Screening the *Toxoplasma* kinome with high-throughput tagging identifies a regulator of invasion and egress

Tyler A. Smith1,2, Gabriella S. Lopez-Perez2, Alice L. Herneisen1,2, Emily Shortt1 and Sebastian Lourido1,2

Protein kinases regulate fundamental aspects of eukaryotic cell biology, making them attractive chemotherapeutic targets in parasites like *Plasmodium* spp. and *Toxoplasma gondii*. To systematically examine the parasite kinome, we developed a high-throughput tagging (HiT) strategy to endogenously label protein kinases with an auxin-inducible degron and fluorophore. Hundreds of tagging vectors were assembled from synthetic sequences in a single reaction and used to generate pools of mutants to determine localization and function. Examining 1,160 arrayed clones, we assigned 40 protein localizations and associated 15 kinases with distinct defects. The fitness of tagged alleles was also measured by pooled screening, distinguishing delayed from acute phenotypes. A previously unstudied kinase, associated with a delayed phenotype, was shown to be a regulator of invasion and egress. We named the kinase Store Potentiating/Activating Regulatory Kinase (SPARK), based on its impact on intracellular Ca2+ stores. Despite homology to mammalian 3-phosphoinositide-dependent protein kinase-1 (PKD1), SPARK lacks a lipid-binding domain, suggesting a rewiring of the pathway in parasites. HiT screening extends genome-wide approaches into complex cellular phenotypes, providing a scalable and versatile platform to dissect parasite biology.

Apicomplexans are widespread parasites that include the causative agents of toxoplasmosis (*Toxoplasma gondii*), cryptosporidiosis (*Cryptosporidium* spp.) and malaria (*Plasmodium* spp.). Kinases provide important insight into parasite biology as key regulators of cellular processes and attractive drug targets1–11. However, sequence-based functional inference is challenging because evolutionary divergence and parasite-specific adaptations drive the rewiring of signalling pathways. Even broadly conserved kinase families have acquired dramatic lineage-specific adaptations12. The apicomplexan kinome also includes phylum-specific families of secreted kinases that evolved to interfere with host cell signalling13,14. While many studies have explored the function of kinases in isolation, systematic approaches can achieve a global view of the contributions of individual enzymes to parasite biology.

Recent high-throughput knockout screens in *T. gondii* and *Plasmodium* spp. have assessed the impact of gene loss on parasite growth15–17. Molecular barcodes have enabled profiling of thousands of genes in a single experiment. Efforts directed at the *Plasmodium berghei* kinome identified genes required for sexual cycle progression18. However, detailed functional characterization and temporal control remain critical challenges for high-throughput screens.

Precise regulation of *T. gondii* gene expression has been achieved through several methods. Inducible transcriptional control of target genes can be mediated by heterologous promoters19–21. Alternatively, conditional recombination is possible using a rapamycin-dimerizable Cre recombinase. However, the effects of transcriptional regulation or recombination may be delayed for several replication cycles22–25. By contrast, the auxin-inducible degron (AID) system confers post-translational regulation, often achieving knockdown of a target protein within 1 h26–28. The AID system is also reversible, retains the target gene’s native promoter and can be paired with a fluorescent-protein tag. Deploying the AID system at scale could provide both subcellular localization and temporal resolution to the functional dissection of target genes.

High rates of non-homologous end joining (NHEJ) typically preclude efficient homologous recombination (HR) in *T. gondii*. Deletion of *KU80*, required for NHEJ, increased the efficiency of HR29–31. High rates of HR in yeast have enabled the generation of arrayed strain collections for the analysis of protein localization and interacting partners32–34. Similar efforts have been undertaken in mammalian cells35–37 and the parasite *Trypanosoma brucei*; however, no such approach has been implemented in apicomplexans38. Stable NHEJ-deficient strains, high rates of transfection and the adaptation of clustered regularly interspaced short palindromic repeats (CRISPR)-based genome engineering39 make *T. gondii* ideal for the development of HiT methods.

We present an HiT strategy amenable to both arrayed and pooled screening in *T. gondii*. We profiled the kinome of *T. gondii*, assessing subcellular localization and defects in cell division and the lytic cycle. Our system provided spatiotemporal resolution that led to the discovery of regulators of various steps in the lytic cycle, including a previously unstudied kinase critical for invasion and egress from host cells. Our system will be a powerful tool to systematically dissect apicomplexan biology.

**Results**

**Development of HiT vectors for *T. gondii***. Pooled knockout screening fails to capture protein localization, expression levels and the timing of phenotype development. To that end, we developed an HiT strategy that uses CRISPR-directed HR to site-specifically integrate exogenous sequences (payloads), such as epitope tags or regulatable elements (Fig. 1a). This strategy is scalable based on

---

1Whitehead Institute for Biomedical Research, Cambridge, MA, USA. 2Department of Biology, Massachusetts Institute of Technology, Cambridge, MA, USA. ✉e-mail: lourido@wi.mit.edu
cloning from entirely synthetic sequences, a modular design and the flexibility to construct multiple vectors in a pooled format. HIT vectors encode a guide RNA (gRNA) and 40-bp homology regions specific to a target site. HR-mediated repair integrates the synthetic construct into the genome, eliminating the gRNA target site. The integrated gRNA sequence provides a molecular barcode to identify each mutant, making it compatible with both pooled and arrayed screening.

To optimize the HIT system for screening, we designed vectors to tag genes with the mNeonGreen (mNG) fluorophore fused to a minimal auxin-inducible degron (mAID). In T. gondii strains containing the heterologous F-box protein TIR1, addition of the
auxin indole-3-acetic acid (IAA) leads to proteosomal degradation of the mAID-tagged protein\textsuperscript{22,23,34,35}. As test cases, we targeted the kinases CDPK1 and CDPK3, which tolerate C-terminal tags\textsuperscript{36–38}. We also constructed scarless clones in which the tag was integrated in the absence of additional exogenous sequences, to calibrate the expression of genes tagged with our HiT vectors. Tagging vectors were cotransfected with a Cas9-expression plasmid. After selection, populations were predominantly mNG-positive (Fig. 1b and Supplementary Fig. 1a). However, HiT-tagged populations were half as fluorescent as their scarless counterparts. Replacing the SAG1 3’ untranslated region (UTR; 3’\textsubscript{\textit{SAG1}}) with the CDPK3 3’ UTR (3’\textsubscript{\textit{CDPK3}}) recovered the expression of the HiT-tagged alleles to near wild-type levels (Fig. 1b, bottom row). The correct localizations and UTR-dependent changes in expression were also apparent by live-cell microscopy (Extended Data Fig. 1a)\textsuperscript{36,38}. Sequencing the gene–tag junctions of isolated clones confirmed correct integration in all the mNG-positive clones (Fig. 1c). Nearly all of the selected parasites integrated the HiT vectors in the correct locus, with most harbouring the tag in-frame—51% of those generated with 3’\textsubscript{\textit{SAG1}} and 90% of those generated with 3’\textsubscript{\textit{CDPK3}}.

To demonstrate the versatility of the HiT vector platform, we generated additional vectors targeting CDPK1. We tagged CDPK1 with a V5 epitope linked to the mKate2 fluorophore via a T2A skip peptide (Fig. 1d). This HiT vector exhibited highly efficient tagging (Fig. 1e–g, Extended Data Fig. 1b and Supplementary Fig. 1b,c), and may be used to select for mutants that incorporate the construct in-frame while avoiding the effect of bulky tags. We also generated a HiT vector for transcriptional regulation, using the U1 system (Fig. 1h)\textsuperscript{31}, which conferred inducible knockdown of CDPK1 (Fig. 1j) and observed complete knockdown (Fig. 1l.m and Extended Data Fig. 1d). The AID system is restricted to downregulation of proteins with cytosolic termini; however, the speed of degradation and compatibility with native regulatory sequences make it the ideal method to target the parasite kinase\textsuperscript{22–24,39}.

**Generation of an array of conditional mutants.** Protein kinases localize to distinct subcellular compartments to control diverse cellular functions\textsuperscript{6}. We designed HiT constructs against 147 protein kinases and 8 control genes, which included genes associated with key lytic cycle transitions, genes previously tagged with the AID system and a dispensable gene (Supplementary Table 1)\textsuperscript{32,39–41}. We excluded kinases with predicted signal peptides, based on the expectation that they are inaccessible to the cytosolic TIR1 and largely dispensable in cell culture\textsuperscript{32}. We designated 40-bp homology regions upstream and downstream from the cut site, which were paired with three different gRNAs per gene, resulting in 465 unique constructs. Homology region 1 (H1) leads into the tag and includes the 40 bp upstream of the stop codon, whereas homology region 2 (H2) starts 6 bp downstream of the cut site to prevent futile cycles of cutting.

Because the screens were performed in parallel to the analysis of UTR function, we generated libraries with both SAG1 and CDPK3 3’ UTRs. Short oligos encoding the gRNA and matched homology regions were synthesized and cloned into an entry vector (Fig. 1n). The library was linearized to introduce the gRNA scaffold and then cloned as a unit into the HiT vector to generate the final libraries (Fig. 2a). The strategy maintains library diversity while generating thousands of tagging vectors in a single reaction.

We carried out arrayed and pooled screens using the constructed libraries (Fig. 2a). Pooling screening is highly scalable, whereas arrayed screening can be used to determine phenotypes through replica-plating and microscopy. To generate the clonal array, the 3’\textsubscript{\textit{CDPK3}} library was transfected into parasites expressing TIR\textsuperscript{22–24,39} and a red-fluorescent inner membrane complex marker (IMC1-tdTomato) to visualize the parasite ultrastructure across the replicative cycle\textsuperscript{44}. Following transfection and selection, the population was subcloned by limiting dilution. A total of 1,160 clonal strains were arrayed and passaged in 96-well plates (Extended Data Fig. 2a).

We employed dual-indexing PCR to sequence the gRNAs from all 1,160 clonal lines in a single next-generation sequencing experiment. We were able to amplify and assign gRNA identities to 917 wells, with 87% (796) of the amplified wells containing a single gRNA representing 49% (228) of gRNAs in the original library and 82% (127) of the targeted genes (Extended Data Fig. 2b). Some 121 of the amplified wells contained two or more gRNAs. Only singly tagged clones were used for phenotype and localization studies.

**Localizations and phenotypes of tagged proteins in the arrays.** We employed high-content imaging to visualize the localization and IAA-induced depletion of tagged proteins, and monitor the consequences of their knockdown. Arrayed clones were replica-plated, treated with IAA or vehicle 3 h post-infection, and imaged the following day. Some 232 clones (29%) displayed a clear mNG signal, enabling unambiguous localization of 40 proteins to diverse subcellular compartments (Fig. 2b, Extended Data Figs. 3,4 and Supplementary Table 2). Some proteins, such as TGGT1_275610, consistently displayed heterogeneous expression, probably indicating cell-cycle regulation. Thirty-six of the 40 proteins localized showed complete depletion after 24 h of IAA treatment, TGGT1_226040, TGGT1_322000, TGGT1_320630 and TGGT1_234950 showed minimal signal reduction under IAA. Localization to the parasitophorous vacuole (TGGT1_320630) or dense granules (TGGT1_234950)\textsuperscript{45}, suggests inaccessibility to TIR1 may account for the lack of degradation. Nevertheless, HiT screening can generally localize parasite proteins with high-throughput.

Knockdown-induced phenotypes were classified based on parasite structure. Mutants in TGGT1_304970, TGGT1_313180, TGGT1_234970 or TGGT1_245580 arrested early in the lytic cycle (Fig. 2c). Degradation of the latter two caused a discontinuity in the IMC marker (singlets II). Accordingly, TGGT1_304970 is a cyclin-related kinase (TgCrk1) required for daughter cell assembly\textsuperscript{46} and TGGT1_234970 is a nuclear tyrosine kinase-like protein (TgTkL2)\textsuperscript{47} related to the human Tousled-like kinase that promotes DNA replication\textsuperscript{48–52}. The two other singlet kinases are also nucleal: TGGT1_313180 is related to a yeast splicing factor\textsuperscript{53}; and TGGT1_245580 is large, found exclusively in coccidians and lacks any other distinguishing features.

The remaining cell division phenotypes could be broadly categorized as exhibiting a continuous (cell division I) or fragmented (cell division II) IMC distribution (Fig. 2c). Within these categories, we found two previously described cyclin-related kinases, TGGT1_256070 (TgCrk4) and TGGT1_229020 (TgCrk5). Knockdown of TgCrk4 causes major morphological abnormalities and decreased plaque size\textsuperscript{46}. The function of TgCrk5 has not been examined previously, although it was shown to interact with the cell-cycle regulator ECR1 at the centrocone\textsuperscript{42}—consistent with our observations (Extended Data Fig. 3a). Indeed, our knockdown of TgCrk5 mirrored the phenotype of a temperature-sensitive allele of ECR1 (ref. 35). Knockdown of the centrosome-associated kinases NIMA-related kinase 1 (TgNek1; TGGT1_292140), MAPK-like protein 1 (TgMAPK-L1; TGGT1_312570) and TgMAPK2 (TGGT1_207820) phenocopied previously characterized conditional mutants\textsuperscript{33–35}. TgMAPK-L1 displayed cell-cycle-dependent localization to diffuse puncta, consistent with its localization to the pericentrosomal matrix. In addition to the reported cytosolic localization, TgMAPK2 was observed at paired puncta in a subset of vacuoles, shedding additional light into the function of this kinase.

Cell-cycle defects were also observed for several kinases that have not been studied previously. Knockdown of TGGT1_240410 phenocopied TgMAPK-L1 disruption, despite being conserved...
Fig. 2 | Deconvolution of protein phenotypes and localizations through high-content imaging of arrayed HiT clones. a, Construction of the V5-mNG-mAID HiT vector library and subsequent screening strategy. Following construction and linearization of the library, the vector was cotransfected with a Cas9-expression plasmid into parasites expressing the TIR1 ligase and a fluorescent peripheral marker (TIR1/IMC1-tdTomato). Following selection, the population was analysed by both pooled and arrayed screening. b, Distribution of subcellular localizations for tagged proteins in the array; number of proteins found in each compartment and clones analysed for each gene indicated in parentheses. Localizations were assigned to a gene if at least half of the uniquely tagged wells for that gene displayed consistent localizations. Representative confocal images of sample clones are displayed with genes numbered based on their unique identifier (for example, TGGT1_210830, labelled 210830). Images are maximum intensity z-projections for mNG (green) and IMC1-tdTomato (magenta). c, Widefield microscopy of representative clones with identified phenotypes. Images are maximum intensity z-projections. The IMC1-tdTomato marker is displayed for cultures treated with either vehicle or IAA for 24 h. Phenotypes were binned into six categories based on their similarity. Number of clones analysed for each gene indicated in parentheses. Scale bar, 15 µm. d, The ability of clones to lyse fibroblasts was assayed by infecting monolayers for 72 h in the presence or absence of IAA. Intact monolayers were visualized by crystal violet staining. Normalized absorbance measurements comparing vehicle- and IAA-treated wells are plotted for each clone. Each plate contained the parental strain (WT) and an AID-tagged CDPK1 clone (CDPK1) as controls. Mean ± s.d. for WT controls are shown.
only among related coccidians. How other kinases regulate the cell cycle is unknown, but they can be associated with cellular functions based on their localization and similarity to well-studied orthologues: TGGT1_235370 and TgMAPK2, TGGT1_224480 and Cdc-like kinases that regulate the mammalian spliceosome7, and TGGT1_210830 and RIO kinase 1 that participates in ribosomal maturation8. As with TGGT1_210830, knockdown of TGGT1_268010 caused an arrest after a single cell cycle, although its conservation is restricted to coccidians. Accumulation of the IMC marker with grossly normal organization resulted from knockdown of TGGT1_278900, for which homology to yeast BUD32 and association of the Plasmodium falciparum orthologue with the EKC/KEOPS complex components suggest conservation of the complex across eukaryotes9.

We also screened for aggregate defects in the lytic cycle by examining monolayer clearance after knockdown (Fig. 2d). Changes in monolayer clearance were compared with positive and negative controls. All positive controls and 109 clones (11%) scored three standard deviations above the average lysis inhibition score for the negative controls. The 109 clones represented 21 genes (17% of genes tested), including every mutant identified by high-content imaging except TgMAPK2, which displayed an incompletely penetrant phenotype. By contrast, clones in which ERK7 or Doc2.1 were tagged displayed defective monolayer clearance but no morphological changes by microscopy, consistent with reported roles in invasion and egress but not replication9,10. TGGT1_306540 shared these phenotypes with ERK7 and Doc2.1, and appears to be a cytosolic ethanolamine kinase (TgEK) necessary for the completion of the lytic cycle. Our results highlight that arrayed HiT screening can capture both localization and detailed cellular phenotypes.

Temporal resolution of phenotypes by pooled screening. Pooled screening offers greater scalability and a more sensitive comparison of mutant fitness than arrayed screening. To perform pooled screens, 3′_SAG or 3′_CDEP HiT vector libraries were transfected into TIR1 or TIR1/IMC1-tdTomato parasites, respectively. After three passages under pyrimethamine selection, the populations were split, treated with vehicle or IAA, and sampled at each passage to quantify gRNA abundances (Fig. 3a). The abundance of gRNAs in the array was strongly correlated to their abundance in the 3′_CDEP pool before IAA treatment (Extended Data Fig. 4e). The relative abundance of a given gRNA could be affected by the efficiency of integration, locus tolerance to tagging, and the consequence of protein degradation by IAA. As expected, gRNAs targeting sequences with NAG protospacer adjacent motifs (PAMs) were less efficiently integrated than those targeting NGG PAMs11,12 (Fig. 3b). To regress the effect of the PAM, we normalized the abundance for NAG gRNAs at each time point by the difference in mean abundance between NAG and NGG guides for genes considered dispensable13 within each sample. We calculated the fold change relative to the second passage, when relative gRNA abundances best reflected the composition of subsequent populations, allowing us to merge the results of both screens.

We compared the trajectories for gRNAs across time and IAA treatment. Using Uniform Manifold Approximation and Projection (UMAP) for dimensionality reduction followed by k-means clustering14,15, the groupings of gRNAs largely agree with previously determined phenotype scores of the target genes (Fig. 3c,d)12. Notably, cluster 1 was enriched in gRNAs that differentially dropped out of the population following IAA treatment (Fig. 3e), suggesting the targeted genes are fitness-conferred and their gene products are successfully depleted upon IAA treatment.

We next calculated the centroid in UMAP space for gRNAs from both screens to measure the performance of individual genes. Unsupervised clustering on the centroids assigned genes to two classifications, one of which, cluster A, included 47 genes and was highly enriched for those contributing to parasite fitness (Fig. 3f).

The arrayed screen captured mainly acute phenotypes and 18 of the 22 genes that had deficits by microscopy or lytic assay were found in cluster A. Thirteen of the cluster A genes were not represented in the array, and the remaining 16 likely display phenotypes difficult to appreciate in isolation or during brief periods of kinase depletion. Discrepancies between the two screens may also result from the analysis of incorrectly integrated vectors in the array; such clones could be theoretically excluded based on lack of tag expression, but that strategy would sacrifice lowly abundant protein kinases. Out-of-frame integration may also be frequent for proteins rendered hypomorphic by the tag and may drive known essential genes like PKG12,16 into cluster B.

Two of the four genes identified by the arrayed screen but not the pooled screen (TGGT1_218720 and TGGT1_250680) are probably false positives in the arrays, because they were only represented by single clones with modest defects in the lytic assay and were dispensable in previous knockout screens42. By contrast, the two other genes missed by the pooled analysis (TGGT1_278900 and TGGT1_240910) are expected to be fitness-conferring43, and TGGT1_278900 degradation was associated with accumulation of the IMC marker (Fig. 2c). Three clones of TGGT1_240910 (Doc2.1; included as a control based on prior studies42) displayed the expected lytic assay phenotype upon knockdown. For some genes (for example, Doc2.1), the discrepancy between screens originated from contradictory results between different gRNAs when the population was treated with IAA; however, we noticed that gRNAs with optimal designs—based on proximity to the coding sequence or use of an NGG PAM—were more likely to conform to expectations.

We next investigated the timing of gRNA loss for genes in cluster A. We sorted genes based on the fraction of the maximum effect from IAA treatment that was observed after a single lytic cycle (Fig. 3g). Fourteen of the 15 genes associated with defects by microscopy (TGGT1_278900 was missed by the pooled screens) dropped out substantially during the first passage in IAA. TGGT1_215700 and TGGT1_270330 (TgCrk7) dropped out acutely but were absent from the array, and the latter is considered essential44. TGGT1_215700 is broadly conserved across eukaryotes and homologous to phosphatidinositol kinases critical for proliferation in other organisms45,46. This temporal resolution was not observed in conventional CRISPR-based screens (Extended Data Fig. 4f). Our results suggest that this analysis can identify genes whose disruption leads to immediate and catastrophic defects in the parasite.

In contrast to the acute defects associated with some genes, gRNAs against other genes were mostly retained during the first lytic cycle (Fig. 3g). Delayed-loss phenotypes were assigned to several genes linked to invasion or egress: CDPK1, ERK7, PKAc1, TKL1 and CAM3 (refs. 44,46,61,62,63). These genes lacked defects during the brief window selected for microscopy. Another delayed-loss gene, CDPK2A, belongs to a kinase family that has been linked to invasion and egress47–49,50,51. TGGT1_249260 (TgCrk3) similarly displayed no defects by microscopy or lytic assay, consistent with the observation that knockdown reduced plaque size but did not impact morphology44. Pooled screens more easily capture subtle defects that accrue over several lytic cycles and may involve processes that accompany egress and invasion.

Two delayed-loss kinases regulate invasion. We characterized four delayed-loss candidates with patterns of gRNA loss that contrasted with the rapid depletion of representative acute-loss genes (Fig. 4a). All four kinases were fitness-confering in genome-wide knockout screens32 and remain largely uncharacterized. The four kinases exhibit different phyletic patterns52: TGGT1_204280 is conserved among several single-celled parasitic phyla, including kinetoplastids and amoebas; TGGT1_268210 and TGGT1_239420 are restricted to Apicomplexa; and TGGT1_239885 is restricted to coccidians. TGGT1_239420 was previously linked to the development of...
was used as a control for the assay. All four mutants were outcompeted by wild-type, demonstrating that the kinases contribute to parasite fitness (Fig. 4b and Supplementary Fig. 2). Three of the mutants also showed clear defects in plaque formation when grown in the presence of IAA (Fig. 4c); knockdown of TGGT1_239420 reduced plaque size, whereas knockdown of TGGT1_268210 blocked plaque formation (Fig. 4d). The lack of a plaquing defect for TGGT1_204280 suggests a subtle or merely competitive defect in fitness.

We assayed the invasion efficiency of all four mutants, as a potential cause of the delayed-loss phenotype. Both TGGT1_268210 and TGGT1_204280 displayed significant invasion defects upon knockdown (Fig. 4e). Because parasites formed normal plaques upon TGGT1_204280 knockdown, the invasion defect may represent a delay rather than a complete block. By contrast, the severe invasion defect of TGGT1_268210, coupled with effects in plaque formation and competition assays, suggests the gene plays a critical role in the lytic cycle.

**SPARK regulates intracellular Ca\(^{2+}\) store release.** We examined TGGT1_268210 (hereafter referred to as SPARK) in greater detail. SPARK belongs to the AGC kinase family. Despite homology to artemisinin resistance in vitro\(^1\). Intriguingly, the *Plasmodium falciparum* orthologue of TGGT1_268210 (PfPDK1; Pf3D7_1121900), was classified as fitness-conferring in previous screens and has been linked to the regulation of protein kinase A (PKA)\(^{12,13}\).

Although three of the candidates were in the array, we rederived conditional mutants to independently validate the screening results. We placed each mutant in competition with TIR1/IMC1-tdTomato parasites under knockdown conditions. A second wild-type strain was used as a control for the assay. All four mutants were outcompeted by wild-type, demonstrating that the kinases contribute to parasite fitness (Fig. 4b and Supplementary Fig. 2). Three of the mutants also showed clear defects in plaque formation when grown in the presence of IAA (Fig. 4c); knockdown of TGGT1_239420 or TGGT1_239885 reduced plaque size, whereas knockdown of TGGT1_268210 blocked plaque formation (Fig. 4d). The lack of a plaquing defect for TGGT1_204280 suggests a subtle or merely competitive defect in fitness.

We assayed the invasion efficiency of all four mutants, as a potential cause of the delayed-loss phenotype. Both TGGT1_268210 and TGGT1_204280 displayed significant invasion defects upon knockdown (Fig. 4e). Because parasites formed normal plaques upon TGGT1_204280 knockdown, the invasion defect may represent a delay rather than a complete block. By contrast, the severe invasion defect of TGGT1_268210, coupled with effects in plaque formation and competition assays, suggests the gene plays a critical role in the lytic cycle.
mammalian PDK1 (Fig. 5a), SPARK lacks the canonical C-terminal phosphoinositide-binding domain, similar to related kinases in yeast and nonvascular plants76. Instead, SPARK and its apicomplexan orthologues possess an N-terminal MKXGFL motif absent from canonical PDK1s (Fig. 5b). Free-living alveolates Vitrella brassicaformis and Chromera velia harbour two PDK1-like kinases, but only one parologue has the N-terminal motif. This suggests the SPARK clade may have arisen from gene duplication prior to the split of the Apicomplexa from other Alveolata, followed by loss of the more closely related PDK1 homologues in the parasitic clade.

SPARK displayed a diffuse cytosolic localization by immunofluorescence and was depleted without affecting replication after 24 h of IAA treatment in the conditional mutant (Fig. 5c,d, Extended Data Fig. 5a and Supplementary Fig. 3). Because egress shares several signalling pathways with invasion, we examined the role of SPARK in this process. We induced parasite egress from host cells with the phosphodiesterase inhibitor zaprinast, which increases cyclic guanosine monophosphate (cGMP) levels, activates protein kinase G (PKG) and triggers the release of intracellular $\text{Ca}^{2+}$ stores77–82. The $\text{Ca}^{2+}$ ionophore A23187 provides an alternative trigger for egress, which also increases cytosolic $\text{Ca}^{2+}$ but circumvents guanylate cyclase activity83–85. Although SPARK knockdown completely blocked zaprinast-induced egress (Fig. 5e), it appeared almost entirely dispensable for A23187-induced egress (Fig. 5f).
These results suggest that SPARK loss interferes with the ability of PKG to trigger intracellular Ca\(^{2+}\) release.

To examine the role of SPARK in Ca\(^{2+}\) release, we tagged the endogenous gene with a HiT vector carrying an mCherry-mAID epitope in parasites expressing TIR1 and the genetically encoded fluorescent Ca\(^{2+}\) sensor GCaMP6f\(^{46}\). Treatment of GCaMP6f/SPARK-AID parasites with IAA attenuated cytosolic Ca\(^{2+}\) fluxes in intracellular parasites following zaprinast-stimulation (Fig. 5g,h). This phenotype was also rescued by A23187. Because cytosolic Ca\(^{2+}\) can originate from multiple sources\(^{67,68}\), we stimulated extracellular parasites in a buffer containing basal Ca\(^{2+}\) concentrations (~100 nM free Ca\(^{2+}\)). SPARK knockdown blocked zaprinast-induced release of intracellular Ca\(^{2+}\) stores (Fig. 5i). By contrast, the response to the Ca\(^{2+}\) ionophores A23187 or ionomycin was unchanged (Extended Data Fig. 5b), indicating that intracellular Ca\(^{2+}\) stores are intact but unresponsive to zaprinast in the absence of SPARK. These results establish SPARK as a regulator of the parasite intracellular Ca\(^{2+}\) store discharge that precedes both invasion and egress. We therefore named the kinase ‘Store Potentiating/Activating Regulatory Kinase’ (SPARK), to describe its proposed role in apicomplexan biology.

**Discussion**

HiT screens benefit from both arrayed and pooled formats. Arrayed screens examine individual clones to characterize subcellular localizations and cellular defects, even in cases in which they are heterogeneous or incompletely penetrant. Pooled screening, by contrast, compares all targeted genes simultaneously, detecting subtle defects that are apparent only in competitive settings. Arrayed screens for protein localization in *Trypanosoma brucei* successfully localized most gene products\(^{51}\). Pooled screens in *Trypanosoma brucei*, *Plasmodium* spp. and *T. gondii* have examined genetic contributions to fitness, but lack temporal or subcellular resolution\(^{40,14,42,43}\). Arrayed and pooled HiT screens work in tandem to achieve protein localization and phenotypic resolution inaccessible to existing platforms. Further, clonality ensures unambiguous localization, uniform knockdown and recovery of individual clones from the arrays for follow-up studies.

Although false positives in the HiT screens were minimal, 29 genes previously reported to be fitness-conferring\(^{17}\) were missed. False negatives probably originate from poor-quality gRNAs, inaccessibility of the tagged protein to TIR1 and tagging-induced hypomorphism. Reduced abundance of low-quality gRNAs also caused skewed sampling in the array. Future screens should use more gRNAs per gene, avoid targeting NAG PAMs and array clones soon after transfection. Alternative HiT payloads can constitute regulatory gRNAs per gene, avoid targeting NAG PAMs and array clones soon after transfection. Further, clonality ensures unambiguous localization, uniform knockdown and recovery of individual clones from the arrays for follow-up studies.

We identified several previously unstudied regulators of the parasite lytic cycle. Knockdown of each of four nuclear kinases caused early arrests, reminiscent of blocks in G\(_i\) or S/M—such kinases probably regulate critical checkpoints. By contrast, later arrests may be associated with a restriction of resources. This is supported by the phenotype of TGGT1_210830, the orthologue of which is critical for ribosomal maturation\(^{15}\). Other phenotypes included abnormalities in parasite morphology. Multiple genes displayed defects similar to those observed previously for TgMAPK-L1 and TgMAPK2 (refs. \(^{53–56}\)). Aberrant morphologies allude to failures in daughter cell assembly or cytokinesis. The visualization of cellular consequences following gene knockdown expands upon general fitness screening leading to specific hypotheses regarding gene function. The cellular defects observed also reflect acute and lethal consequences for the parasites, establishing these kinases as promising therapeutic targets.

The temporal resolution of the HiT screens revealed genes displaying a delayed-loss phenotype, including SPARK (TGGT1_268210). The proposed name for the kinase reflects its role in the regulation of Ca\(^{2+}\) stores during the parasite lytic cycle. SPARK is critical for egress and release of intracellular Ca\(^{2+}\) stores following stimulation of the cGMP pathway—phenotypes rescued by Ca\(^{2+}\) ionophores. This pattern mirrors the regulation of filamentous actin translocation during parasite motility (F-actin flux)\(^{77}\), required for invasion and egress. Regulation of Ca\(^{2+}\) stores by proteins such as SPARK likely precedes microneme discharge, and subsequent egress and invasion. However, SPARK’s phenotype is distinct from PKG, loss of which cannot be compensated by Ca\(^{2+}\) ionophores\(^{53}\). This is consistent with models in which PKG regulates the production of diacylglycerol and inositol trisphosphate\(^{23,40,41,42}\). Inositol trisphosphate-stimulated release of intracellular Ca\(^{2+}\) stores and diacylglycerol conversion to phosphatidic acid are both thought to mediate secretion of microneme contents\(^{16}\). Altering the activity of the cGMP-producing guanylate cyclase blocks egress induced by zaprinast or 5-Benzyl-3-isopropyl-1H-pyrazole[4,3-d]-pyrimidin-7(6H)-one (BIPPO). Reports differ on the effect of A23187 following guanylate cyclase knockdown—ranging from a nearly normal egress to a complete block\(^{30,40,41}\)—making it difficult to definitively place SPARK in the pathway.

The observed phenotypes appear inconsistent with the canonical role of PDK1 activating other AGC kinases\(^{60,66}\). Failure to activate PKG would block both zaprinast- and ionophore-stimulated egress\(^{77}\). Analogously, failure to activate PKA, a negative regulator of egress, would result in premature egress\(^{13,42}\). Whether global downregulation of AGC kinase function would phenocopy the loss of SPARK therefore remains an open question. Our study places SPARK as a positive regulator of invasion and egress via potentiation or activation of intracellular Ca\(^{2+}\) stores, which could be achieved via direct stimulation of Ca\(^{2+}\) channels or modulation of upstream regulators such as PKA. Further work is needed to distinguish between these roles.
models. The characterization of SPARK provides a crucial molecular handle to study the activation of intracellular Ca\textsuperscript{2+} stores—an event that mediates key transitions in the apicomplexan life cycle.

HiT screens expand current platforms and enable the identification of complex cellular phenotypes. This technology can already handle much larger gene sets (~2,000 genes) than the kinome. The AID system is reversible\textsuperscript{22}, which could help distinguish temporary arrests in replication from lethal disruptions. Alternative tags or conditional expression systems can also extend HiT screening to additional questions and make it compatible with secreted
or compartmentalized proteins inaccessible to the AID approach. Concurrently with our work, Jimenez-Ruiz and colleagues developed an alternative strategy for high-throughput phenotypic analysis of T. gondii, which implemented a rapamycin-inducible split Cas9 to precisely time gene disruption99. Together with the HiT screens, these technologies offer unprecedented spatiotemporal resolution to screening in T. gondii and are powerful tools for dissecting the biology of these ubiquitous apicomplexan parasites.

Methods

Data analysis. All data analysis was performed in either Microsoft Excel (v.16.58), GraphPad Prism (v.9.1.2), RStudio (v.1.2.0.5033), Perl (v.5.18), FlowJo (v.10.7), ImageJ (v.2.0.8) or SnapGene (v.5.1.5).

Parasite and host cell culture. T. gondii parasites were grown in human foreskin fibroblasts (HFFs) maintained in DMEM (GIBCO) supplemented with 3% inactivated fetal calf serum (IFS) and 105 fibroblasts (HFFs) maintained in DMEM (GIBCO) supplemented with 3% inactivated fetal calf serum (IFS) and 10 μg ml−1 gentamicin (ThermoFisher Scientific), referred to as D3. Where noted, DMEM supplemented with 10% IFS and 10 μg ml−1 gentamicin was used, referred to as D10.

Parasite transfection. Parasites were passed through 3-μm filters, pelleted at 1,000g for 10 min, washed, resuspended in Cytoxins (10 mM KPO4, 120 mM KCl, 150 mM CaCl2, 5 mM MgCl2, 25 mM HEPS, 2 mM EDTA, 2 mM ATP and 5 mM glutathione) and combined with transfected DNA to a final volume of 400 μl. Electroporation used an ECM 830 Square Wave electroporator (BTX) in 4-mm cuvettes with the following settings: 1.7 kV, two pulses, 176 μs pulse length and 100 ms interval.

Strain generation. Oligos were ordered from IDT. All cloning was performed with Q5 2x master mix (New England Biolabs) and NEBuilder HiFi assembly (New England Biolabs) unless otherwise noted. Primers and plasmids used or generated in this study can be found in Supplementary Table 1. Oligos, plasmids and strains generated within this study are available from the corresponding author by request.

Scarcless CDPK1-mNG-AID. The V5-TEV-mNeonGreen-AID-Ty cassette was PCR amplified from plasmid pBM050 with repair homology arms using primers P108 and P109. Oligos P110 and P111 were duplexed and cloned into plasmid pS051 to create the gRNA/Cas9-expression plasmid. The gRNA/Cas9-expression plasmid was cotransfected with the repair template into TIRI parasites99,107. Following the first lysis, mNG-positive clones were isolated via fluorescence-activated cell sorting. Single clones were obtained by limiting dilution and verified by PCR amplification using primers P114 and P115 and sequencing with primers P112 and P113.

Scarcless CDPK3-mNG-AID. The CDPK3-mNG-AID scarless strain was generated as CDPK1-mNG-AID above, using primers P116 and P117 for validation of clonal isolates, and primers P118 and P119 for assembly of the gRNA/Cas9-expression plasmid, primers P120 and P121 for validation of clonal isolates, and primers P112 and P113 for the sequencing of tag junctions.

TIRI/IMC1-tDmTomato. The sequence pUTB1_ImC1-tDmTomato_DHFR was PCR amplified with primers P96 and P97 to yield a repair template with homology to a defined, neutral genomic locus109. Approximately 2 × 106 extracellular TIRI parasites were transfected with 50 μg of gRNA/Cas9 plasmid targeting the neutral locus and 6 μg of repair template. Single clones were isolated by FACS into 96-well plates containing HFFs. IMC1-tDmTomato positive clones were subsequently identified by microscopy and verified by PCR amplification of the locus using primers P98 and P99.

TGGT1_268210, TGGT1_204280, TGGT1_239885 and TGGT1_239420 AID-tagged lines. HiT vector cutting unit gBlocks (IDT DNA) (P122–125) were cloned into the pGL015 HmG HiT vector backbone. HiT vectors were linearized with BsaI and cotransfected with the pSS014 Cas9-expression plasmid into TIRI/GCaMP6f parasites. Parasite populations were selected with 25 μg ml−1 mycophenolic acid and 30 μg ml−1 xanthine. Single clones were isolated by limiting dilution. Clones were verified by sequencing of the junction between the 3′ end of the gene and 5′ end of the tag protein.

Analysis of CDPK1- and CDPK3-tagged HiT vector populations. Parasites were cotransfected with 40–50 μg of Bsal-linearized HiT 3′ gag, or HiT 3′ crex vectors and the Cas9−expression plasmid pSS014. Twenty-four hours post-transfection, parasite populations were selected with 3 μM pyrimethamine. Following selection population were analysed by flow cytometry with a Miltenyi MACSQuant VYB. Populations were imaged by microscopy using a x60 objective and an Eclipse Ti microscope (Nikon) with an enclosure maintained at 37°C and 5% CO2. For IAA-induced depletion experiments, intracellular parasites were treated with either 30 μM IAA or an equivalent dilution of PBS for 24 h. Following treatment, parasites were passed through a 27-gauge needle, isolated by filtration and analysed by flow cytometry.

Design and cloning of HiT vector libraries. Three gRNA constructs were designed against each gene in the 155 gene library. Because the efficiency of recombination decreases with the distance of the homology regions from the double-stranded break, we selected gRNAs that cut within a 50-bp window downstream of the stop codon. The 3′ end of the genomic sequences (release 36, ToxoDB.org) were scanned for gRNAs containing either NGG or NAG PAMs. gRNAs were ranked based on predicted on-target activity and off-target activity, as determined by the Rule Set 2 and Cutting Frequency Determination calculators99, respectively, and by the distance of the cut site from the stop codon. Because the Rule Set 2 calculator does not take into account efficiencies of different PAMs, the on-target scores of NAG gRNAs were penalized as predicted by the Cutting Frequency Determination calculator. These ranks were used to create an aggregate rank for gRNA selection. Initially, only the highest-ranking gRNAs were selected with cut sites within 30 bp of the stop codon and with a Rule Set 2 score above 0.2. The criteria were progressively relaxed to allow any gRNA within 30 bp, any gRNA within 50 bp and with a Rule Set 2 score above 0.2, and finally any gRNA within 50 bp, until each gene had three gRNAs assigned. A ‘G’ was prepended to gRNAs that did not start with one to ensure proper RNA polymerase III initiation. Synonymous point mutations were introduced to H1 homology regions containing Bsal or Ascl restriction sites to prevent restriction enzyme cutting during the cloning process. The guide library was synthesized by Agilent and each gRNA includes a Grna, 40-bp homology regions, an Ascl restriction site for insertion of the gRNA scaffold and tandem Bsal sites for linearization of the final constructs, all flanked by sequences for cloning into empty HiT vectors (Supplementary Table 1). The HiT 3′ gag and HiT 3′ crex libraries were cloned as described below. The HiT 3′ crex vector library protocol resulted in both a slight increase in correctly assembled products and greater library diversity. All CCR experiments were performed with iProof High-Fidelity DNA polymerase (Bio-Rad) and cloning products were electroporated into MegaXDH10B TiR electrocompetent cells.

HiT 3′ gag library. The synthesized oligo library was PCR amplified with primers P1 and P2. PCR products were cloned into the pTS018 entry vector via Gibson assembly (VWR). The gRNA scaffold was amplified from pLS01 with primers P3 and P4. The amplified scaffold was inserted into Ascl-digested entry vector library via NEBuilder HiFi assembly. Finally, the assembled cutting units were PCR amplified using primers P1 and P2 and cloned into pTS029, the mNG-AID HiT 3′ crex vector, via NEBuilder HiFi assembly. DNA products were isolated from liquid cultures using either a ZymoPURE II Plasmid Maxiprep Kit (Zymo Research) or a Macherey-Nagel Nucleobond Xtra Maxi Kit.

HiT 3′ crex library. To decrease polymerase-induced errors we replaced PCR amplification steps with digestion with the type II restriction enzyme BsaDI. The oligo library was PCR amplified with primers P1 and P2, as in the HiT 3′ gag library. PCR products were cloned into the pTS031 entry vector via NEBuilder HiFi assembly. The gRNA scaffold was isolated from pTS028 via BsaDI digestion and inserted into Ascl-digested entry vector library. Finally, the assembled ‘cutting units’ were BsaDI-digested out of the entry vector library and cloned into pGL013, the mNG-AID HiT 3′ crex vector. DNA products were isolated from solid agar plate cultures using either a ZymoPURE II Plasmid Maxiprep Kit.

Pooled HiT vector screening. For each screen, 500 μg of the HiT vector library was linearized with BsaI-Hf2, cleaned-up using Agencourt RNAcleanX ScreenSaver magnetic beads, and cotransfected with the pSS014 into 5 × 104 TIRI parasites in the HiT 3′ gag screen and 5 × 105 TIRI/IMC1-tDmTomato parasites in the HiT 3′ crex screen. Transfected parasites were used to infect twelve and ten 15-cm2 dishes with HH monolayers in the HiT 3′ gag and HiT 3′ crex screens, respectively; 3 μM pyrimethamine and 10 μg ml−1 DNASel was added 24 h later. The parasites were allowed to egress naturally from host cells, isolated by filtration and passaged onto eight 15-cm2 dishes with fresh monolayers. This process was repeated for two more passages, infecting each dish with approximately 2–3 × 105 parasites. Following the third passage, the population was...
split into three 15-cm² dishes containing fresh monolayers in D10 supplemented with 50 mM IAA and three 15-cm² dishes supplemented with an equal dilution of vehicle (PBS). The populations were maintained and passaged in their respective conditions for four passages in the HIT 3°, screen and for four passages in the HIT 3°, screen. At select passages approximately 10⁴ parasites were pelleted and stored at −80 °C for analysis. Parasite DNA was extracted using the DNeasy Blood and Tissue kit (Qiagen) and integrated gRNA constructs were amplified with primer P5 and barcoding primers P6–22. The resulting libraries were sequenced using single-reads using custom sequencing primer P23 and custom indexing primer P24.

Sequencing reads were aligned to the gRNA library. Read counts were median normalized and gRNAs in the bottom fifth percentile of the input library were removed. To account for differences in NGG and NAG PAM efficiencies, relative abundances for each gRNA were corrected for the PAM used. The PAM efficiencies were calculated by comparing the abundances of only gRNAs in the selected population that target genes identified as dispensable in a previous genome-wide knockout screen. Fold changes were normalized to passage 2. UMAP was used to compare gRNAs in each screen based on their pattern of fold changes for vehicle- and IAA-treated samples. Clusters were calculated by k-means. Gene centroids were calculated in UMAP space and assigned to the dispensable or fitness-conferring class using k-means clustering.

**Arrayed HIT vector screening.** Generation and packaging of array. In parallel to the pooled HIT 3°, screen, single clones were isolated via limiting dilution after four passages of direct propagation. Parasites from four wells were collected as in the pooled screening experiments, using primer P5 and P25 for PCR amplification. Some 1,160 clonal isolates were arrayed into twenty 96-well plates containing HFFs. Included in each plate was a well containing the TIR1/IMC1-tTomato parental line and a well containing the CDPK1-AID-scarless array. Arrays were passaged every 3 d with a multichannel pipette by transferring 5% of the total lysed well volume to 96-well plates containing HFF monolayers.

Individual wells with incomplete lysate were scrapped and passed with 10% of the total well volume.

**Arrayed wildfield microscopy.** Arrays were replica-plated with 12 µl from freshly lysed monolayers used to inoculate 96-well plates of HFFs, maintained in D3 supplemented with either 50 µM IAA or PBS. Each replicate plate contained six uninfection control wells. Replica plates were centrifuged at 150g and 18 °C for 5 min and subsequently incubated at 37 °C and 5% CO₂. At 3 h post-infection, replica plates were supplemented with either PBS or IAA to a final concentration of 50 µM. At 24 h post-IAA or PBS addition, each well was imaged at four adjacent fields-of-view using a x40 objective and an Eclipse Ti microscope (Nikon) with an enclosure maintained at 37 °C and 5% CO₂. Images were acquired using the NIS elements imaging software, W-View Gemini image splitting optics, and a Zyla 4.2 sCMOS camera. FIJI software was used for image analysis and processing.

**Arrayed lytic assays.** Arrays were replica-plated with 10 µl from freshly lysed monolayers used to inoculate 96-well plates of HFFs, maintained in D3 supplemented with either 50 µM IAA or PBS. Each replicate plate contained six uninfection control wells. Plates were centrifuged at 150g and 18 °C for 5 min and incubated for 72 h at 37 °C and 5% CO₂. Plates were washed once with PBS and fixed for 10 min with 100% ethanol. Intact monolayers were visualized by staining the plates for 5 min with crystal violet solution (12.5 g of crystal violet, 125 ml of 100% ethanol and 500 ml of 1% ammonium oxalate) followed by two PBS washes, one water wash and overnight drying. Absorbance at 590 nm was read as a measure of host cell lysis and normalized to the average of a plate’s uninfection control wells.

**Dual-indexed sequencing of arrays.** Parasites were harvested from 100 µl of lysed wells by centrifugation at 1,000g and 18 °C for 10 min. Parasites were resuspended in 25 µl of lysis buffer (1x Q5 buffer supplemented with 0.2 mg m⁻¹ Proteinase K) and lysed using the following conditions: 37 °C for 1 h, 50 °C for 2 h and 95 °C for 15 min.Guides were PCR amplified from gDNA, with each well utilizing a unique combination of 17 index primers (P26–65) and 15 index primers (P66–95). PCR products from an individual plate were pooled and gel extracted using a Zymoclean Gel DNA Recovery Kit and subsequently pooled in equimolar ratios for sequencing. The final PCR product pool was sequenced with a MiSeq v2 kit (Illumina) with single-reads using custom sequencing primer P23 and custom indexing primer P24. Sequencing reads were aligned to the gRNA library. A well was designated as gRNA-containing if a single gRNA had more than 100 reads. Wells containing multiple integrations or mixed populations were defined as those in which a single gRNA-containing population and 1.5 × 10⁴ parasites of the TIR1/IMC1-tTomato strain and 1.5 × 10⁴ parasites of the competitor strain. At 24 h post-infection the media was changed with fresh D10. Populations were assayed by flow cytometry following host cell lysis, and each population was passed to two wells of a six-well plate. At 24 h post-infection the media of one well per strain was changed to D10 and vehicle (PBS) and the media of the second well changed to D10 and 50 µM IAA. Populations were maintained in these conditions for four passages. Following each lysis, the populations were assayed by flow cytometry with a Miltenyi MACSQuant VYB. The fraction of the population that was tdTomato−negative was represented as a ratio of [1% tdTomato−negative in the IAA sample]/[1% tdTomato−negative in the vehicle sample] and was normalized to the initial ratio pre-splitting into ± IAA media.

**Invasion assays.** Strains were each passed to two flasks of HFFs containing D10 media. At 3 h post-infection one flask was supplemented with vehicle (PBS) and the second flask was supplemented with IAA to final concentrations of 50 M. At 27 h post-infection each flask was syringe-lysed and filtered through 5-µm filters. Parasites were pelleted at 1,000g and 18 °C for 10 min. Pellets were resuspended in invasion media (HEPES-buffered DMEM without phenol red) supplemented with 1% FCS to a concentration of 1 × 10⁵ parasites per ml. Then 200 µl of each parasite solution was added to two wells of a clear-bottom 96-well plate containing HFFs. The plate was centrifuged at 290 g and room temperature for 5 min. Plates were incubated for 10 min at 37 °C to stimulate invasion. Following incubation wells were fixed with 4% formaldehyde and extracellular parasites were stained with mouse anti-SAG1 antibody diluted 1:500 (ref. 8). All parasites were stained by permeabilizing with 0.25% Triton X-100 and staining with guinea pig anti-CDPK1 diluted 1:10,000 (Covance). Cells were subsequently stained with anti-guinea pig Alexa-594 antibody diluted 1:1,000 (Invitrogen), anti-mouse Alexa-488 antibody diluted 1:1,000 (Invitrogen) and Hoechst 33258 (Santa Cruz Biotechnology) nuclear dye. Samples were imaged using a BioTek Cytation 3 imaging multimode reader. The number of invaded parasites per field of view was counted and normalized to the number of host cells in the same area. The invasion efficiency for each replicate was normalized to the invaded parasites per host cell nuclei of the parental TIR1 vehicle sample.

**Phylogenetic analysis of SPARK.** SPARK homologues were identified by BLAST search against representative apicomplexan genomes. Protein kinase domains were obtained from EupathDB based on their annotation with Interpro domain IPR011009. Sequences were curated for Thelileria sp., Cryptosporidium parvum, Cryptosporidium hominis, Sarcocystis neurona, Vitrella brassicaformis and Chromera velia to correct errors in the gene model. Domains from the nearest human, mouse and macaque orthologues (as determined by BLAST) were used as outgroups. Individual domains were aligned using ClustalX2, and the phylogenetic tree was generated by neighbour-joining. Visualizations were generated using FigTree (v.1.4.4).
Parasites were passed to 15-cm² dishes containing HFFs and after 6 h were treated for 3 h at which point they were treated with either 50 μM IAA or PBS. At 24 h post-treatment, intracellular parasites were fixed with 4% formaldehyde and permeabilized with 0.25% Triton X-100 in PBS. CDK4 was detected using a guinea pig-derived polyclonal antibody diluted 1:10,000 (Covance®), HA tag was detected using a mouse monoclonal antibody diluted 1:1,000 (Roche). GAP43 was detected using a polyclonal antibody diluted 1:1,000 (Euroclone). Primary antibodies were stained with anti-guinea pig, anti-mouse or anti-rabbit Alexa Flour-labelled secondary antibodies diluted 1:1,000 (Invitrogen). Images were acquired with an Eclipse Ti microscope (Nikon) using the NIS elements imaging software and a Zyla 4.2 sCMOS camera. ImageJ was used for image analysis and processing.

Replication assays. Parasites were inoculated onto coverslips containing HFFs. For AID strains, after 3 h they were treated with either 50μM IAA or PBS. At 24 h post-IAA addition, intracellular parasites were fixed, permeabilized, and stained and imaged as described in the previous section. For each sample, multiple fields-of-view were acquired. The number of parasites per vacuole was calculated from 100 vacuoles. Results are the mean of three independent experiments.

Egress assays. Egress was quantified in a plate-based manner18. HFF monolayers in a clear-bottomed 96-well plate infected with 7.5×10³ parasites per well of parental or SPARK-AID for 3 h were treated with 50μM IAA or PBS for an additional 24 h. Before imaging, the media was exchanged for Fluorescein isothiocyanate (FITC)-supplemented with 10% FBS. Three images were taken before zaprinast (final concentration 500μM) or A23187 (final concentration 8μM) and 4,6-diamidino-2-phenylindole (DAPI; final concentration 5 ng/ml) were added, and imaging of DAPI-stained host cell nuclei continued for an additional 9 min before 1% Triton X-100 was added to all wells to determine the total number of host cell nuclei. Imaging was performed using a 5× objective of a BioTek Cytation 3 imaging software and a Zyla 4.2 sCMOS camera. Image analysis and quantification was done using ImageJ.

Live-cell microscopy of GCaMP6f-expressing parasites. To capture egress, SPARK-AID parasites were grown in HFFs in glass-bottom 35-mm dishes (Ibidi). Imaging was performed in a plate-based manner18. GCaMP6f-expressing parasites were grown in HFFs in glass-bottom 35-mm dishes (Ibidi) and treated for 3 h with either 50μM IAA or PBS. Following a 24 h treatment the cells were washed once with PBS and harvesting in cold Ringer’s buffer prepared without Ca²⁺ (155 mM NaCl, 3 mM KCl, 1 mM MgCl₂, 3 mM NaH₂PO₄, 10 mM HEPES, 10 mM glucose) and supplemented with 1% BSA (w/v) and recorded every 4 s for 220 s with an Eclipse Ti microscope (Nikon) in an enclosure maintained at 37°C and 5% CO₂. Images were acquired using the NIS elements imaging software and a Zyla 4.2 sCMOS camera. Image analysis and quantification was done using ImageJ.

Extracellular zaprinast and Ca²⁺ ionophore treatment of GCaMP6f strains. Parasites were passed to 15-cm² dishes containing HFFs and after 6 h were treated with either 50μM IAA or vehicle (PBS). Following 24 h of treatment the cells were washed once with PBS and harvested in cold Ringer’s Basal Ca²⁺ (155 mM NaCl, 3 mM KCl, 1 mM MgCl₂, 3 mM NaH₂PO₄, 10 mM HEPES, 10 mM glucose, 250μM EGTA, 112μM CaCl₂). Parasites were isolated via syringe lysis and filtration, pelleted and washed once with cold Ringer’s Basal Ca²⁺, and resuspended to 1×10⁶ parasites per ml. Then, 100μl of parasite suspension was added to a clear-bottom 96-well plate and incubated on ice for 5 min. Fluorescence was read with an excitation wavelength of 485 nm and an emission wavelength of 528 nm every 10 s in a BioTek Cytation 3. At 30 s, 50μl of 5× zaprinast (100μM final concentration), A23187 (2μM final concentration), ionomycin (1μM final concentration) or vehicle (dimethylsulfoxide) was added and fluorescence readings were taken for an additional 6 min. Then 50μl of 4x erythrosin (3μg/ml final concentration) and CaCl₂ (2μM final concentration) was added, and the assay plate was incubated at 37°C for 10 min. Fluorescence was read every 1 min for 40 min. The assay plate was shaken for 1 s before each read. The non-fluorescent TIR1 strain was used to perform a baseline subtraction of background fluorescence. Baseline-subtracted values were normalized to initial fluorescence pre-stimulation and maximum fluorescence post-stimulation. Results are the mean of three independent experiments in the case of A23187 and ionomycin and six independent experiments in the case of zaprinast and dimethylsulfoxide.

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

Data availability. All oligos used in this study are available in Supplementary Table 1. All plasmids used or generated in this study are listed with their appropriate GenBank or PMID accession numbers in Supplementary Table 1. Minimally processed pooled and arrayed CRISPR screen sequencing results are available in Supplementary Table 2. Localization assignments, microscopy phenotypes, lytic assay results, and UMAP coordinates and clusters are likewise available in Supplementary Table 2. Source data are provided with this paper. Data from experimental results is available in the source data files. Additional unprocessed data is available from the corresponding author upon request.

Code availability. All code is described in the methods section and available from the corresponding author upon request.

Received: 24 September 2021; Accepted: 11 March 2022; Published online: 28 April 2022

References
1. Cabrera, D. G. et al. Plasmodial kinase inhibitors: License to cure? J. Med. Chem. 61, 8061–8077 (2018).
2. Saurey, C., Ehrenkaufer, G., Shi, D., Deb Nath, A. & Abagyan, R. Antineoplastic kinase inhibitors: A new class of potent anti-amoebic compounds. PLoS Negl. Trop. Dis. 15, e0008425 (2021).
3. Merritt, C., Silva, L. E., Tanner, A. L., Stuart, K. & Pollastri, M. P. Kinases as druggable targets in trypanosomatid protozoan parasites. Chem. Rev. 114, 11280–11304 (2014).
4. Talevich, E., Mirza, A. & Kannan, N. Structural and evolutionary analysis of eukaryotic protein kinases in Apicomplexa. BMC Evol. Biol. 11, 321 (2011).
5. Peixoto, L. et al. Integrative genomic approaches highlight a family of parasite-specific kinases that regulate host responses. Cell Host Microbe 8, 208–218 (2010).
6. Gai, R. Y., Sharp, A. K. & Brown, A. M. Protein kinases in Toxoplasma gondii. Int. J. Parasitol. 51, 415–429 (2011).
7. Beraki, T. et al. Divergent kinase regulates membrane ultrastructure of the Toxoplasma parasitophorous vacuole. Proc. Natl Acad. Sci. USA 116, 6361–6370 (2019).
8. Taylor, S. et al. A secreted serine-threonine kinase determines virulence in the eukaryotic pathogen Toxoplasma gondii. Science 314, 1776–1780 (2006).
9. Fox, B. A. et al. The Toxoplasma gondii rhoptry kinase is essential for chronic infection. mBio 7, e00193-16 (2016).
10. Fleckenstein, M. C. et al. A Toxoplasma gondii pseudokinase inhibits host IRG resistance proteins. PLoS Biol. 10, e1001358 (2012).
11. Niedelman, W. et al. The rhoptry-like proteins ROP18 and ROP5 mediate IRG resistance proteins. PLoS Pathog. 8, e1002784 (2012).
12. Sidik, S. M. et al. A genome-wide CRISPR screen in Toxoplasma identifies essential apicomplexan genes. Cell 166, 1423–1435.e12 (2016).
13. Bushell, E. et al. Functional profiling of a Plasmodium genome reveals an abundance of essential genes. Cell 170, 260–272.e7 (2017).
14. Zhang, M. et al. Uncovering the essential genes of the human malaria parasite Plasmodium falciparum by saturation mutagenesis. Science 360, eaap7847 (2018).
15. Tewari, R. et al. The systematic functional analysis of Toxoplasma gondii eaaap7847 (2018).
16. Meissner, M., Brecht, S., Bujard, H. & Soldati, D. Modulation of myosin A expression by a newly established tetracycline repressor-based inducible system in Toxoplasma gondii. Nucleic Acids Res. 29, E115 (2001).
17. Meissner, M., Schlüter, D. & Soldati, D. Role of Toxoplasma gondii myosin A in powering parasite gliding and host cell invasion. Science 298, 837–840 (2002).
41. Uboldi, A. D. et al. Protein kinase A negatively regulates Ca2+

40. Bullen, H. E. et al. Phosphatidic acid-mediated signaling regulates

39. Long, S., Anthony, B., Drewry, L. L. & Sibley, L. D. A conserved ankyrin

38. Sathyan, K. M. et al. An improved auxin-inducible degron system preserves

37. Weill, U. et al. Genome-wide SW Ap-Tag yeast libraries for proteome

36. Leonetti, M. D., Sekine, S., Kamiyama, D., Weissman, J. S. & Huang, B. A

35. Long, S. et al. Calmodulin-like proteins localized to the conoid regulate

34. Sathyan, K. M. et al. An improved auxin-inducible degron system preserves

33. Nishimura, K., Fukagawa, T., Takisawa, H., Kakimoto, T. & Kanemaki, M.

32. Nishimura, K., Fukagawa, T., Takisawa, H., Kakimoto, T. & Kanemaki, M.

31. Hunt, A. et al. Differential requirements for cyclase-associated protein

30. Leonetti, M. D., Sekine, S., Kamiyama, D., Weissman, J. S. & Huang, B. A

29. Weill, U. et al. Genome-wide SW Ap-Tag yeast libraries for proteome

28. Huh, W.-K. et al. Global analysis of protein localization in budding yeast.

27. Huynh, M.-H. & Carruthers, V. B. Tagging of endogenous genes in a

26. Long, S., Anthony, B., Drewry, L. L. & Sibley, L. D. A conserved ankyrin

25. Huynh, M.-H. & Carruthers, V. B. Tagging of endogenous genes in a

24. Long, S. et al. Calmodulin-like proteins localized to the conoid regulate

23. Hunt, A. et al. Differential requirements for cyclase-associated protein

22. Nishimura, K., Fukagawa, T., Takisawa, H., Kakimoto, T. & Kanemaki, M.

21. Hunt, A. et al. Differential requirements for cyclase-associated protein

20. Pieperhoff, M. S. et al. Conditional U1 gene silencing in toxoplasma gondii.

19. Pieperhoff, M. S. et al. Conditional U1 gene silencing in toxoplasma gondii.

18. van Poppel, N. F. J., Velagen, J., Duisters, R. F. J. J., Vermeulen, A. N. &

17. van Poppel, N. F. J., Velagen, J., Duisters, R. F. J. J., Vermeulen, A. N. &

16. Avalos, C. A., Cox, J. F., Stocker, W. F. & Sibley, L. D. Checkpoints of apicomplexan cell division identified

15. Avalos, C. A., Cox, J. F., Stocker, W. F. & Sibley, L. D. Checkpoints of apicomplexan cell division identified

14. Harding, C. R. et al. Gliding associated proteins play essential roles during

13. Su, X. et al. An improved auxin-inducible degron system preserves

12. Sampels, V. et al. Conditional mutagenesis of a novel choline kinase

11. Mallari, J. P., Okman, A., Vaupel, B. & Goldberg, D. E. Kinase-associated

10. Back, P. S. et al. Ancient MAPK ERK7 is regulated by an unusual inhibitory

9. Donald, R. G. K. et al. Phosphorylation by a calcium-dependent protein kinase

8. Balla, A. & Balla, T. Phosphatidylinositol 4-kinases: old enzymes with

7. Dvorin, J. D. et al. A plant-like kinase in Plasmodium falciparum regulates

6. Kato, R. et al. Cellular function of phosphoinositide 3-kinases: implications for development, homeostasis, and cancer. Annus. Rev. Cell Dev. Biol. 17, 615–675 (2001).

5. McInnes, L., Healy, J. & Melville, J. UMAP: Uniform manifold approximation and projection for dimension reduction. Preprint at https://arxiv.org/abs/1802.03426 (2018).

4. Hartigan, J. A. & Wong, M. A. Algorithm AS 136: A K-means clustering algorithm. J. R. Stat. Soc. Ser. C Appl. Stat. 28, 100–108 (1979).

3. Donald, R. G. K. et al. Optimized sgRNA design to maximize activity and minimize off-target effects of CRISPR-Cas9. Nat. Biotechnol. 34, 184–191 (2016).

2. Sampels, V. et al. Conditional mutagenesis of a novel choline kinase demonstrates plasticity of phosphatidylinositol biogenesis and gene expression in toxoplasma gondii. J. Biol. Chem. 287, 16289–16299 (2012).

1. Doncch, J. G. et al. Optimized sgRNA design to maximize activity and minimize off-target effects of CRISPR-Cas9. Nat. Biotechnol. 34, 184–191 (2016).
81. McRobert, L. et al. Gametogenesis in malaria parasites is mediated by the cGMP-dependent protein kinase G-dependent microneme secretion in Toxoplasma gondii. *J. Biol. Chem.* 294, 8959–8972 (2019).

82. Brown, K. M. & Sibley, L. D. Essential cGMP signaling in Toxoplasma gondii for Cas9 expression in mammalian stem cells using Cas9 ribonucleaseprotein. *eLife* 6, e100334 (2017).

83. Plattner, F. et al. Toxoplasma profilin is essential for host cell invasion and TLR11-dependent induction of an interleukin-12 response. *Cell Host Microbe* 3, 77–87 (2008).

84. Bisio, H., Lunghi, M., Brochet, M. & Soldati-Favre, D. Phosphatidic acid governs natural egress in *Toxoplasma gondii* via a guanylate cyclase receptor platform. *Nat. Microbiol.* 4, 420–428 (2019).

85. Brown, K. M. & Sibley, L. D. Essential cGMP signaling in Toxoplasma gondii is initiated by a hybrid P-Type ATPase-guanylate cyclase. *Cell Host Microbe* 24, 804–816.e6 (2018).

86. Chen, T.-W. et al. Ultrasensitive fluorescent proteins for imaging neuronal activity. *Nature* 499, 295–300 (2013).

87. Pace, D. A., McNicholl, C. A., Liu, J., Jimenez, V. & Moreno, S. N. J. Calcium entry in *Toxoplasma gondii* and its enhancing effect of invasion-linked traits. *J. Biol. Chem.* 289, 19637–19647 (2014).

88. Arzirabalaga, G. & Boothroyd, J. C. Role of calcium during *Toxoplasma gondii* invasion and egress. *J. Int. Parasitol.* 34, 361–368 (2004).

89. Alsford, S. et al. High-throughput phenotyping using parallel sequencing of RNA interference targets in the African trypanosome. *Genome Res.* 21, 915–924 (2011).

90. You, J. et al. A CRISPR platform for targeted in vivo screens identifies *Toxoplasma gondii* virulence factors in mice. *Nat. Commun.* 10, 3963 (2019).

91. Tosetti, N., Dos Santos Pacheco, N., Soldati-Favre, D. & Jacot, D. Three F-actin assembly centers regulate organelle inheritance, cell–cell communication and motility in Toxoplasma gondii. *eLife* 8, e42669 (2019).

92. Brochet, M. et al. Phosphoinositide metabolism links cGMP-dependent protein kinase G to essential Ca²⁺ signaling at key decision points in the life cycle of malaria parasites. *PLoS Biol.* 12, e1001806 (2014).

93. Carruthers, V. B., Moreno, S. N. & Sibley, L. D. Ethanol and acetaldehyde elevate intracellular [Ca²⁺] Ca²⁺ and stimulate microneme discharge in *Toxoplasma gondii*. *Biochem. J.* 342, 379–386 (1999).

94. Lovett, J. L., Marchesini, N., Moreno, S. N. J. & Sibley, L. D. Toxoplasma gondii microneme secretion involves intracellular Ca(2+) release from inositol 1,4,5-triphosphate (IP(3))/ryanodine-sensitive stores. *J. Biol. Chem.* 277, 25870–25876 (2002).

95. Bulen, H. E., Bisio, H. & Soldati-Favre, D. The triumvirate of signaling molecules controlling *Toxoplasma gondii* micrornemecyoctosis: Cyclic GMP, calcium, and phosphatidic acid. *PLoS Pathog.* 15, e1007670 (2019).

96. Leroux, A. E., Schulze, J. O. & Biondi, R. M. AGC kinases, mechanisms of regulation and innovative drug development. *Semin. Cancer Biol.* 48, 1–17 (2018).

97. Mora, A., Komander, D., van Aalten, D. M. F. & Alessi, D. R. PDK1, the master regulator of AGC kinase signal transduction. *Semin. Cell Dev. Biol.* 15, 161–170 (2004).

98. Li, W. et al. A splitCas9 prototypic screen in *Toxoplasma gondii* identifies proteins involved in host cell egress and invasion. *Nat. Microbiol.* https://doi.org/10.1038/s41564-022-01114-y (2022).

99. Markos, B. M., Bell, G. W., Lorenzi, H. A. & Lourido, S. Optimizing systems for Cas9 expression in *Toxoplasma gondii*. *mSphere* 4, e00386-19 (2019).

100. Paquet, D. et al. Efficient introduction of specific homozygous and heterozygous mutations using CRISPR/Cas9. *Nature* 533, 123–129 (2016).

101. Dewari, P. S. et al. An efficient and scalable pipeline for epitope tagging in mammalian stem cells using Cas9 ribonucleaseprotein. *eLife* 7, e35069 (2018).

102. Biell, P., Rivera-Torres, N., Strowe, B. & Knmec, E. B. Regulation of gene editing activity directed by single-stranded oligonucleotides and CRISPR/Cas9 Systems. *PLoS ONE* 10, e0129308 (2015).

103. Liang, X., Potter, J., Kumar, S., Ravinder, N. & Chensnut, J. D. Enhanced CRISPR/Cas9-mediated precise genome editing by improved design and delivery of gRNA, Cas9 nuclease, and donor DNA. *J. Biotechnol.* 241, 136–146 (2017).

104. Burg, J. I., Perelman, 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).

105. Waldman, B. S. et al. Identification of a master regulator of differentiation in toxoplasma. *Cell* 180, 359–372.e16 (2020).

106. Starnes, G. L., Jewett, T. J., Carruthers, V. B. & Sibley, L. D. Two separate, conserved acidic amino acid domains within the *Toxoplasma gondii* Mi2 cyttoplasmic tail are required for parasite survival. *J. Biol. Chem.* 281, 30745–30754 (2006).

107. Plattner, F. et al. *Toxoplasma* profilin is essential for host cell invasion and TLR11-dependent induction of an interleukin-12 response. *Cell Host Microbe* 3, 77–87 (2008).

108. Shwrti, E. & Lourido, S. Plate-based quantification of stimulated *Toxoplasma* egress. *Methods Mol. Biol.* 2071, 171–186 (2020).

Acknowledgements
We thank the Whitehead Institute Bioinformatics and Research Computing Core, especially B. Yuan, for assistance implementing gRNA design pipelines; L.D. Sibley for the TIR1 strain; M. Treck for the DiCre strain; W. Salmon and the W.M. Keck Biological Imaging Facility for confocal microscopy support; P. W. Redden for use of the Illumina MiSeq; R.S. Waldman, E.A. Boydston, C.J. Giuliano, A.W. Chan, S. Sudik and B.M. Markus for technical support in generation of the array; VEuPathDB and all contributors to this resource. This work was supported by funds from a National Institutes of Health grant (R01AI144369) to S.L. and National Science Foundation Graduate Research Fellowships to T.A.S. (2018259980) and A.L.H. (174530).

Author contributions
T.A.S. and S.L. designed the overall study and experiments. H.T. vectors were designed, constructed and tested for their tagging efficiency by T.A.S. and G.S.L.-P. The TIR1/GCaMP6 parasite strain was constructed and validated by A.L.H. and the scarcely tagged CDPPK1 and CDPK3 parasite strains were constructed and validated by E.S. T.A.S. performed all remaining parasite strain construction and experiments. T.A.S. and S.L. wrote the manuscript and all authors reviewed, offered input and approved the manuscript.

Competing interests
The authors declare no competing interests.

Additional information
Extended data is available for this paper at https://doi.org/10.1038/s41564-022-01104-0.

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41564-022-01104-0.

Correspondence and requests for materials should be addressed to Sebastian Lourido.

Peer review information *Nature Microbiology* thanks David Horn and the other, anonymous, reviewer(s) for their contribution to the peer review of this work. Peer reviewer reports are available.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

© The Author(s), under exclusive licence to Springer Nature Limited 2022.
Extended Data Fig. 1 | Transfected populations efficiently incorporate a variety of HiT vectors. a, Fluorescence microscopy of the tagged populations displaying the correct localization of each kinase and expression levels consistent with flow cytometry (Fig. 1b). b, Live microscopy of V5-T2A-mKate2 HiT-tagged population (merged image in Fig. 1g). c, Immunofluorescence microscopy of population tagged with the HA-U1 HiT vector following treatment with rapamycin or vehicle control (merged image in Fig. 1i). d, Flow cytometry of parasite populations tagged with the V5-mNG-mAID HiT vector targeting CDPK1 or CDPK3 and treated with either IAA or vehicle control for 24 h (excerpt shown in Fig. 1j).
Extended Data Fig. 2 | Arrayed screening results. **a**, Results from dual-indexed sequencing of the arrayed clones. A minimum of 100 reads were required to assign a given gRNA to a particular clone. Cases where a second gRNA reached >10% the abundance of the first gRNA were classified as containing multiple integrations. **b**, Histogram showing the distribution of gRNAs and genes contained among single-integrated wells within the array. Genes and gRNAs with no representation are omitted from the plot.
Extended Data Fig. 3 | See next page for caption.
Extended Data Fig. 3 | Representative images from the arrayed screen. a-f, Widefield microscopy of representative clones. Maximum intensity projections for IMC1-tdTomato and mNeonGreen-tagged targets are displayed for cultures treated with either IAA or vehicle for 24 hours. All images are displayed at the same scale. Localizations to the nucleus (a), daughter cell IMC (b), parasitophorous vacuole (c), perinuclear space (d), cytosol (e) or apical end (f) were assigned to a gene if half or more of single-integrated wells for that gene displayed consistent localizations.
Extended Data Fig. 4 | Additional representative images from the arrayed screen and comparisons to the pooled results.  

**a–c**, Widefield microscopy of representative clones. Maximum intensity projections for IMC1-tdTomato and mNeonGreen-tagged targets are displayed for cultures treated with either IAA or vehicle for 24 hours. All images are displayed at the same scale. Localizations to puncta (**a**), the basal end (**b**), or peripheral structures (**c**) were assigned to a gene if half or more of single-integrated wells for that gene displayed consistent localizations.  

**d**, Representative confocal images of a sample of clones. mNeonGreen (green); IMC1-tdTomato (magenta). Images are maximum intensity projections. Genes are numbered based on the unique identifier from ToxoDB (for example, TGGT1_210830, labeled 210830).  

**e**, Comparison of relative gRNA abundances in the array compared to the pooled population that was subcloned. Spearman correlation coefficient = 0.77.  

**f**, Impact of the initial lytic cycles on gRNA abundance for genes with delayed or acute loss phenotypes in the HiT screen. The effect of the first lytic cycle from the HiT screen is plotted against the effect of the first or second lytic cycles for the genome-wide knockout screen (Sidik & Huet, et al. 2016). Genes are paired across their first and second lytic cycles within the genome-wide knockout screen.
Extended Data Fig. 5 | Extended analysis of SPARK depletion. **a**, Replication assay of SPARK-AID parasites. Parasites were treated with either IAA or vehicle at 3 hours post-invasion and imaged 24 hours later. The number of parasites per vacuole were counted for 100 vacuoles per sample. Mean ± S.E. graphed for n = 3 biological replicates. **b**, Extracellular parasites in basal Ca²⁺ buffer stimulated with vehicle or the Ca²⁺ ionophore ionomycin, following 24 h of treatment with vehicle or IAA. Cytosolic Ca²⁺ flux was measured in bulk as GCaMP6f fluorescence normalized to the initial and maximum fluorescence following aerolysin permeabilization in 2 mM Ca²⁺. Mean ± S.E. graphed for n = 3–6 biological replicates.
Reporting Summary

Nature Portfolio wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Portfolio policies, see our Editorial Policies and the Editorial Policy Checklist.

Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

- The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
- A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- The statistical test(s) used AND whether they are one- or two-sided
  - Only common tests should be described solely by name; describe more complex techniques in the Methods section.
- A description of all covariates tested
- A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
- A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted
  - Give P values as exact values whenever suitable.
- For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection
- Flow Cytometry Data: MACSQuantify (Miltenyi)
- Widefield Microscopy: NIS Elements (Nikon)
- Confocal Microscopy: MetaMorph (Molecular Devices)
- Cytation3 Data: Gen5 2.09 (BioTek)
- Next-Generation Sequencing Data: MiSeq (Illumina)

Data analysis
- Data were analysed using Perl (5.18), RStudio (1.2.5033), GraphPad Prism (9.1.2), Microsoft Excel (16.58), SnapGene (5.1.5), FlowJo (10.7), ToxoDB, ImageJ (2.0.0), ClustalX (2.1), and FigTree (1.4.4).

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Portfolio guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:
- Accession codes, unique identifiers, or web links for publicly available datasets
- A description of any restrictions on data availability
- For clinical datasets or third party data, please ensure that the statement adheres to our policy

All oligos used in this study are available in Supplementary Table 1. All plasmids used or generated in this study are listed with their appropriate GenBank or PMID accession numbers in Supplementary Table 1. Minimally processed pooled and arrayed CRISPR screen sequencing results are available in Supplementary Table 2.
Localization assignments, microscopy phenotypes, lytic assay results, and UMAP coordinates and clusters are likewise available in Supplementary Table 2. Data from experimental results is available in the source data files. Additional unprocessed data is available from the corresponding author upon request.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☑ Life sciences  ☐ Behavioural & social sciences  ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | Sample sizes were determined based on effect size from previously established mutants known to affect the relevant phenotype. |
| Data exclusions | No exclusion criteria were predetermined and no data were excluded. |
| Replication | All results in the manuscript were replicated at least twice. Where there was variability between replicates this has been recorded. |
| Randomization | Samples were not randomized but were treated under the indicated conditions side by side with appropriate controls. |
| Blinding | Assignment of localizations was performed prior to gene identification, functionally blinding the assignment for Fig. 2 and Extended Data Fig. 3–4. Other uses of microscopy (e.g. invasion assays, replication assays, or plaque number or size) are not subject to experimenter bias and were therefore not blinded. |

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

| Materials & experimental systems | Methods |
|----------------------------------|---------|
| n/a | Involved in the study |
| ☐ | Antibodies |
| ☐ | Eukaryotic cell lines |
| ☑ | Palaeontology and archaeology |
| ☐ | Animals and other organisms |
| ☐ | Human research participants |
| ☐ | Clinical data |
| ☐ | Dual use research of concern |
| n/a | Involved in the study |
| ☑ | ChIP-seq |
| ☑ | Flow cytometry |
| ☑ | MRI-based neuroimaging |

Antibodies

Primary Antibodies:
mouse anti-V5 (Invitrogen R960-25), guinea pig anti-CDPK1 (Generated by Covance, PMID 31955846), rabbit anti-GAP45 (Provided by Dominique Soldati, PMID 18312842), mouse anti-HA clone 16B12 (BioLegend), rabbit anti-RFP (Invitrogen R10367)

Secondary Antibodies:
Alexa488-conjugated goat anti-mouse (Invitrogen A11029), Alexa594-conjugated goat anti-mouse (Invitrogen A11005), Alexa488-conjugated goat anti-guinea pig (Invitrogen A11073), Alexa594-conjugated goat anti-guinea pig (Invitrogen A11076), Alexa488-conjugated goat anti-rabbit (Invitrogen A11008), IRDye 800CW donkey anti-mouse (LiCor 926-32212), IRDye 680RD donkey anti-guinea pig (LiCor 926-68077), IRDye 680RD goat anti-rabbit (LiCor 926-68071)

Validation

anti-CDPK1 - Waldman, B. S. et al. Identification of a Master Regulator of Differentiation in Toxoplasma. Cell 180, 359–372.e16 (2020).

anti-GAP45 - Plattner F, et al. Toxoplasma profilin is essential for host cell invasion and TLR11-dependent induction of an interleukin-12 response. Cell Host Microbe 3 (2), 77-88 (2008).

anti-V5 - Antibody is described on the manufacturer’s website as validated by both immunofluorescence assay and western blot in human cell lines. In addition, antibody was validated by Invitrogen’s “Relative Expression Antibody Validation” protocol and described on the manufacturer’s website as follows:

“Antibody specificity was demonstrated by detection of different targets fused to V5 tag in transiently transfected lysates tested. Relative detection of V5 tag was observed across different proteins fused with V5 tag in V5-H3-His (Lane 3) and Myc-p65-VS (Lane 4-7), using V5 Tag Monoclonal Antibody (Product # R960-25) in Western Blot. This product has been shown to detect V5 Tag at both
N- and C- termini of a fusion protein.

anti-HA - Antibody is described on the manufacturer's website as validated by both Western blot and immunofluorescence assay in human cell lines.

anti-RFP - Antibody is described on the manufacturer's website as validated by Western blot and immunofluorescence assay in human cell lines.

**Eukaryotic cell lines**

| Policy information about cell lines |
|-------------------------------------|
| **Cell line source(s)** | HFF-1 (ATCC® SCRC-1041™), all T. gondii lines were obtained from published work or created in this study. |
| **Authentication** | HFF-1 were authenticated at the source and subsequently maintained at a low passage. T. gondii mutant lines were validated by PCR and sequencing. |
| **Mycoplasma contamination** | HFF-1 and parental T. gondii cell lines were frequently tested and found negative for mycoplasma. Derived parasite strains were not tested. |
| **Commonly misidentified lines** | No commonly misidentified lines were used. |

(See ICLAC register)

**Flow Cytometry**

**Plots**

- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.

**Methodology**

**Sample preparation**

Parasites were either harvested from lysed host cells or syring-released if intracellular. Extracellular parasites were filtered through a 5 um filter to remove host cell debris, washed, and analyzed.

**Instrument**

Miltenyi MACSQuant VYB

**Software**

FlowJo

**Cell population abundance**

After gating on parasites based on FSC/SSC, and then on single cells (FSC-A/FSC-H and SSC-A/SSC-H) and fluorescent events within chart boundaries, all parasites were included.

**Gating strategy**

Parasites were gated using FSC/SSC and then on single cells (FSC-A/FSC-H and SSC-A/SSC-H) and fluorescent events within chart boundaries. All parasites were included on histograms.

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.