ARTICLE
PUBLISHED ONLINE: 11 AUGUST 2013 | DOI: 10.1038/NCHEMBIO.1315

Small-molecule inhibition of a depalmitoylase enhances *Toxoplasma* host-cell invasion

Matthew A Child[1], Carolyn I Hall[2], Josh R Beck[3], Leslie O Ofori[1], Victoria E Albrow[1], Megan Garland[1], Paul W Bowyer[1], Peter J Bradley[3], James C Powers[4], John C Boothroyd[2], Eranthie Weerapana[5] & Matthew Bogyo[1,2]*

Although there have been numerous advances in our understanding of how apicomplexan parasites such as *Toxoplasma gondii* enter host cells, many of the signaling pathways and enzymes involved in the organization of invasion mediators remain poorly defined. We recently performed a forward chemical-genetic screen in *T. gondii* and identified compounds that markedly enhanced infectivity. Although molecular dissection of invasion has benefited from the use of small-molecule inhibitors, the mechanisms underlying induction of invasion by small-molecule enhancers have never been described. Here we identify the *Toxoplasma* ortholog of human APT1, palmitoyl protein thioesterase-1 (TgPPT1), as the target of one class of small-molecule enhancers. Inhibition of this uncharacterized thioesterase triggered secretion of invasion-associated organelles, increased motility and enhanced the invasive capacity of tachyzoites. We demonstrate that TgPPT1 is a bona fide depalmitoylase, thereby establishing an important role for dynamic and reversible palmitoylation in host-cell invasion by *T. gondii*.

© 2013 Nature America, Inc. All rights reserved.

Apicomplexans such as *T. gondii* and *Plasmodium falciparum* are obligate intracellular parasites and major pathogens of humans and other animals. These parasites use sophisticated strategies for invasion of a host cell[1], and the protective niche provided by this cell is key to their survival and ultimately their virulence within the host. *T. gondii* and members of the apicomplexan phylum use specialized and conserved machinery for the invasion of host cells. Although the identities of many proteins that participate in the process of host-cell invasion are known, the precise signaling events that coordinate their deployment remain poorly defined. Dynamic regulation of this invasion apparatus is essential for success of the parasite within a host, and it is becoming increasingly clear that the use of post-translational modifications (PTMs) to tune processes such as invasion contributes to the level of this success. Here, we describe the application of a small molecule to identify a new regulator of *Toxoplasma* tachyzoite host-cell invasion. We demonstrate that inhibition of a previously uncharacterized thioesterase, TgPPT1, enhanced the ability of the parasite to invade host cells. We genetically and biochemically validate TgPPT1 as the functionally relevant target for the enhancer phenotype and experimentally confirm that the mechanism of action of the substituted chloroisocoumarin enhancers is through covalent inhibition of enzyme activity. These data establish palmitoylation as a key component of the signaling network underlying multiple important biological processes and broadly validates this class of compounds as a tool to study the function of thioesterases in complex biological systems.

RESULTS

Chloroisocoumarins enhance *Toxoplasma* tachyzoite invasion

We recently conducted a forward chemical-genetic screen using a library of compounds designed to covalently target protease and hydrolase enzymes. Using this approach, we identified the target of a peptidic α-β unsaturated ketone that inhibited invasion as the *Toxoplasma* ortholog of the Parkinson's disease-associated protein, TgDJ-1 (ref. 2). To our surprise, in addition to invasion inhibitors, this screen identified a group of substituted chloroisocoumarins (JCP174 (**1**), JCP222 (**2**) and JCP362 (**3**)) that enhanced the ability of the parasite to invade host cells (**Fig. 1a** and **Supplementary Results**, **Supplementary Fig. 1**). Remarkably, although short treatments enhanced invasion, treatment of parasites for longer than 30 min was inhibitory, highlighting the potential to positively and negatively tune this complex biological process. We hypothesized that this phenotype could be the result of activation of signal transduction pathways that trigger invasion[3,4]. Given the unusual nature of this effect, we decided to further investigate the molecular mechanism of these 'enhancers', focusing on JCP174 as it produced the most robustly reproducible data during the compound triaging process undertaken as part of the original high-throughput screen[2].

In light of the overwhelming enhancement of invasion observed under the original screening conditions, in which parasites were treated for 15 min before addition to host cells (**Supplementary Fig. 1**), compound treatment time was reduced to 5 min to enable microscopic quantification of the phenotype. Although the amplitude of the effect was reduced relative to the original observation, treatment of extracellular tachyzoites with a titration of JCP174 confirmed our previous observation; compound treatment enhanced invasion of host cells in a dose-dependent manner (**Fig. 1b**). To address long-term effects following a single treatment of tachyzoites with JCP174, we monitored the gross effect of enhancement on parasite growth through plaque formation on host-cell monolayers[5]. The enhanced invasion of host cells corresponded to an increase in plaque number, indicative of productive parasite growth over multiple lytic cycles (**Fig. 1c**), with no change in final plaque size or shape. This confirmed that JCP174 increased the invasive capacity of the treated tachyzoites with no additional effects upon intracellular replication or general viability. *T. gondii* tachyzoites move by a specialized form of locomotion called gliding motility[6], which is linked to both dissemination away from the site of egress and invasion of host cells[1,6]. JCP174 treatment

[1]Department of Pathology, Stanford University School of Medicine, Stanford, California, USA. [2]Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford, California, USA. [3]Department of Microbiology, Immunology and Molecular Genetics, University of California–Los Angeles, Los Angeles, California, USA. [4]Department of Chemistry, Georgia Institute of Technology, Atlanta, Georgia, USA. [5]Department of Chemistry, Boston College, Chestnut Hill, Massachusetts, USA. *e-mail: mbogyo@stanford.edu

Figure 1 | Substituted chloroisocoumarins enhance *Toxoplasma* host-cell invasion, induce microneme secretion and enhance motility. (**a**) Structures of the substituted chloroisocoumarins identified that enhanced invasion of BSC-1 host cells. (**b**) Invasion assays with a titration of JCP174 using the red-green assay described in the Online Methods. The left graph illustrates data for the total number of parasites (attached and invaded) for each treatment plotted for each concentration of JCP174 tested relative to vehicle-treated control. The right graph illustrates the percentage of parasites that are attached or invaded for each of the individual treatment totals. Asterisk indicates significance as determined by Student's *t*-test; data represent mean ± s.e.m. for $n = 9$ experiments. (**c**) Quantification of enhanced plaque formation following JCP174 treatment. Asterisks indicate significance as determined by Student's *t*-test; data represent ± s.d. for $n = 3$ experiments. (**d**) Representative images for the gliding motility assay described in the methods section. Left, treated with DMSO; right, treated with JCP174. Scale bars, 30 μm. (**e**) Microneme secretion assays monitoring the shedding of MIC2 (sMIC2) into culture supernatant by western blotting at the indicated times after treatment with 50 μM JCP174 or DMSO as a control (full blots are in **Supplementary Fig. 11a**). The samples were also blotted for GRA7 as a loading control for constitutive dense granule secretion.

increased the fraction of parasites that attached and engaged in gliding motility (**Fig. 1d**), as observed by an increase in the density of surface antigen 'trails' deposited onto coverslips. However, it should be noted that these data do not discriminate between an increase in the number of parasites actively engaged in gliding, an increase in trail length or an increase in the overall amount of surface antigen deposited as trails. Both invasion and motility are dependent upon proteins located within specialized organelles called micronemes[7,8]. Microneme secretion is a calcium-triggered event requiring the activity of CDPK1 (refs. 9,10). Upon secretion, microneme proteins such as the adhesin MIC2 are released onto the surface of the parasite and subsequently shed by surface proteolysis[11]. JCP174 treatment induced rapid microneme secretion, measured by detection of shed MIC2 in culture medium (**Fig. 1e**). MIC2 is essential for both motility and invasion[12], and thus the rapid increase in the surface concentration of this surface adhesin following JCP174-induced microneme secretion could be responsible for the enhanced invasive capacity of the treated tachyzoites. However, it should be noted that at this point, we are not able to distinguish whether an increase in the surface concentration of MIC2 or an increase in surface proteolysis of this adhesin functionally contributes to the enhancer phenotype. Moreover, inhibition observed following prolonged treatment might reflect the exhaustion of essential factors located in the micronemes, as previously hypothesized for other secretagogues[13].

A thioesterase is the principal target of JCP174

As the aromatic amine in the 7-substituent position was conserved in all of the enhancers, we synthesized an analog of JCP174 lacking this functional group (JCP174-IA (4); **Fig. 2a**)[14]. This compound did not enhance invasion of host cells (**Supplementary Fig. 2**) and was used as an inactive negative control for all of the subsequent experiments. Substituted chloroisocoumarins are known to be inhibitors of serine hydrolases[15]. Therefore, we first investigated whether the enhancers could compete with a fluorescently labeled broad-spectrum probe, fluorophosphonate-rhodamine (FP-rho), for labeling of serine hydrolase targets[16]. FP-rho labeled a distinct complement of proteins in tachyzoite lysates, and pretreatment of intact tachyzoites with all of the enhancers specifically competed for labeling of a 35-kDa species (**Fig. 2b**), suggesting that the target was likely to be a serine hydrolase. We also synthesized an analog of JCP174 that was derivatized with an alkyne functional group (JCP174-alk (5))[17] to facilitate target identification using tandem orthogonal proteolysis-activity–based protein profiling (TOP-ABPP) methodology[18], in which a cleavable affinity tag is attached to labeled targets using click chemistry (**Fig. 2a**). Notably, this analog retained the ability to enhance host-cell invasion and compete for FP-rho labeling (**Supplementary Fig. 3**). We performed a proteomic identification of both FP-reactive serine

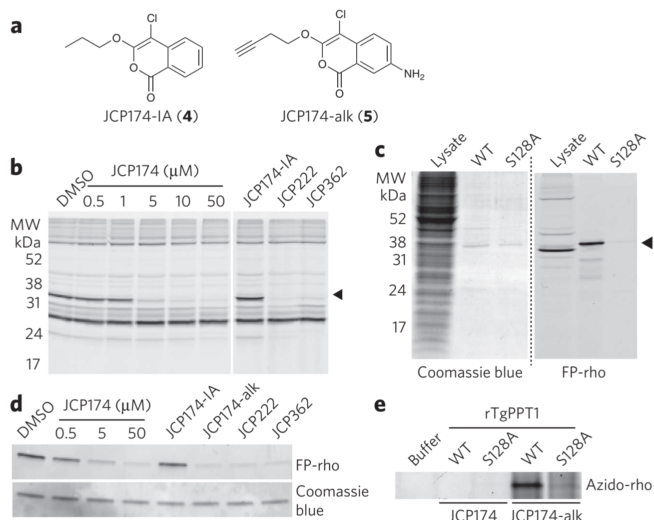

Figure 2 | TgPPT1 is a target of JCP174. (**a**) Structures of the inactive control JCP174-IA and JCP174-alkyne, which was used for affinity purification of labeled targets. (**b**) Activity-based probe competition assays of FP-rho–labeled serine hydrolase targets. Tachyzoite lysates were pretreated with indicated enhancer compounds and controls followed by labeling of residual serine hydrolase activity with FP-rho and analysis by SDS-PAGE and scanning of the gel for rhodamine fluorescence. Arrowhead indicates principal target of competition. MW, molecular weight. (**c**) FP-rho activity–based probe labeling of recombinant $TgPPT1^{WT}$ (WT) and $TgPPT1^{S128A}$ (S128A) compared to labeling of tachyzoite lysate. Arrowhead indicates labeled species at the expected molecular weight for the His_6-tagged protein. (**d**) FP-rho gel-based competition assays using recombinant $TgPPT1^{WT}$ and the panel of enhancer and control compounds. Protein was pretreated with the indicated enhancer or controls (50 μM) followed by labeling with FP-rho. Samples were analyzed by SDS-PAGE followed by scanning of the gel for rhodamine fluorescence (top) and staining with Coomassie blue to show total protein load (bottom). (**e**) Treatment of recombinantly expressed $TgPPT1^{WT}$ (WT) and $TgPPT1^{S128A}$ (S128A) with JCP174 or JCP174-alk followed by click chemistry with azido-rhodamine (full gels are in **Supplementary Fig. 11b**).

© 2013 Nature America, Inc. All rights reserved.

hydrolases and proteins directly labeled by the JCP174-alk probe. By pretreating parasites for 15 min with active or inactive enhancers, we could focus on species that were specifically targeted by the active compounds but not by the inactive control. Combining the data from both approaches (**Supplementary Table 1**) and subsequent triaging to include only proteins that show greater than 90% competition after JCP174 pretreatment in both data sets produced a list of six potential targets (**Supplementary Table 2**). These targets were then considered in terms of their possible contribution to the phenotype and predicted molecular weight. The hit that showed the greatest specific competition by active enhancers and had a predicted size that matched that of the FP-rho–labeled species competed by the enhancers in the in-gel fluorescence analysis was TGGT1_083860. This protein was annotated as a 'putative phospholipase/carboxylesterase' (**Supplementary Tables 1** and **2**), and bioinformatic analysis using BLAST identified it as the likely ortholog of human APT1 (33% identity), a thioesterase responsible for the removal of palmitoyl groups from protein substrates[19]. Although there were no published functional data for this gene product, the locus was previously aligned with rat APT1 and named palmitoyl-protein thioesterase-1 (TgPPT1)[20].

TgPPT1 is the relevant target for the enhancer phenotype

The known important role of APTs in signaling is consistent with TgPPT1 being the relevant target of JCP174, and so we sought to test this hypothesis by direct analysis. We cloned the gene encoding TgPPT1 and recombinantly expressed and purified the product protein from *E. coli* as both the wild-type (rTgPPT1^{WT}) and a catalytically inactive mutant where the conserved catalytic serine, Ser128, was replaced with an alanine, (rTgPPT1^{S128A}) (**Supplementary Fig. 4**). rTgPPT1^{WT} was efficiently labeled with FP-rho, whereas rTgPPT1^{S128A} was not, indicating that Ser128 is necessary for the activity-dependent FP-rho labeling (**Fig. 2c**). Furthermore, in FP-rho competition assays, rTgPPT1 was sensitive to the enhancers and insensitive to JCP174-IA, matching the competition profile of the species competed in parasite extracts (**Fig. 2d**). rTgPPT1^{WT}, but not rTgPPT1^{S128A}, could also be directly labeled and visualized using JCP174-alkyne and azido-rhodamine, confirming that the interaction of JCP174 with TgPPT1 was covalent and dependent upon the putative catalytic serine (**Fig. 2e**).

We next sought to genetically validate TgPPT1 as the target of the enhancers in tachyzoites through a conditional knockdown approach using a destabilization domain[21]. This approach allows the amounts of the corresponding destabilization domain–tagged protein to be regulated using a small molecule. The native TgPPT1-encoding locus was fused with a combined C-terminal hemagglutinin–destabilization domain tag (HAdd; **Fig. 3a**). The tagged gene product was cytosolically localized, similar to the location of human APT1 (ref. 22) (**Supplementary Fig. 5a**) and was degraded following removal of the Shield-1 stabilizing ligand, with ~90% knockdown of total protein quantity achieved after 180 min (**Supplementary Fig. 5b**). Experiments were then performed to test whether the incomplete knockdown of TgPPT1 would be sufficient to enhance the invasive capacity of the transgenic parasites in the absence of Shield-1. Unfortunately, no enhancement was observed under the conditions used, perhaps indicating that residual enzyme activity was sufficient to prevent the accumulation of the palmitoylated substrate (or substrates) required for the manifestation of the enhancer phenotype. To further address this point, we took advantage of the observation that, even when stabilized, amounts of active TgPPT1-HAdd were reduced by ~70% relative to quantities of the FP-rho–labeled active untagged wild-type protein (**Fig. 3a**). Thus, if TgPPT1 were indeed the functionally relevant target of JCP174 for the enhancer phenotype, we would expect parasites expressing a reduced amount of this target to have increased sensitivity to the drug. This was

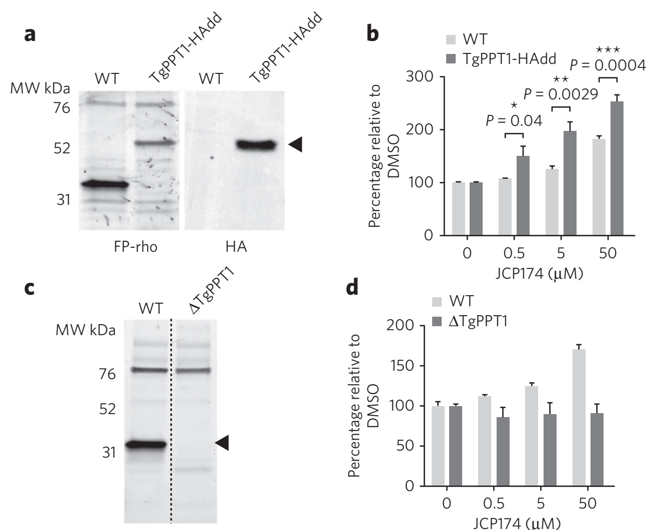

Figure 3 | Conditional knockdown and direct knockout genetically validates TgPPT1 as the target of the enhancers. (**a**) Labeling of parasites expressing TgPPT1 containing a C-terminal HAdd tag. Lysate from wild-type (WT) and HAdd-tagged parasites (TgPPT1-HAdd) were labeled with 2 μM FP-rho, followed by SDS-PAGE and scanning for rhodamine fluorescence. The same samples were also analyzed by western blotting with hemagglutinin-specific antibody (anti-HA). Arrowhead indicates the FP-rho– and HA-positive species in the HAdd-tagged strain. The amount of active destabilization domain–tagged TgPPT1 present in TgPPT1-HAdd was quantified relative to the amount of native untagged species in the wild-type parent (WT) and normalized to the labeling of the fluorophosphonate-reactive species running at 76 kDa, which was used as an internal loading control. (**b**) Comparison of the sensitivity of TgPPT1-HAdd and the untransfected wild-type parasites to JCP174-induced enhancement. Total numbers of parasites from the invasion assay described in the Online Methods are plotted for each concentration of JCP174 tested. Asterisks indicate significance as determined by Student's *t*-test; data represent mean ± s.e.m. for $n = 6$ experiments. (**c**) FP-rho labeling of wild-type and TgPPT1 knockout (ΔTgPPT1) parasites. Arrowhead indicates absence of the FP-rho–labeled species predicted to be TgPPT1. (**d**) Comparison of the sensitivity of ΔTgPPT1 and the untransfected wild-type parasites to JCP174-induced enhancement. Total numbers of parasites from the invasion assay described in Online Methods are plotted for each concentration of JCP174 tested. Data represent mean ± s.e.m. for $n = 6$ experiments.

indeed the case, as observed through increased sensitivity to invasion enhancement (**Fig. 3b**). Next, we generated a parasite line lacking TgPPT1 (ΔTgPPT1) by replacing the native TgPPT1 locus with a drug-selectable marker in the Δ*ku80* parasite line by homologous recombination using targeting sequences 5′ and 3′ of the gene[23] (**Supplementary Fig. 6**). FP-rho labeling of lysates prepared from the ΔTgPPT1 tachyzoites confirmed the loss of the primary labeled species that was competed by JCP174 (**Fig. 3c**). We then tested for sensitivity to enhancement by JCP174 and found that compound treatment had no effect on the knockout cell line (**Fig. 3d**). Notably, the gene itself seemed to be nonessential for parasite survival, with the ΔTgPPT1 parasites having no obvious growth phenotype relative to the wild type. Moreover, in head-to-head invasion assays normalized for the numbers of parasites initially applied to host cells, ΔTgPPT1 parasites were indistinguishable from the wild type both in terms of the total number of parasites present (attached plus invaded) and the fraction of the total able to attach and invade within the experimental conditions used (**Supplementary Fig. 7**). Although unexpected, this is consistent with the knockout of the yeast APT1 ortholog[24] and may

© 2013 Nature America, Inc. All rights reserved.

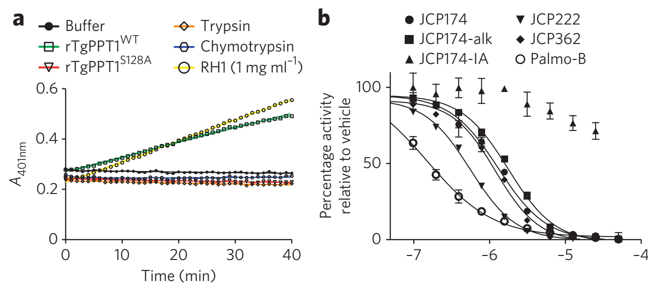

Figure 4 | The enhancers inhibit TgPPT1 activity. (**a**) Activity of recombinant TgPPT1 enzymes or control lysates against the 4-NPO substrate. Controls used were RH1, *T. gondii* RH1 tachyzoite lysate, trypsin and chymotrypsin serine proteases. (**b**) Effect of enhancers and negative control compound treatment on rTgPPT1 activity against the 4-NPO substrate. The human APT1 inhibitor palmostatin B (palmo-B) is also included. Errors bars indicate ± s.d. for $n = 3$ experiments.

be suggestive of functional redundancy in the depalmitoylating machinery similar to that found for some *Toxoplasma* kinase-regulated signaling events[25]. Relatedly, we have identified a second putative depalmitoylase in the *T. gondii* genome (TGME49_254690) and are beginning studies to address whether there is indeed functional redundancy between their enzymatic activities. Regardless, these data confirmed that JCP174 enhances host-cell invasion as a direct result of its targeting of TgPPT1.

Enhancer compounds function by inhibiting TgPPT1 activity

Although the human APT1 protein has been described as a depalmitoylase[19], the function of the ortholog in *Toxoplasma* is not known. To address this, we first tested whether recombinant TgPPT1 was enzymatically active using a commercially available esterase substrate, 4-nitrophenyl octanoate (4-NPO), which was previously shown to be a substrate for the human APT1 enzyme[22]. rTgPPT1^{WT} was able to efficiently process the substrate, whereas the S128A mutant and other serine proteases were not (**Fig. 4a**). These results confirm that rTgPPT1 is enzymatically active and, furthermore, that Ser128 is required for activity. Using this assay, we investigated the effect of the enhancer compounds on enzyme activity. In agreement with the FP-rho competition assays, we found that rTgPPT1 was dose-dependently inhibited by the active enhancers but was unaffected by the inactive control compound (**Fig. 4b**). Furthermore, all of the active enhancers had half-maximum inhibitory concentration (IC_{50}) values in the low-micromolar range, consistent with observed active concentrations in the parasite invasion assay (**Supplementary Table 3**). Notably, a β-lactone, palmostatin B, that was recently shown to be a potent inhibitor of the human APT1 (ref. 22) also inhibited rTgPPT1 activity (**Fig. 4b**) and competed for labeling of the same FP-rho–labeled species targeted by JCP174 (**Supplementary Fig. 8a**). This binding and inhibition of TgPPT1 by palmostatin B also correlated with enhanced host-cell invasion, though to a lesser extent than that observed for the chloroisocoumarins (**Supplementary Fig. 8b**). These data suggested that TgPPT1 might share a conserved function with the human APT1 enzyme. To confirm that TgPPT1, like human APT1, is a depalmitoylase, we tested its ability to remove a palmitate group from a physiologically relevant protein substrate. Gliding-associated protein-45 (GAP45) is an essential component of the glideosome, a protein complex that mediates apicomplexan motility. *P. falciparum* GAP45 is dually acylated with myristate and palmitate[26], and the *Toxoplasma* ortholog is also predicted to be palmitoylated[27]. To confirm this, we metabolically labeled tachyzoites with an alkyne-derivatized fatty acid, 17-octadecynoic acid (17-ODA). This lipid contains an alkyne group, and its addition to proteins can be monitored

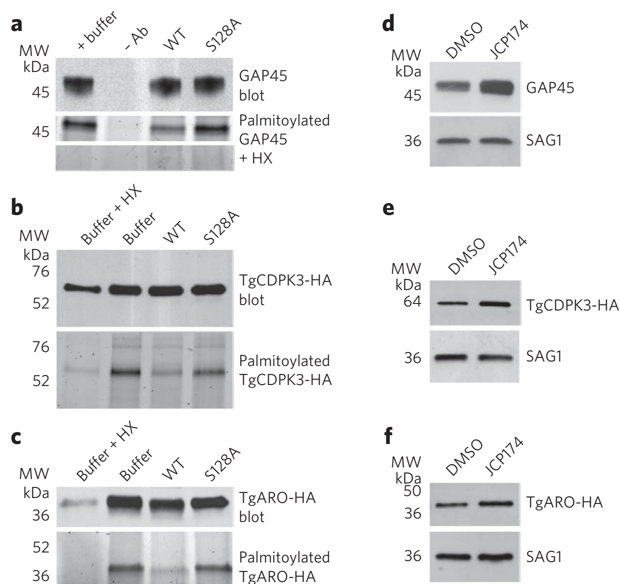

Figure 5 | TgPPT1 is a bona fide thioesterase, and TgPPT1 inhibition increases membrane partitioning of palmitoylated substrates. (**a**) GAP45 is palmitoylated and is a substrate of TgPPT1. GAP45 was immunoprecipitated from parasites metabolically labeled with 17-ODA. Samples were then treated with recombinant TgPPT1^{WT} (WT) or TgPPT1^{S128A} (S128A), and amounts of palmitoylated GAP45 were measured by click chemistry–based labeling of the alkyne palmitate group using azido rhodamine followed by fluorescence scanning of the gel (palmitoylated GAP45). Samples were also treated with hydroxylamine (+ HX) to confirm that the labeling signal was the result of thioester linkage of the palmitate to GAP45. A no-antibody control (− Ab) is also shown. The total amount of precipitated GAP45 is shown in the blot (top panel). MW, molecular weight. (**b**) TgCDPK3 is palmitoylated and a substrate for TgPPT1. Hemagglutinin-tagged TgCDPK3 (TgCDPK3-HA) was immunoprecipitated from parasites metabolically labeled with 17-ODA using anti-HA beads. Samples were then treated with recombinant TgPPT1^{WT} or TgPPT1^{S128A}, and amounts of palmitoylated TgCDPK3 present were visualized as described above for GAP45. Samples were also treated with hydroxylamine (+ HX) to demonstrate the thioester linkage. The total amounts of precipitated TgCDPK3-HA are shown in the blot (top). (**c**) TgARO is palmitoylated and a substrate for TgPPT1. Hemagglutinin-tagged TgARO was immunoprecipitated from parasites metabolically labeled with 17-ODA using anti-HA beads. Samples were then treated with recombinant TgPPT1^{WT} or TgPPT1^{S128A}, and the amount of palmitoylated TgARO present was visualized as described above. Samples were also treated with hydroxylamine (+ HX) to demonstrate the thioester linkage. The total amounts of precipitated ARO-HA are shown in the blot (top). (**d**–**f**) Western blot showing amounts of GAP45 protein (**d**), TgCDPK3 protein (**e**) and TgARO protein (**f**) in the membrane-partitioning fraction after treatment of tachyzoites with DMSO or 50 μM JCP174. SAG1 is shown as a loading control (full gels and blots are in **Supplementary Fig. 11c**).

by click chemistry–mediated attachment of an azido-rhodamine tag (**Supplementary Fig. 9**)[28]. We immunoprecipitated GAP45 from the 17-ODA–labeled parasite membranes and showed that it was labeled by the alkyne fatty acid (**Fig. 5a**), confirming that this protein is in fact acylated in *T. gondii*. Notably, treatment of this acylated GAP45 with rTgPPT1^{WT}, but not the inactive rTgPPT1^{S128A}, catalyzed removal of the associated fatty acid group, as measured as a loss of signal in the fluorescent gel image (**Fig. 5a** and **Supplementary Fig. 10**). This confirmed that a portion of the 17-ODA–labeled GAP45 signal was associated with the protein via an enzyme-accessible thioester linkage and therefore that GAP45 was palmitoylated. The remaining signal may reflect incomplete digestion of substrate under the experimental conditions used

© 2013 Nature America, Inc. All rights reserved. npg

or that, similar to the *Plasmodium* ortholog, TgGAP45 is dually acylated, with the myristate thioether linkage being resistant to activity of a thioesterase. To further validate the depalmitoylating activity of TgPPT1, we chose two other palmitoylated substrates on the basis of their published functional association with aspects of the enhancer phenotype: calcium-dependent protein kinase 3 (TgCDPK3), a *T. gondii* kinase shown to be a key regulator of egress and gliding motility[25,29,30], and armadillo repeat protein (TgARO), recently described as being essential for the correct apical positioning of a cluster of invasion-associated organelles called the rhoptries[31,32]. Transgenic parasite lines expressing hemagglutinin-tagged TgCDPK3 (ref. 29) or hemagglutinin-tagged TgARO[31] were metabolically labeled with 17-ODA, and membrane fractions were prepared. Both TgCDPK3 and TgARO were immunoprecipitated from the labeled membranes and found to be palmitoylated (**Fig. 5b**,**c**). Markedly, rTgPPT1^{WT} was able to catalytically remove the associated 17-ODA group from both TgCDPK3 and TgARO (**Fig. 5b**,**c** and **Supplementary Fig. 10**). These data confirmed that palmitoylated TgCDPK3 and TgARO could be recognized as substrates for recombinant TgPPT1. Thus, TgPPT1 is a bona fide thioesterase with depalmitoylating activity against GAP45, TgCDPK3 and TgARO, all of which are physiologically and phenotypically relevant substrates.

Finally, to confirm that JCP174 binding to TgPPT1 affected palmitoylation in tachyzoites, we monitored the amounts of a palmitoylated protein in response to treatment of extracellular tachyzoites with JCP174. Parasites treated with JCP174 had increased pools of membrane-associated GAP45 (**Fig. 5d**), which is most likely the result of increased palmitoylation of this motility-associated protein. Similarly, JCP174 treatment of tachyzoites resulted in modest increases in the quantities of both TgCDPK3 and TgARO in the membrane fraction (**Fig. 5e**,**f**). However, at this point, further work is required to determine whether the change in the amount of these palmitoylated substrates associated with the membrane fraction functionally contributes to the enhancer phenotype or is instead a reflection of the pleiotropic dysregulation of palmitoylation following inhibition of TgPPT1.

DISCUSSION

Through pharmacological inhibition and genetic manipulation of TgPPT1, we have demonstrated that reversible acylation tunes the invasive capacity of *T. gondii* tachyzoites; inhibition of TgPPT1 and aberrant palmitoylation triggers host-cell invasion, perhaps through dysregulation of microneme fusion in a manner that would mirror palmitate-driven regulation of synaptic vesicle fusion[33].

Microneme secretion is required for motility and invasion[9]. However, it is unclear whether increased microneme secretion would be sufficient to enhance these processes. It is conceivable that it would have to be accompanied by other molecular events, such as the extrusion of an apical complex called the conoid, an event intimately linked to invasion but poorly understood[34]. The potential importance of conoid extrusion for invasion is emphasized by the findings presented in ref. 35, where a small-molecule phenotypic screen identified a compound that inhibited invasion of host cells by *T. gondii* tachyzoites but had no effect upon microneme secretion or parasite motility. Notably, the authors found that the compound blocked conoid extrusion. Furthermore, recent work has indicated that there may in fact be different populations of micronemes, each containing distinct protein cargos[36]. In light of this, it will be interesting to determine whether JCP174-induced microneme secretion is equivalent for all of these populations or whether some micronemes and therefore their protein cargos are preferentially released upon compound treatment.

More generally, palmitoylation is establishing itself as a key organizational PTM in *Toxoplasma* and *Plasmodium*[37], with considerable effort focused on understanding the palmitoyl acyltransferases responsible for the addition of the palmitoyl moiety onto protein targets. Treatment of *T. gondii* tachyzoites with the broad-spectrum, irreversible palmitoyl acyltransferase inhibitor, 2-bromopalmitate, results in reduced invasion[38]. It is intriguing that the opposite effect of increased host-cell invasion was observed by inhibiting a depalmitoylase target. Although a global proteomic description of all of the targets of palmitoylation has yet to be published for *T. gondii*, recent work has validated this PTM having a role in the regulation of a range of protein targets, including TgCDPK3 (refs. 25,29,30) and TgARO[31,32], both of which were confirmed in this study as being *in vitro* substrates of TgPPT1 and whose published functions relate to different aspects of the enhancer phenotype. Given that globally dysregulated depalmitoylation most likely has pleiotropic effects, it is difficult to dissect whether the phenotype is the result of the modulation of the depalmitoylation of a single substrate or whether multiple substrates of TgPPT1 and molecular events contribute to the enhancer phenotype. To definitively address this and identify precisely which substrates of TgPPT1 are responsible for downstream transduction of the enhancer phenotype, we are in the process of generating the *T. gondii* palmitome. Additional insight into the relevant targets will be facilitated by the use of JCP174 and the ΔTgPPT1 parasite line generated herein.

Although a compound that enhances tachyzoite invasion does not immediately present itself as an ideal candidate for anti-*Toxoplasma* therapeutic development, it is interesting to speculate why increased invasive capacity has not been naturally selected. Our data suggests that the enhancer phenotype is due to increased invasion and therefore a reduction in parasite dissemination away from the point of egress. The likely net result of this would be an increase in the number of host cells infected by multiple parasites and a concomitant increase in the competition for resources within any given infected cell. Under normal circumstances, regulation of invasion may serve to promote active dissemination from the point of egress and thus reduce competition for host-cell resources between parasites. Thus, JCP174 could function as a therapeutic agent, but further studies are required to formally address this possibility.

As further validation that an acyl-protein thioesterase homolog was responsible for the enhancer phenotype, we found that palmostatin B, a published inhibitor of the human ortholog of TgPPT1 (HsAPT1) and a chemically distinct compound, was able to enhance the invasive capacity of treated tachyzoites. The amplitude of the effect was reduced compared to that observed for JCP174. This was unexpected as the 4-NPO substrate assays indicated that palmostatin B is a better inhibitor of esterase activity, and the gel competition assays seem to show more effective inhibition of the TgPPT1 target. However, on closer inspection of the gel data for both JCP174 and palmostatin B, we see more residual activity in the palmostatin B–treated parasites. This suggests that palmostatin B is in fact unable to achieve a complete intracellular block of TgPPT1 activity. This incomplete inhibition most likely results in a dampening of the enhancer effect as some amount of TgPPT1-driven depalmitoylation may still be occurring.

A critical feature of the ability of PTMs to modulate cellular processes is their reversibility, which in turn is controlled by proteins that independently add or remove them. Reversible PTMs can be considered to be forcing a target protein to exist in two or more distinct functional states dictated by the presence or absence of the modification. A protein will cycle between these different states, which can be simplistically regarded as 'on' and 'off' modes for the function of a target within a given cellular process. Inhibition of either side of this biochemical equation—PTM addition or removal—will shift the equilibrium and result in the potentiation of whatever state the protein was in. This has been shown for mammalian protein tyrosine phosphatase 1 (PTP1), where phosphatase inactivation by intracellular peroxide results in

© 2013 Nature America, Inc. All rights reserved.

global increases in protein tyrosine phosphorylation (hyperphosphorylation), potentiation of phosphorylative signals and activation of signal transduction pathways[39]. We have now shown that a similar effect occurs following inhibition of a depalmitoylase. This positions TgPPT1 and palmitoylation alongside phosphorylation as critical regulators of the parasite-specific process of host-cell invasion and, more generally, implicates palmitoylation as a regulator of parasite organelle exocytosis.

Received 19 February 2013; accepted 3 July 2013;
published online 11 August 2013

METHODS

Methods and any associated references are available in the online version of the paper.

References

1. Sibley, L.D. Intracellular parasite invasion strategies. *Science* **304**, 248–253 (2004).
2. Hall, C.I. *et al.* Chemical genetic screen identifies *Toxoplasma* DJ-1 as a regulator of parasite secretion, attachment, and invasion. *Proc. Natl. Acad. Sci. USA* **108**, 10568–10573 (2011).
3. Arrizabalaga, G. & Boothroyd, J.C. Role of calcium during *Toxoplasma gondii* invasion and egress. *Int. J. Parasitol.* **34**, 361–368 (2004).
4. Billker, O., Lourido, S. & Sibley, L.D. Calcium-dependent signaling and kinases in apicomplexan parasites. *Cell Host Microbe* **5**, 612–622 (2009).
5. Roos, D.S., Donald, R.G., Morrissette, N.S. & Moulton, A.L. Molecular tools for genetic dissection of the protozoan parasite *Toxoplasma gondii*. *Methods Cell Biol.* **45**, 27–63 (1994).
6. Keeley, A. & Soldati, D. The glideosome: a molecular machine powering motility and host-cell invasion by *Apicomplexa*. *Trends Cell Biol.* **14**, 528–532 (2004).
7. Carruthers, V.B., Giddings, O.K. & Sibley, L.D. Secretion of micronemal proteins is associated with *Toxoplasma* invasion of host cells. *Cell. Microbiol.* **1**, 225–235 (1999).
8. Soldati, D., Dubremetz, J.F. & Lebrun, M. Microneme proteins: structural and functional requirements to promote adhesion and invasion by the apicomplexan parasite *Toxoplasma gondii*. *Int. J. Parasitol.* **31**, 1293–1302 (2001).
9. Carruthers, V.B. & Sibley, L.D. Mobilization of intracellular calcium stimulates microneme discharge in *Toxoplasma gondii*. *Mol. Microbiol.* **31**, 421–428 (1999).
10. Lourido, S. *et al.* Calcium-dependent protein kinase 1 is an essential regulator of exocytosis in *Toxoplasma*. *Nature* **465**, 359–362 (2010).
11. Carruthers, V.B., Sherman, G.D. & Sibley, L.D. The *Toxoplasma* adhesive protein MIC2 is proteolytically processed at multiple sites by two parasite-derived proteases. *J. Biol. Chem.* **275**, 14346–14353 (2000).
12. Huynh, M.H. & Carruthers, V.B. *Toxoplasma* MIC2 is a major determinant of invasion and virulence. *PLoS Pathog.* **2**, e84 (2006).
13. Black, M.W., Arrizabalaga, G. & Boothroyd, J.C. Ionophore-resistant mutants of *Toxoplasma gondii* reveal host cell permeabilization as an early event in egress. *Mol. Cell. Biol.* **20**, 9399–9408 (2000).
14. Heynekamp, J.J. *et al.* Isocoumarin-based inhibitors of pancreatic cholesterol esterase. *Bioorg. Med. Chem.* **16**, 5285–5294 (2008).
15. Powers, J.C. *et al.* Mechanism-based isocoumarin inhibitors for serine proteases: use of active site structure and substrate specificity in inhibitor design. *J. Cell. Biochem.* **39**, 33–46 (1989).
16. Liu, Y., Patricelli, M.P. & Cravatt, B.F. Activity-based protein profiling: the serine hydrolases. *Proc. Natl. Acad. Sci. USA* **96**, 14694–14699 (1999).
17. Haedke, U., Gotz, M., Baer, P. & Verhelst, S.H. Alkyne derivatives of isocoumarins as clickable activity-based probes for serine proteases. *Bioorg. Med. Chem.* **20**, 633–640 (2012).
18. Weerapana, E., Speers, A.E. & Cravatt, B.F. Tandem orthogonal proteolysis-activity-based protein profiling (TOP-ABPP)—a general method for mapping sites of probe modification in proteomes. *Nat. Protoc.* **2**, 1414–1425 (2007).
19. Duncan, J.A. & Gilman, A.G. A cytoplasmic acyl-protein thioesterase that removes palmitate from G protein α subunits and p21(RAS). *J. Biol. Chem.* **273**, 15830–15837 (1998).
20. Santos, J.M., Hedberg, C. & Soldati-Favre, D. Protein acylation: new potential targets for intervention against the apicomplexa in *Apicomplexan Parasites: Molecular Approaches Toward Targeted Drug Development* Vol. 2 (eds. Becker, K. & Selzer, P.M.) 335–357 (Wiley-Blackwell, 2011).
21. Herm-Götz, A. *et al.* Rapid control of protein level in the apicomplexan *Toxoplasma gondii*. *Nat. Methods* **4**, 1003–1005 (2007).
22. Dekker, F.J. *et al.* Small-molecule inhibition of APT1 affects Ras localization and signaling. *Nat. Chem. Biol.* **6**, 449–456 (2010).
23. Fox, B.A., Ristuccia, J.G., Gigley, J.P. & Bzik, D.J. Efficient gene replacements in *Toxoplasma gondii* strains deficient for nonhomologous end-joining. *Eukaryot. Cell* **8**, 520–529 (2009).
24. Duncan, J.A. & Gilman, A.G. Characterization of *Saccharomyces cerevisiae* acyl-protein thioesterase 1, the enzyme responsible for G protein α subunit deacylation *in vivo*. *J. Biol. Chem.* **277**, 31740–31752 (2002).
25. Lourido, S., Tang, K. & Sibley, L.D. Distinct signalling pathways control *Toxoplasma* egress and host-cell invasion. *EMBO J.* **31**, 4524–4534 (2012).
26. Rees-Channer, R.R. *et al.* Dual acylation of the 45 kDa gliding-associated protein (GAP45) in *Plasmodium falciparum* merozoites. *Mol. Biochem. Parasitol.* **149**, 113–116 (2006).
27. Frénal, K. *et al.* Functional dissection of the apicomplexan glideosome molecular architecture. *Cell Host Microbe* **8**, 343–357 (2010).
28. Martin, B.R., Wang, C., Adibekian, A., Tully, S.E. & Cravatt, B.F. Global profiling of dynamic protein palmitoylation. *Nat. Methods* **9**, 84–89 (2012).
29. Garrison, E. *et al.* A forward genetic screen reveals that calcium-dependent protein kinase 3 regulates egress in *Toxoplasma*. *PLoS Pathog.* **8**, e1003049 (2012).
30. McCoy, J.M., Whitehead, L., van Dooren, G.G. & Tonkin, C.J. TgCDPK3 regulates calcium-dependent egress of *Toxoplasma gondii* from host cells. *PLoS Pathog.* **8**, e1003066 (2012).
31. Beck, J.R. *et al.* A *Toxoplasma* palmitoyl acyl transferase and the palmitoylated Armadillo Repeat protein TgARO govern apical rhoptry tethering and reveal a critical role for the rhoptries in host cell invasion but not egress. *PLoS Pathog.* **9**, e1003162 (2013).
32. Mueller, C. *et al.* The *Toxoplasma* protein ARO mediates the apical positioning of rhoptry organelles, a prerequisite for host cell invasion. *Cell Host Microbe* **13**, 289–301 (2013).
33. Prescott, G.R., Gorleku, O.A., Greaves, J. & Chamberlain, L.H. Palmitoylation of the synaptic vesicle fusion machinery. *J. Neurochem.* **110**, 1135–1149 (2009).
34. Del Carmen, M.G., Mondragon, M., Gonzalez, S. & Mondragon, R. Induction and regulation of conoid extrusion in *Toxoplasma gondii*. *Cell. Microbiol.* **11**, 967–982 (2009).
35. Carey, K.L., Westwood, N.J., Mitchison, T.J. & Ward, G.E. A small-molecule approach to studying invasive mechanisms of *Toxoplasma gondii*. *Proc. Natl. Acad. Sci. USA* **101**, 7433–7438 (2004).
36. Kremer, K. *et al.* An overexpression screen of *Toxoplasma gondii* Rab-GTPases reveals distinct transport routes to the micronemes. *PLoS Pathog.* **9**, e1003213 (2013).
37. Jones, M.L., Collins, M.O., Goulding, D., Choudhary, J.S. & Rayner, J.C. Analysis of protein palmitoylation reveals a pervasive role in *Plasmodium* development and pathogenesis. *Cell Host Microbe* **12**, 246–258 (2012).
38. Alonso, A.M. *et al.* Protein palmitoylation inhibition by 2-bromopalmitate alters gliding, host cell invasion and parasite morphology in *Toxoplasma gondii*. *Mol. Biochem. Parasitol.* **184**, 39–43 (2012).
39. Hirakawa, S., Saito, R., Ohara, H., Okuyama, R. & Aiba, S. Dual oxidase 1 induced by T_H2 cytokines promotes STAT6 phosphorylation via oxidative inactivation of protein tyrosine phosphatase 1B in human epidermal keratinocytes. *J. Immunol.* **186**, 4762–4770 (2011).

Acknowledgments

We thank the Bogyo and Boothroyd labs for discussions that shaped the direction of the project; G. Ward (University of Vermont) for discussions and the GAP45 antibody; B. Martin for discussions regarding the 17-ODA metabolic labeling experiments; G. Arrizabalaga (Indiana University) and M. Treeck (Stanford University) for the TgCDPK3-HA parasite line; B. Cravatt (Scripps Research Institute) for FP-rho; T. Wandless (Stanford University) for Shield-1; and the Stanford high-throughput imaging facility for assistance with imaging the attachment and invasion assays. This work was funded by a Burroughs Wellcome Trust New Investigators in Pathogenesis Award (to M.B.), by the US National Institutes of Health grants R01-AI078947 and EB005011 (to M.B.) and RO1 AI21423 (to J.C.B.).

Author contributions

M.A.C. designed and performed the majority of the experiments, analyzed the data, generated the figures and wrote the manuscript. C.I.H. performed the original high-throughput screen and various biochemical and cell biological studies. C.I.H., V.E.A. and P.W.B. characterized the enhancer phenotype. V.E.A. synthesized JCP174-IA and JCP174-alk. J.C.P. and L.O.O. synthesized JCP174, JCP222 and JCP362. J.R.B. generated the TgPPT1-HAdd parasite line under the supervision of P.J.B. M.G. contributed to the CDPK3 experiments. E.W. performed MS experiments. E.W. and J.C.B. intellectually contributed to the decision to pursue TgPPT1 as the functionally relevant target of JCP174. M.B. supervised the project, designed and analyzed experiments and wrote parts of the manuscript.

Competing financial interests

The authors declare no competing financial interests.

Additional information

Supplementary information, chemical compound information and chemical probe information is available in the online version of the paper. Reprints and permissions information is available online at http://www.nature.com/reprints/index.html. Correspondence and requests for materials should be addressed to M.B.

© 2013 Nature America, Inc. All rights reserved.
npg

ONLINE METHODS

Parasite and host-cell maintenance. *T. gondii* strains were maintained by passage through confluent monolayers of human foreskin fibroblasts (HFFs). Host cells were cultured in DMEM supplemented with 10% FBS, 2 mM L-glutamine and 100 µg penicillin and100 µg streptomycin per milliliter, maintained at 37 °C and 5% CO_2. Parasites were harvested for use in assays by either syringe lysis of infected HFF monolayers or collection of parasites from culture supernatant after spontaneous lysis of the monolayer.

Cell assays. For all of the cell assays, compound treatments were performed as follows: intracellular parasites were released from heavily infected host-cell monolayers by syringe lysis. Washed parasites were incubated with compound for 5 min at 37 °C, unless otherwise stated, before being used for the appropriate cell assay.

Invasion assays. Red-green invasion assays were performed as described[35], with compound treatment times adjusted for the rapid kinetics of enhancement (5 min rather than 15 min). For invasion assays using nonfluorescent strains, experiments were performed with the modifications described in ref. 2. Red-green images were collected and processed as described[2], with the following modifications: all of the invasion assays were performed in individual wells of 24-well plates seeded with HFF cells that had been allowed to grow to confluency. Sixteen images per well were collected using an Image-Xpress (Molecular Devices) high-throughput imaging microscope using a 4 × 4 image grid in the center of each well. Each condition was repeated in triplicate (technical replicates), and each experiment was performed in triplicate over the course of three consecutive days unless otherwise stated (biological replicates).

Plaque assays. Toxoplasma plaque formation was assayed as described[5]. Briefly, HFFs were grown to confluency in six-well plates. Treated parasites were counted, and 200 parasites were applied to each well of a six-well plate. Each condition was repeated in triplicate. Infected plates were incubated at 37 °C and 5% CO_2 for 6 d. Infected monolayers were then washed, methanol fixed and stained with crystal violet. Plaques were visualized as nonstained areas and manually counted.

Gliding motility assays. Gliding motility was assessed as described[35]. Briefly, parasites were allowed to settle onto coated glass coverslips at 25 °C for 15 min and were then shifted to 37 °C for 5 min. Coverslips were washed with PBS and fixed with 4% formaldehyde. SAG1-positive trails were stained using mouse anti-SAG1 at 1/1,000 dilution (Thermo Scientific, MA1-83499) and a goat anti-mouse Alexa Fluor-488 secondary antibody diluted to 1/1,000 (Invitrogen Molecular Probes, A11029) and then visualized on an UV epifluorescent microscope.

Microneme secretion assays. Microneme secretion assays quantifying the release of MIC2 into the extracellular medium were performed as described[7], with the modifications described in ref. 2.

FP-rho competition assays. Compound-treated tachyzoites were lysed in lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 0.5% NP40, 0.1% SDS) for 15 min on ice. Lysates were then clarified, and the protein concentration was quantified. For the competition assays, 50 µg lysate was incubated with 2 µM FP-rho for 20 min on ice. Reactions were quenched with reducing SDS sample buffer, and then the entire reaction was resolved by SDS-PAGE. A typhoon flat-bed scanner was used to scan the gel (532-nm laser, 610-nm filter, PMT700). Where necessary, the gel was then western blotted.

MS techniques. The TOP-ABPP approach was used as described[18], with modifications as described previously[2]. For the proteomic profiling of FP- and JCP174-reactive targets in extracellular tachyzoites, parasites were pretreated with DMSO, 50 µM JCP174 or 50 µM JCP174-IA for 15 min at 37 °C. Washed cell pellets were lysed and treated with FP-biotin or JCP-alk for 60 min at 37 °C, and proteins were identified by MS according to the method outlined in ref. 40. For analysis of the data generated by the TOP-ABPP approach, the perfect 'score' was considered to be spectral counts for the JCP174-alk–treated sample that were completely competed away with the pretreatment of the sample with JCP174, that is, 100% competition. For the FP approach, the perfect score was considered to be high spectral counts for the FP-only sample that were completely competed away in the JCP174-treated sample but not in the JCP174-IA–treated sample. Proteins identified using both approaches with fewer than ten spectral counts were discarded (**Supplementary Table 1**). For more stringent analysis, any hits from the TOP-ABPP approach that gave less than 90% competition (JCP174-alk versus JCP174 or JCP174-alk) were discarded, and the resulting short list was ranked in terms of spectral counts for the JCP174-alk treatment (**Supplementary Table 2**).

TgPPT1 recombinant expression. TgPPT1 was amplified from *T. gondii* strain RH and directly cloned into pET-28a with 5′ Nde 1 and 3′ Bam H1 restriction enzyme sites. The conserved putative catalytic serine at position 128 was mutated to an alanine by site-directed mutagenesis (Stratagene). Recombinant proteins were expressed as N-terminal His_6-tagged products in *E. coli* strain BL21 and purified as described in ref. 2, followed by an additional anion-exchange purification using a NaCl gradient for the elution. The protein concentration was quantified, and aliquots were snap-frozen and stored at −80 °C.

TgPPT1-HAdd constructs. Vectors to C-terminal tag the TgPPT1 locus with a combined HA-destabilization domain were designed and constructed as described[21]. A portion of the genomic locus of *Tgppt1* up to but not including the stop codon was PCR amplified from *Toxoplasma* strain RH genomic DNA (primers P1, TACTTCCAATCCAATTTAGCTCCAGTCGACACTCCAGC, and P2, TCCTCCACTTCCAATTTTAGCGTTTGTTGTGAGGACGTTTTC GATG) and inserted into pHA2x-DD.LIC.DHFR by ligation-independent cloning[41]. The resulting plasmid was linearized with AvrII and transfected into the Δ*Ku80* parasite line. Following selection in medium containing pyrimethamine and Shield-1, parasites were cloned by limiting dilution, and a clone expressing the tagged protein of interest was isolated and designated TgPPT1-HAdd.

ΔTgPPT construct and generation of the TgPPT1 knockout. The pTKO knockout vector was designed and constructed as described in ref. 42. Stable populations of transfectants were selected with mycophenolic acid (MPA) and xanthine. Clones were isolated as described below, and integration-specific PCR was used to confirm the expected genomic rearrangements.

Parasite transfection and isolation of single-cell clones. Transgenic parasite strains were made by electroporating the *T. gondii* strain Δ*Ku80* (ref. 23) with 15 µg of linearized plasmid encoding the construct of interest and selecting for HPRT-resistant parasites, as described[43]. Clonal parasites were selected by limiting dilution. Integration was verified using an integration-specific PCR strategy as described[2].

Immunofluorescence assays. Immunofluorescence assays were performed as described[27] with the antibodies indicated **in relevant figure legends**.

Metabolic labeling. Confluent HFF monolayers were heavily infected with *T. gondii* strain RH1 for 24 h and then labeled with 25 µM 17-octadecynoic acid (17-ODA, Cayman Chemicals, >98% purity) for 8 h. Tachyzoites were mechanically released, filtered and washed. Parasite membrane fractionation and click chemistry with the metabolically labeled membranes was performed as described[28].

Esterase and thioesterase activity assays. Purified recombinant $TgPPT1^{WT}$ and $TgPPT1^{S128A}$ were tested for esterase activity against 4-NPO (Sigma) as previously described[22]. For demonstration of thioesterase activity, GAP45 was immunoprecipitated from 17-ODA–labeled membranes in IP buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 0.5% NP40, 0.1% SDS). Bead-associated GAP45 (ba-GAP45) was incubated in digestion buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 10 mM CHAPS) for 30 min on ice. Buffer, 500 nM $TgPPT1^{WT}$ or $TgPPT1^{S128A}$, was then added to the reaction, and incubated at 37 °C for 1 h for 'on-bead' removal of the 17-ODA. ba-GAP45 was then washed 3× with IP buffer, and residual association of 17-ODA with GAP45 was monitored by click reaction with azido-rhodamine, as previously described[28].

Syntheses. Synthetic details and characterization for compounds described in this study can be found in the **Supplementary Note**.

Reagents. All of the reagents were purchased from commercial suppliers and were used without further purifications unless otherwise noted. Solvents used were HPLC grade. All water-sensitive reactions were performed in anhydrous solvents under positive pressure of argon. Reaction progress was monitored by analytical TLC using EM silica gel 60 F-254 precoated glass plates (0.25 mm). Compounds were visualized on the TLC plates with a UV lamp (dual wavelength; λ = 254 nm, λ = 360 nm). Synthesized compounds were purified using flash column chromatography on EM silica gel 60 (230–400) mesh or, alternatively, via preparative reverse-phase HPLC. Products were analyzed

© 2013 Nature America, Inc. All rights reserved.

by LC/MS using an API 150EX single-quadrupole mass spectrometer (Applied Biosystems). Reverse-phase HPLC was conducted with an ÅKTA explorer 100 (Amersham Pharmacia Biotech) using C18 columns. NMR spectra were recorded on a Varian 400 MHz (400/100) or a Varian Inova 500 MHz (500/126 MHz) equipped with a pulsed field gradient accessory. Chemical shifts (δ) are reported in parts per million (p.p.m.) downfield from tetramethylsilane and are referenced to the residual protium signal in the NMR solvents (CD_3Cl, δ = 7.25). Data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, *t* = triplet, m = multiplet and q = quartet), coupling constant (*J*) in Hertz (Hz) and integration.

40. Speers, A.E. & Cravatt, B.F. Activity-based protein profiling (ABPP) and click chemistry (CC)-ABPP by MudPIT mass spectrometry. *Curr. Protoc. Chem. Biol.* **1**, 29–41 (2009).
41. Huynh, M.H. & Carruthers, V.B. Tagging of endogenous genes in a *Toxoplasma gondii* strain lacking Ku80. *Eukaryot. Cell* **8**, 530–539 (2009).
42. Lodoen, M.B., Gerke, C. & Boothroyd, J.C. A highly sensitive FRET-based approach reveals secretion of the actin-binding protein toxofilin during *Toxoplasma gondii* infection. *Cell. Microbiol.* **12**, 55–66 (2010).
43. Donald, R.G. & Roos, D.S. Gene knock-outs and allelic replacements in *Toxoplasma gondii*: HXGPRT as a selectable marker for hit-and-run mutagenesis. *Mol. Biochem. Parasitol.* **91**, 295–305 (1998).

© 2013 Nature America, Inc. All rights reserved.
npg

doi:10.1038/nchembio.1315