Macrophages play an essential role in the early immune response against *Toxoplasma* and are the cell type preferentially infected by the parasite in vivo. Interferon gamma (IFNγ) elicits a variety of anti-*Toxoplasma* activities in macrophages. Using a genome-wide CRISPR screen we identify 353 *Toxoplasma* genes that determine parasite fitness in naïve or IFNγ-activated murine macrophages, seven of which are further confirmed. We show that one of these genes encodes dense granule protein GRA45, which has a chaperone-like domain, is critical for correct localization of GRAs into the PVM and secretion of GRA effectors into the host cytoplasm. Parasites lacking GRA45 are more susceptible to IFNγ-mediated growth inhibition and have reduced virulence in mice. Together, we identify and characterize an important chaperone-like GRA in *Toxoplasma* and provide a resource for the community to further explore the function of *Toxoplasma* genes that determine fitness in IFNγ-activated macrophages.
Toxoplasma gondii is an obligate intracellular parasite that causes disease in immunocompromised individuals, such as HIV/AIDS patients, and when contracted congenitally. It causes lifelong infections by converting from rapidly dividing tachyzoite stages into encysted slow-growing bradyzoites, which mainly localize to the brain and muscle tissues. Once established in the host cell, Toxoplasma resides in a unique replication niche called the parasitophorous vacuole (PV), which is separated from the host cytoplasm by the PV membrane (PVM) and does not fuse with the endolysosomal system.

The cytokine interferon gamma (IFNy) is essential for the control of Toxoplasma replication in host cells. Mice with macrophages that can no longer respond to IFNy are extremely susceptible to Toxoplasma demonstrating their crucial role in IFNy-mediated control of Toxoplasma. IFNy-induced upregulation of immunity-related GTPases (IRGs) and guanylate binding proteins (GBP) are key mechanisms for controlling Toxoplasma in mice. IRGs and GBPVs can bind and vesiculate the PVM, eventually leading to the death of the parasite. It has been hypothesized that the PVM lumen is largely unknown. It has been hypothesized that specific chaperones in the dense granules and in the PV lumen could bind to these hydrophobic GRA effectors and aid their trafficking to the correct destination. Because PVM-localized GRAs are at the host-parasite interface they determine the acquisition of host-derived nutrients, can mediate the evasion of the host cytosolic immune response, and form the components of the translocon that secretes other GRAs into the host cytoplasm. It is therefore likely that these PVM-localized GRAs and the putative chaperones that mediate the correct trafficking of these GRAs to the PVM will be important for parasite fitness in IFNy-activated cells.

Many Toxoplasma genes determining parasite survival in IFNy-activated murine cells were initially identified using genetic crosses between strains that differ in virulence in mice. However, this approach fails to identify strain-independent genes. Here we used a genome-wide loss-of-function screen in the type I (RH) strain, which is resistant to IRGs/GBPVs-mediated killing, to identify 160 Toxoplasma genes that determine fitness in IFNy-activated murine macrophages and 193 genes that determine fitness in naive macrophages. Seven of the high-confidence hits were confirmed by testing single-gene knockouts in functional parasite growth assays in IFNy-activated murine macrophages. We demonstrate that GRA45, one of these hits, has a chaperone-like function and plays an important role in the trafficking and localization of GRA effectors to the PVM and the secretion of GRA effectors beyond the PVM.

Results

A loss-of-function screen identifies Toxoplasma genes that determine fitness in naive and IFNy-activated murine macrophages. We previously identified Toxoplasma genes that determine parasite fitness during infection of human foreskin fibroblasts (HFFs) using a genome-wide loss-of-function CRISPR/Cas9 screen. However, it is likely that this set of fitness-conferring genes varies by host cell type, host species, and after infection of IFNy-activated cells. The challenge in Toxoplasma genome-wide loss-of-function screens is to maintain the complexity of the mutant pool during the selection. It was previously reported that macrophages require stimulation by IFNy along with LPS or TNFα to effectively restrict Toxoplasma growth. Such stimulated macrophages are extremely effective in inhibiting Toxoplasma growth—even of virulent strains like the type I (RH) strain—which would likely create a bottleneck during the selection of the mutant parasite pool. IFNy stimulation by itself was sufficient for a modest but significant inhibition of Toxoplasma growth in C57BL/6J murine bone-marrow-derived macrophages (BMDMs) (Supplementary Fig. 1a). By contrast, IFNy + TNFα stimulation drastically restricted parasite growth (Supplementary Fig. 1b). Thus, we reasoned that a loss-of-function screen in macrophages stimulated with IFNy alone would reduce the random loss of mutants while, at the same time, allowing us to identify mutants that are susceptible to IFNy-mediated growth restriction. A Toxoplasma mutant pool was generated by transfecting RH parasites constitutively expressing Cas9 (RH-Cas9) with a library of sgRNAs containing ten guides for each of the 8156 Toxoplasma genes. This mutant pool was passaged in HFFs to remove mutants with general fitness defects (e.g., invasion and replication) before growing in naive and IFNy-activated BMDMs for three passages (Fig. 1a, b). Continuous passages in HFFs served as a control to identify mutants with fitness defects in naive BMDMs but not in HFFs. The relative abundance of sgRNAs was measured using Illumina sequencing...
Fig. 1 *Toxoplasma gondii* genome-wide loss-of-function screen in naïve or IFN-γ-activated murine bone-marrow-derived macrophages. **a** Screening workflow. At least $5 \times 10^6$ Cas9-expressing RH parasites were transfected with linearized plasmids containing 10 sgRNAs against every *Toxoplasma* gene. Transfected parasites were passaged in HFFs under pyrimethamine selection to remove non-transfected parasites and parasites that integrated plasmids with sgRNAs targeting parasite genes important for fitness in HFFs. Subsequently, the pool of mutant parasites was either continuously passaged in HFFs or passaged for three rounds in murine BMDMs that were left unstimulated or prestimulated with IFN-γ. The sgRNA abundance at different passages, determined by illumina sequencing, was used for calculating fitness scores and identifying genes that confer fitness in naïve or IFN-γ-activated BMDMs.

**b** Timeline for the generation of mutant populations and subsequent selection in the presence or absence of murine IFN-γ. Times at which parasites were passaged (P) are indicated. **c** Correlation between mean parasite gene fitness scores in Naïve BMDMs and HFF control. Orange dots indicate nine high-confidence candidate genes that confer fitness in naïve BMDMs compared to HFFs (Table 1). **d** Correlation between mean parasite gene fitness scores in IFN-γ-activated and Naïve BMDMs. Red dots indicate 16 high-confidence IFN-γ fitness-conferring candidate genes in murine BMDMs and orange dots the nine high-confidence genes conferring fitness in naïve BMDMs compared to HFFs (Table 1).

from the input library and the parasite DNA isolated from the early passage in HFFs (prior to BMDM infection), three additional passages on naïve (referred to as Naïve BMDM) or IFN-γ-activated BMDMs (referred to as IFNγ BMDM), and the late passage in HFFs (referred to as HFF control). We calculated the average log₂ fold change in abundance of sgRNAs targeting a specific gene in the samples (early passage HFF, Naïve BMDM, IFNγ BMDM, or HFF control) relative to the input library and defined it as the “fitness” score for that gene (Supplementary Data 1). The fitness scores of genes after early passage in HFFs were highly correlated ($r = 0.81 \pm 0.03$, mean ± SEM, $n = 3$) with previously determined HFFs “phenotype” scores, highlighting the reproducibility of these screens and demonstrating that up to the point of infecting the BMDMs, the mutant pool was behaving as expected.

Because the intracellular environments between murine BMDMs and HFFs are likely quite different, certain *Toxoplasma* genes could be important for fitness in naïve BMDMs but not HFFs. To identify this set of genes, we determined the probability of being fitness conferring in naïve BMDMs for each gene by comparing the fitness (Naïve BMDM vs. HFF control) distribution to those of 497 control genes from our previous study.\textsuperscript{35}
After removing the genes not expressed in murine macrophages\textsuperscript{36}, we identified 193 parasite genes (Supplementary Fig. 3b), including nine high-confidence hits (Fig. 1c and Table 1), with a significant negative enrichment ($p < 0.05$) and a large effect size (Cohen’s $d \geq 0.8$) compared to control genes (Supplementary Data 1). To determine if these *Toxoplasma* genes shared biological annotation we performed gene set enrichment analysis (GSEA)\textsuperscript{57} and Gene Ontology (GO) enrichment analysis (ToxoDB.org). We found significant enrichment of *Toxoplasma* genes involved in folate metabolism, purine metabolism, and parasite membrane-localized transporters (Supplementary Data 2), suggesting that nutrient availability to *Toxoplasma* in naïve BMDMs and HFFs is variable.

To identify parasite genes that determine fitness in IFNγ-activated BMDMs, we performed three independent screens with minor changes to the IFNγ concentration and time of stimulation: 100 ng/mL of IFNγ for 24 h (referred to as screen 1 or S1), 1 ng/mL of IFNγ for 24 h (referred to as screen 2 or S2), and 100 ng/mL of IFNγ for 4 h (referred to as screen 3 or S3; Fig. 1b). A lower IFNγ concentration (1 ng/mL) induced less parasite growth inhibition (Supplementary Fig. 1a). In addition, IFNγ (100 ng/mL) stimulation of murine BMDMs for 4 h induced a similar but slightly lower expression of gene sets that had $>2$-fold upregulation in IFNγ stimulation for 24 h (Supplementary Fig. 2a, b, and Supplementary Data 3). The screens with a lower IFNγ concentration (S2) or with the high concentration for less time (S3) indeed reduced the bottleneck and associated stochastic loss in *T. gondii* infected murine macrophages of other species\textsuperscript{38}, suggesting that nutrient availability to *Toxoplasma* in naïve BMDMs and HFFs is variable.

Single parasite gene knockouts from hits identified by CRISPR screens have fitness defects in IFNγ-activated BMDMs. To confirm some of the parasite genes that determine fitness in IFNγ-activated BMDMs, we generated individual knockouts of four genes (*TGGT1*\_269620, *GRA45*, *TGGT1*\_232670, and *TGGT1*\_263560) in luciferase-expressing RH parasites (Supplementary Fig. 4a–d). All knockouts had reduced growth compared to wild-type parasites in IFNγ-activated BMDMs as determined by luciferase assays at 24 h post-infection (p.i.) (Fig. 2a–d). We previously determined the contribution to the in vivo fitness of 217 *Toxoplasma* genes\textsuperscript{35}. Of the 31 genes that determined in vivo fitness, $\Delta$gra22 and $\Delta$TGGT1\_269950 parasites were outcompeted by wild-type in the peritoneum\textsuperscript{39}. *GRA22* and *TGGT1*\_269950 have average fitness scores $< -2$ in IFNγ-activated vs. naïve BMDMs (Table 1) suggesting that the in vivo fitness of these genes is likely due to their importance in determining parasite fitness in IFNγ-activated peritoneal macrophages. To confirm this, we tested the growth of $\Delta$gra22, $\Delta$TGGT1\_269950 and wild-type parasites in IFNγ-activated BMDMs and found that IFNγ significantly inhibited $\Delta$gra22 and $\Delta$TGGT1\_269950 parasite growth relative to wild-type parasites (Fig. 2e, f). Most of the knockouts had no, or a minor, fitness defect in naïve murine BMDMs (Supplementary Fig. 5a–f) or mouse embryonic fibroblasts (MEFs) (Supplementary Fig. 5g) confirming that these genes are specific for conferring fitness in the presence of IFNγ. $\Delta$TGGT1\_263560 and $\Delta$gra22 parasites formed larger plaques compared to wild-type parasites in MEFs, although this was only significant for $\Delta$TGGT1\_263560 (Supplementary Fig. 5g). Although the $\Delta$TGGT1\_263560 plaques were larger, they appeared quite different compared to wild-type plaques; the lysis area contained very few parasites and host cells, possibly due to an early egress phenotype (Supplementary Fig. 5h). To test the impact of candidate gene knockouts in a non-luciferase-expressing type I (RH) strain, we performed a growth competition assay, in which growth differences between wild-type and knockouts accumulate over three passages in both naïve and IFNγ-activated BMDMs. Agra22 parasites were outcompeted by wild-type parasites only in the IFNγ-activated BMDMs but not naïve BMDMs (Fig. 2g). Similar to Agra22 parasites, parasites lacking another high-confidence hit, ROM1, (Supplementary Fig. 4h) were outcompeted by wild-type parasites in IFNγ-activated BMDMs (Fig. 2h). Thus, the high-confidence parasite genes identified by our genome-wide loss-of-function screen indeed determine parasite fitness in IFNγ-activated BMDM.

**GRA45 is important for parasite fitness in IFNγ-stimulated macrophages of other species.** Of the seven genes we confirmed to have a fitness defect in IFNγ-activated BMDM, four genes (*GRA22, GRA45, TGGT1*\_263560, and *TGGT1*\_269950) encode known or putative secretory proteins with a predicted signal peptide (ToxoDB.org). GRA22 was previously characterized and...
Table 1 *Toxoplasma* genes that determine fitness in naïve or IFN-γ-activated macrophages.

### Genes with a fitness defect in naïve macrophages

| Gene ID     | Product description                                                                 | Naïve BMDM vs. HFF Control fitness (mean ± SEM) | p value (one-sided Wilcoxon signed-rank test) | Cohen’s d | IFN-γ vs. Naïve BMDM fitness | In vitro phenotype in HFFs | In vivo fitness |
|-------------|-------------------------------------------------------------------------------------|-------------------------------------------------|-----------------------------------------------|-----------|-----------------------------|--------------------------|-----------------|
| TGGT1_249580 | TgApiAT6-3                                                                           | −4.2 ± 1.1                                       | 0.001                                         | 7.6       | −0.2                        | 0.5                      | N/A             |
| TGGT1_247360 | PAP2 superfamily protein                                                             | −3.2 ± 1.0                                       | 0.002                                         | 5.8       | −1.2                        | 1.6                      | N/A             |
| TGGT1_250880 | Kinase, pKB family protein (Adenosine Kinase)                                       | −1.8 ± 0.4                                       | 0.002                                         | 3.4       | 1.2                         | 1.4                      | N/A             |
| TGGT1_290860 | TgApiAT6-2                                                                           | −1.5 ± 1.2                                       | 0.03                                          | 2.8       | 0.5                         | 10                       | N/A             |
| TGGT1_248560 | Hypothetical protein; homology to 26 S Proteasome regulatory subunit RPN2 (Swiss-Model) | −1.5 ± 0.5                                       | 0.003                                         | 2.8       | 0.1                         | 0.3                      | N/A             |
| TGGT1_308075 | Hypothetical protein; homology to DNase Pyocin AP41 (Swiss-Model)                   | −1.3 ± 0.2                                       | 0.003                                         | 2.5       | 0.7                         | 1.5                      | N/A             |
| TGGT1_224840 | 3'5'-cyclic nucleotide phosphodiesterase domain-containing protein                   | −1.3 ± 0.3                                       | 0.004                                         | 2.4       | 0.3                         | 0.4                      | N/A             |
| TGGT1_262650 | WD domain, G-beta repeat-containing protein                                          | −1.1 ± 0.4                                       | 0.007                                         | 2.1       | 0.2                         | 0.4                      | N/A             |
| TGGT1_269390 | CRAL/TRIO domain-containing protein                                                  | −1.0 ± 0.6                                       | 0.03                                          | 2.0       | −0.1                        | 0.1                      | N/A             |

### Genes with a fitness defect in IFN-γ-activated macrophages

| Gene ID     | Product description                                                                 | IFN-γ vs. naïve BMDM fitness (mean ± SEM) | p value (one-sided Wilcoxon signed-rank test) | Cohen’s d | Naïve BMDM vs. HFF control fitness | In vitro phenotype in HFFs | In vivo fitness |
|-------------|-------------------------------------------------------------------------------------|------------------------------------------|-----------------------------------------------|-----------|-------------------------------------|--------------------------|-----------------|
| TGGT1_269620 | DNA repair protein-like (Swiss-model)                                               | −4.0 ± 1.6                              | 0.004                                         | 2.9       | 1.5                                | −0.3                     | N/A             |
| TGGT1_209500 | DNA repair protein-like (HHpred)                                                    | −3.6 ± 1.8                              | 0.006                                         | 2.7       | 0.4                                | 0.1                      | N/A             |
| TGGT1_295340 | UV excision repair protein Rad23 protein                                            | −3.5 ± 1.0                              | 0.003                                         | 2.6       | 1.4                                | −0.4                     | N/A             |
| TGGT1_316250 | GRA45                                                                                | −3.3 ± 0.4                              | 0.002                                         | 2.4       | 1.0                                | 1.2                      | N/A             |
| TGGT1_232670 | Cytohesin-3-like; GEF of Arf6 (Swiss-Model)                                        | −3.3 ± 0.8                              | 0.003                                         | 2.4       | 0.3                                | 1.5                      | N/A             |
| TGGT1_321530 | Cathepsin L (CPL)                                                                   | −3.3 ± 0.9                              | 0.003                                         | 2.4       | 1.7                                | 0.7                      | N/A             |
| TGGT1_263560 | Putative GRA (Supplementary Fig. 5i)                                               | −3.0 ± 1.2                              | 0.006                                         | 2.2       | 1.5                                | 0.5                      | N/A             |
| TGGT1_208370 | GRA46                                                                                | −3.0 ± 1.6                              | 0.008                                         | 2.1       | 1.5                                | 1.8                      | N/A             |
| TGGT1_228300 | CCDC25 protein                                                                       | −2.9 ± 0.6                              | 0.003                                         | 2.1       | 0.3                                | −1.1                     | N/A             |
| TGGT1_215220 | GRA22                                                                                | −2.9 ± 1.8                              | 0.02                                          | 2.1       | 1.1                                | 1.0                      | Peritoneum       |
| TGGT1_314500 | SUB2                                                                                 | −2.8 ± 1.4                              | 0.01                                          | 2.0       | 0.8                                | 0.3                      | N/A             |
| TGGT1_227560 | Putative IWS1 transcription factor                                                  | −2.7 ± 1.7                              | 0.02                                          | 1.8       | 1.3                                | −0.7                     | N/A             |
| TGGT1_200290 | ROM1                                                                                 | −2.4 ± 1.3                              | 0.01                                          | 1.7       | 0.2                                | 0.8                      | N/A             |
| TGGT1_240980 | Cytoplasmic dynein-like (Swiss-Model/HHpred)                                        | −2.4 ± 0.9                              | 0.007                                         | 1.7       | 0.3                                | −1.0                     | N/A             |
shown to contribute to parasite natural egress\textsuperscript{62}, while GRA45 was recently discovered as an ASPS substrate with unknown function\textsuperscript{63}. TGGT1_263560 and TGGT1_269950 were annotated as hypothetical proteins (ToxoDB.org) without previous characterization. We observed that TGGT1_263560 colocalized with PV-resident GRA2 in intracellular parasites (Supplementary Fig. 5i) and considered it as a putative GRA. TGGT1_269950 was predicted as a putative GRA based on published dense granule proteomic analysis\textsuperscript{63} and hyperLOPIT spatial proteomic analysis\textsuperscript{64} (ToxoDB.org). IRG-mediated destruction of the PVM is one of the main mechanisms by which murine cells inhibit Toxoplasma growth. We therefore determined the IRGA6/IRGB6 coating on the vacuole of these knockouts (Fig. 3a, b). We observed slightly increased loading of IRGA6 (Fig. 3a), and a significantly larger fraction of ΔTGGT1_263560 and ΔTGGT1_269950 vacuoles with IRGB6 coating, but no difference in Δgra45 and Δgra22 parasites (Fig. 3b). This indicates that GRA45 and GRA22 employ different mechanisms to counter IFNγ-mediated parasite growth inhibition in murine macrophages.

Macrophages from different species have different mechanisms to control Toxoplasma growth\textsuperscript{65} and therefore knowing if these genes are important for conferring resistance to IFNγ in other species could help us narrow down the possible mechanism by which they do so. To test if these four GRAs are also important for parasite fitness in IFNγ-activated macrophages of other species, primary rat BMDMs and human THP-1 macrophages were stimulated with IFNγ or left unstimulated followed by infection with wild-type, Δgra45, Δgra22, ΔTGGT1_263560, or ΔTGGT1_269950 parasites (Fig. 3c–h). Similar to what was observed in naïve murine BMDMs, these knockouts had no, or a minor but not significant, fitness defects in naïve rat BMDMs and human THP-1 macrophages (Supplementary Fig. 6). Although Δgra22 parasites had significantly higher growth reduction in primary rat BMDMs activated with IFNγ (5 ng/mL) (Fig. 3c), Δgra45 parasites were the only knockout that was significantly more susceptible to IFNγ-mediated growth inhibition compared to wild-type parasites in both rat and human macrophages (Fig. 3c–h) indicating GRA45 plays an important function in the resistance of Toxoplasma to IFNγ in multiple species. We therefore decided to focus on determining the function of GRA45.

GRA45 has structural homology to protein chaperones and maintains the correct localization of GRAs to the PVM. GRA45 was recently shown to interact with GRA44 (TGGT1_221870) and With-No-Gly-loop (WNG)\textsubscript{2} kinase (TGGT1_240090), which also localize to dense granules\textsuperscript{19}. GRA45, GRA44, and WNG\textsubscript{2} have orthologues in coccidian species belonging to the Sarcocystidae (Neospora caninum, Hammondia hammondi, Cystoisospora suis, Sarcocystis neurona) with >40% amino acid identities and Eimeriidae (Eimeria spp. and Cyclospora cayetanensis) with >30% amino acid identities (ToxoDB.org). This is a rather unique phylogenetic profile as most GRAs are only conserved within the Toxoplasmatinae suggesting that these GRAs have a conserved function in these different parasite species. In addition, a GRA44 paralogue (TGGT1_295390) is exclusively expressed in cat intestinal stages, compared to GRA45, which is highly expressed in tachyzoite and bradyzoite stages and has moderate expression levels in sexual stages (ToxoDB.org). Searches for primary sequence homology or known protein domains failed to provide any suggestions for putative GRA45 functions. However, homology searches based on secondary-structure prediction indicated that the N-terminal region of GRA45 (from amino acids 72–225) has structural homology to the α-Crystallin domain (ACD) of small heat shock proteins (sHSPs) (Supplementary
Fig. 2 Validation of candidate genes that determine parasite fitness in IFNγ-stimulated murine BMDMs. a-f Murine BMDMs prestimulated with IFNγ (1 or 5 ng/mL) or left unstimulated for 24 h were infected with luciferase-expressing wild-type (WT) parasites or with parasites in which TGGT1_269620 (a, n = 6), GRA45 (b, n = 5), TGGT1_232670 (c, n = 5), TGGT1_263560 (d, n = 5), GRA22 (e, n = 4), or TGGT1_269950 (f, n = 5) was knocked out (MOI of 0.25). Parasite growth for each strain was measured by luciferase assay at 24 h.p.i. Parasite growth in IFNγ-activated BMDMs is expressed relative to growth in naïve BMDMs. Data are displayed as mean ± SEM with independent experiments indicated by the same color dots. The significance difference between WT and knockout was analyzed with two-tailed paired t-test. g, h Growth competition assay between GFP-positive WT parasites and GFP-negative Δgra22 or Δrom1 parasites was performed in murine BMDMs prestimulated with 1 ng/mL IFNγ or left unstimulated for three passages. The percentage of Δgra22 or Δrom1 was determined at the start of the competition and after three passages by plaque assay measuring the GFP-negative plaques vs. total plaques. Data are displayed as mean ± SEM with independent experiments (n = 3) indicated by the same color dots. The significant difference between the ratio of WT vs. knockout before and after competition was analyzed with two-tailed paired t-test.

Based on its structural homology to sHSPs, we hypothesized that instead of directly neutralizing IFNγ-mediated inhibition, GRA45 might perform a chaperone-like function for other GRA effectors, of which some are known to counteract the murine IFNγ response28,29,38. GRAs with a hydrophobic domain (such as a transmembrane domain or amphipathic α-helices) have peculiar trafficking and do not traffic to the parasite plasma membrane but instead traffic to the dense granules where they exist in partially soluble and aggregated states51. What prevents the aggregation of these hydrophobic domain-containing proteins in dense granules or insertion of their transmembrane domain

Fig. 7a), which are ATP-independent chaperones that maintain the native folding status of proteins under stress conditions. The conserved I/VxI/V motif is present in 90% of sHSPs was conserved in GRA45 and most of its orthologues (Supplementary Fig. 7a). The most significant structural homology was to Aggregation Suppressing Protein A (AgsA) from Salmonella typhimurium45 and HSP20 from Xylella fastidiosa66 (Supplementary Fig. 7a). The GRA45 C-terminal region (amino acids 260–335) had structural sology to DUF1812 domains67 and Mfa268, which share a transthyretin-like fold (Supplementary Fig. 7a). Transthyretin family members are known to bind and transport lipids such as retinol and thyroxine69. In addition, the top 10 models (TM-score 0.9–0.69) with GRA45 structural homology, as predicted by I-TASSER70, are predicted to bind hydrophobic substrates as they have acyltransferase domains. Overall these data indicate GRA45 harbors a potential chaperone-like function with preference for hydrophobic substrates. Consistent with published results that GRA45 remains in the PV lumen after secretion49, we observed that almost all GRA45 colocalized with PV-resident GRA1 and GRA2 in the PV lumen but only partially with PVM-associated GRA5 and GRA7, even though GRA45 colocalized with all these GRAs to dense granules in extracellular parasites (Supplementary Fig. 7b). Thus, GRA45 is unlikely to be a Toxoplasma effector that directly interacts with host proteins to modulate or inhibit IFNγ-induced anti-Toxoplasma activities.

Based on its structural homology to sHSPs, we hypothesized that instead of directly neutralizing IFNγ-mediated inhibition, GRA45 might perform a chaperone-like function for other GRA effectors, of which some are known to counteract the murine IFNγ response28,29,38. GRAs with a hydrophobic domain (such as a transmembrane domain or amphipathic α-helices) have peculiar trafficking and do not traffic to the parasite plasma membrane but instead traffic to the dense granules where they exist in partially soluble and aggregated states51. What prevents the aggregation of these hydrophobic domain-containing proteins in dense granules or insertion of their transmembrane domain...
into the ER membrane or the dense granule membrane is unknown but has been hypothesized to be mediated by dense granule protein chaperone(s)\textsuperscript{51,52}. To test the hypothesis that GRA45 is a dense granule protein chaperone, we first examined the solubility of hydrophobic GRA2, which has two amphipathic helices that mediate its membrane association post secretion\textsuperscript{71}. In parasite lysates generated by freeze/thawing, GRA2 was in both pellet and supernatant fractions in wild-type, Δgra45, and Δgra45 parasites complemented with a C-terminal HA-tagged version of GRA45 (Δgra45 + GRA45HA, Supplementary Fig. 4b, d) and ran according to its corresponding molecular weight (MW) (Fig. 4a). In addition to its predicted size, GRA2 appeared at a much higher MW in the pellet fraction from all strains suggesting insoluble protein aggregates as the bands were not completely separated.
during the gel migration. As a control, we used the parasite plasma membrane protein SAG1, which traffics to the parasite surface independent of the dense granules trafficking pathway. No high MW bands of SAG1 were observed in any of the parasite strains (Supplementary Fig. 10) indicating that the high MW bands of GRA2 were specific to dense granule proteins. After treatment with non-ionic detergents (NP-40), which solubilize membrane proteins by associating with their hydrophobic surface, monomeric GRA2 was completely solubilized and released into the supernatant fraction in all the strains (Fig. 4a). Although the high MW bands were also solubilized after NP-40 treatment, consistently less solubilization of GRA2 high MW bands was observed in Δgra45 parasites compared to wild-type and Δgra45 + GRA45 HA parasites (Fig. 4a and Supplementary Fig. 8a). We then observed the solubility of GRA7, which contains a transmembrane domain but localizes to the dense granule core and does not integrate into the dense granule membrane. In all strains, GRA7 mainly presented in the pellet fraction with both monomeric bands and high MW bands (Fig. 4b). Similar to GRA2, high MW GRA7 in Δgra45 parasites was more resistant to NP-40-induced solubilization (Fig. 4b); however, because of variability between experiments there was no significant difference between wild-type and Δgra45 parasites (Supplementary Fig. 8b). No high MW bands or monomeric GRA7 were observed in Δgra7 parasites (Supplementary Fig. 4i) indicating that the high MW bands were not a specific detection of other Toxoplasma proteins (Supplementary Fig. 8c).

To confirm that transmembrane domain-containing GRAs were affected in Δgra45 parasites, we examined the localization of several GRAs after exocytosis of the dense granules based on their membrane association properties: GRA5, is a PVM-integrated membrane-association property: GRA5, is a PVM-integrated surface protein, we also generated parasites harboring GRA45 with various point mutations including VKV139→AAA together with

The conserved sHSP signatures are critical for the chaperone-like function of GRA45. To confirm the chaperone-like function of GRA45, we generated parasites expressing C-terminal HA-tagged GRA45 without its ACD (ΔACD, Fig. 5a and Supplementary Fig. 4k), which is the central structure of sHSPs and the key element for homodimer formation and substrate binding. The parasite strain expressing GRA45 with C-terminal trans-threonyl-like fold deletion (∆TL) was generated at the same time (Fig. 5a and Supplementary Fig. 4k). To avoid a massive conformational change caused by deletion of a large portion of the protein, we also generated parasites harboring GRA45 with various point mutations including VKV139→AAA together with...
VEV\textsuperscript{162–164}/AAA (referred to as VxV/AAA), IDV\textsuperscript{205–207}/AAA (referred to as 1st IxV/AAA), IDV\textsuperscript{291–293}/AAA (referred to as 2nd IxV/AAA), and IDV\textsuperscript{205–207}/AAA together with IDV\textsuperscript{291–293}/AAA (referred to as IxV/AAA) (Fig. 5a and Supplementary Fig. 4k). These mutant sites are the conserved I/VxI/V motifs of sHSPs, which direct the formation of sHSP oligomers via binding to hydrophobic pockets of the homodimer\textsuperscript{82,83}. We found that in addition to presenting in dense granules of the extracellular parasites all the GRA45 mutants also localized to compartments peripheral to the parasite nucleus (Fig. 5b), which is reminiscent of the organelles in the early secretory pathway, such as endoplasmic reticulum (ER), Golgi and endosome-like compartment. This change of GRA45 localization in the mutant strains was also observed in the intracellular parasites residing in the vacuole (Fig. 5c), suggesting that a higher level of architecture (e.g., oligomer) is needed for its trafficking to dense granules and secretion. Surprisingly, in these GRA45 mutant parasite strains GRA7 completely colocalized with GRA45 in extracellular parasites...
including dense granules as well as the perinuclear compartments (Fig. 5b), suggesting that GRA45 has a chaperone-like function important for the trafficking of GRAs from early secretory organelles to the dense granules. In addition, we noticed that the total amount of GRA7 in ∆gra45 parasites detected by immunofluorescence assay was significantly less compared to GRA45+/+ parasites (Fig. 5b and Supplementary Fig. 8a) although it still localized to dense granules (long exposure in Supplementary Fig. 8a). This could be due to a large portion of GRA7 remaining insoluble (Fig. 4b) and inaccessible to antibodies in ∆gra45 parasites. To determine if the localization of GRAs on the PVM was affected in these GRA45 mutant strains, we observed the localization of MAF1 at 4 h (Fig. 5d) and 24 h p.i. (Supplementary Fig. 9b). Like in ∆gra45 parasites, MAF1 was almost completely retained in the PV lumen of all the mutant strains except for ∆gra45+/+ parasites which had ∼15% of PVM-localized MAF1 (Supplementary Fig. 9c). These mutations in GRA45 also resulted in the absence of translocation of GRA16 and GRA24 to the host nucleus (Fig. 5e, Supplementary Fig. 9d, e). Since these GRA45 mutant strains have a similar phenotype as ∆gra45 parasites, we determined the susceptibility of these parasites to IFNγ-induced parasite growth inhibition in murine BMDMs (Fig. 5f). Except for ∆gra45+/+ parasites, all the GRA45 mutant parasites had significantly reduced growth in IFNγ-activated murine BMDMs. Among the mutants, ∆gra45+/+ parasites had similar level of growth as ∆gra45 parasites suggesting both the ACD and TL domains as well as both 1xV motifs are important for correct trafficking of GRA effector(s) neutralizing the IFNγ responses in murine macrophages. Similar to ∆gra45 parasites, none of the mutants had a growth defect in naive murine BMDMs (Supplementary Fig. 5f). Taken together, GRA45 has conserved SHP functional signatures, which are critical for the trafficking of GRAs (including GRA45 itself) to dense granules and their final destinations.

**GRA45 is important for parasite virulence.** Since the data suggest that GRA45 affects both the correct localization of GRAs to the PVM and the export of GRAs beyond the PVM, it is unclear if the fitness defect of ∆gra45 parasites in IFNγ-activated BMDMs was due to the defect of PV-localized GRAs or exported GRAs. However, the translocon components MYR1/2/3/4 did not emerge as hits in our loss-of-function screen in IFNγ-activated BMDMs (Fig. 6a). GRA45 has a significantly lower IFNγ BMDM vs. naïve BMDM fitness compared to MYR1, MYR2, and MYR3 across the three screens (Fig. 6b). Together with the data that ∆gra45+/+ parasites did not export GRA16 and GRA24 beyond the PVM (Fig. 5e) but only had minor growth reduction in IFNγ-activated murine BMDMs (Fig. 5f) suggests that GRA export is not involved in resistance to IFNγ-induced parasite growth inhibition. To confirm this, we compared side-by-side the susceptibility of ∆gra45 and ∆myr1 parasites to IFNγ-mediated growth inhibition. In murine BMDMs prestimulated with increasing concentrations of IFNγ, we observed that ∆gra45, but not ∆myr1, parasites were more susceptible than either wild-type or ∆gra45+/+ parasites to IFNγ-mediated growth inhibition (Fig. 6c). To determine if these in vitro differences also translated to differences in virulence in mice, we intraperitoneally (i.p.) infected mice with 100 tachyzoites of wild-type, ∆gra45, ∆myr1, ∆gra45+/+ parasites and monitored parasite virulence. Consistent with the in vitro data, GRA45, but not ∆myr1, parasites were significantly less virulent compared to wild-type or GRA45 complemented parasites (Fig. 6d). These data indicate that MYR-component trafficking and function only partially explain the effects of GRA45 on IFNγ resistance and parasite virulence.

**Discussion**

In this study, we performed a genome-wide loss-of-function screen and identified the *Toxoplasma* genes that determine fitness in naïve or IFNγ-activated murine BMDMs. Evading the host immune response at the site of infection and reaching immune-privileged organs is essential for *Toxoplasma* to establish a lifelong chronic infection. It is likely that some of the *Toxoplasma* genes that determined fitness at the site of infection were important for resisting the anti-*Toxoplasma* activities in IFNγ-activated cells or determined parasite growth and survival inside macrophages, the cell type preferentially infected by *Toxoplasma* in vivo. We and another group recently determined the in vivo fitness contribution of a subset of *Toxoplasma* genes encoding secretory proteins, mainly including ROPs and GRAs, in the mouse peritoneum and in distant organs. Five genes (*GRA22*, *TG GT1_269950*, *GRA38*, *TG GT1_205350*, and *TG GT1_269950*) conferring in vivo fitness were also fitness-conferring genes in IFNγ-activated murine macrophages revealing the in vivo phenotype of these genes is likely due to their importance in maintaining parasite growth in IFNγ-activated peritoneal macrophages. One of the most significant parasite genes conferring fitness in IFNγ-activated BMDMs was GRA45. Unlike previously described GRAs that are involved in parasite virulence, GRA45 is not a parasite effector that directly modulates host cell signaling pathways or inhibits host immunity. Our data indicate that GRA45 is an important chaperone-like protein that determines the correct trafficking of PV-localized GRA effectors to their final destination, likely via maintaining their native folding status in the
secretory pathway. Among all the characterized ASP5 substrates, GRA45 best phenocopies the mislocalization of GRAs to PVM caused by the deletion of ASP5. Due to the absence of a TEXEL motif in some of the PVM-localized GRAs, for example GRA7 and MAF1, it is reasonable to believe that the mislocalization of GRA7 and MAF1 in Δasp5 parasites is due to failed GRA45 processing. Many PVM-integrated or -associated GRAs possess a hydrophobic domain and form high MW complexes while trafficking through the secretory pathway and exist as a mix of soluble and aggregated states before dense granule
exocytosis\textsuperscript{31,85,86}. In bacteria and plants, the sHSPs form dynamic assemblies with the early-unfolding intermediates of their substrates to prevent tight protein aggregate formation upon unfolding stress conditions\textsuperscript{82}. The ACD homodimer subunits of sHSP sequester the substrate and preserve them in a complex core structure with a dynamic shell composed of a multitude of diverse oligomerization states driven by the docking of the I/VxI/V motifs to a hydrophobic groove presented in the subunits\textsuperscript{82}. Mutations in the I/VxI/V motifs causes dissociation of oligomerized ACD homodimer subunits resulting in loss of the chaperone-like activity\textsuperscript{83,87}. Since GRA45 deficient in its conserved sHSP domain/motifs caused transmembrane domain-containing GRAs to partially remain in the early secretary compartments and to mislocalize after secretion, it is likely that GRA45 shields the hydrophobic domain of these GRAs to protect them from uncontrolled aggregation during their trafficking in the secretory pathway. The data also suggest that dimerization/oligomerization of GRA45 is required for its own trafficking to dense granules possibly via an ER/Golgi-resident sorting receptor, which only recognizes GRA45/substrate complexes. Thus, deletion of GRA45 had pleiotropic effects, which is the most likely explanation for its importance in conferring fitness in IFN\textgamma-activated macrophages of multiple species. In addition to PVM-localized GRAs, GRA16, and GRA24, which are normally secreted beyond the PVM, are no longer secreted in $\Delta$gra45 parasites. However, it is unlikely that the fitness defect of $\Delta$gra45 parasites was due to the defect in GRA effector export beyond the PVM as $\Delta$myr1 parasites were not significantly more susceptible to IFN\textgamma-activated macrophages than wild-type parasites. The in vivo fitness of $\Delta$gra45 parasites also differed from $\Delta$myr1 parasites, which is consistent with our previous in vivo screen in which MYR1 had no peritoneum fitness defect\textsuperscript{35}. However, studies in the less virulent type II strain showed that individual MYR1 knockout parasites, but not MYR1 knockout parasites in a pool of other mutants, lose their virulence in vivo\textsuperscript{65,84}. This is most likely due to abolished translocation of the GRA effector $\alpha$-Crystallin domain (ACD) and I/VxI/V motifs are critical for the chaperone-like function of GRA45. a Schematic illustration of the constructs used for generation of the parasite expressing full-length GRA45 or $\Delta$gra45 with indicated mutations. ACD and TL indicate predicted $\alpha$-Crystallin domain and transthyretin-like fold, respectively. The conserved VxV or IxV motifs are indicated accordingly. b Extracellular $\Delta$gra45 parasites or $\Delta$gra45 parasites complemented with C-terminal HA-tagged wild-type or indicated mutant version of GRA45 were fixed with methanol and stained with indicated antibodies. The images are representative of results from two independent experiments (scale bar = 2\,µm). c HFFs infected with indicated parasite strains for 24\,h were fixed and stained with antibodies against HA epitope. The images are representative of results from two independent experiments (scale bar = 5\,µm). d HFFs infected indicated parasite strains for 4\,h were stained with antibodies against MAF1. The images are representative of results from two independent experiments (scale bar = 2\,µm). e $\Delta$gra45 or $\Delta$gra45 complemented with wild-type or indicated mutant version of GRA45 were transiently transfected with GRA16-Ty (upper) or GRA24-Ty (lower) expressing plasmids and immediately used to infect HFFs and fixed at 24\,h p.i. and subjected to the immunofluorescent assay with antibodies against Ty epitope. The images are representative of at least 40 host cells containing a single parasitophorous vacuole with four or more parasites (scale bar = 10\,µm). f Murine BMDMs prestimulated with 5\,ng/mL of IFN\textgamma or left unstimulated for 24\,h were infected with indicated parasite strains. Twenty-four hours p.i. parasite growth for each strain was measured by luciferase assay. Parasite growth in IFN\textgamma-activated BMDMs is expressed relative to growth in naïve BMDMs. Data are displayed as mean ± SEM with independent experiments ($n = 3$) indicated by the same color dots. The significant difference between WT and knockout (or mutant strains) was analyzed with one-way ANOVA with Tukey’s multiple comparisons test and the p values are indicated above the columns.
biosynthesis; TGGT1_266640 (Acetyl-coenzyme A synthetase), enzymes involved in fatty acid production and metabolism; GRA38, an orthologue of GRA39 of which the knockout has been shown to accumulate lipid; and ATP-binding cassette transporter ABCG96, which have been shown to mediate cholesterol transport. However, it is well known that Toxoplasma is a glutton for host-derived lipids and takes up more than it can safely store in lipid droplets. Because excess lipids, especially free cholesterol, can be toxic, Toxoplasma might import more cholesterol and the inability to export excess cholesterol in these cells is likely toxic to the parasite. It is, therefore, possible that Toxoplasma needs to modify its acquisition/export or own production of lipids in IFNγ-stimulated BMDMs.

It is worth noticing that there was variation in IFNγ BMDM fitness scores likely resulting in a substantial number of false negatives due to differences in IFNγ concentration and incubation time between the individual screens. For example, deletion of ROP18 resulted in a fitness defect in IFNγ-activated BMDMs in two out of three screens (IFNγ vs. naive BMDM fitness scores of S1, S2, and S3 are −2.03, −0.13, and −1.35, respectively). Given that S1 and S3 were performed in macrophages activated with 100 ng/mL of IFNγ, parasites deficient in ROP18 might only be susceptible to growth inhibition induced with a higher amount of IFNγ. GRA12 had remarkably lower fitness in BMDMs with longer (24 h) IFNγ stimulation (S1 = −3.19 and S2 = −4.15) compared to 4 h of IFNγ incubation (S3 = 0.21) suggesting that the length of IFNγ stimulation probably determined the efficiency of parasite restriction. In addition, our screen likely missed parasite genes that determine fitness in HFFs but that might have an even larger effect on fitness in naïve or IFNγ-activated BMDMs (for example ASP5) because we started our screen after three to four passages in HFFs. At that point, mutants with a fitness defect in HFFs would have already been depleted and therefore, might not have met the threshold to be included in our analysis. The large number of host cells needed to maintain the complexity of the genome-wide library of parasite mutants made it impractical to directly perform the screen in BMDMs. Future focused screens could use the data from our screens as a starting point to identify additional parasite genes that mediate fitness in naïve or IFNγ-activated BMDMs.

![Figure 6](image-url)

Fig. 6 Compared to Δmyr1 parasites, Δgro45 parasites are more susceptible to IFNγ and less virulent in mice. a, Toxoplasma genes that have at least four sgRNAs present after the 3rd passage in naïve BMDM in the three independent screens are rank-ordered according to their IFNγ vs. Naïve BMDM fitness scores. Data are displayed as average fitness scores (black plots) ± SEM (gray lines). b, Mean IFNγ vs. Naïve BMDM fitness of GRA45, MYR1, MYR2, MYR3, and MYR4. Data are displayed as mean ± SEM with independent screens (n = 3) indicated by the same color dots (S1: white; S2: blue; S3: red). The significant difference was analyzed with one-way ANOVA with Tukey’s multiple comparisons test. c, Murine BMDMs prestimulated with or without IFNγ (1, 5, 10, or 50 ng/mL) were infected with luciferase-expressing WT, Δmyr1, Δgro45, or Δgro45 + GRA45HA parasites (MOI of 0.25) for 24 h. Parasite growth for each strain was measured by luciferase assay and the growth in IFNγ-activated BMDMs is expressed relative to growth in naïve BMDMs. Data are displayed as connecting lines with mean of four independent experiments ± SEM. The significant difference was analyzed with two-way ANOVA with Tukey’s multiple comparisons test. d, CD-1 mice were i.p. infected with 100 tachyzoites of WT, Δmyr1, Δgro45, or Δgro45 + GRA45HA parasites and survival of mice was monitored for 30 days. Data are displayed as Kaplan–Meier survival curves (n = 9 mice infected with WT or Δgro45 parasites, n = 5 mice infected with Δmyr1 parasites, and n = 4 mice infected with Δgro45 + GRA45HA parasites). The significant difference was analyzed with Log-rank (Mantel–Cox) test.
from Dr. Anthony Sinai) were cultured in DMEM, 10% fetal bovine serum (FBS), 2 mM L-glutamine, 10 mg/mL gentamicin. Mouse embryonic fibroblasts (MEFs, gift from Dr. Anthony Sinai) were cultured in DMEM, 10% fetal bovine serum (FBS), 2 mM L-glutamine. Human foreskin fibroblasts (HFFs, gift from Dr. John C. Boothroyd) were cultured in Dulbecco’s modified Eagle’s medium (DMEM), 10% fetal bovine serum (FBS), 100 U/mL penicillin/streptomycin, 1× non-essential amino acids, 1 mM sodium pyruvate, 100 U/mL penicillin/streptomycin, 10 mg/mL gentamicin. T-cells (ATCC TIB-202TM, gift from Dr. Daniel A. Bachovchin) were cultured in RPMI-1640, 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin, 10 mg/mL gentamicin. For differentiation, T-cells were stimulated with 10 ng/mL PMA, 1 µM ionomycin, and 3-aceaptate (PMA, Sigma-American) each for a 5-hr incubation in cell culture plates (Sigma-Aldrich) at a housing density of five mice per cage and three rats per cage. The mice and rats were allowed to acclimatize in our vivarium for at least a week undisturbed. The animal room was on a 12-h light/12-h dark cycle, the temperature was maintained at 22–25 °C, and the humidity range was 30–70%. Mice were monitored twice daily by veterinarians, including daily cage cleaning exchanged every two weeks. The mice and rats were housed under pathogen-specific free conditions at the University of California, Davis animal facility. All animal experiments were performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and the Animal Welfare Act, approved by the Institutional Animal Care and Use Committee at the University of California, Davis (UC Davis) (assurance number A-3433-01).
raw read numbers. The abundance of each sgRNA was normalized to the total number of reads. To do the log, transfer, sgRNAs that had zero reads were assigned a pseudo-count of 0.01, which was the lowest sgRNA read in the sample. Only sgRNA whose abundance was above the 5th percentile in the input (library) were further considered for the analysis. The “fitness” score was calculated as the average log₂-fold change of the top five scoring sgRNAs for each given gene, and the mean fitness in the three screens is reported. The Pearson correlation between different screens was calculated using the Pearson correlation coefficient. One-sided Wilcoxon signed-rank test was used to calculate the probability of each gene being fitness conferring by comparing their fitness to the 497 control genes. Cohen’s d calculation was used to measure the effect size of fitness scores for each gene. The fitness scores were defined if they met a significance threshold of p ≤ 0.05 from one-sided Wilcoxon signed-rank test and Cohen’s d ≥ 0.8 with expression in the murine macrophages (FPKM > 1) [3]. The criteria used to identify the high-confidence genes were described in Table 1.

**RNA-seq.** 3 × 10⁶ murine BMDMs were seeded overnight in 6-well plates prior to stimulations. For the stimulated samples, IFNy (100 ng/mL) was added to each well for 4 or 24 h before harvesting the cells for total RNA extractions. The mRNA was purified by polyA-tail enrichment (Dynabeads mRNA Purification Kit, Thermo Fisher, Cat#61006), fragmented into 200–400 basepares, and reverse transcribed into cDNA before adding Illumina sequencing adapters to each end [5]. Libraries were barcoded, multiplexed into four plates per sequencing lane. The Illumina HiSeq 2000, and sequenced from both ends resulting in 50 bp reads trimming off the barcodes. The RNA-sequencing reads were mapped to the mouse genome (mm10) using Bowtie (version 2.0.2) [6] and Tophat (version 2.0.4) [7] and transcript abundance estimated in cufflinks (version 2.2.1) [8].

**Bioinformatic analysis.** GSEA57 was used to determine if Toxoplasma genes that determined fitness in specific conditions were enriched in the functional annotation. GO enrichments were performed using available tools available through the Bioconductor project. In addition, the genes conferring fitness in specific conditions were analyzed using an in-house database that contained information on GO, protein family domains (Interpro), KEGG enzyme EC numbers, localization to specific organelles, amongst others. Pathways that were enriched are indicated in Supplementary Data 2. For enrichment analysis of BMDM pathways stimulated with IFNy vs. unstimulated, the GSEA program (version 4.0.3) and Molecular Signatures Database (MsigDB, version 7.1) was used[57,106,107]. Psi-blast was used to find orthologs of proteins under investigation in other species. Alignments of GRA45 with its orthologs were made using PRALINE[108], and the results (Supplementary Fig. 7a) were used as input for HTRF measurement to predict similarity to secondary structures of other proteins. In addition, the SWISS-MODEL server[109] and I-TASSER analysis[110] were used for the homology modeling of protein structures.

**Plaque assay.** Freshly confluent 24-well plates of HFFs or MEFs were infected with 100 parasites for each well and the plates were incubated at 37 °C without disturbing for 5 days. The total number of plaques in HFFs was observed using a Nikon microscope in an inverted microscope. To measure the parasite growth in HFFs, the area of each individual plaque was captured and analyzed using the above microscope equipped with Hamamatsu ORCA-ER digital camera, and NIH Elements Imaging Software, respectively. For each independent experiment, at least 40 plaques from technical duplicate wells were imaged.

**Luciferase assay.** Luciferase assays were performed to determine the fitness of knockout strains in IFNy-activated macrophages. For murine and rat macrophages, BMDMs in 96-well plates (1 × 10⁵ cells/well) were stimulated with 1 ng/mL or 5 ng/mL murine or rat IFNy (R&D System, Cat#585-IF-100) for 24 h followed by infection with wild-type luciferase-expressing parasites or knockout parasites at two different MOIs (MOI = 0.5 and 1). For human macrophages, PMA-differentiated THP-1 cells in 96-well plates (1 × 10⁶ cells/well) were stimulated with 2.5 ng/mL or 5 ng/mL human IFNy (BIO-RAD, Cat#PHPP050) for 24 h followed by parasite infection. A plaque assay was set up in HFFs at the same time to determine the parasite viability of each parasite strain. After 24 h infection, the cells in 96-well plates were lysed using the lysis buffer of Luciferase Assay System (Promega, Cat#E1500) followed by adding luciferin for the measurement of luciferase activity using a single-channel luminometer (Turner BioSystems). Raw luciferase readings (RLU) of unstimulated infected cells was considered as 100 percent and relative parasite growth in IFNy-activated macrophages was calculated. To make comparisons between wild-type and knockout parasites, “real” MOI was matched against the plaque assay results.

**Growth competition.** The BMDMs were left unstimulated or stimulated with IFNy (1 ng/mL) for 24 h followed by infection with a 1:1 mixed ratio of GFP-expressing wild-type parasites and GFP-negative knockout parasites (Ag22 or Arom1). The mixed parasites were allowed to grow in the BMDMs for three lytic cycles. Plaque assays of the mixed parasites were performed in HFFs before putting into the BMDMs and after the 3rd passage in the BMDMs, and the number of GFP-positive vs. GFP-negative plaques were counted to determine the ratio of wild-type and knockout parasites in naive and IFNy-activated BMDMs.

**Immunofluorescent assay.** To determine the localization of GRA45 in extra-cellular parasites, RHAΔku80hxgprt::GRA45-3xHA tachyzoites released from infected macrophages were fixed for 20 min followed by permeabilization/blocking with PBS containing 3% (v/v) BSA, 5% (v/v) goat serum and 0.1% Triton X-100. Co-localization of GRA45 was detected by anti-HA antibodies along with antibodies against ROP2/3/4, GRA1, GRA2, GRA5, GRA7, or SAG1. To determine the localization of intra-cellular GRA45, HFFs grown on glass coverslips were infected with RHAΔku80hxgprt::GRA45-3xHA parasites for 24 h, fixed with 4% PFA for 20 min, permeabilized/blocked with PBS containing 3% (v/v) BSA, 5% (v/v) goat serum and 0.1% Triton X-100 (or 0.2% Saponin), followed by incubated with antibodies against ROP2/3/4, GRA1, GRA2, GRA5, GRA7, or SAG1 at 4 °C overnight. To check the PVM localization of GRA5 or GRA7, HFFs grown on glass coverslips were infected with wild-type, Δgra45 or Δgra45 + GRA45+HA strains in the RH-Luc+/hxgprt background for 24 h followed by fixation with 4% PFA, permeabilization with 0.2% Saponin and blocking with PBS containing 3% (v/v) BSA, 5% (v/v) goat serum and 0.1% Triton X-100 fixation. The coverslips were incubated with anti-GRA5 antibodies against GRA45 or GRA7 for 1 h at room temperature. MAF1 localization was observed in the HFFs infected wild-type, Δgra45 parasites or Δgra45 parasites complemented with wild-type or mutant versions of GRA45. At 4 or 24 h post-infection, the cells were fixed with 4% PFA, permeabilized/blocked with PBS containing 3% (v/v) BSA, 5% (v/v) goat serum and 0.1% Triton X-100 followed by incubation with MAF1 anti-bodies [5]. To check the PVM localization of GRA23 or export of GRA16 and GRA24, HFFs grown on glass coverslips were infected with wild-type, Δmyr1, Δgra45 parasites or Δgra45 parasites complemented with wild-type or mutant versions of GRA45 transiently expressing GRA44-Myc40 for 20 h were fixed, permeabilized and blocked as described above followed by incubation with anti-Myc antibodies at 4°C overnight. After incubating with Alexa Fluor 488/594 secondary antibodies and DAPI, the coverslips were mounted with Vecta-Shield mounting oil and the microscopy was performed with NIS-Elements software (Nikon) and a digital camera (CoolSNAP EZ, Roper Scient). To quantify the relative localization of GRA5, GRA7, and MAF1 at 24 h p.i., at least 100 vacuoles containing four or more parasites were observed. To quantify the localization of GRA23 and GRA44, at least 50 vacuoles containing two or more parasites were observed.

**IRGA6/IRGB6 coating assay.** 1 × 10⁵ of C57Bl/6J murine BMDMs were plated on coverslips in 24-well plates and rested for 24 h followed by stimulated with 5 ng/mL of murine IFNy for another 24 h. The stimulated BMDMs were infected with freshly lysed wild-type, Δgra45, Δgra22, ΔTG771, 26350T parasites by adding 2 × 10⁵ parasites for 3–5 times to remove uninvaded parasites, fixation with 4% PFA, permeabilization with 0.2% Saponin and blocking with PBS containing 3% (v/v) BSA, 5% (v/v) goat serum and 0.1% Saponin. The coverslips were incubated with anti-SAG1 antibodies along with antibodies against IRGA6 or IRGB6 for 1 h at room temperature followed by incubation with Alexa Fluor 488/594 secondary antibodies and DAPI for 30 min at room temperature. The coverslips were mounted with Vecta-Shield mounting oil and the microscopy was performed as described above. For each independent experiment, at least 200 vacuoles of each strain were observed and the percentage of IRGA6- or IRGB6-positive vacuoles was quantified.

**Cell fractionation.** Purified extracellular parasites were washed with PBS and resuspended in cold PBS containing 1× protease and phosphatase inhibitors (Thermo). Parasites were lysed by freezing and thawing in ice, followed by using a dry ice ethanol bath (~70 °C) and a 37 °C bath. 1/3 of the E/T lysis was kept in PBS, 1/3 in 1% NP-40, and 1/3 in 1% Triton X-100 for 30 min at 4 °C under rotation. The PBS, NP-40, and Triton X-100 treated lysates were separated by high-speed spin 30 min at 20,000 × g. The PBS supernatant was precipitated with TCA (Trichloroacetic Acid) and the 5% HCl fraction of NP-40 or Triton X-100 extraction were cold acetone precipitated.

**Immunoblotting.** Samples from different fractions of the extracellular were boiled for 5 min in sample buffer, separated by SDS-PAGE, and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked in 5% milk in TBS supplemented with 1% Tween-20 (TBS-T) for 30 min at room temperature and then incubated overnight at 4 °C with primary antibody in the blocking buffer. The next day, the blot wassubsequently incubated with HRP-conjugated anti-rabbit secondary antibodies. The GRA7 and SAG1 blots were incubated with HRP-conjugated anti-rabbit secondary antibodies. The HA blot was incubated with...
HRP-conjugated anti-rat secondary antibodies. The HRP was detected using an enhanced chemiluminescence (ECL) kit (Bio-RAD, Cat#1705060). Blot pictures were acquired using a FluorChem E system (ProteinSimple). Between each blot, the membranes were stripped by incubation in stripping buffer (Thermo Fischer, Cat#4628) for 20 min and subsequently washed three times with TBS-T. The absence of residual HRP signal on the membrane was tested by using the ECL kit (Bio-RAD, Cat#1705060) and image acquisition. Then, the membrane was blocked prior to incubation with the next primary antibody. The quantification of the bands intensity was done using the Volume Tools of Image Lab 6.1 software (Bio-RAD).

**In vivo infection.** The mice were randomly assigned to experimental groups (the exact number of mice per group is indicated in the figure legends) at the start of the experiment. *Toxoplasma* tachyzoites were harvested from cell culture and released by passage through a 27-gauge needle, followed by a 30-gauge needle. CD-1 mice were intraperitoneally (i.p.) infected with 100 tachyzoites of each strain and parasite viability of the inocula was determined in a plaque assay after infection. The mice were monitored for 30 days p.i., and the number of dead mice per group was observed every individual day.

**Statistical Tests.** All statistical analyses were performed using Prism (GraphPad) version 8.0. All the data are presented as mean ± standard error of mean (SEM), and the exact n values are mentioned in the figure legends. For all the calculations p < 0.05 are considered as significant. To compare parasite growth of the knockouts vs. wild-type parasites in IFN-γ-activated cells, paired t-test was used. For the data with more than two groups with one variable, One-way ANOVA with Tukey’s multiple comparisons test was used. For one variable test with two groups, the two-way ANOVA with Tukey’s multiple comparisons test was used. Survival experiments were analyzed using the Log-rank (Mantel–Cox) test.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

The authors declare that all data supporting the findings of this study are available within the article and its Supplementary Information files or are available from the authors upon request. CRISPR screen data including raw sequencing read counts are available in Supplementary Data 1. RNA-seq sequencing data have been deposited in BioProject database with the accession code PRJNA664106 and are provided in Supplementary Data 3. The MsigDB are available in https://www.gsea-msigdb.org/gsea/msigdb. All unique materials (e.g., the variety of parasite lines described in this manuscript) and custom code used in the analysis of CRISPR screen data are available from the corresponding author (contact: Jeroen P.J. Saeij, jaeij@ucdavis.edu) upon reasonable request. Source data are provided with this paper.

Received: 17 December 2019; Accepted: 22 September 2020; Published online: 16 October 2020.

**References**

1. Montoya, J. G. & Liesenfeld, O. Toxoplasmosis. *Lancet* **363**, 1965–1976 (2004).
2. Jones, T. C. & Hirsch, J. G. The interaction between *Toxoplasma gondii* and mammalian cells. II. The absence of lysosomal fusion with phagocytic vacuoles containing living parasites. *J. Exp. Med.* **136**, 1173–1194 (1972).
3. Sibley, L. D., Weidner, E. & Krahenbuhl, J. L. Phagosome acidification blocked by intracellular *Toxoplasma gondii*. *Nature* **315**, 416–419 (1985).
4. Suzuki, Y., Orellana, M., Schreiber, R. & Remington, J. Interferon-gamma: the major mediator of resistance against *Toxoplasma gondii*. *Science* **240**, 516–518 (1988).
5. Lykens, J. E. et al. Mice with a selective impairment of IFN-γ-inducible genes with essential, pathogen-specific roles in resistance to infection. *J. Exp. Med.* **194**, 181–188 (2001).
6. Luescher, B. et al. The IFN-γ-inducible GTPase, Irga6, protects mice against *Toxoplasma gondii* but not against *Plasmodium berghei* and some other intracellular pathogens. *PLoS ONE* **6**, e20568 (2011).
7. Saeij, J. P. & Frickel, E.-M. Exposing the house mouse. *elife* **2**, e01298 (2013).
8. Suzuki, Y., Orellana, M., Schreiber, R. & Remington, J. IFN-γ and the major mediator of resistance against *Toxoplasma gondii* and the house mouse. *elife* **2**, e01298 (2013).
9. Suzuki, Y. et al. Interferon-gamma: the major mediator of resistance against *Toxoplasma gondii* in wild-derived mice. *Nat. Commun.* **10**, 1233 (2019).
10. Martens, S. et al. Disruption of *Toxoplasma gondii* parasites in mouse p47-resistance GTPases. *PLoS Pathog.* **1**, e24 (2005).
11. Zhao, Y. O., Khaminets, A., Hunn, J. P. & Howard, J. C. Disruption of the *Toxoplasma gondii* parasites in mouse by IFN-γ-inducible immunity-related GTPases (IRG Proteins) triggers necrotic cell death. *PLoS Pathog.* **5**, e1000288 (2009).
12. Zhao, Y. et al. Virulent *Toxoplasma gondii* evade immunity-related GTPase-mediated parasite vacuole disruption within primed macrophages. *J. Immunol.* **182**, 3775–3781 (2009).
13. Kravets, E. et al. Guanylate binding proteins directly attack *Toxoplasma gondii* via supramolecular complexes. *elife* **5**, e11479 (2016).
14. Adams, L. B., Hibs, J. B. Jr, Taintor, R. R. & Krahenbuhl, J. L. Microbiostatic effect of murine-activated macrophages for *Toxoplasma gondii*. Role in the synthesis of inorganic nitrogene oxides from L-arginine. *J. Immunol.* **144**, 2725–2729 (1990).
15. Appelberg, R. Macrophage nutritive antimicrobial mechanisms. *J. Leukoc. Biol.* **79**, 1117–1128 (2006).
16. Raniga, K. & Liang, C. Interferons: reprogramming the metabolic network against viral infection. *Viruses* **10**, 36 (2018).
17. Stutz, A., Kessler, H., Kaschel, M.-E., Meissner, M. & Dalpke, A. H. Cell invasion and strain dependent induction of suppressor of cytokine signaling-1 by *Toxoplasma gondii*. *Immunobiology* **217**, 28–36 (2012).
18. Hakimi, M. A., Olias, P. & Sibely, L. D. *Toxoplasma* effectors targeting host signaling and transcription. *Clin. Microbiol. Rev.* **30**, 613–645 (2017).
19. Saeij, J. P. et al. Polymorphic secreted kinases are key virulence factors in *Toxoplasmosis*. *Science* **314**, 1780–1783 (2006).
20. Taylor, S. et al. A secreted serine-threonine kinase determines virulence in the mammalian cell. *PLoS Pathog.* **3**, e1000347 (2007).
21. Hermanns, T., Muller, U. B., Konen-Waisman, S., Howard, J. C. & Steinfeld, T. The *Toxoplasma gondii* hsp70-related protein GRA7 effector increases turnover of immunity-related GTPases and regulates host cell death. *Cell Host Microbe* **15**, 537–540 (2014).
22. Alaganan, A., Fentress, S. J., Tang, K., Wang, Q. & Sibely, L. D. *Toxoplasma* GRA7 effector increases turnover of immunity-related GTPases and contributes to acute virulence in the mouse. *Proc. Natl Acad. Sci. USA* **108**, 9625–9630 (2011).
23. Behnke, M. S. et al. Virulence differences in *Toxoplasma* mediated by amplification of a family of polymorphic pseudokinases. *Proc. Natl Acad. Sci. USA* **108**, 9631–9636 (2011).
24. Etheridge, R. D. et al. The *Toxoplasma* pseudokinase ROP5 forms complexes with ROP18 and ROP17 kinases that synergize to control acute virulence in mice. *Cell Host Microbe* **15**, 537–540 (2014).
25. Behnke, M. S. et al. The polymorphic pseudokinase ROP5 controls virulence in *Toxoplasma gondii* by regulating the active kinase ROP18. *PLoS Pathog.* **8**, e1002992 (2012).
26. Fleckenstein, M. C. et al. A *Toxoplasma* pseudokinase inhibits host IRG resistance proteins. *PLoS Biol.* **10**, e1001358 (2012).
27. Niedelmann, W. et al. The rhoptry proteins ROP18 and ROP5 mediate *Toxoplasma gondii* evasion of the murine, but not the human, interferon-gamma response. *PLoS Pathog.* **8**, e1002784 (2012).
28. Selleck, E. M. et al. Guanylate-binding protein 1 (Gbp1) contributes to cell-autonomous immunity against *Toxoplasma gondii*. *PLoS Pathog.* **9**, e1003246 (2013).
29. Vieira Winter, S. et al. Determinants of GBP recruitment to *Toxoplasma gondii* vacuoles and the parasitic factors that control it. *PLoS ONE* **6**, e24434 (2011).
30. Jensen, K. D. et al. *Toxoplasma* polymorphic effectors determine macrophage polarization and intestinal inflammation. *Cell Host Microbe* **9**, 472–483 (2011).
31. Mukhopadhyay, D., Sangare, L. O., Braun, L., Hakimi, M. A. & Saeij, J. P. *Toxoplasma* GRA15 limits parasite growth in IFN-γ-activated fibroblasts through TRAF ubiquitin ligases. *EMBO J* **39**, e103758 (2020).
Kim, E. W. et al. The rhoptry pseudokinase ROP54 modulates parasite growth and pathogenesis. mBio 8, e00589–19 (2017).

Gay, G. et al. Toxoplasma gondii TgSHT co-opts host chromatin repressors dampening STAT1-dependent gene regulation and IFN-γ-mediated innate immune defenses. J. Exp. Med. 213, 1779–1798 (2016).

Olias, P., Etheridge, R. D., Zhang, Y., Holtzman, M. J. & Sibley, L. D. TgSUB2 is a Mi-2/NuRD complex to repress STAT1 transcription and block IFN-γ-mediated gene expression. Cell Host Microbe 20, 72–82 (2016).

Kim, S.-K., Fourest, A. E. & Boothroyd, J. C. Toxoplasma gondii dysregulates IFN-γ-inducible gene expression in human fibroblasts: insights from a genome-wide transcriptional profiling. J. Immunol. 178, 5154–5165 (2007).

Lang, C. et al. Impaired chromatin remodelling at STAT1-regulated promoters leads to global unresponsiveness of Toxoplasma gondii-infected macrophages to IFN-γ. PLoS Pathog. 8, e1002483 (2012).

Rosowski, E. E. & Paej, J. P. Toxoplasma gondii clonal strains all inhibit STAT1 transcriptional activity but polymorphic effectors differentially modulate IFNγ induced gene expression and STAT1 phosphorylation. PLoS ONE 7, e51448 (2012).

Durbin, J. E., Hackenmiller, R., Simon, M. C. & Levy, D. E. Targeted disruption of the mouse Stat1 gene results in compromised innate immunity to viral disease. Cell 84, 443–450 (1996).

Franco, M. et al. A novel secreted protein, MYR1, is central to Toxoplasma gondii’s manipulation of host cells. mBio 7, e00231–15 (2016).

Marino, N. D. et al. Identification of a novel protein complex essential for effector translocation across the parasitoplasmic vacuole membrane of Toxoplasma gondii. PLoS Pathog. 14, e1008628 (2018).

Cogan, A. M. et al. Toxoplasma gondii TgYHR1 identifies three additional proteins within the Toxoplasma gondii parasitoplasmic vacuole required for translocation of dense granule effectors into host cells. mSphere 5, e00858–19 (2020).

Hammond, P. M. et al. Fundamental roles of the golgi-associated Toxoplasma gondii aspartyl protease, ASP5, at the host-parasite interface. PLoS Pathog. 11, e1003211 (2015).

Coffey, M. J. et al. Aspartyl protease 5 matures dense granule proteins that reside at the host-parasite interface in Toxoplasma gondii. mBio 9, e01976–18 (2018).

Curt-Varesano, A., Braun, L., Ranquet, C., Hakimi, M. A. & Boughour, A. The aspartyl protease TgASP5 mediates the export of the Toxoplasma GRA16 and GRA24 effectors into host cells. Cell. Microbiol. 18, 151–167 (2016).

Gendrin, C. et al. Toxoplasma gondii uses unusual sorting mechanisms to deliver transmembrane proteins into the host-cell vacuole. Traffic 9, 1665–1680 (2008).

Beraki, T. et al. Divergent kinase regulates membrane ultrastructure of the Toxoplasma gondii parasitoplasmic vacuole. Proc. Natl Acad. Sci. USA 107, 6361–6370 (2010).

Sidik, S. M. et al. A genome-wide CRISPR screen in Toxoplasma gondii reveals essential apicomplexan genes. Cell 166, 1423–1435.e12 (2016).

Sibley, L. D., Adams, L. B., Fukutomi, Y. & Krahenbuhl, J. L. Tumor necrosis factor-alpha triggers antitoxoplasmal activity of IFN-gamma primed macrophages. J. Immunol. 167, 1491–1512 (2001).

Yang, J. & Zhang, Y. I-TASSER server: new development for protein structure prediction. Nucleic Acids Res. 43, W174–W181 (2015).

Mercier, C., Cesbron-Delawu, M. F. & Sibley, L. D. The amphipathic alpha helices of the toxoplasma protein GRA2 mediate post-secretory membrane insertion. J. Cell Sci. 117, 443–450 (2004).

Jacobs, D., Dubremetz, J. F., Loyens, A., Bosman, F. & Saman, E. Identification and heterologous expression of a new dense granule protein (GRA7) from Toxoplasma gondii. Mol. Biochem. Parasitol. 91, 237–249 (1998).

Fischer, H. G., Stachelhaus, S., Sahim, M., Meyer, H. E. & Reichmann, G. GRA7, an excretory 29 kDa Toxoplasma gondii dense granule antigen released by infected host cells. FEBS Lett. 450, 210–214 (1998).

Lecordier, L. et al. Molecular structure of a Toxoplasma gondii dense granule antigen (GRA 5) associated with the parasitoplasmic vacuole membrane. Mol. Biochem. Parasitol. 59, 143–153 (1993).

Lecordier, L., Mercier, C., Sibley, L. D. & Cesbron-Delawu, M. F. Transmembrane insertion of the Toxoplasma gondii GRA5 protein occurs after soluble secretion into the host cell. Mol. Biol. Cell 10, 1277–1287 (1999).

Coppens, I. et al. Toxoplasma gondii sequesters lysosomes from mammalian hosts in the vacuolar space. Cell 125, 261–274 (2006).

Pernas, L. et al. Toxoplasma effector MAF1 mediates host mitochondria and impacts the host response. PLoS Biol. 12, e1001845 (2014).

Gold, D. A. et al. The Toxoplasma dense granule dense granule protein GRA17 and GRA23 mediate the movement of small molecules between the host and the parasitoplasmic vacuole. Cell Host Microbe 17, 642–652 (2015).

Wang, Y. et al. Three Toxoplasma gondii dense granule proteins are required for induction of Lewis rat macrophage pyroptosis. mBio 10, e02388–1 (2019).

Blakely, W. J., Holmes, M. J. & Arizabalaga, G. The secreted acid phosphatase domain-containing GRA1 protein from Toxoplasma gondii is required for c-Myc induction in infected cells. mSphere 5, e00877–19 (2020).

Coffey, M. J. et al. An aspartyl protease defines a novel pathway for export of Toxoplasma proteins into the host cell. elife 4, e10809 (2015).

Mogk, A., Ruger-Herreros, C. & Bukau, B. Cellular functions and mechanisms of action of small heat shock proteins. Annu. Rev. Microbiol. 73, 89–110 (2019).

Rojas, S., Barrios, R., B接轨, F. & Gómez, M. Characterization of seven Toxoplasma gondii secretory proteins. Parasitol. Res. 100, 1–11 (2009).

Braun, L. et al. Purification of Toxoplasma dense granule proteins reveals that they are in complexes throughout the secretory pathway. Mol. Biochem. Parasitol. 157, 13–21 (2008).

Labruyere, E., Lingnan, M., Mercier, C. & Sibley, L. D. Diverse membrane targeting of the secretory proteins GRA4 and GRA6 within the parasitoplasmic vacuole membrane of Toxoplasma gondii. Mol. Biochem. Parasitol. 132, 31–324 (1999).

Studer, S., Obrist, M., Lenzte, N. & Naberhuber, F. A critical motif for oligomerization and chaperone activity of bacterial alpha-haem motif proteins. J. Biol. Chem. 269, 3578–3586 (1994).

Lee, Y. et al. Initial phospholipid-dependent Ilgno targeting to Toxoplasma gondii vacuoles mediates host defense. Cell. Microbiol. 13, 808–816 (2011).

Ehrenheim, K. et al. Novel roles for ATP-binding cassette G transporters in lipid redistribution in Toxoplasma gondii. Mol. Microbiol. 76, 1232–1249 (2010).

Nolan, S. J., Romano, J. D., Kline, J. T. & Coppens, I. Novel approaches to kill Toxoplasma gondii by exploiting the uncontrolled uptake of unsaturated fatty acids and vulnerability to lipid storage inhibition of the parasite. Antimicrob. Agents Chemother. 62, e00347–18 (2018).
91. Markus, B. M., Bell, G. W., Lorenzi, H. A. & Lourido, S. Optimizing systems for Cas9 expression in Toxoplasma gondii. mSphere 4, e00386–19 (2019).

92. Huynd, M. H. & Carruthers, V. B. Tagging of endogenous genes in a Toxoplasma gondii strain lacking Ku80. Eukaryot. Cell 8, 530–539 (2009).

93. Sadak, A., Taghy, Z., Fortier, B. & Dubremetz, J. F. Characterization of a family of rhoptry proteins of Toxoplasma gondii. Mol. Biochem. Parasitol. 29, 203–211 (1988).

94. Burg, J. L., Pautz, D., Kasper, L. H., Ware, P. L. & Boothroyd, J. C. Molecular analysis of the gene encoding the major surface antigen of Toxoplasma gondii. J. Immunol. 141, 3584–3591 (1988).

95. Dunn, J. D., Ravindran, S., Kim, S. K. & Boothroyd, J. C. The Toxoplasma gondii dense granule protein GRA7 is phosphorylated upon invasion and forms an unexpected association with the rhoptry proteins ROP2 and ROP4. Infect. Immun. 78, 7777–7787 (2010).

96. Kelly, F. D. et al. Toxoplasma gondii MAF1b binds the host cell MIB complex to mediate mitochondrial association. mSphere 2, e00183–17 (2017).

97. Sidik, S. M., Hackett, C. G., Tran, F., Westwood, N. J. & Lourido, S. Efficient genome engineering of Toxoplasma gondii using CRISPR/Cas9. PLoS ONE 9, e100450 (2014).

98. Shen, B., Brown, K. M., Lee, T. D. & Sibley, L. D. Efficient gene disruption in diverse strains of Toxoplasma gondii using CRISPR/CAS9. mBio 5, e01114–e01114 (2014).

99. Rosowski, E. E. et al. Strain-specific activation of the NF-kappaB pathway by GRA15, a novel Toxoplasma gondii dense granule protein. J. Exp. Med. 208, 195–212 (2011).

100. Libenzon, A. et al. Molecular signatures database (MSigDB) 3.0. Bioinformatics 27, 1739–1740 (2011).

101. Libenzon, A. et al. The Molecular Signatures Database (MSigDB) hallmark gene set collection. Cell Syst. 1, 417–425 (2015).

102. Simossis, V. A. & Heringa, J. PRALINE: a multiple sequence alignment toolbox that integrates homology-extended and secondary structure information. Nucleic Acids Res. 33, W289–W294 (2005).

103. Zimmermann, L. et al. A completely reimplemented MPI bioinformatics toolkit with a new HHpred server at its core. J. Mol. Biol. 430, 2237–2243 (2018).

104. Trapnell, C., Pachter, L. & Salzberg, S. L. TopHat: discovering splice junctions with RNA-Seq. Bioinformatics 25, 1105–1111 (2009).

105. Trapnell, C. et al. Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. Nat. Biotechnol. 28, 511–515 (2010).

106. Liberzon, A. et al. Molecular signatures database (MSigDB) 4.0. Bioinformatics 27, 1739–1740 (2011).

107. Liberzon, A. et al. The Molecular Signatures Database (MSigDB) hallmark gene set collection. Cell Syst. 1, 417–425 (2015).

108. Waterhouse, A. et al. SWISS-MODEL: homology modelling of protein structures and complexes. Nucleic Acids Res. 46, W296–W303 (2018).