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A Novel Secreted Protein, MYR1, Is Central to Toxoplasma’s Manipulation of Host Cells

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ABSTRACT The intracellular protozoan Toxoplasma gondii dramatically reprograms the transcriptome of host cells it infects, including substantially up-regulating the host oncogene c-myc. By applying a flow cytometry-based selection to infected mouse cells expressing green fluorescent protein fused to c-Myc (c-Myc–GFP), we isolated mutant tachyzoites defective in this host c-Myc up-regulation. Whole-genome sequencing of three such mutants led to the identification of MYR1 (Myc regulation 1; TGGT1_254470) as essential for c-Myc induction. MYR1 is a secreted protein that requires TgASP5 to be cleaved into two stable portions, both of which are ultimately found within the parasitophorous vacuole and at the parasitophorous vacuole membrane. Deletion of MYR1 revealed that in addition to its requirement for c-Myc up-regulation, the MYR1 protein is needed for the ability of Toxoplasma tachyzoites to modulate several other important host pathways, including those mediated by the dense granule effectors GRA16 and GRA24. This result, combined with its location at the parasitophorous vacuole membrane, suggested that MYR1 might be a component of the machinery that translocates Toxoplasma effectors from the parasitophorous vacuole into the host cytosol. Support for this possibility was obtained by showing that transit of GRA24 to the host nucleus is indeed MYR1-dependent. As predicted by this pleiotropic phenotype, parasites deficient in MYR1 were found to be severely attenuated in a mouse model of infection. We conclude, therefore, that MYR1 is a novel protein that plays a critical role in how Toxoplasma delivers effector proteins to the infected host cell and that this is crucial to virulence.

IMPORTANCE Toxoplasma gondii is an important human pathogen and a model for the study of intracellular parasitism. Infection of the host cell with Toxoplasma tachyzoites involves the introduction of protein effectors, including many that are initially secreted into the parasitophorous vacuole but must ultimately translocate to the host cell cytosol to function. The work reported here identified a novel protein that is required for this translocation. These results give new insight into a very unusual cell biology process as well as providing a potential handle on a pathway that is necessary for virulence and, therefore, a new potential target for chemotherapy.

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Toxoplasma gondii is an obligate intracellular parasite of great medical importance, as infections in immunosuppressed patients often lead to life-threatening encephalitis. While Toxoplasma bradyzoites localize to specific tissues (brain, skeletal muscles, and heart) (1) during the chronic phase, the acute stages of infection involve tachyzoites that are able to infect virtually any nucleated cell. Toxoplasma’s intracellular lifestyle is dependent on the ability of the parasite to regulate host cell processes via its secreted effectors. For example, the rhoptry protein ROP16 regulates the immunologically important host transcription factors STAT1 (2), STAT3 (3), STAT5 (4), and STAT6 (5), causing profound transcriptional changes shortly after invasion. Further examples of Toxoplasma’s ability to specifically regulate host functions are its dense granule effectors GRA15 (6), GRA16 (7), and GRA24 (8), which regulate the NF-κB, p53, and p38 MAP kinase (MAPK) host pathways, respectively.

We have recently discovered that Toxoplasma tachyzoites also specifically and actively induce host c-Myc upon infection (9). c-Myc is a key transcription factor that regulates critical host cell processes such as cell cycle progression, cell metabolism, and apoptosis (10, 11), and several of these c-Myc-regulated processes appear to be modulated in Toxoplasma-infected cells (12). In addition, and likely as a direct consequence of its up-regulation, many of the known gene targets of c-Myc have also been reported to be transcriptionally up-regulated during Toxoplasma infection (9). The up-regulation of c-Myc is likely mediated by one or more novel effectors, since none of the previously identified Toxoplasma effectors appear to play a role (7, 9).

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The mechanism by which secreted effectors reach the host cytosol in Toxoplasma-infected cells is not known. In the related malaria parasites Plasmodium spp., proteins are translocated across the parasitophorous vacuole membrane (PVM) via the PTEX complex (13–15). Toxoplasma has recognizable orthologues of some components of this complex, but recent reports show that these operate in different ways; e.g., the Toxoplasma orthologue of EXP2 is GRA17, but recent work showed that this protein serves as a transporter of small molecules (<3,000 Da), not proteins (16).

To further explore the process by which Toxoplasma effectors operate, we chose a genetic approach that exploits the fact that Toxoplasma tachyzoites up-regulate c-Myc and the existence of faithful c-Myc reporter systems. Here we describe the use of such a screen to identify mutants deficient in a novel Toxoplasma protein that is secreted into the parasitophorous vacuole (PV) and is necessary for host c-Myc induction. We show that mutations in this gene, which we have dubbed MYR1 (Myc regulation 1), are pleiotropic with a pronounced defect in the ability of tachyzoites to manipulate several host pathways, and we propose a role for MYR1 in protein translocation across the PVM.

RESULTS

Isolation of Toxoplasma mutants that fail to induce c-Myc. Forward genetic screens have previously identified important Toxoplasma proteins involved in various functions (17–20). Such an approach is facilitated by the fact that the Toxoplasma genome is haploid, and thus, single mutations often give rise to discrete phenotypes. To further investigate how Toxoplasma tachyzoites interact with the host cell, therefore, we designed a high-throughput genetic screen to isolate mutants defective in their ability to modulate a known and easily studied pathway, the up-regulation of c-Myc. The major requirement for such a screen is a reporter cell that allows easy detection of c-Myc expression levels. For this, we first attempted several plasmid-based systems involving the c-Myc promoter driving reporters such as GFP in established cell lines; unfortunately, however, none showed up-regulation upon infection with Toxoplasma tachyzoites (data not shown). This suggested that the up-regulation required c-myc to be in its natural chromosomal location, where it is known to be regulated by a complex array of enhancers, including at least one that is >400 kbp distant (21).

To overcome the limitation of plasmid-based reporters, we examined bone marrow macrophages (BMMs) from mice engineered to express green fluorescent protein (GFP) from the native c-myc locus (22). BMMs were isolated from the mice and infected at a multiplicity of infection (MOI) of 0.25 with either Toxoplasma RH mCherry or Neospora caninum NC-1 mCherry (9) or were mock-infected. Approximately 24 h postinfection (hpi), flow cytometry was used to distinguish between infected (mCherry-positive) and uninfected (mCherry-negative) cells and to analyze GFP levels in

FIG 1 Genetic screen to isolate Toxoplasma mutants that fail to induce c-Myc. (A) Flow cytometry histogram of BMMs expressing c-Myc–GFP infected with Toxoplasma wild-type tachyzoites (RH) or Neospora tachyzoites or mock-infected. The gate was set on highly infected cells. The y axis shows relative cell count for each population (normalized to mode), and the x axis shows green fluorescence intensity. (B) Selection of mutants that fail to induce c-Myc. BMM c-Myc–GFP reporter cells were infected with a mutagenized population of RH tachyzoites and sorted for low GFP fluorescence. This was repeated until a homogeneous population was obtained that induced low GFP fluorescence. The GFP fluorescence profiles for the mutagenized population after 3, 5, and 7 rounds of selection are shown. (Histograms for first two sorts were similar to the one for sort 3 and were omitted.) The profiles for cells infected with wild-type RH and Neospora tachyzoites are shown for comparison. The x and y axes are as described for panel A.
both populations (Fig. 1A). The results showed that, compared to mock-infected reporter cells, Toxoplasma-infected cells exhibited a marked increase in green fluorescence. Infection with Neospora showed no c-Myc up-regulation, as previously reported (9).

Having identified a reporter system that recapitulates the Toxoplasma-induced up-regulation of c-Myc, we next designed a genetic screen for Toxoplasma mutants that are deficient in this process. Mutagenized Toxoplasma RH parasites expressing mCherry were used to infect the c-Myc–GFP reporter BMMs at an MOI of 0.25. A low multiplicity of infection was used to ensure that most reporter cells were infected with only one parasite, which was confirmed visually by fluorescence microscopy. Toxoplasma wild-type-infected cells and mock-infected cells were used as positive and negative controls for c-Myc induction, respectively. Cells with high levels of red fluorescence but little or no GFP signal were then selected by fluorescence-activated cell sorting (FACS). Neospora-infected cells were used to establish selection gates for the enrichment of mutants. Parasites in the selected cells, representing between 1 and 5% of the total infected population, were expanded by growth in human foreskin fibroblasts (HFFs), which was isolated from the first screen, was also confirmed by the Western blotting. Mock-infected and Neospora-infected cells served as negative controls for c-Myc induction, and cells infected with wild-type Toxoplasma were used as a positive control. The results (Fig. 2A) showed that cells infected with two of the three cloned mutants, MFM1.1 and MFM2.2, showed no detectable c-Myc, indicating the selection strategy was successful in yielding Toxoplasma mutants deficient in c-Myc up-regulation. MFM2.1 had an intermediate phenotype, which is discussed further below.

Whole-genome sequence analysis reveals a candidate gene, TGGT1_254470. To identify the Toxoplasma gene(s) involved in c-Myc up-regulation, the MFM1.1, MFM2.1, and MFM2.2 mutants were analyzed by whole-genome sequencing. Sequence comparison relative to the parental reference revealed 5, 7, and 6 single nucleotide variations (SNVs), respectively, in chromosomal coding regions of MFM1.1, MFM2.1, and MFM2.2 (Table 1). These results showed that although they came from the same screen, MFM2.1 and MFM2.2 are independent mutants, as each contained a different set of SNVs. The independence of MFM1.1, which was isolated from the first screen, was also confirmed by the sequencing. MFM2.1 had no SNVs with a read frequency over 80% but several within the ~40-to-60% range (Table 1). This indicates that the sample containing MFM2.1 is in fact a mix of two
TABLE 1  Coding mutations in mutant strains as detected by whole-genome sequencingα

<table>
<thead>
<tr>
<th>Clone and chromosome</th>
<th>SNV position</th>
<th>% confidence</th>
<th>Codon (WT/mut)b</th>
<th>Amino acid changec</th>
<th>Gene</th>
<th>Predicted function</th>
</tr>
</thead>
<tbody>
<tr>
<td>MFM1.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TGGT1_chrX</td>
<td>6416305</td>
<td>97</td>
<td>tC/tgG</td>
<td>C1296W</td>
<td>TGGT1_214830</td>
<td>Hypothetical protein</td>
</tr>
<tr>
<td>TGGT1_chrVIII</td>
<td>1822637</td>
<td>100</td>
<td>caT/caA</td>
<td>H643Q</td>
<td>TGGT1_232100</td>
<td>RAP domain-containing protein</td>
</tr>
<tr>
<td>TGGT1_chrVIII</td>
<td>2164920</td>
<td>100</td>
<td>ga/g/g/a</td>
<td>E135G</td>
<td>TGGT1_229380</td>
<td>Hypothetical protein</td>
</tr>
<tr>
<td>TGGT1_chrXII</td>
<td>3209479</td>
<td>100</td>
<td>gT/g/Gc</td>
<td>V125G</td>
<td>TGGT1_247350</td>
<td>Thioredoxin domain-containing protein</td>
</tr>
<tr>
<td>TGGT1_chrIII</td>
<td>1740887</td>
<td>100</td>
<td>ta/Ta/tA</td>
<td>Y281*</td>
<td>TGGT1_254470</td>
<td>Hypothetical protein</td>
</tr>
<tr>
<td>MFM2.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>TGGT1_chrVIIb</td>
<td>3288737</td>
<td>42</td>
<td>tCa/Ta</td>
<td>S151L</td>
<td>TGGT1_258580</td>
<td>Rho protein ROP17</td>
</tr>
<tr>
<td>TGGT1_chrIX</td>
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<td>42</td>
<td>Aaa/Gaa</td>
<td>K526E</td>
<td>TGGT1_289190</td>
<td>Tetratricopeptide repeat-containing protein</td>
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<tr>
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<td>43</td>
<td>gG/g/aa</td>
<td>G635E</td>
<td>TGGT1_313370</td>
<td>Hypothetical protein</td>
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<td>46</td>
<td>gG/g/g/T</td>
<td>E294D</td>
<td>TGGT1_239365</td>
<td>Formyl transferase domain-containing protein</td>
</tr>
<tr>
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<td>46</td>
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<td>T781P</td>
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<td>tA/t/gA</td>
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</tr>
<tr>
<td>MFM2.2</td>
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<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
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<td>989081</td>
<td>100</td>
<td>Ggc/Agc</td>
<td>G51S</td>
<td>TGGT1_213520</td>
<td>Peptidase M20D, amidohydrolase</td>
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<td>4035211</td>
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<td>Tat/Cat</td>
<td>Y170H</td>
<td>TGGT1_272370</td>
<td>Hypothetical protein</td>
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<td>100</td>
<td>tgC/tgG</td>
<td>C1296W</td>
<td>TGGT1_214830</td>
<td>Hypothetical protein</td>
</tr>
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<td>S601T</td>
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<td>Hypothetical protein</td>
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<td>Cga/Tga</td>
<td>R312*</td>
<td>TGGT1_254470</td>
<td>Hypothetical protein</td>
</tr>
</tbody>
</table>

α The position and nature of all coding SNVs in chromosomal genes in each mutant are listed. Only mutations that were supported by at least 80% of the reads in MFM1.1 and MFM2.2 or 40% of the reads in MFM2.1 were considered SNVs.

b WT, wild type; mut, mutant. Capital letters indicate the mutated nucleotides.

c *, Stop codon.

lines, consistent with its intermediate phenotype in terms of c-Myc up-regulation, as shown in Fig. 2A.

In addition to the SNVs in chromosomal genes shown in Table 1, mutations might also be present in genes present on relatively short contigs that have yet to be confidently positioned on any chromosome, likely because they are tandemly duplicated genes that are difficult to unambiguously assemble. Identifying mutations in the latter set is particularly challenging because the short sequence reads often cannot be unambiguously assigned to one or other of the tandemly repeated genes and a false assignment may erroneously be taken as an SNV. We did not attempt to resolve SNVs in such contigs and it is possible, therefore, that additional coding SNVs, beyond those shown in Table 1, exist within the mutants.

Of all the identified SNVs, only one Toxoplasma gene (designated TGGT1_254470 in ToxoDB v.10.0; http://www.toxodb.org) carried an alteration in two or more mutants; in fact, it was altered in all three mutants (Table 1), each of which carried a nonsense mutation at a different position (Fig. 2B). Moreover, TGGT1_254470 was the only gene to harbor a nonsense mutation in any of the three mutants (Table 1). These data strongly suggested that TGGT1_254470 is necessary for c-Myc induction in Toxoplasma-infected host cells, and it was therefore dubbed MYR1 for its essential role in c-Myc regulation.

MYR1 is located on chromosome III and is predicted by ToxoDB (v25) to have 4 or 5 introns, depending on the strain. Aggregating RNA-Seq data in ToxoDB, however, clearly show that in most strains, the extra intron intron predicted in TgME49_254470 (positions 1758697 to 1759101) is rarely, if ever, spliced out, and this region is instead fully represented in the bulk of the final mRNA generated. This impacts the predicted open reading frame and extends it 234 bp in the 5’ direction, relative to what is shown for TgME49_254470, resulting in a different predicted start codon (position 1758990, as we have now noted in the comment section for this gene in ToxoDB). Use of this upstream start codon results in the presence of a strongly predicted signal peptide by SignalP 4.1 with a discrimination score (D score) of 0.77, where any score above 0.45 is judged significant. This is consistent with the fact that the MYR1 protein is secreted from the parasite (see below). We conclude, therefore, that the extra intron predicted for MYR1 is typically not spliced out and that the true start site for translation is at nucleotide 1758990.

Expressed sequence tag data, microarray data, and RNA-Seq data, also available on ToxoDB (v25), show that this gene is abundantly expressed across the three major infectious stages of Toxoplasma, tachyzoites, bradyzoites, and sporozoites but is expressed little if at all (<5% of the RPKM [reads per kilobase per million] values for tachyzoites) in the intraepithelial stages in the cat gut (23–27). Its function, therefore, may not be key to the sexual cycle of the parasite that occurs exclusively in felines.

Multiple sequence alignment of nucleotide and protein sequences using the Clustal Omega alignment tool on EMBL-EBI website (http://www.ebi.ac.uk) revealed that this protein is highly conserved (>99% identity) among the three canonical strains, RH (type I), ME49 (type II), and VEG (type III). Likewise, it is expressed at similar levels in tachyzoites from these three strains (ToxoDB [v25]). These similarities were as expected given that all three major types exhibit the c-Myc induction phenotype (9).

MYR1 encodes a novel protein with two predicted transmembrane domains located near its C terminus (Fig. 2B). PsiBLAST analysis of the entire protein sequence revealed no predicted function and no homology to proteins outside its closest relatives,
Hammondia hammondi and Neospora caninum. The orthologue of MYR1 present in the very closely related H. hammondi (HHHA_254470) is 87% identical at the amino acid level, as expected, but in N. caninum (NCLIV_008760), there is only ~45% predicted protein identity relative to the Toxoplasma (GT1) protein (ToxoDB v25). No convincing homologue is detectable in Sarcozystis neurona or the more distantly related apicomplexan genera Eimeria and Plasmodium. These results suggest a function specific to a subset of the tissue-dwelling coccidia.

**MYR1 is necessary for host c-Myc induction.** To confirm that MYR1 is involved in the c-Myc induction phenotype, we generated a targeted deletion of the MYR1 locus in RH type I Toxoplasma and produced a complemented version of this Δmyr1 strain in which a C-terminally hemagglutinin (HA)-tagged version of the MYR1 gene is ectopically expressed in the knockout (RHΔmyr1::MYR1); see Materials and Methods). Successful deletion of the MYR1 locus in the Δmyr1 line was confirmed by PCR (data not shown); Western blots using mouse antiserum raised to a recombinant MYR1 (representing amino acids 155 to 328 of the protein) (Fig. 2B; also, see Materials and Methods) further confirmed both the deletion and successful complementation (Fig. 3A).

HFFs were infected with the deleted and complemented parasites, and lysates of infected cells were prepared at 20 hpi. Wild-type and mock-infected cells served as positive and negative controls for c-Myc induction, respectively. The results (Fig. 3A) showed a much-reduced level of host c-Myc in cells infected with the Δmyr1 parasites compared to cells infected with wild-type or complemented parasites, indicating that MYR1 is indeed necessary for c-Myc induction.

**MYR1 is cleaved in a process dependent on Toxoplasma’s ASP5 protease.** Although the MYR1 protein is predicted to be about 80 kDa after removal of the signal peptide, Western blots of purified wild-type RH (RH-WT) and RHΔmyr1::MYR1 complemented parasites revealed two bands using the anti-MYR1 antibody that migrate as if they were ~105 kDa and ~80 kDa, respectively (Fig. 3B). Western blots of the C-terminally HA-tagged RHΔmyr1::MYR1 line using anti-HA antibodies also yielded a band migrating at ~105 kDa, but instead of the ~80-kDa band, there was a band that migrated at ~32 kDa (Fig. 3C). Given that the recombinant protein used to generate the anti-MYR1 antibodies corresponded to the N-terminal portion (Fig. 2B), and the ~32-kDa band was detected only using the anti-HA antibodies and not with the anti-MYR1 antibodies, these results indicate that MYR1 is initially synthesized as a precursor that migrates as if it is ~105 kDa and that this protein is then proteolytically cleaved to yield fragments corresponding to about the N-terminal three-fourths and C-terminal one-fourth of the protein. The slow migration of the full-length MYR1 and the N-terminal three-fourths could be a result of posttranslational modifications and/or MYR1’s acidic pI (~5.0).

Near the predicted cleavage site based on size is the amino acid sequence RRLSE (Fig. 2B). This is reminiscent of the Plasmodium export element, or PEXEL motif (28, 29), that is processed by plasmepsin V, an aspartyl protease in Plasmodium parasites (30–32). As recently shown by Coffey et al., aspartyl protease 5 (ASP5), a Toxoplasma orthologue of the Plasmodium plasmepsin, is necessary for MYR1 cleavage and mutation of the RRLSE sequence to ARLSE abrogates cleavage, strongly suggesting that MYR1 is cleaved by ASP5 within this pentapeptide, likely between the L and
S, based on the specificity of ASP5 (33). The role of this cleavage is not known, but it could be related to the trafficking and/or biochemical function of MYR1.

**MYR1 is secreted into the PV and associates with the PVM.** The complemented strain used in the studies described above used an ectopically expressed version of MYR1, driven off the GRA1 promoter. GRA1 is a very abundantly expressed dense granule protein, and this strain served the purpose of showing that complementation of the /H9004 m yr1 mutant with ectopic MYR1 rescues the c-Myc regulation phenotype. For determining the true localization of MYR1, however, we needed to be sure the protein was expressed at normal levels and at the correct time in the parasite’s cell cycle. We therefore also created a strain where the native MYR1 locus was engineered to encode a C-terminal HA tag (RH: MYR1-HA). Control experiments confirmed that the expression of this tagged MYR1 were comparable to the levels seen in wild-type parasites (Fig. 4A).

HFFs were infected with RH:MYR1-HA, fixed with methanol at 12 hpi, and examined by confocal microscopy using antibodies to the HA tag (to detect the C-terminal domain) or antibodies to the N-terminal rMR1(155–328). Rabbit antibodies to GRA7 were used as a counterstain and a marker for a secreted protein. The results showed that, like GRA7, both the C- and N-terminal domains of MYR1 are abundantly present within the PV with rare puncta within the parasites themselves (Fig. 4B). Staining for both domains was also seen at the PVM, especially in cells that had vacuoles containing only one or two parasites. Colocalization with anti-GRA7 signal was seen in all three areas although the data did not allow us to reach a firm conclusion on whether MYR1 and GRA7 originate in the same structures (dense granules) within the parasite. This ambiguous, “dense-granule-like” staining is similar to what has been reported for GRA24 (8). Overall, however, these data clearly show that, like GRA7, MYR1 is a secreted protein and that both domains are present in the PV space, with a considerable amount at the PVM itself.

Consistent with these localization results, a recent phospho-proteomics study of tachyzoite-infected fibroblasts by Treeck et al. (24) indicated that MYR1 is specifically phosphorylated outside the parasite (i.e., within the PV and/or the infected host cytosol) (34). The positions of the four phosphorylated serines identified in that study are depicted in Fig. 2B.

**MYR1 is necessary for Toxoplasma’s impact on host cell cycle.** c-Myc is a potent transcription factor whose up-regulation likely affects the host cell in profound ways. One such impact might be on progression through the cell cycle, given c-Myc’s demonstrated role in this key pathway (10, 11, 35–38). To examine this, we used propidium iodide to stain host cells that were first infected with either wild-type RH tachyzoites or RH*/H9004 m yr1. The results (Fig. 5) showed that relative to uninfected cells, RH infection results in a profound dysregulation of the host cell cycle, with many cells showing four times the normal complement of DNA, i.e., 8n. The ploidy profile of host cells infected with the Δm yr1 mutants, on the other hand, was indistinguishable from that of uninfected cells. This indicates that MYR1 is needed for Toxoplasma’s ability to disrupt the host cell cycle, possibly through its role in the up-regulation of c-Myc. Alternatively, the role of MYR1 in this effect on host cell cycle could be through an indirect effect involving other parasite effectors or host proteins other than c-Myc.
MYR1 is a Novel Protein in Toxoplasma Virulence

MYR1 plays a crucial role in the physical translocation of GRA24 from the host cytosol to the host nucleus during Toxoplasma infection. To directly test if MYR1 plays a role in the trafficking of GRA24 to the host nucleus, we introduced a plasmid expressing an epitope-tagged GRA24 into the RH-WT, RH \textit{myr1} mutants to rescue the defect. Hence, either MYR1 is the effector responsible for IRF1 up-regulation, or, like GRA16 and GRA24, the effector involved is dependent on MYR1 for its functionality.

MYR1 is necessary for trafficking of GRA24 to the host nucleus. To directly test if MYR1 plays a role in the trafficking of \textit{Toxoplasma}'s exported effectors, we examined the location of GRA24 in the RH \textit{myr1} mutants by immunofluorescence analysis of infected cells. To do this, we introduced a plasmid expressing an epitope-tagged GRA24 into the RH-WT, RH \textit{myr1} and RH \textit{myr1::MYR1} strains and then stained the infected cells at 20 hpi. The results (Fig. 8) showed that the RH \textit{myr1} mutants are indeed defective in their ability to traffic GRA24 to the host nucleus, whereas RH-WT and the RH \textit{myr1::MYR1} complemented strains show efficient translocation. This indicates that MYR1 plays a crucial role in the physical translocation of GRA24 from the PV into the host cell.

MYR1 mutants exhibit no growth defect \textit{in vitro} but have decreased virulence \textit{in mice}. Given MYR1’s involvement in sev-
eral key pathways involving interactions with the host, we assessed its growth phenotype in vitro and virulence in vivo. The ability of the \( \Delta \text{myr1} \) parasites to grow in HFFs in vitro was first measured. The results (Fig. 9A and C) showed that growth of both the RH\( \Delta \text{myr1} \) and ME49\( \Delta \text{myr1} \) parasites in vitro was indistinguishable from that of wild-type RH and ME49, respectively, consistent with the fact that the pathways shown here to be dependent on MYR1 are generally dispensable for growth in HFFs.

To determine if the mutants had a defect in vivo, C57BL/6 mice were infected intraperitoneally (i.p.) with ME49, ME49\( \Delta \text{myr1} \), or the complemented strain ME49\( \Delta \text{myr1}:\text{MYR1} \), and survival and weight loss were assessed. The results (Fig. 9B) showed that all mice infected with wild-type ME49 parasites succumbed by 9 days postinfection (dpi), whereas all of the mice infected with ME49\( \Delta \text{myr1} \) mutant parasites survived. Similar results were obtained with the RH lines, except that the effect of lacking MYR1 was less dramatic with a reproducible delay only in the mean time to death: infection with the wild type and the complemented RH lines resulted in death by 9 to 10 dpi, versus days 10 to 13 for the RH\( \Delta \text{myr1} \) line (Fig. 9D). All infected mice (including the RH\( \Delta \text{myr1} \)-infected mice) underwent rapid weight loss (an expected symptom in mice infected with Toxoplasma) after about day 5 (data not shown). Thus, MYR1 is necessary for full virulence and is especially important in mouse infections with type II strains.

**DISCUSSION**

Using reporter cells from transgenic mice with a c-Myc–GFP fusion, we were able to isolate Toxoplasma mutants deficient in the induction of host c-Myc. Three such mutants were characterized, and all were found to be deficient in a single secreted protein, dubbed MYR1. Detailed phenotyping of \( \Delta \text{myr1} \) mutants showed a profound defect in the induction of not only host c-Myc but also several other phenotypes involving interaction with the host, including the GRA16-mediated translocation of PP2A, the GRA24-mediated phosphorylation of p38 MAPK, and the induction of IRF1 in the presence of IFN-γ, mediated by an as-yet-unidentified effector. Interestingly, however, the \( \Delta \text{myr1} \) mutants were not defective in host mitochondrial association or NF-κB activation, processes mediated by the dense granule proteins MAF1 and GRA15, respectively. These findings argue that MYR1’s role is for only a subset of the effectors that originate in dense granules. MAF1 is known to operate at the PVM, and GRA15’s final location is not known (it has never been observed outside the PV/PVM), whereas GRA16 and GRA24 clearly traffic to the host nucleus (7, 8). It appears, therefore, that MYR1 functions only in the trafficking of dense granule proteins that physically translocate, in their entirety and as soluble entities, across the PVM.

Our data do not address when or where MYR1 is cleaved into its two domains, and the fact that both give an indistinguishable signal by IFA leaves open the possibility that the cleavage occurs after release from the parasites. The fact that ASP5, the enzyme apparently responsible for the cleavage (33), is found within the Golgi apparatus (33, 41, 42), however, strongly suggests that cleavage occurs prior to release and that the two domains either remain associated or independently target the same ultimate location in the PV/PVM. Both the N- and C-terminal domains are abundant within the PV. The presence of two predicted TM domains in the C-terminal domain strongly suggests that MYR1 is integral to a membrane, likely some combination of the membranous nano-tube system of the intravacuolar network and the PVM, both of which appear normal by electron microscopy in the RH\( \Delta \text{myr1} \) mutants (S. Rastogi and M. Panas, unpublished data). Such an association is consistent with the role of MYR1 in translocating proteins into the host cytosol.

The splicing of the MYR1 transcript appears to differ somewhat between strains based on RNA-Seq data from many labs available on ToxoDB: in lab strains that have been passaged in vitro for many years, such as the common RH, GT1, and ME49 strains, the intron is removed in about 50 to 70% of the 254,470 mRNA species. In “wild” isolates that have not been passaged routinely in the lab, such as the ~20 field isolates analyzed by Minot et al. (43) and the type II Czech isolate used for studies of sexual differentiation in cats (44), this first intron is rarely if ever spliced out. As noted above, splicing out this intron results in a protein that lacks the first 78 amino acids compared to the version encoded by the mRNA with this intron intact, and the resulting N-terminally truncated version of MYR1 would lack a predicted signal peptide and thus presumably not be secreted. This suggests that there may be some cost to secreting a large amount of MYR1 and that repeated passage in vitro selects for decreased expression of secreted MYR1, perhaps because MYR1-mediated transport of effectors into the host cell is crucial only in vivo, where manipulation of the host’s immune response is required.

The precise role played by MYR1 in mediating the effects of GRA16 and GRA24, including transit of GRA24 to the host nucleus, is not clear. Interestingly, BLAST analysis of just the C-terminal domain of MYR1 showed that outside Toxoplasma, Hammondia, and Neospora, the top two matches were a hypothetical protein of Pseudomonas brassicaceae (PMG1108784; E value, 0.005) and TatC, a “Sec-independent periplasmic protein translocon” of Halorhabdus utahensis (E value, 0.64). TatC is part of a complex involving two other proteins, TatA and TatB, that translocates folded proteins containing a twin-arginine motif in their signal peptide across a membrane in a Sec-independent manner (45). This system is evolutionarily conserved, being present in archea, bacteria, chloroplasts, and plant mitochondria. Given that MYR1 appears to play a role in translocating proteins across a membrane (the PVM), and the putative homology was in the region that includes the two predicted transmembrane domains of MYR1, it is tempting to speculate that MYR1 is indeed a Homo-
FIG 7  MYR1 is necessary for the function of several effectors, including GRA16 and GRA24. (A) Effect of MYR1 on GRA24-mediated p38 MAPK activation. An immunofluorescence assay was performed on confluent HFFs that were uninfected or infected with the indicated strains. At 20 hpi, cells were fixed and stained using anti-phospho–p38 MAPK antibody. Bar, 5 μm. (B) Effect of MYR1 on GRA16-mediated PP2A nuclear translocation. The images are as described for panel A except that cells were stained using antibody to the PP2A-B subunit. (C) Role for MYR1 in the block of IFN-γ-induced IRF1 activation. The images are as described for panel A except that HFFs were infected for 14 h with the indicated parasites expressing mCherry, stimulated with 200 units/ml of IFN-γ for 6 h, and fixed with formaldehyde. Cells were stained using anti-IRF1 antibody. White arrowheads highlight the host nuclei in infected cells. UI, uninfected. (D, E, and F) p38 phosphorylation and nuclear accumulation, PP2A nuclear translocation, and IRF1 nuclear accumulation were quantified. Data are means and SEM from three independent assessments. **, P < 0.01.
logue of TatC, even though the E value was weak. BLAST analysis of the Toxoplasma genome with the TatA and TatB proteins did not reveal any apparent homologues (data not shown), but given the very weak extent of similarity between MYR1 and TatC, orthologues may not be readily detected by this method. Future studies aimed at isolation of MYR1-associated proteins will help address this and the question of whether the N- and C-terminal domains of MYR1 associate in infected cells.

Our data do not address whether one or both portions of MYR1 are necessary for the c-Myc up-regulation and the other MYR1-dependent phenotypes described here. Given the tantalizing similarity of the C-terminal domain to TatC, we attempted to ectopically express this domain, including the ASP5-dependent cleavage site, fused to the MYR1 signal peptide, but the resulting protein was not stably expressed. It may be that proper folding, secretion, and/or localization within the PV/PVM of both domains is dependent on their being synthesized as a single protein to start.

Our results showing attenuated virulence of the Δmyr1 mutants indicate a role for MYR1 in the pathogenesis of Toxoplasma in mice, especially for type II strains like ME49. While not directly demonstrated, such a role for MYR1 seems likely to be through its impact on several key pathways that Toxoplasma tachyzoites disrupt in the infected cell: c-Myc, p53, p38 MAPK, and IRF1. All these data are consistent with the fact that MYR1 is dispensable for growth in vitro.

No obvious orthologue of MYR1 is detectable in the related parasite Plasmodium falciparum, which is also known to translocate proteins across the PVM, in this case into the erythrocyte cytosol (13–15). This suggests that either the machinery has di-

FIG 8 MYR1 plays a role in translocation of GRA24 to the host nucleus. HFFs were infected with RH-WT, RHΔmyr1, or RHΔmyr1::MYR1 parasites expressing a Myc-tagged GRA24, and at 20 hpi, the cultures were fixed and stained with antibodies to the Myc tag. Bar, 5 μm.

FIG 9 MYR1 mutants exhibit wild-type growth in vitro but decreased virulence in mice. (A) Growth comparison of ME49 strains in vitro. HFF were infected with the indicated strains of parasites for 22 h, and the number of parasites in each vacuole was then assessed. The percentage of vacuoles demonstrating 2, 4, 8, or >8 parasites per vacuole is displayed. Data are averages for two independent replicates. (B) Kaplan-Meier survival curve for mice infected with ME49 parasites with or without MYR1. Five mice in each group were injected intraperitoneally with PBS or with 100 tachyzoites of Toxoplasma ME49, ME49Δmyr1, or ME49Δmyr1::MYR1. The results are representative of two experiments with similar outcomes. (C) As for panel A, growth of RH strains was assessed in HFFs at 16 h. The percentage of vacuoles demonstrating 2, 4, 8, or >8 parasites per vacuole is displayed. Data are averages for two independent replicates. (D) As for panel B, survival was assessed in mice injected intraperitoneally with PBS or with 100 tachyzoites of Toxoplasma RH (5 mice per group), RHΔmyr1 (6 mice per group), or RHΔmyr1::MYR1 (5 mice per group). The results are representative of two experiments with similar outcomes. *, P < 0.05; **, P < 0.01.
verged in sequence to the extent that it is no longer identifiable by BLAST or different machinery suffices for this purpose. Indeed, *Plasmodium* spp. are known to use a PTEX complex for translocating proteins across the PVM (13–15), and so perhaps that machinery functions without the need for a MYR1-like protein. *Toxoplasma* encodes a clear orthologue of at least one of the key components of the PTEX, EXP2 (GRA17 in fetal bovine serum (FBS), 2 mM L-glutamine, 100 U ml−1 penicillin, 100 µg ml−1 streptomycin, 1 mM N-ethyl-N-nitrosourea (ENU)). After a 2-h incubation at 37°C, mutagenized cells were washed three times for 10 s at room temperature by rinsing the monolayer with 10 ml cold 1× phosphate-buffered saline (PBS), scraped, syringe-lysed into 3 ml PBS, counted, and added to a new flask with HFFs. Mutagenized parasites were expanded for 24 h in HFFs prior to screening for c-Myc mutants. The percent killing that resulted from ENU mutagenesis was assessed by counting plaques resulting from mutagenized and dimethyl sulfoxide (DMSO)-treated parasites. Briefly, 100 parasites were added to a flask containing an HFF monolayer, and plaques were counted after 10 days of incubation at 37°C.

**BMM isolation, culture, and infection.** Mouse bone marrow macrophages (BMMs) were derived from the femurs and tibias of mice via culture for 8 days in cDMEM plus 20% (final concentration) macrophage colony-stimulating factor (M-CSF)-containing medium. Expanded BMMs were infected with *Toxoplasma* or *Neospora* parasites at an MOI of 0.25 and incubated for 20 h at 37°C. Infected cells were washed and incubated in 1× PBS at 4°C for 10 min. BMMs were then harvested by scraping, centrifuged, resuspended in 250 µl PBS containing 0.5% FBS, and filtered using tubes with cell strainer caps (BD Biosciences). Flow cytometry analysis was performed as described below.

**Flow cytometry.** Infected cells were harvested by washing once with PBS and treating with trypsin for 5 min at 37°C. After one PBS wash, cells were resuspended in 0.5% FBS-PBS and filtered using tubes with cell strainer caps (BD Biosciences). Fluorescence was detected by the Falstaff BD Aria II sorter (Stanford FACs facility). Flow cytometry data were analyzed using FlowJo.

**Whole-genome sequencing.** Isolated mutant populations were singly cloned by limiting dilution followed by expansion in HFFs. Genomic DNA from three mutant strains and a wild-type strain was isolated using genomic DNA Clean & Concentrator (Zymo Research).

For the DNA library preparations, 1 µg of gDNA was first sheared down to 200 to 300 bp using the Covaris S2 per the manufacturer’s recommendations. Paired-end sequencing libraries were prepared using Illumina’s TruSeq PCR free sample preparation kit. The target insert size of 200 to 250 bp was size selected using SPRI Ampure XP purification. Following DNA library construction, library size distribution was checked using the Agilent Bioanalyzer high-sensitivity assay. Library quantification was done via qPCR (Stratagene MX3000P). DNA libraries were sequenced using the Illumina HiSeq 2000 in one lane on a flow cell with sequencing paired-end read length at 2 × 50 bp. Reads were demultiplexed using CASAVA (version 1.8.2).

**DNA sequencing data processing and SNV analysis.** The DNA sequencing reads in Fastq format were first checked for quality using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/).

Sequencing adapter sequences present in the reads were trimmed using SeqPrep (https://github.com/jstjohn/SeqPrep). Sequencing reads were then mapped using Bowtie2 (48) with default parameters against the reference genome of *Toxoplasma gondii* GT1 strain downloaded from ToxoDB (release 10.0) (49). Single nucleotide variants (SNVs) that were present in mutant strains and not in the wild type were identified using RADIA (https://github.com/arande/seq/seqPrep) (50). RADIA used the SAMtools (51) (mpileup command [version 0.1.18]) to examine the base calls at each locus of the genome in parallel for the wild type and the mutant sample. The BAM files generated by Bowtie2 were used as input, and output variants were in variant call format (VCF) (https://github.com/samtools/hts-specs). For each base, the following cutoffs were used: a minimum Phred base alignment score of 10 and a maximum Phred mapping quality score of 10. Additional SNV filtering steps were a requirement for an overall read depth of 10 for both the wild type and the mutant strain and a minimum read depth that supported the alternative allele of 4 and 80% of the total read depth.

**Western blotting.** Cell lysates were prepared at 20 h postinfection (hpi), and the total protein concentration of lysates was determined by a Bradford protein assay (Bio-Rad). Samples containing 20 µg of protein were boiled for 5 min, separated by SDS-PAGE, and transferred to polyvinylidene difluoride (PVDF) membranes. c-Myc was detected by incubation of membrane with rabbit anti-N-terminus c-Myc antibody (Y69;
Abcam) and followed by incubation with hors eradish-peroxidase conjugated goat anti-rabbit IgG. The levels of horseradish peroxidase (HRP) were detected using an enhanced chemiluminescence (ECL) kit (Pierce). The expression of MYR1 in parasite strains was detected using mouse anti-MYR1 primary antibodies or anti-hemagglutinin (anti-HA) antibodies (in complemented parasites) and secondary goat anti-mouse IgG. Host GAPDH levels served as a loading control: membranes were stripped with stripping buffer (Thermo Scientific) and stained with mouse anti-GAPDH primary antibody and secondary HRP-conjugated goat anti-mouse IgG antibody. The levels of HRP were measured as stated above. SAG1 levels were used to control for the levels of parasites within the infected cells. Again, membranes were stripped with stripping buffer and stained with rabbit anti-SAG1 followed by staining with secondary HRP-conjugated goat anti-rabbit IgG antibody and detecting the levels of HRP as described above.

**Generation of RHΔmyr1 and ME49Δmyr1 parasites.** To generate RHΔmyr1 parasites, the parental RHΔhxgprt strain, deficient in the hypoxanthine-xanthine-guanine phosphoribosyl transferase (HXGPRT) gene, was used (52). Previously described pTKO2 vector (53), which carries the HXGPRT gene flanked by loxp sites, was modified by flanking the HXGPRT gene with sequences that correspond to 5′ and 3′ genomic regions adjacent to the MYR1 coding region (forward) and 5′-GCCCGCTAGCCCTCTCTTATCTTGCAG-3′ (reverse). The resulting DNA fragment was then inserted into the 3′ multiple cloning site (MCS) flanking the HXGPRT gene in pTKO2 vector using HindIII and Nhel restriction enzymes. Next, the 5′ flanking region of approximately 1–1.2 kb was amplified using the following primers: 5′-GCCCGGTACCCTTCAGCCCTTGAGGTTGAGAAGC-3′ (forward) and 5′-GCCCGCTAGCCCTCTTATCTTGCAG-3′ (reverse). The resulting DNA fragment was then cloned into the 5′ MCS of the pTKO2 vector using Kpn1 and Xhol restriction enzymes. Twenty-five micrograms of the resulting non-linearized pTKO2_MYR1_KO plasmid was transfected into RHΔhxgprt parasites via electroporation. Transfected parasites were cultured in HFFs grown in 24-well plates, and after 24 h, their medium was replaced with selective medium containing 50 µg/ml mycophenolic acid and 50 µg/ml xanthine to select for parasites expressing HXGPRT. After two passages in selective medium, parasites were single-cell cloned by limiting dilution. The resulting parasite strains were tested for HXGPRT integration at the 5′ and 3′ ends, and the absence of MYR1 was confirmed by PCR using primers homologous to the MYR1 open reading frame (data not shown). Ultimately, the loss of MYR1 expression in parasites was confirmed by Western blotting using the mouse anti-MYR1 antibody (Fig. 3). To generate ME49Δmyr1, the above-described transfection was repeated using ME49-lucΔhxgprt (the HXGPRT-deficient type II ME49 strain that expresses luciferase). The same pTKO2_MYR1_KO vector was used to delete MYR1 in ME49-lucΔhxgprt and RHΔhxgprt parasites.

**Ectopic complementation of RHΔmyr1 and ME49Δmyr1 parasites.** To ectopically complement RHΔmyr1 parasites, the HXGPRT selection marker flanked by loxp sites was first removed from the knockout parasites via CRE-mediated DNA recombination as previously described (53). Briefly, myr1-deficient parasites were transiently transfected with a vector carrying CRE recombinase, and Δhxgprt parasites were selected using cDMEM supplemented with 350 µg/ml 6-thioxanthine. Parasites were then single-cloned by limiting dilution and the loss of HXGPRT was confirmed by their inability to grow in media supplemented with mycophenolic acid/xanthine. The resulting RHΔmyr1 Δhxgprt strain was then used to generate the complemented RHΔmyr1::MYR1 parasites by transfecting with a vector carrying the coding region of RH MYR1. This vector was generated by modifying the pGRA1_HA_HXGPRT vector (54): the open reading frame (ORF) of MYR1 lacking the stop codon was amplified from RH genomic DNA using the following primers: 5′-GCCGATGATGTCGCAGGTTCCCTCTCCTGATAACAGCTAGC-3′ (forward) and 5′-GGGCCCATGGCCATTATGCTGACTGACGGAAATGACG-3′ (reverse). The resulting ~4.4-kb fragment was digested with NsiI and Ncol and cloned into the pGRA1_HA_HXGPRT vector, and the resulting Δhxgprt parasites were selected using MPA/XAN selective medium as described above. MYR1 expression in cloned parasites was confirmed by Western blotting using anti-MYR1 and anti-hemagglutinin (anti-HA) antibodies.

To generate ME49Δmyr1::MYR1 complemented parasites, the above-described steps were repeated using the ME49Δmyr1 strain, except that the coding region of MYR1 was amplified from the ME49 genomic DNA using the primers described above.

**C-terminal HA-tagging at the endogenous MYR1 locus.** To construct the vector pTKO2_MYR1_HA for C-terminal endogenous HA-tagging of MYR1, approximately 3,000 bp of the MYR1 coding sequence and hemagglutinin (HA) tag were amplified from the pGRA1_MYR1_HA_HXGPRT complementation plasmid using the primers 5′-GGGCCCATGGCCATTATGCTGACTGACGGAAATGACG-3′ (forward) and 5′-GGCGCAAGCTTCGAGAGACAAATTATCCAGCA-3′ (reverse). The resulting insert was cloned into NotI and EcoRV sites of the pTKO2 mCherry vector. The plasmid was linearized at the SmaI site in the insert, and 25 µg of the linearized plasmid was transfected by electroporation into the RHΔΔhxgprt strain. Parasites were allowed to infect HFFs in T25 flasks for 24 h, after which the medium was changed to complete DMEM supplemented with 50 µg/ml MPA and 50 µg/ml XAN for HXGPRT selection. Parasites were passaged twice before being singly cloned into 96-well plates by limiting dilution. Screening for correct integration into the endogenous locus was performed by PCR using multiple primers, including 5′-GATTCCAGCTCCTCTGACCAT-3′ and 5′-GGCGGatctcGgtcat-3′, as well as by Western blotting.

**Generation of polyclonal anti-MYR1 antibodies.** N-terminal glutathione S-transferase (GST)-fused proteins were expressed using the pGEX-6P1 plasmid (Agilent Technologies). The N-terminal exon region of MYR1 was amplified using the primers 5′-AGATTCTCTAGCCGACAGAACCAGC-3′ and 5′-AGCCGGCCCTGCGTCCCCAGAGAGACCTG-3′ (corresponding to amino acids 155 to 328, inclusive). The resulting recombinant protein was purified from Escherichia coli (Rosetta strain; Novagen/EMD Millipore) and injected intraperitoneally into female BALB/c (Charles River) mice with a Sigma Adjuvant System (Sigma) according to the manufacturer’s instructions. Blood of naive mice was drawn prior to injection, and mice with sera that exhibited the lowest baseline reactivity against monolayers of Toxoplasma-infected cells were selected for antibody generation. Mice were injected with the initial dose of 100 µg protein/mouse followed by boosters of 50 µg protein/mouse on days 21, 40, 65, and 89 after the initial injection. Serum from these mice was isolated on days 33, 51, 75, and 113 after the initial injection. All animal experiments were conducted with the approval and oversight of the Institutional Animal Care and Use Committee at Stanford University.

**Immunofluorescence microscopy.** Infected cells grown on glass coverslips were fixed using 2.5% formaldehyde in PBS for 20 min or using methanol, as indicated in the text. Samples were washed one to three times with PBS and blocked using 3% bovine serum albumin (BSA) in PBS for 1 h at room temperature. Cells were permeabilized with 0.2% Triton X-100–3% BSA–PBS for 10 min at room temperature. MYR1 protein was detected with mouse anti-MYR1 or rat anti-HA primary antibodies and secondary antibodies conjugated to 488-nm fluorochrome. c-Myc was labeled using rabbit anti-c-Myc antibodies and 488-nm-fluorochrome-conjugated goat anti-rabbit IgG. GRA7 protein was detected by mouse anti-GRA7 and 594-nm-fluorochrome-conjugated goat anti-rabbit IgG. NF-kB was detected with SC-109 (Santa Cruz Biotechnology). To assess host mitochondrial association, infected cells were fixed 12 to 14 hpi using 2.5% formaldehyde diluted in prewarmed medium and then permeabilized and blocked as described above. Mitochondria were detected with a
rabit anti-TOM20 antibody (Santa Cruz Biotechnology) used at 1:100. Phospho-p38 MAP kinase and PP2A-B subunit were labeled using rabbit anti-phospho-p38 (Thr180/Tyr182) antibody (no. 9211; Cell Signaling Technologies) and rabbit PP2A B subunit antibody (no. 4953; Cell Signaling Technologies), respectively, followed by 488-nm-fluorochrome-conjugated goat anti-rabbit IgG. Vectashield with 4′,6-diamidino-2-phenylindole (DAPI) stain (Vector Laboratories) was used to mount the coverslips on slides. Fluorescence was detected using either wide-field fluorescence microscopy or by confocal microscopy using an LSM510 or LSM710 inverted confocal microscope (Zeiss). Images were analyzed using ImageJ or FIJI. All images shown for any given condition or staining in any given comparison or data set were obtained using identical parameters.

Cell cycle assessment. RAW264.7 murine macrophage-like cells were infected with parasites at an MOI of 3. At 19 h, cells were harvested and split into two populations, one to assess infection rate and one to assess cell cycle. Infection rates were approximately 97%. Cells intended for cell cycle analysis were fixed and permeabilized with methanol for 20 min and then stained with propidium iodide for 30 min before being assessed on an LSRII flow cytometer.

IRF1 staining. HFFs were infected at an MOI of 0.1 for 14 h before 200 units/ml of human IFN-γ was added. Six hours after the addition of IFN-γ, cells were fixed with formaldehyde and stained with the mouse monoclonal antibody clone 20/IRF1 (catalogue no. 612046; BD Biosciences, San Jose, CA, USA).

GRA24 localization. Tachyzoite-infected HFFs were scraped and lysed using a 27-gauge needle and spun down, and the parasites were then electroporated with 50 μg of the pHTU-GRA24-3XMyc plasmid expressing C-terminal Myc-tagged GRA24 off the tubulin promoter (33). The transfected and mock-transfected parasites were allowed to infect confluent HFFs for 20 h before the monolayers were fixed with 3% formaldehyde and stained using Myc-tag antibody (M4439; Sigma).

Mouse infections. Tachyzoites were grown in HFFs and isolated from infected cells by scraping of the cell monolayer and syringing lysis using a 27-gauge blunt needle. After one wash in PBS, parasites were counted and diluted in PBS. Female C57BL/6 mice were used for studies with ME49 parasites, and female CBA/J mice were used for studies with RH parasites. Each mouse was inoculated via intraperitoneal (i.p.) infection with 100 tachyzoites in a 200-μl volume. Plaque assays were performed on a sample of the inoculum to quantify the number of viable tachyzoites in each sample, and only experiments where comparable numbers were obtained were included in our analyses. All animal experiments were conducted with the approval and oversight of the Institutional Animal Care and Use Committee at Stanford University.

Statistics. Statistical analysis was performed with Prism version 6.04 software. For c-Myc, NF-κB, PP2A, p38, and IRF1, comparisons drawn with the approval and oversight of the Institutional Animal Care and Use Committee at Stanford University provided the Stanford Graduate Fellowship to Nicole D. Marino.

REFERENCES


