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Examination of a Virulence Mutant Uncovers the Ribosome Biogenesis Regulatory Protein of *Toxoplasma gondii*

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Abstract

Several insertional mutants identified in a screen for *Toxoplasma gondii* that were defective in establishing a chronic infection had a common site of plasmid insertion. This insertion site was determined to be 43 bp upstream of the transcription initiation site of a gene whose predicted product has homology to ribosome biogenesis regulatory protein Rrs1p, an essential protein required for ribosome biogenesis in *Saccharomyces cerevisiae*. Northern blot analysis of this locus, termed *TgRRS1*, showed that in the C3 mutant, the full-length transcript is downregulated and at least 1 new smaller transcript is present. Restoration of the intact predicted promoter and locus to *TgRRS1* insertional mutant strain C3 did not restore brain cyst formation to the levels of the parent strain. Epitope-tagged TgRRS1 was found to localize to the parasite nucleolus, in an area corresponding to the granular component region. TgRRS1 can serve as a marker for the sub-nucleolar granular component region of *T. gondii*.

Toxoplasma gondii is an obligate intracellular pathogen capable of establishing a chronic infection in any warm-blooded animal. Acquired by consumption of undercooked contaminated meats or produce, the parasite causes mild flu-like symptoms in immunocompetent hosts. In these cases, acute infection is followed by chronic asymptomatic infection of long-lived muscle and brain cells (Montoya and Liesenfeld, 2004). If the host becomes immunocompromised, the encysted bradyzoite form of the parasite converts to the fast-replicating tachyzoite form, responsible for host cell lysis and damage (Dubey, 1997; Montoya and Liesenfeld, 2004; Dubey and Jones, 2008). To identify genes important for *T. gondii* pathogenesis, a modified signature-tagged mutagenesis approach was used. This technique involved creation of strains identifiable by their tag-bearing plasmid, followed by insertional mutagenesis of these strains with a second plasmid. Resulting mutants were screened for those able to grow in human foreskin fibroblasts (HFF) in vitro, but not form wild-type (WT) numbers of cysts in the brains of experimentally infected mice (Frankel et al., 2007). Of the 39 mutants identified as having at least a 10-fold reduction in the number of cysts formed, 3 shared a common tagged plasmid-bearing parent strain (C3), and, therefore, share insertion within the identical locus. Brain cyst formation

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was determined for tagged strain C3 in comparison to its parental strain Pru *HPT*(WT) following intraperitoneal injection of 2×10^4 tachyzoites into 6-wk-old CBA/J mice. At 22 days post-infection, brain cyst loads were quantified by immunofluorescence microscopy using fluorescein-conjugated *Dolichos biflorus* agglutinin (DbA; Vector Laboratories, Inc., Burlingame, California) as previously described (Frankel et al., 2007). Strain C3 showed reduced numbers of cysts relative to WT (Fig. 1A). Therefore, the in vivo cyst formation defects reported in derivative mutants 19C3, 29C3, and 38C3 may be attributed to parent strain C3, which itself is defective in establishing chronic infection in mice. Strain C3 was capable of forming DbA-reactive cysts in vitro in response to increasing culture medium pH to 8.1 (data not shown). This demonstrates that C3 is able to form cysts, but cannot form or maintain the number of cysts seen during murine infection by the WT strain.

The site of signature-tagged plasmid insertion in strain C3 was determined by plasmid rescue. *Toxoplasma gondii* genomic DNA flanking the plasmid insertion was sequenced and compared to the genome by Basic Local Alignment Search Tool (BLAST) using version 6 of the *T. gondii* genome database (ToxoDB; <http://toxodb.org>; Gajria et al., 2007). The insertion site was then confirmed by PCR using primers flanking the identified insertion site. Results indicate that the plasmid inserted within chromosome III, between 2 predicted genes, annotated as *TgME49_120440* and *TgME49_120450* (Fig. 1D). The plasmid was located within a NotI site 904 bp upstream of the translational start site of *TgME49_120440*, and 472 bp upstream of the translational start site of the oppositely transcribed *TgME49_120450* locus. Interestingly, the insertion site of the signature-tagged plasmid in C3 is identical to that of mutagenic vector pLK47 in 72F2, another strain from the same library of mutants that also displayed a cyst-formation defect (Craver et al., 2010). Identical insertion sites likely arose due to the use of NotI restriction enzyme-mediated insertion for plasmid integration during creation of the library (Frankel et al., 2007).

To assist in determining which gene may be impacted by the plasmid insertion, the transcriptional start site of the most proximal gene, *TgME49_120450*, was determined by rapid amplification of cDNA ends (RACE) using RNA derived from wild-type *T. gondii* (RLM-RACE kit, Ambion, Austin, Texas). The transcriptional start site of *TgME49_120450* was found to be 436 bp upstream of the predicted translational start codon, and 43 bp from the plasmid insertion site, likely affecting *TgME49_120440* transcription. As plasmid insertion occurred just upstream of the transcriptional start site of *TgME49_120450*, we sought to determine if transcription of the locus was affected in the C3 mutant. Total RNA derived from tachyzoites of C3 was compared to WT *T. gondii* for steady-state levels of *TgME49_120450* transcript by northern hybridization (Figs. 1B, D). A transcript of 2.3 kb was detected in mutant and wild-type strains, but was clearly less abundant in the C3 mutant. At least 1 new approximately 1 kb transcript hybridized in the C3 mutant, but not in the WT. When compared to the levels of transcript determined for *T. gondii* housekeeping gene α -tubulin (*TUB1*) as a loading control, the combined *TgME49_120450* transcript levels from the bands of mutant C3 were equivalent to those of the single transcript found in the WT. This result indicates that the plasmid insertion is likely causing addition of an alternative transcriptional start site for the *TgME49_120450* locus.

The *TgME49_120450* transcript was mapped by RT-PCR, using random-hexamer primed cDNA from wild-type *T. gondii* (Superscript III First-Strand Synthesis System, Invitrogen, Carlsbad, California) and sequencing. The determined open reading frame consisted of 5 exons, comprising a 960 bp coding region in agreement with the predicted gene architecture indicated by ToxoDB (Fig. 1D). The 5' UTR as determined by RACE was 436 bp, 32 bases longer than predicted by ToxoDB based on expressed sequence tag evidence. The 3' UTR was unable to be determined by RACE, though expressed sequence tag evidence suggests it is at least 75 bp downstream of the stop codon (ToxoDB). The 2.3 kb transcript size and 4 kb of predicted non-coding region between *TgME49_120450* and the downstream locus suggest the 3' UTR is likely to be approximately 800 bp.

The *TgME49_120450* locus encodes a protein product of 319 amino acids and 35.7 kDa. This predicted protein was searched for conserved domains using Pfam v24.0 (<http://pfam.janelia.org>; Marchler-Bauer et al., 2007), which identified a ribosome biogenesis regulatory protein RRS1 domain (Pfam 04939) from amino acid 24 to 213 (expect value = 6.13×10^{-35}). Rrs1p of *Saccharomyces cerevisiae* is an essential nuclear protein that regulates ribosome synthesis by aiding in maturation of the 25S rRNA, assembly of the 60S ribosomal subunit (Tsuno et al., 2000; Miyoshi et al., 2002; Morita et al., 2002) and is required for export of the 60S subunit from the nucleolus to the cytoplasm (Miyoshi et al., 2004; Zhang et al., 2007). The protein product of *TgME49_120450* was compared to the non-redundant protein sequence database by protein-protein BLAST (<http://www.ncbi.nlm.nih.gov/BLAST/>; Johnson et al., 2008), which identified RRS1 proteins across numerous genera. Given these data and the absence of other RRS1 homologs identified in the *T. gondii* genome, we have named this locus in *T. gondii* *TgRRS1*.

Although *TgRRS1* is not predicted to be a structural component of the mature ribosome, its locus shares sequence similarity with ribosomal protein loci. Two conserved sequence motifs, termed *Toxoplasma* Ribosomal Protein (TRP)-1 and TRP-2 elements, have been identified within the promoter regions of genes encoding *T. gondii* ribosomal proteins (Van Poppel et al., 2006). These 2 motifs alone, or in combination, have been identified in 95% of ribosomal proteins in this parasite, and are located 10 to 330 bases upstream of predicted transcriptional start sites. A perfect consensus TRP-2 element, TGCATGCA, is located 27 bases upstream of the RACE-predicted *TgRRS1* transcriptional start site. This motif matches the *Plasmodium falciparum* PF14_0633 Apetala2 (AP2) DNA-binding protein recognition sequence, suggesting possible transcriptional control by an AP2-like transcription factor (Dixon et al., 2010). While TRP-2 is found in the promoters of ribosomal protein genes, it is also found ubiquitously throughout the *T. gondii* genome (Van Poppel et al., 2006). By contrast, the TRP-1 element is enriched within the upstream of ribosomal protein-encoding loci, and has the consensus sequence TCGGCTTATATTCGG. A similar sequence, CCGGCTTCTTTCTT, is found 155 bases upstream of the RACE-predicted transcriptional start site of *TgRRS1*. This sequence is similar to the TRP-1 consensus, and deviating bases are variants found in previously identified ribosomal protein TRP-1 elements, with the exception of positions 10 and 15 (underlined; Van Poppel et al., 2006). Though the functions of TRP-1 and TRP-2 are not yet characterized, it is interesting to note they are conserved within the promoter of a locus whose product is not predicted to be within the mature ribosome. Sequence elements involved in transcriptional control of ribosomal protein-

encoding genes may also be employed at a locus whose product impacts ribosomal assembly and maturation. This arrangement would allow *TgRRS1* transcription to be coordinately regulated with transcription of structural ribosomal protein loci. In strain C3, plasmid insertion at the *TgRRS1* locus occurred between the predicted TRP-1 and TRP-2 elements, effectively displacing the TRP-1 element from the transcriptional start site by several kilobases.

Inoculation with strain C3 results in a reduced number of cysts in the brains of mice during the early stages of a chronic infection. This phenotype is in contrast to that of a conditional knockdown of *T. gondii* ribosomal structural protein RPS13, which was unable to replicate in vivo due to arrest at the G1 stage of the cell cycle (Hutson et al., 2010). The plasmid insertion upstream of *TgRRS1* may be responsible for cyst formation defect of C3. The shorter *TgRRS1* transcript detected in strain C3 may result from transcription initiation within the coding region, possibly creating a truncated form of TgRRS1 in the C3 mutant. To determine if introduction of additional copies of *TgRRS1* could restore the cyst-formation phenotype to constitutive WT levels, 2 plasmids were generated. One contained the *TgRRS1* cDNA transcribed from the *TUB1* promoter using a derivative of the vector pT230-TUB5/CAT (Kim et al., 1993). The second was comprised of the genomic *TgRRS1* locus, including 1.375 kb upstream of the translational start and 1.88 kb and downstream of the translational stop codon. Analysis by Southern hybridization confirmed C3 transformants. During murine infection, a comparison C3 and WT strains indicated that addition of *TgRRS1* to C3 did not restore cyst-formation to wild-type levels (data not shown). For the other TgRRS1 mutant strain, 72F2, introduction of identical constructs also did not complement its cyst-formation defect (data not shown). Perhaps the truncated form of TgRRS1 in the mutants acts as a dominant negative.

It remained possible expression from the other locus proximal to the plasmid insertion, *TgME49_120440*, may have been impacted by the plasmid insertion and could be responsible for the virulence defect seen in strain C3. As northern hybridization did not reveal a TgME49_120440 transcript (data not shown), quantitative real-time PCR (Q-PCR) was performed on cDNA prepared from WT and C3 tachyzoites to quantify transcripts for *TgRRS1* (using primers 5'-CACCTCCCTTCCTCGACTCT and 5'-TCGTTTCGTGAGGCAGAGCG), *TgME49_120440* (5'-GAGAGACGTTCTTCTTGCCGA and 5'-CCTGCGACTGCTTTCCACTC) and *TUB1*, as previously described (Rooney et al., 2011). Normalized to transcript levels of housekeeping gene *TUB1*, steady state levels of both *TgRRS1* and *TgME49_120440* transcripts were increased in strain C3 relative to WT tachyzoites by 4.4 ± 0.7 -fold and 4.5 ± 0.5 -fold, respectively. Though the region of *TgRRS1* transcript detected by Q-PCR was within exons 1, 2, and 3, whereas the probe used in northern hybridization spanned the 3' end of exon 3 to the stop codon, this difference in detected regions does not explain the contrasting outcomes from these 2 methods of transcript assessment. However, the possibilities that TgRRS1 is overexpressed in C3, or that transcription of both genes proximal to the insertion site are affected, provide explanations as to why addition of TgRRS1 to strain C3 would not restore WT-levels of in vivo cyst formation.

Several member proteins in the RRS1 superfamily were localized to the nucleolus, the site of transcription of ribosomal DNA and maturation of pre-rRNA transcripts (Tsuno et al., 2000; Andersen et al., 2002; Carnemolla et al., 2009). TgRRS1 contains basic stretches sharing similarity to monopartite nuclear localization signals, including the core sequence motif of (R/K)(R/K)X(R/K), which is found repeatedly in several known nucleolar proteins (Fig. 2; Hork et al., 2004). To determine if TgRRS1 has a similar localization pattern, a hemagglutinin (HA) tag was added to the carboxy-terminus of TgRRS1, within the context of a cDNA-based *TgRRS1* construct using the constitutive *TUB1* promoter and its 5' UTR, and a 3' UTR derived from *SAG1*. The resulting plasmid was introduced into strain C3 by electroporation, and clones containing transforming DNA were identified by Southern hybridization. The tagged protein migrated at the expected size of approximately 35 kDa, as determined by western immunoblot using anti-HA antibodies (Fig. 1C).

Tagged-TgRRS1 was also detected by fluorescence microscopy, and was found within bodies in the 4',6-diamidino-2-phenylindole (DAPI) reactive region, highlighting the parasite nucleus. To determine if this region corresponds to the nucleolus, antibodies to fibrillarin, shown to react with parasite and host nucleoli, were also used for localization (Yang et al., 2001; White et al., 2005). TgRRS1 did not precisely colocalize with fibrillarin. While fibrillarin is often used as a nucleolar marker, its expression is not uniform throughout this compartment. The nucleolus is comprised of 3 subcompartments known as the fibrillar centers (FC) that are surrounded by the dense fibrillar component (DFC), which is itself found within the granular component (GC) region (Raska et al., 2006). The FC region is proposed to be a site of inactive r-genes, and has been postulated to exist only in amniotes (Thiry and Lafontaine, 2005). Transcription of rRNA occurs in the FC/DFC boundary, and within the DFC. The DFC is host to cleavage and modification of pre-rRNA transcripts, and is the site of action of fibrillarin. This protein associates with nascent rRNA transcripts and performs ribose 2'-O-methylation in the DFC. Pre-rRNAs are then shuttled to the GC, where the DFC/GC border and GC region are the sites of ribosomal protein association with rRNAs in mammals (Kruger et al., 2007). In most systems, the GC is defined by localization of fluorescent-conjugate of the B23 protein, also known as nucleoplasmin. We were unable to identify a B23 homolog within the *T. gondii* genome and, therefore, define the GC as the area surrounding the DFC. While fibrillarin served as a DFC marker in *T. gondii*, and appeared within compact spots with no apparent FC, TgRRS1 was detected at the DFC/GC border, and was concentrated in a region encircling the DFC that we propose is the GC (Fig. 3).

In the present study, regulator of ribosome synthesis TgRRS1 was identified in *T. gondii*. Expression of this protein was localized to the site of ribosomal protein and transcript association, within the subnucleolar GC and DFC/GC junction. Murine RRS1 is detected in both the DFC and the surrounding region (Carnemolla et al., 2009). Yeast Rrs1p is thought to regulate ribosome biosynthesis within the DFC (Wade et al., 2001). Rrs1p, along with additional assembly factor Rpf2p, recruits and associates with ribosomal proteins rpL5 and rpL11, and the 5S rRNA to form a ribonucleoprotein neighborhood. All 4 proteins are required for the recruitment of the other neighborhood proteins and the 5S rRNA, and for export of preribosomes from the nucleolus to complete their maturation (Zhang et al., 2007). The lack of TgRRS1 detected in the DFC suggests differences in ribosome maturation and

assembly in *T. gondii* relative to model organisms, which include the GC region being site of action of TgRRS1. As the expression pattern of TgRRS1 is distinct from that of TgXPMC2, a suppressor of mitotic defects that localizes uniformly throughout the nucleolus (White et al., 2005), TgRRS1 may also serve as a marker of the subnucleolar granular component region in this parasite.

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LITERATURE CITED

- Andersen JS, Lyon CE, Fox AH, Leung AK, Lam YW, Steen H, Mann M, Lamond AI. Directed proteomic analysis of the human nucleolus. *Current Biology*. 2002; 12:1–11. [PubMed: 11790298]
- Carnemolla A, Fossale E, Agostoni E, Michelazzi S, Calligaris R, De Maso L, Del Sal G, MacDonald ME, Perischetti F. Rrs1 is involved in endoplasmic reticulum stress response in Huntington disease. *Journal of Biological Chemistry*. 2009; 284:18167–18173. [PubMed: 19433866]
- Craver MPJ, Rooney PJ, Knoll LJ. Isolation of *Toxoplasma gondii* development mutants identifies a proteophosphoglycan that enhances cyst wall formation. *Molecular and Biochemical Parasitology*. 2010; 169:120–123. [PubMed: 19879901]
- Dixon SE, Stilger KL, Elias EV, Naguleswaran A, Sullivan WE Jr. A decade of epigenetic research in *Toxoplasma gondii*. *Molecular and Biochemical Parasitology*. 2010; 173:1–9. [PubMed: 20470832]
- Dubey JP. Tissue cyst tropism in *Toxoplasma gondii*: A comparison of tissue cyst formation in organs of cats, and rodents fed oocysts. *Parasitology*. 1997; 115:15–20. [PubMed: 9226953]
- Dubey JP, Jones JL. *Toxoplasma gondii* infection in humans and animals in the United States. *International Journal for Parasitology*. 2008; 38:1257–1278. [PubMed: 18508057]
- Frankel MB, Mordue DG, Knoll LJ. Discovery of parasite virulence genes reveals a unique regulator of chromosome condensation 1 ortholog critical for efficient nuclear trafficking. *Proceedings of the National Academy of Sciences USA*. 2007; 104:10181–10186.
- Gajria B, Bahl A, Brestelli J, Dommer J, Fischer S, Gao X, Heiges M, Iodice J, Kissinger JC, Mackey AJ, et al. ToxoDB: An integrated *Toxoplasma gondii* database resource. *Nucleic Acids Research*. 2007; 36:D553–D556. [PubMed: 18003657]
- Hork S, Reumann K, Schweizer M, Will H, Heise T. Nuclear trafficking of La Protein depends on a newly identified nucleolar localization signal and the ability to bind RNA. *Journal of Biological Chemistry*. 2004; 279:26563–26570. [PubMed: 15060081]
- Hutson SL, Mui E, Kinsley K, Witola WH, Behnke MS, El Bissati K, Muench SP, Rohrmann B, Liu SR, Wollman R, et al. *Toxoplasma gondii* RP promoters and knockdown reveal molecular pathways associated with proliferation and cell-cycle arrest. *Public Library of Science ONE*. 2010; 5:e14057. [PubMed: 21124925]
- Johnson M, Zaretskaya I, Raytselis Y, Merezhuk Y, McGinnis S, Madden TL. NCBI BLAST: A better web interface. *Nucleic Acids Research*. 2008; 36:W5–W9. [PubMed: 18440982]
- Kim K, Soldati D, Boothroyd JC. Gene replacement in *Toxoplasma gondii* with chloramphenicol acetyltransferase as selectable marker. *Science*. 1993; 262:911–914. [PubMed: 8235614]
- Kruger T, Zentgraf H, Scheer U. Intranucleolar sites of ribosome biogenesis defined by the localization of early binding ribosomal proteins. *Journal of Cell Biology*. 2007; 177:573–578. [PubMed: 17517959]
- Marchler-Bauer A, Anderson JB, Derbyshire MK, DeWeese-Scott C, Gonzales NR, Gwadz M, Hao L, He S, Hurwitz DI, Jackson JD, et al. CDD: A conserved domain database for interactive domain family analysis. *Nucleic Acids Research*. 2007; 35:D237–D240. [PubMed: 17135202]

- Miyoshi K, Shirai C, Horigome C, Takenami K, Kawasaki J, Mizuta K. Rrs1p, a ribosomal protein L11-binding protein, is required for nuclear export of the 60S pre-ribosomal subunit in *Saccharomyces cerevisiae*. *FEBS Letters*. 2004; 565:106–110. [PubMed: 15135061]
- Miyoshi K, Tsujii R, Yoshida H, Maki Y, Wada A, Matsui Y, Toh-E A, Mizuta K. Normal assembly of 60 S ribosomal subunits is required for the signaling in response to a secretory defect in *Saccharomyces cerevisiae*. *Journal of Biological Chemistry*. 2002; 277:18334–18339. [PubMed: 11893754]
- Montoya JG, Liesenfeld O. Toxoplasmosis. *Lancet*. 2004; 363:1965–1976. [PubMed: 15194258]
- Morita D, Miyoshi K, Matsui Y, Toh-E A, Shinkawa H, Miyakawa T, Mizuta K. Rpf2p, an evolutionarily conserved protein, interacts with ribosomal protein L11 and is essential for the processing of 27 SB Pre-rRNA to 25 S rRNA and the 60 S ribosomal subunit assembly in *Saccharomyces cerevisiae*. *Journal of Biological Chemistry*. 2002; 277:28780–28786. [PubMed: 12048200]
- Raska I, Shaw PJ, Cmarko D. Structure and function of the nucleolus in the spotlight. *Current Opinions in Cell Biology*. 2006; 18:325–334.
- Rooney PJ, Ayong L, Tobin CM, Moreno SNJM, Knoll LJ. TgVTC2 is involved in polyphosphate accumulation in *Toxoplasma gondii*. *Molecular and Biochemical Parasitology*. 2011; 176:121–126. [PubMed: 21195114]
- Thiry M, Lafontaine DL. Birth of a nucleolus: The evolution of nucleolar compartments. *Trends in Cell Biology*. 2005; 15:194–199. [PubMed: 15817375]
- Tsuno A, Miyoshi K, Tsujii R, Miyakawa T, Mizuta K. *RRS1*, a conserved essential gene, encodes a novel regulatory protein required for ribosome biogenesis in *Saccharomyces cerevisiae*. *Molecular and Cellular Biology*. 2000; 20:2066–2074. [PubMed: 10688653]
- Van Poppel NF, Welagen J, Vermeulen J, Schaap D. The complete set of *Toxoplasma gondii* ribosomal protein genes contains two conserved promoter elements. *Parasitology*. 2006; 133:19–31. [PubMed: 16674839]
- Wade C, Shea KA, Jensen RV, McAlear MA. EBP2 is a member of the yeast RRB regulon, a transcriptionally coregulated set of genes that are required for ribosome and rRNA biosynthesis. *Molecular Cell Biology*. 2001; 21:8638–8650.
- White MW, Jerome ME, Vaishnav S, Guerini M, Behnke M, Striepen B. Genetic rescue of a *Toxoplasma gondii* conditional cell cycle mutant. *Molecular Microbiology*. 2005; 55:1060–1071. [PubMed: 15686554]
- Yang JM, Baserga SJ, Turley SJ, Pollard KM. Fibrillarin and other snoRNP proteins are targets of autoantibodies in xenobiotic-induced autoimmunity. *Clinical Immunology*. 2001; 101:38–50. [PubMed: 11580225]
- Zhang J, Harnpicharnchai P, Jakovljevic J, Tang L, Guo Y, Oeffinger M, Rout MP, Hiley SL, Hughes T, Woolford JL Jr. Assembly factors Rpf2 and Rrs1 recruit 5S rRNA and ribosomal proteins rpL5 and rpL11 into nascent ribosomes. *Genes and Development*. 2007; 21:2580–2592. [PubMed: 17938242]

Figure 1.

(A) C3-infected mice have reduced brain cyst burdens compared to the parental strain, Pru *HPT* (WT). At 22 days post-infection, brains of infected mice were collected, and cysts were quantified based on reactivity to *Dolichos biflorus* agglutinin and immunofluorescence microscopy. Shown are the results from 2 representative experiments performed using 4 mice per strain. Results from individual mice are shown as dots, and average values are shown as bars. (B) Transcription of *TgRRS1* is affected in insertional mutant strain C3. Total RNA from approximately 1×10^8 C3 and Pru *HPT* (WT) tachyzoites was probed with a fragment corresponding to most of the predicted *TgME49_120450* (*TgRRS1*) coding region, and reprobed with the *TUB1* locus as a loading control. Probes are shown at the left, and RNA size standards at the right, in kilobases. (C) TgRRS1 is 35 kDa. Proteins of C3 transformed with a plasmid encoding HA-tagged TgRRS1 (C3/TgRRS1HA) from 1×10^7 intracellular tachyzoites (middle lane) or 2×10^7 lysed, extracellular tachyzoites (right lane) were analyzed in comparison to proteins from 1×10^7 intracellular WT tachyzoites (left lane) by denaturing immunoblot using anti-HA monoclonal antibody clone 16B12 (Covance, Princeton, New Jersey). A reactive band of 35 kDa corresponded with the size predicted from the *TgRRS1* open reading frame, and was found only in the C3 transformant. Size markers on the right side are in kDa. (D) Map of the disrupted region of chromosome III. RT-PCR-confirmed exons and the RACE-identified 5' UTR of *TgME49_120450* (*TgRRS1*) are shown as black and gray boxes, respectively, and the first exon of *TgME49_120440* is shown at left in white. Arrows indicate the transcriptional orientation of these genes, and the site of disruption in strains C3 and 72F2 is marked by a lollipop. Regions included in the cDNA probe for northern hybridization are indicated by white boxes

above the map, and excluded introns are dashed lines. Tick marks every 500 bp are shown below the schematic.

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TgRRS1	1	MASASMPGSPPFLLDSSAPSAGEGNAMESHLNFLLC---IDFSPVLHTDDLSDLTRQNAQS
PfRRS1	1	MD-----IDFCSHLLA---YDNSIINDENEIKAKVEQNLPL
ChRRS1	1	MEEAQLETSP-----LEVCLSNLIG---IDISSINET-EISKSGANNLQL
ScRrs1p	1	MSAEDYKNLPVTVEKPIPVVYDLGNLAAFDSNVLDKNDLDSSNARREEKIKSLTRDNVQL
		* * *
TgRRS1	58	MVAQLCALP-HETTTDDG-----LLVALPPPAKNSTFKLPRMHPAPAAKKLTRWEAFAKE
PfRRS1	35	IFSKINKLQ--KEKEDE-----EDIIYSVTNDN-IFNMPRYSRIPKEKKKTKWQLFAEN
ChRRS1	42	MLNSIWSDLKENTKDG-----TVVSLPSKQE---ILLPRAYPLPAKREKTRWEKFAEL
ScRrs1p	61	LINQLLSLPMKTTTESVGGTGGQSSVMTLLQLPDPTTDLPREKPLPKAKAMTKWEKFAAK
		** * * *
TgRRS1	111	KGIQK KRS -RLVWDANTKDWVPRWGHKGIQOMNALAEAVIEDKDG EYVG RRGR DRGVKK
PfRRS1	86	KLRMKKNKS-GLIYDKVSKGWVRRFQ--KKQIKLNEQKNNFVHEYKN-----
ChRRS1	93	KGIKKRKRS-RMVYDPITDDFVPRWGRNSIKKIQKKHNEAIEIKG-----
ScRrs1p	121	KGIKPKERAGKMIYDEASGEWVPKWG----YKG-----
		* * *
TgRRS1	170	L GKRSK DAQKRRTSQQVECPFEAKAKE KKLR DAQQKLRELNTLEADTDRLP PGVLATL
PfRRS1	132	---KEDIYED-----PFEKEQEEKDIKKMKQKMRELKNKFDQ-----KG-ISTE
ChRRS1	138	---NMDENKD-----PREAINLKRDMMINKQRLRELKNKMNSSKNINNSKSDEFAL
ScRrs1p	150	-----ANKKLDDQWLVEVDDKVKG-----TDNELI
		* *
TgRRS1	230	AAGGEAAAE RRGR AAGLRHRRQTKEELKEVMRRAQTSTASFGQFDRLAKNE K-REK OKTR
PfRRS1	172	DIKYIQRQKRK-----R--ENLIDNLKMAQISSSTFGRKDKKLKKEKKLKLQNDK
ChRRS1	186	GIGNLKDTNVK-----RSKTQVKELFDRVSLSTASYGRRDKALPDED--RTTNKVK
ScRrs1p	175	DPRTLNR AERK-----RLVK-----K-NEK-----
TgRRS1	289	TKGVSLSLDDERSKYRRHLKNILSAAESADV
PfRRS1	220	ITNQKYEKRDLDKDEVQNNRLAALVLKSV
ChRRS1	235	KVKKILDTKNENEKYKSIYSKILKQNSNL
ScRrs1p	194	-----QQRNMKNAL

Figure 2.

TgRRS1 aligned with RRS1 family members. The amino acid sequence of TgRRS1 was aligned with RRS1 proteins from apicomplexan parasites *Plasmodium falciparum* (PfRRS1; accession XP_001347930.1) and *Cryptosporidium hominis* (ChRRS1; accession XP_6653581), as well as the defining family member from *Saccharomyces cerevisiae* (ScRrs1p; accession NP_014937.1) using ClustalW in MacVector v9.0. Amino acid positions are shown at the left of each line. Conserved residues are indicated by asterisks below the alignment. Possible nucleolar localization sequences contained in stretches of sequence similar to monopartite nuclear localization signals, marked by consensus (R/K)X(R/K) are boxed on the TgRRS1 sequence.

Figure 3.

TgRRS1 is localized to the nucleolus, in the region surrounding the dense fibrillar component. Tachyzoites of strains C3 and C3 transformed with a plasmid encoding a hemagglutinin-tagged TgRRS1 were used to infect human foreskin fibroblasts. Infected monolayers were fixed with paraformaldehyde and reacted with anti-HA polyclonal antisera (Zymed/Invitrogen, Carlsbad, California) and anti-fibrillarin monoclonal antibody. Reactivity was detected using fluorescently conjugated secondary antibodies. Images were captured using OpenLabs version 5 (PerkinElmer, Waltham, Massachusetts), and a Zeiss Axioplan 2 microscope equipped with a rear-mounted excitation filter wheel, a triple pass (DAPI/FITC/Texas Red) emission cube, differential interference contrast (DIC) optics, and a Hamamatsu ORCA-AG CCD camera. Volocity version 4 (PerkinElmer) was used to deconvolve images using a constrained iterative algorithm. All images are at the same scale, indicated by a 2.5 μ m bar in the upper left panel. At left are DIC panels showing 2 tachyzoites for each strain. Anti-HA reactivity identifies TgRRS1 (green), while anti-fibrillarin (FIB) is used to highlight the dense fibrillar component of the nucleolus (red). Apicoplast and host and parasite nuclear DNA is shown in the merged image at right by 4–6' diamidino-2-phenylindole reactivity (blue).