LETTERS

Calcium-dependent protein kinase 1 is an essential regulator of exocytosis in *Toxoplasma*

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Calcium-regulated exocytosis is a ubiquitous process in eukaryotes, whereby secretory vesicles fuse with the plasma membrane and release their contents in response to an intracellular calcium surge[1]. This process regulates various cellular functions such as plasma membrane repair in plants and animals[2,3], the discharge of defensive spikes in *Paramecium*[4], and the secretion of insulin from pancreatic cells, immune modulators from lymphocytes, and chemical transmitters from neurons[5]. In animal cells, serine/threonine kinases including cAMP-dependent protein kinase, protein kinase C and calmodulin kinases have been implicated in calcium-signal transduction leading to regulated secretion[1,6,7]. Although plants and protozoa also regulate secretion by means of intracellular calcium, the method by which these signals are relayed has not been explained. Here we show that the *Toxoplasma gondii* calcium-dependent protein kinase 1 (TgCDPK1) is an essential regulator of calcium-dependent exocytosis in this opportunistic human pathogen. Conditional suppression of TgCDPK1 revealed that it controls calcium-dependent secretion of specialized organelles called micronemes, resulting in a block of essential phenotypes including parasite motility, host-cell invasion, and egress. These phenotypes were recapitulated by using a chemical biology approach in which pyrazolopyrimidine-derived compounds specifically inhibited TgCDPK1 and disrupted the parasite's life cycle at stages dependent on microneme secretion. Inhibition was specific to TgCDPK1, because expression of a resistant mutant kinase reversed sensitivity to the inhibitor. TgCDPK1 is conserved among apicomplexans and belongs to a family of kinases shared with plants and ciliates[8], suggesting that related CDPKs may have a function in calcium-regulated secretion in other organisms. Because this kinase family is absent from mammalian hosts, it represents a validated target that may be exploitable for chemotherapy against *T. gondii* and related apicomplexans.

The apicomplexan parasite *T. gondii* has been used as a model for the secretion of numerous proteins from specialized organelles, called micronemes, in response to an increase in intracellular calcium concentration[9]. Microneme secretion can be blocked by broad-spectrum serine/threonine kinase inhibitors, and this is not circumvented by the addition of calcium ionophores, suggesting that kinases mediate the transduction of the calcium signal[10]. CDPKs have been identified in plants, ciliates and apicomplexans but are absent from fungi and animals[11]. CDPKs can respond to calcium when their calmodulin-like domain binds calcium and releases the kinase domain from an inactive conformation[11]. Recent structural studies illustrate a novel mechanism of CDPK activation that results from a large-scale intramolecular rearrangement[12]. Apicomplexans contain a diverse family of CDPKs; some of these have canonical domain structures, whereas others are more diverse[8]. In *Plasmodium*, the causative agent of malaria, gene knockouts of several individual CDPKs have revealed important roles at specific developmental stages[8]. For example, disruption of CDPK4 in asexual stages of *Plasmodium berghei* leads to differentiation defects in male gametocytes[13], a block that currently precludes analysis of its role in other motile stages such as sporozoites. The orthologue of this kinase is called TgCDPK1 in *T. gondii*, and a previous study suggested that the ability of KT5926, a pan-specific S/T kinase inhibitor related to staurosporine, to block cell attachment may result from inhibition of this target[14]. Although KT5926 inhibits TgCDPK1 activity *in vitro*[14], it is unlikely to provide specific inhibition in the parasite, which harbours 11 distinct CDPKs[8] that control largely unexplored cellular pathways.

To precisely define the role of TgCDPK1 in the parasite's life cycle, we generated a conditional knockout (cKO) using the tetracycline transactivator system, previously developed for the study of essential genes in *Toxoplasma*[15]. We first engineered a strain expressing an allele encoding TgCDPK1 tagged with the HA9 epitope (YPYDVPDYA) driven by a modified *TetOSAG1* promoter, permitting suppression of the transgene during growth in anhydrotetracycline (ATc; Fig. 1a). The endogenous *TgCDPK1* gene was then replaced by double homologous recombination, generating the cKO as confirmed by PCR analysis (Fig. 1a, b). Different alleles, expressed under the *SAG1* constitutive promoter, were subsequently introduced into the cKO to test for complementation. Growth of the cKO in ATc resulted in nearly undetectable levels of the HA9-tagged regulatable protein, whereas the c-Myc-tagged constitutive proteins were stably expressed in the complemented strains (Fig. 1c, d). As a first assessment of the essential nature of TgCDPK1, we tested the ability of parasites to form plaques on host cell monolayers. Both the wild-type and cKO lines grew normally in the absence of ATc, whereas the presence of ATc led to a complete block in plaque formation in the cKO only (Fig. 1e). The phenotype was fully rescued when complemented with the wild-type allele (cKO/*WT*; Fig. 1e) but not by a mutant allele in which the catalytic aspartic residue was mutated to alanine (cKO/$D^{174}A$; Fig. 1e), indicating that TgCDPK1 function requires an active kinase.

Motility in apicomplexan parasites depends on a unique system whereby adhesins contained in the micronemes are released onto the apical end of the parasite and translocated to the posterior of the cell, thus propelling the parasite forward[16]. Downregulation of TgCDPK1 by the addition of ATc during intracellular growth did not affect parasite replication, yet parasites harvested from such cultures were significantly impaired in all forms of gliding motility (Fig. 2a). Those few cKO parasites that where able to glide, presumably as a result of leaky suppression, exhibited wild-type speeds of motility, indicating that the motor complex itself was unaffected (Supplementary Fig. 1). Gliding motility is normally a prerequisite for cell invasion[16], and consistent with this was our observation that the cKO experienced a decrease of more than 90% in invasion when grown in the presence of ATc (Fig. 2b). Just as with plaque formation, invasion could be

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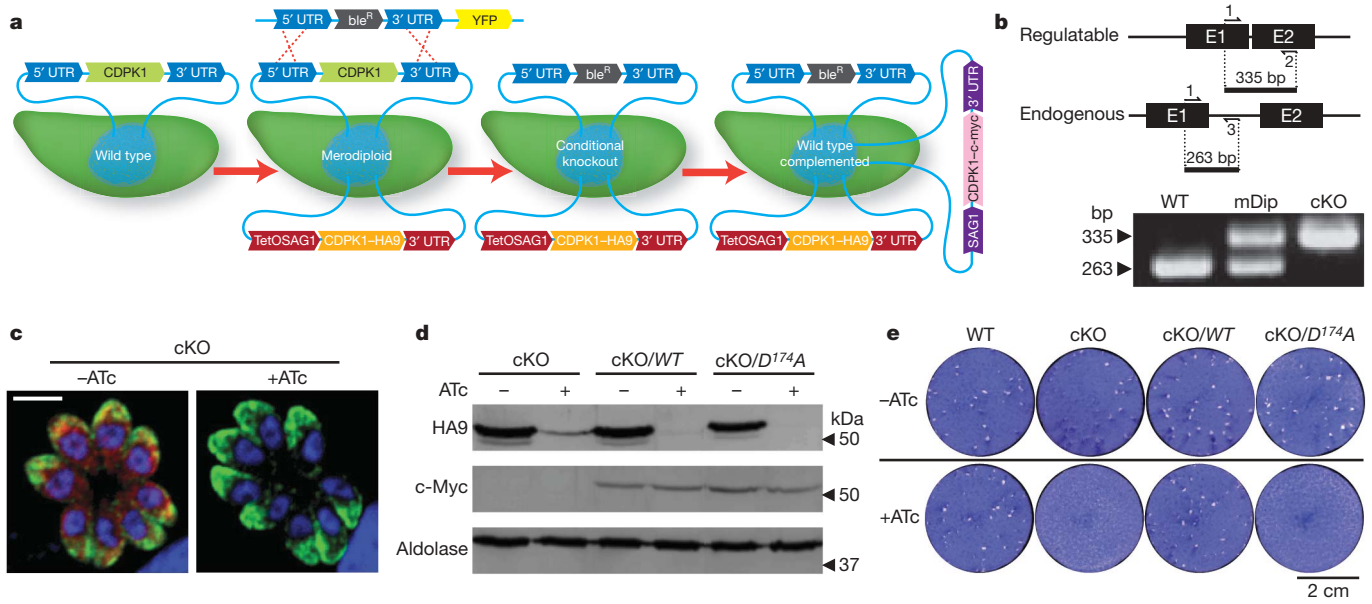

Figure 1 | TgCDPK1 is essential for the lytic cycle. **a**, Regulatable, HA9-tagged TgCDPK1 was added to the wild type (WT) to create a merodiploid (mDip). Endogenous TgCDPK1 was replaced with phleomycin resistance (ble^R) to generate the cKO. Complementation with c-Myc-tagged mutant alleles (denoted by cKO/'*allele*'). UTR, untranslated region; YFP, yellow fluorescent protein. **b**, Multiplexed PCR analysis of TgCDPK1. bp, base pairs. **c**, Immunofluorescence analysis of the cKO in the presence or absence of ATc; green, endogenous MIC2; red, HA9 tag; blue, DNA. Scale bar, 5 μm. **d**, Immunoblot of HA9-tagged regulatable and c-Myc-tagged constitutive TgCDPK1 in cKO and complemented strains in the presence or absence of ATc. Aldolase, loading control. **e**, Plaque formation on fibroblast monolayers, in the presence or absence of ATc for 7 days.

rescued by expression of the constitutive *WT* allele but not the kinase-dead allele (Fig. 2b). Suppression of TgCDPK1 also resulted in a strong decrease in attachment to host cells (Fig. 2b), suggesting that TgCDPK1 affects an early step in invasion.

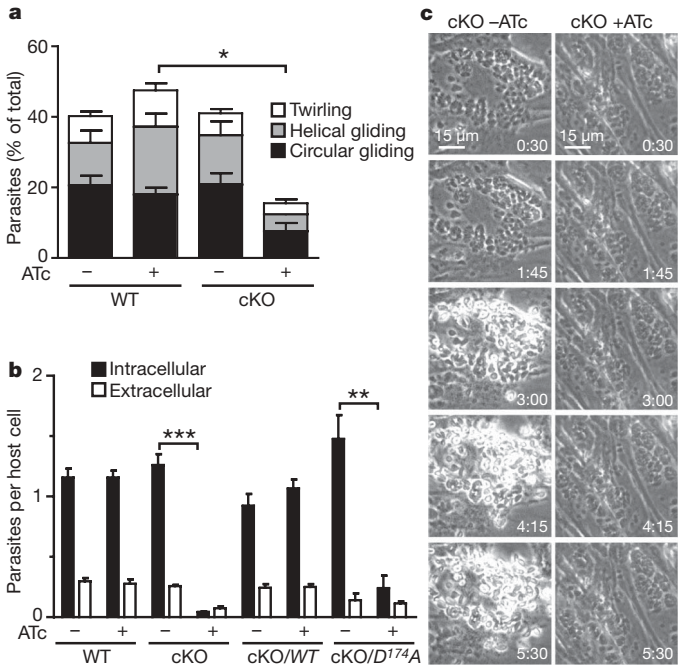

Figure 2 | TgCDPK1 is required for phenotypes associated with microneme secretion. **a**, Types of gliding motility as quantified by videomicroscopy. Student's *t*-test; asterisk, $P < 0.05$; means ± s.e.m. for $n = 4$ experiments. **b**, Invasion of fibroblasts by wild-type, cKO and complemented strains. Extracellular and intracellular parasites were stained differentially and enumerated as described in Supplementary Information. Student's *t*-test; three asterisks, $P < 0.0005$; two asterisks, $P < 0.005$; means ± s.e.m. for $n = 3$ experiments. **c**, Ionophore-induced egress of the cKO in the presence or absence of ATc. The time stamps are coded as minutes:seconds after the addition of calcium ionophore. See Supplementary Movies 1 and 2.

Egress from host cells depends on many of the same cellular pathways required for invasion, and this response may naturally be triggered by accumulation of the plant-like hormone abscisic acid[17]. The cKO parasites grown in the absence of ATc behaved like the wild type and rapidly egressed from host cells in response to treatment with calcium ionophore, an artificial but potent trigger of egress[18] (Fig. 2c, Supplementary Movie 1 and Supplementary Table 1). In contrast, almost all (namely 98%) cKO parasites grown in the presence of ATc did not respond to ionophore, remaining immotile within the vacuole (Fig. 2c, Supplementary Movie 2 and Supplementary Table 1). Taken together, these experiments indicate that TgCDPK1 is essential for the transduction of the calcium signals regulating gliding motility, invasion and egress.

All of the above TgCDPK1-dependent phenotypes share a requirement for adhesins stored in micronemes, which undergo calcium-regulated exocytosis, in contrast to other secretory compartments such as dense granules, which are constitutively released[9]. TgCDPK1 shares a similar expression pattern to that of known microneme proteins, as detected by microarray analysis of synchronized parasites (95% confidence interval; M. Behnke and M. White, personal communication). Taken together, these data suggested that TgCDPK1 might regulate microneme secretion, releasing, among other proteins, the well-studied adhesin microneme protein 2 (MIC2) (ref. 9). After secretion onto the cell surface, MIC2 is translocated to the cell posterior and shed from the parasite surface by proteolysis, permitting the detection of secreted MIC2 in the supernatant[19]. As expected, MIC2 was detected in the supernatant of wild-type parasites stimulated with ethanol, which is another potent secretagogue that is thought to act through phospholipase C (ref. 20) (Fig. 3a). In contrast, the amount of MIC2 secreted by the cKO parasites grown in the presence of ATc was nearly undetectable, demonstrating a severe defect in calcium-regulated exocytosis (Fig. 3a). Secretion of MIC2 was restored to wild-type levels by the constitutive *WT* allele but not by the kinase-dead allele (Fig. 3a). Growth of the cKO in ATc did not affect the release of dense granules (Fig. 3a), demonstrating that TgCDPK1 specifically regulates calcium-dependent exocytosis from micronemes, not other secretory pathways.

Microneme secretion is also important in parasite egress, releasing the perforin-like protein TgPLP1 that aids in permeabilization of the

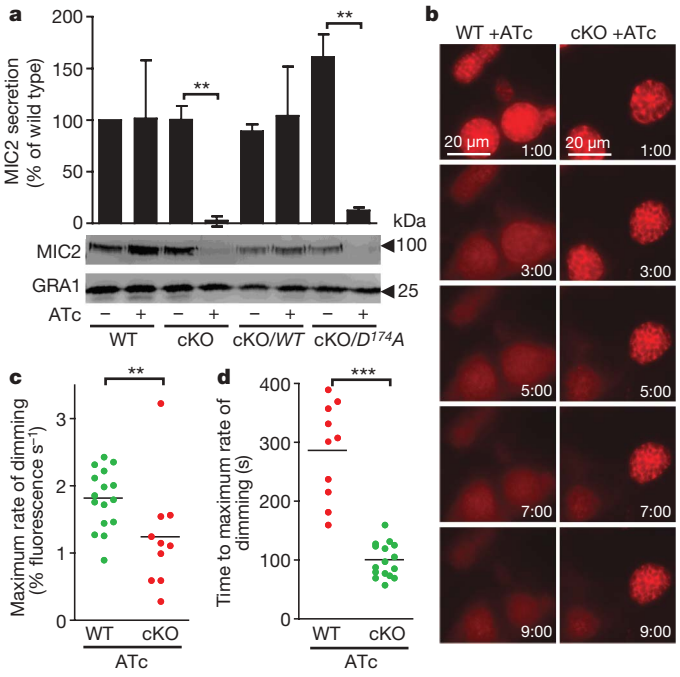

Figure 3 | Calcium-dependent microneme secretion requires TgCDPK1. **a**, Western blot analysis of microneme protein MIC2 secretion after induction with ethanol for 15 min. Dense granule protein-1 (GRA1) shows constitutive secretion of dense granules. Student's *t*-test; two asterisks, $P < 0.005$; means ± s.e.m. for $n = 3$ experiments. **b**, Ionophore-induced permeabilization detected by vacuolar DsRed leakage monitored by fluorescence videomicroscopy of strains after treatment with ATc for 90 h. The time stamps are coded as minutes:seconds after the addition of calcium ionophore. Cytochalasin D was added to prevent egress. **c**, **d**, Quantification of maximal rate (**c**) and timing (**d**) of fluorescence loss from rupturing vacuoles. Mann–Whitney test; three asterisks, $P < 0.0005$; two asterisks, $P < 0.005$; $n = 3$ experiments.

parasitophorous vacuole membrane (PVM)[21]. To assess the role of TgCDPK1 in controlling microneme secretion during egress, we generated wild-type and cKO lines expressing a constitutively secreted form of the fluorescent protein DsRed, permitting the detection of PVM integrity by live videomicroscopy. To avoid premature rupture of the vacuole, parasites were treated with cytochalasin D to immobilize them and allow selective monitoring of the kinetics of PVM rupture by leakage of DsRed. As reported previously[21], wild-type parasites rapidly permeabilized the PVM on treatment with calcium ionophore, releasing DsRed into the host cell's cytoplasm (Fig. 3b and Supplementary Movie 3). All wild-type vacuoles showed rapid PVM permeabilization (1.7 ± 0.5 min (mean ± s.d.)), whereas about 30% of cKO parasites grown in the presence of ATc failed to rupture the PVM (Fig. 3c and Supplementary Movie 4; data not shown). Analysis of those cKO parasite vacuoles that did rupture showed a significant delay in the timing and rate of DsRed release when compared with wild-type vacuoles (Fig. 3c, d). Taken together, these results demonstrate a requirement for TgCDPK1 in controlling the release of microneme contents, including TgPLP1, during egress. Moreover, the inability of calcium ionophore to circumvent the requirement for TgCDPK1 places this kinase as the critical transducer downstream of the calcium signal regulating microneme exocytosis.

Having established the crucial role of TgCDPK1, we took advantage of the atypical nucleotide-binding pocket of TgCDPK1 (ref. 12) to develop a chemical biology approach to further evaluate the essential nature of this kinase. It has been previously reported that the amino-acid residue at the 'gatekeeper' position within the nucleotide-binding pocket radically affects inhibition by pyrazolopyrimidine (PP1) derivatives, which have limited activity against most S/T protein kinases[22]. Insensitivity is conferred by bulky gatekeeper residues in nearly all kinases of both animal and parasite cells; however, kinases can be rendered fully sensitive by mutation to a small gatekeeper[22] (Supplementary Table 2). Fortuitously, TgCDPK1 has a glycine residue at this position, which is unique among canonical CDPKs (Fig. 4a) and all other protein kinases in *T. gondii* (L. Peixoto and D. Roos, personal communication). This finding predicted that wild-type TgCDPK1 would be naturally sensitive to PP1-based inhibitors; consistent with this, a pilot screen of selected derivatives inhibited parasite lytic growth *in vitro* (Supplementary Fig. 2). Furthermore, purified TgCDPK1 enzyme was extremely sensitive to the compound 3-methylbenzyl-PP1 (3-MB-PP1) (Fig. 4b), whereas mutation of the glycine gatekeeper to methionine shifted the sensitivity by more than 4 log units (Supplementary Table 2). Complementation of the TgCDPK1 cKO with either wild-type or gatekeeper-mutant kinase

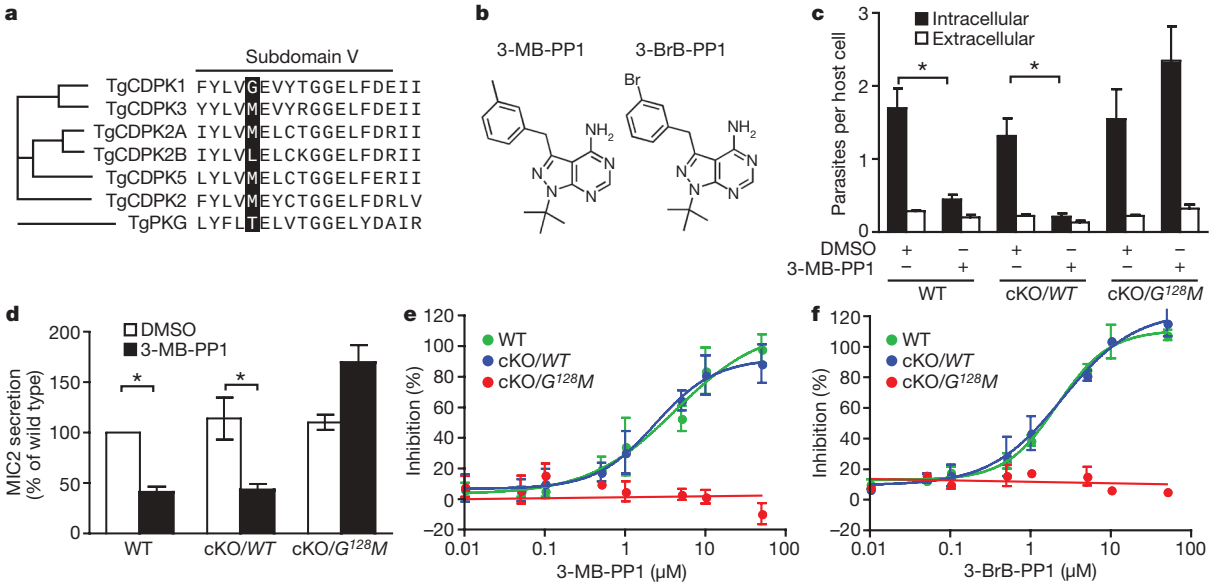

Figure 4 | PP1 derivatives specifically inhibit TgCDPK1 and block microneme-mediated functions. **a**, Alignment of the kinase subdomain V, highlighting the gatekeeper residue. **b**, Structures of 3-MB-PP1 and 3-BrB-PP1. **c**, Effect of 5 µM 3-MB-PP1 on host cell invasion. Student's *t*-test; asterisk, $P < 0.05$; means ± s.e.m. for $n = 3$ experiments. **d**, Effect of 5 µM 3-MB-PP1 on MIC2 secretion. Student's *t*-test; asterisk, $P < 0.05$; means ± s.e.m. for $n = 3$ experiments. **e**, **f**, Effect of PP1 derivatives on host lysis by *T. gondii* in the presence or absence of 3-MB-PP1 (**e**) and 3-Br-PP1 (**f**). Means ± s.e.m. for $n = 3$ experiments.

alleles (cKO/*WT* or cKO/$G^{128}M$, respectively) restored plaque formation, demonstrating that the mutation had no major deleterious effect (Supplementary Fig. 3). When treated with PP1 analogues, both wild-type and cKO/*WT* parasites were strongly inhibited in attachment to and invasion of host cells (Fig. 4c), which is consistent with a recent report that appeared online during the revision of the present work[23]. In contrast with this recent report, which did not provide a quantitative analysis of secretion[23], we observed that microneme secretion by extracellular parasites and ionophore-induced egress (Supplementary Fig. 4) were also strongly inhibited by PP1 analogues (Fig. 4d). The reversal of these phenotypes in the $G^{128}M$ mutant confirms that the primary *in vivo* target of PP1 derivatives is TgCDPK1. Consistent with these effects was our observation that 3-MB-PP1 and the related compound 3-bromobenzyl-PP1 (3-BrB-PP1) blocked the ability of the parasite to lyse host-cell monolayers (Fig. 4e, f), demonstrating the essential role of TgCDPK1 during *in vitro* infection. Chemical genetic studies indicate that TgCDPK1 acts independently of the previously characterized cGMP-dependent protein kinase (PKG), the primary target of tri-substituted pyrrole and imidazopyridine kinase inhibitors that also block microneme secretion in *T. gondii*[24,25]. Correspondingly, PKG is predicted to be insensitive to PP1 derivatives (Fig. 4a)[22]. Taken together, these findings indicate that both kinases are essential for efficient microneme secretion, possibly reflecting a hierarchical control of this important cellular pathway.

Our findings demonstrate that TgCDPK1 acts downstream of the second messenger calcium to regulate exocytosis in *T. gondii*, thus controlling several essential biological steps in the life cycle. Nearly all mammalian protein kinases normally show very low sensitivity to PP1 derivatives[22], making them potential lead compounds for the development of specific anti-parasitic drugs. In addition, CDPKs may regulate calcium-dependent exocytosis in related parasites or other organisms such as ciliates and plants, representing an evolutionary precedent for calmodulin-dependent kinases that regulate exocytosis in animals.

METHODS SUMMARY

Growth of host cells and parasite strains. *T. gondii* tachyzoites were maintained by growth in human foreskin fibroblasts (HFFs), as described previously[26]. Complemented strains were grown in 3 μM pyrimethamine (Sigma), and ATc (Clontech) was added at 1.5 μg ml^{-1} for 72 h unless indicated otherwise. For inhibitor studies, parasites were incubated in the indicated concentration of 3-MB-PP1, 3-BrB-PP1, or dimethylsulphoxide (DMSO) control, for 20 min at room temperature (20–25 °C), before use in assays.

Cellular assays. Plaque formation and invasion assays were performed as described previously[27,28]. Microneme secretion was assayed by monitoring the release of MIC2 into the culture medium after stimulation for 15 min with 3% FBS and 2% ethanol at 37 °C, as described previously[20]. Samples were resolved by SDS–PAGE, blotted and probed with mouse anti-MIC2 (monoclonal antibody 6D10), and mouse anti-GRA1 (monoclonal antibody Tg17-43) and quantified by PhosphorImager analysis. Egress and PVM permeabilization were monitored by videomicroscopy after stimulation with the calcium ionophore A23187 (Calbiochem) at 8 μM. When noted, parasites were pretreated for 10 min with 2 μM cytochalasin D (Calbiochem) to block motility. The extent and rate of egress, and the degree of vacuole permeability, were quantified with Openlab v. 4.1 (Improvision) as described in Supplementary Information. Host cell lysis was assayed by staining monolayers with crystal violet, 3 days after infection at a multiplicity of infection of 1.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions S.L. designed and performed the majority of experiments, analysed the data, generated the figures and wrote the manuscript. J.S. performed the video miscopy measurements of motility and analysed the data. R.H. provided key insight into the regulation of CDPKs by calcium. C.Z. and K.M.S. provided inhibitors and insight into the strategy for chemical biology experiments. L.D.S. supervised the project, assisted with experimental design and analyses, and contributed to writing the manuscript.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of this article at www.nature.com/nature. Correspondence and requests for materials should be addressed to L.D.S. (sibley@borcim.wustl.edu).

METHODS

Growth of host cells and parasite strains. *T. gondii* tachyzoites were maintained by growth in monolayers of HFFs cultured in DMEM medium containing 10% tetracycline-free fetal bovine serum (HyClone), 2 mM glutamine, 10 mM HEPES pH 7.5 and 20 $\mu g\,ml^{-1}$ gentamicin, as described[26]. Chloramphenicol (20 $\mu g\,ml^{-1}$; Sigma), phleomycin (5 $\mu g\,ml^{-1}$; InvivoGen), ATc (1.5 $\mu g\,ml^{-1}$; Clontech) and pyrimethamine (3 μM; Sigma) were added to the medium as indicated, and for the maintenance of merodiploid or complemented strains. When noted, parasites were treated with ATc for 72 h.

Plaque assay. Plaque assays were performed as described previously[27]. Confluent monolayers of HFFs in six-well plates were infected with 200 parasites per well in medium with or without 1.5 $\mu g\,ml^{-1}$ ATc (Clontech). At 24 h after infection, additional medium was added to decrease the concentration of ATc to 1 $\mu g\,ml^{-1}$. Monolayers were fixed 7 days after infection and stained with crystal violet. Experiments were repeated three times with triplicate wells per experiment.

Invasion assay. Parasites were harvested in invasion medium (DMEM containing 20 mM HEPES pH 7.5, supplemented with 3% FBS). Parasites (5×10^6) in a 250 μl volume were added to subconfluent HFF monolayers in 24-well plates and allowed to invade for 20 min. Monolayers were then fixed and stained as described previously[28] to distinguish extracellular from total parasites. Three experimental replicates were performed for each strain in each of three separate experiments, and parasite numbers per field were normalized to host-cell nuclei. For the inhibitor studies, parasites were incubated in 5 μM 3-MB-PP1 or vehicle-only control (DMSO), for 20 min at room temperature, before invasion.

MIC2 secretion assay. Microneme secretion was assayed as described previously[20] by monitoring the release of MIC2 into the culture medium. Secretion was stimulated by treatment for 15 min with 3% FBS and 2% ethanol at 37 °C. Parasite lysis was monitored by the release of actin into the medium and remained undetectable in all experiments presented. GRA1 secretion was used as a control for constitutive secretion. Samples were resolved by SDS–PAGE, blotted and probed with mouse anti-MIC2 (mAb 6D10), rabbit anti-TgACT1 and mouse anti-GRA1 (mAb Tg17-43, provided by M. F. Cesbron). Quantification was performed by densitometry with a FLA-5000 PhosphorImager (Fuji Medical Systems). For the inhibitor studies, parasites were pretreated for 20 min with 5 μM 3-MB-PP1 or vehicle-only control (DMSO) at 37 °C before stimulation.

Strain construction. The TgCDPK1 cKO was constructed with a tetracycline transactivator system[15], as described previously for TgALD1 (ref. 26). In brief, *TgCDPK1* (GenBank accession number AF333958) was cloned with a carboxy-terminal HA9 tag into the *pTetO7SAG1* vector (obtained from D. Soldati), downstream of the inducible promoter, and the *CAT* selectable marker driven by the *SAG1* promoter was introduced at a different site. The TATi-1 strain (obtained from D. Soldati), used as the wild-type background in this study, was transfected with the regulatable construct, and stable merodiploids were selected with chloramphenicol[29] and cloned by limiting dilution. To generate the knockout construct the *Ble* selectable marker[30] was flanked with 1.5 kilobases (kb) upstream of the *TgCDPK1* start codon and 1.5 kb downstream of the stop codon, followed by a YFP expression cassette[26]. The knockout construct was linearized and transfected into the merodiploid strain and stable pools were selected through two rounds of phleomycin selection[30]. Sorting for YFP-negative parasites was used to enrich for successful knockouts, and individual clones were isolated by limiting dilution. Knockout of the endogenous *TgCDPK1* gene was confirmed in clones by PCR, using primers against consecutive exons and the intervening intron, to distinguish between the endogenous and regulatable genes. Complementing plasmids were constructed by cloning *TgCDPK1* with a carboxy-terminal c-Myc tag, under the regulation of the *SAG1* promoter. The *DHFR* selectable marker conferring pyramethamine resistance[31] was cloned into the complementing vectors. For the inhibitor studies, *SAG1* was replaced with the 1.5-kb region preceding the *TgCDPK1* start codon. Co-transfection with *pDHFR-TS* (ref. 31) was used to generate stable clones. Mutations were generated by site-directed mutagenesis. Complementing plasmids were transfected into the cKO, stable lines were selected with pyramethamine, and clones were isolated by limiting dilution. To monitor PVM permeabilization, wild-type and cKO strains were transfected with *p30-DsRed* (ref. 21) (obtained from F. Dzierszinski) and *pDHFR-TS* for isolation of stable transgenic lines as described above.

Host lysis assay. Parasites were harvested and incubated in the indicated inhibitor or DMSO concentrations for 20 min at room temperature, before incubation with confluent monolayers in 96-well plates at a multiplicity of infection of 1. For experiments comparing complemented strains, parasites were grown in 1.5 $\mu g\,ml^{-1}$ ATc for 72 h before harvesting. Parasites were allowed to invade for 1 h, monolayers were washed three to five times, and fresh medium containing 1 $\mu g\,ml^{-1}$ ATc was added. The infection was allowed to progress for 3 days before fixing with 70% ethanol and staining with crystal violet. Host cell lysis was determined by measuring absorbance at 570 nm in an EL800 microplate reader (BioTek Instruments, Inc.).

Immunofluorescence microscopy. Intracellular parasites were stained as described previously[26]. MIC2 staining within the micronemes required permeabilization for 2 min with 100% ethanol on ice. Staining was performed with rabbit anti-HA9 (Invitrogen) and mouse anti-MIC2 (mAb 6D10), followed by Alexa564-goat anti-rabbit IgG (Invitrogen), Cy5-goat anti-mouse IgG (Jackson) and Sytox green (Invitrogen) for the nuclear stain. Images were collected on a Zeiss LSM 510 confocal microscope.

Videomicroscopy and quantification of gliding motility. Gliding and egress were analysed by videomicroscopy as described previously[32]. For gliding, 75 images were taken with exposure times ranging from 50 to 100 ms with 1 s between exposures. Images were collected and combined into composites with Openlab v. 4.1 (Improvision). ImageJ was used to analyse the images. The ParticleTracker plug-in was used to track cell motility and Cell Counter was used to quantify percentage motility.

Ionophore-induced egress and PVM permeabilization. Egress and PVM permeabilization were monitored by videomicroscopy as described above. Where noted, parasites were preincubated for 10 min in medium containing 2 μM cytochalasin D (Calbiochem) at 37 °C. All dishes were allowed to equilibrate for 5 min on the heated stage before the addition of 8 μM calcium ionophore A23187 (Calbiochem). Vacuoles were imaged for up to 10 min after the addition of ionophore. To quantify vacuole permeabilization the fluorescence intensity within an 80-μm^2 region of each vacuole was measured with Openlab. The values for each vacuole were normalized against the starting (100%) and ending (0%) values for that particular vacuole, and the derivative of the curve was used to find the maximal rate of fluorescence loss and the time when that rate occurred. For the inhibitor studies, parasites were pretreated for 20 min with 5 μM 3-MB-PP1 or vehicle-only control (DMSO) at 37 °C before the addition of ionophore.

***In vitro* determination of IC_{50}.** Full-length His-tagged TgCDPK1 was cloned into the pET-22b(+) vector (Novagen) and expressed in *Escherichia coli* BL21. Point mutations were generated by QuickChange site-directed mutagenesis (Stratagene). Proteins were induced with isopropyl β-D-thiogalactoside and purified by nickel-affinity chromatography. Activities of *WT* and $G^{128}M$ TgCDPK1 were determined with the CycLex CaM Kinase II Assay Kit (MBL International Corporation) in accordance with the manufacturer's instructions. c-Src proteins were expressed and assayed in the presence of various concentrations of the inhibitors as described previously[33]. IC_{50} was determined by fitting the dose–response curve with GraphPad Prism software.

Statistics. Experiments were repeated three or more times and statistical analyses were conducted in Excel with Students's *t*-test (unpaired, equal variance, two-tailed test) for comparisons with data that fit a normal distribution or the Mann–Whitney test for non-parametric comparisons.

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