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Two palmitoyl acyltransferases involved sequentially in the biogenesis of the inner membrane complex of *Toxoplasma gondii*

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Abstract

The phylum Apicomplexa includes a number of significant human pathogens like *Toxoplasma gondii* and *Plasmodium* species. These obligate intracellular parasites possess a membranous structure, the inner membrane complex (IMC), composed of flattened vesicles apposed to the plasma membrane. Numerous proteins associated with the IMC are anchored via a lipid post-translational modification termed palmitoylation. This acylation is catalysed by multi-membrane spanning protein S-acyl-transferases (PATs) containing a catalytic Asp-His-His-Cys (DHHC) motif, commonly referred to as DHHCs. Contrasting the redundancy observed in other organisms, several PATs are essential for *T. gondii* tachyzoite survival; 2 of them, TgDHHC2 and TgDHHC14 being IMC-resident. Disruption of either of these TgDHHCs results in a rapid collapse of the IMC in the developing daughter cells leading to dramatic morphological defects of the parasites while the impact on the other organelles is limited to their localisation but not to their biogenesis. The acyltransferase activity of TgDHHC2 and TgDHHC14 is involved sequentially in the formation of the sub-compartments of the IMC. Investigation of proteins known to be palmitoylated and localised to these sub-compartments identified TgISP1/3 as well as TgIAP1/2 to lose their membrane association revealing them as likely substrates of TgDHHC2, while these proteins are not impacted by TgDHHC14 depletion.

KEYWORDS

cytoskeleton biogenesis, inner membrane complex, palmitoylation, protein S-acyl transferase, *Toxoplasma gondii*

1 | INTRODUCTION

The phylum Apicomplexa comprises numerous obligate intracellular parasites of medical and veterinary importance. Among them, *Toxoplasma gondii* establishes a chronic infection in about one third of the human population and can lead to severe complications in immunocompromised individuals or in case of congenital infection. Apicomplexans exhibit an atypical cytoskeleton organised around a membranous structure, named inner membrane complex (IMC), and composed of flattened sacs and a meshwork of intermediate filament-like proteins connected to the cortical microtubules (Mann & Beckers, 2001). The IMC is central in the biology of these parasites, being critical for their motility, replication, polarity and integrity (Anderson-White et al., 2012). The fast replicating stage of *T. gondii*, called the tachyzoite, divides by endodyogeny, a process in which the IMC provides the scaffold for the two daughter progenies to develop within the mother cell. In *T. gondii* and related coccidian parasites, freeze-fracture studies from the 70s revealed a high degree of organisation of the IMC (Dubremetz & Torpier, 1978; Porchet & Torpier, 1977). At the apical end, one truncated cone-shaped plate

constitutes the apical cap in which are anchored, for instance, the acylated proteins TgISP1 (IMC Sub-compartment Protein 1), TgGAP70 (gliding-associated protein 70 kDa) and TgPhIL1 (photosensitised INA-labelled protein 1) (Beck et al., 2010; Frénal et al., 2010; Gilk et al., 2006). The rest of the IMC is composed of three rows of rectangular plates joined together by sutures and constituting the central and basal sections of the IMC (Figure 1a) (Beck et al., 2010). So far, mainly acylated proteins have been found associated to these regions with TgISP2 and TgISP4 in the central part and TgIAP1 (IMC-Associated Protein 1) in the basal part as well as in the basal ring at the edge of the IMC (Figure 1a) (Beck et al., 2010; Frénal, Marq, Jacot, Polonais, & Soldati-Favre, 2014; Fung, Beck, Robertson, Gubbels, & Bradley, 2012). More recently, proximity labelling experiments have been carried out with IMC-resident proteins and identified new proteins localised to the apical cap, central and basal regions, as well as longitudinal or transverse sutures (Chen et al., 2015, 2017; Long, Anthony, Drewry, & Sibley, 2017; Long, Brown, et al., 2017).

Surprisingly, among them, very few are embedded in the membrane of the organelle by transmembrane domain(s) while several are potentially anchored to the outer leaflet by predicted acylation, and especially by palmitoylation.

Palmitoylation refers to the covalent attachment of a palmitate moiety onto cysteine residues of peripheral and integral membrane proteins via a thioester bond, which increases membrane affinity of the substrates, and thereby modulating their localisation, conformation, stability and interaction. Palmitoylation is the only acylation to be enzymatically reversible and thus allows a dynamic regulation of cellular processes (Blaskovic, Blanc, & van der Goot, 2013; Linder & Deschenes, 2007). It is widespread in eukaryotes and represents around 10% of the human proteome (Blanc et al., 2015). In *T. gondii*, palmitoylation has been proved to be critical for cell division (Beck et al., 2010), pellicle integrity (Frénal et al., 2010), egress of the parasites from the infected host cell (McCoy et al., 2017) as well as for the apical positioning of secretory organelles called rhoptries whose
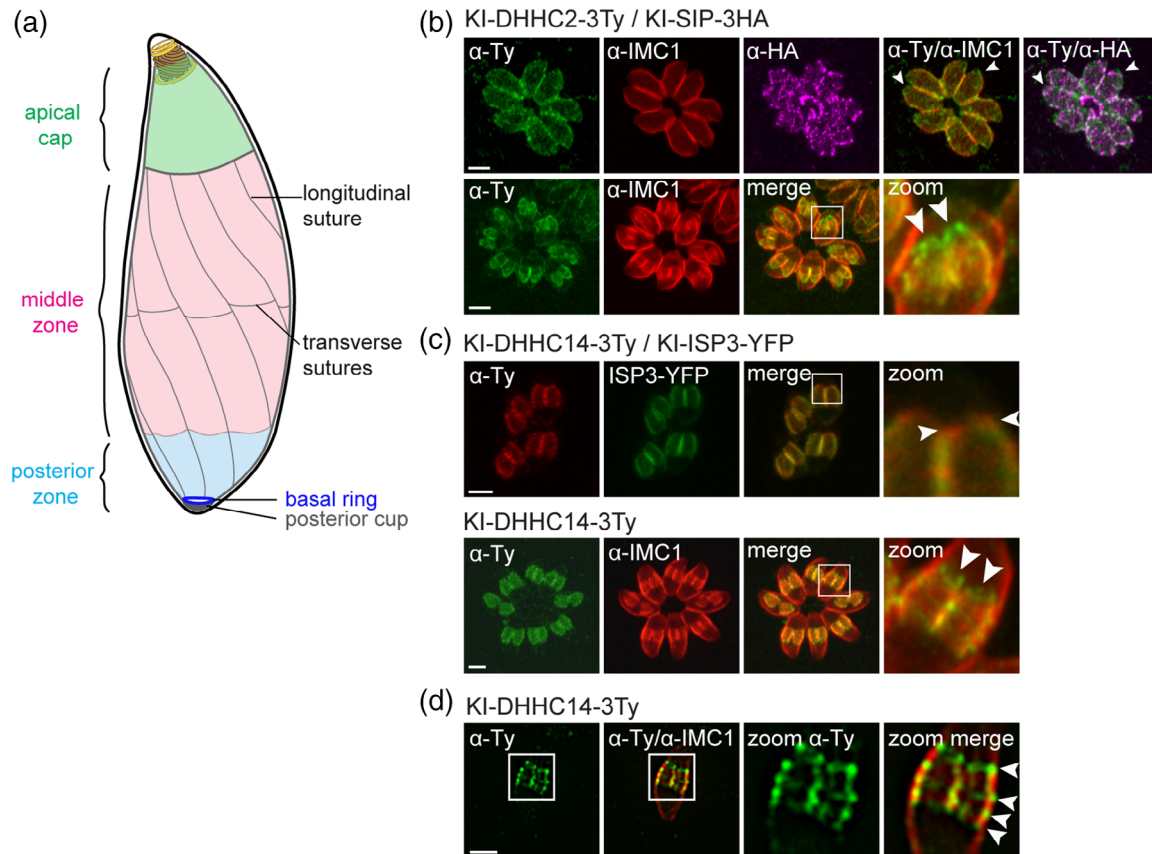
FIGURE 1 DHHC2 and DHHC14 occupy distinct sub-compartments of the IMC. (a) Schematic representation of a tachyzoite and its pellicle formed by the plasma membrane and the underlying IMC. The IMC is composed of three sub-compartments, the apical cap (in green), the middle zone (in pink) and the basal region (in blue). Under the apical cap, the IMC is composed of a patchwork of rectangular plates joint by longitudinal and transversal sutures. (b) Immunofluorescence assay (IFA) performed on intracellular tachyzoites expressing the endogenously tagged TgDHHC2 (TgDHHC2-3Ty) and TgSIP (TgSIP-3HA). TgDHHC2 localises to the full IMC in contrast to TgIMC1 and TgSIP which are absent from the apical cap (arrowheads, upper panel). As the daughter cells grow, TgDHHC2 is barely undetectable in the mother IMC (lower panel). (c) IFA on intracellular tachyzoites expressing the endogenously tagged TgDHHC14 (TgDHHC14-3Ty). TgDHHC14 is mainly concentrated in the growing daughter cells and is not detected in the apical cap of the IMC, like TgISP3. Co-staining between TgDHHC14 and TgIMC1 shows that TgDHHC14 localisation is not homogeneous in the IMC. (d) Super-resolution revealed that TgDHHC14 is actually found at the transverse sutures of the IMC. Scale bars: 2 μm

content is secreted at the time of invasion and participates in host cell penetration and establishment of the intracellular lifestyle (Beck et al., 2013; Mueller et al., 2013). Overall, the global analysis of palmitoylated proteins (palmitoylomes) in *T. gondii* and the related parasite *Plasmodium falciparum* revealed the presence of over 300 and 400 proteins, respectively, including previously experimentally confirmed ones (Foe et al., 2015; Jones, Collins, Goulding, Choudhary, & Rayner, 2012). This is significantly more than the palmitoylomes reported before for other unicellular eukaryotes and suggests a large use of this post-translational modification by the Apicomplexans to regulate critical processes (Emmer et al., 2011; Roth et al., 2006). Palmitoylation is mediated by multi-membrane spanning protein S-acyltransferases (PATs) containing a catalytic Asp-His-His-Cys motif facing the cytoplasm and thus commonly referred to as DHHCs (Linder & Deschenes, 2007). The recently elucidated structure of two DHHCs from human and zebrafish established that the active site, catalysing the thioester exchange from the palmitoyl-coA to the substrate, is located at the membrane-cytosol interface and hence explains why the palmitoylated sites of integral protein substrates reside close to the transmembrane domains (Rana et al., 2018).

T. gondii genome encodes 18 DHHCs, 16 of them being expressed in the tachyzoite stage (Frénal et al., 2013). As in other organisms, including humans, most of the DHHCs have been localised to the Golgi apparatus, the endoplasmic reticulum and the plasma membrane but three of them were found in the membrane of apicomplexan-specific organelles, TgDHHC2 and TgDHHC14 in the IMC and TgDHHC7 in the rhoptries (Beck et al., 2013; Frénal et al., 2013). Surprisingly, and in contrast to other organisms in which none of the PATs were individually essential (Emmer et al., 2011; Roth et al., 2006), five DHHCs have been refractory to deletion in *T. gondii* including the three aforementioned ones (Frénal et al., 2013). In this study, we investigate the role of TgDHHC2 and TgDHHC14 for the biology of the tachyzoite. These two PATs are conserved across the phylum Apicomplexa and likely not redundant within the IMC (Frénal et al., 2013; Wetzel et al., 2015). While TgDHHC2 appears as a canonical PAT predicted to have four transmembrane domains with the catalytic region located between the second and the third transmembrane domain, TgDHHC14 exhibits a longer N-terminus encompassing ankyrin repeats. PfDHHC1, the homologue of TgDHHC14 in *P. falciparum*, has been localised to the IMC of the blood stages, following the PfISP1 and PfISP3 staining during schizogony (Wetzel et al., 2015). In addition, during gametocytogenesis, PfDHHC1 appears in the transversal sutures of the developing IMC. Surprisingly, in *P. berghei*, PbDHHC2, the homologue of TgDHHC2, is cytoplasmic in the blood stages and ookinete (Santos et al., 2015). PbDHHC2 is likely essential since the gene could not be disrupted but a promoter swap cell line revealed its critical role for the ookinete development, downstream of the fertilisation step, suggesting an involvement in the morphogenesis of this zygote stage. Both *PfDHHC2* and *PfDHHC1* appear non-mutable in a *piggyBac* transposon screen of the *P. falciparum* genome (Zhang et al., 2018), suggesting a conserved and essential role of these PATs in apicomplexan parasites.

Here, we show that TgDHHC2 and TgDHHC14 have distinct localisations within the IMC of the tachyzoite. Depletion of either TgDHHC2 or TgDHHC14 severely compromised IMC biogenesis without impacting the replication process and the biogenesis of other organelles. The collapse of the IMC is due to the loss of the acyl-transferase activity of the enzymes. Investigation of proteins of the IMC known to be palmitoylated identified proteins of distinct sub-compartments as probable substrates of TgDHHC2, while no substrates of TgDHHC14 have been identified.

2 | RESULTS

2.1 | Two PATs localised into distinct sub-compartments of the IMC

To refine the timing of expression and localisation of TgDHHC2 and TgDHHC14 in *T. gondii* tachyzoite, the strains endogenously tagged with 3Ty by knock-in strategy have been scrutinised by immunofluorescence assay (IFA) using different markers associated with the IMC. TgDHHC2 was detected in mature parasites (Figure 1b) as well as in dividing parasites in which the protein is mainly concentrated in the growing daughter cells. TgDHHC2 localises to the three sub-compartments of the IMC as shown in mature parasites by its staining that extends up to the apical end beyond the staining of TgIMC1 that is excluded from the cap and TgSIP (Stripes IMC Protein, [Lentini et al., 2015]) which decorates the transverse sutures of the IMC plates and therefore delineates the base of the apical cap (Figure 1b, upper panel). This localisation is more clearly visible during the budding of the daughter cells in which the staining of TgDHHC2 is detected in the apical cap before the appearance of TgISP3 (Figure S1a in Data S1) and above TgIMC1 (Figure 1b, lower panel). In contrast, TgDHHC14 is nearly undetectable in mature parasites and only visible in the daughter cells where its staining always starts as a ring that elongates along the developing progenies (Figure S1b in Data S1). Co-staining with TgISP3 and TgIMC1 (Figure 1c) shows that TgDHHC14 is absent from the apical cap and exhibits a non-homogenous signal in the rest of the IMC. Super-resolution images from structured illumination microscopy (SR-SIM) revealed that TgDHHC14 specifically localises to the transverse sutures of the IMC plates (Figure 1d). In addition, both TgDHHC2 and TgDHHC14 appear before the staining of TgIMC1 is observed in the budding parasites (Figure S1 in Data S1).

These data show that the two palmitoyl transferases of the IMC, TgDHHC2 and TgDHHC14, are mainly expressed during the division process of the parasites and have distinct localisations within the IMC, with TgDHHC2 found in the full organelle while TgDHHC14 is restricted to the transverse junctions of the IMC.

2.2 | TgDHHC2 and TgDHHC14 are critical for the life cycle of T. gondii tachyzoite

We previously failed to individually disrupt these genes, suggesting that despite some overlapping localisation between TgDHHC2 and TgDHHC14, these enzymes are likely not redundant and have a

different substrate specificity within the IMC (Frénal et al., 2013). Therefore, to investigate their function, inducible knock-down (iKD) cell lines have been generated by replacing the endogenous promoter with a tetracycline-repressive promoter using a CRISPR-based genome editing strategy to enhance the integration (Figure S2a,b and Table S2 in Data S1). Subsequently, a 3Ty tag has been added to the 3′ end of the coding sequence by knock-in. The localisation of TgDHHC2 and TgDHHC14 is maintained in these strains (Figure S2c in Data S1) and their expression is tightly down-regulated under anhydrotetracycline (ATc) treatment, reaching a drastic reduction after 16 hr of treatment (Figure 2a) and undetectable levels by western blot within 24 hr for TgDHHC14 and 48 hr for TgDHHC2 (Figure 2b). Depletion of each PAT strongly impairs the growth of the parasites as shown by plaque assays performed on TgDHHC2-iKD, TgDHHC14-iKD and the parental ΔKU80 strains (Figure 2c). In contrast to the control, very small (TgDHHC14-iKD + ATc) or no plaques (TgDHHC2-iKD + ATc) were visible on the fibroblast monolayer after 9 days in presence of ATc, indicating a defect in one or more steps of the tachyzoite lytic cycle.

Altogether, these results confirm the critical nature of TgDHHC2 and TgDHHC14 for the tachyzoite survival that was previously suggested by the inability to knock-out these genes (Frénal et al., 2013). It also corroborates the low fitness score (−4.36 and −3.21, respectively) attributed to these genes in the recent CRISPR-based genome-wide screen performed in *T. gondii* tachyzoite (Sidik et al., 2016).

2.3 | Depletion of TgDHHC2 or TgDHHC14 alters the IMC biogenesis but not the replication process and the biogenesis of the other organelles

We examined the intracellular development of TgDHHC2- and TgDHHC14-depleted tachyzoites by IFAs after 16 hr of ATc treatment. Since the two enzymes are localised to the IMC, we first focused our attention on the fate of the IMC during the cell division process using the transmembrane protein TgGAP50 endogenously tagged at its C-terminus with a YFP. In both knock-down strains, initiation of new daughter cells can be observed with the budding occurring in random orientations. However, TgGAP50 staining highlights a strong alteration of the biogenesis and morphology of the IMC later on as well as a strong defect in parasite organisation within the parasitophorous vacuole (Figure 3a). In addition, although the nucleus divides, it is not always associated to an IMC structure that usually encapsulates the newly formed progenies (Figure 3a, arrowheads). Nonetheless, the nuclei remain associated with the centrosomes as shown by the centrin 1 staining (TgCEN1, Figure S3a in Data S1). The IMC of the daughter cells likely collapses earlier in TgDHHC2-depleted parasites than in TgDHHC14-depleted since we do not observe very advanced progenies in this cell line compared to the TgDHHC14 knock-down. In both cases, with the successive rounds of division, the shape of the parasites is more and more altered preventing their normal proliferation in the host cell monolayer. Of

note, we sometimes observed intact parasites in the case of TgDHHC14 knock-down cell line that could explain the very small plaques monitored previously. This could be either due to a leakiness of the inducible system in which the residual level of enzyme could be sufficient to ensure the PAT activity or the critical but not totally essential function of TgDHHC14.

In parallel, we examined other organelles that are packaged in the developing daughter cells in a timely orchestrated process during the cell division (Nishi, Hu, Murray, & Roos, 2008). Secretory organelles such as micronemes and rhoptries are made de novo at each round of division. After their trafficking through the endoplasmic reticulum (ER) and the Golgi apparatus, the secreted proteins are sorted to the apical micronemes or rhoptries through the endosome-like compartment (ELC) (Nishi et al., 2008; Sangaré et al., 2016). Staining of TgMIC2 in the micronemes and TgROP7 in the rhoptries shows that the biogenesis of these organelles is not impacted by the depletion of either TgDHHC2 or TgDHHC14 and they remain fairly associated with apical structures within the deformed parasites (Figure S3a,b in Data S1). Organelles that are inherited during the cell division such as the apicoplast and the mitochondrion were also looked at. In both TgDHHC2- and TgDHHC14-depleted parasites, the apicoplast divides and is most of the time found in close proximity to the nucleus (Figure S3c in Data S1), likely because the organelle associates with the centrosomes during division (Striepen et al., 2000). We also checked the fate of the mitochondrion in both knock-down cell lines since this organelle was reported to make contact sites with the IMC (Ovciarikova, Lemgruber, Stilger, Sullivan, & Sheiner, 2017) and to be inherited late during the division cycle, crawling along the IMC of the daughter cells (Nishi et al., 2008). Surprisingly, no problem of the mitochondrion inheritance was observed 16 hr post ATc treatment (Figure S3d in Data S1).

The IMC is critical to maintain the shape of the parasite since it is connected to the cortical microtubules, likely through transmembrane proteins of the GAPM (glideosome-associated protein with multiple-membrane spans) family (Bullen et al., 2009; Harding et al., 2019). Staining of the microtubules with the anti-acetylated α-tubulin revealed that they emerge with the growing daughter cells (Figure 3b), likely associated with the IMC previously observed with GAP50-YFP. However, again, no elongated daughter cells stained for the microtubules are observed for TgDHHC2-depleted parasites in contrast to TgDHHC14 knock-down parasites.

To have a better understanding of the morphology defect of the down-regulated parasites, the two strains were observed by electron microscopy after 24 hr ±ATc (Figure 3c and Figure S4 in Data S1). In both cases, TgDHHC-depleted parasites are multinucleated cells containing micronemes, rhoptries, dense granules and mitochondria. However, they present a strong alteration of the IMC which is discontinued and detached from the plasma membrane at the level of the mother cell pellicle. Fragments of IMC still connected to microtubules are also observed floating in the cytoplasm (Figure 3c).

Taken together, the close examination of the parasite morphology shows that depletion of TgDHHC2 or TgDHHC14 induces strong defects in the IMC biogenesis with a collapse of the structure in the developing daughter cells likely occurring earlier in the case of

TgDHHC2 knock-down compare to TgDHHC14. Despite this severe defect, the cell division process as well as the biogenesis of the other organelles remain apparently unaffected.

2.4 | The loss of acyl-transferase activity is responsible for the collapse of the IMC

To determine whether TgDHHC2 and TgDHHC14 are active enzymes and if the observed phenotypes were due to a loss of the acyl-transferase activity, the two iKD strains were complemented with either the wild type TgDHHC (cpt-DHHC2/14-WT) or with a mutant in which the DHHC motif was substituted by DHHS (cpt-DHHC2-C194S and cpt-DHHC14-C618S), a point mutation preventing the acyl-transferase activity (Lobo, Greentree, Linder, & Deschenes, 2002; Zhang, Wu, Kelly, Nurse, & Hang, 2013). An additional complementation was performed for TgDHHC14 with a copy lacking the ankyrin repeats (cpt-DHHC14-ΔANK). All these complemented constructs were inserted into the UPRT locus, expressed under the control of their endogenous promoter and tagged with 4 c-Myc (Figure S5a and Table S2 in Data S1).

For each PAT, the complemented proteins were all expressed at similar levels except TgDHHC14-ΔANK (Figure 4a) that was well integrated in the genome (Figure S5b,c in Data S1) but likely not targeted to the IMC and therefore unstable and never detected either by western-blot or by IFA. In contrast, the WT and the catalytically dead mutants were localised at the IMC like the endogenously tagged copies (Figure 4b). In TgDHHC2- and TgDHHC14-depleted parasites, only the WT but not the mutated version of the enzyme was able to prevent the collapse of the IMC as shown by IFA (Figure 4b). In accordance, plaque assays performed over 7 days with the complemented strains showed that the growth phenotype of TgDHHC2 and TgDHHC14 depletion can be rescued by the WT copy of TgDHHC2 and TgDHHC14 but not with the single point mutants (Figure 4c).

These results indicate that TgDHHC2 and TgDHHC14 are active enzymes of the IMC and that the collapse of the structure observed upon their depletion is therefore likely due to the non-palmitoylation of substrates contributing to the biogenesis of the IMC.

2.5 | Exploration of potential substrates of TgDHHC2 and TgDHHC14

To investigate potential substrates of TgDHHC2 and TgDHHC14, we focused on proteins known to be palmitoylated and localised at the IMC. We first followed the acylated proteins recruited to the different sub-compartments of the IMC by myristoylation and palmitoylation of their N-terminus, namely the early marker for bud formation TgISP1 at the apical cap, and the later markers TgISP2 in the middle zone and TgISP3 in the central and posterior regions of the IMC (Beck et al., 2010). Upon TgDHHC2 depletion, TgISP1 signal almost totally disappears from the parasites as shown by IFA (Figure 5a). Sequential fractionations corroborate this phenomenon with a total amount of

TgISP1 highly reduced suggesting a strong destabilisation of the protein. Concomitantly, TgISP1 becomes partially soluble in PBS and disappears from the pellet fraction likely because in absence of TgDHHC2, TgISP1 is not palmitoylated and therefore its myristoylation is not enough to strongly anchor the protein to the IMC membrane (Figure 5b). Similarly, TgISP2 and TgISP3 signal associated to membranous material decreases upon TgDHHC2 depletion and appears partially cytoplasmic by IFA (Figure 5a). However, no variation of the total amount of protein can be observed in the fractionation experiments and only TgISP3 profile changes with ATc treatment with less protein found in the pellet and more in the soluble fraction (Figure 5b). In contrast, upon depletion of TgDHHC14, the anchoring of TgISP1 at the apical cap is still observed as well as TgISP2 and TgISP3 along the parasite except at the basal pole (Figure 5a). The profiles of TgISP1, TgISP2 and TgISP3 do not change during sequential fractionations in the TgDHHC14-iKD strain treated or not with ATc (Figure 5b).

These results suggest that TgISP1 and TgISP3 are substrates of TgDHHC2 since their localisation and solubility are impacted upon TgDHHC2 depletion while none of the TgISPs seem to be substrates of TgDHHC14.

We then examined IMC proteins that are only palmitoylated. The only reported protein is TgIAP1 (IMC-associated protein) that is part of the MyoC-glideosome at the basal pole of the parasite and anchored in the basal sub-compartment of the IMC by N-terminal palmitoylation (Frénal et al., 2014). We also identified a distant homologue of TgIAP1 that we named TgIAP2 (TGGT1_297130) that presents no particular domain or motif but four predicted palmitoylation sites that are conserved with TgIAP1 (Figure S6 in Data S1). In addition, TgIAP2 is found in the *T. gondii* palmitoylome like TgIAP1 (Foe et al., 2015). TgIAP1 and TgIAP2 have been endogenously tagged in both TgDHHC2- and TgDHHC14-iKD strains. TgIAP1 localises to the basal pole as expected, while TgIAP2 localises to the IMC but is absent from the apical cap (Figure 6a). Strikingly, upon depletion of TgDHHC2, TgIAP1 signal completely disappears and the signal of TgIAP2 associated with membranes decreases, as observed by IFA. We observe a concomitant change of protein profile in sequential fractionations, with both proteins visible in the PBS soluble fraction. In contrast, with depletion of TgDHHC14, the membrane association of TgIAP1 and TgIAP2, as seen by IFA, as well as the protein profile in the fractionations appears unaltered. These results suggest that both TgIAP1 and TgIAP2 could be substrates of TgDHHC2.

3 | DISCUSSION

The large repertoire of PATs in Apicomplexa likely highlights the importance of the role of palmitoylation in these parasites. This assumption is further supported by the reported palmitoylomes of *T. gondii* and *P. falciparum* that identified more than 300 proteins involved in multiple aspects of the parasite biology (Caballero et al., 2016; Foe et al., 2015; Jones, Collins, et al., 2012; Jones, Tay, & Rayner, 2012). However, there is comparatively little knowledge

about the enzyme-substrate relationships. The rhoptry-localised protein named armadillo repeat only (TgARO), anchored to the surface of the organelle by acylation, is the only substrate shown to be modified by the conserved TgDHHC7, also localised in the membrane of the rhoptry (Beck et al., 2013; Frénal et al., 2013; Mueller et al., 2013). Here, we have investigated the functionality of two other conserved PATs, TgDHHC2 and TgDHHC14, and explored some potential substrates.

In tachyzoites, these two enzymes are mainly expressed in the IMC of the budding daughter cells, but with restricted sub-compartmentalisation. TgDHHC2 spans the entire IMC like other transmembrane domain proteins of the IMC such as TgGAP40 and TgGAP50. On the other hand, TgDHHC14 is restricted to the transverse sutures of the IMC like its counterpart in *P. berghei* gametocytes (Wetzel et al., 2015). These latest orthologs constitute the only transmembrane proteins identified in these sutures and it remains to be determined how they can be specifically targeted to these structures.

The distinction of their spatial localisation suggests that they plausibly act on different substrates, though some overlapping substrate specificity cannot be ruled out. TgDHHC2 is observed before the signal of TgISP3 was detected while both TgDHHC2 and TgDHHC14 appear before the staining of TgIMC1. These results give a first insight on the timing of recruitment of IMC-associated proteins during the assembly of the organelle.

We uncovered the critical role of TgDHHC2 and TgDHHC14 for the proper biogenesis of the tachyzoite IMC during endodyogeny. A close examination of all the organelles showed that neither of them appears to be required for the division of the nucleus, the apicoplast and the mitochondrion. Their depletion has also no impact on the biogenesis of the micronemes and rhoptries although their localisation is altered since the integrity of the parasites is lost. Depletion of TgDHHC2 or TgDHHC14 induces a strong defect in the IMC biogenesis that is dependent on the palmitoyl transferase activity. In both cases, initiation of the daughter cell budding can be observed but the
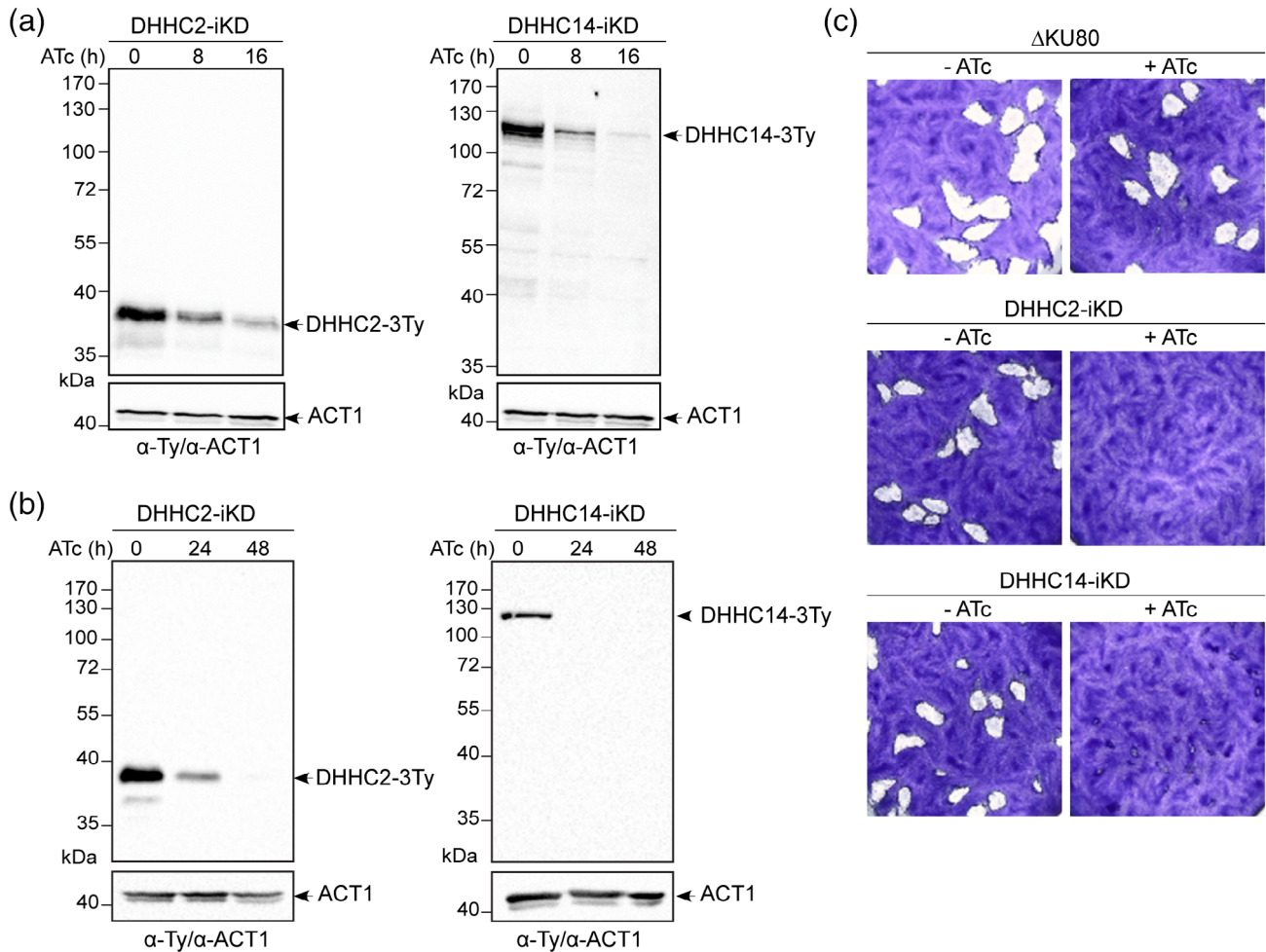
FIGURE 2 DHHC2 and DHHC14 are critical for the lytic cycle of the tachyzoite. (a) The inducible knock-down of TgDHHC2 and TgDHHC14 (TgDHHC2-3Ty-iKD and TgDHHC14-3Ty-iKD), detected at the expected size of 40 and 110 kDa, respectively by western-blot and tightly downregulated upon addition of anhydrotetracycline (ATc) with almost no protein detectable after 16 hr of treatment. (b) TgDHHC2 is undetectable at 48 hr of ATc treatment while TgDHHC14 is already not detected after 24 hr. (c) Plaque assay performed over 9 days. In contrast to the parental strain (ΔKU80), no lysis plaques are formed for TgDHHC2-iKD and TgDHHC14-iKD in presence of ATc, attesting for a defect in one or several steps of the lytic cycle in absence of the corresponding enzyme
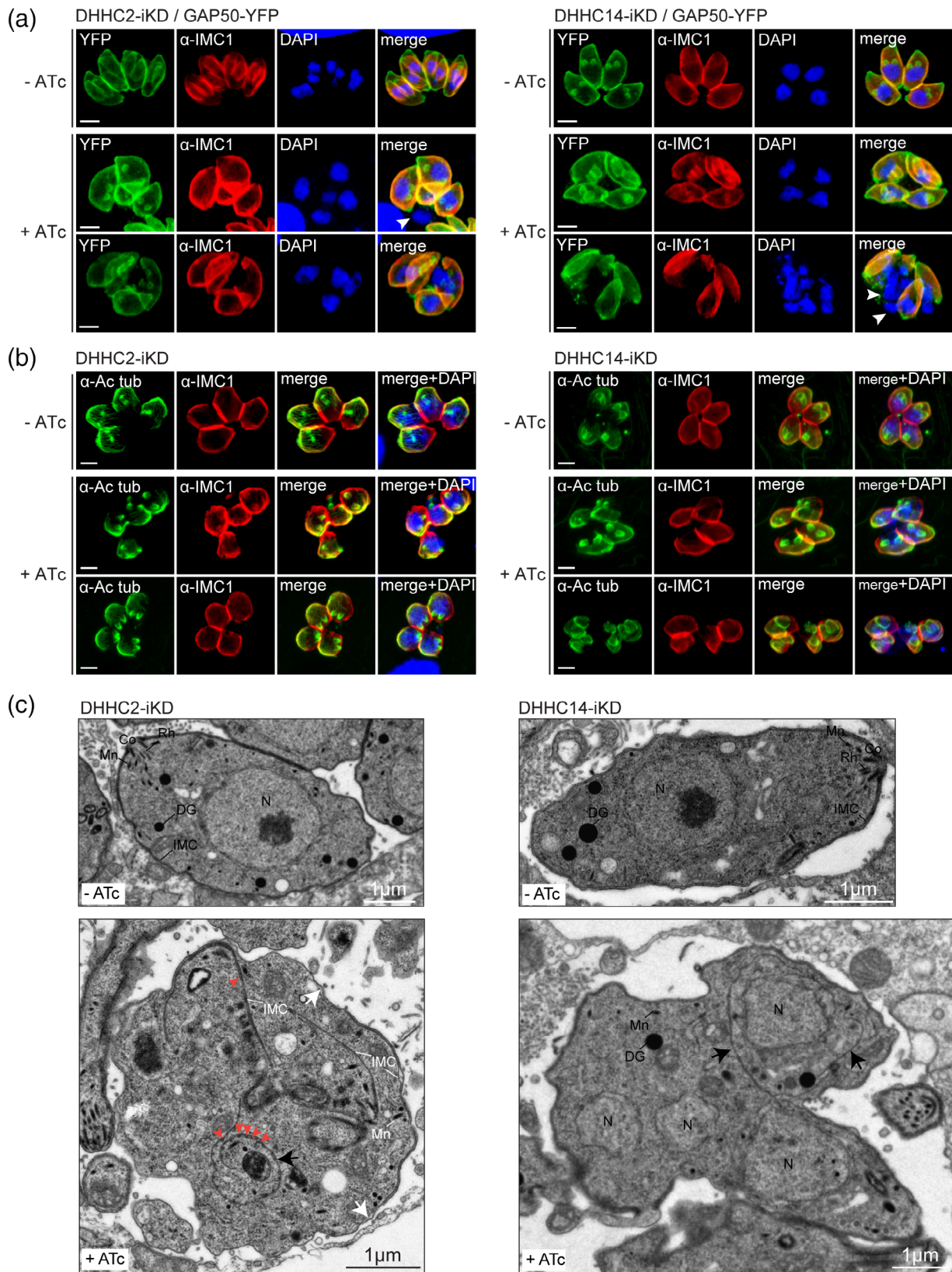
FIGURE 3 TgDHHC2 and TgDHHC14 are critical for the morphology of the parasites. TgDHHC2- and TgDHHC14-iKD tachyzoites expressing the endogenously tagged TgGAP50-YFP (a) or stained with the anti-acetylated tubulin antibodies (b) were treated for 16 hr ± ATc before fixation. TgIMC1 stains the alveolin network. Scale bars: 2 μm. (c) Electron microscopy pictures of TgDHHC2- and TgDHHC14-iKD tachyzoites treated ± ATc for 24 hr. In TgDHHC2 depleted parasites, the IMC is not always associated to the plasma membrane of the mother cell (white arrows) and IMC material (black arrow) can be seen inside the parasite associated to microtubules (red arrowhead). Co, conoid; DG, dense granule; IMC, inner membrane complex; Mn, microneme; N, nucleus; Rh, rhoptry

collapse of the developing IMC occurs earlier upon the TgDHHC2 knock-down compare to TgDHHC14 indicating a sequential requirement of these enzymes to build the sub-compartments of the IMC.

The strong phenotype observed upon the depletion of TgDHHC2 and TgDHHC14 likely results from the simultaneous destabilisation of several palmitoylated proteins associated with the IMC. We have investigated few of them that have been shown experimentally to be palmitoylated and/or have been identified in the global palmitoylome of *T. gondii* (Beck et al., 2010; Chen et al., 2015, 2017; Foe et al., 2015). Among them, TgISP1-3 are dually acylated at their N-terminus by myristoylation that occurs co-translationally on the N-terminal glycine after cleavage of the methionine and by palmitoylation. The myristoylation can confer membrane association to the protein but is not strong enough to stabilise the protein into the membrane that therefore goes on and off the membrane (Peitzsch & McLaughlin, 1993). Immunofluorescence assays combined with solubilisation experiments revealed that TgISP1 and TgISP3 are likely substrates of TgDHHC2 while no TgISPs seem to be impacted by TgDHHC14 depletion. A similar trend is observed for the palmitoylated proteins TgIAP1 and TgIAP2, upon the down-regulation of TgDHHC2, suggesting an enzyme-substrate relationship between these proteins. The proportion of the potential substrates still associated with the membranous structure upon enzyme depletion can be due to the presence of other acylation and/or the residual expression of the enzyme 16 hr after the ATc treatment. Although TgISP1 and TgISP3 are not individually essential for the tachyzoite survival (Beck et al., 2010; Sidik et al., 2016), their combined destabilisation could account for the observed phenotype upon TgDHHC2 depletion. It is also legitimate to speculate that other substrates participate to this phenotype such as alveolins (TgIMCs) which compose the intermediate filamentous meshwork (Anderson-White et al., 2011) or the IMC transmembrane protein TgGAP40 for example. Indeed, our data are reminiscent of the TgGAP40-iKO phenotype (Harding et al., 2016) and TgGAP40 has been isolated in the palmitoylome of both *T. gondii* and *P. falciparum* (Foe et al., 2015; Jones, Collins, et al., 2012). The predicted palmitoylation site is on the cytoplasmic face just after the fourth transmembrane domain and could modulate the role of TgGAP40 in the biogenesis of the IMC. Further experiments will be needed to test this hypothesis.

While the spatial localisation of PATs confers their association with substrates, protein motifs in these enzymes are important for potentiating enzyme-substrate interactions as well. TgDHHC14 contains at its N-terminus an ankyrin repeat domain, a 33-residue motif which mediates protein–protein interactions. The ankyrin repeat domain of human DHHC17, also known as huntingtin interacting proteins 14 (Hip14) has been shown to confer its palmitoylating activity on the huntingtin proteins, and also to transfer the substrate specificity when fused with another DHHC (Huang et al., 2009). Deletion of the ankyrin domain appears to affect the stability of TgDHHC14, leading to disappearance of the protein as evaluated by western blot. This domain likely plays a role in determining its localisation along the IMC sutures or in providing substrate specificity, thereby regulating the palmitoylating activity of TgDHHC14. Despite our attempts, none of the palmitoylated proteins tested seem to be affected by the depletion of TgDHHC14. The potential substrates likely reside in the transverse sutures of the IMC and only six proteins have been localised there so far (Chen et al., 2017; Lentini et al., 2015). Out of them, only TgTSC5, is predicted to affect the tachyzoite fitness upon depletion and is found in the *T. gondii* palmitoylome (Foe et al., 2015; Sidik et al., 2016). This protein is predicted to have three transmembrane domains but no palmitoylation site can be found in silico so further work will be needed to functionally characterise this candidate and establish if it is a substrate of TgDHHC14.

4 | EXPERIMENTAL PROCEDURES

4.1 | Preparation of *T. gondii* genomic DNA and cDNA

All genomic DNA (gDNA) have been prepared from tachyzoites (RH strain) using the Wizard SV genomic DNA purification system (Promega).

4.2 | Cloning of DNA constructs

All amplifications for cloning were performed with the Q5 polymerase (New England Biolabs) and the primers used are listed in Table S1 in Data S1. All amplifications for screening were performed with the GoTaq DNA polymerase (Promega) and the primers listed in Table S2 in Data S1.

4.2.1 | Tet-inducible knockdown of TgDHHC2 (DHHC2-iKD)

A 586 bp gDNA fragment of the N-terminus of *TgDHHC2* (TGME49_278850) was amplified by PCR with primers TgDHHC2-5174/TgDHHC2-5175, digested with *Avr*II and *Spe*I restriction enzymes and cloned into a modified version of the 5′COR-TATi1-HX-tetO7S1MycCOR vector (Salamun, Kallio, Daher, Soldati-Favre, & Kursula, 2014). Then, a 535 bp gDNA fragment of the 5′ flanking region of *TgDHHC2* was amplified using primers TgDHHC2-5172/TgDHHC2-5173 and cloned into the *Nco*I and *Nhe*I sites. To direct the insertion of the plasmid digested with *Avr*II and *Nhe*I before transfection, a specific gRNA vector has been generated using the Q5 site-directed mutagenesis kit (New England Biolabs) with the vector pSAG1::CAS9-GFP-U6::sgUPRT as template (Shen, Brown, Lee, & Sibley, 2014) and the primer pair TgDHHC2-5171/gRNA-4883.

4.2.2 | Tet-inducible knockdown of TgDHHC14 (DHHC14-iKD)

A PCR fragment encoding the TATi trans-activator, the HXGPRT cassette and the TetO7S1 promoter was generated using the

KOD DNA polymerase (Novagen, Merk) with the vector 5′COR-TATi1-HX-tetO7S1MycCOR as template and the primers TgDHHC14-5362 and TgDHHC14-5363 that also carry 30 bp homology with the 5′ end of *TgDHHC14*. To direct the insertion of the PCR product at the start of *TgDHHC14* (TGME49_293730), a specific sgRNA vector has been generated as described above using the primer pair TgDHHC14-5181/gRNA-4883.

4.2.3 | C-terminal tagging of TgDHHC2 and TgDHHC14

To endogenously tag *TgDHHC2* and *TgDHHC14*, the HXGPRT cassette of the KI-DHHC2-3Ty-HX and KI-DHHC2-3Ty-HX vectors (Frénal et al., 2013) has been replaced by the DHFR-TS cassette subcloned into the two *Sac*II sites.

4.2.4 | Complementations of DHHC2-iKD and DHHC14-iKD

To complement the DHHC2-iKD and DHHC14-iKD with a wild type copy and a dead enzyme mutant, the UPRT-pRON5-G13-4myc vector (Guérin et al., 2017) has been used to subclone the endogenous promoter and the coding sequence of each gene. For *DHHC2*, the promoter was amplified from gDNA using the primer pair TgDHHC2-8626/TgDHHC2-8627 and cloned between the *Not*I and *Pac*I sites. Then the coding sequence amplified from cDNA with the primers TgDHHC2-8624/TgDHHC2-8625 was cloned between the *Pac*I and *Eco*RV sites. To generate the DHHC2-C194S mutant, the previous UPRT-pDHHC2-DHHC2-4myc vector was used as template for Q5 site directed mutagenesis using the primer pair TgDHHC2-8707/TgDHHC2-8708. For *DHHC14*, the intermediate vector 5′DHHC14-pDHHC14-loxP-DHHC14-3Ty-loxP-YFP-3′DHHC14 vector was used as a template to amplify pDHHC14-loxP-DHHC14 with the primers TgDHHC14-8628/TgDHHC14-8629. The fragment was assembled into the UPRT-pRON5-G13-4myc vector digested with *Not*I and *Eco*RV using the NEBuilder HiFi DNA Assembly kit to generate the UPRT-pDHHC14-DHHC14-4myc vector. This last vector was then used as template for Q5 site directed mutagenesis using the primer pair TgDHHC14-8703/TgDHHC14-8704 to generate the DHHC14-C618S mutant and using the primers TgDHHC14-8705/ TgDHHC14-8706 to generate the DHHC14-ΔANK mutant.

4.2.5 | pGAP50-YFP-CAT

The 3′ end of the Tg*GAP50* gene (gDNA, TGME49_219320) was amplified using the primers TgGAP50-2900/TgGAP50-2901 and then cloned in frame with the YFP in the pYFP-LIC-CAT vector as previously described using the ligation independent cloning (LIC) technology (Huynh & Carruthers, 2009).

4.2.6 | pKI-IAP1-Myc

A gDNA fragment corresponding to the 3′ end of *TgIAP1* (TGME49_283510) was amplified using the primers pair TgIAP1-3631/TgIAP1-3617 and cloned in the vector pT8-ARO-Myc-Bleo (Mueller et al., 2013) between the *Kpn*I and *Nsi*I sites.

4.2.7 | pKI-IAP2-2Ty

To tag *Tg*IAP2 (TGME49_297130) at its C-terminal extremity, a PCR fragment containing 32 bp homology with the 5′ end of *TgIAP2* has been amplified using the vector pLinker-2xTy-DHFR-LoxP as template and the primers pair TgIAP2_8120/TgIAP2_8121. To direct the insertion of the PCR product at the 5′ end of *IAP2*, a specific sgRNA vector has been generated as described above using the primer pair TgIAP1-8122/gRNA-4883.

4.3 | *Toxoplasma gondii* tachyzoite cultures

T. gondii tachyzoites strains were grown in confluent human foreskin fibroblasts (HFF) maintained in Dulbecco´s Modified Eagle´s Medium (Gibco DMEM) supplemented with 5% fetal calf serum, 2 mM glutamine and 25 μg/mL gentamicin. Conditional expression of the Tet-inducible constructs was performed using 1 μg/ml anhydrotetracycline (ATc) (Meissner, Schlüter, & Soldati, 2002).

## 4.4 | Parasite transfection and selection of stable transformants

Parasite transfections were performed as previously described (Soldati & Boothroyd, 1993). RHΔKU80 (Huynh & Carruthers, 2009) and derivative strains have been transfected with 15–20 μg of the linearised knock-in constructs. Either mycophenolic acid (MPA, 25 mg/ml) and xanthine (50 mg/ml) or pyrimethamine (1 μg/ml) was used to select the resistant parasites carrying the HXGPRT or the DHFR cassette respectively. For the CRISPR-Cas9 strategy, 15 μg of the circular gRNA-specific CRISPR/CAS9 vector was transfected into RHΔKU80 together with the product of 2 PCR reactions. For complementation, 40 μg of the complemented vectors, digested by *Nde*I for DHHC14 vectors and by *Apa*I and *Bam*HI for DHHC2 plasmids, were transfected with 15 μg of the UPRT-specific sgRNA plasmid pU6-UPRT (Guérin et al., 2017), a gift from Dr Maryse Lebrun.

4.5 | Antibodies

The antibodies used in this study were previously described as follow: polyclonal rabbit: α-IMC1 (1:1000 WB and IFA) and α-GAP45 (1:10000 WB and IFA) (Frénal et al., 2014), α-Catalase (1:2000 WB) (Ding, Clayton, & Soldati, 2000) and α-PRF (1:2000 WB, [Plattner
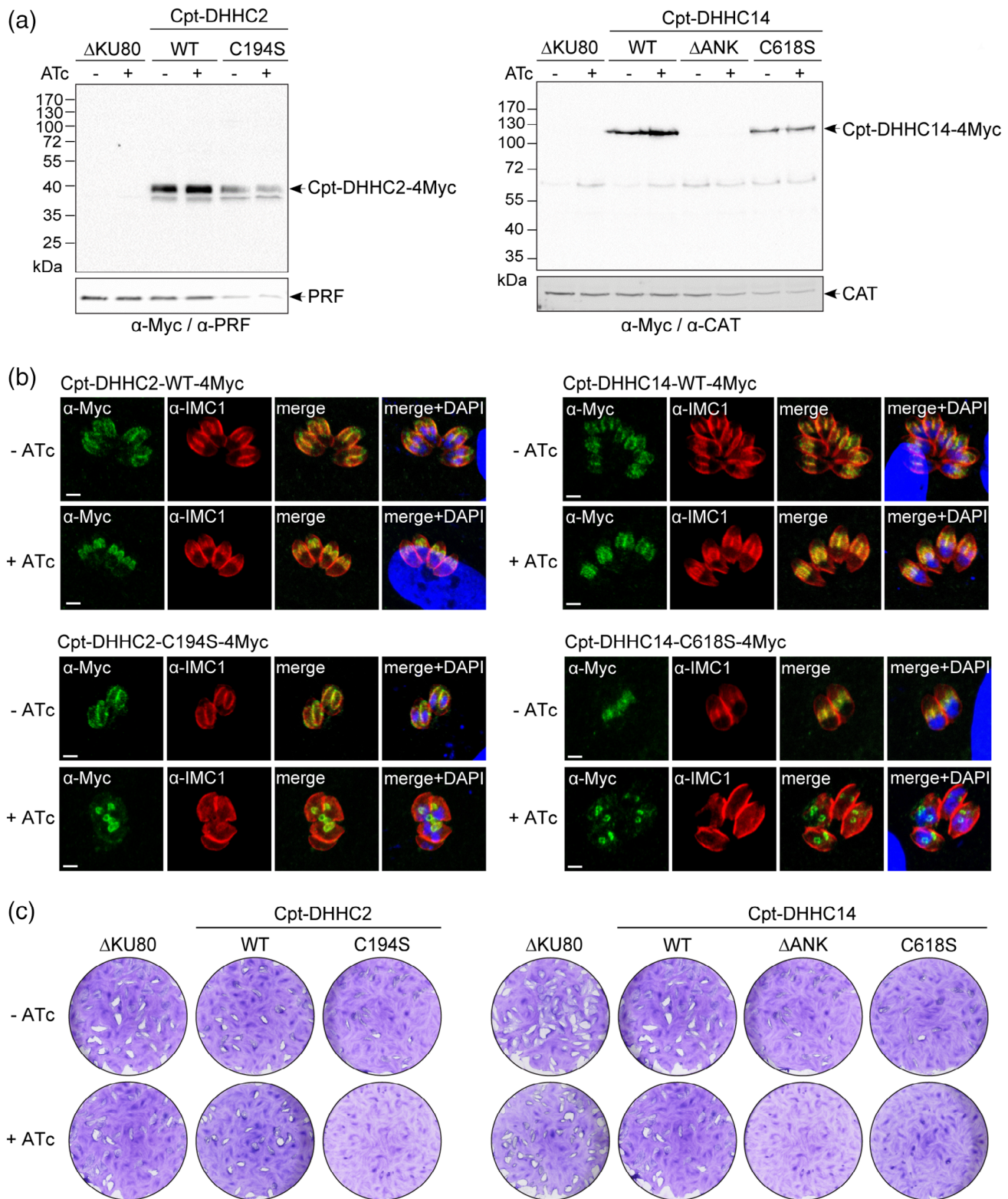
FIGURE 4 The activity of TgDHHC2 and TgDHHC14 is critical for the IMC biogenesis. (a) TgDHHC2-iKD and TgDHHC14-iKD strains, complemented with either the wild type (cpt-DHHC2/14-WT) or the mutants (cpt-DHHC2-C194S, cpt-DHHC14-C618S, cpt-DHHC-ΔANK) were treated for 24 hr ± ATc and analysed by western blot. Catalase (CAT)/Profilin (PRF) was used as loading control. (b) The same strains were also analysed by IFA. Scale bars: 2 μm. (c) The drastic impairment of the lytic cycle seen in TgDHHC2-iKD and TgDHHC14-iKD strains, as assessed by plaque formation after 7 days in the presence of ATc, is rescued by complementation with the wild type DHHCs but not the acyl-transferase mutants
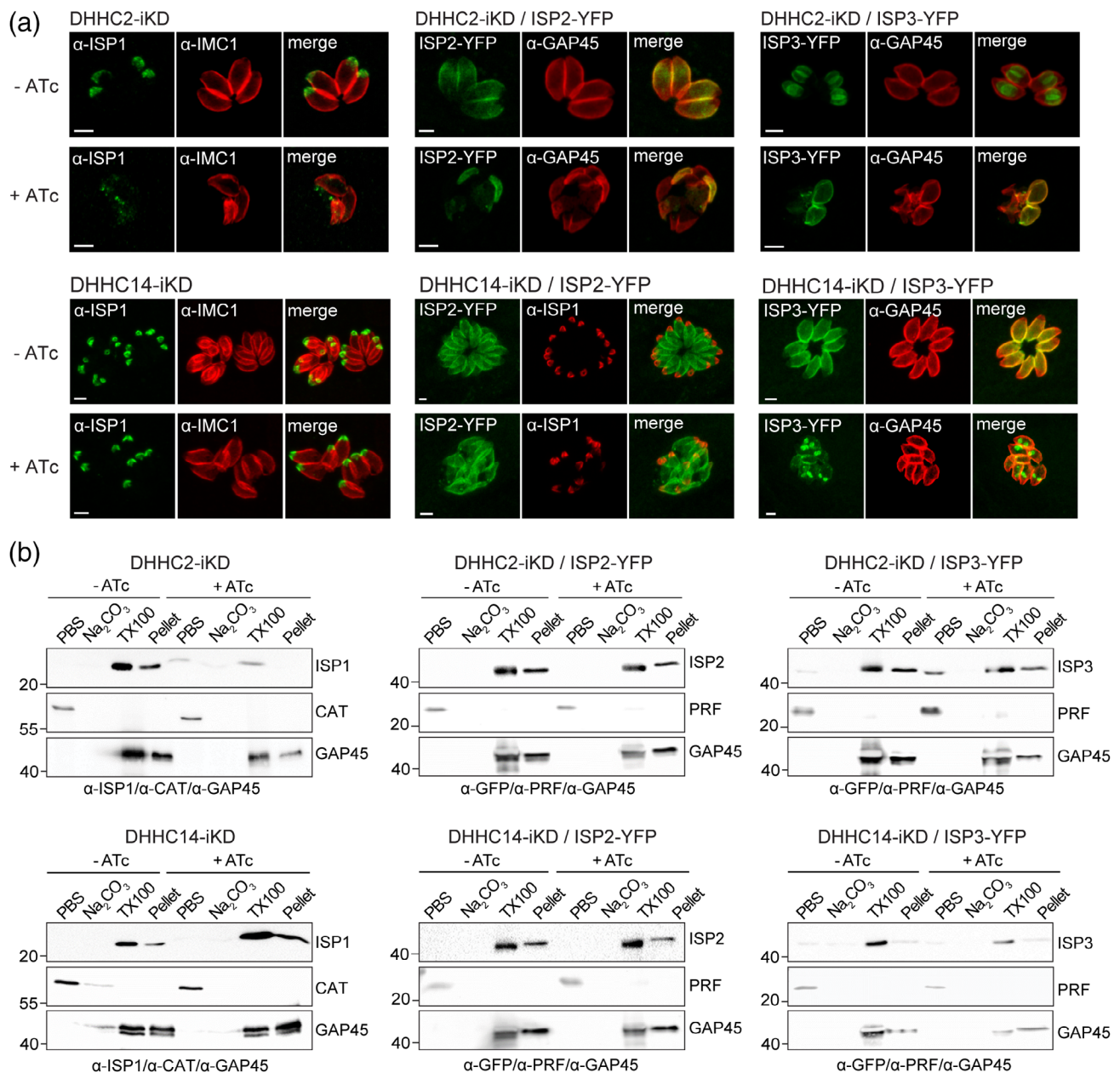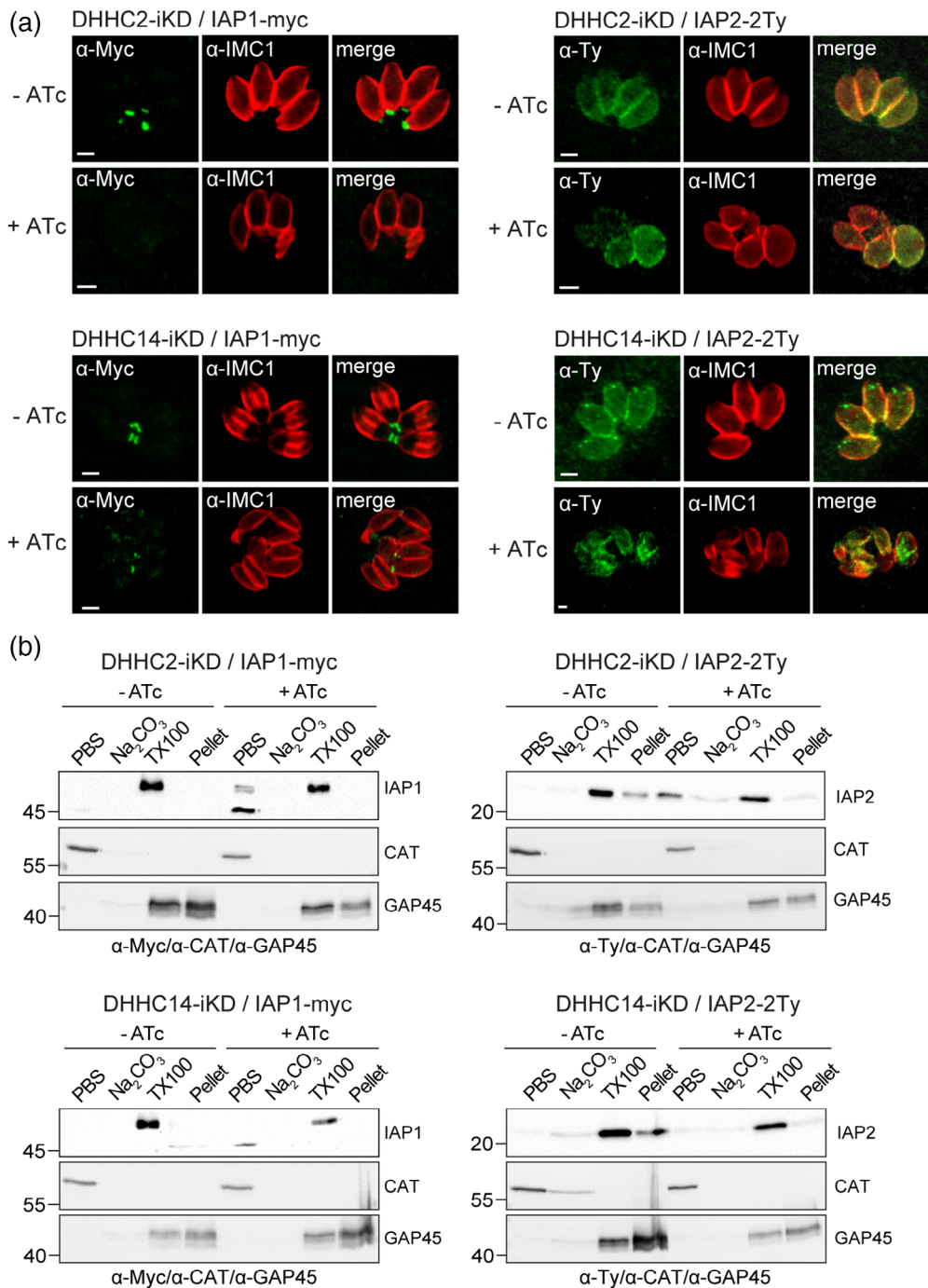
FIGURE 5 Evaluation of TgISPs as substrates of TgDHHC2 and/or TgDHHC14. (a) The localisation of the IMC sub-compartment proteins (TgISPs) was evaluated in TgDHHC2-iKD and TgDHHC14-iKD strains, following treatment for 16 hr ± ATc and fixation. TgISP1 localisation was assessed using specific antibodies while TgISP2 and TgISP3 were endogenously tagged at the C-terminus with YFP. TgIMC1 stains the alveolin network, and TgGAP45 stains the pellicle. Scale bars: 2 μm. (b) The solubility of TgISPs in the TgDHHC2-iKD and TgDHHC14-iKD, treated for 16 hr ± ATc, was analysed by western blot after serial fractionation assay. TgISPs were detected using anti-TgISP1 or anti-GFP antibodies. The cytosolic profilin (TgPRF) and the pellicular TgGAP45 were used as controls of solubilisation

et al., 2008]); monoclonal mouse, α-ACT (supernatant of hybridoma, 1:10 WB [Herm-Götz et al., 2002]), α-ISP1 (1:2000 WB and IFA, (Beck et al., 2010)), α-Myc (9E10, supernatant of hybridoma, 1:10 WB and IFA), α-Ty (BB2, supernatant of hybridoma, 1:10 WB and IFA [Bastin, Bagherzadeh, Matthews, & Gull, 1996]), monoclonal rat α-HA (1:1000 IFA, Roche), α-acetylated α-tubulin (6-11B-1, 1:1000 IFA, Abcam). For western blot analyses, secondary peroxidase conjugated goat α-rabbit/mouse antibodies (Sigma) were used. For immunofluorescence analyses, the secondary antibodies Alexa Fluor 488- and

Alexa Fluor 594-conjugated goat α-mouse/rabbit antibodies (1:3000 IFA, Thermofisher) were used.

4.6 | Immunofluorescence assay (IFA)

Parasite-infected HFF cells seeded on cover slips were fixed with 4% PFA/0.05% glutaraldehyde (PFA/GA) in PBS for 10 min or with methanol for 8 min for anti-tubulin-Ac and then processed as previously described

FIGURE 6 Evaluation of TgIAP1/2 as substrates of TgDHHC2 and/or TgDHHC14. (a) The localisations of the IMC associated proteins (TgIAPs) were evaluated in TgDHHC2-iKD and TgDHHC14-iKD strains, following treatment for 16 hr ± ATc and fixation. TgIMC1 stains the alveolin network. TgIAP1 and TgIAP2 were endogenously tagged at the C-terminus with 4 c-Myc or 3 Ty tags, respectively. (b) The solubility of TgIAPs in the TgDHHC2-iKD and TgDHHC14-iKD, treated for 16 hr ± ATc, was analysed by western blot after serial fractionation assay using anti-Myc and anti-Ty antibodies. TgPRF and TgGAP45 were used as controls for the cytosolic and the cytoskeletal fractions, respectively

(Hettmann et al., 2000). Confocal images were generated with a Zeiss LSM700 laser scanning confocal microscope using an apochromat 63×/1.4 oil objective at the Bioimaging core facility of the Faculty of Medicine, University of Geneva. Stacks of sections were processed with ImageJ and projected using the maximum projection tool.

supernatant was then collected and mixed with SDS–PAGE loading buffer under reducing conditions. Separated proteins were transferred to nitrocellulose membranes and probed with appropriate antibodies in 5% non-fat milk powder in PBS-0.05% Tween20. Bound secondary peroxidase conjugated antibodies were visualised using the ECL system.

4.7 | Western blot analysis

Parasites were lysed in RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris pH 7.5), incubated on ice for 15 min and then centrifuge for 30 min at 14,000 rpm at 4°C. The

4.8 | Plaque assay

Confluent HFF cells were infected with freshly egressed parasites and treated ± ATc if necessary. After 7 days, the cells were fixed with PFA/GA and stained with a crystal violet solution (Sigma).

4.9 | Transmission electron microscopy

Freshly egressed parasites were inoculated on confluent HFFs and allowed to grow for 24 hr ± ATc Infected host cells were washed with 0.1 M phosphate buffer pH 7.4, then fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.4, scrapped and pelleted. Samples were then treated as previously described (Frénal et al., 2010). Thin sections were analysed using a Technai 20 electron microscope (FEI Company) at the "Pôle Facultaire de Microscopie Ultrastructurale (PFMU)" of the Faculty of medicine of Geneva.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.