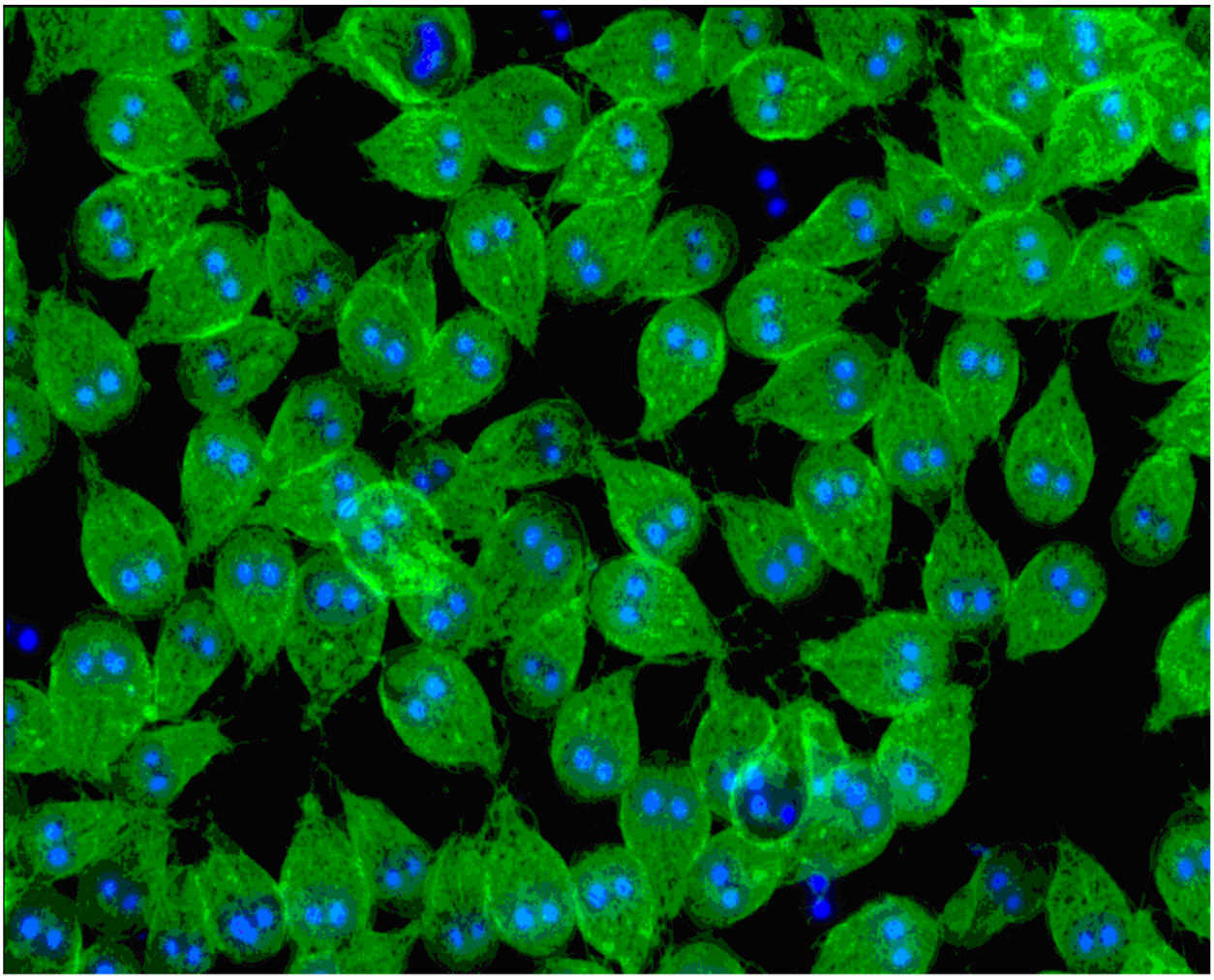
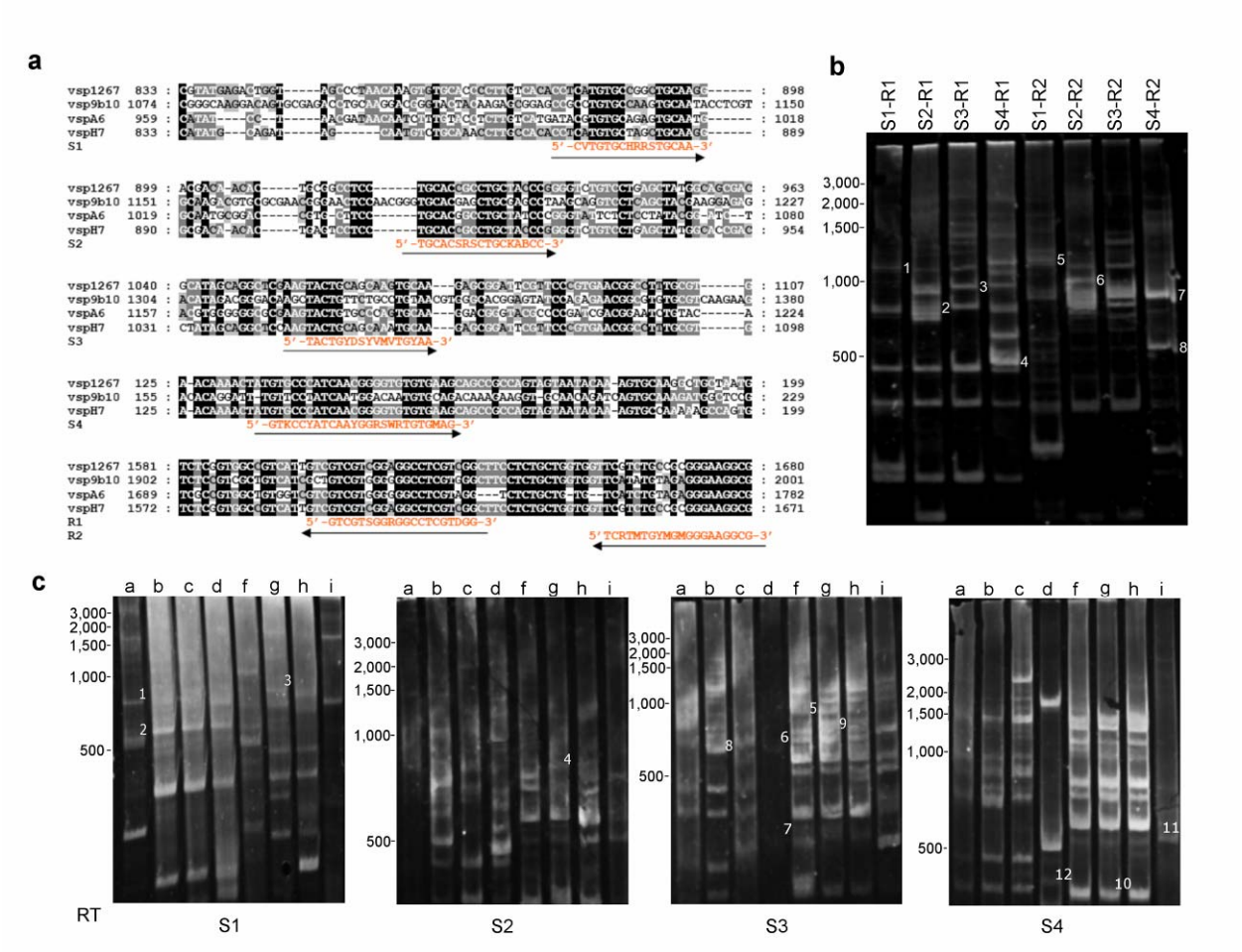
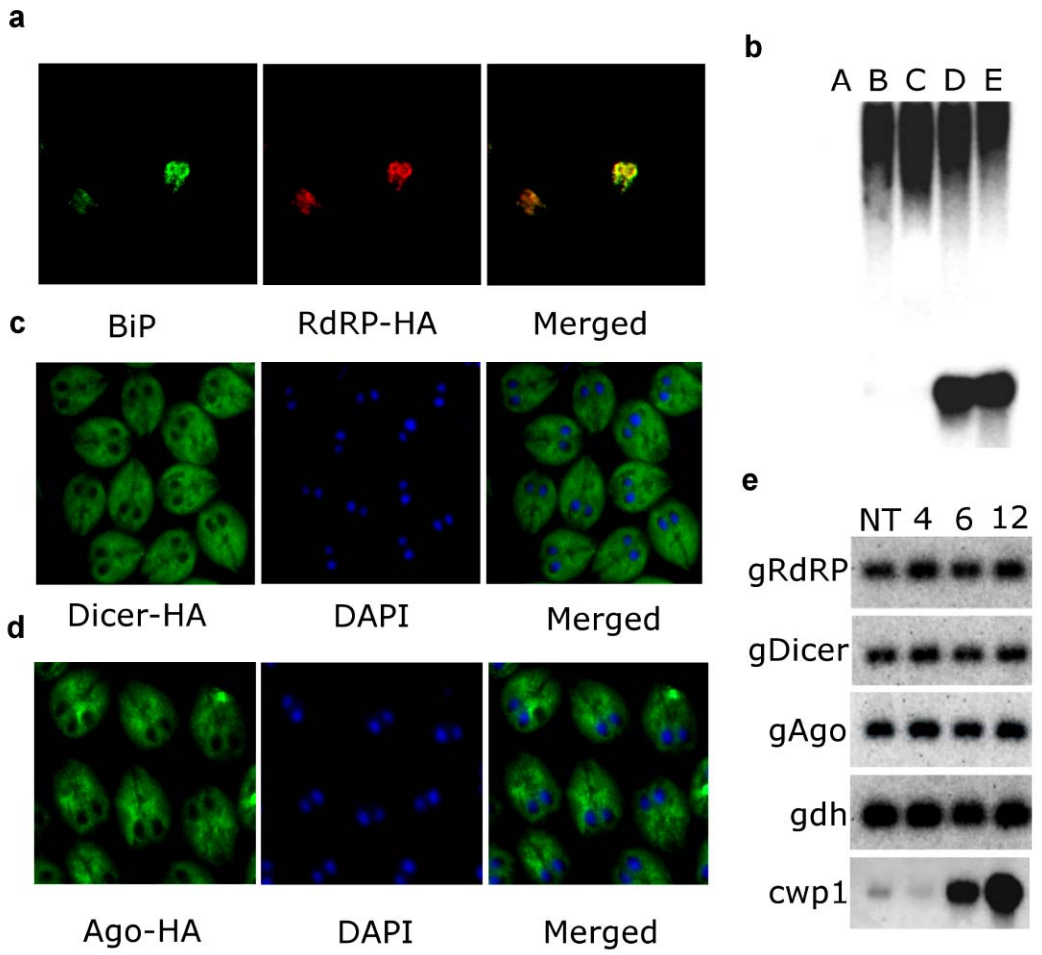
SUPPLEMENTARY INFORMATION



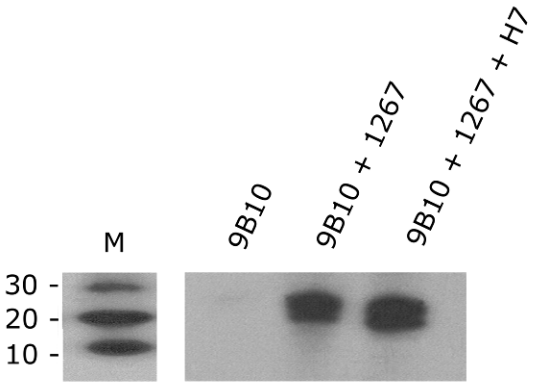
Supplementary Figure 1 | Representative control of *Giardia* culture homogeneity. *Giardia* trophozoites were cultured in TYI-S-33 medium. Continuous cloning was performed weekly by limiting dilution and selection based on immunofluorescence assays using specific monoclonal antibodies. Immunofluorescence image showing that almost 100% of trophozoites express on their surface VSP9B10, as determined by reactivity with VSP9B10-specific antibody (cell surface in green) and counterstained with DAPI (nuclei in blue).



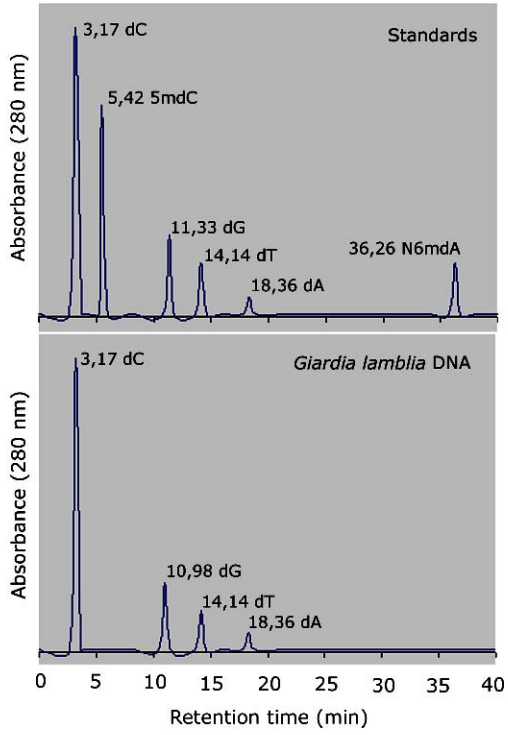
Supplementary Figure 2 | Novel tools for the study of antigenic variation in *G. lamblia*. **a,** Oligonucleotide primers suitable to amplify by PCR a large number of *vsp* genes were generated. Sequences from four previously known VSPs belonging to *G. lamblia* isolate WB (VSP1267, VSP9B10, and VSPA6) and GS (VSPH7) were selected since we possess specific monoclonal antibodies that allow the continuous control of clone homogeneity[36-40]. These four *vsp* sequences were aligned and conserved regions useful for primer design identified. Arrows below oligonucleotide sequences highlight the domains where *vsp* generic primers (S1-S4 and R1-R2) were targeted. **b,** Combinations of four sense (S1-S4) and two reverse (R1, R2) primers were then employed in PCR analysis on genomic DNA derived from *Giardia* clone WB9B10, which only expresses VSP9B10 on its surface (Figure S1). Several DNA fragments were amplified regardless of the primer combinations used. Fifty nine major products of these reactions were isolated, cloned, and sequenced revealing that all of them encode *vsp* fragments (results not shown). Several of these products (labeled 1 to 8 in white) were subsequently used as probes to screen a genomic DNA library, which allowed us to identify ORFs encoding novel proteins with typical features of *Giardia* VSPs (VSPS1-S8; GenBank accession numbers: AY142122 to AY142129). Size markers in nt are indicated on the left. **c**, To test whether silencing of *Giardia vsp* transcripts involves the generation of *vsp* antisense RNAs, we searched for these molecules in RNA preparations isolated from trophozoites expressing a single VSP. RT-PCRs were performed with the same *vsp* primer combinations previously utilized on genomic DNA, but using sense primers during the reverse transcription (RT) reaction. Sense primers (S1 to S4) are shown on top. Lanes (a) to (h) indicate the primer combinations used in each PCR reaction: (a) S1-R1, (b) S2-R1, (c) S3-R1), (d) S4-R1, (e) S1-R2, (f) S2-R2, (g) S3-R2, and (h) S4-R2. Negative controls without Reverse Transcriptase (-RT) are depicted. Thirty eight products amplified under these conditions were isolated, cloned and sequenced, demonstrating that they encode antisense *vsp*s (results not shown). Twelve of these products (labeled 1 to 12 in white) were also used as probes to screen a genomic library, which allowed us to obtain the full-length sequence of novel *vsp* genes (VSPAS1-VSPAS12, GenBank accession numbers AY143130-AY142141), indicating that the antisense transcripts target functional *vsp* genes.



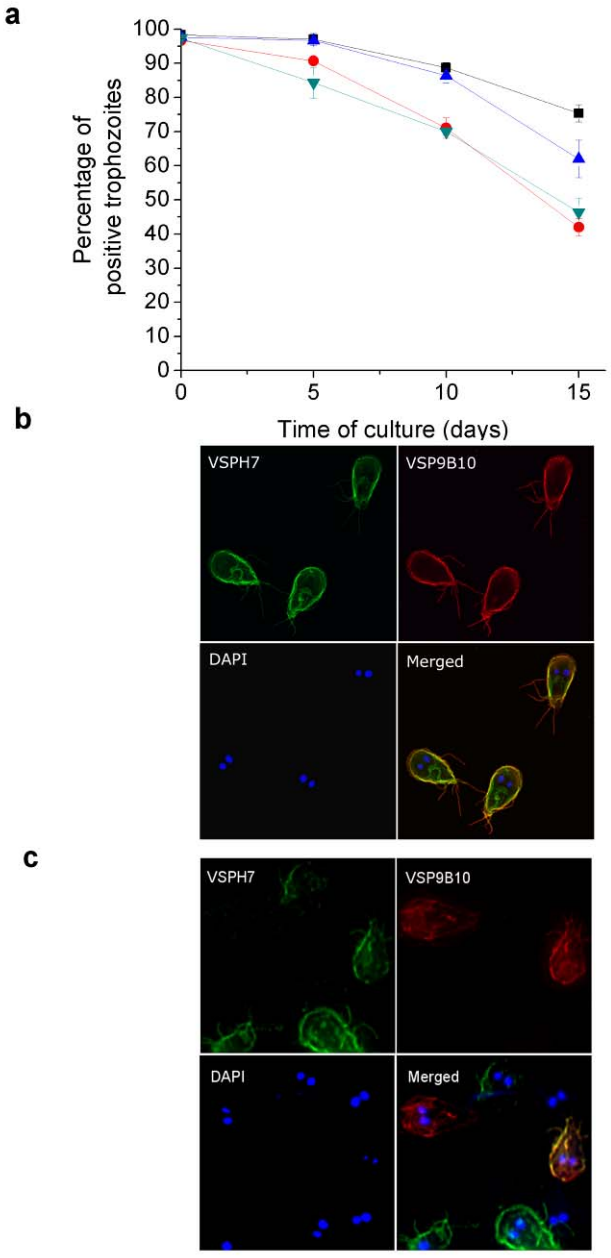
Supplementary Figure 3 | *Giardia lamblia* RNA-dependent RNA-polymerase, Dicer and Argonaute. Using *Drosophila* Dicer-1 sequence to search for homolog genes in the *Giardia* Genome Database, we were able to identify several putative clones with high degree of homology to Dicer domains. By PCR and subsequent screening of the genomic library, we obtained two independent ORF containing domains present in known Dicer enzymes[49]: An Argonaute protein with PIWI and PAZ domains (gAgo, GenBank accession number AY142142), and a bidentate RNase III (gDicer, GenBank accession number AY142144) containing both a PAZ domain and a leucine zipper motif probably involved in enzyme interaction with other components of RISC and with RNA, respectively. **a**, Immunolocalization of a HA-tagged version of gRdRP expressed in trophozoites of clone WB9B10. The enzyme (in red) localizes to a region surrounding both nuclei of the parasite. This region mostly corresponds to the rough endoplasmic reticulum (yellow) as seen by co-Immunolocalization with the ER-chaperone BiP (mAb 9C9, in green). Analysis of different *Giardia* clones indicates that this is not a result of gRdRP over-expression in this population (not shown). **b**, RdRP activity. Different combination of RNA substrates were used (**A**, **B** and **D**, *vsp*9B10 and *vsp*1267; **C** and **E**, *vsp*9B10, vsp1267, and vspH7), in the absence (**A-C**) or presence of primer R1 (**D**) or R2 (**E**). **A** is a control without purified RdRP. High molecular weight products are observed both in the presence or absence of specific primers, indicating that the purified enzyme is active in the presence of homologous VSP transcripts and that there is no requirement of priming for activity. In the presence of only one transcript, the enzyme is not active (not shown). **c**, Immunolocalization of a HA-tagged version of gDicer expressed in trophozoites of clone WB9B10. The enzyme (in green) localizes in the cytoplasm of the cell. Nuclei are stained with DAPI (blue). **d**, Immunolocalization of a HA-tagged version of gAgo expressed in trophozoites of clone WB9B10. The enzyme (in green) localizes in the cytoplasm of the cell. Nuclei are stained with DAPI (blue). **e**, Northern blotting with probes for *gRdRP*, gAgo, gDicer, GDH and CWP1 on total RNA extracted from clone WB9B10 trophozoites induced to encyst for 4, 6 or 12 h, and trophozoites maintained in growth medium (NT*)* for 12 h. Results show the constitutive expression of these PTGS components.



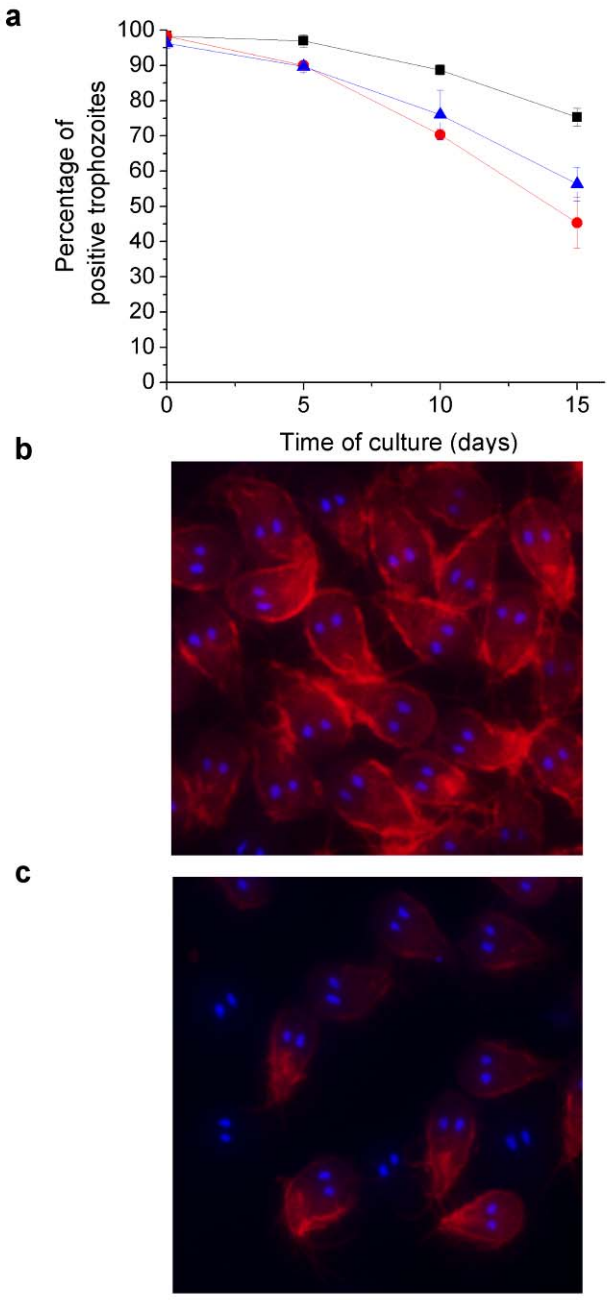
Supplementary Figure 4 | Generation of small RNAs by incubation of *vsp* riboprobes with *Giardia* extracts from clone WB1267. One, two, or three different *vsp* mRNAs (*vsp9B10, vsp1267, vspH7*) were mixed and confronted to *Giardia* trophozoite extracts. [^{32}P]-labelled small RNAs were produced whenever more than one transcript is present. RNA size markers in nt are on the left.



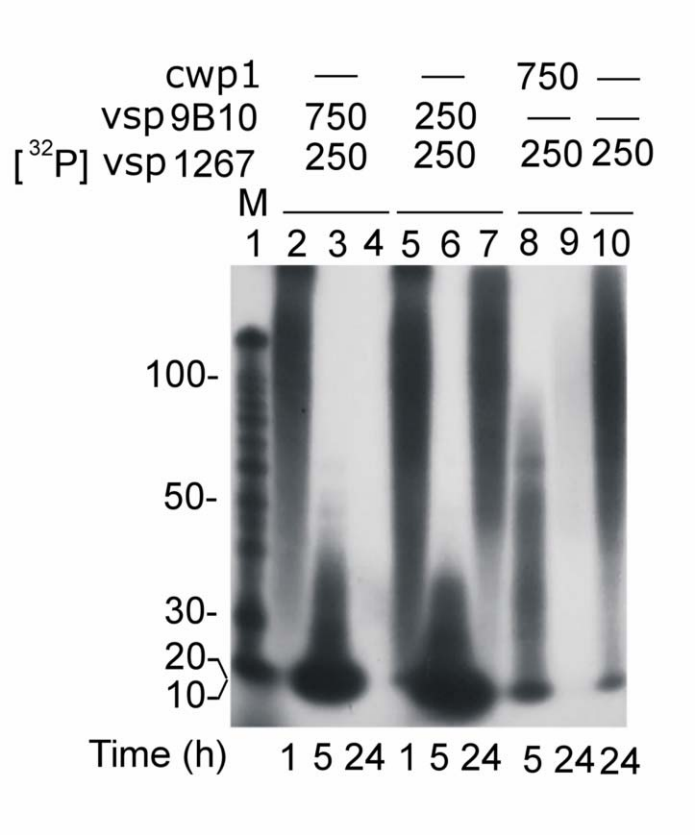
Supplementary Figure 5 | *Giardia lamblia* DNA is not methylated. Figure shows representative chromatograms illustrating the detection and separation of deoxynucleosides by HPLC. **a**, Standard mixture of deoxynucleosides, in order of appearance: deoxycytosine, 5-methyl deoxycytosine, deoxyguanosine, deoxytimidine, deoxyadenosine, and N-6-methyldeoxyadenosine. **b**, *G. lamblia* DNA digest obtained from non-encysting trophozoites shows no peaks at the positions of 5mdC and N6mdA. Similar results were obtained using DNA from encysting cells (not shown).



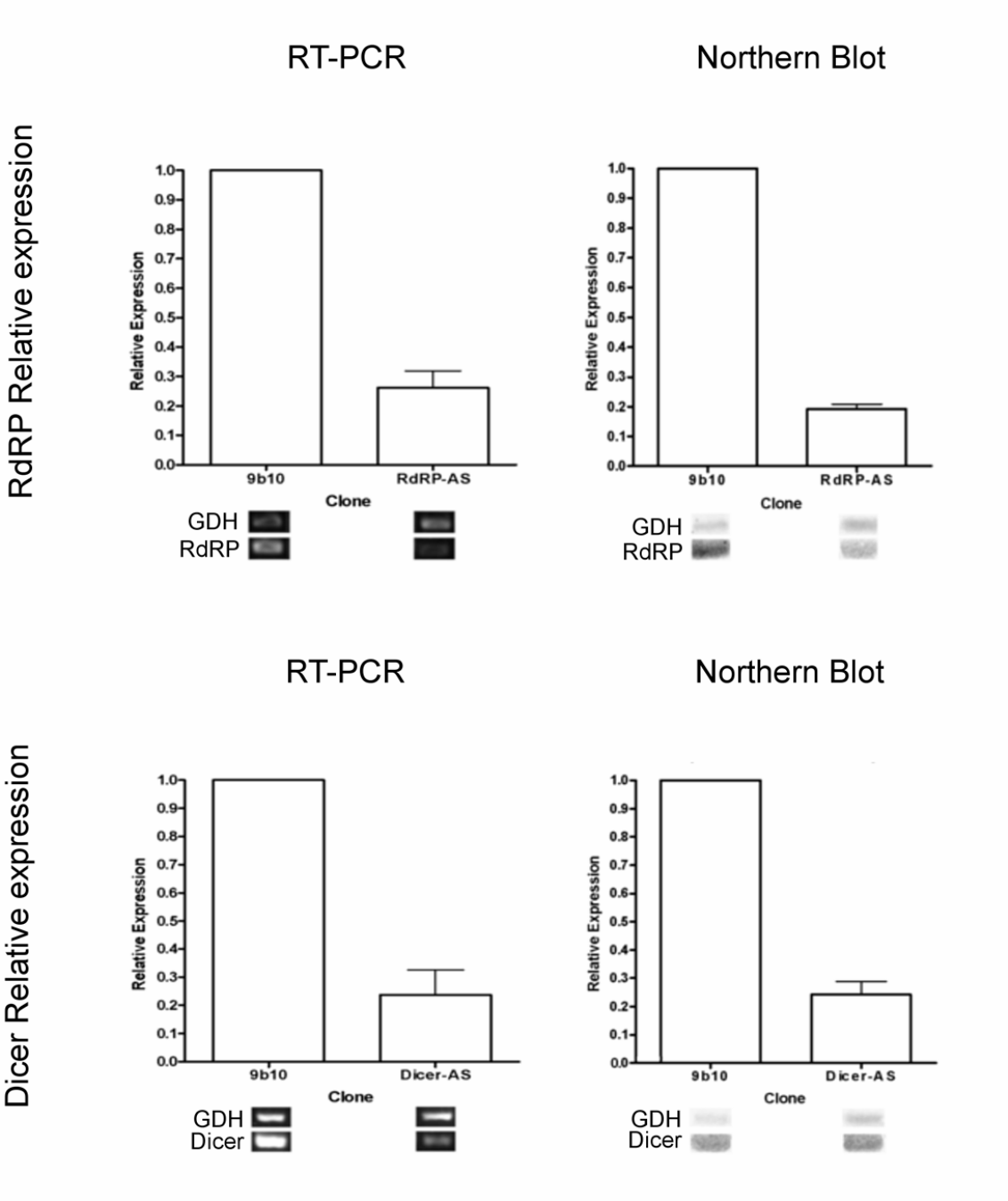
Supplementary Figure 6 | Antigenic switching in WB9B10 trophozoites overexpressing VSPH7. a, Trophozoites from clone WB9B10 were transfected with either the pTubCWP1.Pac vector or the pTubH7.Pac vector, which drive the expression of CWP1 or VSPH7 under the control of the α-tubulin promoter. After cloning by limiting dilution and under continuous selection with Puromycin, cell cultures were evaluated at different time point by indirect immunofluorescence assays using specific anti-VSPs monoclonal antibodies. PTubCWP1.Pac-transfected GS trophozoites expressing VSPH7 were also used as a control (green triangles). In control-transfected WB9B10 trophozoites, VSP9B10 decreases over the time (black squares) due to spontaneous antigenic switching. WB9B10 trophozoites constitutively expressing *vspH7* show that expression of either VSP9B10 (red circles) or VSPH7 (blue triangles) diminishes faster that the respective control. Results represent the mean percentage of three independent experiments ± S.D. **b**, Representative image of WB9B10 trophozoites expressing VSPH7 at time 0. All cells express simultaneously on their surface VSP9B10 and VSPH7. **c**, Representative image of WB9B10 trophozoites expressing VSPH7 after 15 days in culture. Some cells express on their surface VSP9B10 and VSPH7, others only VSP9B10 or only VSPH7, and others none of them. Nuclei are labeled with DAPI in Blue.



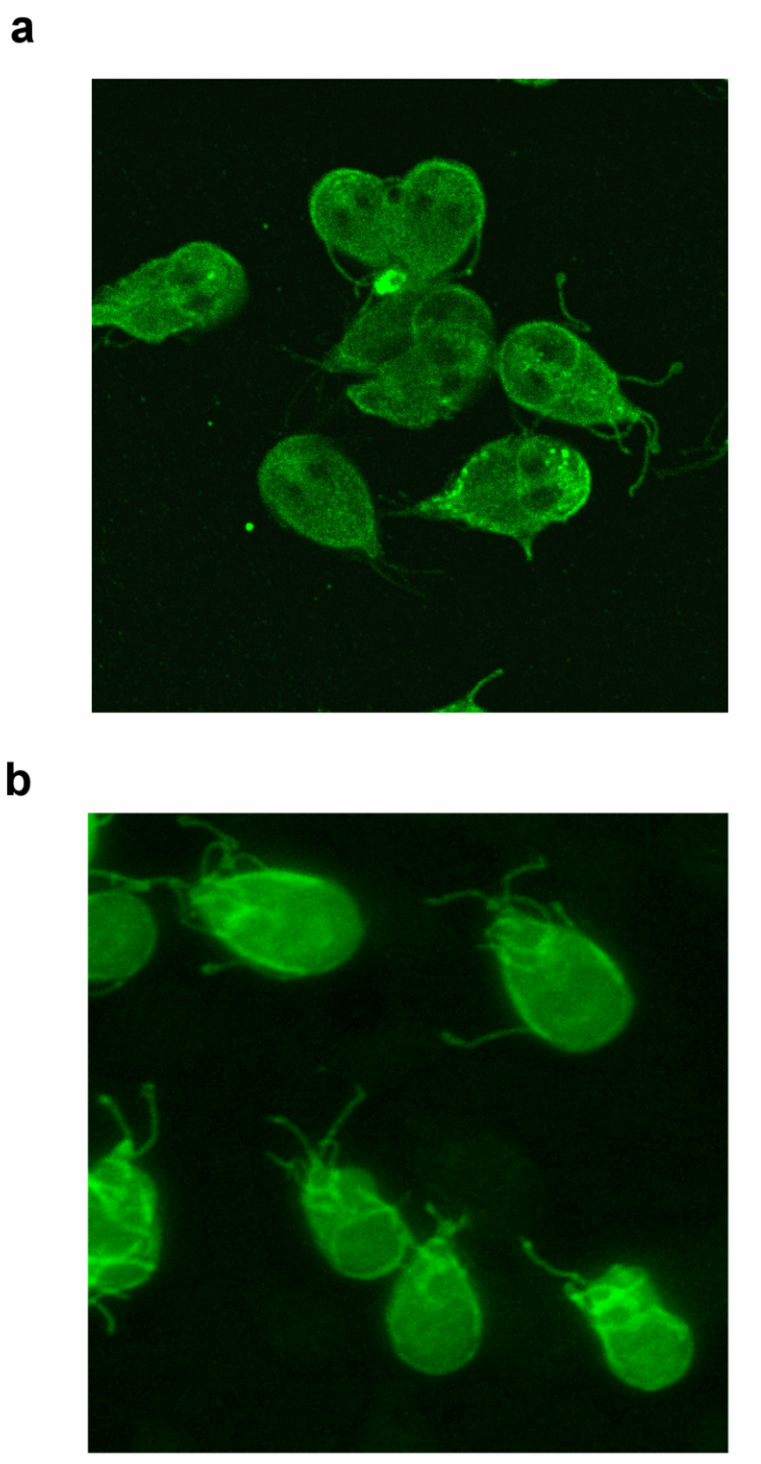
Supplementary Figure 7 | Antigenic switching in WB9B10 trophozoites expressing antisense *vsp9B10* fragments. a, Trophozoites from clone WB9B10 were transfected with either the pTubCWP1.Pac vector (black squares) or the pTubPac vector including the 5´(red circle) or 3´(blue triangle) antisense half sequence of *vsp9B10*. After cloning by limiting dilution, the cultures were assessed for the expression of VSP9B10 in the surface of the parasite using mAb 9B10 as described. Results represent the mean value of three independent experiments ± S.D and indicate that the number of VSP9B10-expressing trophozoites diminishes faster than the control. **b**, Representative image of WB9B10 trophozoites transfected with a non-VSP gene. All cells express on their surface VSP9B10. **c**, Representative image of WB9B10 trophozoites expressing the 3' antisense fragment of *vsp9B10* after 15 days in culture. VSP9B10 is present in approximately 50% of the population. Nuclei are labeled with DAPI in Blue.



Supplementary Figure 8 | Variations in concentration of different transcripts may determine which one evades the silencing system. *vsp1267*, *vsp9B10*, and *cwp1* were cloned into the pGEM-T-easy vector and *in vitro* transcribed in the presence or absence of ^{32}P-UTP. Different concentrations of unlabeled *vsp9B10* and *cwp1* transcripts were then incubated for different periods with WB1267 cytoplasmic extracts containing a fixed concentration of radiolabeled *vsp1267* RNA. Lane 1: Decade Marker (Ambion). Lanes 2, 3 and 4: Incubation of 750 ng of *vsp9B10* and 250 ng of *vsp1267* (3:1 ratio), 1, 5 and 24 h, respectively. Lane 5, 6 and 7: Incubation of equal amounts of *vsp9B10* and *vsp1267* (250 ng each; 1:1 ratio), 1, 5 and 24 h, respectively. Lanes 8 and 9: Incubation of 750 ng of *cwp1* and 250 ng of *vsp1267*, 5 and 24 h, respectively. Lane 10: Incubation of 250 ng of *vsp1267* transcript. At short incubation times (1 h), little degradation of *vsp1267* takes place with clone WB1267 extracts regardless of the amount of *vsp9B10* added to the mixture (lanes 2 and 5). In contrast, after longer incubations (5 h) the appearance of radiolabeled *vsp1267* siRNAs augments by the presence of *vsp9B10* (compare lane 10 to 3, 6, and 8). The presence of the unrelated transcript c*wp1* has no effect, even at high concentration (lane 8). At 24 h of incubation, most of the radiolabeled transcript is completely degraded. These results suggest that a *Giardia* extract is programmed to keep unprocessed a particular *vsp* transcript, but it can start degradation of that RNA when a different *vsp* is in higher concentration (processing of a given transcript may depend on the cytoplasmic relative concentration of each *vsp*).



Supplementary Figure 9 | Knock-down of gDicer and gRdRP in *Giardia* trophozoites. Selected *Giardia* clones of Dicer-AS and RdRP-AS present a reduction of the steady-state RNA levels between 65% and 75% compared to untransfected WB9B10 trophozoites as measured by densitometry of bands obtained by RT-PCR and Northern blotting assays performed in quintuplicate. Results represent the mean value ± s.d. qRT-PCR showed almost identical results (not shown).



Supplementary Figure 10 | Monoclonal antibody that recognizes the VSP conserved five-amino acid tail. Immunofluorescence assay showing that: **a**, mAb 12F1 clone G3 (generated to the CRGKA domain of VSPs) reacts with all trophozoites in an uncloned population. **b**, mAb 9B10 (specific for VSP9B10) only labels the surface of a percentage of the cells in the same population.

Supplementary Table

Supplementary Table 1 | Small RNAs generated *in vitro* by gDicer activity on VSP transcript mixtures are 22-25-nt long. The products of VSP processing as shown in Figure 2, which have been generated by Dicer activity of *Giardia* extracts on mixtures of riboprobes for VSP9B10, VSP1267 and VSPH7 were further characterized by analyzing the capability of these molecules to be ligated to adaptors for subsequent PCR amplification, cloning, and sequencing. The results showed that (a) those small RNAs are able to be ligated and cloned similar to siRNAs that have 5'-P and 3'-OH (except when they were pretreated with alkaline phosphatase or subjected to periodate oxidation followed by β-elimination, respectively), (b) they are 22-25 nt-long, and (c) they codify for sense and antisense VSP fragments.

VSP9B10 sense small RNAs	VSP9B10 antisense small RNAs
GTTTTGTTCTCGCGGGGGTACTCGT	ACCCCCGCGAGAACAAAACTGCC
AGAGCGCGCGGCTCAATGCGCAG	TCCTGCCCATGCAATCTGGACGA
GATTGCATGGGCAGGAAAAGCAA	ATTGAGCCGCGCGCTCTGTTGCTTT
TCTCGATGTAACACAGGATTTGT	ATGCTTCCTCTGCGCAATTAGTGT
GGACAATGTGCAGACNNAGAAGG	CAATACAATTTACCACCGATCAG
AAAGATGGCTCCGGAGGCGATACA	TGCACATTGTCCATTGATAGGAA
CAGACCTGTGGACAGTGCGCCGAG	CTGATCAGCTGTATCGCCTCCGG
CTTTCATGTACAAGGGCGGCTGT	GTAACAGCCGCCCTTGTACATGA
CGAAGCANCCCAGCAGCCCGGACAG	CATCTGCCGCCNGACANNTGGTC
GCAAGGATACTTCGTGCCGCCGG	CCCGGCGGCACGAAGTATCCTTG
ACCAATCGGTCATACCATGCGGAG	ACCGATTGGTGAGAGGCGTCTGC
ACGATAAAAAGTACAAGGGCGTGCT	ACTTTTTATCGTTCTTAACTGTTA
ACCGGCACCAAGACGTGCAAGAC	TGTGGGAGCGTAACACCGAGTGC
TGCGACGTGCGAGAAGGGCGCCGA	CACGCAGTACACGTGGCGGCCTT
GCCCGACCCNNAGTGCAACACCCCC	CACGCAGGAGGTGGCTGAGTCCT
GCTGCAAGACGTGCAGTGAGCCGA	GTGCCGGTGCACTCTTCTTCTGT
AGACAAGCAAGGAGGTGTGCNCA	TTGTTTGGCTGGACCTTCTTATT
ACGGTTGTGAGCACCTGGAAGGC	GCACTCACCGTCTCCGGGCACTTT
CCTGTGCCAAGTGCAATACCTCG	GCTTCTTCGTGCACCCGGTGCCC
AGCTACGAAGGAGAGGGCACGGGG	CTGGTGCCCTCGTAGTNNCCTNCC
TCGGCCCGCACAGCCTCCTGCCAG	TGAGGACCTGCTTAGGCTCGCAG
ACGAAACGACCAANCTCCCTGGAA	CTTCTTGACNCACACGCCGTTCTC
TGAATAATGGCGCGCTCATCACTTG	TAGCAGCCCCCGTTCATGCGGAA
GATGTAAGACGTGCACCAGCCAG	ACCTCCTCACAGACGCTCTTTCCA
TACTACCTGTCCAAAGAAAAGTG	GCTTGTATCCGTCGGCCGGAGTC
CCCCCAACCAACAATAAAGGGCC	CACTCGGAGCACCCAGTGGCGCA
ACCTCATACAGAACANNAACAGG	CACTTGGTGGCGTCGTCCGCATTG
GGGATCTCCGTCGCTGTCATCGC	ACAGCGNCGGAGATCCCCGCTATGG
TGCTGGTGGTTCATATGTAGNGG	AGAGGAAGCCCACGAGGCCCCC

VSP1267 sense small RNAs	VSP1267 antisense small RNAs
GCAAGCACTCTTGCAGGAGCTT	GGCAATTAATTAATAGAAACAT
GCTCTACGACTCAGGCTAATTGT	GCTATTAGGCAATTAATTAATAG
CAACGGGGTGTGTGAAGCAGCCGC	TTCCGCAACACAATTAGCCTGAG
GGCTGCTAATGGTAGTGATAACG	TGCACTTTGTATTACTACTGGCG
GTAAGAAGTGCCTTCTGCAAACC	GCACTTCTTACAAGTCTGATCAG
CAAACCTTCATGTTCAAGGGCGG	TTAAATTACCAGTNNCTCCCGCT
TGATGCTGCCTCTGGTACTACTGG	ATTACTCTCACCAATCGTGACCCC
GCGGCTGATACCACGGATTCCTGT	GCGTNATCATTAGGGAAATATCC
CAACTGGGGTCACGATTGGTGAG	TTAAGGGNNCTCAGGCTATTCGTG
ACTAATTGCGTTNNGTGTACCAAA	TCAGGACAGACCCNGGGTAGCAG
GAGTGTGCTTCCAATCTGTATCTG	CGTCGGCGCAGTTTTCCAAATAC
GAAACTTGCAAGACAGGATATTTCC	AGTGCAAACTCCGTTGGTTTTATCC
TTTCCNTAATGATAACGCTGATA	GCATCGGCTGTCGTGCACAACGT

CCGGTGCTATTCTTATCACCTGCA	GCCGCTGGTCTNGACGTAGCAGG
GCAAGGACGACAACACTGCGGCC	
GTATCGCAGAGTGCACGGGAANG	
GGCAGTGCACAGCTAGCATAGCAG	
CCTTTGCGTGTCGGCCGAAACAG	
TAAAACCAACGGAGTTTGCACTGCC	
CTATCAGGCTGAGAAGTTTCCTG	
GCAGGAAAGTGCACGACCTGTGCG	

VSPH7 sense small RNAs	**VSPH7 antisense small RNAs**
GGAAACCTTGGTAGGATTATTTGC	AGTCGTAGAGCAAGCTCCTGCAAG
AATGCTAATCTGTACCTGAAGGCT	ACCCCGTTGATGGGCACATAGTTT
GGCATAGATGGGTGCTCTGCATG	CTTCCGTCTTNNAAATAATCCTACC
ACAAAGGGAACATGCATTGCAGA	GAATCGTAACCCCGGTTGCGTCG
ACGCCGGATAANACCAACGGAGTT	ACTTAGCACAACCGGCCACACCC
TGTGCAANNGATAACACTAANA	CTTATCAGCCGTACTACAGGTAAG
CTCGTCGGCTTCCTCTGCTGGTG	ACGGTGCTAGCCCTAGTTGTAGA
	CGTTCTTCAAGGNNCTCAGATTGTT
	CTCTGCAATGCATGTTCCCTTT
	CGACCCGGGTGGTGCCGCTCTTGC
	CCATTGCTGTCTNTATCTTGCCC
	GTGTTATCTTN-GCACACGATGC
	GACGGGAGTAGAACTCTGAGGAGA

Supplementary Full Methods

Cell culture conditions. *Giardia lamblia* trophozoites, strain WB clones 9B10[39], 1267[40] and A6[41], and strain GS, clone H7[42], were used in this study.

PCR. Total DNA from *G. lamblia* trophozoites was isolated as described[43]. *vsp* primers were designed as shown in Supplementary Figure 1: Sense primers S1 (5´-CVT GTG CHR RST GCA A-3´), S2 (5´-TGC ACS RSC TGC YAB CC-3´), S3 (5´-TAG TGY DSY VMV TGY AA-3´) and S4 (5´-CGA TCA TGA CGG GCT TCT-3´). Anti-sense primers R1 (5´-CCB ACG AGG CCY CCS ACG AC-3´) and R2 (5´-CGC CTT CCC KCK RCA KAY GA-3´). PCR conditions were: denaturalization at 94°C for 40 s, annealing at 53°C for 40 s and elongation at 72°C for 90 s, using High Fidelity *Taq* polymerase (Invitrogen) for a total of 35 cycles.

RT-PCR. Sense *vsp* primers (S1-S4) were added to 1 μg of total RNA and heated to 70°C for 5 min. Samples (2 μl) from the reverse transcription reaction were amplified using all possible combinations between sense/anti-sense primers listed above or using *vsp1267* and *vsp9B10* specific primers (1267_F, 5'-ATG TTG TTG ATA GCC TTC TAT C-3'; 1267_R, 5'-CTA CGC CTT CCC CCT GCA TAT G-3'; 9B10_F, 5'-ATG TTT GGC AGT TTT GTT CTC-3'; 9B10_R, 5'-TCA CGC CTT CCC TCT ACA TAT G-3'). RT-PCR products were analyzed by electrophoresis and purified using the Qiaex II Gel Extraction Kit (Qiagen). To study the expression of different Giardia genes during trophozoite differentiation or in knock-down experiments, RT-PCR was using the following specific primer pairs: gDicer (645 bp): HL160, 5'-TGG CGG CGT CGT ATC AGT TAT-3', HL161, 5'-TCC CCG CAC GCA AGA AGA A-3'; gAgo (912 bp): HL164, 5'-ATT GCC CCC TAC GGT GTC-3', HL165, 5'-CTC TGC CGG CCT TCC TAC-3', gRdRP (569 bp): HL187, 5'-CAT GGG TTG CAG TTT CTT GAC GA-3', HL188, 5'-AGC CCC TTA TCT GTT GCC TCC TTC-3'; and the control of differentially expressed CWP1 (533 bp): HL183, 5'-TCG CCC TGG ATG TTT CGG ACA T-3', HL184, 5'-AGG CGG GTG AGG CAG TA-3', and the constitutively expressed GDH (407 bp): HL185, 5'-AGT GGG GCG GGT CTT TAC TCA-5', HL186, 5'-TGT TCG CGC CCA TCT GGT AGT TCT-3'. The products of these reactions were also isolated, labelled, and used as probes for Northern blot, as indicated below.

Northern hybridization. Total RNA (10-15 μg) was fractionated on a 1.2% agarose-formaldehyde gel, transferred to a Hybond N+ (GE) and fixed with a UV Crosslinker (UVP) by standard procedures. The conserved C-terminal end fragment (anti-sense primer R2) was radioactively labelled with T4 polynucleotide kinase using γ-[^{32}P]-ATP according (5'-End Labelling System, Promega). *grdrp,* as well as other DNA fragments, were uniformly labelled by random priming (Prime-A-Gene Labelling System, Promega).

Nuclear run-on analysis. 1 x 10^7-1 x 10^8 cells were resuspended in 1 ml of ice-cold lysis buffer (10 mM Tris-HCl pH 8.4, 1.5 mM $MgCl_2$, 0.14 M NaCl, and Complete™ protease inhibitor cocktail) at 4°C; 2.25 μl of Nonidet P-40 were added and the suspension was incubated for 15 min on ice. Nuclei were recovered by centrifugation at 2,000*g* for 1 min and washed twice in 1 ml of ice-cold nuclei wash buffer (20 mM Tris-HCl pH 8.4, 140 mM KCl, 10 mM $MgCl_2$, 20% (V/V) glycerol and 14mM β-mercaptoethanol) at 4° C. Then, nuclei were resuspended in 50 μl of labeling buffer (20 mM Tris-HCl (pH 8.4 at 4° C), 140 mM KCl, 10 mM $MgCl_2$, 20% (V/V) glycerol and 14 mM β-mercaptoethanol, 1 mM each of ATP, GTP, and CTP, 10 mM phosphocreatine, 100 μg/ml phosphocreatine kinase and 0.1 μM [^{32}P]UTP, 5000 μCi/ml) and incubated

for 40 min at 37°C. VSP products generated by RT-PCR and 3 μg of *vsp9B10*, *vsp1267*, *vspH7*, and *vspA6* cloned into p-GEM T-easy vector (Promega) were transferred onto Hybond N+ using a slot blot apparatus (BioRad). Additionally, sense and antisense transcripts generated in the *in vitro* transcription reaction were also blotted under similar conditions.

Detection of small RNA. The detection of small RNA was performed as reported[35]. Briefly, 15 μg of *G. lamblia* total RNA was denatured for 10 min at 65°C in 1X loading buffer, and loaded on a 15% polyacrylamide/7 M urea gel and electrophoresed. After separation, RNA was electroblotted in 0.5X Tris-borate-EDTA buffer (pH 8) onto a Hybond N+ membrane for 45 min at 100 V in TBE 0.5X, and finally UV fixed. [^{32}P]-labelled riboprobes were transcribed *in vitro* by T7 or SP6 RNA polymerase using VSPs genes 9B10, 1267 and H7 cloned into p-GEM T-easy vector (Promega). Labelled RNA was partially hydrolyzed during 1 h by incubation at 60°C in the presence of 80 mM $NaHCO_3$ and 160 mM Na_2CO_3. Each hydrolyzed VSP transcript was hybridized in 25% formamide, 0.5 NaCl, 25 mM EDTA, 1X Denhardt's solution and 150 μg/ml denatured salmon sperm DNA and incubated at 42°C overnight. After hybridization the membranes were washed twice in 2X SSC, 0.5% SDS for 30 min and once in 0.5X SSC, 0.5% SDS for 15 min at 45°C. Subsequently, each reverse hydrolyzed *vsp* transcript was hybridized in the same way and the membranes signals were detected by exposure to Kodak XAR films al –70° C or a phosphoimager (Amersham). Length standards were from a commercial source (Decade™ RNA Markers, Ambion).

Endonuclease activity. Dicer activity was analyzed by incubating dsRNA molecules with cytoplasmic extracts of *Giardia* clone WB9B10, WBA6, or WB1267. *vsp9B10*, *vsp1267, vspH7*, *cwp2,* and *gdh* genes cloned into p-GEM T-easy vector (Promega) were transcribed *in vitro* to produce full-length sense [^{32}P]-labelled RNA probes, which were purified and tested for the absence of small RNA contaminants. Pure or mixed *vsp* transcripts were incubated for 1 h at 37°C with *Giardia* extracts. dsRNA was produced by annealing equal amounts *in vitro* transcribed sense and antisense RNAs (*vsp1267*, *vsp9B10*, *cwp2* and *gdh*), labelled or not with [^{32}P]UTP. These dsRNAs were resuspended in Tris-HCl (pH 7.5)/20mM NaCl, heated at 95°C for 1 min and cooled down at room temperature for 12 h. Cell lysates were generated from 1 x 10^7-1 x 10^8 cells, which were resuspended in 500 μl of buffer (25 mM Tris-HCl pH 7.5, 250 mM Sucrose, and containing Complete™ protease inhibitor cocktail), sonicated, and centrifuged at 2,000*g* for 15 min to separate unbroken cells and nuclei, and then incubated with dsRNA at 37°C for 1 h. Then, total RNA was extracted, electrophoresed, and transferred as indicated above for small RNA. Selection of low molecular weight RNAs was made by filtration through Microcon-100 filter units. The filtrate, containing small RNAs, was precipitated with 300 mM NaCl/0.6 ml of isopropanol, loaded on a 20% polyacrylamide/7 M urea gel, and electrophoresed.

To determine the effects of ATP on the endonuclease activity, ATP was depleted by incubating *Giardia* cytoplasmic extracts with 2 mM glucose/0.1 U/μl hexokinase (Sigma) for 30 min at 35°C. Then, [^{32}P]UTP-labelled *vsp*1267 dsRNA was added to the lysate in the presence or absence of 10 mM phosphocreatine, 100 μg/ml phosphocreatine kinase, or both, for 1 or 3 h at 37°C. Total RNA was extracted using Trizol and the RNA sample was RNA samples were enriched for low molecular weight RNAs using the Microcon-100 filtration unit as described above. Samples were electrophoresed and products detected as above.

The products of dsRNA processing using *Giardia* extracts were gel purified, ligated, amplified, cloned, and sequenced[36]. To determine the nature of the small RNAs, they were treated with alkaline phosphatase (to demonstrate the presence of 5' phosphates) or subjected to periodate oxidation followed by β-elimination (to confirm the presence of 3' hydroxyls), as described[44].

RdRP cloning, sequencing, and activity. For RT-PCR, cDNA synthesis was performed using total RNA extracted from trophozoites and oligo$(dT)_{20}$ as primers. Alignment of the known RdRP from several organisms, in conjunction with codon usage knowledge in *Giardia*, allowed the design of moderately degenerated primers: RdRP_F: (5'-TA(T/C) GT(T/C) TTT AC(T/C) GAT GGC G(C/G)A GG)-3') and RdRP_R: (5'-TCA CC(A/G) TCC AGG TC(G/A) CTG CC)-3'). The PCR product generated using those oligonucleotides was electrophoresed, gel purified, radiolabeled by random priming, and used to screen a *G. lamblia* cDNA library in λgt22a as reported[44]. λZAP gDNA library screening was performed as described[44]. DNA fragments were cloned into pBlueScript SKII$^+$ and submitted for automatic sequencing.

5'-RACE was performed using a commercial kit from Invitrogen and the primers 5'-CTT GTG CAT AGT AAA CAA AG-3' and 5'-CAA ATG GTC GAT GCT GGG-3'. For gRdRP activity *in vitro*, HA-tagged RdRP was purified from transfected trophozoites by affinity using antiHA-sepharose (Sigma). Enzyme activity was assayed at 35°C for 60 min in 20 µl reaction mixture containing 50 mM Hepes pH 7.6, 20 mM ammonium acetate, 5 mM MgCl2, 0.1% Triton X-100, 1 mM each of four ribonucleoside triphosphate (including [α-^{32}P] UTP), and 1 u/µl RNasin, plus the addition of ssRNA substrates (250 µg/ml) prepared by *in vitro* transcription as described, with or without the presence of VSP specific primers. Reaction products were analyzed by agarose gel electrophoresis followed by transfer and autoradiography**.**

Transfection and Immunofluorescence Assays. The plasmid PTubPac[37] was modified to introduce the entire gRdRP, gDicer, gAgo, and VSPH7 coding regions and, when corresponding, the Influenza Haemaglutinin epitope (HA) before the TAA stop codon[23]. gAgo coding region was introduced into the plasmid **pTubNterPac** in which the gene is introduced after the Haemaglutinin coding region to avoid possible interference of the HA tag with the PIWI domain. This plasmid is a modification of the **pTubHAPac** with the substitution of the original multiple cloning site by a new one (MCSnewSense: 5´GAT TCC GGG CCC AGA TCT ATC GAT ACG CGT ATG CAT TCG CGA GAT ATC TGC 3´ MCSnewAntisense: 5´GCG GCC GCA GAT ATC TCG CGA ATG CAT ACG CGT ATC GAT AGA TCT GGG CCC G 3´). Transfection of *G. lamblia* trophozoites was done by electroporation as previously described[38]. Cells were incubated on ice for 10 min, cultured in growth medium overnight at 37°C and selected as puromycin-resistant cells. Indirect immunofluorescence assays using an anti-HA monoclonal antibody (Sigma) was performed on non-encysting trophozoites fifteen days after transfection as described[23]. Previously reported anti-VSP monoclonal antibodies were used to test the expression on different VSP on fixed trophozoites as described elsewhere[23.45]. Novel monoclonal antibodies generated in this study were used similarly. Confocal images were collected using a Zeiss LSM5 Pascal laser-scanning confocal microscope equipped with an argon/helium/neon laser and a X100 (numerical aperture = 1.4) oil immersion objective (Zeiss Plan-Apochromat). Single confocal sections of 0.3 µm were taken parallel to the coverslip (z sections). Images were acquired using a Zeiss charge-coupled device camera and processed with LSM and Adobe Photoshop software.

For functional analysis of gRdRP, gDicer, and gAgo, and VSP9B10 specific sense primers containing an *Eco RV* site and antisense primers with an *Nco I* site were used to amplify by PCR the a portion of the ORF of each gene. PCR products were purified, restricted, and cloned into the vector **pTubHAPac**[37]. In this manner, genes were inversely inserted inside **pTubHAPac**, giving the antisense construct that was then used for inhibition of expression[23]. Sequences were always confirmed by dye terminator cycle sequencing. Gene knock-downs were confirmed by RT-PCR and qRT-PCR using the gene specific primers indicated above on total RNA extracted from transfected trophozoites and compared to control of cells transfected with vector only or with the same vector expressing an HA-tagged version of each molecule.

Production of monoclonal antibodies to the 5-amino acid tail of VSPs and to individual VSPs. Six-week-old female BALB/c mice were immunized subcutaneously with either 200 µg of (a) an HPLC-purified preparation of NH2-CRGKA-COOH peptide conjugated to KLH using the sMBS cross-linker, (b) the synthetic multiple antigen peptide [NH2-CRGKA]8-[K]7-bAla-OH (both from Biosynthesis, Inc.), or protein extract of cultured trophozoites derived from the WB isolate, emulsified in Sigma adjuvant system (Sigma). Mice were boosted subcutaneously after 21 days with 200 µg of the same preparations, and 20 days later boosted intravenously with 100 µg of the antigen preparations. Three days later, the mice were euthanized and the spleen cells used for fusion to NSO myeloma cells. Hybridomas secreting antibodies were screened by ELISA using the original peptides and by indirect immunofluorescence using non-encysting and encysting trophozoites[31,41]. Monoclonal antibodies against VSP were generated using entire trophozoites as previously reported[43].

DNA methylation. DNA from *G. lamblia* clone 1267 was purified by extraction with phenol and chloroform/isoamyl alcohol (24/1 % v/v), incubated with ARNase (Roche) to eliminated ARN contamination, and precipitated with ethanol. The presence of methyl deoxyribonucleosides was determined by high-performance liquid chromatography[45,46]. The separation of deoxyribonucleosides was performed on a Phenomenex Luna 5 µm C18, 4.6 x 150 mm. The method was calibrated based on the absorption of standard deoxyribonucleosides of known concentration.

Methods References

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