Molecular Characterization of the Schistosoma mansoni Zinc Finger Protein SmZF1 as a Transcription Factor

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Introduction

Schistosomiasis is a disease caused by trematode worms, mainly Schistosoma mansoni, S. haematobium and S. japonicum. According to World Health Organization, this parasitic disease affects 200 million people throughout the world [1]. Although the level of schistosome-associated morbidity is unclear, some recent studies have demonstrated that the illness is a more serious problem than it was previously thought to be [2,3]. Therefore, emphasis should be focused on mechanisms that could not only prevent, but also cure schistosomiasis. A useful approach to fight the disease should include infrastructure and educational components, as well as the development of vaccines and new drugs [4]. Luckily we are living a special moment, with the recent publication of both S. mansoni [5] and S. japonicum [6] genomes, which will bring to the scientific community an enormous amount of data to be mined in the search for new therapeutic targets and vaccine development. Lastly, additional effort should also be dedicated to studies regarding the biology and development of the parasite.

During its life cycle, S. mansoni is exposed to different environmental conditions: water, intermediate molluscan host, and a definitive vertebrate host. As a consequence, this parasite...
S. mansoni adult worms in this study were recovered from infected mice. Lung-stage schistosoma were prepared according to Harrop and Wilson [37]. Cercariae were obtained from Biomphalaria glabrata by exposing the infected snails to light for 2 h to induce shedding of parasites.

Sections of Omnifix (AnCon Genetics Inc., Melville, NY, USA) fixed, paraaffin-embedded adult male or female worms were deparaffinized using xylol, hydrated with an ethanol series, fixed, paraffin-embedded adult male or female worms were used the same solution and condition. Samples were incubated in PBS (Phosphate Buffered Saline – 130 mM NaCl, 2 mM KCl, 8 mM Na2HPO4, 1 mM KH2PO4), then emulsified with Complete Freund Adjuvant and used for the primary intramuscular injection into a rabbit or with Incomplete Freund Adjuvant for the two subsequent boosts (15 and 30 days after the first immunization). Pre-immune serum was obtained before the first immunization and rabbit serum containing anti-SmZF1 antibodies was collected 15 days after the third immunization.

S. mansoni immunohistochemistry assays

S. mansoni adult worms used in this study were recovered from perfused mice. Lung-stage schistosoma were prepared according to Harrop and Wilson [37]. Cercariae were obtained from Biomphalaria glabrata by exposing the infected snails to light for 2 h to induce shedding of parasites.

Sections of Omnifix (AnCon Genetics Inc., Melville, NY, USA) fixed, paraaffin-embedded adult male or female worms were deparaffinized using xylol, hydrated with an ethanol series, washed in PBS and then incubated in a blocking solution (0.05% Tween 20, 1% w/v BSA (Bovine Serum Albumin) in PBS pH 7.2) overnight at 4°C. Samples were reacted for 1 h with either the anti-SmZF1 or a control, pre-immune rabbit serum, both diluted 1:30 in 10x diluted blocking solution. Sections were then washed in PBS and reacted for 1 h with a 1:400 diluted goat anti-rabbit IgG-Cy-5 conjugate (Jackson Immunoresearch Labs Inc., West Grove, PA, USA) in 10x diluted blocking solution, which also contained Alexa Fluor 488 phalloidin (Invitrogen, Carlsbad, CA, USA) diluted 1:100 to stain actin filaments, and Alexa Fluor 594 Phalloidin (Invitrogen, Carlsbad, CA, USA) diluted 1:100 to stain actin filaments. Sections were then washed, incubated for 10 min with 1:3000 diluted propidium iodide (Sigma-Aldrich, St. Louis, MO, USA) diluted 1:100 to stain actin filaments, and Alexa Fluor 594 Phalloidin (Invitrogen, Carlsbad, CA, USA) diluted 1:100 to stain actin filaments. Sections were then washed, incubated for 10 min with 1:3000 diluted propidium iodide (Sigma-Aldrich, St. Louis, MO, USA) in 10x diluted blocking solution to stain nuclei and then washed with PBS.

For experiments using cercariae and lung-stage schistosoma, a whole-mount protocol was chosen. Omnifix fixed cercariae were treated with a permeabilizing solution (0.1% Triton X-100, 1% w/v BSA and 0.1% w/v sodium azide in PBS pH 7.4) for 3 h at 4°C under constant agitation. Subsequent immunostaining steps used the same solution and condition. Samples were incubated overnight with the anti-SmZF1 antibody diluted 1:50, washed
several times and reacted for 4 h with the goat anti-rabbit IgG-Cy-5 conjugate diluted 1:1200 in solution containing Alexa Fluor 488 phallodin (1:500). The cercariae were then incubated for 20 min with propidium iodide diluted 1:6000 and washed once more. The schistosomulum immunohistochemistry assays were carried out with cercariae, with the following modifications: lung stage schistosomula were treated with permeabilizing solution overnight and then incubated with the anti-SmZF1 antibody (1:90) for 2 h. The secondary antibody was used at a 1:1000 dilution, and the phallodin at a 1:100 dilution for 2 h.

Samples (adult male and female worms, schistosumula and cercariae) were prepared with a mounting solution (90% glycerol, 10% tris-HCl 1 M, pH 8.0) and the fluorescence images were captured with a Carl Zeiss LSM 510 META confocal microscope using a 63x oil-immersion objective lens in the Center of Electron Microscopy (CEMEL-ICB/UFMG). Images were analyzed with Zeiss LSM Image Browser software and edited with Adobe Photoshop CS.

All research protocols involving mice in the course of this study were reviewed and approved by the local Ethics Committee on Animal Care at Universidade Federal de Minas Gerais (CETEA – UFMG N° 023/05).

RNA extraction and Real-Time PCR
Adult worms recovered from perfused mice were manually separated and pooled according to their sex. Total RNA of both male and female worms was extracted using Trizol reagent (Invitrogen) and treated with DNase using ILLstra RNApin Mini RNA Isolation Kit (GE Healthcare, Waukesha, WI, USA) according to the manufacturer’s instructions. RNA was then quantified using a NanoDrop Spectrophotometer ND-1000 (Thermo Scientific, Waltham, MA, USA). cDNA was synthesized using 0.3 to 1.0 mg total RNA and SuperScript III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen) according to the manufacturer’s protocol. For q-PCR reactions, the primers SmZF1_real2_forw 5’-ACCTCTCTACAAATCCAGCCT-3’ and SmZF1_real2_rev 5’-TGGAGGAGATTTTTACATCTGGTGT-3’ were used at a 900 nM initial concentration.

The Transcription Factor SmZF1
Plasmid constructs and COS-7 cells culture
The SmZF1 cDNA was PCR amplified in a reaction mixture prepared in a 50 mL final volume containing 25 ng of template DNA, 0.2 pmol/μL of each primer (SmZF1-5’-start-Xba: 5’-CAGTCCTAGAATCTAATGGAAAT-3’ and SmZF1-3’-stop-Apa: 5’-CAGGGGCCCCTCAGGGAAAGGTTGAGA-3’), or SmZF1-5’-start-Sac: 5’-CAGGAGCTCCTTTAATCTGGAAAT-3’ and SmZF1-3’-stop-Hind: 5’-CAGAAGCTTTACATCGGGAAGCGTGAG-3’), 200 mM dNTPs, and 5 U of Taq DNA polymerase (Phoneutria, Belo Horizonte, MG, Brazil) in the appropriate buffer (50 mM KCl, 10 mM Tris-HCl pH 8.0, 0.1% Triton X-100, 1.5 mM MgCl2). The fragments obtained were double-digested with XbaI and ApaI or SacI and HindIII restriction enzymes (New England Biolabs) and purified using a Wizard SV Gel and PCR Clean-up System (Promega, Madison, WI, USA) following the manufacturer’s instructions. The fragments were then inserted, respectively, into the commercial vectors pcDNA4/TO/myc-His (Invitrogen) or pEYFP-c1 (Clontech, Mountain View, CA, USA), generating the constructs pCDNA4-SmZF1 and pEYFP-SmZF1, which express the recombinant proteins SmZF1-myc tag and YFP-SmZF1, respectively. In addition, the viral thymidine kinase (tk) promoter region was inserted (Neo/Neo) into the commercial vector pGL3-basic (Promega), generating the vector pGL3-tk-luc, with the luciferase (lac) reporter gene under control of the thymidine kinase promoter. Subsequently, an oligonucleotide containing four repetitions of the putative SmZF1 DNA binding site, D1-3DNA [36], was inserted (Neo/Neo) upstream of the minimal tk promoter, producing the vector pGL3-tk-luc. The oligonucleotide sequence was as follows: 5’-CAAGAAACAGCTATGACCGGAGGGAGTACGCCAGAGGATGTCCGGAGGGAGTTCGTGACTGGGAAAACCCCTGGCGG-3’ (specific binding sites D1-3DNA are indicated in bold).

The above plasmid constructs were used either to transfect or co-transfect COS-7 cells using Lipofectamine TM 2000 Transfection Reagent (Invitrogen), according to the manufacturer’s protocol. COS-7 cells were maintained at 37°C, 5% CO2 in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum and 1% glutamine (Invitrogen).

COS-7 cells fluorescence microscopy and Western blot assays
The plasmids pEYFP-c1 (control) or pEYFP-SmZF1 were transfected (as above) into COS-7 cells for transient protein expression studies. Forty-eight hours after transfection the culture medium was carefully removed and cells were fixed (15 min) with 3% paraformaldehyde in PBS, washed and then quenched using PBS plus 10 mM NH4Cl (10 min). Cells were washed three times with PBS and incubated for 7 min with 0.1% Triton X-100. After another wash in PBS, COS-7 cells nuclei were stained (4 min) with 5 μL of 1 mM Hoechst 33342 dye (Sigma-Aldrich). The fluorescence was directly observed using a confocal microscope.
For Western blot assays, COS-7 cells (0.5 x 10^6) transfected either with pCDNA4-SmZF1 or pEYFP-SmZF1 and control cells transfected either with pEYFP or pCDNA were washed and resuspended in 200 µL of cold TNE (150 mM NaCl, 50 mM Tris-HCl pH 7.5 and 1 mM ethylenediaminetetraacetic acid (EDTA)). A 50 µL aliquot of cells was centrifuged (700 g, 4 min, 4°C) and the pellet resuspended in 500 µL of 2x SDS gel-loading buffer (100 mM Tris-HCl pH 6.8, 200 mM dithiothreitol, 4% SDS, 0.2% bromophenol blue, 20% glycerol) and boiled for 5 min, generating the total extract. The remaining 150 µL of cells was centrifuged (700 g, 4 min, 4°C) and the pellet resuspended in 40 µL of lysis buffer (10 mM Tris-HCl pH 7.5, 10 mM NaCl, 2 mM MgCl₂, 1 mM phenylmethysulphonylfluoride (PMSF), one dissolved tablet of Complete Protease Inhibitor Cocktail (Roche, Basel, Switzerland), 1 mM Na₃VO₄ and 1 mM NaF) plus 100 µL of 1% Nonidet P-40 (Sigma-Aldrich) in 50 mM Tris-HCl pH 7.5. Samples were incubated in an ice bath for 10 min and centrifuged (700 g, 4 min, 4°C). Ninety-five microliters of 2x SDS gel-loading buffer was added to the supernatant, which was boiled for 5 min, generating the cytoplasmic fraction. The pellet was washed twice with cold TNE, centrifuged (700 g, 4 min, 4°C), resuspended in 50 µL of 2x SDS gel-loading buffer and boiled for 15 min, generating the nuclear fraction. COS-7 total, cytoplasmic and nuclear extracts, normalized at equal volume percentage, were separated using 10% SDS-PAGE and blotted (2 h, 20 mA) onto nitrocellulose membranes (Whatman GmbH, Dassel, Germany) using a semi-dry blot system (GE healthcare). Antibody reactions were performed as described by Koritschoner and colleagues [39]. Briefly, membranes were blocked overnight in TBS (25 mM Tris-HCl pH 7.4, 137 mM NaCl, 5 mM KCl, 0.6 mM Na₂HPO₄, 0.7 mM CaCl₂, 0.5 mM MgCl₂) plus 1% BSA in this stage using this technique (Figures 1F–I and Supporting Information, Figure S2). No SmZF1 staining was observed in the negative controls (Figures 1E, J, O, T) in which only the rabbit pre-immune serum was used. These results suggest that SmZF1 is a nuclear protein present in the nuclei of cells from diverse developmental stages where it may act as a transcription factor.

Electrophoretic mobility shift assay

For the electrophoretic mobility shift assay (EMSA), 20 pmol of the D1-3DNA oligonucleotide (5'-CGAGGGGAGT-3') was incubated with 1 µg of the total extract of COS-7 cells transfected with plasmids pEYFP-c1 (control) or pEYFP-SmZF1. Extracts were produced as follows: cells (0.5 x 10^6) were washed in PBS and resuspended in 100 µL of TDBG solution (20 mM Tris-HCl pH 7.5, 2 mM dithiothreitol, 400 mM KCl, 5 µg/ml leupeptin, 5 µg/ml aprotinin, 20 µg/ml EDTA, 1 mM Na₃VO₄, 0.05% Tween-20 and 3% BSA) followed by two washes with 100 mM Tris-HCl pH 8.0, 200 mM NaCl, 0.2% Tween-20 (wash buffer). Samples were reacted with anti-myc, anti-GFP or anti-c-erbB-2 (1:1000) peroxidase conjugated antibodies (BD Biosciences, Franklin Lakes, NJ, USA) in blocking buffer for 1 h. Subsequently, blots were washed and developed with ECL enhanced chemiluminescence reagents (GE Healthcare) and exposed to X-ray film. The exclusively cytoplasmic protein c-erbB-2 was used as a quality control for extracts.

Transient co-transfections and luciferase activity assays

Plasmid DNA co-transfections of COS-7 cells were carried out in 24-well plates (Corning Inc., Corning, NY, USA). The day before transfection, 8 x 10^4 COS-7 cells were plated in 0.5 mL of medium/well. For each well, 2 µL of Lipofectamine™ 2000 Transfection Reagent was mixed with 1.2 µg of the plasmid DNA of interest and 300 ng of TK-Renilla reporter plasmid in serum-free Opti-MEM (Invitrogen) to allow the formation of DNA-Lipofectamine™ 2000 Transfection Reagent complexes. The complexes were added to the respective wells and mixed by gently rocking the plate back and forth. Cells were incubated in a 5% CO₂ incubator at 37°C for 48 h and then lysed with 60 µL of reporter lysis buffer (Promega). Luciferase activity (Relative Light Units – RLU) was assayed with 20 µL of lysate and 80 µL of luciferase assay reagent (Promega) in a TD20/20 luminometer (Promega) using a 10 s measurement period. Each transfection was performed in triplicate. Transfection efficiency was normalized to TK-Renilla luciferase reporter plasmid. Statistical analysis of the data was carried out with Minitab Version 1.4 using Student’s t test with Welch’s correction. Only p values<0.05 were considered as significant.

Results

SmZF1 has a nuclear localization at diverse *S. mansoni* developmental stages

SmZF1 (GenBank accession AF316828) was initially identified during a screen of an adult worm *S. mansoni* cDNA library [35]. Although it has also been detected in cDNA libraries of other developmental stages of this parasite (i.e., egg, 3 h schistosomulum and cercaria), the biological function of the protein coded by this gene remains to be elucidated. The SmZF1 protein contains three C₂H₂-type zinc finger motifs and binds specific DNA oligonucleotides, as do similar nuclear proteins involved in gene transcriptional regulation [35,36]. Therefore, to investigate whether SmZF1 is present in the nucleus, where it could act as a transcription factor, we decided to verify its subcellular localization at diverse *S. mansoni* life stages.

We carried out *in situ* immunohistochemistry experiments using an anti-SmZF1 antibody on *S. mansoni* collected at various stages during its life cycle. Western blot assays using the recombinant SmZF1 protein previously separated from its MBP portion, as well as fractionated extracts form adult worms revealed that this polyclonal antibody is specific to SmZF1 (Supporting information, Figure S1). The immunohistochemistry assays showed that SmZF1 protein localizes in the cells nuclei of adult male worms (Figures 1A-D), cercariae (Figures 1K-N) and lung stage schistosomula (Figures 1P-S). Although we have performed three different experiments in which we analyzed various paraffin sections of female adult worms, the protein could not be detected in this stage using this technique (Figures 1F-I and Supporting information, Figure S2). No SmZF1 staining was observed in the negative controls (Figures 1E, J, O, T) in which only the rabbit pre-immune serum was used. These results suggest that SmZF1 is a *S. mansoni* protein present in the nuclei of cells from diverse developmental stages where it may act as a transcription factor. Plus, SmZF1 expression might be sex-specific since it could not be detected in adult female worms.
The SmZF1 mRNA is equally expressed in adult male and female worms

We were unable to confirm the results from the immunohistochemistry experiments showing differences in expression of SmZF1 between male and female by Western blot, since nuclear protein extraction from single sex pooled S. mansoni worms did not provide sufficient material necessary for SmZF1 detection. Therefore, we decided to verify gene expression by comparing the transcript levels between adult male and female worms. Total RNA extraction was performed in separate pools of male or female worms and q-PCR analyses were carried out using primers specifically designed for SmZF1 amplification. We detected no difference in SmZF1 expression (p = 0.22) between male and female worms when comparing the amplification profile, indicating that the SmZF1 mRNA is equally present in both genders (data not shown). These results suggest that although the SmZF1 gene is transcribed in female worms, a post-transcriptional regulatory mechanism could be occurring to block SmZF1 protein production in adult female worms.

SmZF1 goes to the nucleus when expressed in mammalian cells

After demonstrating the nuclear localization of SmZF1 in S. mansoni cells, the next step in the protein characterization was to heterologously express it in a mammalian system to test its ability to activate the transcription of a reporter gene. To accomplish this, we initially transfected COS-7 cells with the pEYFP-SmZF1 construction and forty-eight hours after transfection, we verified the presence of the YFP-SmZF1 recombinant protein mainly in the cells nuclei using fluorescence microscopy. However, a low level of fluorescent staining remained in the cytoplasm. In some cases, the protein was also visualized as fibrous material in the perinuclear region, probably associated with the cytoskeleton or Golgi complex. The YFP protein (negative control) was visualized diffusely distributed throughout the cells area (Figure 2A).

Since part of the fusion protein still remained in the cytoplasm of the cells, a second construction lacking YFP (SmZF1-myc tag) was used to confirm the SmZF1 nuclear localization in mammalian cells. Western blot assays using equal amounts of total, cytoplasmic

![Figure 1. Native SmZF1 displays a nuclear localization at diverse stages of S. mansoni development.](image-url)
and nuclear extracts of COS-7 cells expressing the proteins YFP, YFP-SmZF1 or SmZF1-myc tag were performed. Fractions were analyzed using either anti-GFP (which also recognizes YFP) or anti-myc antibodies (Figure 2B). The results corroborated those obtained by fluorescence microscopy (Figure 2A), showing that YFP-SmZF1 is present in both nuclear and cytoplasmic extracts, with a slight enrichment of the protein in the nuclear extract (Figure 2B). However, the recombinant protein SmZF1-myc tag is only present in the nuclear COS-7 extract, suggesting that YFP may be interfering in the transport of the fusion protein to the nucleus. The quality of the fractionation was confirmed by the localization of the cytoplasmic protein c-erbB-2 in the total and cytoplasmic fractions only (Figure 2B).

**YFP- SmZF1 is able to bind specific DNA oligonucleotides and activate transcription in COS-7 cells**

In previous experiments using purified recombinant SmZF1 protein expressed in bacteria, we demonstrated the nucleic acid binding ability and specificity of SmZF1, its preference for DNA as compared to RNA, and its putative best DNA binding sequence (D1-3DNA) [36].

To verify whether the recombinant protein YFP-SmZF1 expressed in mammalian COS-7 cells was able to interact with D1-3DNA binding site in a manner comparable to its recombinant prokaryotic counterpart, EMSA assays were performed. Total extracts of COS-7 cells transfected with either pEYFP-c1 or pEYFP-SmZF1, expressing YFP or YFP-SmZF1, respectively, were incubated with the D1-3DNA oligonucleotide. To confirm the SmZF1/D1-3DNA interaction, supershift assays using anti-GFP and anti-SmZF1 antibodies were also performed. Extracts of cells expressing the YFP-SmZF1 recombinant protein were able to shift the oligonucleotide migration in the gel (Figure 3, lane 5). Additionally, both anti-GFP and anti-SmZF1 antibodies were able to supershift D1-3DNA migration, confirming that the YFP-SmZF1 protein was responsible for the oligonucleotide binding (Figure 3, lanes 6 and 7). Extracts of cells expressing only the YFP protein (Figure 3, lanes 2–4), as well as anti-GFP and anti-SmZF1 antibodies (Figure 3, lanes 8 and 9), were not able to shift the D1-3DNA migration.

Although new vectors which will allow transfection of schistosome cells are under development [40–42], it is still not possible to continuously cultivate schistosome cell lineages in vitro. Accordingly, some authors describe the use of mammalian cells to study aspects of *S. mansoni* gene regulation processes, such as testing transcription factor activities or mapping promoter regions of genes [28,43,44]. Thus, a luciferase system assay in COS-7 cells...
mammalian cells expressing YFP-SmZF1 fusion protein was used here to test SmZF1 ability to regulate gene transcription. COS-7 cells co-transfected with the expression vector pEYFP-SmZF1 and the construction pGL3-zf-tk-luc, which contains four repetitions of the SmZF1 D1-3DNA binding site and a thymidine kinase minimal promoter upstream of the luciferase coding gene, were able to increase gene transcription by 2-fold (p < 0.003) when compared to negative controls, using the Student’s t test (Figure 4). These results suggested that SmZF1 positively affects the transcriptional activity of the minimal thymidine kinase promoter in COS-7 cells.

Discussion

Schistosomiasis is one of 13 neglected tropical diseases that together affect 1 billion people worldwide. The disease is considered the second most socioeconomically devastating parasitic disease, the first being malaria [45]. According to Chirac and Torreele, in the past 30 years the number of drugs which target these neglected diseases is about 1% of all the new chemical entities commercialized by the pharmaceutical industry [46].

*S. mansoni* presents a variety of interesting biological regulatory processes, such as transcriptional control, which can be used to allow its adaptation to the diverse biotic and abiotic environments [8]. Description of genes expressed in a stage- or sex-specific manner may help to elucidate the events used by the parasite to deal with these potentially adverse conditions. In turn, this information may also help to develop suitable vaccines and chemotherapeutic drugs against this organism [7]. As stated in the recent and high quality review on schistosome genomics by Han and colleagues [47], some potential drug targets should include proteins involved in DNA replication, transcription and repair systems. This suggestion is also corroborated by a chemogenomics screening approach described as part of the up-to-date *S. mansoni* genomic analysis, in which the authors used a strategy to find significant matches between parasite proteins and proteins known to be targets for drugs in humans and human pathogens. That study revealed 26 putative *S. mansoni* protein targets and their potential drugs. Of these 26 targets, three proteins are involved in DNA metabolism and two others are involved in chromatin modification (histone deacetylase 1 and 3) [5]. These two examples emphasize the importance of nuclear proteins as potential drug targets.

According to the authors of the *S. mansoni* transcriptome project [48], 2.4% of the categorized ESTs (Expressed Sequence Tags) under the Molecular Function in Gene Ontology (GO) encode transcriptional regulators. A search for conserved domains using the Pfam database in a subset of those transcripts showed that 5%
of them consist of zinc fingers of the C2H2 group [48]. Moreover, most of the 15 Pfam domains found were from proteins involved in either intercellular communication or transcriptional regulation. These findings reinforce the importance of this class of regulatory proteins for *S. mansoni* biology. In addition, using the SAGE approach, Ojopi and colleagues found that 9.7% of the most abundant genes (genes containing more than 500 tags) from *S. mansoni* adult worms comprise those from the nucleic acid binding GO functional category [49].

The present study defines the SmZF1 protein as a *S. mansoni* transcription factor. SmZF1 is a C2H2 zinc finger protein able to specifically bind to RNA and DNA, but with higher affinity for DNA molecules. Its transcript was identified in the cercaria, egg, schistosomulum and adult worm stages, suggesting its importance as a regulatory protein [35,36].

To define SmZF1 activity as a transcription factor, we first verified its subcellular localization, since this class of proteins is preferentially located or able to go to the cell nucleus, this import being a central step to regulate gene transcription [50,51]. *In silico* analyses of the SmZF1 amino acid sequence did not predict any classical potential nuclear localization signal (NLS), but did reveal positively charged amino acids within the zinc finger motifs [35]. It has been demonstrated that zinc finger motifs are sufficient and sometimes essential for nuclear localization of ZF proteins, even without any canonical NLS detected in their amino acid sequences [51,52]. Moreover, it is well known that small proteins (<40 Kda), like SmZF1, are sometimes able to passively diffuse into the nucleus [50].

Immunohistochemical analysis of the diverse parasite developmental stages demonstrated that SmZF1 was indeed localized in the nucleus of *S. mansoni* cercariae, schistosomula and adult male worms. This confirms previous results obtained by SmZF1 cDNA amplification [35] and reinforces our hypothesis that the protein is a transcription factor. An unexpected result was the lack of detection of SmZF1 protein in adult female worms when assayed by this technique. This differs from available transcriptome data, given the existence of one EST sequence (GenBank accession number BF936884) derived from an adult female worm cDNA library presenting 99% identity with SmZF1. Also, studies using oligonucleotide microarrays in which the SmZF1 sequence was spotted on the slide did not reveal this transcript as being differentially expressed between adult male and female worms [16,20]. Based on these observations, we performed q-PCR experiments to analyze the SmZF1 mRNA expression. We were not able to detect differences in the levels of SmZF1 transcripts between adult male and female worms, indicating that the SmZF1 gene is being equally transcribed in adult female as it is in adult male worms. The fact that SmZF1 protein was not detected in adult female worms by immunofluorescence experiments suggests that a post-transcriptional mechanism regulates the gene. It is important to note that, apparently, SmZF1 mRNA levels are low in all parasite life cycle stages, as demonstrated by the number of ESTs matching SmZF1 cDNA present at dbEST (Table S1). Since the SmZF1 protein is highly abundant at the various stages, as verified by immunohistochemistry assays (except for the femaleadult worm), it can be hypothesized that the protein has a long half life and that the few existing mRNAs may possess a high translational rate. However, this picture might be different for female adult worms, in which the transcript could be less translated or translated in a non-efficient way. As a second hypothesis, the protein in females may present a higher turnover. Future experiments need to be done in order to clarify these points. In a recent study concerning *S. japonicum*, Liu and colleagues analyzed data obtained using either transcriptome or proteome approaches and found several genes with no direct correlation in their expression when comparing these two techniques [53]. The authors explained this fact by limitations in sensitivity of the proteomic technologies they employed, but also highlighted that some transcripts may be relatively stable, persisting throughout several stages and being translated in a shorter window. This could contribute to the discrepancy between the proteomic and transcriptomic data [53].

According to Hokke and colleagues, investigating proteins differentially associated with each sex could reveal important clues concerning the formation of sexually mature schistosomes and consequently leading to the description of novel chemotherapeutic targets acting in the maturation process [54]. Recently, different groups have used a myriad of approaches to describe schistosome genes expressed in a gender- or stage-enriched/specific fashion, emphasizing the importance of identification and characterization of proteins that may be controlling the transcription of these genes [8,16–26]. Moreover, the sex-specific presence of a protein potentially capable of regulating the expression of a large number of other genes, as in the case of SmZF1, becomes undoubtedly important in this context. One molecule, SmLIMPETin appears to medulate gene expression in *S. mansoni* [55]. SmLIMPETin gene is less expressed in sexually mature adult females when compared to sexually immature adult females and sexually mature and immature adult males [55]. These observations suggest that the sex-specific expression of a transcription factor may be a common feature involved in the maintenance of this parasite life cycle.

The ability of SmZF1 to activate/repress transcription of a luciferase reporter gene in a cellular context was assessed using COS-7 cells. The first step was to confirm the expression, localization and activity of the fusion protein YFP-SmZF1, used for the assay. YFP-SmZF1 was clearly visualized in the COS-7 cells nuclei using fluorescent microscopy; however, the protein was also visualized as fibrous filaments dispersed at the perinuclear region, probably associated with the cells cytoskeleton. Furthermore, Western blot assays showed the preferential nuclear localization for YFP-SmZF1, although it was also detected to a lesser degree in the cytoplasmic extract fraction. One possible explanation for this finding is that the YFP portion of the fusion protein, considering its larger size, is interfering with the efficiency of its transport to the nucleus. Conversely, recombinant SmZF1-myc tag, a smaller protein, is detected exclusively in the nuclear portion of COS-7 cells.

The second step was to verify the protein activity, i.e., if the recombinant protein YFP-SmZF1 was able to bind to its target DNA. EMSA assays were performed using total COS-7 extracts incubated with the putative SmZF1 best binding sequence, D1-3DNA. The experiments showed that cell extracts expressing the YFP-SmZF1 recombinant protein retarded the migration of D1-3DNA in the gel. When anti-GFP or anti-SmZF1 antibodies were added to the extract-DNA samples, a supershift was observed, confirming the binding of YFP-SmZF1 to its target.

The transcriptional activity of SmZF1 was further tested using a luciferase reporter system. The results showed a 2-fold increase on the luciferase gene expression in COS-7 cells co-transfected with the putative SmZF1 best binding sequence, D1-3DNA. The experiments showed that cell extracts expressing the YFP-SmZF1 recombinant protein retarded the migration of D1-3DNA in the gel. When anti-GFP or anti-SmZF1 antibodies were added to the extract-DNA samples, a supershift was observed, confirming the binding of YFP-SmZF1 to its target.
binding partner was present, the increase in the transcriptional activation would probably be much more substantial. A similar scenario has been reported for the protein SmNR1 from *S. mansoni*. In a recent work, Wu and colleagues demonstrated that the SmNR1 protein alone is able to activate the transcription of a reporter gene in COS-7 cells, but when another protein already known to interact with it (SmRXR1) is present, this activation increases approximately 2-fold [28]. In order to better characterize SmZF1 action as a transcription factor, future experiments designed to detect the protein binding partners will be necessary.

In addition to the DNA/RNA specific binding ability of SmZF1 [36], the evidence of its nuclear localization, as well as its capacity to activate gene transcription, strongly suggest that SmZF1 is a *S. mansoni* transcriptional regulator. Additional experiments aimed at determining SmZF1 biological role are being performed. Recently our group used RNAi to conduct an *in vitro* phenotypic screening of 32 *S. mansoni* genes, including SmZF1, known to be expressed at the sporocyst stage [57]. In this study, miracidia were cultivated *in vitro*, transformed into sporocysts in the presence of specific dsRNAs and observed during 7 days, in order to evaluate phenotypic changes. The treatment of the *S. mansoni* larvae with SmZF1-dsRNA induced a reduction of 30% on the SmZF1 transcript levels, when assayed by q-PCR. This modest reduction on the transcript levels was accompanied by a shortening at the sporocyst length in two out of three independent experiments, when compared to a negative control in which a GFP-dsRNA was used. These results show that, even with a small reduction at the transcript levels the parasite phenotype was altered, demonstrating the importance of the SmZF1 gene expression for the parasite larval stage. We believe that the significance of these findings can be extended for the other life cycle stages.

**Supporting Information**

**Figure S1** Anti-SmZF1 polyclonal antibody recognizes the SmZF1 protein. *S. mansoni* fractionated protein extracts, as well as the recombinant MBP-SmZF1 protein previously cleaved from its MBP portion, were submitted to SDS - PAGE 10% and blotted onto a nitrocellulose membrane. The anti-SmZF1 antibody was used to specifically recognize the protein. TE - total extract, NE - nuclear extract, CE - cytoplasmic extract.

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