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Short communication

Protein palmitoylation inhibition by 2-bromopalmitate alters gliding, host cell invasion and parasite morphology in *Toxoplasma gondii*

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ABSTRACT

Protein palmitoylation is the reversible covalent attachment of palmitic acid onto proteins. This post-translational modification has been shown to play a part in diverse processes such as signal transduction, cellular localization and regulation of protein activity. Although many aspects of protein palmitoylation have been identified in mammalian and yeast cells, little is known of this modification in *Toxoplasma gondii*. In order to determine the functional role of protein palmitoylation in *T. gondii*, tachyzoites were treated with the palmitoylation inhibitor 2-bromopalmitate (2-BP). Parasites treated with 2-BP displayed a significant increase in non-circular trails which were longer than those trails left by non-treated parasites. Furthermore, 2-BP treatment reduced the invasion process to the host cells. Long-term treatment of intracellular tachyzoites resulted in major changes in parasite morphology and shape in a dose-dependent manner. These results suggest that palmitoylation could be modifying proteins that are key players in gliding, invasion and cytoskeletal proteins in *T. gondii*.

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S-acylation is the covalent attachment of fatty acids onto proteins usually via a thioester bond [1]. Palmitic acid (C16:0) is the fatty acid most commonly found to be linked in this manner and as such, S-acylation is frequently referred to as palmitoylation. Both cytosolic and transmembrane proteins can be palmitoylated. Protein palmitoylation increases the hydrophobicity of the targeted protein promoting its association to membranes and often to membrane microdomains known as lipid rafts [2]. Other important functions include the regulation of enzymatic activity [3], trafficking of lipid-modified signal transducers [4,5] and recently a novel role has been described in the regulation of gene expression [6]. Protein palmitoylation is a reversible modification and as such, can actively modulate the location and function of specific proteins. Furthermore, it has been shown that during its life-time, a protein goes thorough several cycles of palmitoylation-depalmitoylation [7], giving physiological importance to this process.

The use of a general palmitoylation inhibitor provides a tool to evaluate the functional significance of this modification in a given system. The general protein palmitoylation inhibitor 2-bromopalmitate (2-BP) is a non-metabolizable palmitate analog that blocks palmitate incorporation onto proteins. The exact mechanism responsible for 2-BP mediated inhibition of protein palmitoylation is not known. Although 2-BP behaves as palmitate, it is speculated that it binds to palmitoyl acyl-transferases – enzymes that carry out protein palmitoylation – but the bromide group prevents the transfer of 2-BP to the acceptor proteins [8]. In the last few years, the importance of protein palmitoylation in different protozoan parasites has arisen especially in *Plasmodium* spp., *Trypanosoma* spp. and *Giardia* spp. [9]. Up to date, there are a few reports on protein palmitoylation in *T. gondii* [10–12]. Interestingly, all these proteins (GAP45, MLC, ISPs) are localized at the inner membrane complex (IMC) where the gliding machinery is anchored. Furthermore, we have proposed that protein palmitoylation may play a key role in the invasion process in *T. gondii* since many proteins involved in this process are predicted to be palmitoylated including AMA1, RON4, ROP2, MIC2, MIC3, MIC4, MIC7 and MLC [9]. The *T. gondii* genome encodes for 17 DHHC-containing proteins that share homology with human PATs (palmitoyl-acyl transferases) [9], indicating that this parasite possesses all the machinery required for palmitoylation to take place.

The purpose of this study was to better understand the functional significance of protein palmitoylation in invasion, gliding

Abbreviations: 2-BP, 2-bromopalmitate and 2-bromohexadecanoic acid; AMA1, apical membrane antigen 1; RON4, rhoptry neck protein 4; ROP2, rhoptry protein 2; MIC2, micronemal protein 2; MIC3, micronemal protein 3; MIC4, micronemal protein 4; MIC7, micronemal protein 7; MLC, myosin light chain; ISPs, IMC sub-compartment proteins; PATs, palmitoyl-acyl transferases.

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and replication. Here we report that treatment of tachyzoites with 2-BP results in an altered pattern in gliding motility trajectory, a reduction in host–cell invasion and defects in parasite morphology of intracellular tachyzoites. The results obtained here suggest that palmitoylated proteins seem to be necessary for an adequate regulation of these processes.

We first tested if treatment of extracellular tachyzoites with 2-BP produced significant changes in parasite integrity and morphology. Parasites (2×10^6) were treated with 0, 50 and 100 μM of 2-BP during 2 h at 37 °C (in darkness with rotation) and were analyzed by indirect immunofluorescence study and XTT methods (XTT is a method based on measuring the mitochondrial dehydrogenase activity of living cells). Fig. 1A and B shows that extracellular parasites under 2-BP treatment conserved their physical shape and membrane integrity. This observation is in agreement with a study reporting that treatment of cells with 100 μM of 2-BP is non-toxic for the cells and as such, is used to study specifically inhibition of protein palmitoylation [13].

In order to examine if 2-BP treatment could have any impact on parasite invasion to the host-cell, we incubated fresh tachyzoites with increasing concentrations of 2-BP as mentioned above. Then the parasites were washed three times in PBS and the red/green invasion assay was performed [14], in which untreated or treated parasites were allowed to infect host cells for a 1-h time period and extracellular and intracellular parasites were detected by staining and microscopic counting. A 48% reduction in penetration was observed in parasites treated with 50 and 100 μM 2-BP compared to the control (Fig. 1C). This decrease in penetrated parasites was concomitant with an increase in the attached parasites that failed to invade the host cells at the concentrations tested. As such, this experiment demonstrates that a/some step/s in the invasion process but not the initial attachment is/are impaired by 2-BP treatment.

T. gondii actively moves among tissues employing a unique parasite-dependent mechanism termed gliding motility, which is also involved in host cell invasion [15]. As the parasite glides, it leaves behind a trail that can be visualized by staining for proteins found at the plasma membrane such as SAG1. The effect of 2-BP (0, 50 and 100 μM of 2-BP during 2 h at 37 °C in darkness with rotation) on parasite gliding was assessed by indirect immunofluorescence studies employing anti-SAG1 antibody. The observed trails were grouped according to their trajectories as being circular or non-circular [16]. We found that the percentage of parasites associated to trails was similar at all the concentrations tested (Fig. 1D), being 31.6 ± 2.98, 27.4 ± 6.30 and 23.6 ± 6.55 for the control, 50 μM and 100 μM, respectively. This result indicates that after the treatment the parasites are still motile. Helical gliding is thought to be the principal form of gliding [16] and is shown by parasites leaving a wide-arching or straight trails (non-circular). If we consider only the parasites attached to trails, we found that the percentage of non-circular gliding increased significantly ($p < 0.05$) at 50 μM (Fig. 1E), suggesting that the pattern of motility was altered by the palmitoylation inhibitor favoring the non-circular gliding. We also observed that the length of non-circular trails left increased as well in a dose-dependent manner (Fig. 1F and Fig. S1). At 50 μM the increment in trail length was 1.7 times longer that of those left by non-treated parasites, whereas at 100 μM the increment reached 2.5 times. In addition to the type of gliding and the trail length, a more eclectic path pattern in parasites treated with 2-BP could be observed (Fig. S1). This result suggests that 2-BP did not prevent gliding motility but rather had an effect on the motility profile and speed. It is important to note that in order to have a more thorough knowledge of the effect of 2-BP on tachyzoite gliding, time-lapse video microscopy should be used.

T. gondii divides through the internal budding process known as endodiogeny, in which two daughters develop internally within the parental cell. Therefore, 2, 4, 8, etc. tachyzoites *per* vacuole are found after several rounds of replication [17]. In order to assess whether protein palmitoylation could play a role in replication, parasites (8×10^5) were allowed to invade a confluent monolayer of *human foreskin fibroblasts* (HFF) cells for 1 h at 37 °C in 5% CO_2. The wells were then washed three times with PBS, fresh media containing 0, 3.125, 6.25, 12.5, 25, 50 and 100 μM of 2-BP was added to the cells and were incubated for an additional 16–18 h. Dividing cells were detected by indirect immunofluorescence by using anti-SAG1 and anti-IMC1 antibodies and DAPI staining of nuclear material. Treatment with 2-BP did not seem to prevent parasite replication since more than one parasite could be detected in the parasitophorous vacuoles (Fig. 2A and Fig. S2). Moreover, multinucleated cell were common and were encapsulated by the IMC (Fig. 2A, arrowhead). In addition, IMC1, which was predicted to be palmitoylated [9], did not present an evident pellicle delocalization. Intracellular parasites presented an alteration in their morphology that was dose-dependent (Fig. 2A and S2). However, in spite of the altered morphology of the mature tachyzoites, the nascent daughter cells appeared normal, including at later stages of segregation (Fig. 2A, arrowhead). The alteration in morphology, which appeared as aberrant parasites in the parasitophorous vacuoles, was dose-dependent and clearly observed in almost all of the 2-BP treated intracellular parasites at 50 and 100 μM (Fig. 2B and S2). Since the cytoskeleton is essential for cell morphology and shape, this result suggests that some cytoskeletal proteins would be altered by depalmitoylation. The cytoskeletal/IMC proteins IMC1, tubulin α-chain, GAP45, HSP20 and Morn-1 were predicted to be palmitoylated in *Toxoplasma* and may be affected by 2-BP [9].

Here we demonstrated that protein depalmitoylation affected two linked processes (gliding and invasion) and intracellular morphology. In vitro studies revealed that at 100 μM of 2-BP almost complete inhibition of palmitoyl acyl-transferases is achieved [8]. Regarding gliding motility, the presence of 2-BP did not prevent this process; instead, it increased the non-circular motility, with a marked eclectic trajectory as well as an increase in the length of parasite path. Non-circular gliding is linked to invasion [16], but our results showed that protein depalmitoylation showed a strong impairment on parasite invasion rate. It is possible that the gliding motility defect observed in parasites treated with 2-BP could be due to a defect in the adhesion to the substrate, a process determined by different steps that are tightly regulated [18]. As such, 2-BP treatment may lead to an uncoordinated or unregulated gliding motility. If this were the case, the invasion could be suffering an interference or disengagement in the penetration process, which is in agreement with our findings.

The alteration in invasion and gliding could be explained by the effect of 2-BP on several proteins of the glideosome either known to be palmitoylated such as TgGAP45 [11] or predicted to be, including MLC [9]. Being the glideosome at the space between the plasma membrane and the IMC, other proteins localized in this space could also have an impact on motility. It has been demonstrated that the lack of expression of palmitoylated proteins such as MyoA or TgGAP45 are essential for gliding [19,20]. Thus, protein palmitoylation could have an effect on their functioning and as such, the observed results are consistent with the published literature. Whether this effect is direct or indirect (through the effect on some gliding modulators) will need to be address in the future.

Long-term (16–18 h) incubation of intracellular parasites with 2-BP did not impair parasite replication since multinucleated and budding cells could be visualized in treated parasites, but their morphology and shape were altered significantly. Daughter cells are encapsulated by the IMC and assembled by cortical cytoskeleton (sub-pellicular microtubules and intermediate-filament like proteins) as well as other proteins like Morn-1 [21–23]. It seems that during this process palmitoylation does not play any

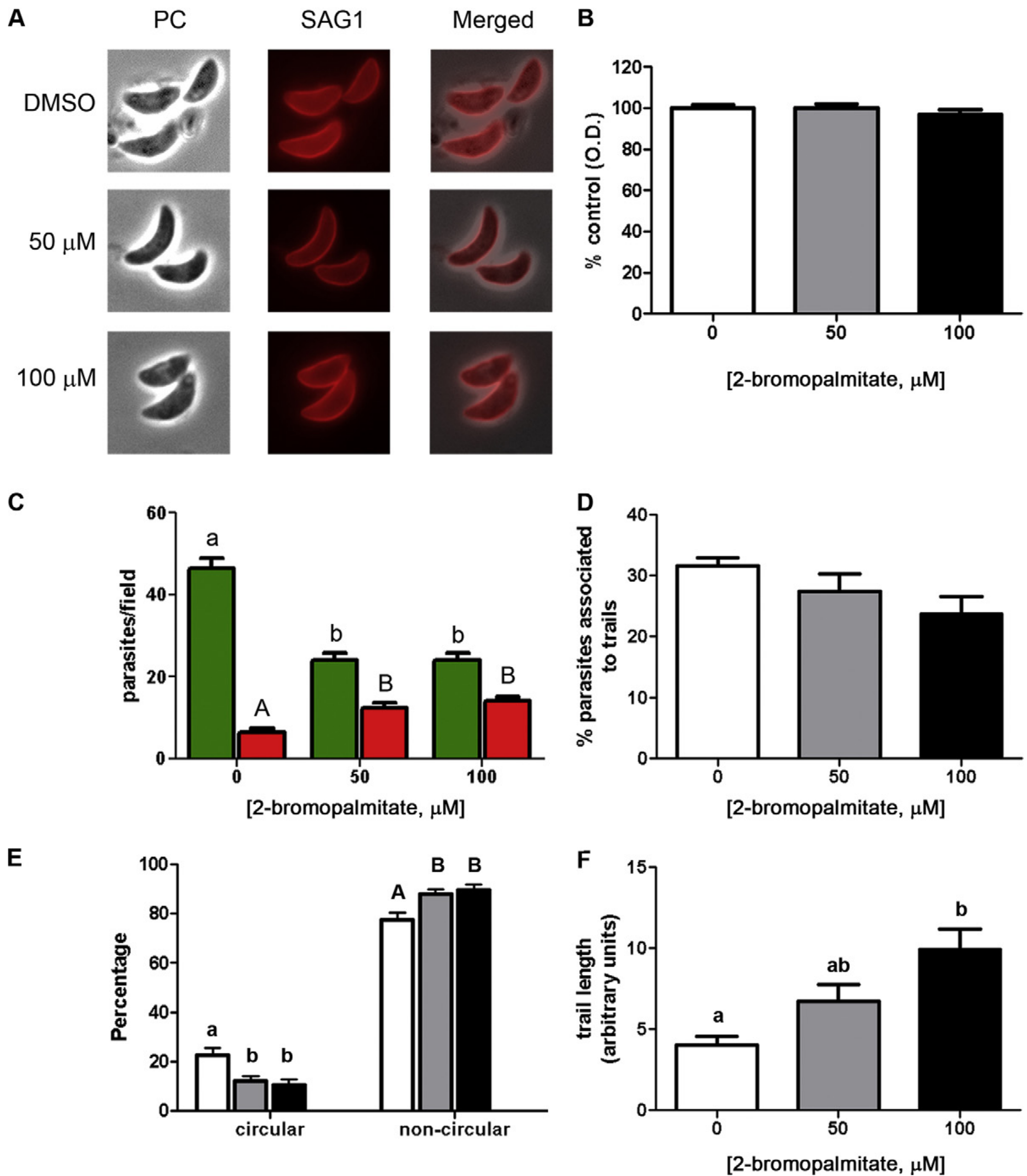
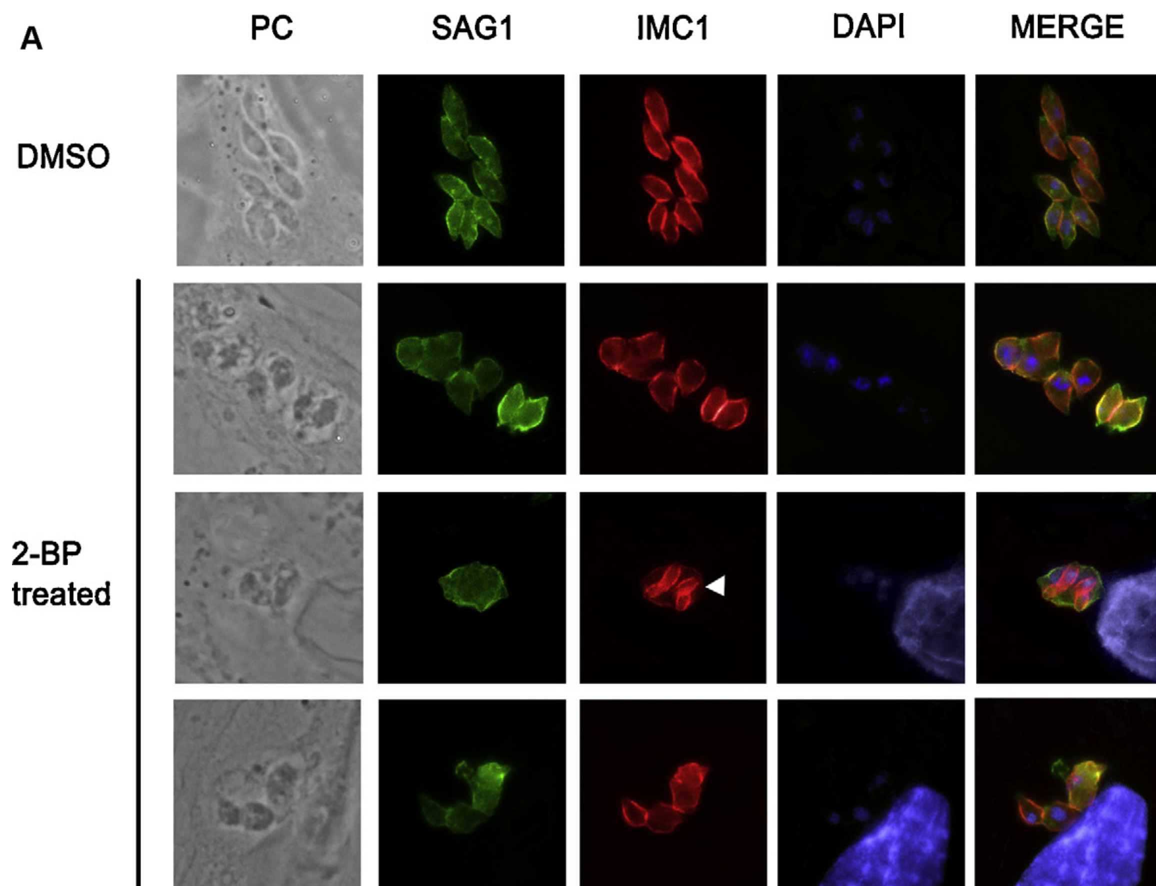


Fig. 1. Analysis of protein depalmitoylation on extracellular tachyzoites. (A) Parasites (2×10^6) were treated either with DMSO (control), 50 μM or 100 μM of 2-BP for 2 h and resuspended in 150 μl DMEM + P + S and subjected to immunofluorescence studies using anti-SAG1 antibody. All observations were performed on a Nikon E600 epifluorescence microscope. Adobe Photoshop (Adobe Systems) was used for image processing. PC: phase contrast. A: 100X. (B) After 2 h of 2-BP treatment, *T. gondii*'s viability was assessed by the XTT method, following the manufacturer's instructions. The percentage of optical density was calculated in respect to control. Three independent experiments in quintuplicate were performed and analyzed. White bar: control (DMSO treated); grey bar: 50 μM 2-BP; black bar: 100 μM 2-BP. (C) Parasites were treated for 2 h with 2-BP, washed and allowed to invade fibroblast monolayers for 1 h at 37 °C. They were then fixed and incubated with rabbit anti-SAG1 as extracellular parasite labeling antibody. After that, the samples were permeabilized by including 0.1% (v/v) TX-100 in the PBS–BSA block and incubated with murine anti-IMC1 antibody. Secondary antibodies used were Alexafluor goat anti-mouse 488 and Alexafluor goat anti-rabbit 594 (Invitrogen). For each treatment, at least 250 total parasites were counted from nine random fields per sample, and values are presented as internal (green) or external (red) parasites per field. Data are mean values ± SEM (error bars) for three independent experiments performed in duplicates. Different letters indicate significant differences with a $p < 0.05$. Intracellular and extracellular parasites were analyzed separately using one-way ANOVA followed by Tukey's multiple comparison tests. Prism GraphPad software was used for statistical analysis. (D) Trails left for each of the concentration tested after 2 h 2-BP treatment were analyzed by indirect immunofluorescence using anti-SAG1 antibody and performed on poly-l-lysine coated slides. Percentage of the parasites associated to trails in regards to the total of parasites did not differ significantly at the concentration tested. Three independent experiments in duplicate were performed and 100 fields were analyzed each time. (E) From all the parasites associated to trails, the percentage of circular versus non-circular trails was calculated. Significant differences could be observed at 50 μM 2-BP. White bar: control (DMSO treated); grey bar: 50 μM 2-BP; black bar: 100 μM 2-BP. Different letters indicate significant differences with a $p < 0.05$. Three independent experiments in duplicate were performed and an average of 100 trails was analyzed. Non-circular trails (10 for each concentration) were chosen at random and analyzed (see Fig. S1). (F) Quantification of trail-lengths left after treatment with 2-BP. Significant differences could be observed in a dose-dependent manner. White bar: control (DMSO treated); grey bar: 50 μM 2-BP; black bar: 100 μM 2-BP. Different letters indicate significant differences with a $p < 0.05$. ANOVA analysis, followed by Tukey's multiple comparison tests was performed with the data obtained at least in three independent experiments with duplicates in each.



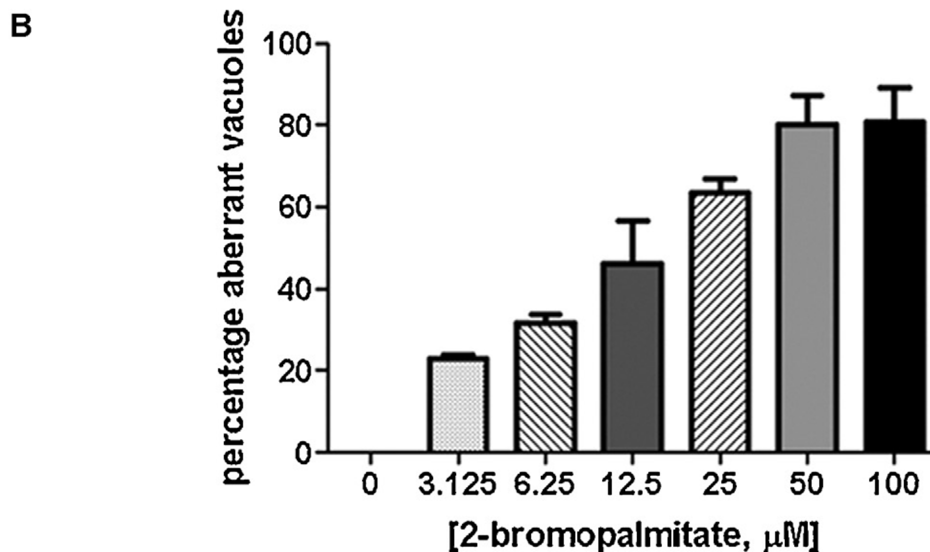


Fig. 2. Analysis of protein depalmitoylation on intracellular tachyzoites. (A) Untreated tachyzoites were incubated with a monolayer of HFF cells. After 1 h at 37 °C in 5% CO_2, the wells were washed three times with PBS and fresh media containing 0, 3.125, 6.25, 12.5, 25, 50 and 100 μM of 2-BP in DMSO was added to the cells. Sixteen hours post-infection cells were fixed and permeabilized. Dividing tachyzoites were visualized as budding cells labeled with anti-IMC1 antibody, whereas nuclei were visualized with DAPI. Morphology could be observed by anti-SAG1 antibody. PC: phase contrast. A: 100X. (B) Percentage of intracellular parasites with aberrant morphology was plotted. This figure is representative of six independent experiments performed in duplicates where at least 200 vacuoles were counted. All the experiments presented similar results (see Fig. S2 to observe aberrant vacuoles at each concentration tested).

significant role. However, palmitoylation is important to maintain the morphology of the mature intracellular tachyzoite, suggesting that some cytoskeletal proteins could be palmitoylated, at least in the mature tachyzoite, being this modification important for parasite shape. Recently it was found that the IMC of mature tachyzoites is composed of three sections were certain proteins could be palmitoylated by different PATs [12]. It is possible that some of these PATs could associate to the IMC at a later stage of

parasite replication or only in mature tachyzoites, regulating the final step of parasite morphology. Future studies should be carried out to confirm this point.

Concluding remarks

This work provides the first evidence of the functional role of protein palmitoylation in *T. gondii* by using the global palmitoylation inhibitor 2-BP. Inhibition of protein palmitoylation showed to alter gliding motility patterns (circular versus non circular trajectories) as well as trail-length. Moreover, the invasion process was severely reduced. Therefore, these results provide important evidence that palmitoylation is determinant for the biological role of gliding/motility associated proteins. Furthermore, intracellular parasites also presented an aberration which was observed as deformed mature tachyzoites. A global study of the targeted proteins affected by this post-translational modification should be addressed in further studies to asses the importance of palmitoylation in *T. gondii*'s biology.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molbiopara.2012.03.006.

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