the drawing is not to scale) and alignment of the three motifs characteristic of 3'-5' exonucleases of the DEDDh superfamily from E. coli DnaQ [25], N. crassa QIP [20], and T. brucei RIF4. Asterisks and bold characters indicate conserved residues.

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domain of DnaQ [25]. Besides the exonuclease motifs, TbRIF4 and \(\text{NQIP}\) do not share any other sequence similarity. TbRIF5 is specific to the trypanosomatid lineage and bioinformatic analysis did not reveal any known domains (Figure S2).

TbRIF4 is essential for RNAi in both the cytoplasm and the nucleus. First, \(\text{nfs}^{-/-}\) cells (Figure S3A) had a much reduced ability to respond to transfection with \(\alpha\)-tubulin dsRNA (Table 1, Figure 2A), an assay that monitors the cytoplasmic RNAi response and in wild-type cells results in degradation of \(\alpha\)-tubulin mRNA. Second, in \(\text{nfs}^{-/-}\) cells the levels of long and heterogeneous repeat-derived (CIR147) transcripts were substantially increased as compared to wild-type cells (Figure 2B, lane 2, and Figure 2C, lane 3), a previously-reported hallmark of nuclear RNAi deficiency [16]. A \(\text{TbRIF4-GFP}\) cassette, integrated into the tubulin locus of \(\text{Tb}\) \(\text{rif}4\) cells (Figure S3A), restored \(\text{TbRIF4}\) levels and complemented the mutant phenotypes, leading to wild-type response to dsRNA transfection (Table 1, Figure 2A) and restoration of long retropon- and repeat-derived transcript levels (Figure 2B, lane 3, and Figure 2C, lane 4). Consistent with the involvement of \(\text{TbRIF5}\) in both the nuclear and cytoplasmic arms of the RNAi pathway, the \(\text{TbRIF4-GFP}\) fusion protein was distributed uniformly between the nucleus and cytoplasm (Figure 2D).

\(\text{TbRIF5}\), on the other hand, only appears to be required for the cytoplasmic branch of the RNAi pathway. \(\text{nfs}^{-/-}\) cells (Figure S3B) transfected with double-stranded \(\alpha\)-tubulin RNA showed a severe defect in the cytoplasmic RNAi response (Table 1). However, levels of CIR147 transcripts were not increased by \(\text{TbRIF5}\) ablation (Figure 2B, lane 7), suggesting that nuclear RNAi was unaffected. A \(\text{TbRIF5-BB2-HA}\) cassette, integrated into the tubulin locus of \(\text{nfs}^{-/-}\) cells (\(\text{rif}5\)c cell line, Figure S3B), complemented the cytoplasmic RNAi defect (Table 1). Due to the low level of expression of the epitope-tagged protein, the cellular localization of \(\text{TbRIF5}\) could not be ascertained by IFA. However, cell fractionation experiments indicated that most of the protein partitioned in the cytoplasm (data not shown).

\(\text{TbRIF4}\) functions downstream of the dicer step of RNAi, while \(\text{TbRIF5}\) acts with \(\text{TbDCL1}\)

To determine the stage in the RNAi pathway at which \(\text{TbRIF4}\) and \(\text{TbRIF5}\) act, we first asked whether siRNAs were produced in their absence. Northern blot analysis revealed that in \(\text{nfs}^{-/-}\) cells (Figure 3A, lane 2) ingi- and CIR147-derived siRNAs accumulated to levels greater than wild-type (lane 1) or \(\text{rif}4\)c (lane 3) trypanosomes, although their size distribution was more heterogeneous than in wild-type cells, possibly due to nucleasenibbling as these siRNAs are not associated with \(\text{TbAGO1}\) (see below). An \textit{in vitro} dicing assay programmed with long dsRNA [16] indicated that siRNA processing was as active in \(\text{nfs}^{-/-}\) as in wild-type cell extracts (Figure 3B), suggesting that \(\text{TbRIF4}\) acts downstream of the dicing step.

siRNAs also accumulated in \(\text{nfs}^{-/-}\) parasites. However, as in a \(\text{dcl1}^{-/-}\) cell line [16], while siRNAs derived from the CIR147 tandem repeats accumulated in \(\text{nfs}^{-/-}\) cells to a level comparable to wild-type or \(\text{rif}5\)c trypanosomes (Figure 3A), SLACS retroposon siRNA levels were reduced in the mutant cell line (lane 5), but were restored by complementation (lane 6). A similar phenotype was observed for the ingi-derived siRNAs (data not shown). These data suggested a link to the cytoplasmic Dicer and the following experiments provided corroborating evidence. Firstly, the \textit{in vitro} dicing assay showed that the siRNAs generated by a \(\text{nfs}^{-/-}\) cell extract were the same size as those created by a \(\text{dcl1}^{-/-}\) extract, which, as has been shown previously by our group, are one nucleotide smaller than those generated by a \(\text{dcl2}^{-/-}\) extract [16]; Figure 3B). Secondly, we co-immunoprecipitated the two proteins (Figure S4) and found that a proportion of \(\text{TbDCL1}\) is in a complex with \(\text{TbRIF5}\). We expressed \(\text{TbDCL1-BB2-FLAG}\) and \(\text{TbRIF5-BB2-HA}\) in the same cell line. Immunoprecipitation of \(\text{TbDCL1}\) (top panel) or \(\text{TbRIF5}\) (bottom panel) was carried out using anti-FLAG or anti-HA antibodies, respectively, and the fate of both \(\text{TbDCL1}\) and \(\text{TbRIF5}\) was monitored by Western blotting for the common BB2 epitope. It should be noted that \(\text{TbDCL1-BB2-FLAG}\) is at least two to three times more abundant than \(\text{TbRIF5-BB2-HA}\) in this cell line (see input lane in Figure S4). When \(\text{TbDCL1}\) was pulled down, the majority of \(\text{TbRIF5}\) was found in the immunoprecipitated fraction. In contrast, when \(\text{TbRIF5}\) was immunoprecipitated, approximately 50% of \(\text{TbDCL1}\) was detected in the precipitate, in agreement with the lower abundance of \(\text{TbRIF5}\) (the identity of the immunoprecipitated proteins was verified by Western blotting with HA monoclonal antibody for \(\text{TbRIF5}\), or FLAG monoclonal antibody for \(\text{TbDCL1}\); data not shown). The interaction of these two factors was further supported by: i. co-fractionation on a Superdex-200 sizing column; ii. mass-spectrometry of FLAG-immunoprecipitated \(\text{TbDCL1}\), which revealed several peptides derived from \(\text{TbRIF5}\) as well as \(\text{TbDCL1}\) (data not shown). Thus, we concluded that \(\text{TbDCL1}\) and \(\text{TbRIF5}\) interact either directly or indirectly.

\section*{Table 1. Analysis of cytoplasmic RNAi in \textit{nfs}^{-/-} and \textit{nfs}^{-/-} cell lines.}

| dsRNA | Cell line | 0 pg | 1 pg | 2 pg | 5 pg |
|-------|-----------|------|------|------|------|
| wt    |           | <5   | 39   | 56   | 100  |
| \(\text{nfs}^{-/-}\) |       | <5   | <5   | <5   | <5   |
| \(\text{rif}4\)c |        | <5   | 40   | 47   | 94   |
| \(\text{nfs}^{-/-}\) |       | <5   | <5   | <5   | <5   |
| \(\text{nfs}^{-/-}\) |       | <5   | 25   | 37   | 93   |
| \(\text{ago}1^{-/-}\) |       | <5   | <5   | <5   | <5   |

Cell lines were transfected with the indicated amounts of dsRNA. The FAT cell phenotype was scored 16 hr post-transfection and is represented as % FAT cells; the response of wild-type cells transfected with 5 pg dsRNA was set at 100% and the values for the other cell lines were normalized accordingly. For each cell line the experiments were repeated three times, and the average value is shown. doi:10.1371/journal.ppat.1002678.t001

siRNAs in \(\text{TbRIF4}\) null trypanosomes accumulate in duplex form

An essential step in the RNAi pathway is the formation of the RNA-induced silencing complex or RISC [26], consisting of an AGO “slicer”, an endonuclease of the RNase H family, in a complex with a siRNA, which guides target cleavage by AGO. Biochemical studies have shown that human and \textit{Drosophila} AGO2 are initially loaded with duplex siRNAs and, following passenger strand nicking by AGO, the resulting fragments dissociate [27]. Specific factors required for this transition have been identified, such as the multicentric endonuclease \textit{C3PO} (Component 3 Promoter of RISC) in \textit{Drosophila} and humans [28,29] or the \textit{Neurospora} 3'-5' exonuclease QIP [20,30,31]. Thus, to test whether \(\text{TbRIF4}\) and/or \(\text{TbRIF5}\) were involved in RISC formation, we next asked whether the \(\text{nfs}^{-/-}\) and \(\text{nfs}^{-/-}\) derived siRNAs were single- or double-stranded. We purified RNA from wild-type, \(\text{nfs}^{-/-}\), \(\text{rif}4\)c, \(\text{nfs}^{-/-}\), and \(\text{nfs}^{-/-}\) \(\text{S100}\) extracts and analyzed the samples on a native polyacrylamide gel with or without
denaturation at 100°C (Figure 3C). A radiolabelled double-stranded synthetic siRNA was used as a control (lanes 7 and 8). Following Northern blot hybridization with a CIR147 probe, most siRNAs from wild-type cells appeared single-stranded (lane 1, see [22]). A similar result was obtained with siRNAs from rif5−/− and rif5c cells (lanes 11 and 13). In contrast, by this analysis non-denatured rif4−/−-derived siRNAs displayed the mobility expected for duplex molecules (compare lanes 3 and 7), but were converted

Figure 2. Analysis of *Tb*RIF4- and *Tb*RIF5-mutant cell lines. (A) Response of rif4−/− cells and cells expressing *Tb*RIF4 catalytic mutants or HsAGO2 to transfection of α-tubulin dsRNA. Cells were electroporated with different amounts (in micrograms) of α-tubulin dsRNA, as indicated above each lane or with 5 µg poly(I-C) (first lane in each panel set), and total RNA was prepared 2 h after electroporation and analyzed by Northern hybridization with an α-tubulin-specific probe (α-tubulin panels). α-tubulin mRNA hybridization was quantitated by PhosphorImager analysis, normalized to the load control and expressed as % mRNA remaining, setting as 100% the amount of α-tubulin mRNA present in the samples that received poly(I-C). Load; hybridization to fumarate hydratase mRNA. (B) Steady-state levels of repeat-derived (CIR147) transcripts are increased in rif4−/− cells but not rif5−/− cells. Total RNA isolated from various cell lines, as indicated above each lane, was analyzed by Northern hybridization with a CIR147-specific probe. Load; hybridization to α-tubulin mRNA. Filled squares indicate the positions of the large ribosomal RNAs, as determined by methylene blue staining of the membranes. (C) Semi-quantitative RT-PCR analysis of CIR147 transcript levels. cDNA derived from various cell lines (as indicated above each lane; lanes 1–10) was used as a template for 22 cycles of PCR using oligonucleotides that amplify the CIR147 tandem repeat transcripts, producing a ladder of fragments (top two panels), or a portion of histone H4 (bottom two panels). Dilutions of genomic DNA were used as a positive control and an indicator for non-saturated PCR (lanes 11–13); mock cDNA synthesis without reverse transcriptase (second and fourth panels, -RT) served as a negative control. (D) Cells were visualized using differential interference microscopy (DIC), GFP imaging, and Hoechst (Nucleus and Kinetoplast are indicated by N and K, respectively); GFP and Hoechst images were merged (merge).

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to ss-siRNAs upon denaturation (lane 4), whereas siRNAs from the rif4 sample again reproduced the single-stranded nature present in wild-type cells (lane 5).

To provide additional evidence that siRNAs isolated from rif4/2 trypanosomes were double-stranded, we incubated the RNA samples at different temperatures (20°C to 100°C) prior to loading them on a native gel (Figure S5A). Ingi siRNA strands dissociated between 60°C and 65°C, consistent with a duplex structure. Lastly, we assessed the sensitivity of siRNAs isolated from various cell lines, as indicated above each lane, were resolved on a native polyacrylamide gel without (−) or with (+) heating the samples to 100°C for 2 minutes prior to electrophoresis and analyzed by Northern blotting with a CIR147 probe. A radiolabelled synthetic RNA was included as a control (lanes 7 and 8). Load; hybridization to 5S rRNA.

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The above results strongly indicated that rif4/2 siRNAs are predominantly in duplex form. We think it is unlikely that the duplex siRNAs are an artefact of the RNA isolation procedure, since very little duplex siRNA can be observed in wild-type samples prepared under the same conditions (Figure 3C, lane 1). We showed previously by Northern blotting [32] that both sense and antisense siRNAs from the retroposons ingi and SLACS accumulate in T. brucei. Recent deep-sequencing of siRNAs associated with TbAGO1 confirmed these early observations and extended them to include CIR147 (unpublished observations). Although wild-type siRNAs have the potential to anneal to form
siRNAs are not associated with TbAGO1 in Tb Rif4 null cells

Next we asked whether siRNAs in rif4−/− cells were associated with TbAGO1. To this end, we introduced TAP-tagged TbAGO1, which fully complements TbAGO1 deficiency [14], into rif4−/− cells. It should be noted that the level of TAP-tagged TbAGO1 was much lower in rif4−/− cells as compared to wild-type trypanosomes (Figure 4A, top panel, compare lanes 4 and 8). This dramatic diminution of TbAGO1 levels was also observed for endogenous TbAGO1 in rif4−/− cells (Figure 4B, middle panel, compare lanes 1 and 2), but returned to normal upon complementation (lane 3). By semi-quantitative Western blot analysis we estimated that the level of endogenous TbAGO1 was reduced approximately 20-fold in rif4−/− cells (data not shown).

TAP-tagged TbAGO1 was immunoprecipitated from wild-type and rif4−/− S100 extracts and varying amounts from the different samples were analysed by Western (Figure 4A, top panel) or Northern blotting (middle panel). This experiment showed that when roughly similar amounts of TbAGO1 protein were loaded (compare 90% of the immunoprecipitated material from rif4−/− cells with 9% or even 1% of the material from the wild-type sample), no siRNAs could be detected in the pellet from the rif4−/− sample (lanes 6–8), although these molecules were clearly visible in the wild-type immunoprecipitate (lanes 2–4). We concluded that the majority of CIR147 siRNAs in rif4−/− cells were not associated with TbAGO1.

Biochemical investigation of Tb Rif4 exonuclease activity

Our experiments so far suggested that Tb Rif4 plays a role in the conversion of double- to single-stranded siRNAs. To test whether the predicted 3′-5′ exonuclease domain in the C-terminus of Tb Rif4 was involved in this process, we generated recombinant GST-Tb Rif4 fusion proteins (Figure S6A) and tested its activity in vitro. Incubation of 25-nt duplex α-tubulin siRNA-315 [16], labelled at the 3′ end of the sense strand, with increasing amounts of GST-Tb Rif4 produced a distinct ladder of fragments that progressively decreased in size with increasing protein concentration (Figure 5A, lanes 4–11) and with time (Figure S6B, lanes 2–7). Mutation of conserved exonuclease active site residue H472 to alanine drastically reduced the detected exonuclease activity (Figure 5A, lane 5 and Figure S6B, lane 8). With the above substrate we observed accumulation of a 7-nt fragment, but this was not the case when the antisense strand of the duplex substrate was labelled (Figure S6C), indicating that digestion by Tb Rif4 is somewhat affected by the substrate sequence. Nevertheless, these results suggested that in vitro both strands are accessible to Tb Rif4 digestion and that the enzyme has a distributive rather than processive mode of action.

To further characterize Tb Rif4 activity, we first used substrates with no, single or double 3′ nucleotide overhang (the label was placed at the 5′ end of the sense strand) and found that they were also processed, suggesting that the 2-nt 3′ extension is not important for substrate recognition by recombinant Tb Rif4 in vitro (Figure S6D), consistent with a distributive mode of action. Secondly, we showed that recombinant Tb Rif4 acted preferen-

Figure 4. siRNAs are not associated with TbAGO1 in rif4−/− cells. (A) Cytoplasmic extracts from cells expressing TAP-tagged TbAGO1 in a wild-type (lanes 1–4) or rif4−/− (lanes 5–8) background were immunoprecipitated with IgG beads, and the indicated amounts of supernatant (S, lanes 1 and 5) and immunoprecipitated material (P, lanes 2–4 and 6–8) were analyzed by Western blotting with a polyclonal anti-TbAGO1 antibody (upper panel, TbAGO1). CIR147 siRNAs in the soluble and precipitated materials were revealed by Northern hybridization (middle panel, CIR147 siRNA). RNA loading was quantified by hybridization to 5S RNA (bottom panel, 5S RNA). (B) Tb Rif4 and TbAGO1 steady-state levels in various cell lines. Total protein extracts from 5×10⁶ cells from various cell lines, as indicated above each lane, were resolved by SDS-PAGE and Western blotted with a polyclonal anti-Tb Rif4 antibody (top panel, Tb Rif4) or a polyclonal anti-TbAGO1 antibody (middle panel, TbAGO1). Load; a cross-reacting band to the TbAGO1 antibody (bottom panel). doi:10.1371/journal.ppat.1002678.g004

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ationally on duplex as opposed to single-stranded RNA (Figure S6E) and that catalysis required magnesium ions (Figure S6F) and a free 3’ end (Figure S6G), as processing of the RNA substrate was inhibited by EDTA or by blocking the 3’ end with a 2’,3’-dideoxyctosine, respectively. Thirdly, we asked whether GST-TbRIF4 can convert the RNA duplex into ss-RNA. Recombinant TbRIF4 does not exhibit clear strand selectivity (Figure S6C), thus either strand can be degraded and any released ssRNA will base-pair with available complementary products to form dsRNA. To circumvent this problem, we carried out an in vitro assay where one strand of the double-stranded RNA substrate was labelled at the 5’ end and the 3’ end of the same strand was blocked by dideoxy-C to prevent it from being a substrate for TbRIF4 (Figure 5B). This mimics the effect of a bound protein protecting one end of the duplex, a likely in vivo scenario. The products of this reaction were separated on a native gel, showing that many of the molecules had been converted to the single-stranded form. Thus, TbRIF4 has an intrinsic 3’-5’ exonuclease activity in vitro, which acts preferentially on double-stranded RNA, and its activity is dependent on a predicted active site residue.

Active site residues are important for TbRIF4 function in vivo

Next we assessed whether TbRIF4’s conserved DnaQ exonuclease family DEDDh residues (Figure 1) were essential for in vivo function by mutating each amino acid to alanine in the context of the TbRIF4-GFP complementation cassette, and introducing the resulting mutants into nfk1−/− cells. With the exception of mutant E296A (Figure 4B, lane 5), the other proteins (D294A, D392A, H472A and D477A, lanes 4, 6, 7 and 8) were expressed close to wild-type levels in nuclear RNAi (lanes 5–7). Finally, expression of active-site TbRIF4 mutants D294A, D392A, H472A and D477A failed to restore TbAGO1 to wild-type levels (Figure 4B, middle panel, lanes 4–8), although the amount of TbAGO1 was increased as compared to nfk1−/− cells.

TbRIF4 is part of a complex containing TbAGO1 and siRNAs

The finding that siRNAs were not detectably associated with TbAGO1 in nfk1−/− cells could indicate a physical interaction between the two proteins. To test this hypothesis, we performed immunoprecipitations. Pull-downs of either wild-type or H472A TbRIF4-GFP revealed the presence of TbAGO1 in the immunoprecipitates (Figure 6A), with more TbAGO1 in the H472A TbRIF4-GFP sample compared to the wild-type sample (compare lanes 3 and 6, bottom panel). Next we asked whether siRNAs could be immunoprecipitated with wild-type or H472A TbRIF4-GFP (Figure 6B). In cells expressing wild-type TbRIF4-GFP, CIR147 siRNAs were below the limit of detection (lane 2). In contrast, a fraction of CIR147 siRNAs were found in the immunoprecipitated material after pull-down of H472A TbRIF4-GFP (lane 4). These results indicated that a proportion of a catalytically inactive TbRIF4 was in a complex containing TbAGO1 and siRNAs. We take these results as supportive evidence that TbRIF4 and TbAGO1 interact in vivo, either directly or indirectly, and that the interaction can be stabilized by mutation of the TbRIF4 exonuclease active site, although the significance of the latter observation remains to be investigated further.
TbRIF4 substitutes for the slicer activity of human AGO2 in passenger strand removal

Our past and present data indicated that the transition from ds- to ss-siRNAs in T. brucei is uncoupled from TbAGO1 slicer function and is carried out by the TbRIF4 exonuclease. We previously reported that human AGO2 slicer (HsAGO2), but not HsAGO1, which lacks target transcript slicing activity, can partially complement TbAGO1 null cells [17]. Since HsAGO2 slicing activity is involved in the cleavage of both the passenger strand of duplex siRNAs and the target RNA [27,33], we hypothesized that the restoration of the RNAi response by HsAGO2 may occur by a TbRIF4-independent mechanism. Thus, HA-FLAG-tagged HsAGO2 was introduced into rif4<sup>−/−</sup>:ago1<sup>−/−</sup> cells. Expression of HsAGO2 resulted in a partial cytoplasmic RNAi response measured by transfection with dsRNA (Figure 2A), as previously described for cells expressing HsAGO2 and TbRIF4, but nuclear RNAi was not restored (Figure 2C, lane 9), as evidenced by the accumulation of CIR147 transcripts. To monitor the physical state of the siRNAs, HsAGO2 was immunoprecipitated and the associated siRNAs were analyzed by native gel electrophoresis (Figure 6C). In agreement with the partial RNAi response, a fraction of the siRNAs was single-stranded (lane 3). These results indicated that in trypanosomes expressing HsAGO2 maturation of siRNAs from duplex- to ss-form, takes place independently of TbRIF4 and underscored a model where TbRIF4 in a complex with TbAGO1 degrades the siRNA passenger strand, thus substituting for HsAGO2 passenger cleavage activity in ss-siRNA biogenesis in T. brucei.

Discussion

The availability of genome sequences from trypanosomatids that are either RNAi proficient or not, provided us with an opportunity to tease out genes specific to the RNAi mechanism. Quite surprisingly, the comparative genomics analysis revealed a limited set of five genes: the previously characterized TbAGO1, TbDCL1 and TbDCL2, as well two additional candidates, TbRIF4 and TbRIF5. Here, we showed that both TbRIF4 and TbRIF5 are essential for RNAi, and carry out distinct functions. TbRIF4 is implicated in the formation of a stable TbAGO1/guide siRNA complex through conversion of duplex siRNAs to their single-
stranded form, whereas TbRIF5’s function is less clearly defined due to a lack of recognizable domains, but this factor appears to be working in concert with TbDCL1. The latter conclusion is based on the observations that TbRIF5’s ablation resulted in cytoplasmic RNAi deficiency and that the protein associated with TbDCL1, the cytoplasmic Dicer. We therefore propose a role for TbRIF5 as a cofactor in TbDCL1-mediated processing of long dsRNA. It is unlikely that TbRIF5 is involved in nuclear RNAi as CIR1/7 siRNAs, which are generated by TbIDCL2, are not affected by TbRIF5 ablation, and we have recently shown that RNAi of small nucleolar RNAs similarly requires TbDCL2, but is independent of TbDCL1 [34]. The specific reduction of SLACS and ingi siRNAs in TbRIF5 null cells points to the possibility that TbRIF5 is required at some point in the biogenesis of such siRNAs in the cytoplasm, either by stimulating TbDCL1 cleavage activity and/or by functioning as a bridge between TbDCL1, the siRNAs and TbAGO1. RNase III enzymes, such as Dicer and Drosha, in general require the assistance of dsRBP cofactors [12]. For instance, in Drosophila R2D2 forms a complex with DCR-2, enhances the incorporation of siRNAs into RISC [35] and acts as a sensor for strand asymmetry of siRNAs [36]. In human cells the interaction of DGCR8 with Drosha is essential for excision of premiRNAs from primary miRNA containing transcripts [37]. Although TbRIF5 lacks a recognizable dsRNA binding domain, it may be that such a motif exists but is highly divergent. 

TbRIF4 contains a predicted 3’-5’ exonuclease domain. In vivo TbRIF4 knockout results in both nuclear and cytoplasmic RNAi deficiency and in the accumulation of duplex siRNAs that are not detectably associated with TbAGO1 by co-immunoprecipitations. As predicted from its domain structure, TbRIF4-GST is endowed with in vitro 3’-5’ exonuclease activity, and processes duplex siRNAs. Complementation of rif4 cells with TbRIF4-GFP carrying mutations of catalytic residues D294A, D392A and H472A, which also impair exonuclease activity in vitro (data not shown), did not restore RNAi competency of TbRIF4 null cells, indicating that TbRIF4 catalytic activity is required for RNAi function.

TbRIF4 and TbAGO1 interact, either directly or indirectly, and form a complex that can be recovered in greater amounts from mutant H472A TbRIF4-GFP than from wild-type cells and contains a small proportion of siRNAs. Although at present it is unclear how this observation relates to TbRIF4 function, it indicates that mutant TbRIF4 becomes “stuck” in a complex with TbAGO1. It is possible that the interaction between the two wild-type proteins is transient and that after the siRNA is converted to single-stranded form within this complex (either before or after loading into TbAGO1), TbRIF4 dissociates from TbAGO1. TbRIF4 on its own does not appear to show any strand selectivity; therefore, the strand removed is possibly determined by the orientation in which the duplex is presented by Dicer, as has been reported in Drosophila [38]. The use of chaperone proteins, for example heat shock proteins, in RISC loading has been reported in higher eukaryotes [39-42]. It is possible that TbRIF4 acts in this way in addition to its role as an exonuclease. We also observed that TbAGO1 levels are diminished in cells lacking functional TbRIF4. However, we note that there was no straightforward correlation between the amount of TbAGO1 present in the various mutant cell lines and their degree of RNAi competency, suggesting that catalytically impaired TbRIF4s can to some extent promote TbAGO1 accumulation through a mechanism that is presently unknown. As TbAGO1 mRNA levels are only marginally affected by TbRIF4 deletion (data not shown), either TbAGO1 translation is severely inhibited or the protein is made and rapidly degraded. So far inhibition of the proteasome and lysosome degradation pathways has not restored TbAGO1 levels (unpublished observations), pointing to the possibility that translational control regulates the amount of TbAGO1 that is synthesized.

T. brucei RIF4 shares with Neurospora QIP a C-terminal 3’-5’ exonuclease domain of the DnaQ superfamily. Although both proteins are essential RNAi factors and interact with the corresponding Argonaute, they are functionally distinct in several respects. First, in Neurospora qip cells duplex siRNAs are stably loaded into the AGO homolog QDE-2 and, after QDE-2-slicer-mediated cleavage of the passenger strand, remain bound to QDE-2 in duplex, nicked form [20]. In contrast, siRNAs can be successfully loaded into TbAGO1 and TbRIF4 can accomplish the transition from double- to single-stranded siRNAs in the absence of TbAGO1 slicer activity [21,22]. Secondly, we showed that TbRIF4 activity is required for TbAGO1 protein accumulation. This finding is not mirrored in Neurospora or Drosophila, where mutation of passenger strand fragment removal factors QIP or C3PO has no effect on the corresponding AGO levels [20,29]. In light of these data, it is evident that TbRIF4 and TbQIP are functionally distinct RNAi factors.

In trypanosomes expressing HsAGO2, maturation of siRNAs takes place independently of TbRIF4, suggesting a model where TbRIF4 substitutes for AGO slicing activity in the generation of ss-siRNAs. This would represent a departure from strategies of RISC maturation in higher eukaryotes, which favor slicer activity marking the passenger strand for degradation, although bypass pathways can operate in the absence of slicing activity [27,43,44]. Taken together with our earlier studies, the results presented here provide a detailed depiction of the core RNAi machinery in the early divergent parasitic protozoan T. brucei. In these organisms, the RNAi pathway is initiated by two distinct Dicer-like enzymes, namely TbDCL1, mostly found in the cytoplasm [15], and TbDCL2, a mainly nuclear Dicer responsible for processing dsRNAs originating from retroposons and satellite-like repeats [16], siRNAs generated by both Dicers are channelled into a single member of the Argonaute family of proteins, namely TbAGO1 [13,14]. The identification and characterization of TbRIF4 and TbRIF5 most likely completes the collection of core components shared by RNAi-positive trypanosomatids. However, it is likely that, as described in other organisms, other cellular factors interact with the core RNAi machinery. Some of these factors may perform housekeeping functions, for instance the Hsp90/70 family of chaperons that functions in RISC assembly in higher eukaryotes, and be present in all trypanosomatids irrespective of their RNAi proficiency, while others may be organism-specific. Indeed, we have recently discovered that the T. brucei homologue of the plant HEN1 2’-O methyltransferase [45] is required for siRNA 3’ end modification (unpublished observation). However, siRNA modification is not an absolute requirement for trypanosomatid RNAi, as in L. braziliensis the HEN1 gene is absent, and deletion of TbHEN1 is still compatible with a robust RNAi response (unpublished observations). It could be argued at this point that attempting to reconstitute RNAi in RNAi-deficient pathogens is quite daunting with a minimum of five core components. However, our past and current mechanistic studies have highlighted that not all components are necessary. In particular, we have shown previously that TbDCL2-deficient cells are more responsive to RNAi triggers than wild-type cells [16] suggesting that this factor does not need to be included in a reconstitution attempt. In addition, in the present study we have shown that human AGO2 can functionally replace both TbAGO1 and TbRIF4, indicating that the TbRIF4 function can be bypassed by using an appropriate AGO protein. It will be interesting to see
whether human AGO2 can similarly function in *L. braziliensis*. The factor remaining a mystery is *TbRIF5* and its function will be challenging to ascertain due to a complete lack of a possible functional indicator. Nevertheless, the available proof of principle that RNAs can be reconstituted in *S. coenensis* by using only AGO1 and DCR1 from *S. castellii* [9] in combination with our functional studies raises the intriguing possibility that a similar strategy might be applicable in RNAi-negative trypanosomatids.

**Materials and Methods**

**Standard methods**

Previously published procedures were followed for culturing trypanosome YTat 1.1 cells [46], generation of knockout cell lines by PCR-based methods [47], Northern blots of total RNA [32], Western blots [14], S100 preparation [32], immunoprecipitations [14], and the Dicer *in vitro* assay [16].

**RT-PCR**

5 μg of total RNA from various cell lines was treated with 2 units of DNase RQ (Promega), phenol extracted and ethanol precipitated. DNase-treated RNA was used in a reverse transcription reaction using the manufacturer’s protocol for the Superscript II enzyme (Invitrogen), phenol extracted, ethanol precipitated, and resuspended in 100 μl water. Mock reactions without the reverse transcriptase enzyme were carried out in parallel. 22 cycles of PCR were performed using the Phusion enzyme (Finnzyme) and the GC buffer provided, according to manufacturer’s instructions: 50°C annealing temperature, 10 sec extension time for the histone 4 reactions.

**Preparation of small RNAs**

Small RNAs were extracted from S100 extracts by incubation in polysome buffer plus 20 mM EDTA, 2% SDS and 0.1 μg/μl protease K for 1 hour at room temperature. An equal volume of phenol and 1/10 volume 20 mM glycylglycine (Ambion) was added and the mixture was centrifuged at 13,200 g for 5 min. The supernatant was incubated with 600 μl isopropanol and 30 μg GlycoBlue (Ambion) at −20°C for 30 min and centrifuged at 13,200 g for 10 min at 4°C. The pellet was washed with 70% ethanol, dried and resuspended in water (for native gels) or urea loading buffer (for denaturing gels), which was allowed to cool to room temperature, twice prior to use. RNA was loaded in the lane compared to the other two lanes.

**Preparation of probes**

DNA oligos were 5’ end-labelled with T4 polynucleotide kinase (NEB) using [γ-32P]ATP and purified using P6 columns (BioRad). Hybridizations were carried out in ExpressHyb solution (Clontech) overnight at 30°C. Washes in 2× SSC, 0.2% SDS were carried out at room temperature. Blots were analyzed by PhosphorImager.

**Purification of recombinant protein**

*TbRIF4* was cloned into the pGEX4T-1 vector. Inductions of 500 ml DH5α bacteria were carried out by adding 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) overnight at 16°C. Cells were washed in PBS containing protease inhibitors, lysed by sonication in 5 ml PBS containing protease inhibitors and GST-*TbRIF4* was purified using a SpinTrap column (GE). Proteins were eluted in 50 mM Tris-HCl [pH 8.0], 10 mM glutathione, before being applied to a desalting column and stored in assay buffer (20 mM HEPES-KOH [pH 7.9], 150 mM sucrose, 20 mM potassium L-glutamate, 3 mM MgCl2, 2 mM DTT).

**Exonuclease assays**

Exonuclease assays were carried out for 30 min at 28°C in 10 μl assay buffer including RNase Inhibitor (Roche) and 0.01 μg/μl bovine tRNA. Reactions were terminated by the addition of 4× SET, 20 mM EDTA, 30 μg GlycoBlue and isopropanol, stored at −20°C for 30 min and spun at 13,200×g for 10 min at 4°C. The pellet was washed with 70% ethanol, dried and resuspended in urea loading buffer. Samples were resolved on a 20% denaturing polyacrylamide gel and analyzed by PhosphorImager.

**Supporting Information**

- **Figure S1 TbRIF4 sequence analysis.** (A) Sequence alignment of *TbRIF4* protein sequences from *T. brucei* (Tbru), *T. congolense* (Tcon), *T. vivax* (Tviv) and *L. (V.) braziliensis* (Lbra) created using ClustalX (www.clustal.org) and shaded using boxshade (http://www.ch.embnet.org/software/BOX_form.html). Conserved motifs are outlined, and conserved active site residues are indicated by asterisks. (B) Ribbon diagram of the secondary structure of *E. coli* DnaQ (left) adapted from [25] and of the predicted model of the exonuclease domain of *TbRIF4* (right) using the alignment interface of SWISS-MODEL [24]. (TIF)

- **Figure S2 TbRIF5 sequence analysis.** Sequence alignment of *TbRIF5* protein sequences from *T. brucei* (Tbru), *T. congolense* (Tcon), *T. vivax* (Tviv) and *L. (V.) braziliensis* (Lbra) created using ClustalX (www.clustal.org) and shaded using boxshade (http://www.ch.embnet.org/software/BOX_form.html). (TIF)

- **Figure S3 TbRIF4 and TbRIF5 knockout and complementation cell lines.** (A) rif4ΔΔ cells do not express *TbRIF4* mRNA (top panel) or protein (bottom panel). Total RNA isolated from various cell lines, as indicated above each lane, was resolved by formaldehyde-agarose gel electrophoresis and analyzed by Northern blot hybridization with a *TbRIF4* specific probe (top panel). Load: α-tubulin hybridization. Total protein extracts were resolved by SDS-PAGE and probed with a polyclonal anti-*TbRIF4* antibody (third panel). Load: a cross-reacting band. (B) rif5ΔΔ cells do not express *TbRIF5* mRNA (top panel). Total RNA isolated from various cell lines, as indicated above each lane, was resolved by formaldehyde-agarose gel electrophoresis and analyzed by Northern blot hybridization with a *RIF5*-specific probe (top panel). Load: α-tubulin hybridization. Ten-fold less RNA was loaded in the rif5ΔΔ lane compared to the other two lanes. (TIF)

- **Figure S4 Physical association of TbDCL1 and TbRIF5.** Cytoplasmic extracts from rif4ΔΔ cells expressing DCL1-FLAG-BB2 and RIF5-HA-BB2 were subjected to immunoprecipitation with anti-FLAG antibody (upper panel) or anti-HA antibody (lower panel). Equal numbers of cell equivalents of the input (I), supernatant (S), and immunoprecipitated material (P) were analyzed by Western blotting with a monoclonal anti-BB2 antibody. (TIF)

- **Figure S5 siRNAs in rif4ΔΔ trypanosomes are double-stranded.** (A) Native gel analysis of siRNAs in rif4ΔΔ cells. RNA isolated from a rif4ΔΔ S100 extract was resolved on a native
polyacrylamide gel after heating the samples at a range of temperatures as indicated above each lane for 2 minutes prior to electrophoresis and analyzed by Northern blotting with an ingi probe. (B) siRNAs in nfe/− cells are protected from digestion with RNase A. (C) Recalculated wild-type (lanes 1–3) and nfe/− (lanes 4–6) cultures were treated with RNase T1 (5 U/ml) and RNase A (20 mg/ml) for 1 hr at 25°C, either without (lanes 2 and 5) or with (lanes 3 and 6) denaturation by boiling prior to enzyme addition. RNAs incubated with buffer alone (lanes 1 and 4) were included as a control. Following digestion with proteinase K, the samples were electrophoresed on a 16% denaturing gel and analyzed by Northern hybridization with a Cir147 probe (top panel). Load; hybridization to 5S rRNA (bottom panel).

Figure 6 Recombinant TbRIF4 3′–5′ exonuclease activity in vitro. (A) Purification of GST-TbRIF4. 30 pmol of recombinant GST-TbRIF4 was resolved by SDS-PAGE and stained with Coomassie Brilliant Blue. (B) Time-course of GST-TbRIF4 activity. 1 pmol of a 25 nt-long, 5′-end labelled synthetic dsRNA was incubated with 30 pmol GST-RIF4 for 1 to 30 min (lanes 2–7). The activity of GST-TbRIF4 carrying mutation H472A incubated for 30 min is shown in lane 8, and a no-protein control is in lane 1. (C) GST-TbRIF4 activity is not restricted to one of the duplex RNA strands. 1 pmol of synthetic siRNA-315 labelled at the 5′ end of either the “sense” or “antisense” strand was incubated with wild-type GST-TbRIF4 (wt; lanes 2 and 5) or GST-TbRIF4 carrying mutation H472A (H472A; lanes 3 and 6) or buffer alone (lanes 1 and 4). (D) Action of GST-TbRIF4 on siRNA-like substrates with shorter 3′ overhangs. 1 pmol of a 25 nt-long, 5′-end labelled synthetic dsRNA with 3′ overhangs of 2, 1 or 0 nt was incubated with 0.04 to 25 pmol GST-TbRIF4 (lanes 2–6, 8–12 and 14–18) or buffer alone (lanes 1, 7 and 13). (E) GST-TbRIF4 preferentially cleaves double-stranded RNA. 1 pmol synthetic siRNA (lanes 1 and 2) or dsRNA (lanes 3 and 4) was incubated with (+; lanes 2 and 4) or without (−; lanes 1 and 3) 30 pmol wild-type GST-TbRIF4. (F) GST-TbRIF4 activity is abolished by addition of EDTA. 1 pmol synthetic dsRNA was incubated with 30 pmol wild-type GST-RIF4 (wt; lanes 2 and 5), GST-TbRIF4 carrying mutation H472A (H472A; lanes 3 and 6) or buffer alone (lanes 1 and 4). 5 mM EDTA was added in the reactions for lanes 4–6. (G) GST-TbRIF4 activity requires a free 3′ end. 1 pmol synthetic dsRNA either without (lanes 1–3) or with (lanes 4–6) a terminal 2′,3′-cyclicadenosine residue was incubated with 30 pmol GST-TbRIF4 (wt; lanes 2 and 5), GST-TbRIF4 carrying mutation H472A (H472A; lanes 3 and 6) or buffer alone (lanes 1 and 4).

Figure 7 Efficient pull-down of TbRIF4-GFP. Cytoplasmic extracts from nfe/− cells expressing wt TbRIF4-GFP were subjected to immunoprecipitation with anti-GFP antibody. Equal volumes of the input (I), supernatant (S) and immunoprecipitated material (P) were analyzed by Western blotting with a polyclonal anti-TbRIF4 antibody.

Author Contributions
Conceived and designed the experiments: RLB HS NGK CT EU. Performed the experiments: RLB HS NGK CT EU. Analyzed the data: RLB HS NGK CT EU. Wrote the paper: RLB CT EU.

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