Title

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

Journal

Nature Chemical Biology, 9(10)

Authors

Child, Matthew
Hall, Carolyn
Beck, Josh
et al.

Publication Date

2013-10-01

DOI

10.1038/nchembio.1315

Peer reviewed

HHS Public Access

Author manuscript

Nat Chem Biol. Author manuscript; available in PMC 2014 April 01.

Published in final edited form as:

Nat Chem Biol. 2013 October ; 9(10): 651–656. 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], and **Matthew Bogyo**[1,2]

[1]Department of Pathology, Stanford University School of Medicine, Stanford, CA, 94305 USA

[2]Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford, CA, 94305 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, GA USA

[5]Department of Chemistry, Boston College, Chestnut Hill, Massachusetts 02467, USA

Abstract

While 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* orthologue 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*.

*Correspondence and requests for materials should be addressed to: M.B. (mbogyo@stanford.edu).

Supplementary Information is linked to the online version of the paper.

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. synthesized JCP174, JCP222, 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 the mass-spectrometry 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.

The authors declare no competing financial interests.

Introduction

Apicomplexans such as *Toxoplasma gondii* and *Plasmodium falciparum* are obligate intracellular parasites and major pathogens of humans and other animals. These parasites employ 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 novel 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 alpha-beta unsaturated ketone that inhibited invasion as the *Toxoplasma* orthologue of the Parkinson's disease-associated protein, TgDJ-1[2]. To our surprise, in addition to invasion inhibitors, this screen identified a group of substituted chloroisocoumarins (**1 (**JCP174), **2 (**JCP222), **3 (**JCP362)) that *enhanced* the ability of the parasite to invade host cells (Fig. 1a, Supplementary Results, Supplementary Fig. 1). Intriguingly, although short treatments enhanced invasion, treatment of parasites for longer than 30 minutes 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 prior to addition to host cells (Supplementary Fig. 1), compound treatment time was reduced to 5 minutes 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 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[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 media (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 if an increase in the surface concentration of MIC2 or an increase in surface proteolysis of this adhesin functionally contribute 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

Since the aromatic amine in the 7-substituent position was conserved in all the enhancers, we synthesized an analogue of JCP174 lacking this functional group (**4**, JCP174-IA); 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 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 pre-treatment 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 (**5**, JCP174-alk)[17] to facilitate target identification using the TOP-ABPP methodology[18] in which a cleavable affinity tag is attached to labeled targets using click chemistry (Fig. 2a). Importantly, 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 hydrolases and proteins directly labeled by the JCP174-alk probe. By pre-treating parasites for 15 minutes 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 Tables 1) and subsequent triaging to include only proteins that show greater than 90% competition after JCP174 pre-treatment in both data sets produced a list of 6 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 which 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 orthologue of human APT1 (33% identity), a thioesterase responsible for the removal of palmitoyl groups from protein substrates[19]. Although there was 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 *Tg-ppt1* gene 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, S128, 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 S128 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 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 (dd)[21]. This approach allows the levels of the corresponding dd-tagged protein to be regulated using a small molecule. The native *Tg-ppt1* locus was fused with a combined C-terminal hemaglutinin-dd tag (-HAdd) (Fig. 3a). The tagged gene product was cytosolically localized, similar to the location of human APT1[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/s

required for the manifestation of the enhancer phenotype. To further address this point we took advantage of the observation that even when stabilized, levels 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 level of this target to have increased sensitivity to the drug. This was 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-rhodamine 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). Interestingly, the gene itself appeared to be non-essential 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 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 surprising, this is consistent with the knockout of the yeast APT1 orthologue[24], and may be suggestive of functional redundancy in the depalmitoylating machinery similar to that found for some *Toxoplasma* kinase-regulated signaling events[25]. In relation to this, 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 homolog 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) that 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 S128 is required for activity. Using this assay, we investigated the effect of the enhancer compounds on enzyme activity. In agreement with the FP-rhodamine 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 the active enhancers had IC_{50} values in the low micromolar range, consistent with observed active concentrations in the parasite invasion assay (Supplementary table 3). Interestingly, a beta-lactone, palmostatin-B, that was recently shown to be a potent inhibitor of the human APT1[22] also inhibited rTgPPT1 activity (Fig. 4b) and competed for labeling of the same FP-rhodamine 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 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* orthologue 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 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*. Importantly, 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 (Figure 5a, 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, or that similar to the *Plasmodium* orthologue, TgGAP45 is dually acylated, with the myristate thioether linkage being resistant to activity of a thioesterase. To further validate the depalmitoyating activity of TgPPT1, two other palmitoylated substrates were chosen on the basis of their published functional association with aspects of the enhancer phenotype: calcium-dependent 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 HA-tagged TgCDPK3[29] or HA-tagged TgARO[31] were metabolically labeled with 17-ODA, and membrane fractions prepared. Both TgCDPK3 and TgARO were immunoprecipitated from the labeled membranes and found to be palmitoylated (Fig. 5b and c). Importantly, $rTgPPT1_{WT}$ was able to catalytically remove the associated 17-ODA group from both TgCDPK3 and TgARO (Fig. 5b and c, 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 levels 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), likely the result of increased levels of 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 and f). However, at this point further work is required to determine if 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].

Micronemes secretion is required for motility and invasion[9], however it is unclear if 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 [35], where a small molecule phenotypic screen identified a compound that was invasion inhibitory to *T. gondii* tachyzoites, but had no effect upon microneme secretion or parasite motility. Interestingly, the authors found that the compound blocked conoid extrusion. Furthermore, recent work has indicated that there may in fact be different populations of micronemes, with 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 these populations, or if 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 significant effort focused on understanding the palmitoyl acyltransferases (PATs) responsible for the addition of the palmitoyl moiety onto protein targets. Treatment of *T. gondii* tachyzoites with the broad-spectrum, irreversible PAT inhibitor, 2-bromopalmitate (2-BP), results in reduce 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 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[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 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 if 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 homologue was responsible for the enhancer phenotype we found that palmostatin-B, a published inhibitor of the human orthologue (HsAPT1) and chemically distinct compound, was able to enhance the invasive capacity of treated tachyzoites. The amplitude of the effect was significantly less than observed for JCP174. This was surprising as the 4-NPO substrate assays indicated that palmostatin-B is a better inhibitor of esterase activity, and the gel competition assays appear 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 significantly 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 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 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.

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/100 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 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 minutes 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 minutes rather than 15 minutes). For invasion assays using non-fluorescent strains, experiments were performed with the modifications described in [2]. Red/green images were collected and processed as described[2], with the following modifications: All invasion assays were performed in individual wells of 24 well plates seeded with HFF cells that had been allowed to grow to confluency. 16 images/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 performed in triplicate over the course of 3 consecutive days unless otherwise stated (biological replicates).

Plaque assays—*Toxoplasma* plaque formation was assayed as described[5]. Briefly, HFFs were grown to confluency in 6 well plates. Treated parasites were counted and 200 parasites were applied to each well of a 6 well plate. Each condition was repeated in triplicate. Infected plates were incubated at 37°C and 5% CO_2 for 6 days. Infected monolayers were then washed, methanol fixed, and stained with crystal violet. Plaques were visualized as non-stained 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 minutes, and then shifted to 37°C for 5 minutes. Coverslips were washed with PBS and fixed with 4% formaldehyde. SAG1-positive trails were stained using mouse SAG1 antibodies and a goat anti-mouse alexafluor-488 secondary antibody, and then visualized on an UV epifluorescent microscope.

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

FP-rho competition assays

Compound treated tachyzoites were lysed in lysis buffer (50 mM Tris pH7.4, 150 mM NaCl, 0.5% NP40, 0.1% SDS) for 15 minutes on ice. Lysates were then clarified and the protein concentration quantified. For the competition assays 50 μg lysate was incubated with 2 μM FP-rhodamine for 20 minutes on ice. Reactions were quenched with reducing SDS sample buffer, and then the entire reaction 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 blotted for Westerns.

Mass spectrometry 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 pre-treated with DMSO, 50 μM JCP174 or 50 μM JCP174-IA for 15 minutes at 37°C. Washed cell pellets were lysed, treated with FP-biotin or JCP-alk for 60 min at 37°C and proteins identified by MS according to the method outlined[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 are then completely competed away with the pretreatment of the sample with JCP174, i.e. 100% competition. For the FP approach, the perfect "score" was considered to be high spectral counts for the FP-only sample that are completely competed away in the JCP174 treated sample, but not competed by JCP174-IA. Proteins identified using both approaches with fewer that 10 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 vs. JCP174/JCP174-alk) were discarded, and the resulting short-list 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 hexa-his tagged products in *E. coli* strain BL21 and purified as described in [2], followed by an additional anion-exchange purification using a NaCl gradient for the elution. Protein concentration was quantified, and aliquots 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: TCCTCCACTTCCAATTTTAGCGTTTGTTGTGAGGACGTTTTCGATG) 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 media 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 [42]. Stable populations of transfectants were selected with mycophenolic acid (MPA)/xanthine. Clones

were isolated as described below and integration-specific PCRs 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*[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.

Metabolic labeling

Confluent HFF monolayers were heavily infected with *T. gondii* strain RH1 for 24 hours, and then labeled with 25 μM 17-octadecynoic acid (17-ODA, Cayman Chemicals, >98% purity) for 8 hours. 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-nitrophenyl octanoate (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 pH7.4, 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 hour for ‘on-bead’ removal of the 17-ODA. ba-GAP45 was then washed 3x with IP buffer, and residual association of 17-ODA with GAP45 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 associated Supplementary notes.

Reagents

All and reagents were purchased from commercial suppliers and used without further purifications unless otherwise noted. Solvents used were HPLC grade. All water-sensitive reactions were preformed in anhydrous solvents under positive pressure of argon. Reaction progress was monitored by analytical thin-layer chromatography (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 reversed phase HPLC. Products were analyzed 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 (ppm) downfield from tetramethylsilane and 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank the Bogyo and Boothroyd labs for discussions that shaped the direction of the project; Gary Ward for discussions and the GAP45 antibody; Brent Martin for discussions regarding the 17-ODA metabolic labeling experiments; Gustavo Arrizabalaga and Moritz Treeck for the TgCDPK3-HA parasite line; Ben Cravatt for FP-rhodamine; Tom Wandless for Shield-1 and the Stanford high-throughput imaging facility for assistance with imaging the attachment/invasion assays. This work was funded by a Burroughs Wellcome Trust New Investigators in Pathogenesis Award (to MB), by the NIH grants R01-AI078947 and EB005011 (to MB) and RO1 AI21423 (to JCB).

References

1. Sibley LD. Intracellular parasite invasion strategies. Science. 2004; 304:248–53. [PubMed: 15073368]
2. Hall CI, et al. Chemical genetic screen identifies Toxoplasma DJ-1 as a regulator of parasite secretion, attachment, and invasion. Proc Natl Acad Sci U S A. 2011; 108:10568–73. [PubMed: 21670272]
3. Arrizabalaga G, Boothroyd JC. Role of calcium during Toxoplasma gondii invasion and egress. Int J Parasitol. 2004; 34:361–8. [PubMed: 15003496]
4. Billker O, Lourido S, Sibley LD. Calcium-dependent signaling and kinases in apicomplexan parasites. Cell Host Microbe. 2009; 5:612–22. [PubMed: 19527888]
5. Roos DS, Donald RG, Morrissette NS, Moulton AL. Molecular tools for genetic dissection of the protozoan parasite Toxoplasma gondii. Methods Cell Biol. 1994; 45:27–63. [PubMed: 7707991]
6. Keeley A, Soldati D. The glideosome: a molecular machine powering motility and host-cell invasion by Apicomplexa. Trends Cell Biol. 2004; 14:528–32. [PubMed: 15450974]
7. Carruthers VB, Giddings OK, Sibley LD. Secretion of micronemal proteins is associated with toxoplasma invasion of host cells. Cell Microbiol. 1999; 1:225–35. [PubMed: 11207555]
8. Soldati D, Dubremetz JF, Lebrun M. Microneme proteins: structural and functional requirements to promote adhesion and invasion by the apicomplexan parasite Toxoplasma gondii. Int J Parasitol. 2001; 31:1293–302. [PubMed: 11566297]
9. Carruthers VB, Sibley LD. Mobilization of intracellular calcium stimulates microneme discharge in Toxoplasma gondii. Mol Microbiol. 1999; 31:421–8. [PubMed: 10027960]
10. Lourido S, et al. Calcium-dependent protein kinase 1 is an essential regulator of exocytosis in Toxoplasma. Nature. 2010; 465:359–62. [PubMed: 20485436]
11. Carruthers VB, Sherman GD, Sibley LD. The Toxoplasma adhesive protein MIC2 is proteolytically processed at multiple sites by two parasite-derived proteases. J Biol Chem. 2000; 275:14346–53. [PubMed: 10799515]
12. Huynh MH, Carruthers VB. Toxoplasma MIC2 is a major determinant of invasion and virulence. PLoS Pathog. 2006; 2:e84. [PubMed: 16933991]

13. Black MW, Arrizabalaga G, Boothroyd JC. Ionophore-resistant mutants of Toxoplasma gondii reveal host cell permeabilization as an early event in egress. Mol Cell Biol. 2000; 20:9399–408. [PubMed: 11094090]
14. Heynekamp JJ, et al. Isocoumarin-based inhibitors of pancreatic cholesterol esterase. Bioorg Med Chem. 2008; 16:5285–94. [PubMed: 18353652]
15. Powers JC, et al. Mechanism-based isocoumarin inhibitors for serine proteases: use of active site structure and substrate specificity in inhibitor design. J Cell Biochem. 1989; 39:33–46. [PubMed: 2654146]
16. Liu Y, Patricelli MP, Cravatt BF. Activity-based protein profiling: the serine hydrolases. Proc Natl Acad Sci U S A. 1999; 96:14694–9. [PubMed: 10611275]
17. Haedke U, Gotz M, Baer P, Verhelst SH. Alkyne derivatives of isocoumarins as clickable activity-based probes for serine proteases. Bioorg Med Chem. 2012; 20:633–40. [PubMed: 21454080]
18. Weerapana E, Speers AE, Cravatt BF. Tandem orthogonal proteolysis-activity-based protein profiling (TOP-ABPP)--a general method for mapping sites of probe modification in proteomes. Nat Protoc. 2007; 2:1414–25. [PubMed: 17545978]
19. Duncan JA, Gilman AG. A cytoplasmic acyl-protein thioesterase that removes palmitate from G protein alpha subunits and p21(RAS). J Biol Chem. 1998; 273:15830–7. [PubMed: 9624183]
20. Santos, JM.; Hedberg, C.; Soldati-Favre, D. Protein Acylation: New Potential Targets for Intervention Against the Apicomplexa. In: Becker, K.; Selzer, PM., editors. Apicomplexan Parasites: Molecular Approaches toward Targeted Drug Development. Vol. 2. Wiley-Blackwell; 2011.
21. Herm-Gotz A, et al. Rapid control of protein level in the apicomplexan Toxoplasma gondii. Nat Methods. 2007; 4:1003–5. [PubMed: 17994029]
22. Dekker FJ, et al. Small-molecule inhibition of APT1 affects Ras localization and signaling. Nat Chem Biol. 2010; 6:449–56. [PubMed: 20418879]
23. Fox BA, Ristuccia JG, Gigley JP, Bzik DJ. Efficient gene replacements in Toxoplasma gondii strains deficient for nonhomologous end-joining. Eukaryot Cell. 2009
24. Duncan JA, Gilman AG. Characterization of Saccharomyces cerevisiae acyl-protein thioesterase 1, the enzyme responsible for G protein alpha subunit deacylation in vivo. J Biol Chem. 2002; 277:31740–52. [PubMed: 12080046]
25. Lourido S, Tang K, Sibley LD. Distinct signalling pathways control Toxoplasma egress and host-cell invasion. EMBO J. 2012; 31:4524–34. [PubMed: 23149386]
26. Rees-Channer RR, et al. Dual acylation of the 45 kDa gliding-associated protein (GAP45) in Plasmodium falciparum merozoites. Mol Biochem Parasitol. 2006; 149:113–6. [PubMed: 16750579]
27. Frenal K, et al. Functional dissection of the apicomplexan glideosome molecular architecture. Cell Host Microbe. 2010; 8:343–57. [PubMed: 20951968]
28. Martin BR, Wang C, Adibekian A, Tully SE, Cravatt BF. Global profiling of dynamic protein palmitoylation. Nat Methods. 2012; 9:84–9. [PubMed: 22056678]
29. Garrison E, et al. A forward genetic screen reveals that calcium-dependent protein kinase 3 regulates egress in Toxoplasma. PLoS Pathog. 2012; 8:e1003049. [PubMed: 23209419]
30. McCoy JM, Whitehead L, van Dooren GG, Tonkin CJ. TgCDPK3 regulates calcium-dependent egress of Toxoplasma gondii from host cells. PLoS Pathog. 2012; 8:e1003066. [PubMed: 23226109]
31. Beck JR, 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. 2013; 9:e1003162. [PubMed: 23408890]
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. 2013; 13:289–301. [PubMed: 23498954]
33. Prescott GR, Gorleku OA, Greaves J, Chamberlain LH. Palmitoylation of the synaptic vesicle fusion machinery. J Neurochem. 2009; 110:1135–49. [PubMed: 19508429]
34. Del Carmen MG, Mondragon M, Gonzalez S, Mondragon R. Induction and regulation of conoid extrusion in Toxoplasma gondii. Cell Microbiol. 2009; 11:967–82. [PubMed: 19416276]

35. Carey KL, Westwood NJ, Mitchison TJ, Ward GE. A small-molecule approach to studying invasive mechanisms of Toxoplasma gondii. Proc Natl Acad Sci U S A. 2004; 101:7433–8. [PubMed: 15123807]

36. Kremer K, et al. An Overexpression Screen of Toxoplasma gondii Rab-GTPases Reveals Distinct Transport Routes to the Micronemes. PLoS Pathog. 2013; 9:e1003213. [PubMed: 23505371]

37. Jones ML, Collins MO, Goulding D, Choudhary JS, Rayner JC. Analysis of protein palmitoylation reveals a pervasive role in Plasmodium development and pathogenesis. Cell Host Microbe. 2012; 12:246–58. [PubMed: 22901544]

38. Alonso AM, et al. Protein palmitoylation inhibition by 2-bromopalmitate alters gliding, host cell invasion and parasite morphology in Toxoplasma gondii. Mol Biochem Parasitol. 2012; 184:39–43. [PubMed: 22484029]

39. Hirakawa S, Saito R, Ohara H, Okuyama R, Aiba S. Dual oxidase 1 induced by Th2 cytokines promotes STAT6 phosphorylation via oxidative inactivation of protein tyrosine phosphatase 1B in human epidermal keratinocytes. J Immunol. 2011; 186:4762–70. [PubMed: 21411736]

40. Speers AE, Cravatt BF. Activity-Based Protein Profiling (ABPP) and Click Chemistry (CC)-ABPP by MudPIT Mass Spectrometry. Curr Protoc Chem Biol. 2009; 1:29–41. [PubMed: 21701697]

41. Huynh MH, Carruthers VB. Tagging of endogenous genes in a Toxoplasma gondii strain lacking Ku80. Eukaryot Cell. 2009; 8:530–9. [PubMed: 19218426]

42. Lodoen MB, Gerke C, Boothroyd JC. A highly sensitive FRET-based approach reveals secretion of the actin-binding protein toxofilin during Toxoplasma gondii infection. Cell Microbiol. 2010; 12:55–66. [PubMed: 19732057]

43. Donald RG, Roos DS. Gene knock-outs and allelic replacements in Toxoplasma gondii: HXGPRT as a selectable marker for hit-and-run mutagenesis. Mol Biochem Parasitol. 1998; 91:295–305. [PubMed: 9566522]

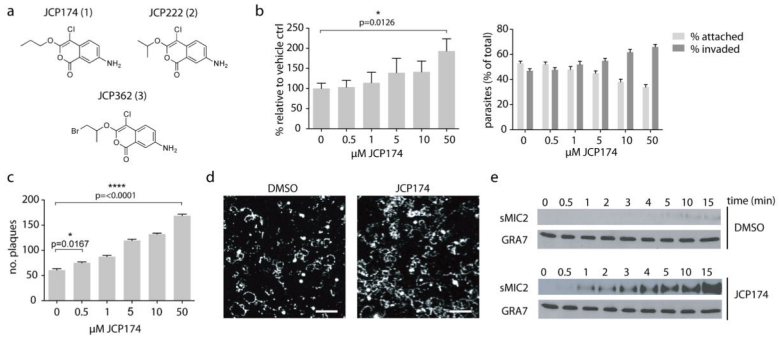

Figure 1. Substituted chloroisocoumarins enhance *Toxoplasma* host-cell invasion, induce microneme secretion and enhance motility

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 methods section. 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 fraction of parasites that are attached or invaded for each of the individual treatment totals. Student's *t*-test; asterisk indicates significance with the P value shown; means +/− SEM for $n = 9$ experiments. **c**, Quantification of enhanced plaque formation following JCP174 treatment. Student's *t*-test; asterisk indicates significance, with the P value shown; +/− SD for $n = 3$ experiments. **d**, Representative images for the gliding motility assay described in the methods section. Scale bar, 30 μm. **e**, Microneme secretion assays monitoring the shedding of MIC2 (sMIC2) into culture supernatant by Western blot at the indicated times after treatment with 50 μM JCP174 or DMSO as a control (full blots in Supplementary Fig. 11a). The samples were also blotted for GRA7 as a loading control for constitutive dense granule secretion.

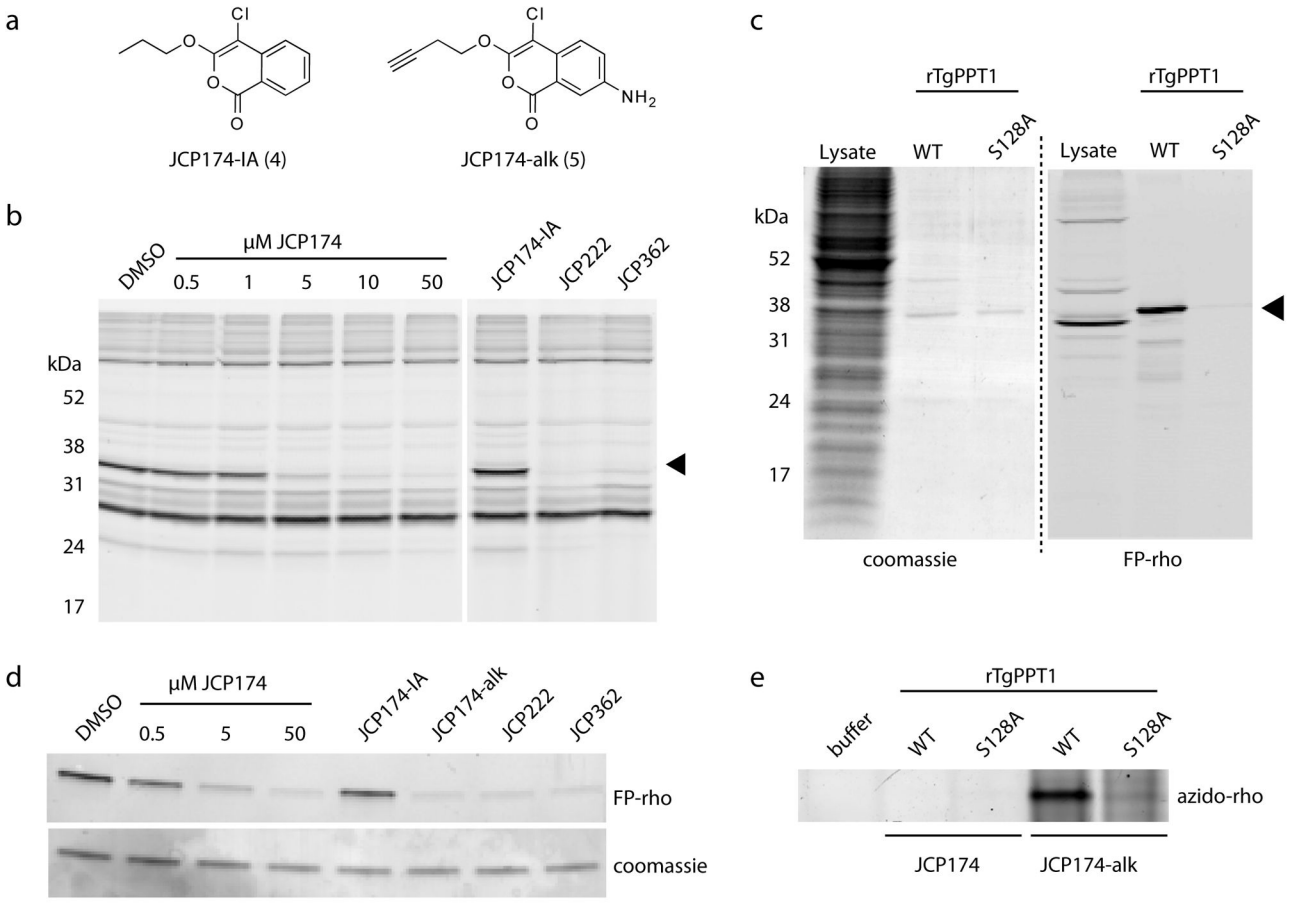

Figure 2. TgPPT1 is a target of JCP174

a, Structures of the inactive control JCP174-IA and JCP174-alkyne that was used for affinity purification of labeled targets. **b**, Activity-based probe (ABP) competition assays of FP-rhodamine (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. **c**, FP-rho ABP labeling of recombinant $TgPPT1_{WT}$ (WT) and $TgPPT1_{S128A}$ (S128A) compared to labeling of tachyzoite lysate (RH1). Arrowhead indicates labeled species at the expected MW for the His6-tagged protein. **d**, FP-rho gel-based competition assays using recombinant $TgPPT1_{WT}$ and the panel of enhancer and control compounds. Protein was pre-treated 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 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 in Supplementary Fig. 11b).

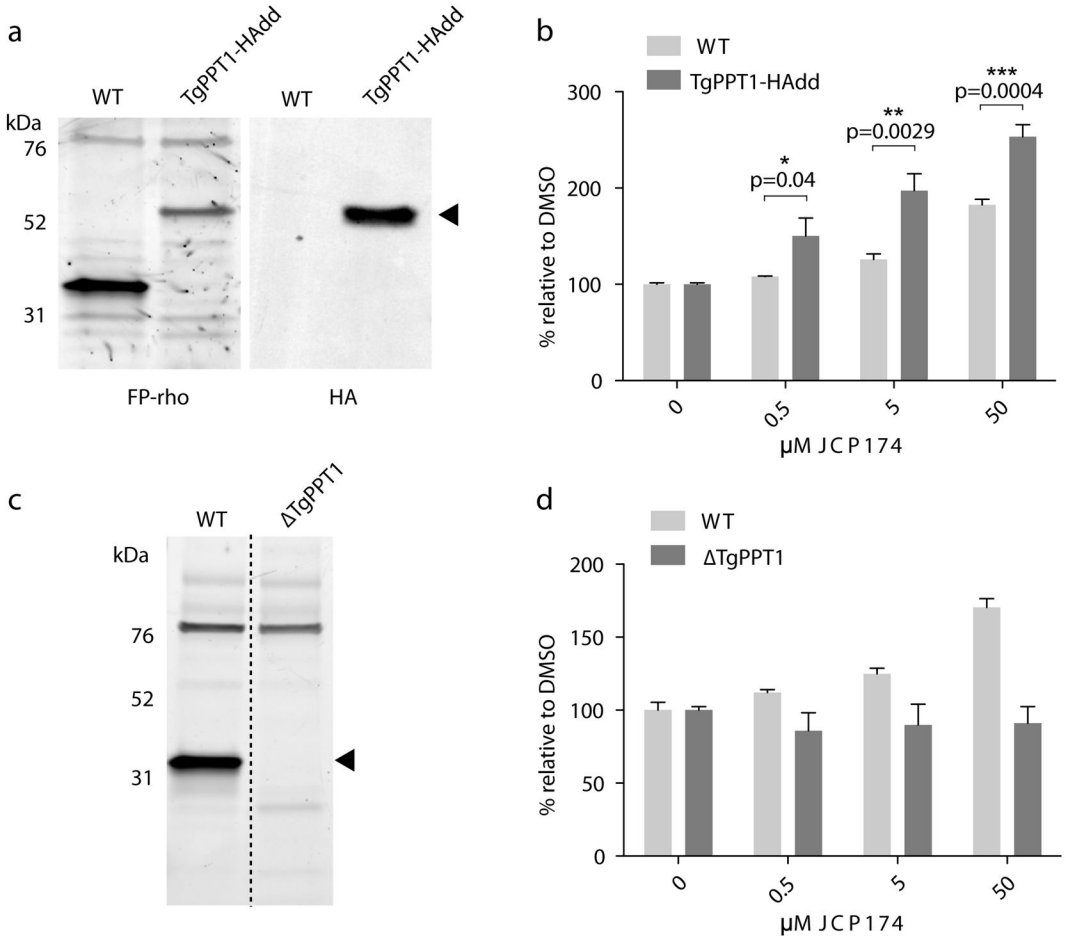

Figure 3. Conditional knockdown and direct knockout genetically validates TgPPT1 as the target of the enhancers

a, Labeling of parasites expressing TgPPT1 containing a C-terminal HA-destabilization domain tag (-HAdd). Lysate from wild-type (WT) and HAdd-tagged parasites (TgPPT1-HAdd) were labeled with 2 μM FP-rhodamine (FP-rho) followed by SDS-PAGE and scanning for rhodamine fluorescence. The same samples were also analyzed by anti-HA Western blot (HA). Arrowhead indicates the FP-rho/HA positive species in the HAdd-tagged strain. The amount of active DD-tagged TgPPT1 present in TgPPT1-HAdd was quantified relative to the untagged species in wild-type parent (WT), normalized to the labeling of the FP-reactive species running at 76 kDa that was used as an internal loading control. **b**, Comparison of the sensitivity of TgPPT1-HAdd and the untransfected WT parasites to JCP174-induced enhancement. Total numbers of parasites from the invasion assay described in the methods section are plotted for each concentration of JCP174 tested. Student's *t*-test; asterisk indicates significance with the P value shown; means +/− SEM for $n = 6$ experiments. **c**, FP-rhodamine labeling of wild-type (WT) and TgPPT1 knockout (ΔTgPPT1) parasites. Arrowhead indicates absence of the FP-rhodamine labeled species predicted to be TgPPT1. **d**, Comparison of the sensitivity of ΔTgPPT1 and the untransfected WT parasites to JCP174-induced enhancement. Total numbers of parasites from the invasion assay described in the methods section are plotted for each concentration of JCP174 tested.

Student's *t*-test; asterisk indicates significance with the P value shown; means +/− SEM for $n = 6$ experiments.

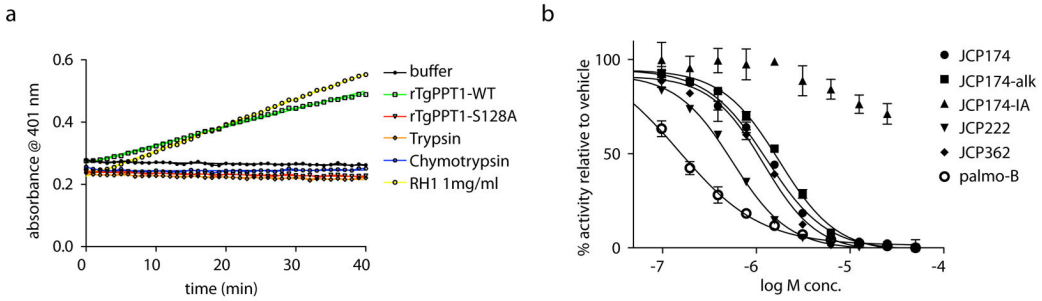

Figure 4. The enhancers inhibit TgPPT1 activity

a, Activity of recombinant TgPPT1 enzymes or control lysates against the 4-nitrophenyl octanoate (4-NPO) substrate. Controls; RH1, *T. gondii* RH1 tachyzoite lysate. Trypsin, 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.

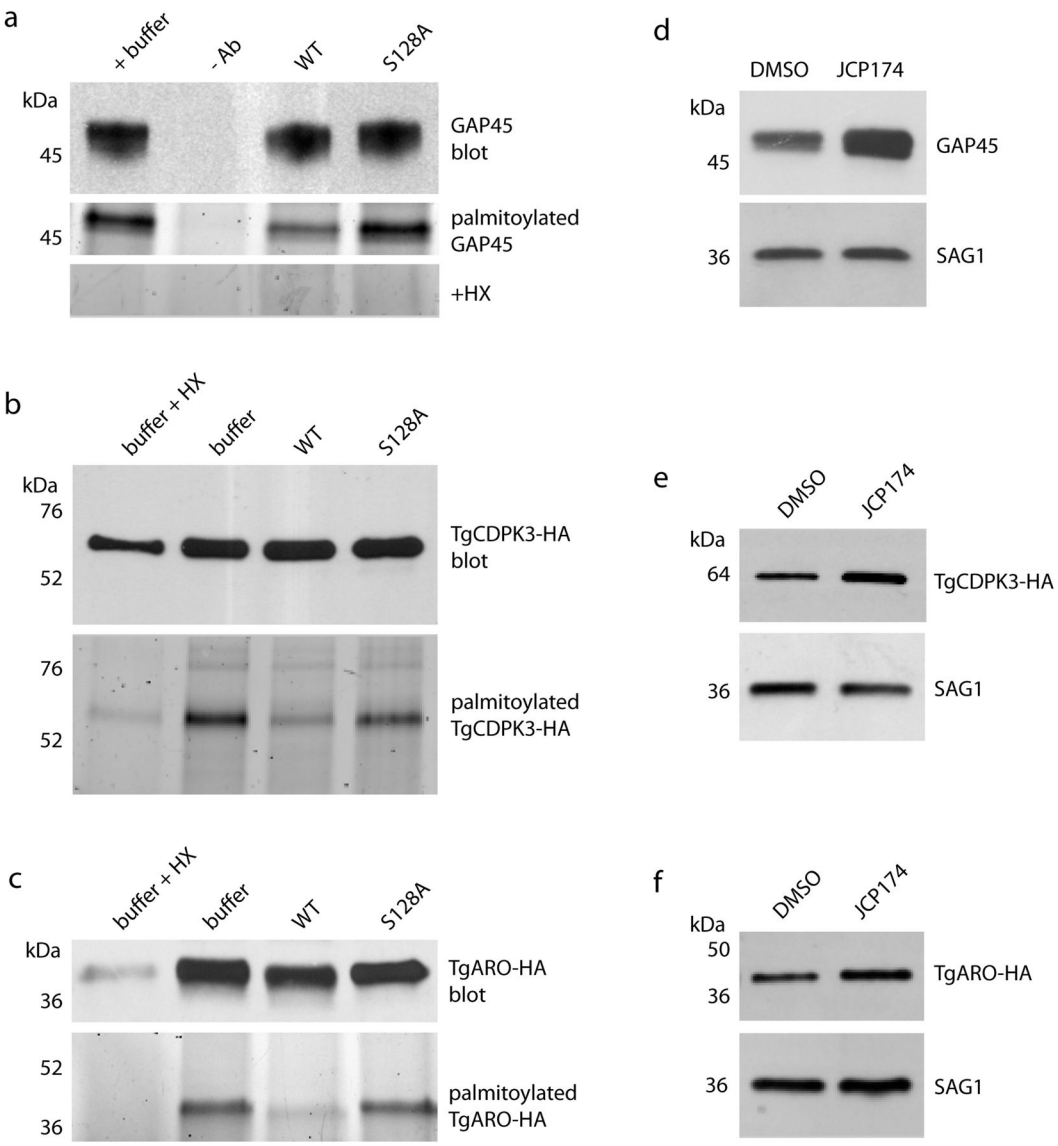

Figure 5. TgPPT1 is a *bona fide* thioesterase, and TgPPT1 inhibition increases membrane partitioning of palmitoylated substrates

a, GAP45 is palmitoylated and is a substrate of TgPPT1. GAP45 was immunoprecipitated from parasites metabolically labeled with 17-octadecynoic acid (17-ODA) using a specific antibody. Samples were then treated with recombinant $TgPPT1_{WT}$ (WT) or $TgPPT1_{S128A}$ (S128A) and levels of palmitoylated GAP45 measured by Click 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 the GAP45 protein. A no antibody control (–Ab) is also shown. Total GAP45 levels immunoprecipited are shown in the blot (top panel). **b**, TgCDPK3 is palmitoylated and a substrate for TgPPT1. HA-tagged TgCDPK3 was immunoprecipitated from parasites metabolically labeled with 17-octadecynoic acid (17-ODA) using a anti-HA beads. Samples were then treated with recombinant $TgPPT1_{WT}$ (WT) or $TgPPT1_{S128A}$ (S128A) and levels of palmitoylated

TgCDPK3 present visualized as described above for GAP45. Samples were also treated with hydroxylamine (+HX) to demonstrate the thioester linkage of the palmitate to TgCDPK3. Total TgCDPK3-HA levels immunoprecipited are show in the blot (top panel). **c**, TgARO is palmitoylated and a substrate for TgPPT1. HA-tagged TgARO was immunoprecipitated from parasites metabolically labeled with 17-octadecynoic acid (17-ODA) using a anti-HA beads. Samples were then treated with recombinant $TgPPT1_{WT}$ (WT) or $TgPPT1_{S128A}$ (S128A) and levels of palmitoylated TgARO present visualized as described above. Samples were also treated with hydroxylamine (+HX) to demonstrate the thioester linkage of the palmitate to TgARO. Total ARO-HA levels immunoprecipited are shown in the blot (top panel). **d**, Western blot showing levels of GAP45 protein in the membrane partitioning fraction after treatment of tachyzoites with DMSO or 50 μM JCP174. SAG1 is shown as a loading control. **e**, Western blot showing levels of TgCDPK3 protein in the membrane partitioning fraction after treatment of tachyzoites with DMSO or 50 μM JCP174. SAG1 is shown as a loading control. **f**, Western blot showing levels of TgARO protein 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 in Supplementary Fig. 11c).